# Fermentation of Vitamin K2 (Menaquinone-7): Development of an Optimal Fermentation Process to Selectively Enhance the Production of the Biologically Significant All-*Trans* Isomer

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# Abstract

Vitamin K is a lipid-soluble vitamin first discovered in 1929 by the Danish nutritional biochemist Carl Peter Henrick Dam as an antihaemorrhagic factor capable of correcting dietary-induced bleeding disorders in chicks. The vitamin K family encompasses a series of structurally related compounds, namely vitamin K1 (phylloquinone), vitamin K2 (menaquinones), and vitamin K3 (menadione), which share a common 2-methyl-1,4-naphthoquinone moiety but differ in the structure of a lateral isoprenoid chain at the 3-position. Despite their structural similarities, all K vitamers have different pharmacokinetic properties attributable to the length and degree of saturation of their isoprenoid side chain.

All vitamin K isoforms are involved in the activation of hepatic and extrahepatic vitamin K-dependent proteins (VKDPs) and play a central role in blood coagulation and haemostasis. Additionally, it has been established that the potential health benefits of vitamin K are far more diverse. In particular, menaquinones (MKs) offer more significant health gains than phylloquinone (PK) and have been associated with the prevention of osteoporosis and cardiovascular diseases (CVDs) and decreasing the risk of and improving the outcomes associated with several illnesses and health conditions, including cancer, neurological diseases, type 2 diabetes mellitus, chronic kidney disease, immune disorders, obesity, and coronavirus disease 2019 (COVID-19).

Of the various MKs, menaquinone-7 (MK-7) has superior bioavailability due to its long plasma half-life. Thus, it is considered the most notable form of vitamin K2 and has the greatest efficacy with respect to extrahepatic biological functions. However, MK-7 exists at low concentrations in limited foods, especially those with universal appeal. This has incentivised the development of MK-7 dietary supplements and functional food products to complement natural sources and satisfy the daily intake requirements of this essential vitamin.

Recently, it has been recognised that MK-7 exists as geometric isomers, comprising the bioactive all-*trans* isomer and various *cis* forms of the compound, which lack or have considerably compromised biological significance. This is a salient aspect worthy of attention, as the effectiveness of MK-7 nutritional supplements is primarily determined by the content of all-*trans* MK-7, and all other isomers of the vitamin are essentially impurities that lack therapeutic value.

MK-7 can be produced synthetically from chemical reaction methods or naturally from bacterial fermentation. The latest market trend promoting natural and organic alternatives over synthetic products has rendered natural fermentation-based synthesis more favourable from a consumer's perspective. Furthermore, microbial production is a more sustainable approach for the large-scale synthesis of MK-7; hence, natural fermentation techniques can satisfy both the market demand and sustainable development goals.

The MK-7 isomer composition of the final product is influenced by many factors, predominantly the methods used for its production and the purification of the post-reaction mixture, as well as exposure to certain environmental factors and storage conditions. It is important to appreciate that while studies analysing the isomer profile of MK-7 dietary supplement preparations of various origins have been carried out, the isomer composition resulting from fermentation processes has not been elucidated.

In light of the fact that only all-*trans* MK-7 sustains biological activity and fermentation-based synthesis is superior from both the viewpoint of consumers and the environment, the MK-7 isomer profile attained from fermentation warrants further investigation. Therefore, the fundamental aim of this research was to evaluate the MK-7 isomer composition resulting from fermentation processes employing different synthesis conditions to explicate the optimal fermentation method for the production of the biologically significant all-*trans* form of the vitamin. A holistic approach was adopted to systematically follow a typical fermentation process from its foundational stages to the final fermented product while considering the MK-7 isomer profile obtained from fermentation under various conditions and the bioactivity and therapeutic value of fermented MK-7 consumer end products. Accordingly, key aspects of upstream and downstream fermentation were explored, along with nanobiotechnological approaches, to potentially address the major challenges accompanying the fermentation and downstream processing of MK-7. The isomer composition and stability of all-*trans* MK-7 resulting from exposure to possible conditions and environmental factors encountered during the manufacture, transportation, and storage of fermented bioactive MK-7 goods available on the market were also assessed.

The selection of nutrients, particularly carbon, nitrogen, and salt sources, and their respective concentrations in the fermentation media are a central aspect of any fermentation process, as they influence microbial growth and metabolism and, ultimately, the process productivity and product yield. Thus, the first step was to consider the impact of the media composition on MK-7 isomer production and ascertain the optimum media combination to favour the synthesis of all-*trans* MK-7. Several nutrient sources were screened, and the concentration of the effective media components was optimised to obtain the ideal fermentation media, which contained 1% (*w/v*) glucose, 2% (*w/v*) yeast extract, 2% (*w/v*) soy peptone, 2% (*w/v*) tryptone, and 0.1% (*w/v*) calcium chloride ($CaCl_2$). The optimal fermentation media resulted in an all-*trans* isomer concentration of 36.37 mg/L and a *cis* isomer concentration of 1.23 mg/L. It was also established that only a single *cis* isomer is obtained from fermentation under the investigated conditions. Moreover, different concentrations of the *cis* isomer were achieved when the selection and concentration of nutrients constituting the fermentation media were varied, implying that the media composition is instrumental in determining the nature of the extracellular environment in the fermentation broth, and this likely influences the extent to which all-*trans* MK-7 isomerises to the *cis* form.

Various fermentation parameters and operating conditions also play an indispensable part in fermentation processes, as they contribute to the microbial growth environment and, hence, impact product formation and the efficacy of the fermentation system. Consequently, the subsequent focus was to explore the effect of crucial fermentation parameters, specifically the inoculum size, fermentation temperature, agitation speed, and length of fermentation, on the MK-7 isomer profile. These factors were optimised to enhance the synthesis of the biologically important all-*trans* isomer and minimise the concentration of *cis* MK-7 while using the previously developed fermentation media. The optimum fermentation conditions consisted of an inoculum size of 2% (*v/v*), a fermentation temperature of 40 °C, an agitation speed of 200 rpm, and a fermentation period of 7 days and enabled an all-*trans* and *cis* isomer concentration of 53.29 mg/L and 1.22 mg/L, respectively. An approximately 46.5% greater all-*trans* MK-7 concentration was attained from fermentation utilising the optimal media, inoculum size, temperature, agitation speed, and duration compared to fermentation with only the optimum media composition, which emphasises the pivotal role of the fermentation environment in regulating the production of the desired isomer.

Although fermentation is the preferred method for MK-7 synthesis, its low yield and large number of downstream processing steps increase production expenses and the cost of the final product. The high price of fermented MK-7 supplements reduces their widespread accessibility. While optimisation of various aspects of the fermentation process, such as the fermentation format, nutrient selection and media composition, and value of key fermentation parameters and operating conditions, serves to enhance the production and yield of MK-7, it offers little opportunity to refine the fermentation system through process intensification. Therefore, there is a need for innovative approaches to not only boost the process yield but also streamline the overall fermentation procedure by decreasing the complexity and number of unit operations involved in the downstream processing of the vitamin. It is also essential to ensure that all-*trans* MK-7 is produced almost exclusively or in the most significant proportion during fermentation. In this regard, bacterial cell immobilisation with biocompatible magnetic iron oxide nanoparticles (IONs) was investigated to assess their influence on the MK-7 isomer profile obtained from fermentation. Three types of IONs, including one uncoated (naked) and two coated (amine-functionalised) IONs, were synthesised using the co-precipitation method and characterised using several techniques, and their effect on microbial growth and the production and yield of MK-7 isomers was considered. Naked IONs were initially examined, and the results were compared with the findings for the 3-aminopropyltriethoxysilane (APTES)- and L-lysine (L-Lys)-coated IONs (IONs@APTES and L-Lys@IONs). The optimum concentration of naked IONs was 300 µg/mL, and it improved the process output and resulted in an all-*trans* MK-7 concentration of 28.78 mg/L and a 1.6-fold greater all-*trans* isomer yield relative to the control. In comparison to the naked IONs, the amine-functionalised IONs had superior properties and provided more positive outcomes. The optimal IONs@APTES and L-Lys@IONs concentrations were 300 µg/mL and

400 μg/mL, enabling a maximum all-*trans* MK-7 concentration of 41.93 mg/L and 32.08 mg/L, respectively. In addition, the yield of the biologically effective isomer compared to the control increased by 3.1-fold for the IONs@APTES and 2.1-fold for the L-Lys@IONs. Of all three forms of IONs that were explored, it was determined that 300 μg/mL of IONs@APTES was the most beneficial to enhance the all-*trans* isomer concentration and minimise the synthesis of *cis* MK-7. The magnetic nature of IONs also increases the scope for process intensification through magnetic separation technology, which has the potential to improve the productivity of MK-7 fermentation.

It is of interest to note that the greatest all-*trans* MK-7 concentration obtained in the presence of IONs (41.93 mg/L) was less than that achieved in their absence when applying the same fermentation media and operating conditions as previous experiments (53.29 mg/L). This disparity can be attributed to the different fermentation volumes used in the two studies, as even the untreated (control) samples in the experiments involving IONs resulted in considerably lower concentrations of the all-*trans* isomer (less than 15 mg/L). All prior investigations were carried out on a small scale (6 mL), whereas the studies employing IONs required a slightly greater volume (approximately 20 mL) to accommodate the inclusion of IONs in the fermentation medium and the related measurements. It is apparent that even a small increase in the volume and, consequently, the scale of the fermentation process has a noticeable impact on the concentration of the target product. These observations highlight the central role of the fermentation volume in determining the concentration of all-*trans* MK-7 attained on a larger scale and present basic knowledge of likely challenges associated with the scale-up of fermentation processes targeting the production of the bioactive isomer.

It has been proposed that exposure to various environmental conditions influences the isomer composition of MK-7 products. Awareness of these factors and their impact on the proportion of MK-7 isomers is vital to preserve the content of the biologically significant all-*trans* isomer and prevent its transformation to *cis* MK-7 during the storage of fermented MK-7 supplements and fortified or functional foods. In this respect, the effect of short-term exposure to common environmental factors and storage conditions, such as light, atmospheric oxygen, and different temperatures, on the MK-7 isomer profile was considered to establish the optimum conditions to conserve the quantity of the all-*trans* isomer during the storage of fermented MK-7 preparations. It was ascertained that the vitamin is reasonably heat-stable but extremely light-sensitive. Long-term storage at a low temperature with minimal oxygen exposure in the absence of light resulted in a negligible change in the concentration of the all-*trans* isomer and was, thus, the optimal environment for the prolonged storage of fermented all-*trans* MK-7. These findings provide a deeper understanding of the impact of different environments on the stability of the biologically efficacious isomer and are a valuable step forward in establishing ideal storage conditions to maintain the concentration of all-*trans* MK-7 in fermented nutritional supplements.

Overall, this research presents novel insights into the MK-7 isomer profile achieved from fermentation in various contexts, a previously unexplored field, and has laid the foundation for

future studies. Although it is desirable to exclusively produce the bioactive isomer from fermentation, the collective experimental findings have revealed that obtaining small quantities of the *cis* isomer alongside all-*trans* MK-7 is largely unavoidable under the circumstances considered. However, it is possible to maximise the concentration of the all-*trans* isomer and minimise the amount of the biologically insignificant isomer, which is a reasonable compromise. Appreciation of the key factors that influence the MK-7 isomer composition resulting from fermentation will allow their manipulation to attain a more favourable MK-7 profile in the final preparation, which can be included in nutraceuticals, dietary supplements, and functional food products or used in purified form. Furthermore, the outcomes of this study have the potential to aid the development of an industrial fermentation process that specifically targets the production of the biologically active and therapeutically valuable all-*trans* MK-7 isomer. The prospect for process intensification through the innovative use of magnetic IONs will likely streamline the production system and decrease the related expenses. This will help reduce the price and increase the accessibility of fermented bioactive MK-7 supplements and fortified or functional foods. Moreover, a greater understanding of the environmental factors and storage conditions that affect the isomer composition of fermented MK-7 preparations is likely to improve the standard of MK-7 nutritional products by preserving the quantity of the all-*trans* isomer. The widespread availability and consumption of high-quality bioactive MK-7 products by a range of consumers are expected to raise the vitamin K status of individuals and decrease the risk and progression of several age-related disorders and diseases of global significance. This holds great promise for reducing the disease burden and alleviating the socioeconomic consequences of an ageing population.

# Dedication

This dissertation and all my academic achievements are dedicated with love to Toffee, my forever study buddy. I am eternally grateful for your unconditional love, support, and companionship. You are the key to my success. I hope I have made you proud.

Also, thank you to my parents for always being there for me. I would not have been able to complete this endeavour and accomplish all I have without your everlasting support and encouragement.

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# List of Abbreviations

| | |
|---|---|
| AI | Adequate intake |
| ALP | Alkaline phosphatase |
| Al | Aluminium |
| APTES | 3-aminopropyltriethoxysilane |
| $NH_4OH$ | Ammonium hydroxide |
| ANOVA | Analysis of variance |
| IONs@APTES | APTES-coated IONs |
| APCI | Atmospheric pressure chemical ionisation |
| $BaCO_3$ | Barium carbonate |
| BMD | Bone mineral density |
| BMP-2 | Bone morphogenic protein 2 |
| BMP-4 | Bone morphogenic protein 4 |
| $BF_3O(C_2H_5)_2$ | Boron trifluoride etherate |
| Ca | Calcium |
| $CaCO_3$ | Calcium carbonate |
| $CaCl_2$ | Calcium chloride |
| $Ca_3(C_6H_5O_7)_2$ | Calcium citrate |
| $CO_2$ | Carbon dioxide |
| Gla | γ-carboxyglutamic acid |
| dp-cMGP | Carboxylated, dephosphorylated MGP |
| cOC | Carboxylated OC |
| p-cMGP | Carboxylated, phosphorylated MGP |
| CVDs | Cardiovascular diseases |
| CCF | Central composite face-centred |
| CAD | Charged aerosol detector |
| $CoQ_{10}$ | Coenzyme $Q_{10}$ |
| CFU/mL | Colony forming units/mL |
| CI | Confidence interval |
| $CuCO_3$ | Copper carbonate |
| CuO | Copper oxide |
| COVID-19 | Coronavirus disease 2019 |
| DFT | Density functional theory |
| DOE | Design of experiments |
| DAD | Diode array detector |
| $K_2HPO_4$ | Dipotassium hydrogen phosphate |
| EC | Electrochemical |

| | |
|---|---|
| ESI | Electrospray ionisation |
| ER | Endoplasmic reticulum |
| ECM | Extracellular matrix |
| EPS | Extracellular polymeric substances |
| $Fe^{3+}$ | Ferric ions |
| $FePO_4$ | Ferric pyrophosphate |
| $Fe^{2+}$ | Ferrous ions |
| FL | Fluorescence |
| FDA | Food and Drug Administration |
| FA | Formaldehyde |
| FTIR | Fourier-transform infrared |
| GRAS | Generally recognised as safe |
| GMOs | Genetically modified organisms |
| GRP | Gla-rich protein |
| Glu | Glutamic acid |
| GGCX | γ-glutamyl carboxylase |
| GA | Glutaraldehyde |
| α-FeOOH | Goethite |
| Au | Gold |
| GAS6 | Growth arrest-specific protein 6 |
| α-$Fe_2O_3$ | Hematite |
| HPLC | High-performance liquid chromatography |
| INPs | Inorganic NPs |
| $FeCl_3 \cdot 6H_2O$ | Iron (III) chloride hexahydrate |
| $Fe_2O_3$ | Iron oxide |
| IONs | Iron oxide nanoparticles |
| FeOOH | Iron oxyhydroxide |
| $FeSO_4 \cdot 7H_2O$ | Iron (II) sulphate heptahydrate |
| L-Arg | L-arginine |
| LC | Liquid chromatography |
| LSF | Liquid-state fermentation |
| L-Lys@IONs | L-Lys-coated IONs |
| L-Lys | L-lysine |
| LDL | Low-density lipoproteins |
| γ-$Fe_2O_3$ | Maghemite |
| Mg | Magnesium |
| MgO | Magnesium oxide |
| $Fe_3O_4$ | Magnetite |

| | |
|---|---|
| MS | Mass spectrometry |
| MGP | Matrix Gla protein |
| MK-4 | Menaquinone-4 |
| MK-6 | Menaquinone-6 |
| MK-7 | Menaquinone-7 |
| MK-8 | Menaquinone-8 |
| MK-10 | Menaquinone-10 |
| MK-11 | Menaquinone-11 |
| MKs | Menaquinones |
| MM | Molecular mass |
| MLR | Multiple linear regression |
| NCs | Nanoclays |
| NEs | Nanoemulsions |
| NMs | Nanomaterials |
| NPs | Nanoparticles |
| $N_2$ | Nitrogen |
| NF-κB | Nuclear factor-kappa B |
| NMR | Nuclear magnetic resonance |
| OD | Optical density |
| ONPs | Organic NPs |
| $OsO_4$ | Osmium tetroxide |
| OC | Osteocalcin |
| Osc | Osterix |
| $O_2$ | Oxygen |
| PLF | Periostin-like factor |
| p-ucMGP | Phosphorylated, uncarboxylated MGP |
| PK | Phylloquinone |
| PBD | Placket Burman design |
| PCS | Plastic composite support |
| Pt | Platinum |
| PUFAs | Polyunsaturated fatty acids |
| KBr | Potassium bromide |
| PXR | Pregnane X receptor |
| PRGPs | Proline-rich Gla proteins |
| QTOF | Quadrupole time-of-flight |
| ROS | Reactive oxygen species |
| RANKL | Receptor activator of nuclear factor-kappa B ligand |
| RDA | Recommended dietary allowance |

| | |
|---|---|
| RDI | Recommended dietary intake |
| RRT | Relative retention time |
| RSM | Response surface methodology |
| SEM | Scanning electron microscopy |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus 2 |
| $SiO_2$ | Silica |
| Si | Silicon |
| Ag | Silver |
| $Ag_2O$ | Silver oxide |
| $C_2H_6AsNaO_2$ | Sodium cacodylate |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| SSF | Solid-state fermentation |
| SD | Standard deviation |
| SE | Standard error |
| SXR | Steroid and xenobiotic receptor |
| MS/MS | Tandem MS |
| 2θ | 2-theta |
| $TiO_2$ | Titanium dioxide |
| TMGPs | Transmembrane Gla proteins |
| TEM | Transmission electron microscopy |
| TNF-α | Tumour necrosis factor-alpha |
| UHPLC-DAD-CAD-QTOF | Ultra-HPLC-DAD-CAD-QTOF |
| UV | Ultraviolet |
| dp-ucMGP | Uncarboxylated, dephosphorylated MGP |
| ucMGP | Uncarboxylated MGP |
| ucOC | Undercarboxylated OC |
| UrAc | Uranyl acetate |
| VSMCs | Vascular smooth muscle cells |
| VKDPs | Vitamin K-dependent proteins |
| KO | Vitamin K epoxide |
| VKOR | Vitamin K epoxide reductase |
| K | Vitamin K hydroquinone |
| $k_La$ | Volumetric mass transfer coefficient |
| FeO | Wustite |
| XG | Xanthan gum |
| XRD | X-ray powder diffraction |
| Zn | Zinc |

| | |
|---|---|
| ZnO | Zinc oxide |

# 1

# Introduction

# Chapter 1 - Introduction

## 1.1 Background information

Several studies have demonstrated the superior health benefits of MK-7 compared to the other K vitamers due to its high bioavailability. Consequently, there has been a strong drive to improve the dietary intake of MK-7 through the development of nutritional supplements and functional food products. Although MK-7 can be produced from chemical reaction schemes, which tend to be more cost-effective, natural fermentation-based synthesis is preferable from a consumer's point of view, largely owing to the recent market trend favouring natural and organic alternatives over synthetic preparations. Additionally, microbial synthesis methods are more sustainable for the large-scale production of MK-7, and natural fermentation techniques can fulfil both the market demand and environmental sustainability goals.

It has recently been acknowledged that MK-7, like most biological molecules, occurs as geometric isomers. Only the all-*trans* (all-*E*) configuration is the active form of the vitamin, and the *cis* isomers have relatively little or no biological significance, which is an important consideration from a health, nutritional, and therapeutic outlook. Significant attention has been dedicated to enhancing the MK-7 concentration and yield obtained from fermentation processes and reducing the production cost of the vitamin to make it more affordable and accessible for consumers, and comparatively less recognition has been given to assessing the proportion of the biologically active isomer in preparations.

The identification and quantification of MK-7 isomers is a fairly recent and emerging area of interest. Hence, limited research has been conducted in this field, and a great deal is yet to be explored. Most studies focusing on MK-7 isomers have only evaluated the isomer composition of dietary supplements, and the isomer profile obtained from fermentation methods remains to be elucidated. Furthermore, the number and type of different *cis* isomers that are potentially attainable is uncertain, as very few studies have considered this aspect. The isomer composition in the final product is influenced by numerous factors, including the techniques used for its production and purification and exposure to certain environmental factors and storage conditions, which have also not been investigated. The MK-7 profile obtained from the various production methods has not yet been ascertained, and the ideal approach for synthesising the all-*trans* form of the vitamin is also debatable.

In view of the differing biological efficacy of MK-7 isomers and the advantages of microbial fermentation for MK-7 production over chemical procedures, with respect to its ability to satisfy the desire of consumers and support sustainable development initiatives, the isomer composition resulting from fermentation processes deserves greater consideration. To date, this subject has received little attention, and there is a significant gap in the literature regarding the impact of fermentation methods on the production of MK-7 isomers. Acquiring a deeper and more

comprehensive understanding in this area will advance the current knowledge of the production of MK-7 isomers from fermentation, and it will also provide valuable insight for the development of effective fermented MK-7-enriched functional food products and dietary supplements that predominantly contain the biologically active and therapeutically significant all-*trans* isomer. The extensive availability and uptake of such products by a range of consumers are expected to improve the vitamin K status of individuals and reduce the progression and burden of CVDs, osteoporosis, age-related disorders, and other diseases of global relevance. This has the potential to ease the socioeconomic effects of demographic ageing and decrease the negative consequences of prevalent health problems worldwide.

## 1.2 Research objectives

The fundamental objective of this research was to evaluate the MK-7 isomer profile of fermented samples obtained from different synthesis conditions to elucidate the ideal fermentation method to enhance the production of the biologically active all-*trans* isomer. This study sought to methodically investigate key facets of a standard fermentation process from its foundational steps to the final consumer product to develop a fermentation process that selectively targets the production and preserves the quantity of all-*trans* MK-7 while minimising the concentration of the biologically inefficacious *cis* isomer. Essential aspects of upstream fermentation were first explored before focusing on the isomer composition and synthesis of bioactive MK-7 in a broader fermentation context to overcome the inherent challenges of MK-7 fermentation and the downstream processing of the vitamin using nanobiotechnological strategies. Factors that have the ability to influence the isomer profile of fermented MK-7 preparations during production, transportation, storage, and consumption were also assessed to ascertain the optimal conditions to maintain the concentration of the biologically effectual isomer in fermented MK-7 consumer end products.

The specific aims of this research encompassed the following:

1. Upstream fermentation – Investigate the effect of the media composition on the isomer profile obtained from fermentation and determine the optimum media combination.
    - Screen a diverse range of carbon, nitrogen, and salt sources and optimise the concentration of the significant nutrients to enhance the production of the all-*trans* isomer and decrease the concentration of *cis* MK-7.
2. Upstream fermentation – Assess the impact of important fermentation parameters on the isomer composition.
    - Optimise the inoculum size, fermentation temperature, agitation speed, and length of fermentation and establish the optimum conditions to increase the synthesis of bioactive MK-7 and minimise the production of the *cis* isomer.

3. Approaches to address fundamental challenges relating to the fermentation and downstream processing of MK-7 – Determine the effect of bacterial cell immobilisation with magnetic IONs on microbial growth and the production and yield of MK-7 isomers.
    - Synthesise and characterise naked and amino acid-coated IONs and evaluate their impact on cell growth and ability to increase the concentration and yield of all-*trans* MK-7 and diminish the production of the *cis* isomer.
4. Finished fermented MK-7 consumer end products – Consider the influence of environmental factors and storage conditions on the isomer composition of fermented MK-7.
    - Explore the short- and long-term effects of exposure to light, atmospheric oxygen, and different temperatures on the isomer profile to assess the stability and preserve the quantity of all-*trans* MK-7 in the final preparation obtained from fermentation.

## 1.3 Thesis overview

This thesis is a Doctor of Philosophy (PhD) with publication submitted in fulfilment of the requirements set out at The University of Waikato. The thesis comprises nine chapters, and the key objectives of this study are addressed in five research chapters (Chapters 4–8), which have been prepared as independent articles for submission to peer-reviewed journals. Consequently, these papers inherently contain repetitive elements, especially in the introductory and methodological sections. The journal publications are preceded by a short overview of the contribution of each paper to the entire thesis, and the relevant findings of each chapter are applied to subsequent chapters to link the various aspects of this study and enable a complete analysis. The overall thesis consists of an introduction, a literature review, a comprehensive materials and methods section, five peer-reviewed journal articles, and a concluding discussion emphasising the important findings and significance of this research and recommendations for future investigation.

Chapter 1 provides a general introduction to the topics considered and discussed in this thesis and an overview of the research problem and objectives.

Chapter 2 presents a critical review of the literature pertaining to this study. The properties, health benefits, dietary sources, and intake of vitamin K, specifically MK-7, are discussed, along with MK-7-enriched functional food products and dietary supplements. Additionally, the current knowledge and understanding of MK-7 isomers and their biological activity, safety, and toxicity are evaluated. Furthermore, the methods of MK-7 synthesis and the production of *cis* and *trans* isomers are reviewed. The issues associated with MK-7 fermentation and the ability of nanoparticles (NPs) to potentially address these production challenges are also

examined. Finally, methods for analysing and measuring MK-7 isomers are presented, and the possible difficulties likely to be encountered during the analysis of MK-7 isomers are considered.

Chapter 3 details the materials, equipment, methodology, and experimental procedures employed throughout this research.

Chapter 4 considers the effect of various media components on MK-7 isomer production. The media composition plays a crucial role in microbial growth and metabolism, which ultimately influences the efficiency of the fermentation process and the product concentration and yield. Therefore, the selection of carbon, nitrogen, and salt sources and their concentrations in the fermentation media must be tailored to suit the growth and metabolic requirements of the microorganism of interest. Accordingly, different carbon, nitrogen, and salt sources were initially screened to identify the nutrients that have a significant impact on MK-7 isomer production. The key media components were further analysed in an optimisation study to develop the ideal fermentation media to maximise the production of the bioactive isomer and minimise the concentration of *cis* MK-7. A monitoring investigation was then conducted to examine the variation in bacterial growth, MK-7 isomer production, and pH over a time-course fermentation study employing the optimal media. This chapter is written in the form of a journal article, which has been published in Bioprocess and Biosystems Engineering. The article is open access, and permission has been granted to reproduce this work and include a PDF version of the published paper in this thesis under the Creative Commons Attribution 4.0 International License (CC BY).

Chapter 5 evaluates the influence of important fermentation parameters on the MK-7 isomer profile. Since each microorganism has specific growth and metabolic demands, selecting the appropriate fermentation conditions to promote microbial growth and product formation is essential. Consequently, an optimisation study was carried out to determine the ideal inoculum size, fermentation temperature, agitation speed, and fermentation period to enhance the concentration of all-*trans* MK-7 and decrease the production of the *cis* isomer. This chapter is written in the form of a journal article, which has been published in Biocatalysis and Agricultural Biotechnology. Permission has been granted by the publisher (Elsevier) to reproduce this work and include a PDF version of the published paper in this thesis.

Chapter 6 investigates the impact of bacterial cell immobilisation with magnetic IONs on bacterial growth and the MK-7 isomer composition and yield obtained from fermentation. Although the fermentation-based synthesis of MK-7 is preferable to other methods of production, it entails several challenges, primarily the low fermentation yield and large number of tedious unit operations involved in the complex downstream processing of the vitamin. Both factors increase production costs and result in an expensive final product that is not widely accessible to all consumer groups. Nanobiotechnological approaches have the potential to overcome these obstacles by improving the production and yield of MK-7 and providing an opportunity to streamline the fermentation system through process intensification. In this respect, naked (uncoated) IONs were synthesised via the co-precipitation method and characterised using several

techniques. Subsequently, the effect of bacterial cell immobilisation on cell growth and the yield of all-*trans* and *cis* MK-7 was evaluated. The ability of IONs to promote the production of the bioactive isomer and reduce the formation of the ineffectual isomer was assessed, and the optimal naked ION concentration was investigated in a time-course fermentation study to explore its influence on the microbial growth curve, MK-7 isomer concentrations, and medium pH. This chapter is written in the form of a journal article, which has been published in Nanomaterials. The article is open access, and permission has been granted to reproduce this work and include a PDF version of the published paper in this thesis under the Creative Commons Attribution 4.0 International License (CC BY).

Chapter 7 builds on the previous chapter and examines two biocompatible IONs functionalised with amino acid coatings (IONs@APTES and L-Lys@IONs), which have superior properties to naked IONs and promote more favourable interactions with microbial cells. IONs@APTES and L-Lys@IONs were synthesised and characterised, and the impact of bacterial cell immobilisation with each type of amine-functionalised ION on microbial growth and the production and yield of MK-7 isomers was evaluated. The ideal amino acid-coated ION and its optimum concentration, concerning the investigated parameters, were determined. The optimal amine-functionalised ION concentration was further analysed in a monitoring study to examine its effect on the cell growth, MK-7 isomer, and pH profiles. This chapter is written in the form of a journal article, which has been published in Molecular Biotechnology. The article is open access, and permission has been granted to reproduce this work and include a PDF version of the published paper in this thesis under the Creative Commons Attribution 4.0 International License (CC BY).

Chapter 8 explores the influence of different environmental factors and storage conditions on the MK-7 isomer composition and stability of the all-*trans* isomer. It has been recognised that exposure to light, atmospheric oxygen, and elevated temperatures can alter the quality of vitamin preparations. Thus, the effect of these factors on the concentration of MK-7 isomers and stability of all-*trans* MK-7 was investigated with the aim of preserving the quantity of the biologically significant isomer from the perspective of fermented MK-7 end products available to consumers. This chapter is written in the form of a journal article, which has been published in Processes. The article is open access, and permission has been granted to reproduce this work and include a PDF version of the published paper in this thesis under the Creative Commons Attribution 4.0 International License (CC BY).

Chapter 9 summarises the conclusions drawn from this study and recommends possible directions for further research.

The co-authorship forms outlining the contribution of all authors to the publications comprising this thesis are provided in Appendix C.

# 2

# Literature Review

# Chapter 2 – Literature Review

## 2.1 Vitamin K

Vitamin K was first discovered in 1929 by the Danish nutritional biochemist Carl Peter Henrik Dam [1, 2]. During his research on cholesterol metabolism at the Biochemical Institute of the University of Copenhagen, he discovered an antihaemorrhagic factor capable of correcting dietary-induced bleeding disorders in chicks [1-5]. This fat-soluble vitamin, distinct from vitamins A, D, and E, was termed vitamin K based on the word "Koagulation" from the German and Scandinavian languages [6]. Following its discovery, the American biochemist Edward Albert Doisy successfully isolated vitamin K and elucidated its 2-methyl-3-phytl-1,4-naphthoquinone chemical structure in the late 1930s [1, 7, 8]. In 1943, both Dam and Doisy were awarded the Nobel Prize for Physiology or Medicine for their valuable contribution to the discovery and determination of the chemical structure of vitamin K [1, 2].

Vitamin K collectively refers to a group of lipid-soluble compounds that contain a 2-methyl-1,4-naphthoquinone moiety (menadione) but differ in the structure of a lateral isoprenoid chain at the 3-position (Figure 2-1) [9]. Vitamin K1 or PK and vitamin K2 or MKs are naturally occurring K vitamers, whereas vitamin K3 or menadione is a synthetic form of vitamin K and is often regarded as a pro-vitamin [10, 11]. All differences in the properties of the various isoforms of vitamin K are attributable to the length and degree of saturation of the isoprenoid side chain [12-14]. PK is a single compound with a side chain comprising four isoprenoid residues, three of which are saturated [12]. Conversely, MKs have side chains of varying length and degree of unsaturation, and this can be represented by the general form MK-*n*, where *n* denotes the number of unsaturated isoprenoid units in a chain, which is usually between four and thirteen [12, 15, 16]. Thus, the most commonly occurring MKs have four to thirteen unsaturated isoprenoid units. Unlike PK and MKs, menadione does not contain an isoprenoid side chain.

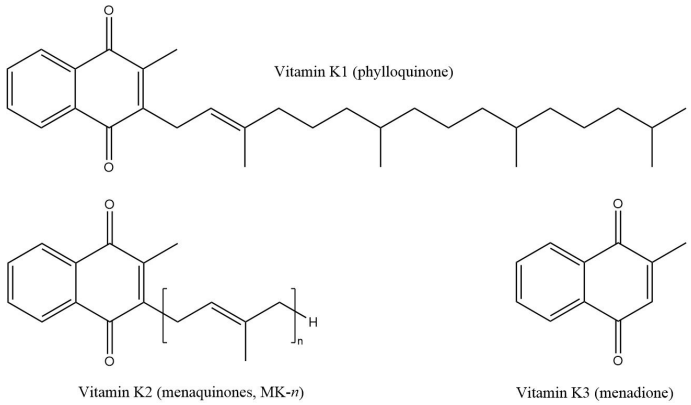


Figure 2-1 The different types of vitamin K and their chemical structure (adapted from Szterk et al. [17])

PK is present in photosynthetic plants and algae and is ubiquitous within the chloroplasts, where it serves as an electron carrier in photosystem I [18-20]. In contrast, MKs are predominantly of microbial origin and are produced by both Gram-positive and Gram-negative bacteria, such as *Bacillus subtilis* and *Escherichia coli*, to function as electron acceptors in the respiratory chain [12, 20, 21]. It has been suggested that besides microbial fermentation, menaquinone-4 (MK-4) can also be synthesised in humans and animals from the tissue-specific conversion of PK and/or menadione [2, 11, 22, 23]. Menadione is commonly incorporated in animal feed as a source of vitamin K; however, it is unsuitable for human consumption, as it generates harmful reactive oxygen species (ROS) [10, 11]. Consequently, PK and MKs constitute the major classes of vitamin K that play an essential role in human health and nutrition.

## 2.2 Health benefits of vitamin K

Vitamin K is primarily involved in blood coagulation and haemostasis. However, numerous studies have demonstrated that the possible health benefits of vitamin K extend well beyond the activation of hepatic coagulation factors. In particular, vitamin K intake has been associated with an improvement in bone and cardiovascular health. Additionally, recent investigations have established other potential functions and health benefits of vitamin K, specifically vitamin K2. These are illustrated in Figure 2-2 and include the prevention of cancer by inducing cell cycle arrest to inhibit cell proliferation; the suppression of Parkinson's disease through electron transfer to recover mitochondrial dysfunction; assisting the functional recovery of the liver; and decreasing the risk of type 2 diabetes mellitus, chronic kidney disease, immune disorders, neurological diseases, and obesity [20, 24-36].

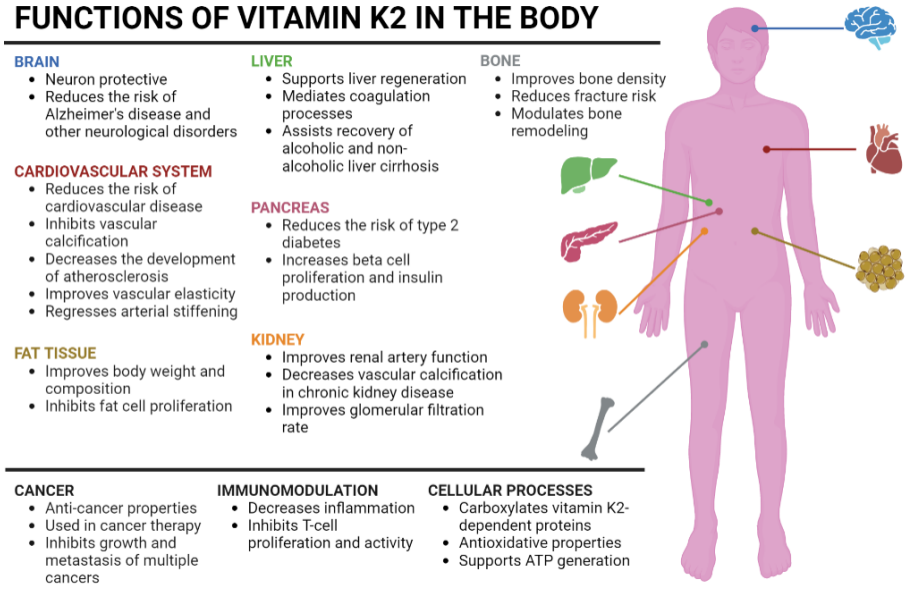


Figure 2-2 The various roles and functions of vitamin K2 in the body (adapted from Halder et al. [28])

PK and all forms of MKs play a fundamental role in the activation of both hepatic and extrahepatic VKDPs [37]. As illustrated in Figure 2-3, vitamin K acts as a cofactor for the enzyme

γ-glutamyl carboxylase (GGCX), which participates in the post-translational modification of VKDPs, catalysing the conversion of protein-bound glutamic acid (Glu) residues to γ-carboxyglutamic acid (Gla) through the addition of carbon dioxide ($CO_2$) [15, 17, 38-42]. During this process, vitamin K hydroquinone (K), the active form of the vitamin, is converted to vitamin K epoxide (KO). KO is subsequently reduced to K in a two-step reaction catalysed by the enzymes vitamin K epoxide reductase (VKOR) and quinone reductase [1]. Therefore, the γ-carboxylation process is a cyclical transformation, where the oxidised and reduced forms of vitamin K act as the driving factors for the reactions [1, 28]. The vitamin K cycle occurs in the endoplasmic reticulum (ER) and exerts its function at the cell surface or in the extracellular matrix (ECM) of specific tissues [43].

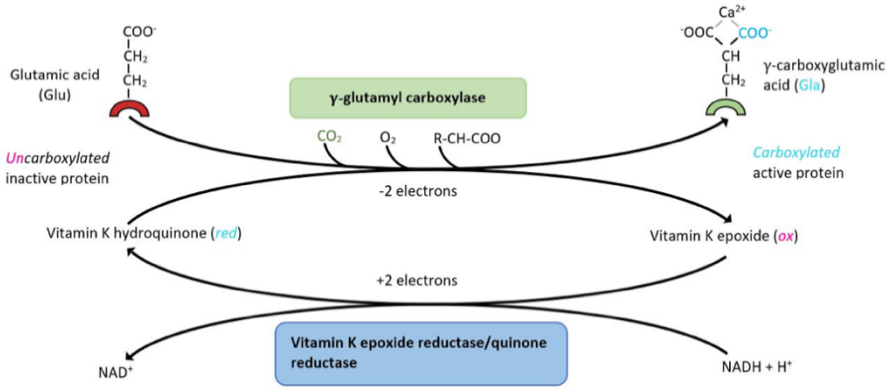


Figure 2-3 Vitamin K cycle (adapted from Gröber et al. [1])

Currently, seventeen VKDPs have been elucidated, and these can be grouped into different categories depending on the biological processes in which they are involved [12, 44-46]. The Gla protein family includes (i) seven proteins pertaining to the coagulative cascade, which are all synthesised in the liver: prothrombin, factor VII, factor IX, factor X, protein C, protein S, and protein Z; (ii) four proteins related to the regulation of bone and vascular mineralisation: osteocalcin (OC; present in bone), matrix Gla protein (MGP; mostly occurs in cartilage and vessel walls), growth arrest-specific protein 6 (GAS6), and Gla-rich protein (GRP); and (iii) two proline-rich Gla proteins (PRGPs), two transmembrane Gla proteins (TMGPs), periostin, and periostin-like factor (PLF) [12, 44, 45]. The presence of Gla residues on proteins increases their affinity for calcium (Ca) ions and facilitates Ca binding, which determines their biological activity [5, 17, 47, 48]. The Gla family of proteins participate in important metabolic processes, including the blood coagulation pathway, preventing arterial and vascular calcification, and increasing bone mineralisation, which are the prominent health benefits associated with the sufficient intake of vitamin K [15, 17, 37, 38, 49].

Structural diversities between the distinct classes of vitamin K significantly influence their metabolism. The various forms of the vitamin, especially PK and MKs, have different cofactor activities and function differently in processes such as absorption, transport, cellular uptake, tissue distribution, bioavailability, and turnover as a result of the differences in the

structure and length of their isoprenoid side chain [5, 41]. Such dissimilarities arise due to the different lipophilicities of the various side chains and the diverse food matrices in which they occur [5]. The differences in the pharmacokinetics between the many types of vitamin K also result in contrasting plasma half-life times, which impact their ability to activate VKDPs and perform extrahepatic functions [12, 50]. It has been demonstrated that with respect to extrahepatic biological functions, MKs, mainly long-chain MKs, are a more active form of vitamin K (relative to PK) owing to their longer half-life in the blood [15, 17].

Of the several K vitamers, PK, MK-4, and MK-7 are the most common in food supplements [50]. The structures of PK and MK-4 are comparable, as they both contain four isoprenoid residues (three of which are saturated in PK but contain a double bond in MK-4); hence, they display like physicochemical properties and have a similar plasma half-life of 1-2 hours [50-53]. Conversely, MK-7 is unique, as it is comparatively more hydrophobic and has greater extrahepatic availability, resulting in a significantly longer half-time of 72 hours [52-54]. Following intestinal absorption, all K vitamins are taken up in the triglyceride fraction, after which they are rapidly cleared by the liver; however, only higher MKs, including MK-7, are incorporated into low-density lipoproteins (LDL) [50]. This allows for the redistribution of long-chain MKs, such as MK-7, to extrahepatic tissues, leading to an extended plasma half-life and greater efficacy concerning extrahepatic functions [43, 50]. Furthermore, the prolonged intake of MK-7 enables its accumulation to much higher levels and results in stable serum levels, which enhances its bioavailability and effectiveness [50, 51, 53]. Therefore, MK-7, due to its greater bioavailability and longer half-life, is more effectual than the other vitamin K homologues regarding preventative and therapeutic characteristics [1, 26, 28].

MK-7 has gained widespread interest in the biotechnology field, as recent studies have demonstrated that adequate MK-7 consumption improves bone and cardiovascular health and protects against osteoporosis and CVDs [2, 5, 10, 12, 26, 39, 41, 49, 55]. Essentially, MK-7 promotes a state of homeostasis by establishing a functional equilibrium between the cardiovascular and skeletal systems [53, 56]. MK-7 facilitates the proper utilisation of Ca in the body by activating MGP and OC. This enables both MGP and OC to bind Ca ions. OC directs and binds Ca to bones, and MGP inhibits Ca deposition in the arteries and soft tissues. Thus, when active, the synergistic action of OC and MGP plays a vital role in maintaining cardiovascular and bone health.

In the context of CVDs, MGP is particularly important, as it is a potent inhibitor of soft tissue and vascular calcification [1, 5, 57, 58]. MGP is produced by vascular smooth muscle cells (VSMCs) and chondrocytes. MGP contains both Gla and serine residues and requires (vitamin K-dependent) carboxylation and phosphorylation for its activity [33, 58]. Many different forms of MGP exist, depending on their state of carboxylation and phosphorylation. These include uncarboxylated MGP (ucMGP); carboxylated, dephosphorylated MGP (dp-cMGP); phosphorylated, uncarboxylated MGP (p-ucMGP); and uncarboxylated, dephosphorylated MGP

(dp-ucMGP), which are the inactive forms of MGP, and the active form of MGP is both carboxylated and phosphorylated (p-cMGP) [58]. When active, MGP (negatively charged) has a high affinity for and binds to free Ca ions (positively charged), forming an inactive complex [33]. Active MGP also inhibits the activity of bone morphogenic proteins 2 and 4 (BMP-2 and BMP-4), supressing the transdifferentiation of VSMCs to osteoblasts in blood vessel walls [46, 56]. The overall activity of functional MGP impedes Ca deposition in the vasculature and prevents soft tissue calcification. The fully inactive form of MGP (dp-ucMGP) is considered to reflect the vitamin K status more accurately compared to the other inactive forms of MGP, and it contributes to the pathogenesis of vascular calcification [55, 58]. Several studies have investigated the effect of vitamin K consumption on active MGP levels and the incidence of vascular calcification and CVDs. The observations from the majority of these studies indicate that the intake of MKs, especially MK-7, considerably decreases the level of dp-ucMGP and reduces the occurrence of vascular calcification and CVDs [47, 49, 55, 57-61].

Various VKDPs, such as OC, MGP, protein S, and periostin, play an essential role in bone metabolism [26]. OC is produced by osteoblasts and, when carboxylated, regulates Ca deposition in bone and contributes to dilatational band formation, which affects the mechanical properties of bone [26, 46, 62]. OC is commonly associated with bone health, and the ratio of undercarboxylated OC (ucOC) to carboxylated OC (cOC) is a sensitive marker of the (bone) vitamin K status. It has been found that a greater concentration of cOC promotes bone health, while a higher level of ucOC is often linked to poor bone health, low bone mineral density (BMD), and an increased risk of fractures [1, 10, 26, 50, 63-66]. Vitamin K2 also regulates bone metabolism through mechanisms independent of OC activation [46, 67]. Specifically, vitamin K2 inhibits the cytokine-induced (receptor activator of nuclear factor-kappa B ligand (RANKL) and tumour necrosis factor-alpha (TNF-α)) activation of nuclear factor-kappa B (NF-κB) [46]. Inhibition of NF-κB stimulates the expression of alkaline phosphatase (ALP) and the transcription factors RunX and Osterix (Osc) in osteoblast precursor cells and promotes their differentiation into mineralising osteoblasts, which encourages osteoblastogenesis [46]. On the contrary, in osteoclast precursor cells, vitamin K2-induced inhibition of NF-κB reduces cell differentiation and prevents osteoclastogenesis and bone resorption [46]. Additionally, vitamin K2 is involved in the activation of the pregnane X receptor (PXR) or steroid and xenobiotic receptor (SXR) and regulates the expression of genes that encode ECM proteins (TSK and MATN2), thereby facilitating collagen synthesis and matrix formation in osteoblasts [46]. Numerous studies have investigated the effect of vitamin K on bone metabolism, fractures, and osteoporosis [26, 54, 63, 66, 68-75]. Of the different vitamin K homologues, it has been demonstrated that MK-7 has exceptional bioavailability, enabling more effective γ-carboxylation of OC and positively affecting bone metabolism [26, 50, 69]. Many studies have also concluded that vitamin K supplementation, including MK-7, has little influence on enhancing BMD. However, it

significantly improves bone strength and quality, decreasing bone loss and reducing the incidence of fractures and osteoporosis.

Moreover, it has been proposed that reduced vitamin K status is a potentially modifiable risk factor for severe COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [76, 77]. In particular, MK-7 deficiency may be associated with manifestations of COVID-19 and comorbidities related to the acute form of the disease [78, 79]. Respiratory failure and thromboembolism are frequently observed in critically affected patients. Vitamin K depletion exacerbates elastic fibre degradation (due to calcification, which promotes lung fibrosis) and thrombosis in acute COVID-19 as a result of the impaired activation of MGP and endothelial (anticoagulant) protein S, respectively [76]. It has also been proposed that the mechanisms leading to these conditions are linked to inflammatory responses rather than the specific properties of the virus, and vitamin K, predominantly MK-7, owing to its anti-inflammatory properties, which are independent of its role as an enzyme cofactor, is likely to improve the health outcomes of COVID-19 [77, 78, 80, 81]. Individuals who develop serious infections often have comorbid conditions, such as diabetes, hypertension, and CVDs, which are also attributable to a compromised level of vitamin K (MK-7) [76-78, 82]. The study conducted by Mangge et al. [83] assessed the serum vitamin K (PK, MK-4, and MK-7) and KO levels in patients hospitalised with COVID-19-related pneumonia, non-COVID-19 pneumonia patients, and healthy individuals with a typical western lifestyle, and it was determined that COVID-19 patients had significantly lower levels of MK-7 in comparison to patients with pneumonia and healthy controls. The findings of this investigation were largely supported by studies carried out by Dofferhoff et al. [76], Anastasi et al. [80], Desai et al. [84], and Linneberg et al. [85], which indicate that a low extrahepatic vitamin K status (usually denoted by elevated dp-ucMGP levels) may be connected to greater disease severity and poorer health outcomes associated with COVID-19. It has been suggested that high circulating levels of dp-ucMGP in COVID-19 patients may arise due to premorbid vitamin K insufficiency in combination with enhanced utilisation during infection [77]. Additionally, it is anticipated that during SARS-CoV-2 infection, there is a greater demand for active MGP as a result of profound proteolytic activity in the lungs. This accelerates elastic fibre degradation and increases their vulnerability to Ca ions, leading to an up-regulation of MGP synthesis and the depletion of extrahepatic vitamin K stores [77]. Thus, vitamin K, especially MK-7, supplementation will likely benefit COVID-19 patients and reduce the morbidity and mortality associated with the disease. However, clinical intervention trials are needed to establish whether vitamin K, specifically MK-7, administration plays a crucial role in the treatment and prevention of severe COVID-19 [77, 78]. Legacy et al. [86] have established a study protocol to conduct a randomised, double-blind, placebo-controlled clinical trial to evaluate the effect of various dietary supplements, including vitamin K2 (MK-7), on reducing the severity and duration of symptoms in individuals with COVID-19. However, the study is still in its early stages, and suitable participants are being recruited; hence, more insightful conclusions can be inferred upon completion. Furthermore, a

randomised, controlled clinical trial was carried out in the Netherlands from February 2021 to March 2022 to investigate the safety and effects of vitamin K2 (MK-7) supplementation in COVID-19 patients requiring hospitalisation [87]. In this study, patients were either administered a placebo or a dietary supplement containing MK-7 for 14 days or until discharge (whichever occurred first), and the outcomes with regard to safety, pulmonary damage, and coagulation were assessed. Although this study has been completed, the results are yet to be published.

Essentially, MK-7 has excellent health benefits and is more therapeutically significant compared to the other K vitamers for the treatment and prevention of various age-related and other health conditions and globally relevant diseases. Therefore, widespread MK-7 consumption is likely to be instrumental in managing pressing health issues worldwide and alleviating the socioeconomic consequences of an ageing population.

## 2.3 Dietary sources and intake of vitamin K

Vitamin K can be obtained in the diet from a variety of food sources, and the vitamin K content of commonly consumed food items is listed in Table 2-1.

PK is the main source of dietary vitamin K, accounting for 75-90% of all vitamin K consumption, and is synthesised by plants, green algae, and certain species of cyanobacteria [39, 88-90]. The major dietary sources of PK are green vegetables, such as broccoli, iceberg lettuce, and spinach, and vegetable oils, including soybean, canola, cottonseed, and olive oils [12, 17, 88, 89]. Margarines, spreads, salad dressings, and other products derived from plant oils also contain vitamin K1 [88, 91].

MKs are primarily of microbial origin. Long-chain MKs, such as menaquinone-6 (MK-6), MK-7, menaquinone-8 (MK-8), menaquinone-10 (MK-10), and menaquinone-11 (MK-11), can also be produced by the gut microbiota. Although, it is anticipated that their absorption is restricted, as they are tightly bound to bacterial membranes and require bile salts for their absorption and solubilisation; thus, they do not contribute substantially to daily vitamin K requirements [2, 5, 26, 92]. Hence, food products are the dominant source of vitamin K2. However, compared to vitamin K1, vitamin K2 is present at lower concentrations in fewer dietary sources [12]. MKs, particularly long-chain MKs, largely occur in fermented foods, including cheese, curd, sauerkraut, cheonggukjang, and natto, and the type of microorganism employed in the fermentation process influences the MK composition of the resulting food product [12, 15, 17, 69, 88, 93-97]. Additionally, dairy products, such as milk, yoghurt, and cream, contain appreciable quantities of MKs, and the total amount of vitamin K is often proportional to the fat content [98]. The form, range, and quantity of MKs present in dairy goods are usually diverse and are most likely determined by the microbial species used as the starter culture, as different bacterial strains produce a distinct spectrum of MK compounds through fermentation [97-99]. Certain animal products, such as meat and eggs, are also a source of MK-4, synthesised from the menadione included in animal feed [12, 69, 88, 93, 97].

Table 2-1 The vitamin K composition of assorted foods

| | Vitamin K Content (ng/g) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Food Source** | **PK** | **MK-4** | **MK-5** | **MK-6** | **MK-7** | **MK-8** | **MK-9** | **MK-10** | **Reference(s)** |
| **Vegetables** | | | | | | | | | |
| Wakame (seaweed) | 12930 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| Kale | 6180-8170 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| Parsley | 3600-5480 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| Spinach | 2700-5750 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| Laver (seaweed) | 4130 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| Carrot (raw) | 55-132 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | [97] |
| Iceberg lettuce (raw) | 241 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | [97] |
| Cabbage | 1270-3390 | 0-10 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| Broccoli (boiled) | 1100-3070 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| Brussels sprouts | 1220-2890 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| **Fruits** | | | | | | | | | |
| Apples | 27-34 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [91] |
| Bananas | 2-4 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [91] |
| Kiwi fruit | 343 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| Blueberries | 193 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | [97] |
| Blackberries | 198 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | [97] |
| Black currant | 300 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Avocado | 10-200 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| Grapes (green) | 83-190 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| **Breads** | | | | | | | | | |
| Rye | 5-9 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [91] |
| Wheat | 10-12 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [91] |
| Sourdough | 9-11 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [91] |
| **Beverages** | | | | | | | | | |
| Tea | 2-4 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [91] |
| Coffee | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [91] |
| Orange juice | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [91] |
| **Plant Oils and Fats** | | | | | | | | | |
| Olive oil | 300-800 | 0-4 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| Sunflower oil | 55-59 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [91] |
| Soybean oil | 1310-2340 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| Vegetable oil (mixed) | 1340-1640 | n.d. | n.d. | n.d. | 10 | n.d. | n.d. | n.d. | [67] |
| Rapeseed oil (refined) | 920-1500 | n.d. | n.d. | n.d. | 0-30 | n.d. | n.d. | n.d. | [67] |
| Corn oil | 27-31 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [91] |
| Margarine | 120-1100 | 0-3 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67, 91] |
| Butter | 132-159 | 135-159 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [91] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Fermented Foods** | | | | | | | | | |
| Natto | 200-450 | 0-10 | 72 | 124 | 9000-12300 | 824 | 0 | 0 | [67, 100] |
| Sauerkraut | 224 | 4.3 | 8.6 | 15.9 | 2.3 | 8.9 | 15 | 0 | [100] |
| **Cheeses** | | | | | | | | | |
| Brie | 49.2 | 125 | 0 | 0 | 0 | 0 | 0 | 0 | [97, 100] |
| Boursin | 45.5 | 89.3 | 0 | 1.1 | 3.3 | 8.2 | 9.1 | 0 | [97, 100] |
| Camembert | 25 | 79.5 | 13.4 | 10.1 | 32.4 | 151 | 395 | 0 | [97, 100] |
| Roquefort | 65.6 | 131 | 6.4 | 4.8 | 11.6 | 50.9 | 176 | 0 | [97, 100] |
| Munster | 20.6 | 102 | 4.5 | 4.6 | 83.7 | 412 | 194 | 0 | [97, 100] |
| Cheddar | 21.6 | 51.2 | 0 | 2.8-33.5 | 0-23.4 | 8.3-62.6 | 0-254.8 | 0-67.5 | [97, 100] |
| Stilton | 36.2 | 100 | 9.4 | 6 | 14 | 66.3 | 298 | 0 | [97, 100] |
| Feta | 13.5 | 1 | 0 | 3.5 | 11.8 | 23.3 | 76.9 | 0 | [97, 100] |
| Mozzarella | 15 | 53.1 | 1.6 | 0 | 0 | 0 | 7.5 | 0 | [97, 100] |
| Parmesan | 20.6 | 0 | 0 | 0.5 | 1 | 1.5 | 0 | 0 | [97, 100] |
| Gorgonzola | 17.3 | 111 | 0 | 1.7 | 30.7 | 2.4 | 2.5 | 5.1 | [97, 100] |
| Pecorino | 55.6 | 93.7 | 0 | 0 | 0 | 0 | 0 | 0 | [97, 100] |
| Emmental | 24.1-52 | 34-89.5 | 21.5 | 0-19 | 0 | 0 | 0 | 0-322 | [97, 100] |
| Gruyere | 25-58 | 51.5-98 | 13.8 | 0 | 0 | 0 | 0 | 0 | [97, 100] |
| Raclette | 15.5-26 | 30-86 | 0-4 | 0-219 | 0-826 | 18-100 | 50-290 | 0 | [97, 100] |
| Edam | 17.8-37.6 | 30.5-113 | 0-11.2 | 0-6.1 | 0-13.4 | 74.6-112.5 | 274-459 | 0-9.9 | [97, 100] |
| Gamalost | 1.8 | 10.3 | 6.2 | 2.9 | 9.7 | 51.2 | 440 | 22 | [97, 100] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Norvegia | 43.7 | 51 | 0 | 3 | 13.3 | 52.5 | 295 | 0 | [97, 100] |
| Cottage cheese (4% fat) | 2-4 | 2-4 | 3-7 | 4-6 | 4-8 | 18-32 | 66-94 | 2-6 | [97, 98] |
| Cottage cheese (reduced-fat) | n.d. | n.d. | n.d. | n.d. | n.d. | 4-12 | 17-29 | 1-5 | [97, 98] |
| **Other Dairy Products** | | | | | | | | | |
| Milk (4% fat) | 3 | 8 | n.d. | n.d. | n.d. | n.d. | 160 | 20 | [98] |
| Milk (2% fat) | n.d. | 5 | n.d. | n.d. | n.d. | n.d. | 50 | 6 | [98] |
| Regular yoghurt (full-fat) | 3-5 | 4-10 | n.d. | n.d. | n.d. | n.d. | 84-180 | 10-22 | [98] |
| Regular yoghurt (fat-free) | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [98] |
| Greek yoghurt (full-fat) | 2-4 | 7-9 | n.d. | n.d. | n.d. | n.d. | 126-170 | 12-24 | [98] |
| Greek yoghurt (fat-free) | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [98] |
| Cream (heavy) | 23-25 | 85-101 | n.d. | n.d. | n.d. | n.d. | 4118-4722 | 743-961 | [98] |
| Cream (light) | 12 | 53 | n.d. | n.d. | n.d. | n.d. | 1030 | 130 | [98] |
| Half-and-half | 7-9 | 19-27 | n.d. | n.d. | n.d. | n.d. | 231-577 | 20-70 | [98] |
| **Animal Products** | | | | | | | | | |
| Whole egg | 3-25 | 56-250 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Minced meat | 10.9 | 76.1 | 0 | 0 | 0 | 0 | 0 | 0 | [100] |
| Pork cutlet | 0 | 10.5 | 0 | 1.1 | 0 | 0 | 0 | 0 | [100] |
| Beef meat | 0.2 | 13.9 | 0 | 0 | 1.3 | 3.7 | 0 | 0 | [100] |
| Beef liver | 18-58 | 7-8 | 0 | 11.2 | 49.9 | 16 | 14.6 | 18.3 | [67, 100] |
| Pork meat | 0 | 13.6 | 0 | 0 | 0 | 0 | 0 | 0 | [100] |
| Pork liver | 0 | 2.8 | 0 | 10.5 | 5.1 | 0 | 0 | 0 | [100] |
| Chicken meat | 0-20 | 89-600 | 0 | 0 | 0 | 0 | 0 | 0 | [67, 100] |
| Chicken liver | 0-25 | 40-141 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | [67] |
| Deer back | 24.3 | 8.8 | 0 | 0 | 0 | 0 | 0 | 0 | [100] |
| **Seafood** | | | | | | | | | |
| Mackerel | 5.1 | 6.2 | 0 | 0 | 0 | 0 | 0 | 0 | [100] |
| Eel | 13 | 631 | 0 | 0 | 0 | 0 | 0 | 0 | [100] |
| Plaice | 0 | 3.8 | 0 | 0 | 0 | 49 | 0 | 0 | [100] |
| Prawn | 0 | 1.9 | 0 | 0 | 0 | 0 | 0 | 0 | [100] |
| Salmon | 1.3 | 5.7 | 0 | 0 | 0 | 0 | 0 | 0 | [100] |
| Herring | 1.1 | 0.7 | 0 | 0 | 0 | 0 | 0 | 0 | [100] |

Note: n.d. = not detected; n.a. = not available

Of the several forms of vitamin K2, MK-7 has exceptional biological activity and is the most therapeutically significant; therefore, sufficient intake of MK-7 is important from a dietary perspective. However, MK-7 is present in limited food sources, and its concentration is very low. The digestion and utilisation of MK-7 from foods also become less efficient with ageing [94, 101]. The richest source of MK-7 is natto, a Japanese fermented soybean, with approximately 800-1000 µg of MK-7 per 100 g of natto [94, 101, 102]. Nevertheless, many consumers find natto unpalatable due to its strong taste and intense aroma [94]. The daily recommended vitamin K intake differs between countries, and the exact value varies based on age, gender, and other factors. Moreover, there are no specific guidelines available for MK-7. In the United States of America, the recommended dietary intake (RDI) of vitamin K is 90 µg for (adult) females and 120 µg for (adult) males [103], whereas in the United Kingdom and other European countries, an adequate intake (AI) of 1 µg/kg of body weight is suggested [104, 105]. In Australia and New Zealand, the recommended dietary allowance (RDA) for vitamin K is 60 µg for (adult) females and 70 µg for (adult) males [106]. As a result, meeting the dietary intake requirements would entail the consumption of impractically large quantities of MK-7-rich foods; consequently, obtaining adequate levels of MK-7 from regular food products is not feasible [94, 107].

Thus, the low availability of sufficient concentrations of MK-7 in the diet necessitates the development of dietary supplements and fortified or functional food products to complement the amount of MK-7 acquired from natural sources.

## 2.4 MK-7-enriched fortified or functional food products and dietary supplements

Of the different K vitamers, MK-7 offers the most health benefits and has the greatest remedial value. Nonetheless, it can only be obtained in the diet at relatively low concentrations from few food sources. This creates a lucrative market for MK-7-enriched fortified or functional food products and dietary supplements to accompany natural food sources and satisfy the recommended daily intake requirements.

Functional foods encompass whole, fortified, enriched, or enhanced products that provide additional health benefits beyond their nutritional value [108, 109]. The primary advantage of functional food products is that they enable essential nutrients to be delivered to large portions of the population without the need for drastic changes in food consumption patterns [109]. Assorted food matrices can be used to develop functional food products, the type of which is dependent on their suitability for the target population, the production process, and the desired health outcomes. Items such as cereals and cereal-based products, milk and dairy products, fats and oils, tea and other beverages, and salt, sugar, soy sauce, and other condiments are common food matrices that can be employed for the manufacture of functional foods [109]. Ideally, for it to be effective in achieving the intended health benefits, a functional food item should be widely available and easily incorporated into an individual's dietary routine.

The development of a palatable MK-7-rich functional food product, which can either be consumed directly or fortified into other food matrices, from the fermentation of food substrates is likely to be beneficial in reducing the incidence of CVDs, osteoporosis, and other diseases of global relevance [110, 111]. Various studies have explored the production of fermented MK-7-enriched food products and have considered screening different food substrates and optimising the fermentation media and operating conditions to enhance MK-7 production for this application [110-113].

The fermentation-based synthesis of MK-7 has gained substantial interest over the last few years. Despite significant advancements in this area, the low fermentation yield and high production cost, due to the numerous downstream processing steps, result in an expensive final product with a market price of approximately USD 5 million/kg [110-112]. Consequently, it is not readily available to consumers at an affordable price. Therefore, fermented MK-7-enriched functional food products are a promising alternative. The primary advantage of which is fewer downstream processing steps and lower production costs, as the fermentation medium can be used directly as a food ingredient after appropriate pre-treatment rather than producing pure MK-7 as the final product [110, 111].

Southee et al. [111] and Novin et al. [112] studied the development of a functional dairy product rich in MK-7 using milk as the fermentation substrate. The rich nutritional profile and high Ca content of milk make it a suitable medium for producing a functional food product to address osteoporosis, CVDs, and other health conditions associated with MK-7 deficiency. The synergistic effect of Ca and MK-7 is also reported to benefit bone and cardiovascular health. Southee et al. [111] assessed the feasibility of milk as a fermentation medium and the impact of nutrients on the MK-7 yield. It was found that milk, especially standard milk, was a promising medium for MK-7 production using *B. subtilis natto*. A maximum MK-7 concentration of 11.22 mg/L was achieved for a medium containing standard milk and 1% (*w/v*) glycerol after 48 hours of fermentation with *B. subtilis natto* at 40 °C, 200 rpm, and 1 vvm. On the other hand, Novin et al. [112] examined the effect of agitation and aeration on MK-7 biosynthesis by *B. subtilis natto* using milk as the fermentation media, and it was determined that the optimum agitation and aeration rate were 525 rpm and 5 vvm, respectively, which enabled a MK-7 yield of 3.54 mg/L. A sensory evaluation of the MK-7-enriched milk product was also conducted, and it was found that the fermented milk product was very salty and had a cheesy aroma relative to non-fermented milk. Hence, it was not as well accepted by the panellists.

In contrast, Singh et al. [113] considered the development of a MK-7-enriched soy nutraceutical through the fermentation of *B. subtilis* using soybeans. Various nutrients were explored to optimise the fermentation media and produce a large quantity of MK-7 within a short period. Nine nutrient sources were evaluated, and a MK-7 concentration of 3.039 µg/g was attained after 24 hours of fermentation employing the optimised medium, which consisted of 20

g of soybeans, 40 mL/kg of glycerol, 60 g/kg of mannitol, 4 g/kg of yeast extract, 8 g/kg of malt extract, and 4 g/kg of $CaCl_2$.

Ma et al. [110] also investigated the potential to utilise food ingredients for the production of MK-7-enriched functional foods. As part of this process, they screened a range of food products for their suitability for MK-7 biosynthesis and determined that a combination of soy protein and glycerol was ideal for MK-7 production. The developed process was also scaled-up, and a dual-feeding strategy was implemented to overcome foaming and achieve a high MK-7 yield. This enabled a MK-7 titre of 99 mg/L and a productivity of 2.1 mg/L/h, among the highest values reported in the open literature. The resulting product was then formulated into soymilk to examine the stability of MK-7 in food formulation, and it was established that approximately 75% of the MK-7 remained after 24 weeks of storage at room temperature. Despite the significant advancements in this area, further development is likely required before a suitable, palatable, and organoleptically appealing fermented MK-7-enriched functional food product is available on the market.

Several studies have also demonstrated the ability to fortify a range of food matrices with vitamin K, including milk, yoghurt, yoghurt-based drinks, eggs, and olive oil, to produce a fortified functional food product to enhance the vitamin K intake of individuals and achieve better health outcomes [54, 114-119]. Brugè et al. [54], Kanellakis et al. [114], Knapen et al. [115], Knapen et al. [116], and Kruger et al. [117] evaluated the effect of different dosages and forms of vitamin K, mainly PK and MK-7, on the cardiovascular and bone health of adults of different age groups. These studies also considered the inclusion of other vitamins (B, C, D, and E), minerals (Ca and magnesium (Mg)), and nutrients (*n*-3 polyunsaturated fatty acids (PUFAs) and coenzyme $Q_{10}$ ($CoQ_{10}$)), along with vitamin K, to provide further health benefits. Various biochemical parameters were analysed to determine the effectiveness of the fortified products and their capacity to produce the intended health outcomes by improving the nutritional status of vitamin K. This involved measuring the typical markers of vitamin K status, specifically the circulating levels of ucOC and dp-ucMGP, and directly evaluating the plasma PK or MK-7 concentration. All studies demonstrated the successful absorption of the fortified vitamin K compound and established the potential of fortified food products to effectively increase the vitamin K status of individuals (as determined by a reduction in ucOC and/or dp-ucMGP levels) to achieve the desired health gains. Additionally, Cirilli et al. [119] studied the bioavailability of MK-7 in a low-fat milk formulation and compared two different fortification strategies, namely enrichment by directly dissolving a MK-7 powder or with a pre-prepared oil-water MK-7 dispersion. MK-7 bioavailability was evaluated by quantifying plasma MK-7 levels over a specified timeframe, and it was determined that the fortification method strongly influences the bioavailability of MK-7. Fortification with an oil-water MK-7 emulsion enabled greater homogeneity, stability, and bioavailability of the enriched milk product, and it is a promising approach to increase the solubility of lipophilic MK-7 in reduced-fat dairy products.

Conversely, O'Sullivan et al. [118] considered the biofortification of chicken eggs to enhance their vitamin K content by increasing the quantity of vitamin K3 in hen feed. High-quality biofortified chicken eggs, which had at least twice the vitamin K content of commercially available eggs, were produced in this study. Unlike the previously mentioned investigations, this study did not involve human participants and did not assess the ability of the biofortified eggs to increase the vitamin K status of individuals to enable greater health benefits. Instead, the primary focus was to improve the vitamin K content of a regularly consumed food item (eggs) to increase the dietary vitamin K intake of the general population. This study successfully illustrated an alternative method to boost the vitamin K content of foods, especially non-dairy items, without directly adding vitamin K to the desired food product.

Although many studies have demonstrated the successful fortification of a range of food matrices to produce functional food products rich in vitamin K (including MK-7) to address various health needs, the availability of such products is scarce. Therefore, since vitamin K-enriched functional foods are currently not readily accessible to consumers, they are unlikely to improve the vitamin K status of large populations presently and in the immediate future.

Alternatively, assorted vitamin K2 dietary supplements are available on the market, and the primary manufacturers and their product details are outlined in Table 2-2. Due to the prominent health benefits associated with MK-7, the majority of commercial vitamin K2 supplements exclusively contain or comprise a substantial proportion of MK-7. Currently, this is the most common approach to compensate for the low availability of MK-7 in natural food sources. The vitamin K2 in such dietary supplements can be obtained from fermentation or chemical methods. Gnosis, NattoPharma, Viridis Biopharma, and Sungen Bioscience are the notable producers of naturally derived vitamin K2 nutritional supplements synthesised from microbial fermentation [20]. In contrast, Kappa Bioscience is the leading manufacturer of synthetic vitamin K2 supplements obtained from chemical reactions [20].

However, such dietary supplements are often expensive as a result of the numerous complex steps involved in the manufacturing process. Thus, compared to MK-7-enriched functional food products, they are likely not as widely accessible. It has also been observed that there is significant diversity in the MK-7 content of dietary supplements, and in some cases, the actual MK-7 concentration is lower than that declared on the label [15, 17]. Furthermore, different excipients in MK-7 supplements influence the purity profile and stability of the finished product, particularly during storage [120]. Certain excipient compounds, such as L-arginine (L-Arg) and magnesium oxide (MgO), encourage the deterioration of MK-7 [120]. This degradative effect is more profound with MgO and is most likely associated with alkalinisation, which promotes the instability and degradation of MKs [120]. Such occurrences may explain the low MK-7 content (below the declared concentration) in certain supplements. Moreover, it is also possible for dietary supplements to contain additional impurities and other compounds that are produced during the

technological processes used to manufacture capsules and hard tablets, which is undesirable [15, 17].

Table 2-2 Different producers of vitamin K2 dietary supplements and their product details (adapted from Ren et al. [20])

| Manufacturer | Location | Product Trademark | Synthesis Technique |
|---|---|---|---|
| Kappa Bioscience | Norway | K2VITAL | Chemical |
| NattoPharma | Norway | MenaQ7 | Fermentation |
| Gnosis | Italy | VitaMK7 | Fermentation |
| DSM | Netherlands | Quali-K | Chemical |
| Viridis Biopharma | India | MenaquinGold | Fermentation |
| Frutarom | Israel | UniK2 | Fermentation |
| DuPont Nutrition & Health | USA | ActivK | Chemical |
| GeneFerm Biotechnology | Taiwan | NattoMena | Fermentation |
| Sungen Bioscience Co. Ltd | China | ESSEK2 | Fermentation |
| Eisai Co. Ltd | Japan | MK-4 | Chemical |
| Gusheng | China | MK-4 | Chemical |

Essentially, both MK-7 dietary supplements and fortified or functional food products are possible approaches to complement natural sources and satisfy dietary intake requirements. It must be noted that although MK-7 dietary supplements are presently more accessible, there are some prominent drawbacks associated with this form of MK-7 supplementation. Conversely, MK-7-enriched fortified or functional food products are still being developed and are not readily available on the market. However, a nutritional food supplement rich in MK-7 has the additional advantage that it can be made available to a wide range of consumers at a relatively low cost, which is likely to reduce the occurrence of globally significant diseases and health conditions that can be alleviated through the sufficient intake of MK-7.

## 2.5 The current knowledge and understanding of MK-7 isomers

In light of the numerous health benefits of MK-7, the development of nutritional supplements and functional food products to accompany natural food sources and increase the dietary intake of MK-7 has become progressively widespread. However, many people have not yet realised that MK-7 can exist as geometric isomers [13, 15, 17, 121-123]. The all-*trans* isomer is the natural form of the vitamin, whereas the *cis* isomers of MK-7 are not naturally occurring compounds and are produced from the isomerisation of the all-*trans* isomer under various

conditions. It has been recognised that the chemical structure of MK-7 influences its ability to interact with subcellular structures and, therefore, determines its biological activity [13, 122, 123]. Only the all-*trans* isomer of MK-7 is biologically significant [124, 125], which is an important consideration for the development of MK-7-enriched functional foods and dietary supplements to improve human health.

The molecular structure of MK-7 comprises seven double bonds, and in the all-*trans* isomer, all seven double bonds have the *trans* configuration. Individual double bonds in the isoprenoid units of MK-7 can adopt the *cis* conformation. Depending on the number and location of *cis* bonds (in addition to the *trans* bonds) and their various combinations in the side chain of MK-7, numerous *cis/trans* isomers (containing double bonds in both the *cis* and *trans* arrangements) can potentially exist. It may also be possible for all seven double bonds to adopt the *cis* configuration, giving rise to the all-*cis* isomer. However, the stability of the different structures and shapes that result from the various organisations of double bonds is likely to differ between isomers, as some forms of *cis* MK-7 may be less energetically favourable and are, consequently, less likely to occur than the other more stable conformations.

The number and type of *cis* isomers that can be achieved are ambiguous, as very few studies have considered this aspect, and those that have often present conflicting information. Marles et al. [48] suggest that the natural all-*E* form of MK-7 can isomerise to the mono-*cis* form, which has the *Z* (*cis*) configuration at the 2' position (2-*Z*) instead of the 2-*E* arrangement (with regard to the first double bond in the molecular structure and the 3' methyl group of the side chain), as a result of exposure to certain conditions. This implies that only a single *cis* isomer is attainable. On the other hand, the studies conducted by Szterk et al. [17], Szterk et al. [15], Sitkowski et al. [13], Orlando et al. [120], and Bus et al. [126] have established the existence of more than one *cis* MK-7 isomer in dietary supplements and preparations of different origins. Szterk et al. [17], Szterk et al. [15], and Sitkowski et al. [13] have identified five *cis/trans* MK-7 isomers, besides all-*trans* MK-7, and Orlando et al. [120] have observed various unidentified peaks (speculated to correspond to the *cis/trans* isomers of MK-7) in different preparations. More recently, Bus et al. [126] have isolated and determined the chemical structure of thirteen new MK-7 isomers and confirmed the identity of three previously known MK-7 compounds, specifically all-*trans* MK-7 ((*E6, ω* )-MK-7) and two *cis* isomers, one of which is produced from photoisomerisation ((*Z,E5,ω*)-MK-7) and the other ((*E,Z3,E2,ω*)-MK-7) has been previously characterised [13], from a synthetic mixture of MK-7. It must be acknowledged that most studies postulating the existence of multiple *cis* forms of MK-7 have explored dietary supplements or similar formulations, and thus far, investigations considering fermented samples have not been carried out.

MK-7 is traditionally produced from bacterial fermentation; however, of late, synthetic methods for MK-7 preparation have been introduced. Although the all-*trans* form of MK-7 is likely to be the most viable, the exact ratio of the different MK-7 isomers obtained from natural

and chemical production methods has not been elucidated, and the isomeric forms of MK-7 attained from the different synthesis techniques are also unclear. In particular, there is insufficient material in the literature outlining the MK-7 profile achieved from fermentation, as different fermentation conditions may produce variable proportions of isomers [13, 15, 17].

The ideal method for synthesising all-*trans* MK-7 is also debatable, as different producers have different opinions. Kappa Bioscience, the leading manufacturer of synthetic MK-7, argues that natural fermentation-based synthesis results in the production of a mixture of *cis* and *trans* isomers, whereas synthetic methods favour the production of the all-*trans* form of the vitamin [122]. In contrast, NattoPharma, one of the prominent producers of naturally derived MK-7 preparations, claims that their natural MK-7 products have high purity and contain the all-*trans* isomer [127].

It is suggested that the production of *cis/trans* isomers during fermentation-based synthesis occurs during the purification processes implemented to isolate the target vitamin product from other impurities [122, 123]. However, this aspect has not been widely investigated, and there is no clear evidence to support this claim. This may be a potential reason certain producers prefer synthetic production methods, and it may also be cheaper than natural synthesis techniques. Nevertheless, it must be noted that most consumers prefer naturally derived products, as they are deemed to confer a greater health benefit and are regarded as a more natural and organic alternative to synthetic preparations. Naturally derived MK-7 is also produced from fermentation with generally recognised as safe (GRAS) bacterial strains and is considered non-toxic. On the contrary, chemically synthesised MK-7 products could contain traces of harmful solvents, which are an environmental hazard and may pose a health risk and raise product safety concerns, especially for food supplements. As a result, consumers are often prepared to pay more for natural products, which hence have a greater demand relative to synthetic formulations.

Further investigation in this area is necessary to develop an understanding of the profile of *cis* and *trans* MK-7 isomers in various fermented products, together with the important production processes and purification techniques that may influence the proportion of isomers attained. Additionally, knowledge of the key factors that impact the isomer composition may enable their manipulation to obtain a superior MK-7 profile in the final fermented product.

## 2.6 Biological activity of MK-7 isomers

Like most biological molecules, MK-7 can occur as geometric isomers, existing in the all-*trans* and various *cis* forms [13, 15, 17, 122, 123]. Differences in the isomeric structures of MK-7 relate to the arrangement of double bonds in the isoprenoid units of the side chain [13, 128]. All double bonds in naturally occurring MKs are in the *trans* configuration; however, some commercial preparations of the vitamin contain a mixture of compounds with both *cis* and *trans* configurations of individual double bonds in isoprenoid units [13, 128].

The organisation of double bonds in MK-7 molecules determines their shape and biological activity [13, 122, 123, 125]. The all-*trans* form of MK-7 consists of an extended system of isoprenoid units with double bonds in the *trans* configuration, resulting in a linear organisation (Figure 2-4) [121-123]. In contrast, the *cis* isomers of MK-7 have a non-linear shape (Figure 2-4) due to the presence of one or more double bonds in the *cis* arrangement, which creates a bend in the molecular structure [121-123]. This impairs the interaction of *cis* MK-7 isomers with subcellular structures, such as vitamin K2-dependent enzymes and proteins, reducing their ability to perform their biological function [13, 125].

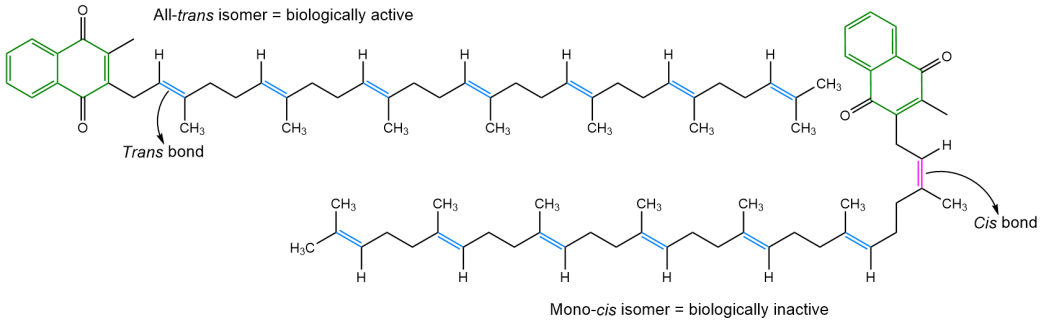


Figure 2-4 The chemical structure of *cis* and *trans* MK-7 isomers (adapted from Lal and Berenjian [129])

Therefore, the different geometric shapes of MK-7 influence its biological activity, and it has been demonstrated that the *cis* forms of vitamin K merely exhibit 1% of the biological activity of the all-*trans* isomer [124, 125]. Furthermore, a recent comparison of the carboxylative efficacy and biological function of MK-7 isomers has revealed that while not completely ineffective, *cis* MK-7 has substantially reduced carboxylative capacity and compromised bioactivity relative to the all-*trans* isomer [90]. Consequently, consideration of the isomeric composition of MK-7 preparations obtained through both natural and chemical methods is crucial in the development of MK-7 dietary supplements and functional foods, as only the all-*trans* isomer is significant from a biological perspective.

## 2.7 Safety and toxicity of MK-7 isomers

It is essential to account for the possibility for the various isomers of MK-7 to have different cytotoxic or toxic characteristics [13, 17]. The cytotoxic or toxic properties of the *cis* forms of MK-7 have not been broadly researched, and little information is available [13, 17].

It has been established that no safety concerns are linked to the consumption of MKs and that moderate doses of MKs in nutritional supplements do not pose a health risk [12, 14, 39, 43, 48, 93, 130]. The study carried out by Pucaj et al. [14] (using rats) demonstrated that there is no toxicity associated with the oral administration of synthetic MK-7 in a single dose of up to 2000 mg/kg or over 90 days at a dose of 10 mg/kg per day [12]. Ravishankar et al. [130] also conducted an extensive safety assessment on the suitability of MK-7 for human nutrition. The study examined the acute and subacute toxicity of MK-7 and its potential for genotoxicity and

mutagenicity through the analysis of various biochemical, haematological, urine, and histopathological parameters. The results of this report largely supported the observations made by Pucaj et al. [14] and determined that the oral administration of 2000 mg/kg of MK-7 does not induce toxicity in both male and female rats. The subacute toxicity analysis also revealed the safety and compatibility of prolonged MK-7 treatment over 90 days [130]. In addition, the study demonstrated that MK-7 does not cause cellular damage or possess any genotoxic or mutagenic qualities [130]. Overall, the investigations carried out by Pucaj et al. [14] and Ravishankar et al. [130] indicate the suitability of MK-7 for food-related applications and the production of dietary supplements.

Given the lack of toxicity associated with MK-7 compounds, the probability for the *cis* isomers of MK-7 to demonstrate cytotoxic or toxic properties or have any significant adverse effects on human health is likely low. Nevertheless, their presence in various food and other supplementary sources is an impurity, as they sustain no biological activity.

## 2.8 Methods of MK-7 synthesis and the production of *cis* and *trans* isomers

As a nutraceutical and dietary supplement, MK-7 can be produced naturally by fermentation or synthetically from chemical processes. The MK-7 profile achieved depends on several aspects, primarily the processes used for its synthesis and the purification of the crude reaction mixture [13, 15, 17, 122]. Particular environmental and storage conditions can also result in the geometric isomerisation of the isoprenoid units in the chemical structure of MK-7, which also impacts the isomer composition of the final product [13, 15, 17, 128].

### 2.8.1 Microbial fermentation

MK-7 can be synthesised from fermentation using both wild-type and engineered microorganisms, including members of the *Bacillus* species, lactic acid bacteria, and various other bacterial strains, such as *Flavobacterium* and *E. coli* [39, 94, 96, 113, 131-136]. Although many types of bacteria can synthesise MK-7, safety concerns often limit their suitability for producing microbial-derived MK-7 as a food supplement. In order to be acceptable for the manufacture of fermented MK-7 products intended for human consumption, microbial production hosts must be safe and receive GRAS accreditation [96]. Accordingly, *B. subtilis natto*, which has been awarded the GRAS status and enables a high MK-7 yield (MK-7 accounts for at least 90% of all vitamin K isoforms synthesised by the bacterium), is considered the ideal microorganism for the industrial production of MK-7 and is preferentially used to manufacture MK-7 supplements and functional food products [39, 102, 132, 134, 137-141].

Many studies examining the fermentation-based synthesis of MK-7 have focused on liquid-state fermentation (LSF), where fermentation is carried out in a liquid medium. However, more recently, solid-state fermentation (SSF) methods involving fermentation on a solid substrate

have gained significant interest. SSF techniques, in comparison to LSF, are likely to present a more viable and economical alternative, as they consume less pre-processing energy, enable superior productivity, generate less wastewater, and result in improved product recovery [142, 143]. Moreover, SSF fermentation methods can enable the crude fermented product to be used directly as a food supplement, eliminating the need to extract and purify the vitamin, thereby reducing the number of processing steps and the associated cost [20, 142, 144].

The MK-7 yield obtained from fermentation processes varies considerably and is determined by several physicochemical and biochemical parameters. The type of microorganism and the nature of the fermentation substrate, as well as the substrate particle size, medium components, pH, substrate pre-treatment methods, initial substrate moisture content, inoculum size, incubation temperature, relative humidity, fermentation period, aeration rate, and supplementation of trace elements and additional nutrients, are the most prominent factors that influence the MK-7 yield attained in SSF and LSF processes [37, 39, 94, 137, 143]. Additionally, numerous studies have investigated the effect of different substrates and nutrients, dynamic versus static fermentation conditions, various chemical and physical treatments (surfactants and ultrasound), and genetic manipulation on the fermentation yield [96, 101, 131, 135, 137, 142, 145, 146].

When static fermentation conditions are applied in traditional SSF and LSF techniques, the absence of adequate agitation and aeration creates a non-homogeneous environment, which results in operational issues, such as heat and mass transfer inefficiencies; thus, scale-up becomes a challenge [147-151]. These operational issues arise due to the tendency of *Bacillus* strains, especially *B. subtilis natto*, to form pellicles and biofilms [147-150, 152]. Biofilm formation is an example of passive immobilisation and occurs when planktonic cells, in response to harsh environmental conditions, undergo certain genetic changes, allowing them to colonise a suitable surface to form multicellular communities [148-150]. The formation of biofilms by *B. subtilis natto* has been correlated with the production of MK-7; consequently, many studies aiming to enhance the MK-7 yield have employed static fermentation conditions in both SSF and LSF [148]. However, static fermentation conditions lead to operational issues due to biofilm formation, limiting the suitability of such processes for the large-scale production of MK-7 [148]. Hence, biofilm reactors present a promising alternative to traditional SSF and LSF methods to improve MK-7 production, as they facilitate biofilm formation under controlled conditions while employing robust agitation and aeration [148, 151, 153]. Several studies have been conducted by Mahdinia et al. [147-154] to explore and optimise different aspects of biofilm reactors for the commercial production of MK-7. These investigations have demonstrated that *B. subtilis natto* is the best strain to enhance MK-7 production and that a plastic composite support (PCS) consisting of 50% polypropylene, 40% soybean hulls, 5% soybean flour, 5% yeast extract, and minor salts is ideal for the construction of biofilm reactors [148]. Both batch and fed-batch strategies and the use of glucose- and glycerol-based media have been compared to optimise the fermentation

conditions in various studies [148-151, 154]. Overall, biofilm reactors hold great promise for the large-scale production of MK-7. Nonetheless, the development of biofilm reactors for such applications is still in the preliminary stages, and further optimisation of the important fermentation parameters and improvement in reactor design are likely to enable the industrial production of MK-7 using this approach in the future [20, 148].

Generally, bacterial fermentation is assumed to produce the all-*trans* isomer of MK-7, but no studies have specifically considered this aspect. Therefore, no evidence outlining the ratio of MK-7 isomers obtained from microbial metabolic pathways is available in the literature [13, 15, 17]. Szterk et al. [15] and Szterk et al. [17] demonstrated that dietary supplements comprising MK-7 derived from natural natto extracts included varying proportions of *cis* isomers in addition to all-*trans* MK-7. These observations may indicate the potential dishonesty of producers in declaring the source of the MK-7 contained in their dietary supplements (that is, the MK-7 has been acquired from chemical synthesis techniques rather than from natural natto extracts) or the presence of *cis* isomers may be a result of the processes used to manufacture dietary supplements in the form of hard tablets or capsules [15, 17]. The MK-7 profile of fermented MK-7-enriched functional foods is yet to be elucidated, as most investigations have only analysed the MK-7 composition of dietary supplements achieved from natural and chemical production methods.

### 2.8.2 Chemical synthesis

MK-7 can also be produced synthetically, which is often the case for MK-7 dietary supplements unless derived from natural sources. Several methods exist for the chemical synthesis of MK-7 [155]. These generally involve the introduction of an isoprenoid functionality into the aromatic naphthoquinone nucleus, and the required side chain can be attained from various compounds, such as geraniol, farnesol, phytol, or solanesol [156, 157]. The locus of functionalisation and the degree of control over the stereochemical outcome of the introduced moiety are central factors in the chemical preparation of MKs [156].

Typically, MKs have been synthesised from the condensation of 2-methyl-1,4-naphthoquinol with an appropriate allylic alcohol in the presence of an acid catalyst, the most effective being boron trifluoride etherate ($BF_3O(C_2H_5)_2$) [156]. The resulting menaquinol product is then converted to the corresponding quinone product through mild oxidation with oxygen ($O_2$), ferric ions ($Fe^{3+}$), or silver oxide ($Ag_2O$) [156]. These reaction conditions usually avoid side chain isomerisation and chromanol cyclisation and have been optimised to avoid 2-alkylation [156]. However, the fundamental limitation of this reaction pathway is the inherent instability of the allylic alcohol component owing to the acidic conditions employed [156].

Another standard method for the introduction of the isoprenoid side chain into the 3-position of 2-methyl-1,4-naphthoquinone is the Friedel-Crafts alkylation of 2-methylnaphthalene-1,4-diol or 1-monoester derivatives with phytol or polyprenyl alcohols [157]. A key disadvantage

of this method is chromanol formation and side chain cyclisation, which make it difficult to isolate the final product [158].

In addition, Sato et al. [157] demonstrated MK synthesis through the activation of the side chain component as a nucleophile via a π-allyl-nickel intermediate to enable direct addition to a quinone or coupling with a protected 2-methyl-3-bromonaphthoquinol, followed by oxidation to achieve the corresponding prenylated quinone [16, 156].

Baj et al. [159] have also explored the chemical synthesis of MK-7 using a stereoselective approach comprising a "1 + 6" convergent strategy involving the condensation of two building blocks, a menadione monoprenyl derivative (fragment "1") with hexaprenyl bromide (fragment "6"). This synthetic pathway resulted in the production of MK-7 exclusively in the all-*trans* configuration [159].

Therefore, the procedures used for the production of synthetic MK-7 preparations are an important consideration with respect to the proportion of MK-7 isomers obtained, as during the chemical synthesis of MK-7, *cis/trans* isomers, relative to the all-*trans* form, can be acquired in a ratio of 1:3, 1:2, or not at all, depending on the method of synthesis [15].

### 2.8.3 Chemical transformations and isomerisation

The geometric isomerisation of isoprenoid units in the structure of MK-7 can occur under numerous conditions, particularly as a result of the methods used in the chemical synthesis of the vitamin and various technological processes, purification techniques, environmental factors, and storage conditions [13, 15, 17, 120, 122, 128].

Isomerisation largely occurs due to light exposure, notably ultraviolet (UV) radiation [13, 15, 17, 128, 160]. In addition, oxidation catalysed by high temperatures or the effect of radicals promotes the formation of epoxides and *cis* double bonds at various locations in the isoprenoid side chain of MK-7 [13, 15, 17, 158]. These processes may occur during the preparation of microcapsules containing MK-7, a precursor of dietary supplements in the form of hard tablets and capsules [13, 15, 17].

In the case of naturally derived MK-7 dietary supplements, isomerisation of the all-*trans* isomer of the vitamin may arise as a consequence of the technological procedures used to manufacture hard tablets and capsules and/or due to auto-oxidation processes promoted by exposure to atmospheric oxygen, light, and elevated temperatures during the storage of the supplement [13, 15, 17]. However, it is yet to be determined if similar processes occur for MK-7-enriched functional foods obtained from bacterial fermentation.

## 2.9 Issues associated with MK-7 production

Although MK-7 can be produced chemically, which is more economical, the recent market trend endorsing natural and organic alternatives over synthetic formulations has rendered fermentation-based synthesis a more favourable option from a consumer's perspective. Hence,

consumers often prefer naturally derived products, which are considered to provide greater health benefits compared to synthetic preparations. Fermentation can also naturally enhance the nutrient profile and sensory characteristics of various products, increasing their appeal to consumers [102, 161-163]. Furthermore, microbial fermentation is a more sustainable process for the large-scale production of MK-7, and using natural production methods can satisfy the market demand and sustainable development goals [96, 164, 165].

However, several challenges are linked to the natural synthesis of MK-7, the main problem being the low fermentation yield. Additionally, the large number of tedious unit operations required in the downstream processing of the vitamin increase its cost of production and raise the price of the final product, reducing its accessibility [166]. Thus, there is a need for innovative methods and technologies to enhance the fermentation yield and/or reduce the number of unit operations involved, as well as ensure that the bioactive all-*trans* isomer is produced almost exclusively or in the most significant proportion. In this regard, employing nano-sized materials with novel properties in the fermentation process is a promising technique that can overcome the barriers of MK-7 production.

## 2.10 Nanomaterials (NMs)

NMs are materials with structural components smaller than 1 μm (1000 nm) in at least one dimension and are often referred to as particulate dispersions or solid particles with a size of 10-1000 nm [167, 168]. NMs have gained extensive interest due to their exceptional physicochemical and biological properties, which make them suitable for different purposes in several sectors, such as science, technology, and medicine. The nanoscale size of NMs provides a large surface-area-to-volume ratio and confers unique physical, chemical, biological, mechanical, electrical, structural, morphological, and optical properties not observed in the corresponding bulk material [167, 169-172]. Such distinctive properties and novel characteristics play a fundamental role in determining the suitability of nanomaterials for an assortment of innovative applications. NMs can be classified into three broad categories, NPs, nanoclays (NCs), and nanoemulsions (NEs), which have different structures and can be synthesised using various methods [173].

NPs have been employed in a vast range of industrial and other uses in numerous fields, such as electronics, optics, agriculture, wastewater treatment, catalysis, sensing, and biomedicine, as well as in the food, cosmetic, and chemical industries [170-175]. In addition, NPs can be applied to the fermentation-based synthesis of MK-7 to address the major issues associated with MK-7 production. NPs can be implemented to improve the productivity of the process by enhancing the metabolic efficiency of the cells and/or decrease the number of downstream unit operations (process intensification) through bacterial cell immobilisation. The subsequent sections provide a brief overview of the types of NPs and discuss the potential of NPs to overcome the challenges of MK-7 fermentation.

### 2.10.1 Types of NPs

Depending on their composition and chemical characteristics, NPs can be classified as either organic or inorganic [173, 176]. The two forms of NPs differ in their methods of synthesis, and variation in important chemical and physical parameters, including the temperature, pH, solvent type, and precursors, enables the production of NPs with different morphologies and properties that can be customised for specific applications [172, 173, 176].

#### 2.10.1.1 Organic NPs (ONPs)

ONPs are largely composed of organic materials, such as lipids, proteins, carbohydrates, and other organic compounds, and are commonly employed to enhance the nutrient value of food systems and the delivery of essential nutrients or pharmaceuticals [173, 176-178]. Several synthesis techniques can be used for the fabrication of ONPs, and these can be classed as top-down (high-energy) or bottom-up (low-energy) procedures (Figure 2-5). The two approaches can also be combined to produce ONPs.

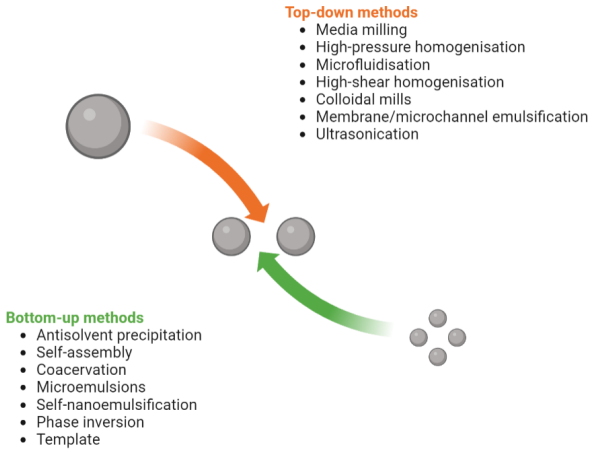


Figure 2-5 Top-down and bottom-up methods typically used to produce ONPs (adapted from Pan and Zhong [177])

#### 2.10.1.2 Inorganic NPs (INPs)

INPs are synthesised from inorganic materials, such as metals, metal oxides, or metal carbonates [174, 176]. Silver (Ag) and gold (Au) NPs are the most prevalent metallic NPs, which possess antimicrobial, antiviral, and antifungal activity and have extensive use in environmental remediation and food and biomedical applications [174, 176, 179-186]. Zinc oxide (ZnO), copper oxide (CuO), iron oxide ($Fe_2O_3$), magnetite ($Fe_3O_4$), silica ($SiO_2$), and titanium dioxide ($TiO_2$) are the most widespread metal oxide NPs, which are employed for the synthesis of industrial products and have numerous food, biomedical, agricultural, environmental, electrical, catalytic, gas sensing, and energy-related uses [174, 176, 180, 187-191]. Calcium carbonate ($CaCO_3$), barium carbonate ($BaCO_3$), and copper carbonate ($CuCO_3$) are the most common metal carbonate NPs

that serve a range of biomedical [192-194], catalytic [195-197], and antimicrobial [198, 199] functions.

Two general approaches can be implemented for the synthesis of INPs, top-down and bottom-up techniques, and these can be further grouped into three broad categories, namely physical, chemical, and biological processes (Figure 2-6) [191, 200].

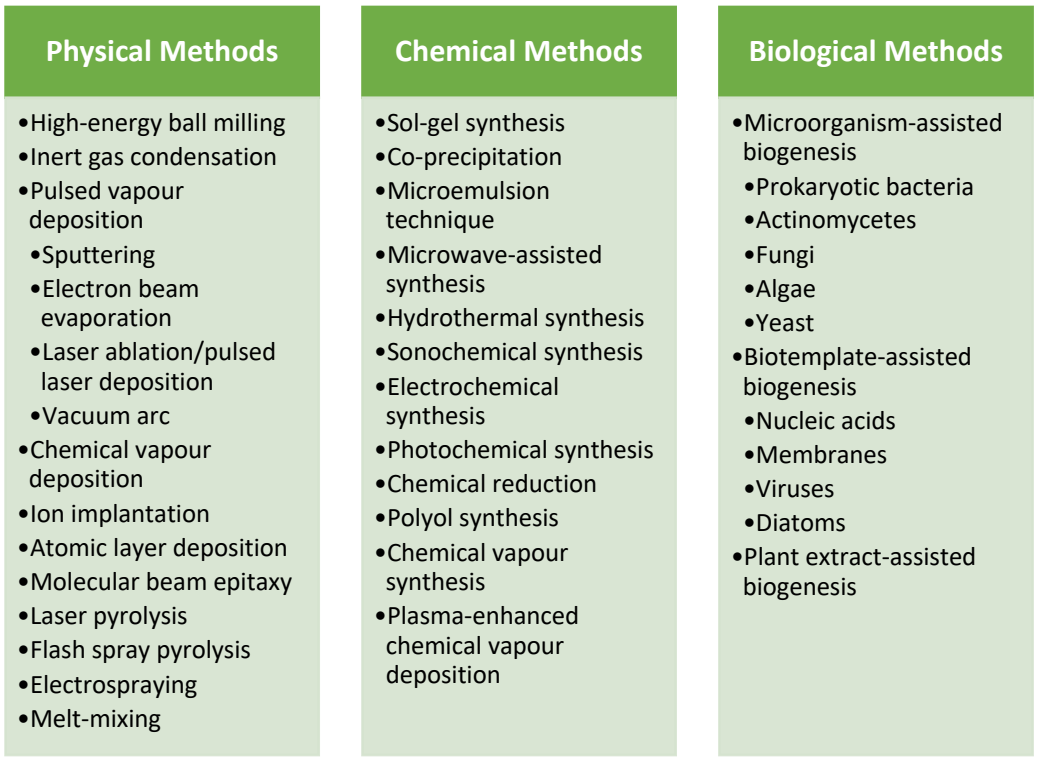


Figure 2-6 Physical, chemical, and biological approaches commonly employed to synthesise INPs

In the context of MK-7 biosynthesis, INPs are the most relevant to enhance the MK-7 concentration and yield, and they also present a favourable approach to refine the industrial production of MK-7 through bioprocess intensification.

### 2.10.2 The potential of NPs to address the challenges of MK-7 production

Of the various INPs, iron-based NPs, specifically IONs and iron oxyhydroxide (FeOOH) NPs, have the ability to boost the MK-7 yield and improve the productivity of the fermentation process. IONs also possess superparamagnetic properties, which can be exploited to aid bacterial cell recovery, making them a prospective novel tool to address the current challenges of industrial MK-7 fermentation [166]. Alternatively, FeOOH NPs are food grade and can be used to enhance the MK-7 content of MK-7-enriched functional food products [201]. Thus, depending on their nature and properties, iron-based NPs can be tailored to aid MK-7 production in different applications.

### 2.10.2.1 Bacterial cell immobilisation and the use of IONs to facilitate MK-7 production

#### *2.10.2.1.1 Bacterial cell immobilisation*

Bacterial cell immobilisation involves the physical confinement of viable microbial cells to a defined region of space in order to limit free migration [202].

##### *2.10.2.1.1.1 IONs and magnetic cell immobilisation*

There are many types of IONs, such as $Fe_3O_4$, hematite ($\alpha$-$Fe_2O_3$), maghemite ($\gamma$-$Fe_2O_3$), wustite (FeO), $\varepsilon$-$Fe_3O_4$, and $\beta$-$Fe_3O_4$, and of these, $Fe_3O_4$ is typically used to enhance MK-7 production [203, 204]. Of the myriad pathways available for the synthesis of IONs, the co-precipitation of ferrous ($Fe^{2+}$) and $Fe^{3+}$ ions by an alkali, usually ammonium hydroxide ($NH_4OH$) or sodium hydroxide (NaOH), in an aqueous solution tends to be the preferred (wet chemical) method and is frequently employed to manufacture $Fe_3O_4$ NPs [205, 206]. The size, shape, and composition of the resulting IONs are dependent on several key synthesis parameters, including the nature of the salts used, the reaction pH and temperature, the ionic strength of the media, and the ratio of $Fe^{2+}$ and $Fe^{3+}$ ions present in the solution [204, 207]. These factors can also be altered to achieve different properties and allow the surface functionalisation of IONs to promote specific interactions with bacterial cells [203]. The main advantages of the co-precipitation method are that it is fairly simple and efficient and enables the synthesis of large quantities of NPs; however, difficulties in controlling the particle size distribution and the formation of impurities, such as goethite ($\alpha$-FeOOH) and $\gamma$-$Fe_2O_3$, are the primary drawbacks of this technique [204, 208, 209].

IONs have gained considerable interest in bacterial cell immobilisation due to their unique physicochemical properties, such as superparamagnetism, large surface-area-to-volume ratio, biocompatibility, and simple separation methodology [203]. Magnetic cell immobilisation involves the decoration of bacterial cells with magnetic NPs or the entrapment of bacterial cells within clusters of magnetic NPs [203]. The surface of bacterial cells can be easily decorated with IONs through various non-specific interactions, including hydrogen bonds, Van der Waals forces, electrostatic attractions, and hydrophobic interactions [166]. This technique, unlike the other common immobilisation methods, enables a higher specific yield, does not impede mass transfer, and combines the benefits of immobilisation with free-cell fermentation. The cells can also be readily separated from the fermentation broth with an external magnetic field, which does not significantly compromise the viability and reusability of the immobilised cells and allows simple downstream processing with fewer steps [166].

##### *2.10.2.1.1.2 Bacterial responses to magnetic cell immobilisation*

Bacterial attachment to a solid surface, such as IONs, is influenced by various characteristics of microbial cells, including the age and physiological state of cells, the surface

charge and hydrophobicity, the presence of extracellular polymeric substances (EPS), the existence of surface proteins and glycocalyx, and the occurrence of cell wall structures, such as pili and fimbriae [202]. The environmental and culture conditions also play a role in determining the success and efficiency of the magnetic immobilisation procedure. The most notable factors are temperature, pH, oxygen concentration, hydrodynamic interactions, adhesive forces, nutrient availability, flow velocity, rheology, and the presence of antimicrobial agents, cations, and anions [202]. Additionally, the nature and properties of IONs, including their hydrophobicity, porosity, roughness, superficial charge, and toxicity, impact bacterial attachment and the efficacy of magnetic immobilisation [202]. The particle size, concentration, shape, and surface functionality of IONs are also key parameters that influence the success of magnetic immobilisation. Therefore, synthesising IONs with characteristics appropriate for the desired application and optimising various factors are of utmost importance for the success of this technique and achieving a high immobilisation efficiency.

It is also essential to consider the effect of IONs on bacterial cells, as the interaction between IONs and the bacterial cell surface may or may not be favourable, depending on the metabolic response(s) exhibited by the bacterial cells upon interaction with the NPs. The many possible metabolic responses of bacterial cells to immobilisation with IONs are summarised in Figure 2-7, and the specific metabolic response(s) observed following magnetic immobilisation with IONs can vary with the bacterial species, the properties of the IONs, and the culture conditions [203, 204].

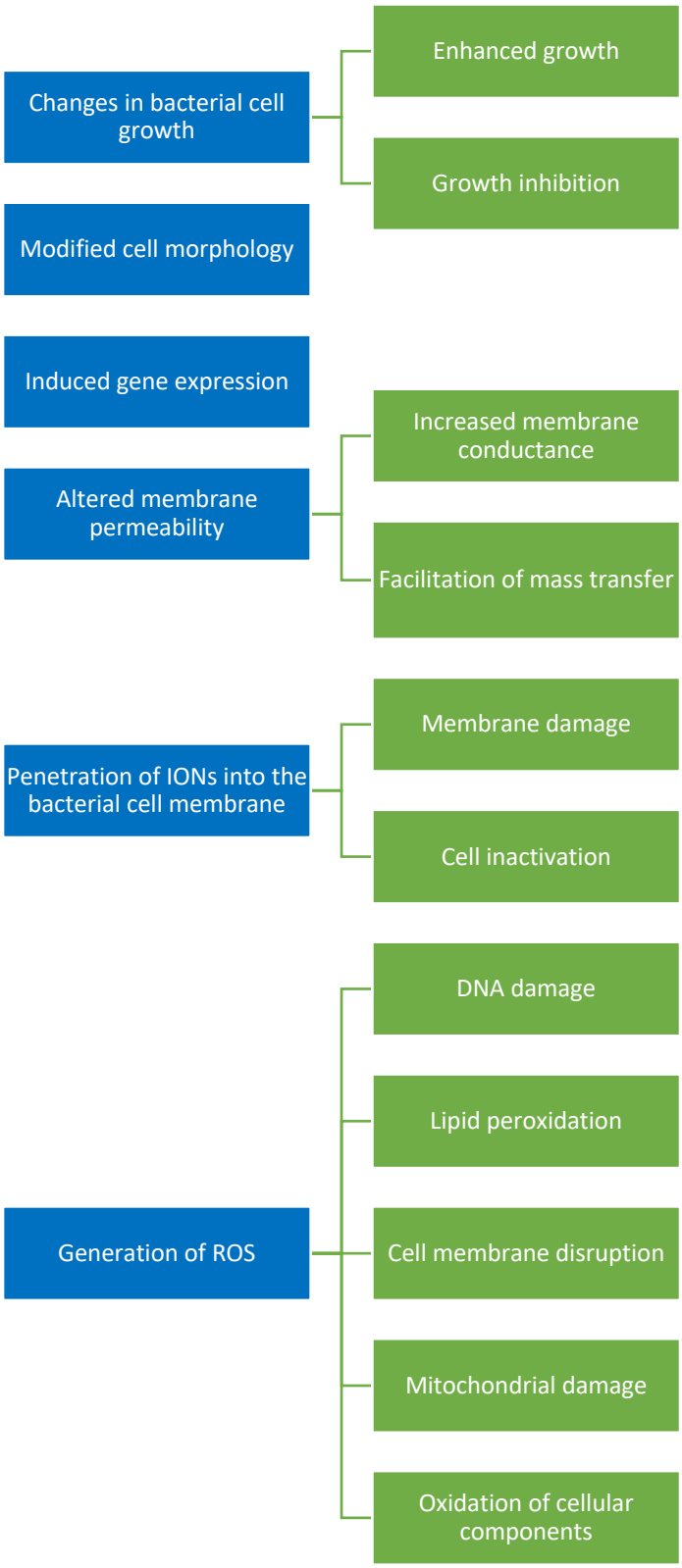


Figure 2-7 Potential metabolic responses of bacterial cells to immobilisation with IONs (adapted from Ranmadugala et al. [203])

#### *2.10.2.1.2 The use of IONs to facilitate MK-7 production*

Several studies have investigated the ability of bacterial cell immobilisation with IONs to enhance MK-7 production and aid cell recovery for process intensification [166, 210, 211]. In

these studies, the surface of *B. subtilis natto* cells was decorated with IONs via non-specific interactions.

Ebrahiminezhad et al. [166] and Ebrahiminezhad et al. [210] considered four different concentrations of IONs (0 µg/mL, 50 µg/mL, 100 µg/mL, and 150 µg/mL) and examined the impact of magnetic immobilisation on cell growth, MK-7 production, and the possibility for in situ product recovery and cell recycling. The findings from both investigations were comparable, and it was determined that MK-7 production increased over the five-day fermentation period for all tested concentrations of IONs. The majority of MK-7 was synthesised during the bacterial growth phase, while a smaller amount was produced during the stationary phase. This demonstrated that MK-7 production is partly growth-associated. It was also observed that IONs slightly inhibited bacterial growth; however, they did not have a negative effect on MK-7 production. Moreover, a higher MK-7 specific yield was noted for the magnetically immobilised bacterial cells in comparison to the untreated cells, suggesting that magnetically immobilised cells are more metabolically efficient due to the interactions between the IONs and the bacterial cell surface. It has been proposed that the attachment of NPs to the bacterial cell surface improves the permeability of the cell membrane. This is facilitated by the non-specific interactions between the IONs and the membrane compounds, which promote disorganisation of the lipid packing and increase the membrane permeability. Hence, although the presence of IONs slightly decreased bacterial growth, the enhanced metabolic efficiency of the cells facilitated greater MK-7 secretion into the fermentation medium, resulting in a higher MK-7 specific yield.

The IONs synthesised in both studies also exhibited superparamagnetism, which plays a crucial role in cell separation and dispersion through the application and removal of an external magnetic field. This is advantageous as MK-7 is an extracellular product; thus, it can be easily recovered by cell removal using an external magnetic field. The cell separation efficiency in both reports demonstrated a dose-dependent increase in the number of captured microorganisms, with the best capture efficiency (more than 90%) attained at an ION concentration of 150 µg/mL. This was mainly attributable to more magnetic particles on the surface of the bacterial cells at higher concentrations. The prospect of running successive recycle batches was also explored in both investigations, and it was noticed that the capture efficiency and MK-7 production achieved in consecutive cycles were not substantially compromised. This indicates that magnetic immobilisation with IONs is a promising approach for process intensification, as magnetic separation technology is scalable and can be effectively incorporated into a recycle loop in a bioreactor to allow the rapid recovery of bacterial cells during fermentation. In this regard, designing intensified bioreactors, with the aid of bacterial cell immobilisation, enables the integration of product formation and cell recovery, which has various advantages, such as cell reusability, simple equipment requirements, and low energy consumption [166]. Therefore, bacterial cell immobilisation with IONs is a favourable technique that can be employed to overcome the limitations of industrial MK-7 production.

It is essential to appreciate that Ebrahiminezhad et al. [166] considered naked IONs, whereas Ebrahiminezhad et al. [210] synthesised L-Lys@IONs. Naked IONs and L-Lys@IONs tend to display similar properties; however, naked IONs exhibit high non-specific binding, low physicochemical stability, and microbial toxicity [208, 210]. Biocompatible coatings, especially amino acids, could eliminate such unfavourable effects. Amino acids are an ideal coating material due to their chemical stability, surface activity, and biocompatibility [210]. L-Lys is particularly desirable, as it does not have any detrimental effects on the important characteristics of IONs. It also introduces amine functional groups to the structure of the NPs, which improves their interaction with negatively charged cell membrane domains and increases the potential for surface interactions [210]. Consequently, coated IONs present a better alternative to uncoated IONs, as although both coated and naked IONs enable a similar MK-7 yield, coated IONs have superior properties and excellent biocompatibility.

Ranmadugala et al. [211] have also assessed the effect of amine-functionalised NPs, specifically IONs@APTES, on bacterial growth and MK-7 production. The APTES coating confers the same properties as L-Lys but offers additional benefits, as it prevents the oxidation of NPs and preserves their crystalline structure [211]. In the study carried out by Ranmadugala et al. [211], it was established that IONs@APTES enhanced the production and yield of MK-7. The experimental conditions employed in this investigation were similar to the studies conducted by Ebrahiminezhad et al. [166] and Ebrahiminezhad et al. [210]. However, it was found that compared to naked IONs and L-Lys@IONs, IONs@APTES allowed a higher MK-7 concentration to be achieved (relative to the untreated cells) over a five-day course of fermentation. Of the IONs@APTES concentrations considered (0 µg/mL, 100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL, 500 µg/mL, 600 µg/mL, and 700 µg/mL), a concentration of 500 µg/L resulted in maximal MK-7 production (41 mg/L), which was approximately twice that of the untreated cells (22 mg/L). The MK-7 specific yield was also higher for the IONs@APTES in comparison to the untreated cells, and it was determined that an IONs@APTES concentration of 200 µg/L was the optimum, as it enabled the greatest MK-7 specific yield and resulted in high overall productivity during *B. subtilis* fermentation. In addition, the IONs@APTES were compatible with *B. subtilis* cells and did not impede bacterial growth within the tested concentrations. This was in contrast to naked IONs [166] and L-Lys@IONs [210], which hindered the growth of *B. subtilis* over the fermentation period.

In another study, Ranmadugala et al. [212] evaluated the impact of IONs@APTES on biofilm formation and the growth and viability of *B. subtilis* cells. Biofilm formation is one of the dominant issues in industrial fermentation, as it leads to many process and operational problems that decrease the performance of the process, reduce the yield and quality of the desired product, and increase the process and equipment-related costs [212]. This investigation compared the effect of naked IONs and IONs@APTES with untreated cells, and it was determined that 100 µg/mL of IONs@APTES significantly decreased biofilm formation without compromising cell

growth and viability. In contrast, naked IONs had a negative effect on cell viability at higher concentrations and showed no notable reduction in biofilm formation. Therefore, IONs@APTES appear to be the most promising to boost the MK-7 yield, promote cell growth and viability, reduce biofilm formation, decrease production costs, and increase the overall productivity of the fermentation system.

Essentially, the results of these studies imply that IONs are a valuable tool to enhance the MK-7 yield and overcome the obstacles in the large-scale production of the vitamin.

#### 2.10.2.2 Food grade NPs and the development of MK-7-enriched functional food products

NPs have been extensively used for a range of food-related applications, such as food preservation, fortification, and packaging, as well as for the development of food grade NP-based delivery systems and for improving the shelf life, quality, and safety of food products [169-171, 173, 213-215].

While many studies [166, 210, 211] have demonstrated the ability of iron NPs to enhance the MK-7 yield in fermentation processes, they have not explicitly explored this in the context of food-related applications. Instead, they have largely focused on overcoming the primary challenges accompanying the industrial fermentation and purification of MK-7, such as increasing MK-7 production, decreasing the number of downstream processing steps, and reducing biofilm formation. However, more recently, Novin et al. [201] have considered the application of iron (FeOOH) NPs to boost MK-7 production for the development of a MK-7-enriched functional dairy product.

It is essential to note that in order for NPs to be suitable for inclusion in functional food products, they must be biocompatible and should not possess any harmful properties that could pose a health risk, as, unlike magnetic NPs, they are free-floating and remain in the final product. Uncoated iron NPs are toxic to biological systems and environments and have insufficient physicochemical stability, including poor solubility and biocompatibility [201, 216]. Hence, it is beneficial to manipulate their surfaces using coatings or other components to improve their biocompatibility and eliminate any detrimental properties. NPs with biocompatible coatings have been approved by the Food and Drug Administration (FDA) for biomedical applications [201, 216-218]. Although assorted materials can be implemented to modify NPs for food-related applications, polysaccharides, particularly microbial polysaccharides, are preferable, as they have better water solubility and stability and result in fewer side effects on the organism [219]. Of the various microbial polysaccharides, xanthan gum (XG) is the most commercially favourable due to its unique rheological properties and stability over a range of temperature and pH conditions [220, 221]. It is also a safe and non-toxic polymer (FDA-approved) widely used in the food industry as an emulsifier, thickener, and stabiliser in food products [220, 221]. Thus, the

characteristics of XG make it an ideal coating agent for synthesising biocompatible NPs for food-related applications.

In the study by Novin et al. [201], naked and XG-coated FeOOH NPs were synthesised and compared regarding their ability to boost the MK-7 yield for the production of a fermented functional dairy product. It was demonstrated that 3 mg/L of XG-coated and 12 mg/L of naked FeOOH NPs significantly increased bacterial growth and MK-7 biosynthesis relative to the control samples. While the naked FeOOH NPs also enhanced the MK-7 yield, a greater concentration was required than the XG-coated FeOOH NPs. Furthermore, naked iron NPs are toxic and unsuitable for human consumption, whereas XG-coated NPs are biocompatible. The iron-polysaccharide complex also provides additional benefits, such as good tolerability, improved bioavailability, and a higher percentage of iron. Overall, the results of this research illustrate the potential for biocompatible XG-coated FeOOH NPs to be used for the development of fermented functional food products rich in MK-7.

### 2.10.3 NPs and the production of MK-7 isomers

Although various iron-based NPs have been extensively investigated to determine their ability to enhance MK-7 biosynthesis and establish their suitability for process intensification or the development of MK-7-enriched fermented functional food products, their influence on the production of MK-7 isomers has not been examined. While it has been recognised that iron-based NPs can increase the MK-7 yield, the proportion of *cis* MK-7 isomers obtained, together with the all-*trans* form, in the presence of iron-based NPs remains to be elucidated. Considering that only the all-*trans* isomer is biologically active, using NPs to improve the MK-7 concentration and yield is only valuable if the all-*trans* isomer is attained in the most significant proportion. Accordingly, it would be advantageous to assess the impact of magnetic and/or biocompatible iron-based NPs on the concentration of all-*trans* MK-7 achieved from fermentation with regard to applications relating to bioprocess intensification or the development of MK-7-enriched functional food products.

## 2.11 Analysis and measurement of MK-7 isomers

The analysis of MK-7 isomers encompasses two steps, the separation of MK-7 compounds and the identification and quantification of the different isomers [13, 15, 17, 18]. Additionally, the location of *cis* bonds in the isoprenoid side chain and the chemical structure of the isomers can be determined using nuclear magnetic resonance (NMR) spectroscopy [13, 15, 17].

Numerous methods are available for quantifying the vitamin K series in different matrices. Most studies have solely considered the analysis of PK and MKs and have not assessed the isomer profile of the constituent compounds. Only a few investigations have evaluated vitamin K1 isomers, and a comparatively smaller number have specifically focused on the quantification of MK-7 isomers. The analytical techniques used in several vitamin analysis studies are summarised

in Table 2-3, and the following sections provide an overview of the key analytical procedures and highlight some of the possible challenges involved in the analysis and measurement of MK-7 isomers.

Table 2-3 Summary of the analytical methods employed in various vitamin analysis studies

| Sample Type(s) | Compound(s) Determined | Column(s) | Analytical Technique(s) | Reference |
|---|---|---|---|---|
| Dietary supplements | MK-7 isomers | COSMOSIL cholester column (150 mm × 2 mm × 3 μm) | UHPLC-DAD-CAD-QTOF (ESI in the positive ionisation mode) | [15] |
| Foods commonly consumed in Australia | PK, MK-4, and MK-7 | Reversed-phase Accucore PFP HPLC column (100 mm × 2.1 mm × 2.6 μm) | LC-MS/MS (ESI in the positive ionisation mode) | [222] |
| Plasma | PK | ProntoSil $C_{30}$ column (250 mm × 4.6 mm × 5 μm) | LC-APCI-MS (APCI in the positive ionisation mode) | [223] |
| Food items frequently consumed in Japan | PK and MKs | Capcell Pak $C_{18}$ column (250 mm × 4.6 mm × 5 μm) | HPLC-FL 320 nm or 240 nm/430 nm, reduction with Pt (reactor 15 mm × 4 mm) | [224] |
| Serum | PK, MK-4, and MK-7 | SB-$C_8$ (100 mm × 2.1 mm × 1.8 μm) | LC-MS/MS (ESI in the positive ionisation mode) | [42] |
| Faeces, serum, and food | PK and MKs | Phenomenex Kinetex $C_{18}$ column (150 mm × 3 mm × 2.6 μm) | LC-APCI-MS (APCI in the positive ionisation mode) | [225] |
| Assorted products | Vitamin K1 isomers | YMC $C_{30}$ column (250 mm × 4.6 mm × 3 μm) | LC-APCI/IM-MS (APCI in the positive ionisation mode) | [226] |
| Plasma | MK-7 | Phenomenex Kinetex $C_{18}$ column (100 mm × 4.6 mm × 2.6 μm) | HPLC-FL 335 nm/430 nm (CQ-R 20 mm × 2 mm) | [119] |

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|---|---|---|---|---|
| Selected plant foods | Carotenoids and fat-soluble vitamins, including the vitamin K series | ProntoSil $C_{30}$ column (250 mm × 4.6 mm × 5 µm) | LC-DAD-MS/MS (APCI in the positive ionisation mode) | [227] |
| Various cheeses, meat, fish, and vegetables | Vitamin K | Reversed-phase $C_{18}$ column | HPLC-FL 246 nm/430 nm, reduction with Zn | [100] |
| Fermented foods | PK and MKs | Phenomenex Kinetex $C_{18}$ column (100 mm × 2.1 mm × 1.7 µm) | UHPLC-APCI-MS/MS (APCI in the positive ionisation mode) | [18] |
| Synthetic MK-7 preparation | MK-7 isomers | ACE 5 $C_{18}$ column (250 mm × 4.6 mm × 5 µm) and COSMOSIL 5$C_{18}$-MS-II column (250 mm × 4.6 mm × 5 µm) | Semi-preparative HPLC with silver complexation (detection at 268 nm) and NMR spectroscopy (ESI in the positive ionisation mode) | [126] |
| Infant formulas | Vitamin K1 isomers | YMC $C_{30}$ column (150 mm × 4.6 mm × 3 µm) | UPLC-ESI-MS/MS (ESI in the positive ionisation mode) | [128] |
| Serum | Vitamin K analogues | Shodex $C_{18}$ column (250 mm × 4.6 mm × 5 µm) | HPLC-EC, reduction with a Pt catalyst (column 10 mm × 4.6 mm) (EC detector operated in the oxidation mode) | [228] |
| Animal products | PK and MKs | Vydac 201 TP54 $C_{18}$ column (250 mm × 4.6 mm × 5 µm) | HPLC-FL 238 nm/425 nm, reduction with Zn (reactor 50 mm × 2.1 mm) | [229] |
| Plasma | Carotenoids and PK | YMC $C_{30}$ column (250 mm × 4.6 mm × 3 µm) | LC-APCI-MS (APCI in the positive ionisation mode) | [230] |
| Dietary supplements | MK-7 isomers | COSMOSIL cholester column (250 mm × 10 mm × 5 µm) | UHPLC-DAD-CAD-QTOF (ESI in the positive ionisation mode) | [17] |

| | | | | |
|---|---|---|---|---|
| Rat tissues | Vitamin K1 isomers | YMC $C_{30}$ column (250 mm × 4.6 mm × 3 µm) | HPLC-FL 243 nm/430 nm, reduction with Zn (reactor 20 mm × 4 mm) | [231] |
| Plasma | Vitamin K1 | Alltima $C_{18}$ column (150 mm × 2.1 mm × 3 µm) | LC-APCI-MS/MS (APCI in the positive ionisation mode) | [232] |
| Various fermented dairy products | MKs | Phenomenex Gemini $C_{18}$ column (100 mm × 4.6 mm × 3 µm) | HPLC-FL 220 nm/436 nm, reduction with Zn (reactor 50 mm × 4 mm) | [233] |
| Fruits and vegetables | PK | Phenomenex Kinetex PFP column (100 mm × 2.1 mm × 2.6 µm) | LC-APCI-MS/MS (APCI in the positive ionisation mode) | [234] |
| Danish cheese products | PK and MKs | Ascentis Express $C_{18}$ guard column (5 mm × 2.1 mm × 2.7 µm) Ascentis Express $C_{18}$ HPLC column (100 mm × 2.1 mm × 2.7 µm) | LC-ESI-MS/MS (ESI in the positive ionisation mode) | [99] |
| Plasma | Vitamin K homologues | Capcell Pak $C_{18}$ column (250 mm × 4.6 mm × 5 µm) | LC-APCI-MS/MS (APCI in the positive ionisation mode) | [235] |
| Serum and staple foods consumed by the Indian population | PK and MK-7 | Phenomenex Kinetex $C_{18}$ column (100 mm × 4.6 mm × 2.6 µm) | HPLC-FL 248 nm/430 nm, reduction with Zn (reactor 30 mm × 4 mm) | [236] |
| Avocados | Fat-soluble vitamins and carotenoids | YMC Carotenoid $C_{30}$ column (150 mm × 4.6 mm × 3 µm) | LC-MS (ESI in the positive ionisation mode) | [237] |
| Margarines | PK and dihydro-vitamin K1 | YMC $C_{30}$ column (250 mm × 4.6 mm × 3 µm) | HPLC-FL 243 nm/430 nm, reduction with Zn (reactor 20 mm × 4 mm) | [238] |

| | | | | |
|---|---|---|---|---|
| Plasma | Vitamin K1 isomers | COSMOSIL cholester column (75 mm × 3 mm × 2.5 µm) | UFLC-APCI-MS/MS (APCI in the positive ionisation mode) | [239] |
| Hazelnut, broccoli, cheese, and pork | PK, MK-4, MK-7, and MK-9 | Ascentis Express $C_{18}$ HPLC column (100 mm × 2.1 mm × 2.7 µm) | LC-MS/MS (APCI and ESI were compared in the positive ionisation mode) | [92] |
| Plasma | PK | Hypersil BDS $C_{18}$ column (150 mm × 3.2 mm × 3 µm) | HPLC-FL 244 nm/430 nm, reduction with Zn (reactor 50 mm × 2.1 mm) | [240] |
| Dietary supplements | MK-7 isomers | COSMOSIL cholester column (250 mm × 10 mm × 5 µm) | UHPLC-QTOF and NMR spectroscopy (ESI in the positive ionisation mode) | [13] |
| Oils, margarines, and butter | PK | Vydac 201 TP54 $C_{18}$ column (250 mm × 4.6 mm × 5 µm) | HPLC-EC (dual-electrode EC detector operated in the redox mode) | [241] |
| Wide range of food products | Vitamin K1 isomers | YMC $C_{30}$ column (250 mm × 4.6 mm × 3 µm) | HPLC-FL 243 nm/430 nm, reduction with Zn (reactor 20 mm × 4 mm) | [19] |
| Plasma | Vitamin K1 | Sepax GP $C_8$ column (250 mm × 4.6 mm × 5 µm) | HPLC-APCI-MS (APCI in the positive ionisation mode) | [242] |
| Vegetables, fruits, and berries | PK | Vydac 201 TP54 $C_{18}$ column (250 mm × 4.6 mm × 5 µm) | HPLC-EC (dual-electrode EC detector operated in the redox mode) | [243] |
| Meat, fish, fruits, vegetables, dairy, oils, | PK and MKs | Reversed-phase $C_{18}$ column | HPLC-FL 246 nm/430 nm, post-column EC reduction | [91] |

| | | | | |
|---|---|---|---|---|
| margarines, bread, and beverages | | | | |
| Honey | MKs | Waters Acquity BEH $C_{18}$ column (150 mm × 2.1 mm × 1.8 µm) | UPLC-UV-MS (ESI in the positive ionisation mode) | [244] |
| Serum | Fat-soluble vitamins | Agilent Pursuit PFP column (100 mm × 3 mm × 3 µm) | LC-MS/MS (ESI in the positive ionisation mode) | [245] |
| MK-7 formulations of different origins | MK-6 and MK-7 isomers | Phenomenex Kinetex $C_{18}$ column (100 mm × 4.6 mm × 2.6 µm) Acclaim $C_{30}$ column (250 mm × 2.1 mm × 3 µm) | HPLC-UV (detection at 268 nm and 248 nm) HPLC-FL 245 nm/430 nm, reduction with Zn (reactor 30 mm × 4 mm) | [120] |
| Infant formula | Vitamin K1 isomers | YMC $C_{30}$ column (150 mm × 4.6 mm × 3µm) | ID-LC-MS/MS (APCI and ESI were compared in the positive ionisation mode) | [246] |
| Assorted food products | Vitamins D and K | Zorbax Eclipse ODS non-endcapped $C_{18}$ column (250 mm × 4.6 mm × 5 µm) | LC-DAD and LC-APCI-MS (APCI in the negative ionisation mode followed by the positive ionisation mode) | [247] |
| Plasma | Vitamin K1 | Spherisorb Ultrasphere ODS Beckman $C_{18}$ column (125 mm × 4.6 mm × 5 µm) | HPLC-FL 244 nm/430 nm, reduction with Zn (reactor 50 mm × 4.6 mm) | [248] |

### 2.11.1 Chromatographic separation

High-performance liquid chromatography (HPLC) is the preferred method for the analysis of vitamin K compounds [18, 39, 249]. HPLC is ideal for this purpose, as it operates at a moderate temperature and protects samples from exposure to light during the chromatographic run, which decreases the potential for sample damage [39, 249]. It also enables high sensitivity and significant resolution [39, 249, 250]. In addition, HPLC is a highly versatile technique, as various combinations of stationary phases, columns, and detection methods can be used to suit the analytical requirements of different compounds [249].

#### 2.11.1.1 Column

It has been noted that the type of chromatography column used to separate vitamin K isomers plays a vital role in determining the efficiency and degree of separation achieved [128]. Several columns are available for chromatographic separations. The reversed-phase $C_{18}$ and $C_{30}$ columns are frequently used to separate fat-soluble vitamins due to the hydrophobic nature of the stationary phase in these columns [251]. $C_{18}$ columns are the most commonly used for separating and analysing K vitamers; however, they cannot discern geometric isomers [39, 128, 249]. In contrast, $C_{30}$ columns can effectively separate geometric and positional isomers of structurally similar molecules, including the *cis* and *trans* isomers of vitamin K compounds [18, 19, 238, 252, 253]. $C_{30}$ columns usually have a stationary phase consisting of $C_{30}$ alkyl silane, which enables high shape selectively and is, thus, ideal for the separation of hydrophobic structurally related isomers [18, 19, 160, 238, 252, 253]. Huang et al. [128] compared the ability of $C_{18}$ and $C_{30}$ columns to separate *cis* and *trans* vitamin K1 isomers in infant formulas, and it was found that only the $C_{30}$ column was able to accomplish complete separation. Numerous other investigations considering the separation of vitamin K isomers and other fat-soluble vitamins, such as the studies by Cook et al. [238], Fu et al. [223], Gentili and Caretti [227], Lee et al. [246], Woollard et al. [19], and Xiao et al. [226], have also reported the use of a $C_{30}$ column for this purpose.

Szterk et al. [17], Szterk et al. [15], Sitkowski et al. [13], and Bus et al. [126] employed a COSMOSIL cholester column to separate *cis* and *trans* MK-7 isomers in MK-7 dietary supplements and synthetic preparations. COSMOSIL cholester columns are reversed-phase columns that contain silica-bonded cholesteryl groups as the stationary phase, which provide increased stereoselectivity and improved resolution for geometric isomers relative to alkyl-bonded materials [239, 254]. Therefore, compared to regular $C_{30}$ columns, COSMOSIL cholester columns will likely enable the superior separation of *cis* and *trans* MK-7 isomers.

#### 2.11.1.2 Detection methods

Many detection methods are available to identify the eluted compounds following post-column derivatisation [18]. Standard techniques include UV, fluorescence (FL), and electrochemical (EC) detection [18, 39, 229, 238, 240, 241, 243, 248].

Although EC detection offers greater sensitivity and selectivity, it is rarely used for analysing vitamin K in food products owing to the oxygen sensitivity of this method, which results in a high background current [39, 228]. It is also not possible to efficiently separate different vitamin K compounds in a reasonable timeframe with an EC detector [229, 255]. Thus, UV and FL detectors are more favourable for the detection of K vitamers in a range of samples [39].

FL detectors have high sensitivity and are suitable for analysing samples where vitamin K is present at low concentrations, such as in plasma [39, 229]. It must be acknowledged that vitamin K compounds do not exhibit natural FL. Consequently, its naphthoquinone ring must be reduced to the highly fluorescent hydroquinone analogue for it to be detected using the FL method [39, 234, 235]. This can be achieved either through EC reduction or post-column reduction with a zinc (Zn) or platinum (Pt) catalyst [39, 234, 235]. Although FL detectors have a high sensitivity, extensive sample preparation requirements and chemical derivatisation may decrease the accuracy and precision of this method [246].

While FL detection is typically used for the determination of vitamin K compounds in different matrices, such as various food items and biological material, UV detection is regularly employed for the quantification of MKs, including MK-7, in fermented samples [37, 101, 112, 149, 211, 256]. The method outlined in the USP Monograph also recommends the use of UV detection for the analysis of MK-7 [257], and Jedynak et al. [258] have further developed the suggested approach to obtain an optimised HPLC-UV procedure for the evaluation of MK-7 in samples of different purities. This technique is superior to that outlined in the USP Monograph, as it enables enhanced sensitivity, selectivity, and accuracy, and it also reduces the analytical time and the consumption of both sample and solvent. UV detection is generally carried out at a wavelength of 248 nm or 268 nm, and most studies have used a wavelength of 248 nm, as it results in the highest absorbance [249, 258]. However, 248 nm may be a somewhat non-selective wavelength; hence, other wavelengths, such as 254 nm, 270 nm, or both, can be used for greater selectivity [249]. Despite its comparatively lower selectivity, UV detection offers several advantages relative to the previously discussed detection methods, as it is a reasonably straightforward and time-efficient process and does not require elaborate sample preparation procedures or post-column reactions to facilitate the detection of MK-7. Therefore, based on the success of previous studies employing UV detection for the determination of MK-7 and the merits of this method, UV detection appears to be preferable for the analysis of MK-7, especially in fermented samples.

However, this technique is likely not as suitable to independently discern the geometric isomers of vitamin K due to its inability to accurately identify the separated compounds without a specific analytical standard for each isomer. Conventional HPLC practices rely on reference standards to distinguish the separated compounds, which is accomplished by comparing the unknown peaks from the sample with the peaks from the standard to determine the compound(s) of interest. Although the different isomers have variable retention times owing to their slight

structural dissimilarities (which result in their differing ability to move through the column), the retention time alone is not sufficient to confirm their identity in the absence of distinct reference standards for the *cis* isomers. Consequently, various mass spectrometric methods have been developed in conjunction with liquid chromatography (LC) techniques to enable greater specificity and sensitivity, particularly for analysing isomeric compounds [18].

### 2.11.2 Identification and quantification using mass spectrometry (MS)

LC-MS methods, in comparison to traditional chromatography detection techniques, provide much greater selectivity and sensitivity and are widely used for vitamin analysis [18, 128, 234].

Numerous studies have reported the use of MS techniques with HPLC for the identification and quantification of various vitamin compounds, including vitamin K, in different types of samples [13, 15, 17, 18, 42, 92, 128, 223, 225-227, 230, 232, 234, 235, 237, 239, 242, 245-247]. Several investigations employing standard MS have utilised a single quadrupole mass spectrometer in selected ion monitoring mode [223, 225, 230, 242, 247]. However, tandem MS (MS/MS) systems offer improved selectivity and sensitivity compared to a single quadrupole MS instrument [18, 234]. Dunovska et al. [42], Gentili and Caretti [227], Huang et al. [128], Jäpelt and Jakobsen [234], Jensen et al. [92], Nannapaneni et al. [239], and Tarvainen et al. [18] have implemented MS/MS for the analysis of vitamins in fruits, vegetables, meat, dairy products, infant formulas, human serum and plasma, and fermented foods. However, the majority of these studies centred on the determination of vitamin K1 isomers or the quantity of vitamin K and other fat-soluble vitamins in different sources and did not explicitly consider the quantification of MK-7 isomers.

It has also been noted that the choice of ionisation method in LC-MS and LC-MS/MS applications is an important consideration due to its influence on the observed matrix effects and the assay sensitivity [239]. Electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) are the two most widely used ionisation methods. APCI is the most frequently used ionisation method, and the majority of investigations involving the analysis of vitamin compounds have employed APCI in the positive ionisation mode [18, 128, 223, 225-227, 232, 234, 235, 239, 259]. It has also been suggested that relative to the ESI method, APCI is less susceptible to matrix effects [246]. Many studies have also successfully demonstrated the use of positive ESI in vitamin analysis procedures [13, 15, 17, 42, 128]. ESI is a viable alternative to APCI, as vitamin K compounds are quinones, containing two lone pairs of electrons on the oxygen atoms, and have significant hydrogen-bonding capacity [128]. Furthermore, greater sensitivity has been reported with the ESI method compared to APCI [42, 92].

Various investigations have also used quadrupole time-of-flight (QTOF) MS, which offers the advantage of better mass resolution but has a lower dynamic range than other MS detectors [13, 15, 17, 18]. However, QTOF, in combination with other detectors, such as a diode

array detector (DAD) or a charged aerosol detector (CAD), can provide valuable structural information and is likely to be ideal for the analysis of isomers [18]. This has been effectively demonstrated in investigations using ultra-HPLC-DAD-CAD-QTOF (UHPLC-DAD-CAD-QTOF) to determine MK-7 isomers in dietary supplements [13, 15, 17, 18].

Therefore, in addition to conventional chromatographic approaches that utilise standard detection methods, LC-MS or LC-MS/MS techniques are promising for the determination of vitamin K isomers, including *cis* and *trans* MK-7 isomers, in different sources, such as fermented and other food products and dietary supplements, as they offer greater selectivity and sensitivity in the absence of specific reference standards.

### 2.11.3 Structure determination of MK-7 isomers

The location of *cis* bonds and the specific chemical structure of MK-7 isomers can be determined through NMR spectroscopy [13, 15, 17, 126]. Sitkowski et al. [13] used NMR methods supported by density functional theory (DFT) computations to establish the chemical structure of one of the *cis/trans* isomers of MK-7 ((*E,Z3,E2,ω*)-MK-7) in dietary supplements. Nonetheless, several assumptions and simplifications were required, as this task is relatively complex due to the presence of many repeating isoprenoid units and numerous rotatable single bonds in the structure of MK-7 [13]. Consequently, other researchers have made limited attempts to determine the complete chemical structure of the various *cis/trans* MK-7 isomers [13]. More recently, Bus et al. [126] employed argentation chromatography and NMR techniques to assign chemical shifts and elucidate the structure of thirteen previously unidentified MK-7 isomers and verify the identity of three known isomers (all-*trans* MK-7, (*Z,E5,ω*)-MK-7, and (*E,Z3,E2,ω*)-MK-7) originating from a synthetic MK-7 preparation. The findings of these investigations further support and confirm the existence of multiple *cis* forms of the vitamin in dietary supplements and synthetic formulations. However, it is not yet clear whether the same holds true for fermented products.

Even though it is possible to ascertain the full chemical structure of MK-7 isomers, for the purpose of determining the quality and biological function of natural and synthetic MK-7 preparations, differentiation between MK-7 subtypes and quantification of the proportion of isomers present in samples from different sources are sufficient [15, 17]. Hence, complete structure determination of the identified isomers is not essential.

### 2.11.4 Potential challenges associated with the analysis of MK-7 isomers

Compared to conventional MK-7 analysis, which does not consider the isomer composition of samples, the analysis and measurement of MK-7 isomers entail several challenges. The primary and most significant obstacle is the lack of reference standards for the various *cis* isomers, as only the all-*trans* MK-7 standard is available [15, 17]. Without suitable reference standards, identifying and quantifying *cis* MK-7 isomers in analytical samples are not as simple. Since the *cis* isomers are not naturally occurring compounds and numerous *cis/trans* isomers are

potentially attainable, it is not feasible to have a specific reference standard for each *cis* form of MK-7, which is a likely explanation for the lack of analytical standards for the *cis* isomers.

The series of studies carried out by Szterk et al. [17], Szterk et al. [15], and Sitkowski et al. [13] successfully illustrated the use of only the all-*trans* MK-7 standard for the analysis of MK-7 isomers in dietary supplements. The samples were initially analysed using a stereoselective chromatography column to separate the MK-7 isomers, and the peaks in the resulting chromatograms were compared with those for the all-*trans* MK-7 reference standard. This allowed the all-*trans* isomer to be identified. Compounds pertaining to the peaks that had a similar retention time to all-*trans* MK-7 were speculated to be the *cis/trans* isomers of MK-7. However, identifying the separated compounds based on the retention time alone was unreliable due to the lack of appropriate reference standards; thus, the identity of the compounds had to be confirmed using an alternative approach. Accordingly, MS techniques were used to verify the presence of *cis/trans* isomers by comparing their molecular mass (MM) and fragmentary spectra with those of the all-*trans* MK-7 analytical standard. The MS analysis determined that the fragmentary spectra of the separated compounds were equivalent to the all-*trans* MK-7 reference standard. The isolated compounds also ionised similarly to all-*trans* MK-7 and had a theoretical mass of 649.5 g $mol^{-1}$, corresponding to the MM of MK-7. These observations confirmed that the separated compounds were indeed the *cis/trans* geometric isomers of MK-7.

It must be appreciated that while MS techniques offer high selectivity and sensitivity and are beneficial for the analysis of MK-7 isomers, they are likely to be less convenient than standard HPLC techniques for routine sample analysis. Evaluation of the chromatographic retention time of the peaks representing all-*trans* and *cis* MK-7 can enable the identification of MK-7 isomers using typical HPLC detection methods, such as UV detection. This approach has been recommended in the USP Monograph [257] and is exemplified in the study conducted by Jedynak et al. [258], where the relative retention time (RRT) of the *cis* isomer (the ratio of the retention time of the *cis* isomer to the all-*trans* isomer) is used to distinguish the *cis* isomer from all-*trans* MK-7. The RRT is likely a constant value that is unique for a particular separation process (the RRT for the procedure outlined in the USP Monograph [257] is 1.1, whereas that for the method implemented by Jedynak et al. [258] is 1.15). Any differences in the RRT between sources can be credited to variations in the chromatographic conditions and analytical techniques used. Thus, when developing a new separation methodology, MS can initially be applied to verify the identity of the isolated compounds and establish the RRT for the compound(s) of interest using available reference standards. Subsequently, the RRT can be used to distinguish the target compounds employing conventional HPLC with UV detection, which is likely preferable and far more straightforward than LC-MS for regular analysis. This method will likely be suitable for identifying and quantifying MK-7 isomers present in fermented samples without specific analytical standards for the *cis* forms of the vitamin.

Essentially, limited studies have been devoted to exploring MK-7 isomers, and the production of all-*trans* and *cis* geometric isomers from fermentation has not been assessed. Therefore, there is a notable gap in the literature regarding the effect of fermentation processes on the production of MK-7 isomers. Considering the differing bioactivity of the various isomeric forms of MK-7 and the advantages of microbial fermentation for MK-7 production, the geometric isomer profile achieved from fermentation processes deserves further attention. Accordingly, this research was performed to gain insight into the impact of different synthesis conditions on the MK-7 isomer composition obtained from fermentation and develop an optimal fermentation method to enhance the production of the biologically significant all-*trans* isomer.

## 2.12 References


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# 3

# Materials and Methods

# Chapter 3 – Materials and Methods

This chapter provides a detailed account of the experimental methods and analytical procedures employed in this research.

## 3.1 Chemicals and materials

A wide range of chemicals were used for the various aspects of this study, including the preparation of the bacterial spore suspension, NP synthesis, fermentation experiments, and analytical techniques. All media components were microbiology grade, and all solvents were analytical grade.

Glucose and iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) were obtained from Ajax Finechem Pty Ltd (Taren Point, NSW, Australia), and yeast extract, peptone, tryptone, and soytone were acquired from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Glycerol, soy peptone, dipotassium hydrogen phosphate ($K_2HPO_4$), ethanol, methanol, 2-propanol, *n*-hexane, and $NH_4OH$ (32%) were purchased from Merck Millipore (Burlington, MA, USA). $CaCl_2$, iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), APTES, L-Lys, glutaraldehyde (GA) (25%), and sodium cacodylate ($C_2H_6AsNaO_2$) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Sodium chloride (NaCl) was provided by a domestic supplier, and nutrient (BHI) agar plates were acquired from Fort Richard Laboratories (Auckland, New Zealand). The all-*trans* MK-7 analytical standard (98.1% purity) was purchased from ChromaDex (Los Angeles, CA, USA).

## 3.2 Experimental methods

### 3.2.1 Microorganism and inoculum preparation

*B. subtilis natto* was used for the fermentation experiments, as it is GRAS and deemed the ideal strain for the fermentation-based synthesis of MK-7. The *B. subtilis natto* strain was prepared as described previously [1]. The cells were cultivated in a liquid culture medium containing 0.5% (*w/v*) yeast extract, 1% (*w/v*) tryptone, and 1% (*w/v*) NaCl before streaking on nutrient agar plates. The plates were incubated at 37 °C for 48 h. Following incubation, the cells were scraped off the plates and immersed in a sterilised saline solution (0.9% (*w/v*) NaCl). The solution was then placed in a water bath (PolyScience, IL, USA) at 80 °C for 30 min to inactivate the vegetative cells and induce the production of spores. The cell debris was removed by centrifugation (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 3000 rpm for 10 min. The resulting spore suspension was stored in the refrigerator and used as the inoculum for the fermentation experiments.

Spores were preferred over vegetative cells, as they are a form of long-term cell survival. The bacterial genome is preserved inside the spore, which is metabolically dormant and able to withstand various stresses and survive in nutrient-free and harsh environments. In response to nutrients and favourable environmental conditions, spores can quickly germinate and return to the vegetative state. In contrast, vegetative cells may have reduced viability and be susceptible to mutation during long-term storage, compromising the quality of the inoculum for the fermentation studies and, ultimately, the experimental results.

The plate counting method was used to estimate the number of bacterial spores. A 1 mL aliquot of the bacterial spore suspension was appropriately diluted before streaking on nutrient agar plates. The plates were incubated at 37 °C for 24 h. Subsequently, the number of isolated colonies was counted, and the results were expressed as colony-forming unit/mL (CFU/mL).

## 3.2.2 NP synthesis and characterisation

### 3.2.2.1 Synthesis of naked and surface-functionalised IONs

The co-precipitation technique was used to synthesise the IONs employed in this research, as it is a simple and convenient way to produce a reasonably large quantity of NPs with only a few chemicals. Furthermore, the specific methods that were selected have been used in previous MK-7 fermentation studies to fabricate the same types of IONs for a similar purpose and, thus, are known to be successful for synthesising naked IONs, IONs@APTES, and L-Lys@IONs for the desired application. Following synthesis, the resulting NP powders were stored in the refrigerator and preserved under nitrogen ($N_2$) until required for the characterisation studies and bacterial cell immobilisation procedures.

#### *3.2.2.1.1 Naked IONs*

Naked (uncoated) IONs were synthesised from the co-precipitation of $Fe^{2+}$ and $Fe^{3+}$ ions by an alkali ($NH_4OH$) under an inert atmosphere (Figure 3-1), as described by Ranmadugala et al. [2]. Accordingly, 0.74 g of $FeSO_4 \cdot 7H_2O$ and 1.17 g of $FeCl_3 \cdot 6H_2O$ were dissolved in 50 mL of distilled water. The solution was briskly stirred for 1 h at 70 °C in a $N_2$ atmosphere to prevent oxidation. Afterwards, 5 mL of $NH_4OH$ was quickly added to the reaction mixture, and the solution was stirred for another 1 h until precipitation occurred. Magnetic IONs were produced according to the following reaction (Eq. 3-1):

$$Fe\ (II) + 2Fe\ (III) + 8\ OH \rightarrow Fe_3O_4 + 4\ H_2O \qquad \text{(Eq. 3-1)}$$

A permanent magnet was used to separate the magnetic particles from the non-magnetic particles, and the black precipitate was washed with hot distilled water to remove impurities and dried in an oven (Contherm Thermotec 2000, Contherm Scientific Ltd, Wellington, New Zealand) at 50 °C overnight (24 h).

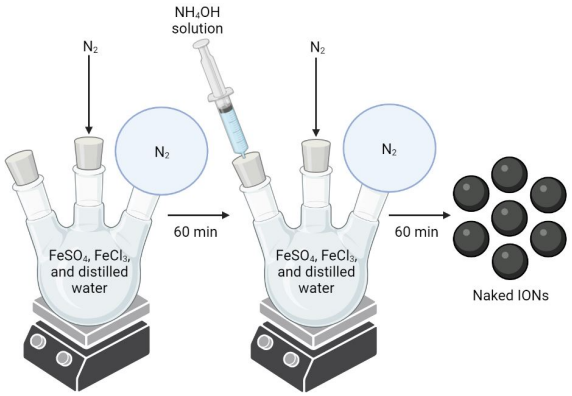


Figure 3-1 Schematic representation of the steps involved in the synthesis of naked IONs using the co-precipitation method

#### *3.2.2.1.2 IONS@APTES*

APTES coating was carried out following the approach implemented by Ebrahiminezhad et al. [3] (Figure 3-2). Uncoated IONs (0.7 g) were dissolved in 25 mL of an absolute ethanol and distilled water mixture (1:1 (*v/v*)) and sonicated (Qsonica-Q800R, Newtown, CT, USA) while kept in an ice bath for 2 min to achieve a uniform dispersion. Subsequently, 2.8 mL of APTES solution was added to the reaction in a $N_2$ atmosphere, and the mixture was rapidly stirred at 40 °C for 2 h. The coated particles were separated with a permanent magnet and washed with absolute ethanol and double-distilled water to remove contaminants. The precipitate was then dried overnight (24 h) in an oven (Contherm Thermotec 2000, Contherm Scientific Ltd, Wellington, New Zealand) at 50 °C.

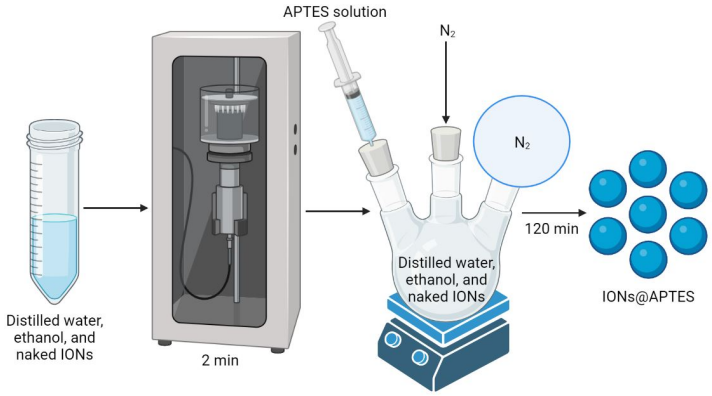


Figure 3-2 Schematic illustration of the APTES functionalisation of naked IONs

#### *3.2.2.1.3 L-Lys@IONs*

The method proposed by Ebrahiminezhad et al. [4] was used to synthesise the L-Lys@IONs (Figure 3-3). $FeSO_4 \cdot 7H_2O$ (0.74 g), $FeCl_3 \cdot 6H_2O$ (1.17 g), and L-Lys (1.6 g) were dissolved in distilled water (50 mL), and the solution was vigorously mixed for 1 h at 70 °C in a $N_2$ atmosphere. After 1 h, 5 mL of $NH_4OH$ was added to the reaction, and the solution was stirred for a further 1.5 h. The magnetic particles were then magnetically collected, and the black

precipitate was washed with hot distilled water to exclude impurities. The particles were dried in an oven (Contherm Thermotec 2000, Contherm Scientific Ltd, Wellington, New Zealand) at 50 °C overnight (24 h).

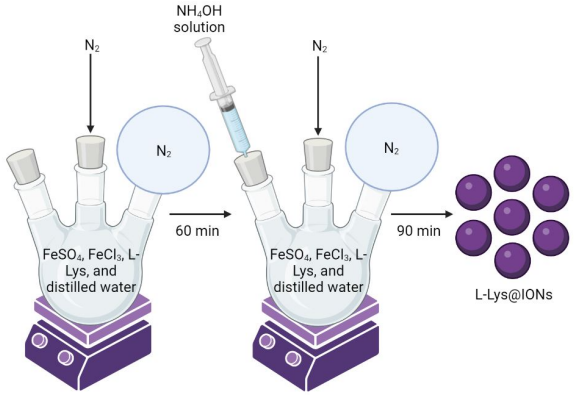


Figure 3-3 Schematic description of the synthesis of L-Lys-functionalised IONs

### 3.2.2.2 Characterisation of the synthesised NPs

#### *3.2.2.2.1 Transmission electron microscopy (TEM)*

The size and morphology of the synthesised NPs were ascertained by TEM (Philips, CM 10, Philips Electron Optics, Eindhoven, The Netherlands). For the TEM analysis, a NP dispersion was prepared in distilled water, and a drop of the solution was put on a carbon-coated copper grid. Images were captured at HT 100 kV.

#### *3.2.2.2.2 Fourier-transform infrared (FTIR) spectroscopy*

FTIR spectroscopy (Bruker VERTEX 70 FTIR spectrometer, Bruker, Kassel, Germany), in the range of 4000-400 $cm^{-1}$, was used to establish the presence of key functional groups and chemical bonds. A pellet, with a NP-to-potassium bromide (KBr) ratio of 1%, was prepared before the FTIR procedure and placed in a hydraulic press for 10 min to create a solid disc. The samples were analysed by the FTIR instrument at room temperature.

#### *3.2.2.2.3 X-ray powder diffraction (XRD)*

The crystal structure of the NPs was determined by XRD (Siemens D5000, Munich, Germany), with a 2-theta (2θ) between 20-90°. The dried powder was packed on a zero-background silicon (Si) holder, and the excess was removed using a brush and straight edge. The analysis was carried out at ambient temperature, 40 mA, and 45 kV, using an exploration range (2θ) between 20-90° and a step size of 0.0530°.

#### *3.2.2.2.4 Scanning electron microscopy (SEM)*

SEM (Hitachi Regulus SU8230 FE-SEM, Tokyo, Japan) was used to visualise the surface structure of the NPs and their interaction with the bacterial cells. The pure IONs (naked,

IONs@APTES, and L-Lys@IONs) were observed in their powdered state, whereas the samples involving bacterial cells were fixed on glass coverslips, as outlined below. Before SEM analysis, all samples (NP powders and fixed bacterial cells) were mounted on aluminium (Al) stubs and coated with Pt using a sputter coater (Hitachi, E1030, Tokyo, Japan). SEM images of pure IONs, free-floating bacterial cells, and cells immobilised with naked and coated IONs were taken at 3 kV.

#### *3.2.2.2.4.1 Cell fixation and sample preparation for SEM*

Bacterial cells in the presence and absence of NPs were fixed on glass coverslips to view the cell morphology and attachment of NPs to the bacterial cell surface via SEM. Although several chemical agents, including formaldehyde (FA), GA, osmium tetroxide ($OsO_4$), and uranyl acetate (UrAc), are available for cell fixation, GA was selected as the fixative agent in this study due to its accessibility and suitability for SEM studies. Previous MK-7 studies [2, 5] have also used GA for cell fixation to visualise the interactions between bacterial cells and various IONs. The surface structure of both the bacterial cells and NPs was preserved and successfully visualised using SEM, and no notable negative impact on the quality of the samples was observed.

Cell fixation was carried out at room temperature using the procedure explained by Ebrahiminezhad et al. [5]. A small drop (approximately 10 µL) of the cell suspension was placed on a glass coverslip. The coverslip was swirled in a circular motion to achieve a thin smear, and the liquid was heat-fixed by passing the bacterial smear through the flame of a Bunsen burner. The cells were chemically fixed using 2.5% (*v/v*) GA in 0.1 M $C_2H_6AsNaO_2$ buffer for 45 min and rinsed with saline (0.9% (*w/v*) NaCl) for 15 min. Cell dehydration was conducted by keeping the coverslip in a series of ethanol concentrations (30, 50, 70, 80, 90, and 95%) for 10 min each. The coverslip was then stored in absolute ethanol for 20 min and subjected to critical point drying (Polaron E3000, Quorum Technologies, East Sussex, England, UK) for 2 h, as opposed to air drying, to protect the surface structure of the cells.

## 3.2.3 Experimental design and statistical analysis

### 3.2.3.1 Preliminary experiments

A preliminary investigation with a linear experimental design was carried out to assess the effect of selected nutrient sources and fermentation conditions on the resulting MK-7 isomer composition. Aspects such as the type and concentration of media components, the fermentation temperature, the length of fermentation, dynamic versus static fermentation, and the fermentation volume were considered at an elementary level to observe the general trends, acquire a basic understanding of the effect of the different variables on MK-7 isomer production, and inform decisions regarding the range of each factor to be examined in greater detail in the optimisation experiments. As this preliminary analysis was rudimentary and purely observational, the isomer concentrations resulting from fermentation were not quantified. Instead, rough conclusions were

drawn from the appearance of all-*trans* and *cis* MK-7 isomer peaks in the LC chromatograms corresponding to each sample (Appendix A).

Three sets of media (Table 3-1) were used for the preliminary experiments. The compositions of media 1 and 3 were arbitrarily derived with guidance from various sources [6, 7], whereas media 2 was obtained from Berenjian et al. [1] and is known to result in a high MK-7 concentration.

Table 3-1 Media compositions used for the preliminary studies

| **Media 1** | **Media 2** | **Media 3** |
|---|---|---|
| Tryptone = 17 g/L | Yeast extract = 5% (*w/v*) | Peptone = 1% (*w/v*) |
| Soytone = 3 g/L | Soy peptone = 18.9% (*w/v*) | NaCl = 0.25% (*w/v*) |
| Glucose = 52.5 g/L | Glycerol = 5% (*v/v*) | Glucose = 0.5% (*w/v*) |
| NaCl = 5 g/L | $K_2HPO_4$ = 0.06% (*w/v*) | |
| $K_2HPO_4$ = 2.5 g/L | | |
| Yeast extract = 8 g/L | | |

#### *3.2.3.1.1 Experiment 1*

The media described in Table 3-1 was prepared in duplicate (A and B), and distilled water was added to achieve a fermentation volume of 50 mL (in a 100 mL shake flask). After sterilisation, all samples were inoculated with 2% (*v/v*) of the bacterial spore suspension and fermented at 37 °C and 120 rpm for 2 days (bioline incubator shaker 8500, Bioline Global Pty Ltd, NSW, Australia). Following fermentation, 3 mL of each sample was extracted for analysis and reconstituted in 3 mL of methanol. These parameters were selected at random and based on past observations and experience.

Definite MK-7 peaks were not observed in the chromatograms for all three types of media. Although the size of the peaks was too small to be recognised by the instrument, the chromatograms pertaining to media 1 (A and B) showed a slight all-*trans* MK-7 peak at approximately 21 min. The lack of clearly defined MK-7 peaks for all samples was speculated to be due to the following:

- Short fermentation time (2 days) – A longer fermentation time may be necessary to enable greater MK-7 production, such that the instrument can discern the all-*trans* and *cis* MK-7 peaks.
- Large fermentation volume (50 mL) – Due to the large fermentation volume, only part of the total sample could be extracted, and all the MK-7 produced during fermentation was not available for analysis.
- Small sample volume used for extraction (3 mL) – A greater sample volume may be required to ensure that a sufficient quantity of MK-7 is obtainable for HPLC.

#### *3.2.3.1.2 Experiment 2*

The above procedure was repeated, and all aspects were identical to experiment 1, except the fermentation time was increased to 4 days to explore the effect of a longer fermentation time on the MK-7 isomer profile.

Overall, the results of the second trial were preferable to the first, as noticeably larger all-*trans* MK-7 peaks were observed for samples A and B of media 1, and both samples for media 2 also showed obvious (although very small) peaks for the all-*trans* isomer. Additionally, while they were not recognised by the instrument, minor all-*trans* MK-7 peaks at around 21 min were visible for media 3 (both samples A and B). These results imply that a longer fermentation time is ideal, as it allows adequate time for a measurable quantity of MK-7 to be produced. It is important to note that a *cis* MK-7 peak was not detected for any of the samples. However, the chromatograms for samples A and B of media 1 showed a small peak at about 24 min, which likely corresponds to the *cis* isomer.

Further to the points noted in section 3.2.3.1.1, the following could also be possible reasons for the small all-*trans* MK-7 peaks that were achieved and the absence of distinguishable peaks for the *cis* isomer:

- Small inoculum volume (2% (*v/v*)) – There may not be enough bacteria to produce sufficient MK-7.
- Low fermentation temperature – Although a temperature of 37 °C is appropriate for the fermentation of *B. subtilis natto*, it may be too low to promote optimal bacterial growth and metabolism.

#### *3.2.3.1.3 Experiment 3*

A third study was conducted employing the same fermentation and analysis procedures as those outlined in section 3.2.3.1.1. However, a few changes were made based on the findings of the first two experiments:

- Fermentation time = 6 days
- Inoculum volume = 5% (*v/v*)
- Fermentation temperature = 40 °C

The results obtained in this experiment were inferior to the previous trial, as the all-*trans* MK-7 peak was smaller for media 1 (A and B) and media 2B, no *cis* isomer peaks were visible for any of the samples, and MK-7 peaks were absent for media 2A and media 3 (A and B). These observations implied that the conditions employed in trial 2 were better than trial 3. However, since more than one variable was different between experiments 2 and 3, it was not possible to ascribe the poor results to any factor in particular, and further investigation was necessary before any reliable conclusions could be drawn.

#### *3.2.3.1.4 Experiment 4*

The outcomes of earlier trials were used to design another experiment. The general approach was identical to the preceding investigations, but an additional media composition was considered, and a few parameters were altered to achieve more promising results. The media compositions are provided in Table 3-2, and the fermentation conditions are detailed below.

Table 3-2 Media compositions employed for trial experiment 4

| **Media 1** | **Media 2** | **Media 3** | **Media 4** |
|---|---|---|---|
| Tryptone = 17 g/L | Yeast extract = 5% (*w/v*) | Peptone = 1% (*w/v*) | Tryptone = 17 g/L |
| Soytone = 3 g/L | Soy peptone = 18.9% (*w/v*) | NaCl = 0.25% (*w/v*) | Soytone = 3 g/L |
| Glucose = 52.5 g/L | Glycerol = 5% (*v/v*) | Glucose = 0.5% (*w/v*) | Glucose = 52.5 g/L |
| NaCl = 5 g/L | $K_2HPO_4$ = 0.06% (*w/v*) | | NaCl = 5 g/L |
| $K_2HPO_4$ = 2.5 g/L | | | $K_2HPO_4$ = 2.5 g/L |
| Yeast extract = 8 g/L | | | Yeast extract = 8 g/L |
| | | | Glycerol = 50 mL/L |

- Fermentation time = 4 days
- Inoculum volume = 5% (*v/v*)
- Fermentation temperature = 37 °C
- Fermentation volume = 6 mL
- Agitation conditions = dynamic/120 rpm (sample A) and static (sample B)
- Extraction volume = 6 mL
- Methanol volume = 1 mL

The results of this trial were encouraging, as potential MK-7 peaks were observed for all samples. Detectable all-*trans* MK-7 peaks at approximately 21 min were noted for each media composition (while not completely discerned by the instrument, a small peak was visible for sample B of media 3). This contrasts previous experiments, where the peak representing the all-*trans* isomer was not obtained for all samples. A *cis* MK-7 peak at around 24 min was also evident for both samples of media 2 (A and B) and sample A for media 4.

Unlike the earlier investigations, the small fermentation volume used in this experiment allowed the entire sample to be extracted. Thus, all the MK-7 produced during fermentation was analysed rather than just a fraction of the total quantity, which was the case when using a larger fermentation volume (50 mL). The methanol volume was also reduced to 1 mL to avoid substantially diluting the MK-7. Both of these factors likely contributed to increasing the amount of MK-7 available for analysis and improved the detection of MK-7 peaks by the HPLC system, especially for the *cis* isomer, which is synthesised in much smaller quantities relative to all-*trans* MK-7.

The effect of glycerol on MK-7 isomer production was considered in this experiment by comparing the peaks in the chromatograms of media 1 and 4, which had the same composition,

except media 4 also contained glycerol. Glycerol is known to have a positive impact on MK-7 production, but this has not been explored from the perspective of MK-7 isomers. The size of the all-*trans* MK-7 peak for samples A and B of media 1 was slightly greater than that for the respective samples of media 4, indicating that glycerol does not enhance the production of the bioactive isomer. The influence of glycerol on MK-7 isomer production may be different when combined with other types and concentrations of nutrients, as one or more media components may exert a collective effect on the response; however, it was not possible to examine this in a linear study.

Furthermore, the impact of dynamic versus static fermentation conditions was evaluated by fermenting one sample (A) for each media at 120 rpm (dynamic) and the other (B) in the absence of agitation (static). The all-*trans* MK-7 peak for most of the A samples was larger than the B samples for each kind of media, suggesting that dynamic fermentation conditions improve MK-7 production compared to static conditions. Agitation during fermentation reduces heat, oxygen, and mass transfer gradients, resulting in a homogenous fermentation environment relative to static conditions, facilitating cell growth, metabolism, and product formation.

Although the results achieved in this trial experiment were the most favourable, they cannot be attributed to any specific factor, as many variables were altered from the previous trial. Moreover, the reliability of the results may be compromised due to the lack of replicate samples, and additional studies are required to establish more accurate and reliable trends in the data.

#### *3.2.3.1.5 Experiment 5*

The concentration range of each nutrient for the screening study was initially assessed in a trial experiment to determine the appropriate design space in which to screen and optimise the concentration of significant media components. Ten different nutrients (glucose, glycerol, yeast extract, soy peptone, peptone, tryptone, soytone, $K_2HPO_4$, $CaCl_2$, and NaCl) were selected using the composition of media 1-4 as a guide.

Generally, the concentration of carbon and nitrogen sources in fermentation media is greater than salt sources. Since the studied media (media 1-4) had a diverse concentration of nutrients, a wide concentration range was first explored to gain a broad perspective. Accordingly, for the selected media components, the concentration of carbon and nitrogen sources was considered between 0.5-10% (*w/w* for all dry nutrients and *v/v* for glycerol), and the salt sources were examined between 0.05-2% (*w/w*).

A basic design of experiments (DOE) plan was created (Table 3-3) and used to prepare the samples (6 mL). The samples were sterilised, inoculated with 5% (*v/v*) of the microbial spore suspension, and fermented at 37 °C and 120 rpm for 6 days (bioline incubator shaker 8500, Bioline Global Pty Ltd, NSW, Australia). Following fermentation, the whole sample was extracted and dissolved in 1 mL of methanol for HPLC analysis.

Table 3-3 Basic DOE plan to determine a suitable range of nutrient concentrations for the screening and optimisation studies

| | Media Components (g or µL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Sample** | **Glucose** | **Glycerol** | **Yeast Extract** | **Soy Peptone** | **Peptone** | **Tryptone** | **Soytone** | **$K_2HPO_4$** | **$CaCl_2$** | **NaCl** |
| 1 | 0.6 | 30 | 0.03 | 0.03 | 0.6 | 0.03 | 0.03 | 0.12 | 0.12 | 0.003 |
| 2 | 0.6 | 600 | 0.03 | 0.03 | 0.03 | 0.6 | 0.03 | 0.003 | 0.12 | 0.12 |
| 3 | 0.6 | 600 | 0.6 | 0.03 | 0.03 | 0.03 | 0.6 | 0.003 | 0.003 | 0.12 |
| 4 | 0.6 | 600 | 0.6 | 0.6 | 0.03 | 0.03 | 0.03 | 0.12 | 0.003 | 0.003 |
| 5 | 0.03 | 600 | 0.6 | 0.6 | 0.6 | 0.03 | 0.03 | 0.003 | 0.12 | 0.003 |
| 6 | 0.6 | 30 | 0.6 | 0.6 | 0.6 | 0.6 | 0.03 | 0.003 | 0.003 | 0.12 |
| 7 | 0.03 | 600 | 0.03 | 0.6 | 0.6 | 0.6 | 0.6 | 0.003 | 0.003 | 0.003 |
| 8 | 0.6 | 30 | 0.6 | 0.03 | 0.6 | 0.6 | 0.6 | 0.12 | 0.003 | 0.003 |
| 9 | 0.6 | 600 | 0.03 | 0.6 | 0.03 | 0.6 | 0.6 | 0.12 | 0.12 | 0.003 |
| 10 | 0.03 | 600 | 0.6 | 0.03 | 0.6 | 0.03 | 0.6 | 0.12 | 0.12 | 0.12 |
| 11 | 0.03 | 30 | 0.6 | 0.6 | 0.03 | 0.6 | 0.03 | 0.12 | 0.12 | 0.12 |
| 12 | 0.6 | 30 | 0.03 | 0.6 | 0.6 | 0.03 | 0.6 | 0.003 | 0.12 | 0.12 |
| 13 | 0.03 | 600 | 0.03 | 0.03 | 0.6 | 0.6 | 0.03 | 0.12 | 0.003 | 0.12 |
| 14 | 0.03 | 30 | 0.6 | 0.03 | 0.03 | 0.6 | 0.6 | 0.003 | 0.12 | 0.003 |
| 15 | 0.03 | 30 | 0.03 | 0.6 | 0.03 | 0.03 | 0.6 | 0.12 | 0.003 | 0.12 |
| 16 | 0.03 | 30 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.003 | 0.003 | 0.003 |
| 17 | 0.315 | 315 | 0.315 | 0.315 | 0.315 | 0.315 | 0.315 | 0.0615 | 0.0615 | 0.0615 |
| 18 | 0.315 | 315 | 0.315 | 0.315 | 0.315 | 0.315 | 0.315 | 0.0615 | 0.0615 | 0.0615 |
| 19 | 0.315 | 315 | 0.315 | 0.315 | 0.315 | 0.315 | 0.315 | 0.0615 | 0.0615 | 0.0615 |

**Carbon and Nitrogen Sources:**
0.03 g and 30 µL = 0.5% (*w/v* or *v/v*)
0.315 g and 315 µL = 5.25% (*w/v* or *v/v*)
0.6 g and 600 µL = 10% (*w/v* or *v/v*)

**Salt Sources:**
0.003 g = 0.05% (*w/v*)
0.0615 g = 1.025% (*w/v*)
0.12 g = 2% (*w/v*)

**Total Sample Volume = 6 mL**

Exploring an appropriate range of nutrient concentrations is essential when developing an optimal fermentation media. Low nutrient concentrations may not adequately support microbial growth, metabolism, and product formation; conversely, high concentrations can decrease the water activity and place osmotic stress on the bacterial cells, resulting in cell death.

MK-7 peaks were not visible for the majority of the samples. The all-*trans* isomer peak was observed for samples 11, 15, 16, and 18, and only samples 15 and 16 showed a *cis* MK-7 peak. Sample 16 had the lowest concentration of all nutrients and the largest all-*trans* MK-7 peak. The second largest all-*trans* isomer peak was obtained for sample 15, which had two nitrogen and two salt sources at their highest concentration. In contrast, sample 11 had three nitrogen and all salt sources at their highest concentration, and sample 18 had all nutrients at their intermediate concentration. These conditions resulted in a very small all-*trans* MK-7 peak for both samples. In addition, samples 17 and 19, identical to sample 18, did not display any MK-7 peaks, suggesting that the results have low reliability and reproducibility at these nutrient concentrations.

The lack of MK-7 peaks in samples with high nutrient concentrations implies that the cumulative effect of many nutrients at high concentrations likely subjects the cells to osmotic stress. It is evident that the investigated range of concentrations were too high and inapt to support MK-7 production; hence, a lower maximum concentration of all nutrient sources is potentially ideal for maintaining osmotic balance and promoting bacterial growth, metabolism, and MK-7 synthesis.

Overall, the results of the preliminary investigation (experiments 1-5) indicate that a small fermentation volume (to enable extraction of the entire sample), an inoculum volume of 5% (*v/v*), a fermentation temperature of 37 °C, an agitation speed of 120 rpm, a fermentation time of 4-6 days, and a lower span of nutrient concentrations provide a suitable basis for further studies.

#### 3.2.3.2 Optimisation of the fermentation media and key fermentation parameters

Despite the value of linear experiments in establishing a basic understanding of the influence of different variables on the MK-7 isomer profile attained from fermentation, it only allows the impact of each factor to be assessed independently and does not account for the possible combined or interactive effects of the factors. Examining the interactions between variables is imperative, as the contribution of each factor to the overall fermentation process is not isolated. Instead, the impact of the different variables on the production of the desired product is often interrelated and collectively affects the fermentation efficiency. Therefore, a DOE approach was employed to consider both the individual and interactive effects of the various factors to develop the ideal fermentation media and ascertain the optimal value of important fermentation parameters.

The MODDE version 13 software (Sartorius, Gottingen, Germany) was used to create the design matrices, develop the regression models, and determine the optimum level of the media components, inoculum concentration, fermentation temperature, agitation speed, and length of

fermentation to maximise the all-*trans* MK-7 concentration and minimise the concentration of the *cis* isomer.

The optimal value of the media components and operating conditions was established using multiple linear regression (MLR), in which a line of best fit was determined for the data by minimising the sum of squares of the errors (residuals) resulting from differences in the observed experimental values and the values predicted by the developed model, such that the vertical distance from the data points to the regression line was minimised. Any outlying data points were removed to improve the accuracy and fit of the regression model.

A second-order polynomial regression model (Eq. 3-2) was generated for each response using the experimental data.

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j \qquad \text{(Eq. 3-2)}$$

Where $Y$ represents the all-*trans* or *cis* MK-7 isomer concentration; $b_0$ is a constant term; $b_i$, $b_{ii}$, and $b_{ij}$ are the coefficients of the linear, quadratic, and synergistic effects, respectively; and $X_i$ and $X_j$ correspond to the significant factors.

The $R^2$-value was used to express the quality of the fit for the developed regression models, and statistical significance was determined using the analysis of variance (ANOVA) test and accepted at $p < 0.1$. While it is appreciated that significance is commonly accepted at $p < 0.05$ (95% confidence interval (CI)), a significance level of $p < 0.1$ (90% CI) was selected for the optimisation experiments (fermentation media and key fermentation parameters). The $p$-value for most variables was borderline (slightly more than 0.05) and not deemed statistically significant based on the experimental results. However, the significance of these factors in MK-7 fermentation has been established in past studies. Thus, ignoring the potential significance of these variables simply due to a low $p$-value would reduce the validity of the overall analysis. Accordingly, a significance level of $p < 0.1$ was preferred for these investigations, as it provided meaningful results and was more suitable for the factors considered.

#### *3.2.3.2.1 Fermentation media*

##### *3.2.3.2.1.1 <u>Screening study</u>*

Assorted medium components (carbon, nitrogen, and salt sources) that enhanced MK-7 production in the preliminary studies and previous investigations [1, 7-13] were initially screened within a suitable range of concentrations, and their impact on the MK-7 isomer composition was evaluated. A Placket Burman design (PBD) was used to examine the individual effects of the different factors on the all-*trans* and *cis* isomer concentrations attained from fermentation. A PBD is a standard two-level fractional factorial screening design that allows the main effects of each factor to be estimated and the maximum amount of information to be extracted with the lowest number of experimental runs, saving time and resources. This design was deemed suitable for screening, as it allowed the important nutrients that significantly impact MK-7 isomer production

to be determined from 10 possible nutrient choices in the smallest number of experimental runs (19), reducing the number of nutrients investigated in the optimisation study. However, a PBD only allows the main effects of each factor to be examined and does not account for the possible interactions between variables. Nevertheless, estimating interactive effects was not essential during the screening stage, and this was considered in the optimisation study. Each factor was assessed at three levels (low, intermediate, and high), and the experimental plan for the screening study is outlined in Table 3-4.

Table 3-4 DOE plan for the screening of various carbon, nitrogen, and salt sources

| | Media Components (g or µL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Sample** | **Glucose** | **Glycerol** | **Yeast Extract** | **Soy Peptone** | **Peptone** | **Tryptone** | **Soytone** | **$K_2HPO_4$** | **$CaCl_2$** | **NaCl** |
| 1 | 0.12 | 0 | 0 | 0 | 0.12 | 0 | 0 | 0.06 | 0.06 | 0 |
| 2 | 0.12 | 120 | 0 | 0 | 0 | 0.12 | 0 | 0 | 0.06 | 0.06 |
| 3 | 0.12 | 120 | 0.12 | 0 | 0 | 0 | 0.12 | 0 | 0 | 0.06 |
| 4 | 0.12 | 120 | 0.12 | 0.12 | 0 | 0 | 0 | 0.06 | 0 | 0 |
| 5 | 0 | 120 | 0.12 | 0.12 | 0.12 | 0 | 0 | 0 | 0.06 | 0 |
| 6 | 0.12 | 0 | 0.12 | 0.12 | 0.12 | 0.12 | 0 | 0 | 0 | 0.06 |
| 7 | 0 | 120 | 0 | 0.12 | 0.12 | 0.12 | 0.12 | 0 | 0 | 0 |
| 8 | 0.12 | 0 | 0.12 | 0 | 0.12 | 0.12 | 0.12 | 0.06 | 0 | 0 |
| 9 | 0.12 | 120 | 0 | 0.12 | 0 | 0.12 | 0.12 | 0.06 | 0.06 | 0 |
| 10 | 0 | 120 | 0.12 | 0 | 0.12 | 0 | 0.12 | 0.06 | 0.06 | 0.06 |
| 11 | 0 | 0 | 0.12 | 0.12 | 0 | 0.12 | 0 | 0.06 | 0.06 | 0.06 |
| 12 | 0.12 | 0 | 0 | 0.12 | 0.12 | 0 | 0.12 | 0 | 0.06 | 0.06 |
| 13 | 0 | 120 | 0 | 0 | 0.12 | 0.12 | 0 | 0.06 | 0 | 0.06 |
| 14 | 0 | 0 | 0.12 | 0 | 0 | 0.12 | 0.12 | 0 | 0.06 | 0 |
| 15 | 0 | 0 | 0 | 0.12 | 0 | 0 | 0.12 | 0.06 | 0 | 0.06 |
| 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | 0.06 | 60 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.03 | 0.03 | 0.03 |
| 18 | 0.06 | 60 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.03 | 0.03 | 0.03 |
| 19 | 0.06 | 60 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 | 0.03 | 0.03 | 0.03 |

**Carbon and Nitrogen Sources:**
0 g and 0 µL = 0% (*w/v* or *v/v*)
0.06 g and 60 µL = 1% (*w/v* or *v/v*)
0.12 g and 120 µL = 2% (*w/v* or *v/v*)

**Salt Sources:**
0 g = 0% (*w/v*)
0.03 g = 0.5% (*w/v*)
0.06 g = 1% (*w/v*)

**Total Sample Volume = 6 mL**

#### *3.2.3.2.1.2 Optimisation study*

A central composite face-centred (CCF) design, a type of response surface design with star points at the centre of each face of the factorial space, was employed to optimise the significant variables determined from the screening stage, and response surface methodology (RSM) was applied to analyse the results. Since there were fewer nutrients, a more comprehensive study design was appropriate to investigate possible factor interactions and develop a quadratic model for the responses (all-*trans* and *cis* isomer concentrations). The experimental plan for the optimisation study is shown in Table 3-5.

Table 3-5 DOE plan to optimise the concentration of significant nutrients

| | **Media Components (g)** | | | | |
|---|---|---|---|---|---|
| **Sample** | **Glucose** | **Yeast Extract** | **Soy Peptone** | **Tryptone** | **$CaCl_2$** |
| 1 | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 |
| 2 | 0.12 | 0.06 | 0.06 | 0.06 | 0.006 |
| 3 | 0.06 | 0.12 | 0.06 | 0.06 | 0.006 |
| 4 | 0.12 | 0.12 | 0.06 | 0.06 | 0.06 |
| 5 | 0.06 | 0.06 | 0.12 | 0.06 | 0.006 |
| 6 | 0.12 | 0.06 | 0.12 | 0.06 | 0.06 |
| 7 | 0.06 | 0.12 | 0.12 | 0.06 | 0.06 |
| 8 | 0.12 | 0.12 | 0.12 | 0.06 | 0.006 |
| 9 | 0.06 | 0.06 | 0.06 | 0.12 | 0.006 |
| 10 | 0.12 | 0.06 | 0.06 | 0.12 | 0.06 |
| 11 | 0.06 | 0.12 | 0.06 | 0.12 | 0.06 |
| 12 | 0.12 | 0.12 | 0.06 | 0.12 | 0.006 |
| 13 | 0.06 | 0.06 | 0.12 | 0.12 | 0.06 |
| 14 | 0.12 | 0.06 | 0.12 | 0.12 | 0.006 |
| 15 | 0.06 | 0.12 | 0.12 | 0.12 | 0.006 |
| 16 | 0.12 | 0.12 | 0.12 | 0.12 | 0.06 |
| 17 | 0.06 | 0.063 | 0.063 | 0.063 | 0.033 |
| 18 | 0.12 | 0.063 | 0.063 | 0.063 | 0.033 |
| 19 | 0.063 | 0.06 | 0.063 | 0.063 | 0.033 |
| 20 | 0.063 | 0.12 | 0.063 | 0.063 | 0.033 |
| 21 | 0.063 | 0.063 | 0.06 | 0.063 | 0.033 |
| 22 | 0.063 | 0.063 | 0.12 | 0.063 | 0.033 |
| 23 | 0.063 | 0.063 | 0.063 | 0.06 | 0.033 |
| 24 | 0.063 | 0.063 | 0.063 | 0.12 | 0.033 |
| 25 | 0.063 | 0.063 | 0.063 | 0.063 | 0.006 |
| 26 | 0.063 | 0.063 | 0.063 | 0.063 | 0.06 |
| 27 | 0.063 | 0.063 | 0.063 | 0.063 | 0.033 |

| | | | | | |
|---|---|---|---|---|---|
| 28 | 0.063 | 0.063 | 0.063 | 0.063 | 0.033 |
| 29 | 0.063 | 0.063 | 0.063 | 0.063 | 0.033 |

**Carbon and Nitrogen Sources:**
0.06 g = 1% (*w/v*)
0.063 g = 1.05% (*w/v*)
0.12 g = 2% (*w/v*)

**Salt Sources:**
0.006 g = 0.1% (*w/v*)
0.033 g = 0.55% (*w/v*)
0.06 g = 1% (*w/v*)

**Total Sample Volume = 6 mL**

#### *3.2.3.2.1.3 Validation and monitoring study*

The optimum concentration of the media components to maximise the concentration of the all-*trans* isomer and minimise the production of *cis* MK-7 was determined by solving the regression equations within the design space. Three replicate samples were prepared with the nutrient concentrations proposed by the MODDE software and fermented under the conditions used in the screening and optimisation experiments. The all-*trans* and *cis* isomer concentrations obtained from fermentation were compared with the software prediction to validate the experimental results.

A time-course study was then conducted to monitor trends in MK-7 isomer production, bacterial growth, and pH during fermentation employing the optimum media composition. The fermentation conditions were the same as the screening and optimisation experiments and validation study. The samples were prepared in triplicate, and three were harvested each day of fermentation (days 0-6) to measure the all-*trans* and *cis* MK-7 isomer concentrations, microbial growth, and pH.

### *3.2.3.2.2 Key fermentation parameters*

A CCF design and RSM were used to evaluate and optimise the value of essential fermentation parameters, specifically the inoculum concentration, agitation speed, fermentation temperature, and duration of fermentation. Since these factors are known to have a significant impact on the product concentration, screening was not carried out, unlike the media experiments. The range for each fermentation parameter was derived from the preliminary studies and prior investigations [1, 6, 9, 12, 14-18], and the DOE plan is shown in Table 3-6.

Table 3-6 DOE plan for the optimisation of key fermentation parameters

| Sample | Inoculum Size (% (*v/v*)) | Temperature (°C) | Agitation Speed (rpm) | Fermentation Time (days) |
|---|---|---|---|---|
| 1 | 2 | 35 | 100 | 4 |
| 2 | 10 | 35 | 100 | 4 |
| 3 | 2 | 45 | 100 | 4 |
| 4 | 10 | 45 | 100 | 4 |
| 5 | 2 | 35 | 200 | 4 |
| 6 | 10 | 35 | 200 | 4 |

| | | | | |
|---|---|---|---|---|
| 7 | 2 | 45 | 200 | 4 |
| 8 | 10 | 45 | 200 | 4 |
| 9 | 2 | 35 | 100 | 10 |
| 10 | 10 | 35 | 100 | 10 |
| 11 | 2 | 45 | 100 | 10 |
| 12 | 10 | 45 | 100 | 10 |
| 13 | 2 | 35 | 200 | 10 |
| 14 | 10 | 35 | 200 | 10 |
| 15 | 2 | 45 | 200 | 10 |
| 16 | 10 | 45 | 200 | 10 |
| 17 | 2 | 40 | 150 | 7 |
| 18 | 10 | 40 | 150 | 7 |
| 19 | 6 | 35 | 150 | 7 |
| 20 | 6 | 45 | 150 | 7 |
| 21 | 6 | 40 | 100 | 7 |
| 22 | 6 | 40 | 200 | 7 |
| 23 | 6 | 40 | 150 | 4 |
| 24 | 6 | 40 | 150 | 10 |
| 25 | 6 | 40 | 150 | 7 |
| 26 | 6 | 40 | 150 | 7 |
| 27 | 6 | 40 | 150 | 7 |

**Level of Each Factor:** **Total Sample Volume = 6 mL**

Low

Intermediate

High

### 3.2.3.3 NP experiments

The optimal media composition and fermentation conditions were applied to prepare the sample media and conduct fermentation. A larger media volume (20 mL) was used to facilitate the attachment of NPs to the surface of the bacterial cells and accommodate sample measurements. A NP stock solution (0.01 g/mL) was prepared with distilled water. A different volume of the stock solution was added to each sample to achieve the various NP concentrations considered (0-600 µg/mL), and a suitable volume of distilled water was added to all samples to obtain an equal volume. The components comprising the different samples and their respective quantities are listed in Table 3-7. The samples for each type of ION (naked, IONs@APTES, and L-Lys@IONs) were prepared separately and in triplicate (three samples for each NP concentration).

Table 3-7 Sample composition for each NP concentration

| Sample | Media Volume (mL) | NP Stock Volume (mL) | Distilled Water Volume (mL) | Total Volume (mL) |
|---|---|---|---|---|
| **0 µg/mL (control)** | 20 | 0.0 | 1.2 | 21.2 |
| **100 µg/mL** | 20 | 0.2 | 1.0 | 21.2 |
| **200 µg/mL** | 20 | 0.4 | 0.8 | 21.2 |
| **300 µg/mL** | 20 | 0.6 | 0.6 | 21.2 |
| **400 µg/mL** | 20 | 0.8 | 0.4 | 21.2 |
| **500 µg/mL** | 20 | 1.0 | 0.2 | 21.2 |
| **600 µg/mL** | 20 | 1.2 | 0.0 | 21.2 |

ANOVA was used to evaluate statistical significance, and the mean values of different groups were compared with a two-sample *t*-test. The data were described as the mean ± standard error (SE) of three replicate samples, and statistical significance was accepted at $p < 0.05$. The typical significance level of 0.05 was fit for these studies, as it was appropriate for the variables examined and allowed noteworthy results, in agreement with previous reports, to be attained.

A time-course monitoring study was conducted to assess the variation in MK-7 isomer production, microbial growth, and pH during fermentation in the presence of the optimal naked and amine-functionalised ION concentration. The fermentation conditions were identical to the initial experiments. The samples were prepared in triplicate, and three were harvested each day of fermentation (days 0-7) to analyse the all-*trans* and *cis* MK-7 isomer concentrations, bacterial growth, and pH.

### 3.2.3.4 Environmental factors and storage conditions study

The optimum media composition and value of key fermentation parameters were employed to prepare and ferment the samples, and MK-7 was extracted from the samples, as discussed in section 3.2.5. The extracted MK-7 samples (contained in transparent McCartney bottles) were then exposed to various environmental and storage conditions to explore the short- and long-term impact of these factors on the MK-7 isomer composition.

Several temperature (low (4 °C), ambient (20 °C), and high (100 °C)), light (no light/dark, ambient light, and UV light), and oxygen (exposed to atmospheric oxygen and not exposed to atmospheric oxygen) conditions were selected to simulate likely storage environments for fermented MK-7 consumer end products, such as MK-7-enriched fortified or functional foods and dietary supplements. Possible conditions that fermented MK-7 could be exposed to during the manufacture of these products were also considered.

The effect of short-term exposure to the different temperature, light, and oxygen conditions on the isomer profile of fermented MK-7 was first examined. The samples were

prepared in triplicate and subjected to the factors outlined in Table 3-8 for 0, 3, 6, and 9 days to investigate the variation in the isomer composition over a brief timeframe for all conditions.

Table 3-8 Environmental and storage conditions for the short-term exposure study

| Sample | Conditions |
|---|---|
| **1** | Low temperature (4 °C) and exposed to atmospheric oxygen = stored in the fridge with the lid off |
| **2** | Low temperature (4 °C) and not exposed to atmospheric oxygen = stored in the fridge with the lid on (purged with $N_2$) |
| **3** | High temperature (100 °C) and exposed to atmospheric oxygen = stored in the oven with the lid off |
| **4** | High temperature (100 °C) and not exposed to atmospheric oxygen = stored in the oven with the lid on (purged with $N_2$) |
| **5** | No light and exposed to atmospheric oxygen = stored in the dark with the lid off at ambient temperature (by default) |
| **6** | No light and not exposed to atmospheric oxygen = stored in the dark with the lid on (purged with $N_2$) at ambient temperature (by default) |
| **7** | Ambient light and exposed to atmospheric oxygen = stored in ambient light (lamp) with the lid off at ambient temperature (by default) |
| **8** | Ambient light and not exposed to atmospheric oxygen = stored in ambient light (lamp) with the lid on (purged with $N_2$) at ambient temperature (by default) |
| **9** | UV light and exposed to atmospheric oxygen = stored in UV light (lamp) with the lid off at ambient temperature (by default) |
| **10** | UV light and not exposed to atmospheric oxygen = stored in UV light (lamp) with the lid on (purged with $N_2$) at ambient temperature (by default) |

The optimum storage conditions, which resulted in the least deterioration of all-*trans* MK-7, determined from the short-term investigation, were further analysed in a monitoring study to explore the stability of the all-*trans* isomer and variation in the isomer profile over an extended period. Accordingly, the samples were prepared in triplicate and stored at a low temperature (4 °C) with minimal oxygen exposure in the absence of light for 8 weeks. The MK-7 isomer composition was analysed after 0, 1, 2, 3, 4, 5, 6, 7, and 8 weeks of storage.

Statistical significance was determined by ANOVA, and a two-sample *t*-test was used to compare the mean values of different groups. The data were reported as the mean ± standard deviation (SD) of three replicates, and statistical significance was accepted at $p < 0.05$. Similar to the NP studies, the standard significance level of 0.05 was considered appropriate to achieve meaningful outcomes for the variables assessed in the storage investigation.

### 3.2.4 Fermentation process

The fermentation media was either prepared individually in McCartney bottles or in bulk in a Schott bottle before dispensing into suitable fermentation vessels (McCartney bottles or shake flasks), depending on the nature of the experiments. The media, fermentation vessels, and all relevant equipment required for sample inoculation were sterilised in an autoclave (TOMY SX-700E, Tokyo, Japan) at 121 °C for 20 min. The media was allowed to cool before sample preparation and inoculation with the bacterial spore suspension. The experimental samples were then fermented aerobically in an environment relevant to the aspect(s) studied. The overall experimental workflow is outlined in (Figure 3-4).

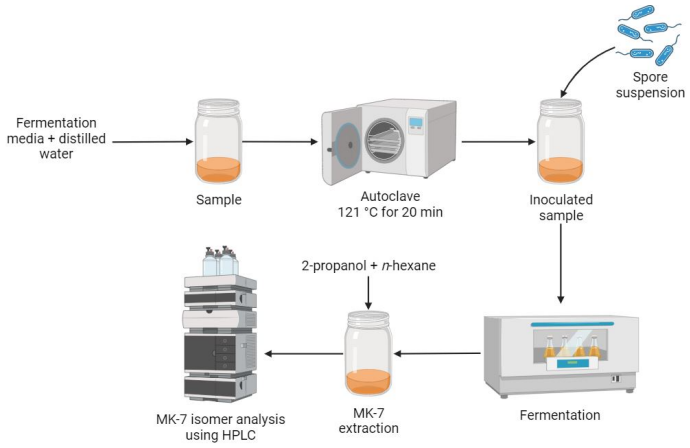


Figure 3-4 Experimental workflow

#### 3.2.4.1 Screening and optimisation experiments

All screening and optimisation experiments were carried out in McCartney bottles with a fermentation volume of 6 mL to allow extraction of the entire sample and eliminate any errors associated with sampling from the fermentation media. The samples were prepared separately and sterilised before inoculating with the bacterial spore suspension, as described above. All samples were fermented in a shaker incubator (bioline incubator shaker 8500, Bioline Global Pty Ltd, NSW, Australia) according to the DOE plan.

#### 3.2.4.2 NP studies

The fermentation experiments involving NPs were performed in 50 mL shake flasks with a sample volume of just over 20 mL. The media was prepared and sterilised in bulk, as previously outlined, and added to each sample prior to inoculation.

The bacterial cells were immobilised with uncoated and amine-functionalised IONs (IONs@APTES and L-Lys@IONs). A schematic representation of the decoration of bacterial cells with naked IONs, IONS@APTES, and L-Lys@IONs is illustrated in Figure 3-5. Each type of NP was individually dispersed in sterilised distilled water by sonication (Qsonica-Q800R, Newtown, CT, USA) for 2 min to prepare a stock solution (0.01 g/mL), and different

concentrations (0, 100, 200, 300, 400, 500, and 600 µg/mL) of the NP stock were added to the samples. The samples for each type of NP were prepared separately and fermented under optimum conditions in a shaker incubator (bioline incubator shaker 8500, Bioline Global Pty Ltd, NSW, Australia).

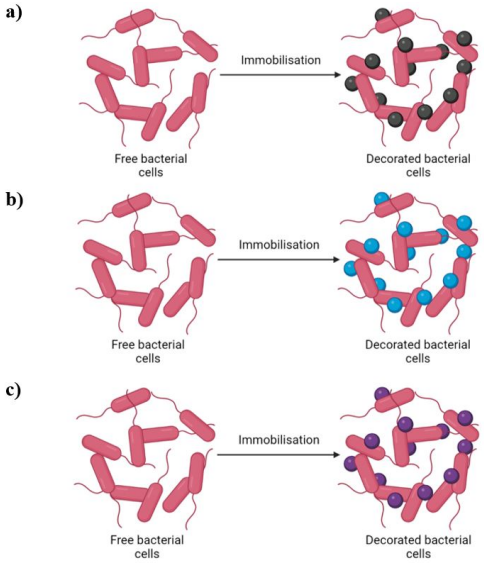


Figure 3-5 Bacterial cell immobilisation with a) naked IONs, b) IONs@APTES, and c) L-Lys@IONs

### 3.2.4.3 Storage investigations

The samples for both the short- and long-term exposure studies were fermented in McCartney bottles. Owing to the large number of samples required for the storage experiments, the media was prepared and sterilised in bulk. After sterilisation, 10 mL of the media was added to each McCartney bottle before inoculating with the bacterial spore suspension. A slightly greater fermentation volume was selected for these experiments to account for sample losses due to evaporation and ensure that a sufficient quantity remained for extraction. The samples were fermented in an optimal environment in a shaker incubator (bioline incubator shaker 8500, Bioline Global Pty Ltd, NSW, Australia).

## 3.2.5 MK-7 extraction

MK-7 was extracted from the samples prior to analysis using a mixture of 2-propanol and *n*-hexane in a ratio of 1:2 (*v*/*v*) and a liquid-to-organic ratio of 1:4 (*v*/*v*) [1]. The mixture was vigorously shaken for 2 min using a vortex mixer and centrifuged (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 3000 rpm for 10 min to separate the two phases. Since MK-7 is a lipid-soluble vitamin, it preferentially dissolves in *n*-hexane, a non-polar solvent. Therefore, the upper hexane layer was removed from the aqueous phase and evaporated under a vacuum to recover the extracted MK-7. The extracted samples were reconstituted in 0.75 mL or 1 mL of methanol, filtered through a 0.2 µm syringe filter, and dispensed in HPLC vials for analysis.

The amount of methanol used for dilution depended on the fermentation volume relative to the volume extracted and the factors investigated in each study. A methanol volume of 1 mL was used for the optimisation experiments, as the small fermentation volume enabled the entire sample to be extracted. This reduced any errors due to sampling from the fermentation broth, and the concentration of MK-7 isomers in these samples was adequately detected by the HPLC system when diluted with 1 mL of methanol. Since the fermentation volume was large for the NP investigations, only a fraction of the total sample was extracted; consequently, not all the MK-7 produced during fermentation could be analysed. Hence, the methanol volume was reduced to 0.75 mL to increase the concentration of all-*trans* and *cis* MK-7 in the HPLC samples and allow the corresponding peaks to be distinguished by the instrument. For the short- and long-term storage studies, although the small fermentation volume allowed the whole sample to be extracted, exposure to the different environmental factors and storage conditions decreased the concentration of MK-7 isomers, especially the *cis* isomer, in the samples over the investigated timeframes. Thus, a methanol volume of 0.75 mL was the most appropriate to permit the equipment to detect low concentrations of the isomers. It was not feasible to use a methanol volume less than 0.75 mL, as at lower volumes, the liquid level in the HPLC vial was insufficient to enable sampling by the instrument.

### 3.2.6 Analytical methods

#### 3.2.6.1 HPLC

A MK-7 calibration curve (Figure 3-6) was constructed based on the peak area corresponding to known concentrations of the analytical standard and used to infer the MK-7 concentration of the fermented samples. The MK-7 calibration curve was linear between 0.1 mg/L and 50 mg/L ($R^2$ = 0.99). A single curve was suitable to determine both the all-*trans* and *cis* MK-7 concentrations due to the isomeric nature of these compounds.

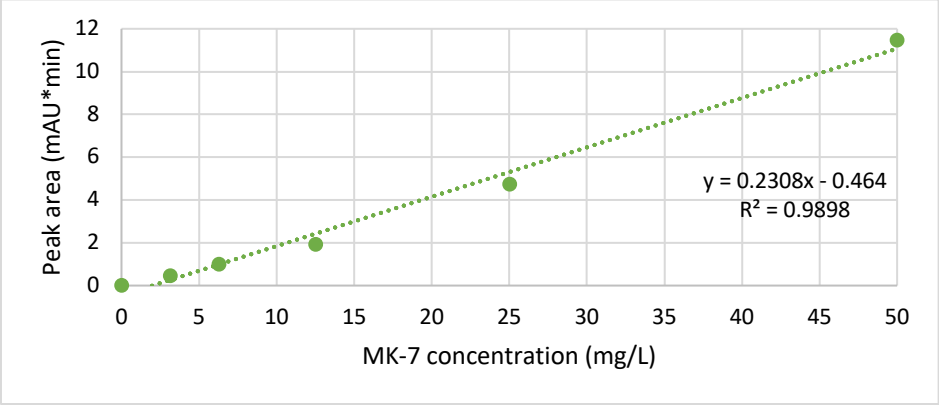


Figure 3-6 MK-7 calibration curve

MK-7 analysis was carried out using the method summarised by Berenjian et al. [19] with minor alterations to accommodate the requirements of the chromatography column used in this

study. A Dionex HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with four pumps (P680), an automated sample injector (ASI-100), a thermostatted column compartment (TCC-100), and a photodiode array UV detector (UVD340U) was employed to analyse and determine the concentration of MK-7 isomers in the fermented samples. A reversed-phase COSMOSIL Cholester packed column (100 mm × 2 mm × 2.5 µm; Nacalai Tesque Inc., Kyoto, Japan) was used to separate the compounds at 40 °C. Methanol constituted the mobile phase, and the compounds were eluted isocratically at a flow rate of 0.2 mL/min. The injection volume, autosampler temperature, analytical wavelength, and run-time were 10 µL, 10 °C, 248 nm, and 30 min, respectively. The Chromeleon 7 software (Thermo Fisher Scientific, Waltham, MA, USA) was used for data acquisition.

### 3.2.6.2 LC-MS

Compared to conventional MK-7 analytical methods, which do not evaluate the isomer composition of samples, the analysis and measurement of MK-7 isomers are more elaborate due to the lack of specific reference standards for the various possible *cis* forms of the vitamin. Therefore, it is necessary to apply LC-MS techniques to verify the presence and confirm the chromatographic retention times of the all-*trans* and *cis* MK-7 isomers present in fermented samples using the all-*trans* MK-7 reference standard. The approach employed by Szterk et al. [20] was used as a guide, and although the central principle was similar, particular aspects of the process were adapted to comply with the LC-MS system used in this study. The LC chromatograms and MS data for the all-*trans* MK-7 reference standard and an experimental sample are provided in Appendix B.

The LC-MS platform comprised a Dionex Ultimate 3000 UHPLC system and a QExactive mass spectrometer with a HESI II source (Thermo Fisher Scientific, Waltham, MA, USA). The Thermo XCalibur 4.3 application (Thermo Fisher Scientific, Waltham, MA, USA) was used to control the system, and data handling was carried out using the Chromeleon 7.3 program (Thermo Fisher Scientific, Waltham, MA, USA). Separation was performed utilising the chromatographic conditions discussed in section 3.2.6.1, except the injection volume and run-time were altered to 5 µL and 37 min, respectively, to accommodate the requirements of the LC-MS system. Data collection was conducted in the positive ionisation mode with a MS1 scan range of 150-1000 *m/z*, a resolution of 70000, an AGC target of $3 \times 10^6$, and a maximum injection time of 200 ms. The MS data were analysed using the Thermo FreeStyle 1.6 package (Thermo Fisher Scientific, Waltham, MA, USA).

### 3.2.6.3 Cell density and pH measurements

Bacterial growth was estimated from the cell density, which was determined by measuring the optical density (OD) at 600 nm using a UV-vis spectrophotometer (Shimadzu UV-1900, Kyoto, Japan) after appropriate dilution with distilled water. The pH was directly measured

in the cultivation medium with a standard laboratory pH meter (CyberScan pH 100, Eutech Instruments, Paisley, UK).

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# 4

# Optimisation of the Fermentation Media to Enhance the Production of the Bioactive Isomer of Vitamin Menaquinone-7

A journal article published in

***Bioprocess and Biosystems Engineering***
***Volume 45, Issue 8, Springer, 2022***

By

N. Lal, M. Seifan, and A. Berenjian

# Chapter 4 – Optimisation of the Fermentation Media to Enhance the Production of the Bioactive Isomer of Vitamin Menaquinone-7

This chapter focuses on the fermentation media, which is at the heart of the upstream stage of any fermentation process and is the first step in designing a targeted fermentation method to enhance the concentration of the all-*trans* isomer and reduce the production of *cis* MK-7. The assortment of nutrients comprising the fermentation media and their respective concentrations play an essential role in microbial growth and metabolism and, consequently, influence the efficiency of the fermentation process and the concentration and yield of the desired product. It is widely recognised that *B. subtilis natto* is the model microorganism for MK-7 fermentation; hence, it was deemed the most suitable for this study. Therefore, the media composition must be optimised to meet the growth and metabolic demands of the *B. subtilis natto* strain with regard to the objectives of this research.

A DOE approach was used to develop the ideal fermentation media. Several media components, including a variety of carbon, nitrogen, and salt sources, known to be beneficial in MK-7 fermentation using *B. subtilis natto* were initially assessed in a screening study to distinguish those that have a significant effect on MK-7 isomer production. The identified nutrients were then considered in an optimisation study to determine the optimum concentration of each component to favour the production of bioactive MK-7 and minimise the concentration of the biologically insignificant isomer. A time-course fermentation analysis was subsequently carried out to monitor changes in bacterial growth, the concentration of MK-7 isomers, and the pH of the medium during the different stages of the process when employing the optimal fermentation media.

The findings of this investigation were novel and are applied in the following chapters (Chapters 5–8) to provide the necessary framework to further develop and enhance the overall fermentation process in the specific context of each chapter.

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RESEARCH PAPER

# Optimisation of the fermentation media to enhance the production of the bioactive isomer of vitamin menaquinone-7

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**Abstract**
Menaquinone-7 (MK-7) offers significant health benefits; however, only the all-*trans* form is biologically active. MK-7 produced through fermentation can occur as all-*trans* and *cis* isomers, and the therapeutic value of the resulting MK-7 is exclusively determined by the quantity of the all-*trans* isomer. Therefore, this study aimed to investigate the effect of the media composition on the isomer profile obtained from fermentation and determine the optimum media combination to increase the concentration of the all-*trans* isomer and diminish the production of *cis* MK-7. For this purpose, design of experiments (DOE) was used to screen the most effective nutrients, and a central composite face-centred design (CCF) was employed to optimise the media components. The optimum media consisted of 1% (*w/v*) glucose, 2% (*w/v*) yeast extract, 2% (*w/v*) soy peptone, 2% (*w/v*) tryptone, and 0.1% (*w/v*) $CaCl_2$. This composition resulted in an average all-*trans* and *cis* isomer concentration of 36.366 mg/L and 1.225 mg/L, respectively. In addition, the optimised media enabled an all-*trans* isomer concentration 12.2-fold greater and a *cis* isomer concentration 2.9-fold less than the unoptimised media. This study was the first to consider the development of an optimised fermentation media to enhance the production of the bioactive isomer of MK-7 and minimise the concentration of the inactive isomer. Furthermore, this media is commercially promising, as it will improve the process productivity and reduce the costs associated with the industrial fermentation of the vitamin.

**Keywords** Menaquinone-7 isomers · Bioactivity · Fermentation · Media optimisation

## Introduction

Vitamin K is a fat-soluble vitamin first discovered in 1929 by the Danish nutritional biochemist Carl Peter Henrik Dam as an antihaemorrhagic factor capable of correcting dietary-induced bleeding disorders in chicks [1–3]. The vitamin K series consists of a group of compounds that contain a 2-methyl-1,4-naphthoquinone moiety (menadione) but differ in the structure of a lateral isoprenoid chain at the 3-position (Fig. 1) [4]. The length and degree of saturation of the isoprenoid side chain influence the properties of the various forms of vitamin K [5–7].

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Vitamin K1 (phylloquinone) and vitamin K2 (menaquinones) are the two naturally occurring forms of vitamin K [9, 10]. Phylloquinone (PK) is a single compound and is the dominant source of vitamin K in the diet and can be obtained from green vegetables, vegetable oils, and products derived from these plant oils [5, 11, 12]. Whereas menaquinones (MK) are primarily of microbial origin and comprise side chains of varying length and degree of saturation; this is described by the general representation MK-*n*, where *n* denotes the number of unsaturated isoprenoid units in a chain, which is typically between four and thirteen [5, 8, 13]. The intestinal microbiota also contribute to the synthesis of MK in the human body, and MK-4, the most common isoform in humans, can also be produced from the tissue-specific conversion of PK [14].

All forms of vitamin K are involved in the blood coagulation pathway and haemostasis; however, numerous studies have established that the potential health gains of vitamin K extend well beyond the activation of hepatic coagulation factors. In particular, vitamin K intake has been associated with improved bone and cardiovascular

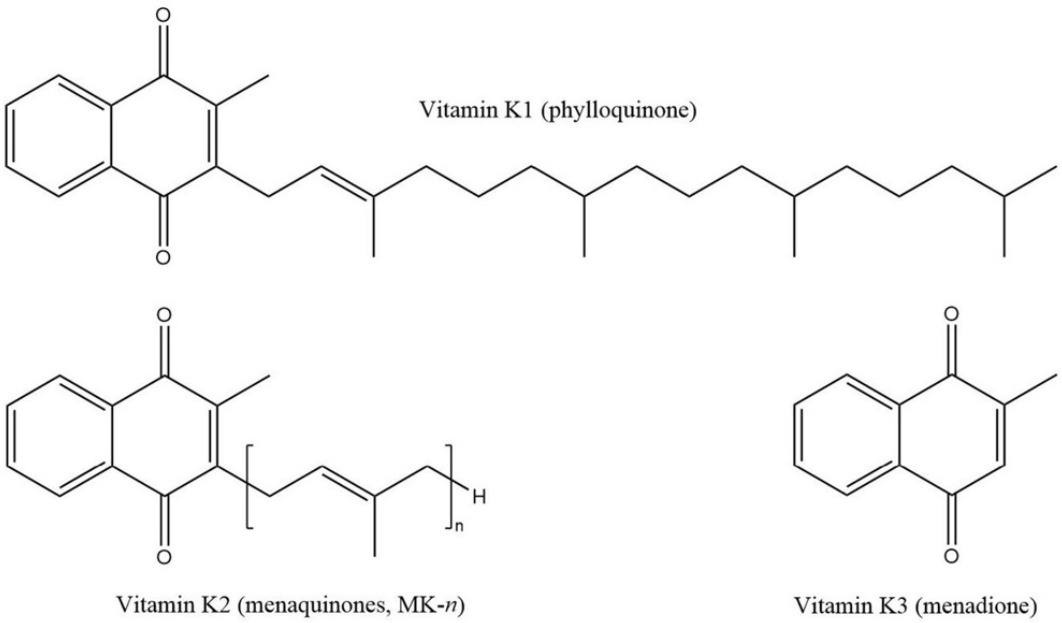


**Fig. 1** The different forms of vitamin K and their chemical structure (adapted from Szterk et al. [8])

health [5, 9, 15–22]. In addition, several more recent investigations have established other possible functions and health benefits of vitamin K, specifically, vitamin K2, which include the prevention of cancer, the suppression of Parkinson's disease, assisting the functional recovery of the liver, and decreasing the risk of type 2 diabetes mellitus, chronic kidney disease, immune disorders, neurological disease, and obesity [21, 23–30]. Furthermore, it has been suggested that reduced vitamin K status is a possible modifiable risk factor for severe coronavirus disease 2019 (COVID-19) and may be linked to manifestations of COVID-19 and comorbidities related to the acute form of the disease [31, 32]. Hence, vitamin K supplementation will likely reduce the morbidity and mortality associated with COVID-19.

Of the various forms of vitamin K, MK-7 is the most notable and provides the greatest health benefits owing to its longer half-life in the body and superior extrahepatic availability [22, 33]. MK-7 supplementation can also facilitate anticoagulant therapy involving vitamin K antagonists, as low doses of MK-7 supplements can help improve anticoagulant management in patients [14]. Although, due to its low concentration in limited food products, obtaining adequate levels of MK-7 from regular food items is not feasible [34, 35]. Therefore, in light of the various health advantages of MK-7, the development of nutritional supplements and functional food products to complement natural food sources and improve the dietary intake of MK-7 has become increasingly widespread.

However, it is essential to note that, like most biological molecules, MK-7 can exist as geometric isomers, of which only the naturally occurring all-*trans* isomer is bioactive [6, 8, 36]. The double bond arrangement in the isoprenoid side chain of MK-7 molecules determines its shape and, consequently, its biological activity [6, 37]. The all-*trans* form of MK-7 has a linear molecular structure (Fig. 2), as all the double bonds in its side chain have the *trans* configuration [38]. Whereas in the *cis* isomers, the presence of one or more double bonds in the *cis* arrangement creates a bend in the isoprenoid chain, and this causes the *cis* isomers to adopt a non-linear structure (Fig. 2) [38]. The altered molecular structure of the *cis* isomers compromises their ability to carry out their biological function, and it has been demonstrated that the *cis* forms of vitamin K merely exhibit 1% of the biological activity of the all-*trans* isomer [6, 37, 39]. The bioactivity of MK-7 isomers is an important consideration from a health, nutritional, and therapeutic perspective.

The concept of MK-7 isomers is a relatively recent and emerging area of interest; thus, insufficient research has been conducted on this aspect, and a great deal is yet to be explored. MK-7 can be produced from fermentation [15, 41] or through chemical reaction methods [13, 42–45], and the isomer composition that is obtained is dependent on a variety of factors, primarily the methods used for the synthesis of the vitamin and purification of the post-reaction mixture [6, 8, 36, 38]. The molecular structure of MK-7 comprises seven double bonds, and in the all-*trans* isomer, all seven double bonds have the *trans* configuration. Individual double

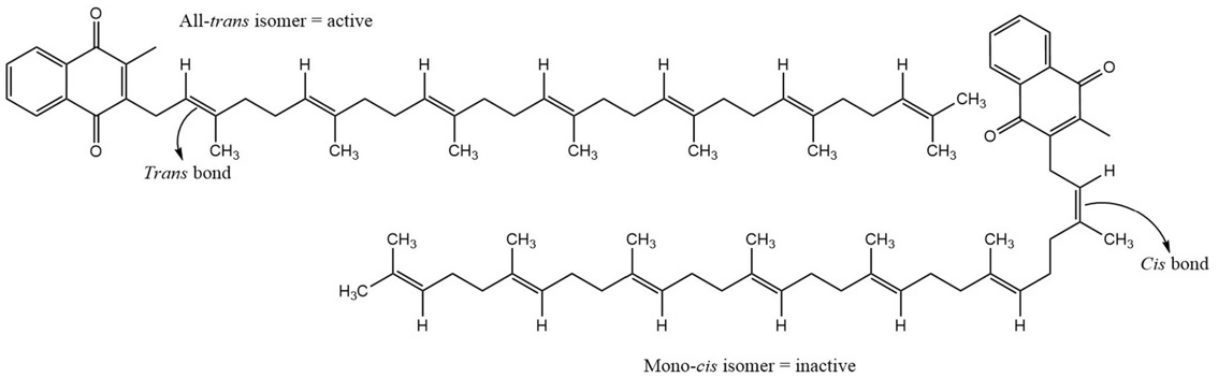


**Fig. 2** The chemical structure of *cis* and *trans* MK-7 isomers (adapted from Lal and Berenjian [40])

bonds in the isoprenoid chain of the molecule can also adopt the *cis* arrangement, and various *cis/trans* isomers, with different combinations of double bonds in the *cis* and *trans* configurations, may be attainable. However, the stability of the different isomers is likely to vary depending on the organisation of double bonds in the isoprenoid chain, which results in diverse molecular structures and shapes, as some forms of *cis* MK-7 may be less energetically favourable and are, therefore, less likely to exist than other more stable conformations.

Moreover, the number and type of different *cis* isomers that can potentially be achieved are ambiguous, as very few studies have considered this aspect and often present conflicting information. The majority of investigations that have focused on analysing the MK-7 isomer composition have explored dietary supplements or similar formulations [6, 8, 36, 46], and thus far, studies examining fermented samples have not been carried out. Additionally, the number and type of *cis* isomers that can be obtained from fermentation are unknown, as although fermentation is believed to produce the all-*trans* isomer selectively, the MK-7 isomer profile of fermented samples is yet to be elucidated.

The fermentation-based synthesis of MK-7 is superior, as most consumers prefer naturally derived products, which hence have a greater demand than synthetic formulations. Fermentation can also naturally enhance the nutrient profile and sensory characteristics of various products, thereby increasing their appeal to consumers [47, 48]. Furthermore, microbial fermentation is a more sustainable process for the large-scale production of MK-7, and the use of natural production methods can help satisfy the market demand and sustainable development goals [49]. Many Gram-positive and Gram-negative bacterial strains can synthesise MK, which function as electron carriers in the respiratory chain [5, 23, 50]. Both wild-type and engineered microorganisms have been used for MK-7 production via fermentation of which *Bacillus* strains, lactic acid bacteria, and various other types of microorganisms, such as *Escherichia coli*, *Flavobacterium meningosepticum*, *Enterobacter agglomerans, Enterococcus faecium,* and *Serratia marcescens*, tend to be the most common [49]. Nevertheless, members of the *Bacillus* species, such as *Bacillus subtilis natto* [51], *Bacillus licheniformis* [52], and *Bacillus amylolyquifaciens* [53], are the most notable [54]. Of the several suitable strains, *B. subtilis natto* is considered to be the most ideal for the industrial production of MK-7 and is preferentially used for the manufacture of MK-7 supplements and functional food products, as it is generally recognised as safe (GRAS) and enables a high MK-7 yield [15, 55–60]. As a result, there are no safety concerns associated with the *B. subtilis natto* strain, and it is suitable for the production of microbial-derived MK-7 products that are intended for human consumption.

Numerous studies have explored and enhanced different aspects of the fermentation process in various contexts, including liquid-state fermentation (LSF) [55, 58, 60–63], solid-state fermentation (SSF) [64–69], and biofilm reactors [54, 70–76], to improve MK-7 production using *B. subtilis natto*. The fermentation media, in particular, has been of considerable interest, as the selection of carbon, nitrogen, and salt sources and presence of growth factors, vitamins, minerals, bioactives, and other essential nutrients play a crucial role in microbial growth and metabolism [77, 78], which ultimately influence the productivity of the fermentation process and the MK-7 yield. Recently, several investigations have focused on improving the MK-7 yield by optimising the fermentation media using DOE and response surface methodology (RSM) [54, 61, 62, 69, 79, 80]. This approach allows both the individual and interactive effects of nutrients to be considered to determine the optimum media composition for a specific fermentation process, unlike the conventional method in which components are varied and analysed independently. However, although fermentation medium engineering to improve MK-7 productivity has been the subject of many studies, the focus has predominantly

been to enhance MK-7 production without regard to the proportion of the bioactive all-*trans* isomer obtained from fermentation under the investigated conditions. The isomer composition achieved through fermentation is a key aspect worthy of attention, as the effectiveness of MK-7 nutritional supplements and functional food products is only determined by the content of the all-*trans* isomer, and all other isomeric forms of the vitamin are essentially impurities that lack biological significance.

Therefore, the primary objective of this study was to investigate the effect of the media composition on the MK-7 isomer profile and determine the optimum combination of nutrients and their required concentrations to enhance the fermentation yield of the all-*trans* isomer and reduce the production of *cis* MK-7. As part of this process, ten different nutrient components, including carbon, nitrogen, and salt sources, were initially screened using a Plackett–Burman design (PBD) to identify the important nutritional factors that significantly impact the isomer concentration. A CCF design and RSM were then employed to optimise the concentration of the significant nutrients determined from the screening stage to develop the ideal fermentation media to enhance the production of the bioactive all-*trans* isomer and minimise the yield of the biologically insignificant *cis* isomer. This study will create new opportunities to develop an industrial fermentation process that targets the synthesis of the all-*trans* MK-7 isomer, which is commercially attractive, as it will refine the production process and decrease the related costs. This will be a valuable step forward in increasing the accessibility of biologically active fermented MK-7 supplements and functional food products to consumers, which will likely ameliorate the dietary intake of MK-7 and improve health outcomes.

## Materials and methods

### Chemicals and materials

The all-*trans* MK-7 analytical standard (98.1% purity) was purchased from ChromaDex (Los Angeles, CA, USA). Glucose was obtained from Ajax Finechem Pty Ltd (Taren Point, NSW, Australia), and yeast extract, peptone, tryptone, and soytone were acquired from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Glycerol, soy peptone, $K_2HPO_4$, methanol, 2-propanol, and *n*-hexane were purchased from Merck Millipore (Burlington, MA, USA). NaCl was obtained from a domestic supplier, and $CaCl_2$ was acquired from Sigma-Aldrich (St. Louis, MO, USA). Nutrient agar plates were purchased from Fort Richard Laboratories (Auckland, New Zealand).

### Microorganism and inoculum preparation

The *B. subtilis natto* strain was prepared as described previously [61]. Briefly, the cells were cultivated in a liquid culture medium containing tryptone, yeast extract, and NaCl before streaking on nutrient agar plates. The plates were incubated at 37 °C for 48 h. The cells were then scraped off the plates and suspended in a sterilised saline solution (0.9% (*w/v*) NaCl). The suspension was subsequently placed in a water bath at 80 °C for 30 min to inactivate the vegetative cells and induce the production of spores before centrifuging (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 3000 rpm for 10 min to remove the cell debris. The resulting spore suspension ($4.8 \times 10^6$ CFU/mL) was used as the inoculum for the fermentation experiments.

### Experimental design and statistical analysis

Ten different nutrients, which have been shown to enhance the MK-7 yield, were selected based on previous studies [54, 60–63, 69, 80, 81], and the effect of various carbon, nitrogen, and salt sources on isomer production was considered. Glucose and glycerol were the two carbon sources explored; yeast extract, soy peptone, peptone, tryptone, and soytone were selected as potent complex nitrogen sources; and $K_2HPO_4$, $CaCl_2$, and NaCl were determined to be effective salt sources. All carbon and nitrogen sources were investigated in the range of 0–2% (*w/v*), while the salt sources were considered between 0–1% (*w/v*). These concentration spans were derived from the literature and preliminary experiments.

A PBD was implemented to examine the individual effects of the selected nutritional factors on the all-*trans* and *cis* MK-7 isomer concentrations achieved during fermentation. Each factor was considered at three levels (high, intermediate, and low). A CCF design was employed to optimise the significant variables identified from the screening step, and RSM was used to analyse the results. The experimental values were scaled factors, and the response was described by a quadratic equation. The MODDE 13 software (Sartorius, Gottingen, Germany) was used to create the design matrices, develop a model, and determine the optimum level of the media components to achieve the highest concentration of all-*trans* MK-7 and minimise the concentration of the *cis* isomer. The experimental data was then used to generate a second-order polynomial regression model (Eq. 1) for each response.

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j \quad (1)$$

Where $Y$ represents the all-*trans* or *cis* MK-7 isomer concentration; $b_0$ is a constant term; $b_i$, $b_{ii}$, and $b_{ij}$ are the

coefficients of the linear, quadratic, and synergistic effects, respectively; and $X_i$ and $X_j$ correspond to the significant factors. The $R^2$ value was used to express the quality of the fit for the developed regression models, and statistical significance was determined using the analysis of variance (ANOVA) test and accepted at $P < 0.1$.

### Fermentation procedure

For both the screening and optimisation experiments, the fermentation media was prepared according to the DOE plan and sterilised using an autoclave (TOMY SX-700E, Tokyo, Japan) at 121 °C for 20 min. Each sample was then inoculated with 5% (*v*/*v*) of the pre-prepared *B. subtilis natto* spore suspension. Fermentation was conducted aerobically at 37 °C under dynamic conditions (120 rpm) for six days. The inoculum volume and operating conditions were selected based on preliminary studies. A small fermentation volume (6 mL) was used to enable the extraction of the whole sample, as it allowed all of the MK-7 produced during fermentation to be analysed and eliminated any errors associated with sampling from the fermentation media.

### MK-7 extraction

MK-7 was extracted from the samples prior to analysis using a mixture of 2-propanol and *n*-hexane in the ratio of 1:2 (*v*/*v*) and a liquid-to-organic ratio of 1:4 (*v*/*v*) [61]. The mixture was vigorously shaken for 2 min using a vortex mixer and centrifuged (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 3000 rpm for 10 min to separate the two phases. Afterwards, the upper hexane layer was separated from the aqueous phase and evaporated under a vacuum to recover the extracted MK-7.

### MK-7 analysis

MK-7 analysis was carried out using the method outlined by Berenjian et al. [35] with minor alterations to accommodate the requirements of the chromatography column used in this study. A Dionex high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with four pumps (P680), an automated sample injector (ASI-100), a thermostatted column compartment (TCC-100), and a photodiode array UV detector (UVD340U) was employed to determine the MK-7 isomer concentration in the fermented samples. A COSMOSIL Cholester packed column (100 mm × 2 mm × 2.5 µm; Nacalai Tesque Inc., Kyoto, Japan) operated at 40 °C was used to separate the compounds. Methanol, at a flow rate of 0.2 mL/min, was used as the mobile phase (isocratic elution), and the analytical wavelength, injection volume, autosampler temperature, and run-time were 248 nm, 10 µL, 10 °C, and 30 min, respectively. The Chromeleon 7 software (Thermo Fisher Scientific, Waltham, MA, USA) was used for data acquisition. The MK-7 calibration curve was linear between 0.1 mg/L and 50 mg/L ($R^2 = 0.99$).

Liquid chromatography-mass spectrometry (LC–MS) techniques were applied to confirm the presence and verify the chromatographic retention times of the all-*trans* and *cis* MK-7 isomers. The method developed by Szterk et al. [8] was employed as a guide for the LC–MS analysis, and although the fundamental concept was similar, specific aspects of the procedure were tailored to suit the requirements of the LC–MS system that was used in the present study. The LC–MS platform consisted of a Dionex Ultimate 3000 ultra-high-performance liquid chromatography (UHPLC) system and a QExactive mass spectrometer with a HESI II source (Thermo Fisher Scientific, Waltham, MA, USA). The Thermo XCalibur 4.3 software (Thermo Fisher Scientific, Waltham, MA, USA) was used to control the system, and data handling was carried out using the Chromeleon 7.3 software (Thermo Fisher Scientific, Waltham, MA, USA). Separation by liquid chromatography was performed using the chromatographic conditions outlined above, except the injection volume was altered to 5 µL, and the run-time was extended to 37 min to adapt to the requirements of the LC–MS system. Data collection was carried out in the positive ionisation mode with an MS1 scan range of 150–1000 *m/z*, a resolution of 70,000, an AGC target of $3 \times 10^6$, and a maximum injection time of 200 ms. The mass spectrometry (MS) data were analysed using the Thermo FreeStyle 1.6 software (Thermo Fisher Scientific, Waltham, MA, USA).

### Cell density and pH measurements

Bacterial growth was inferred from the cell density, which was determined by measuring the optical density (OD) at 600 nm using a UV–vis spectrophotometer (Shimadzu UV-1900, Kyoto, Japan) after appropriate dilution with distilled water. The pH was directly measured in the cultivation medium with a standard laboratory pH meter (CyberScan pH 100, Eutech Instruments, Paisley, UK).

## Results and discussion

### LC–MS analysis

Relative to traditional MK-7 analytical techniques, which do not consider the isomer composition of samples, the analysis and measurement of MK-7 isomers present various difficulties. The fundamental issue is the absence of specific analytical standards for the potential *cis* forms of the vitamin, as

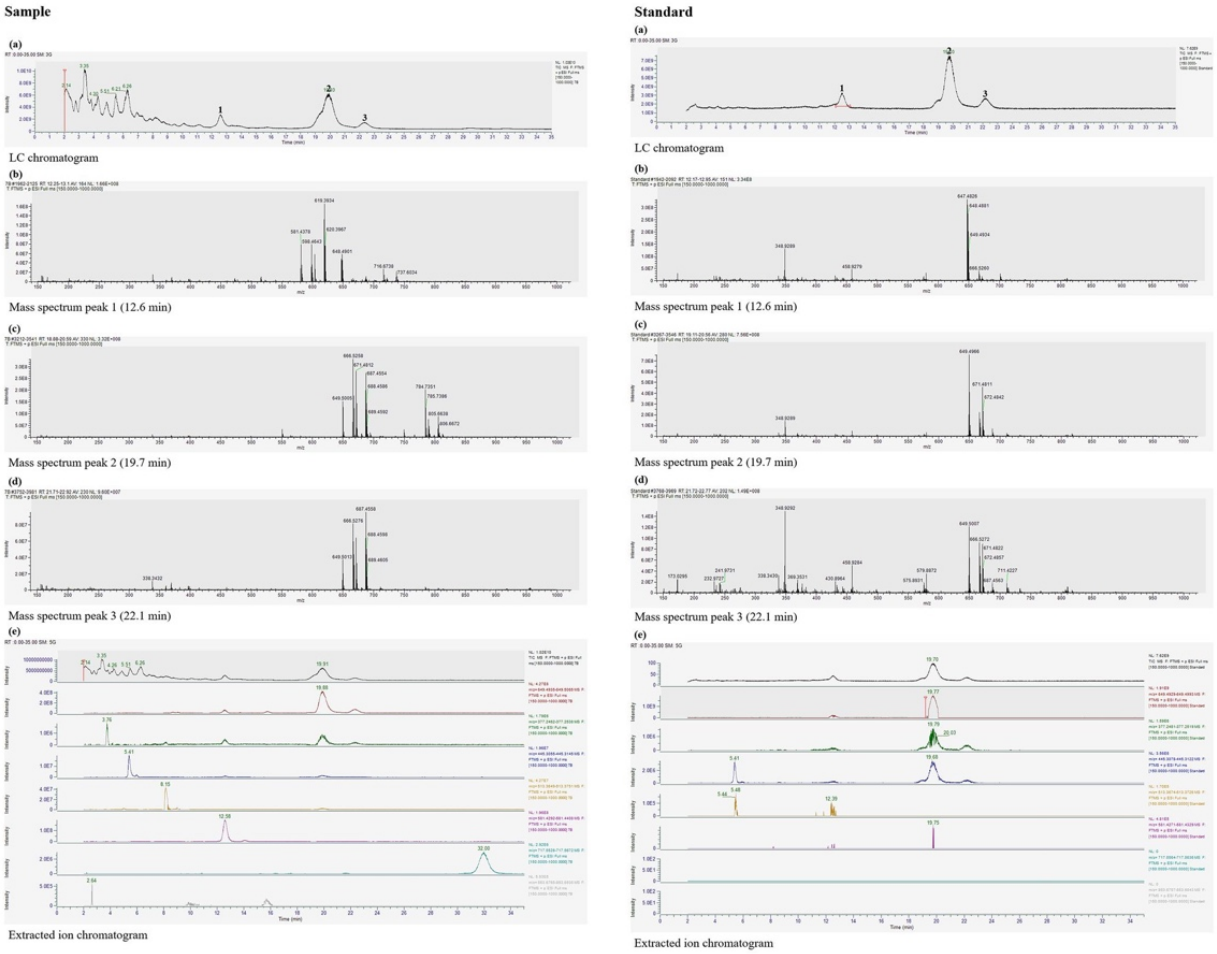


**Fig. 3** Liquid chromatography (LC) chromatograms **a**, MS data **b-d**, and extracted ion chromatograms **e** for an experimental sample and the all-*trans* MK-7 reference standard

only the all-*trans* MK-7 standard is commercially available [8, 36]. As a result, the identification and quantification of *cis* MK-7 pose a challenge due to the lack of appropriate reference standards. In addition, very few studies have considered the concept of MK-7 isomers [6, 8, 36, 46, 82], and the primary focus of these investigations has been to analyse the isomer composition of MK-7 dietary supplements and other similar preparations. Therefore, the nature of the MK-7 isomer profile obtained from fermentation is unknown, as fermented samples are yet to be explored.

This study used the all-*trans* MK-7 reference standard to identify and quantify the all-*trans* and *cis* MK-7 isomers present in fermented samples. The retention time of the peaks in the analytical standard was used to identify the corresponding peaks in the samples, which consistently appeared at approximately 12.6 min, 19.7 min, and 22.1 min. The compounds pertaining to these peaks were speculated to be MK-7 isomers. Due to the unavailability of suitable reference standards, MS techniques were used to verify the identity of the isomers by confirming the presence of a peak at approximately 649.5 *m/z* (molecular mass of MK-7) in the MS data and comparing the mass spectra of the samples to that of the all-*trans* MK-7 standard. This approach was valid, as the compounds of interest are isomers and have the same molecular mass (approximately 649.5 *m/z*), and this, together with the mass spectra, can be used to ascertain whether the three chromatographic peaks represent MK-7 isomers. A similar strategy has been successfully demonstrated in the series of studies conducted by Szterk et al. [8], Szterk et al. [36], and Sitkowski et al. [6] for the analysis of MK-7 isomers present in dietary supplements.

The MS analysis determined that the compounds relating to the peaks at approximately 19.7 min and 22.1 min were MK-7 isomers, while the peak at approximately 12.6 min was not (Fig. 3). The MS data for the peaks at approximately 19.7 min and 22.1 min were comparable

and showed a similar set of mass peaks at each retention time across all samples. The MS data for both peaks also showed a mass peak at approximately 649.5 *m/z*. Although other mass peaks, which may indicate impurities or other possible MK of different chain lengths, were observed at these time points, they were not as significant, and evaluation of the MS data obtained from the samples and standard was sufficient to establish that the compounds corresponding to the peaks at approximately 19.7 min and 22.1 min were MK-7 isomers. The chromatographic peak appearing at approximately 19.7 min was notably larger than that at approximately 22.1 min. Consequently, it is anticipated to correspond to the all-*trans* isomer, as all-*trans* MK-7 is the naturally occurring form of the vitamin and is, thus, likely to be produced in a greater quantity relative to the *cis* isomer, which is represented by the peak at approximately 22.1 min. A larger peak at approximately 19.7 min was also observed for the standard, which is a commercial sample of high purity (98.1% all-*trans* MK-7) and, as a result, contains a significantly greater proportion of the all-*trans* isomer.

From the LC–MS analysis of the fermented samples, it was also established that only a single *cis* MK-7 isomer is produced during fermentation (under the conditions employed). In contrast, it may be possible for samples of chemical or other origins to contain more than one *cis* MK-7 isomer, which may arise due to the synthesis procedures or purification techniques implemented in the production of the vitamin or vitamin product. Additionally, while these compounds are isomers, they have slightly different retention times owing to minor differences in their structure, resulting in their differing ability to move through the stereoselective column. The retention time of the all-*trans* and *cis* MK-7 isomers is consistent with the analytical standard and across a range of fermented samples, including samples from preliminary experiments that had different media components and compositions. It has also been established through these experiments that when employing the previously mentioned chromatography conditions, the relative retention time (RRT) of the *cis* isomer, which is the ratio of the retention time of the *cis* isomer to that of the all-*trans* isomer, is always constant (1.12) for all samples and is identical to that for the reference standard. Use of the RRT to distinguish the *cis* MK-7 isomer has been recommended in the USP Monograph [83] (RRT of 1.1) and demonstrated in the investigation carried out by Jedynak et al. [82] (RRT of 1.15). Although the suggested values for the RRT vary slightly between these sources and the present study, these minor differences are likely to be due to the different instruments, columns, solvents, chromatographic conditions, and analytical procedures that have been employed in each case to achieve separation. Therefore, the RRT is a reliable method to identify the all-*trans* and *cis* MK-7 isomers in fermented samples using HPLC, which is far more convenient and avoids the need to recurrently use LC–MS techniques for the analysis of samples in subsequent experiments.

## Screening study

The selected media components, consisting of different carbon, nitrogen, and salt sources, were initially screened using a PBD to determine the important nutritional factors that significantly affect the fermentation yield of the all-*trans* and *cis* isomers. The carbon and nitrogen sources were considered between 0–2% (*w/v*), while the salt sources were explored in the range of 0–1% (*w/v*). The concentration span of each nutrient was selected based on the literature and from the unfavourable results that were obtained from preliminary experiments, which examined higher nutrient concentrations (up to 10% (*w/v*) for the carbon and nitrogen sources and up to 2% (*w/v*) for the salt sources). The unsatisfactory results may be attributed to the high nutrient concentrations, which are likely to decrease the water activity and induce osmotic stress, consequently inhibiting cell growth, metabolism, and MK-7 production [53, 61]. Hence, it was decided to investigate lower nutrient concentrations in the screening study. Due to the large number of factors being investigated, it was not feasible to consider both the individual and interactive effects of the different nutrients. Therefore, only the individual effects of the media components on the isomer concentration were evaluated to ensure that the number of experimental runs was practical. The experimental design and the isomer concentrations are outlined in Table 1, and the statistical analysis is presented in Table 2.

## Carbon sources

Carbon sources are essential for MK-7 synthesis, as the pathway for producing the isoprene side chain and quinone skeleton (1,4-naphthoquinone) of MK-7 relies on the presence of carbon sources in the fermentation media [69]. Numerous carbon sources, including glucose, glycerol, sucrose, molasses, inulin, mannose, starch, dextrose, fructose, corn syrup, and maltose, have been extensively examined in previous investigations focusing on improving the MK-7 yield in a range of contexts and applications, such as SSF, LSF, biofilm reactors, and the development of MK-7 enriched nutraceuticals, functional foods, and animal feed [15, 53, 54, 58, 61–63, 69, 70, 72, 73, 81, 84–86]. However, of these, the majority of studies have determined glucose and glycerol to be the most effective at enhancing MK-7 production. Therefore, simply glucose and glycerol were considered in the screening process.

Of the two evaluated carbon sources, only glucose had a significant effect on the isomer concentration ($P < 0.1$). In

**Table 1** Experimental design and measured responses from the screening study

| Nutrient factors (% *w/v*) | | | | | | | | | | | Measured responses (mg/L) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Run | Glucose | Glycerol | Yeast extract | Soy peptone | Peptone | Tryptone | Soytone | $K_2HPO_4$ | $CaCl_2$ | NaCl | All-*trans* MK-7 concentration | *Cis* MK-7 concentration |
| 1 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 1 | 0 | 0.000 | 0.000 |
| 2 | 2 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 1 | 11.940 | 1.064 |
| 3 | 2 | 2 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 37.494 | 2.302 |
| 4 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 57.336 | 3.343 |
| 5 | 0 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 1 | 0 | 10.201 | 0.869 |
| 6 | 2 | 0 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 1 | 40.551 | 2.480 |
| 7 | 0 | 2 | 0 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 53.771 | 3.512 |
| 8 | 2 | 0 | 2 | 0 | 2 | 2 | 2 | 1 | 0 | 0 | 43.654 | 2.723 |
| 9 | 2 | 2 | 0 | 2 | 0 | 2 | 2 | 1 | 1 | 0 | 21.600 | 1.285 |
| 10 | 0 | 2 | 2 | 0 | 2 | 0 | 2 | 1 | 1 | 1 | 0.666 | 0.000 |
| 11 | 0 | 0 | 2 | 2 | 0 | 2 | 0 | 1 | 1 | 1 | 19.768 | 1.330 |
| 12 | 2 | 0 | 0 | 2 | 2 | 0 | 2 | 0 | 1 | 1 | 21.402 | 1.385 |
| 13 | 0 | 2 | 0 | 0 | 2 | 2 | 0 | 1 | 0 | 1 | 5.246 | 0.609 |
| 14 | 0 | 0 | 2 | 0 | 0 | 2 | 2 | 0 | 1 | 0 | 20.425 | 1.481 |
| 15 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 1 | 0 | 1 | 12.763 | 0.996 |
| 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.511 | 0.000 |
| 17 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.5 | 0.5 | 0.5 | 18.32 | 1.486 |
| 18 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.5 | 0.5 | 0.5 | 19.118 | 1.584 |
| 19 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.5 | 0.5 | 0.5 | 30.664 | 2.387 |

addition, the positive coefficient for glucose for the all-*trans* and *cis* MK-7 isomer concentrations implies that glucose has a significant positive effect on the two responses and, thus, enhances the concentration of both isomers. Considering that the all-*trans* isomer is the biologically active form of the vitamin, it is beneficial to increase the production of the all-*trans* isomer, whereas, in comparison, *cis* MK-7 has very little or no bioactivity; hence, it is necessary to minimise its fermentation yield.

It has been suggested that glucose promotes cell growth, while glycerol, although not beneficial for cell growth, facilitates MK-7 biosynthesis and secretion in *B. subtilis natto* and increases MK-7 productivity on a per-cell basis [23, 51, 62, 71, 72, 87]. Therefore, glycerol has commonly been used to enhance the MK-7 yield and is often reported as the most efficient carbon source in MK-7 fermentation [53, 61, 62, 69, 81, 88]. However, various investigations, including more recent studies involving biofilm reactors, have demonstrated glucose to be an effective carbon source for MK-7 biosynthesis [54, 71, 73]. Interestingly, only glucose has a significant positive effect on the isomer concentration in the present study. A possible explanation for this could be that since glucose, compared to glycerol, is a more preferable and readily metabolisable carbon source for *B. subtilis* strains, it facilitates rapid microbial growth and, consequently, has a more substantial contribution to the MK-7 yield, as MK-7 production is partially growth-associated [35, 54, 58, 61, 63, 74, 84, 87–89]. Moreover, prior investigations have only explored the overall fermentation yield of MK-7 without any regard to the isomer profile; thus, the impact of glucose and glycerol may differ when considering MK-7 isomers.

In light of the aims of this study, glucose acts to enhance the production of all-*trans* MK-7, which is favourable; however, it also increases the concentration of the *cis* isomer, which is undesirable. Therefore, it is necessary to balance the glucose concentration in the fermentation media to regulate isomer production.

## Nitrogen sources

The selection of nitrogen sources in the fermentation media plays a crucial role in microbial growth and metabolism and the production of proteins involved in the cellular respiration processes of *B. subtilis*, such as haem [54, 61, 88]. The nature of the nitrogen source tends to influence the fermentation yield of MK-7, as inorganic and complex nitrogen sources supply different concentrations and types of amino acids, which may act as precursors or feedback inhibitors of the shikimate pathway that is involved in the synthesis of MK-7 and other MK [61].

**Table 2** Statistical analysis of the variables from the screening study

| All-*trans* MK-7 concentration | | | |
|---|---|---|---|
| Term | Coefficient | Standard error | *P*-value |
| Constant | 22.391 | 2.276 | 9.592e-06 |
| Glucose | 6.914 | 2.480 | 0.024 |
| Glycerol | 2.449 | 2.480 | 0.352 |
| Yeast extract | 6.429 | 2.480 | 0.032 |
| Soy peptone | 7.341 | 2.480 | 0.018 |
| Peptone | − 0.397 | 2.480 | 0.877 |
| Tryptone | 4.786 | 2.480 | 0.090 |
| Soytone | 4.139 | 2.480 | 0.134 |
| $K_2HPO_4$ | − 2.204 | 2.480 | 0.400 |
| $CaCl_2$ | − 9.083 | 2.480 | 0.006 |
| NaCl | − 3.604 | 2.480 | 0.184 |
| *Cis* MK-7 concentration | | | |
| Term | Coefficient | Standard error | *P*-value |
| Constant | 1.5177 | 0.1541 | 9.5025e-06 |
| Glucose | 0.3616 | 0.1679 | 0.0634 |
| Glycerol | 0.1618 | 0.1679 | 0.3634 |
| Yeast extract | 0.3548 | 0.1679 | 0.0675 |
| Soy peptone | 0.4388 | 0.1679 | 0.0310 |
| Peptone | − 0.0139 | 0.1679 | 0.9359 |
| Tryptone | 0.3493 | 0.1679 | 0.0711 |
| Soytone | 0.2493 | 0.1679 | 0.1759 |
| $K_2HPO_4$ | − 0.1754 | 0.1679 | 0.3266 |
| $CaCl_2$ | − 0.5344 | 0.1679 | 0.0129 |
| NaCl | − 0.1904 | 0.1679 | 0.2896 |

$R^2 = 0.855$; $R^2$ (adj.) = 0.673; significance accepted at $P < 0.1$

$R^2 = 0.818$; $R^2$ (adj.) = 0.590; significance accepted at $P < 0.1$

Several inorganic and complex nitrogen sources, including $NaNO_3$, $(NH_4)_2SO_4$, $KNO_3$, urea, soy protein, yeast extract, malt extract, peptone, tryptone, soy peptone, and soytone, have been considered in earlier studies focusing on MK-7 fermentation [15, 23, 51, 54, 58, 60, 62, 63, 69, 81, 87, 88]. Essentially, it has been determined that complex nitrogen sources are superior to inorganic nitrogen sources and are more efficient in supporting *B. subtilis* growth, various microbial processes, and MK-7 synthesis, as they tend to provide an assortment of amino acids and other essential growth factors, such as polypeptides and coenzymes [60, 90, 91]. In particular, yeast extract, soy peptone, peptone, tryptone, and soytone have frequently been employed in MK-7 fermentation studies and are deemed to be the most efficacious complex nitrogen sources for enhancing the yield of the vitamin [15, 54, 58, 62, 63, 85, 88]. Accordingly, only complex nitrogen sources, consisting of yeast extract, soy peptone, peptone, tryptone, and soytone, were included in the screening study.

From the five nitrogen sources that were screened, all but peptone and soytone had a significant effect on the yield of both isomers ($P < 0.1$). Yeast extract, soy peptone, and tryptone all have positive coefficients for the two isomers, suggesting that all of these complex nitrogen sources have a significant positive effect on the concentration of the all-*trans* and *cis* isomers. Since only the all-*trans* isomer sustains biological activity, it is advantageous to improve its productivity and reduce the proportion of the *cis* isomer achieved during fermentation.

The findings of the present study are supported by observations from prior investigations, which have commonly identified yeast extract, soy peptone, and tryptone as potent nitrogen sources that enhance MK-7 production [54, 58, 61–63, 80, 88, 92]. Yeast extract, soy peptone, and tryptone provide a broad spectrum of nitrogen compounds, such as

short-chained and simple amino acids, that are easily metabolised by *B. subtilis* strains [54]. This is important from the perspective of MK-7 biosynthesis, which is an elaborate process that comprises several interrelated steps, namely the shikimate pathway to form the aromatic ring, the methylerythritol 4-phosphate (MEP) and isopentenyl diphosphate route to produce the isoprenoid tail, and the MK pathway in which the two separately synthesised structures are combined to generate MK-7 [23, 93–95]. The complexity of the MK-7 biosynthetic pathway is likely to have high energy requirements, mainly due to the need to produce the amino acids involved in MK-7 synthesis. The energy requirements can be reduced if the bacteria are supplied with the necessary amino acids, eliminating the need for their synthesis and thereby accelerating MK-7 production.

It has also been established that yeast extract and soy peptone support microbial growth and metabolism and have an interactive influence on MK-7 productivity [58, 61, 96], possibly due to the beneficial effect of the combined range and concentration of amino acids and nutrients, and are, thus, often used in conjunction in MK-7 fermentation [35, 58, 61, 88]. Moreover, the yeast extract, soy peptone, and tryptone used in this study have different origins (the yeast extract is derived from autolysed yeast cells, the soy peptone is papain-digested, and the tryptone is a pancreatic digest of casein) and, hence, are likely to supply a diverse range and concentration of amino acids, growth factors, and other vital compounds that synergistically promote microbial growth and metabolism and, ultimately, MK-7 biosynthesis.

Considering the bioactivity of the two MK-7 isomers and the objectives of this investigation, the effect of yeast extract, soy peptone, and tryptone on the production of all-*trans* MK-7 is beneficial, whereas their impact on the *cis* isomer concentration is not ideal. Consequently, it is important to ensure that the fermentation media contains the correct amounts of these nitrogen sources to control isomer production.

### Salt sources

Salts are an important source of trace and essential elements that perform vital functions during cell growth, metabolism, and product formation [91]. Common trace elements include iron ($Fe^{2+}$ and $Fe^{3+}$), zinc ($Zn^{2+}$), potassium ($K^{+}$), magnesium ($Mg^{2+}$), manganese ($Mn^{2+}$), molybdenum ($Mo^{2+}$), sodium ($Na^{+}$), cobalt ($Co^{2+}$), calcium ($Ca^{2+}$), and copper ($Cu^{2+}$), which are often supplied in small quantities in the form of mineral salts [91].

Previous experiments have explored the effect of various salts, such as $K_2HPO_4$, $CaCl_2$, $MgSO_4.7H_2O$, $MnCl_2.4H_2O$, and NaCl, on MK-7 production, and of these, $K_2HPO_4$, $CaCl_2$, and NaCl are deemed to be the most effective [35, 51, 61, 63, 69, 80, 85, 86, 90, 92]. Therefore, $K_2HPO_4$, $CaCl_2$, and NaCl were considered in the screening study, and only $CaCl_2$ had a significant effect on MK-7 isomer production ($P < 0.1$). Additionally, its negative coefficient indicates that $CaCl_2$ has a significant adverse effect on the isomer concentration. This is not advantageous for the all-*trans* MK-7 yield, as the goal of this study is to maximise its production; conversely, $CaCl_2$ has a favourable influence on the *cis* isomer concentration, as the intention is to minimise its synthesis during fermentation.

The majority of previous investigations have reported that $K_2HPO_4$ is an important phosphate source that facilitates the production of primary and secondary metabolites and has a positive effect on MK-7 production [34, 35, 51, 61, 63, 80, 88], which is not consistent with the observations from the present study. However, prior investigations have exclusively focused on the overall fermentation yield of MK-7 and have not considered the proportion of MK-7 isomers that are obtained; therefore, $K_2HPO_4$ may have an insignificant effect on the production of MK-7 isomers. Inversely, it has been demonstrated in the study conducted by Singh et al. [69] that $CaCl_2$ has a significant positive effect on MK-7 production, and it has also been established that an adequate concentration of $Ca^{2+}$ benefits MK-7 biosynthesis [23]. It is important to note that in the present study, while it has been determined that $CaCl_2$ has a significant effect on the MK-7 isomer yield, it acts to decrease the concentration of both isomers. The contrasting effect of $CaCl_2$ in the two investigations is likely to be a result of the differing aims of each study, as while $CaCl_2$ may have a positive effect on MK-7 production holistically, it may have the opposite effect when considering the MK-7 isomer profile.

Despite the negative impact of $CaCl_2$ on the MK-7 isomer concentration, its presence in the fermentation media is imperative to decrease the yield of the *cis* isomer. Therefore, given the biological significance of the different MK-7 compounds and the purpose of this study, the concentration of $CaCl_2$ needs to be optimised to reduce the synthesis of the *cis* isomer without significantly diminishing the productivity of the all-*trans* isomer.

### Optimisation of the fermentation media

The screening study concluded that glucose, yeast extract, soy peptone, tryptone, and $CaCl_2$ significantly impact MK-7 isomer production. The presence of glucose, yeast extract, soy peptone, and tryptone is advantageous to improve the all-*trans* MK-7 isomer concentration; however, they also increase the concentration of *cis* MK-7, which is unfavourable. Whereas $CaCl_2$ decreases the production of all-*trans* MK-7, which is not ideal but it is also beneficial, as it reduces the *cis* isomer yield. As a result, given the synonymous nature of the effect of the significant nutrients on the two responses, it is likely not to be possible to eliminate the

**Table 3** Experimental design and responses from the optimisation study

| Run | Nutrient factors (% *w/v*) | | | | | Observed responses (mg/L) | | Predicted responses (mg/L) | |
|---|---|---|---|---|---|---|---|---|---|
| | Glucose | Yeast extract | Soy peptone | Tryptone | $CaCl_2$ | All-*trans* MK-7 concentration | *Cis* MK-7 concentration | All-*trans* MK-7 concentration | *Cis* MK-7 concentration |
| 1 | 0.1 | 0.1 | 0.1 | 0.1 | 1 | 2.984 | 0.585 | 3.643 | 0.641 |
| 2 | 2 | 0.1 | 0.1 | 0.1 | 0.1 | 7.892 | 1.078 | 8.403 | 1.076 |
| 3 | 0.1 | 2 | 0.1 | 0.1 | 0.1 | 10.306 | 1.254 | 11.113 | 1.338 |
| 4 | 2 | 2 | 0.1 | 0.1 | 1 | 22.264 | 2.437 | 22.222 | 2.444 |
| 5 | 0.1 | 0.1 | 2 | 0.1 | 0.1 | 5.605 | 0.843 | 5.521 | 0.829 |
| 6 | 2 | 0.1 | 2 | 0.1 | 1 | 20.102 | 2.545 | 19.169 | 2.453 |
| 7 | 0.1 | 2 | 2 | 0.1 | 1 | 15.006 | 1.791 | 14.368 | 1.785 |
| 8 | 2 | 2 | 2 | 0.1 | 0.1 | 24.138 | 2.639 | 23.353 | 2.575 |
| 9 | 0.1 | 0.1 | 0.1 | 2 | 0.1 | 6.345 | 0.922 | 7.607 | 1.003 |
| 10 | 2 | 0.1 | 0.1 | 2 | 1 | 17.065 | 2.091 | 17.478 | 2.094 |
| 11 | 0.1 | 2 | 0.1 | 2 | 1 | 10.233 | 1.265 | 10.942 | 1.355 |
| 12 | 2 | 2 | 0.1 | 2 | 0.1 | 18.555 | 2.172 | 19.116 | 2.203 |
| 13 | 0.1 | 0.1 | 2 | 2 | 1 | 10.708 | 1.407 | 10.525 | 1.398 |
| 14 | 2 | 0.1 | 2 | 2 | 0.1 | 33.937 | 3.512 | 33.606 | 3.445 |
| 15 | 0.1 | 2 | 2 | 2 | 0.1 | 19.399 | 1.223 | 19.364 | 1.242 |
| 16 | 2 | 2 | 2 | 2 | 1 | 27.300 | 1.635 | 26.416 | 1.577 |
| 17 | 0.1 | 1.05 | 1.05 | 1.05 | 0.55 | 10.343 | 0.980 | 7.846 | 0.679 |
| 18 | 2 | 1.05 | 1.05 | 1.05 | 0.55 | 17.190 | 1.471 | 18.681 | 1.713 |
| 19 | 1.05 | 0.1 | 1.05 | 1.05 | 0.55 | 14.031 | 1.109 | 12.718 | 1.152 |
| 20 | 1.05 | 2 | 1.05 | 1.05 | 0.55 | 17.528 | 1.452 | 17.835 | 1.350 |
| 21 | 1.05 | 1.05 | 0.1 | 1.05 | 0.55 | 18.174 | 1.521 | 13.296 | 1.171 |
| 22 | 1.05 | 1.05 | 2 | 1.05 | 0.55 | 15.898 | 1.274 | 19.771 | 1.565 |
| 23 | 1.05 | 1.05 | 1.05 | 0.1 | 0.55 | 17.384 | 1.467 | 17.890 | 1.498 |
| 24 | 1.05 | 1.05 | 1.05 | 2 | 0.55 | 24.060 | 1.734 | 22.548 | 1.645 |
| 25 | 1.05 | 1.05 | 1.05 | 1.05 | 0.1 | 26.125 | 1.738 | 24.220 | 1.670 |
| 26 | 1.05 | 1.05 | 1.05 | 1.05 | 1 | 22.905 | 1.666 | 23.805 | 1.675 |
| 27 | 1.05 | 1.05 | 1.05 | 1.05 | 0.55 | 16.755 | 1.218 | 18.375 | 1.336 |
| 28 | 1.05 | 1.05 | 1.05 | 1.05 | 0.55 | 13.270 | 1.030 | 18.375 | 1.336 |
| 29 | 1.05 | 1.05 | 1.05 | 1.05 | 0.55 | 21.077 | 1.524 | 18.375 | 1.336 |

synthesis of *cis* MK-7; nevertheless, its yield relative to the all-*trans* isomer can be minimised to achieve the objectives of this study.

The key nutrients identified from the screening study were then further optimised using a CCF design and RSM to determine the ideal fermentation media to enhance the production of the biologically significant MK-7 isomer and reduce the yield of the inactive *cis* isomer. The range of all the factors was altered slightly to reposition the design space near the probable optimum. Table 3 illustrates the CCF design matrix and the observed and predicted all-*trans* and *cis* MK-7 isomer concentrations corresponding to each sample. The statistical analysis for the optimisation study is outlined in Table 4. Polynomial regression models were developed based on the significant model terms to predict the yield of the all-*trans* (Eq. 2) and *cis* (Eq. 3) MK-7 isomers as a function of the glucose, yeast extract, soy peptone, tryptone, and $CaCl_2$ concentrations.

$$Y_1 = 18.375 + 5.417X_1 + 2.559X_2 + 3.237X_3 + 2.329X_4 - 5.112X_1^2 + 5.637X_5^2 - 1.731X_2X_4 \quad (2)$$

$$Y_2 = 1.336 + 0.517X_1 + 0.197X_3 + 0.337X_5^2 - 0.132X_1X_2 - 0.217X_2X_3 - 0.294X_2X_4 - 0.186X_4X_5 \quad (3)$$

Where $Y_1$ represents the concentration of the all-*trans* MK-7 isomer; $Y_2$ corresponds to the *cis* isomer concentration; and $X_1$, $X_2$, $X_3$, $X_4$, and $X_5$ refer to glucose, yeast extract, soy peptone, tryptone, and $CaCl_2$, respectively.

**Table 4** Statistical analysis of the optimisation study

| All-*trans* MK-7 concentration | | | |
|---|---|---|---|
| Term | Coefficient | Standard error | *P*-value |
| Constant | 18.375 | 1.156 | 2.454e-07 |
| $X_1$ | 5.417 | 0.828 | 1.803e-04 |
| $X_2$ | 2.559 | 0.828 | 0.015 |
| $X_3$ | 3.237 | 0.828 | 0.004 |
| $X_4$ | 2.329 | 0.828 | 0.023 |
| $X_5$ | − 0.207 | 0.828 | 0.809 |
| ${X_1}^2$ | − 5.112 | 2.244 | 0.052 |
| ${X_2}^2$ | -3.099 | 2.244 | 0.205 |
| ${X_3}^2$ | − 1.842 | 2.244 | 0.435 |
| ${X_4}^2$ | 1.844 | 2.244 | 0.435 |
| ${X_5}^2$ | 5.637 | 2.244 | 0.036 |
| $X_1X_2$ | − 1.003 | 0.879 | 0.287 |
| $X_1X_3$ | 1.178 | 0.879 | 0.217 |
| $X_1X_4$ | 0.605 | 0.879 | 0.511 |
| $X_1X_5$ | 0.308 | 0.879 | 0.735 |
| $X_2X_3$ | − 0.724 | 0.879 | 0.434 |
| $X_2X_4$ | − 1.731 | 0.879 | 0.084 |
| $X_2X_5$ | 0.333 | 0.879 | 0.715 |
| $X_3X_4$ | 1.109 | 0.879 | 0.242 |
| $X_3X_5$ | − 1.213 | 0.879 | 0.205 |
| $X_4X_5$ | − 1.584 | 0.879 | 0.109 |
| *Cis* MK-7 concentration | | | |
| Term | Coefficient | Standard error | *P*-value |
| Constant | 1.336 | 0.088 | 3.501e-07 |
| $X_1$ | 0.517 | 0.063 | 3.635e-05 |
| $X_2$ | 0.099 | 0.063 | 0.156 |
| $X_3$ | 0.197 | 0.063 | 0.014 |
| $X_4$ | 0.073 | 0.063 | 0.277 |
| $X_5$ | 0.002 | 0.063 | 0.972 |
| ${X_1}^2$ | − 0.140 | 0.171 | 0.437 |
| ${X_2}^2$ | − 0.085 | 0.171 | 0.633 |
| ${X_3}^2$ | 0.032 | 0.171 | 0.854 |
| ${X_4}^2$ | 0.235 | 0.171 | 0.205 |
| ${X_5}^2$ | 0.337 | 0.171 | 0.084 |
| $X_1X_2$ | − 0.132 | 0.067 | 0.083 |
| $X_1X_3$ | 0.082 | 0.067 | 0.254 |
| $X_1X_4$ | 0.023 | 0.067 | 0.740 |
| $X_1X_5$ | − 0.094 | 0.067 | 0.199 |
| $X_2X_3$ | − 0.217 | 0.067 | 0.012 |
| $X_2X_4$ | − 0.294 | 0.067 | 0.002 |
| $X_2X_5$ | − 0.027 | 0.067 | 0.696 |
| $X_3X_4$ | − 0.071 | 0.067 | 0.319 |
| $X_3X_5$ | − 0.112 | 0.067 | 0.133 |
| $X_4X_5$ | − 0.186 | 0.067 | 0.024 |

$X_1$ = glucose; $X_2$ = yeast extract; $X_3$ = soy peptone; $X_4$ = tryptone; $X_5 = CaCl_2$; $R^2 = 0.929$; $R^2$ (adj.) = 0.753; significance accepted at $P < 0.1$

$X_1$ = glucose; $X_2$ = yeast extract; $X_3$ = soy peptone; $X_4$ = tryptone; $X_5 = CaCl_2$; $R^2 = 0.948$; $R^2$ (adj.) = 0.817; significance accepted at $P < 0.1$

**Table 5** ANOVA for the quadratic models

| All-*trans* MK-7 concentration | | | | | | |
|---|---|---|---|---|---|---|
| Source of variation | DF | SS | MS | *F*-value | *P*-value | SD |
| Total corrected | 28 | 1399.696 | 49.989 | – | – | 7.070 |
| Regression | 20 | 1300.898 | 65.045 | 5.267 | 0.011 | 8.065 |
| Residual | 8 | 98.798 | 12.350 | – | – | 3.514 |
| *Cis* MK-7 concentration | | | | | | |
| Source of variation | DF | SS | MS | *F*-value | *P*-value | SD |
| Total corrected | 28 | 10.971 | 0.392 | – | – | 0.626 |
| Regression | 20 | 10.399 | 0.520 | 7.271 | 0.004 | 0.721 |
| Residual | 8 | 0.572 | 0.072 | – | – | 0.267 |

*DF* degree of freedom, *SS* sum of squares, *MS* mean sum of squares, *SD* standard deviation

The linear term for glucose ($X_1$) is especially significant ($P < 0.001$) for both responses, which suggests that the concentration of glucose has a direct relationship with MK-7 production in this particular media. This may be attributed to the critical role of glucose in facilitating microbial growth and metabolism and, consequently, MK-7 production. Additionally, the quadratic terms ${X_1}^2$ and ${X_5}^2$ and the interactive term $X_2X_4$ were significant ($P < 0.1$) for the all-*trans* MK-7 isomer concentration, whereas the quadratic term ${X_5}^2$ and the interactive terms $X_1X_2$, $X_2X_3$, $X_2X_4$, and $X_4X_5$ were significant ($P < 0.1$) for the *cis* MK-7 isomer concentration.

The ANOVA analysis (Table 5) demonstrated that the developed models were consistent with the experimental results, as the standard deviation of the regression was considerably greater than the standard deviation of the residuals for both the all-*trans* and *cis* MK-7 isomer concentrations. This is also evident from the significant *P*-value (0.011 and 0.004) and high *F*-value (5.267 and 7.271) for each response. An $R^2$ value of 0.929 for the all-*trans* MK-7 response and 0.948 for the *cis* MK-7 response indicate a good model fit, as only 7.1% and 5.2% of the total variation, respectively, is not explained by the proposed models.

Interactions between the different media components and their effect on the production of MK-7 isomers can be visualised using contour plots for each pair of nutrients, while the concentration of the remaining nutrients is fixed at their intermediate value. Ten contour plots were generated for each response, and these are illustrated in Fig. 4 for the all-*trans* isomer concentration and Fig. 5 for the *cis* isomer concentration. Overall, the response surface plots depict a complex scenario, as the two responses are interrelated. The objective of this study was to enhance the all-*trans* isomer yield and decrease the production of the *cis* isomer, and the response surface plots demonstrate an intermediate glucose concentration, high yeast extract, soy peptone, and tryptone concentration, and low $CaCl_2$ concentration best satisfy this aim.

## Validation study

The ideal concentration of the media components to promote the synthesis of the all-*trans* isomer and reduce the production of *cis* MK-7 was determined by solving the regression equations within the design space. The optimum media contained 1% (*w/v*) glucose, 2% (*w/v*) yeast extract, 2% (*w/v*) soy peptone, 2% (*w/v*) tryptone, and 0.1% (*w/v*) $CaCl_2$, and the experimental concentrations (mean ± standard deviation (SD)) of all-*trans* and *cis* MK-7 achieved using the optimised media from triplicate samples were 36.366 ± 2.232 mg/L and 1.225 ± 0.063 mg/L, respectively. Although the experimental concentrations differed from the values envisaged by the model (30.109 mg/L of all-*trans* MK-7 and 1.941 mg/L of *cis* MK-7), the all-*trans* isomer concentration was higher and the *cis* isomer concentration was lower than the concentrations anticipated by the model. Thus, the experimental observations were superior to the model prediction, and the overall results were in accordance with the fundamental aim of this experiment, which was to maximise the production of the all-*trans* isomer and minimise the concentration of *cis* MK-7.

The development of an optimised media to enhance the production of the bioactive MK-7 isomer and decrease the concentration of the inactive isomer is significant from a commercial outlook, as it will improve the process productivity and reduce or eliminate the steps involved in the removal of the *cis* isomer.

## Monitoring the isomer composition and the fermentation process using the optimal fermentation media

The all-*trans* and *cis* MK-7 isomer concentrations, bacterial growth, and pH were analysed each day over the course of fermentation for the optimised media (Fig. 6). The trends

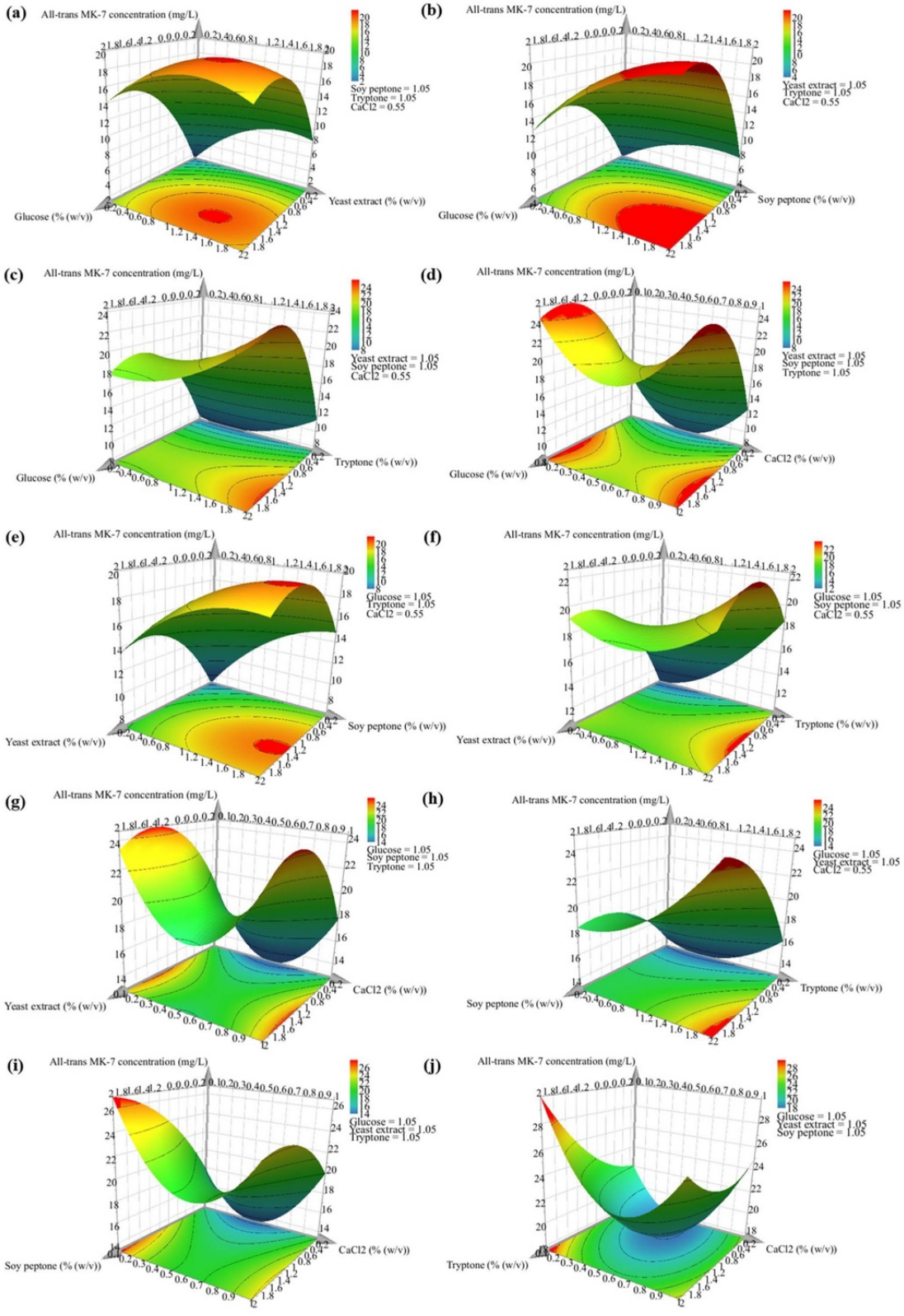


**Fig. 4** Response surface plots **a-j** demonstrating the effect of each pair of nutrients on the all-*trans* MK-7 isomer concentration

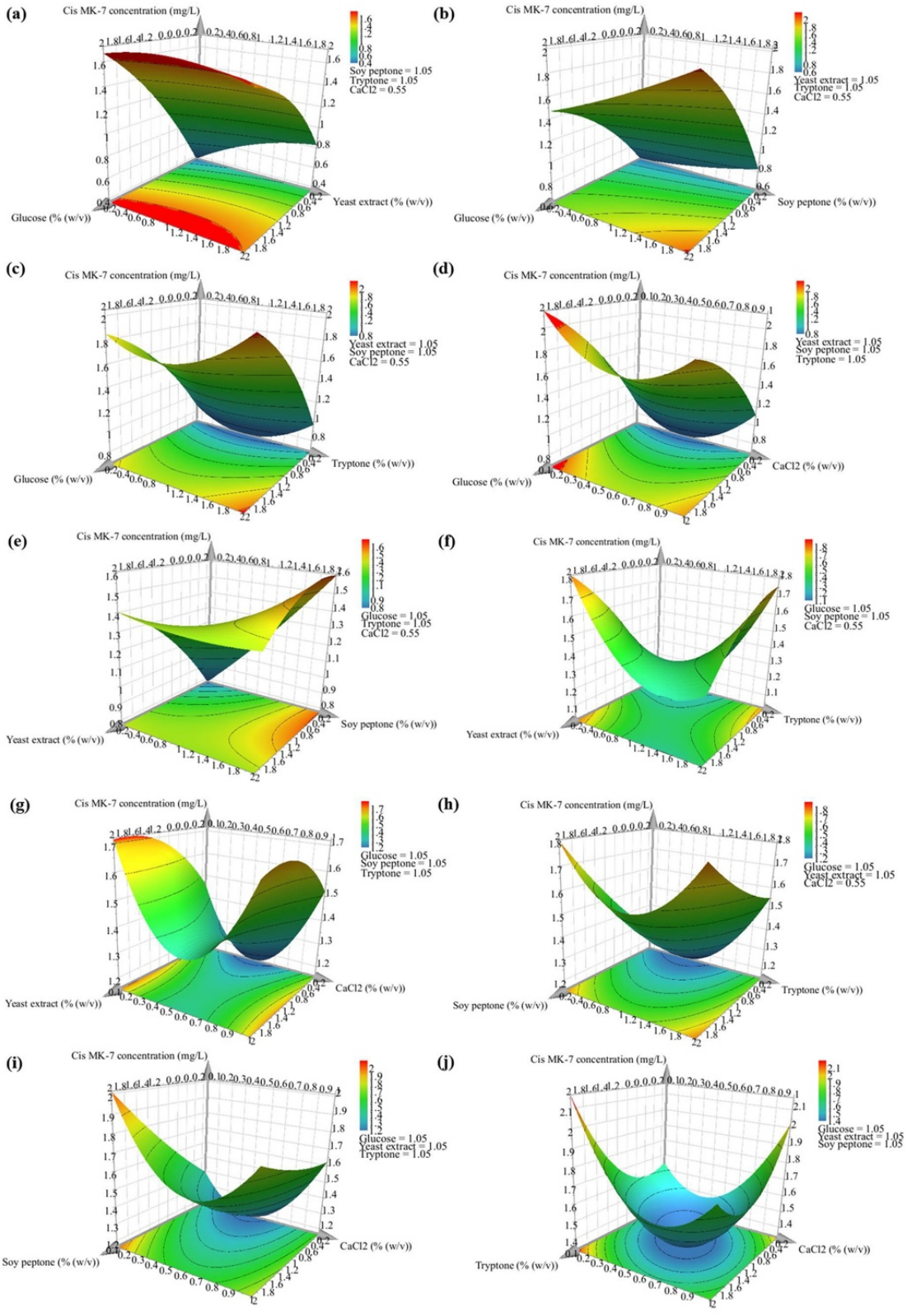


**Fig. 5** Response surface plots **a-j** demonstrating the effect of each pair of nutrients on the *cis* MK-7 isomer concentration

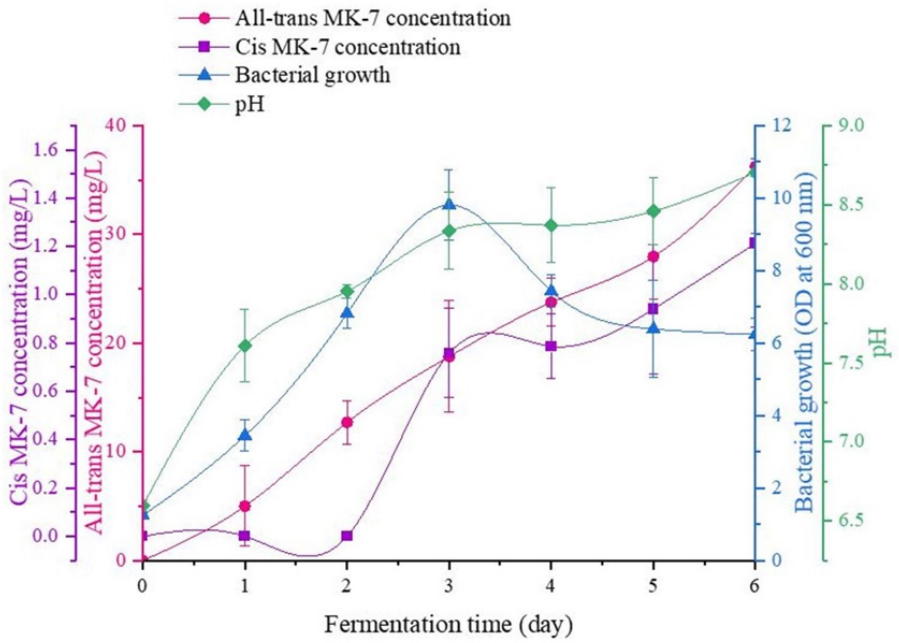


**Fig. 6** Changes in the all-*trans* and *cis* MK-7 isomer concentrations, bacterial growth, and pH over the fermentation period for the optimised media, which comprised 1% (*w/v*) glucose, 2% (*w/v*) yeast extract, 2% (*w/v*) soy peptone, 2% (*w/v*) tryptone, and 0.1% (*w/v*) $CaCl_2$ (the error bars represent the SD calculated from four replicate samples for each response)

observed in MK-7 isomer production, particularly the all-*trans* isomer, closely reflected the cell growth pattern, congruous with accounts from previous investigations [61, 84, 89, 97]. The OD increased slowly over the first day of fermentation, after which it escalated to 9.81 on day 3 and decreased to 7.43 on day 4, ultimately reaching a value of 6.24 on day 6. The variation in the OD measurements corresponds to the bacterial growth profile, and days 0 to 1 are likely to represent the lag phase, between days 1 and 3 correlate with the exponential growth period, and days 4 to 6 appear to denote the stationary phase.

It is interesting to note that although the overall variation in the concentration of the two isomers over the investigated timeframe is similar, the day on which their production began was different. Production of the all-*trans* isomer commenced on day 1 and increased over the fermentation period at a rate compliant with the bacterial growth profile, reaching a final concentration of 36.232 mg/L on day 6. Correspondingly, 13.83% of the total all-*trans* MK-7 was noted during the lag phase, 37.95% was synthesised during the logarithmic growth phase, and 48.22% was produced during the stationary phase. In comparison, production of the *cis* isomer initiated on day 3 and increased according to the bacterial growth curve, resulting in a concentration of 1.214 mg/L at the end of fermentation. Accordingly, 0% of the total *cis* MK-7 was observed during the lag phase, 62.47% was produced during the exponential growth phase, and 37.53% was detected during the stationary period. The delay in the production of the *cis* isomer, relative to all-*trans* MK-7, is an intriguing observation, as it would generally be expected for both isomers to be synthesised simultaneously. There are two probable explanations for this finding. Since the all-*trans* isomer is the naturally occurring form of the vitamin, it is produced in a greater quantity than the *cis* isomer, and thus, the concentration of *cis* MK-7 may be too low to be detected by the instrument. Alternatively, it is possible for the *cis* isomer to be produced from the isomerisation of all-*trans* MK-7 due to the culture or environmental conditions, and this may occur after a few days of fermentation once a sufficient amount of the all-*trans* isomer has been synthesised. The trend in both the all-*trans* and *cis* isomer concentrations seems to continue to increase at the end of the fermentation period, which contrasts with the trend in the OD measurements.

Prior studies [61, 84, 97] have reported that the MK-7 concentration plateaus during the stationary phase, suggesting that MK-7 production correlates with bacterial growth. Whereas Xu and Zhang [92], Sato et al. [87], and Song et al. [63] have observed a substantial rise in the MK-7 concentration when the bacterial culture enters the stationary phase, which may be ascribed to the release of intracellular MK-7 as a result of cell lysis [92]. MK-7 is synthesised intracellularly and is secreted from the cell as a soluble complex with an acidic binding factor [92, 95, 98–100]. It has been noted that the amount of MK-7 inside *B. subtilis* cells is greater than the extracellular quantity [53, 92]. Therefore, during the stationary phase, it is anticipated for the extracellular MK-7 concentration to rapidly increase due to cell rupture and the successive release of the intracellular contents [92]. However, when bacterial growth enters the death phase,

the MK-7 concentration is likely to decrease, as over an extended timeframe, proteases, other enzymes, and various cellular components released during cell lysis may degrade the MK-7 present in the fermentation broth. Consequently, it is potentially advantageous to terminate fermentation before the onset of the death phase to ensure maximal all-*trans* MK-7 production.

Consolidating the findings from prior studies and the observations from this investigation, MK-7 is a mixed metabolite, as its production is partly growth-associated. The majority of MK-7 biosynthesis possibly occurs during the logarithmic phase, resulting in a gradual rise in the MK-7 concentration during this stage as some intracellular MK-7 is secreted into the extracellular fraction. A significant increase in the MK-7 concentration may only be noted during the stationary phase when the intracellular MK-7 is released due to cell lysis. Further extension of the fermentation period beyond the stationary phase and into the death phase might not be ideal, as it may lead to a drop in the MK-7 concentration. However, in the present study, it is necessary to prolong fermentation and observe the trends in bacterial growth and MK-7 production beyond day 6 to draw accurate conclusions in this regard.

Additionally, the pH of the media progressively increased from an initial value of 6.60 to 8.71 at the end of the fermentation period. This change in pH is verified by previous investigations [61, 92, 97] and may be attributed to protein hydrolysis and the subsequent release of ammonia by *B. subtilis* [61, 97].

## Conclusions

This study was the first to explore the production of MK-7 isomers in a fermentation context and consider the development of an optimised media to enhance the concentration of the biologically significant all-*trans* isomer and minimise the production of the inactive *cis* isomer. A PBD was initially employed to screen ten different carbon, nitrogen, and salt sources to determine the most effective nutrients that have a notable effect on the MK-7 isomer concentration. Glucose, yeast extract, soy peptone, tryptone, and $CaCl_2$ were then further analysed in an optimisation study using a CCF design and RSM to ascertain the optimum fermentation media. An experimental all-*trans* isomer concentration of 36.366 mg/L and a *cis* isomer concentration of 1.225 mg/L were achieved using the ideal fermentation media, which consisted of 1% (*w/v*) glucose, 2% (*w/v*) yeast extract, 2% (*w/v*) soy peptone, 2% (*w/v*) tryptone, and 0.1% (*w/v*) $CaCl_2$. The experimental concentrations were superior to the values predicted by the model. This media presents a commercially attractive alternative to MK-7 synthesis using fermentation media that does not specifically target the production of the bioactive isomer and, thus, necessitates the separation and removal of *cis* MK-7 from the desired all-*trans* product. Although the *cis* isomer is inactive, it may not be possible to eliminate its production during fermentation, as it is synthesised in small quantities alongside the all-*trans* isomer. Therefore, MK-7 synthesis using an optimised fermentation media, which enhances the yield of the biologically important all-*trans* isomer and minimises the concentration of *cis* MK-7, is likely to entail fewer downstream processing steps and reduce the costs associated with the industrial fermentation of the vitamin.

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## Declarations

**Conflict of interest** The authors declare that they have no conflicts of interest associated with this publication.



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# 5

# The Impact of Key Fermentation Parameters on the Production of the All-*Trans* Isomer of Menaquinone-7

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By

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# Chapter 5 – The Impact of Key Fermentation Parameters on the Production of the All-*Trans* Isomer of Menaquinone-7

This chapter draws attention to essential parameters that determine the fermentation conditions during upstream processing and evaluates their effect on the production of MK-7 isomers. Like the fermentation media, the factors defining the fermentation conditions have a direct impact on the growth and metabolic characteristics of the fermentation microorganism. Thus, optimisation of key fermentation parameters to suit the growth and metabolic requirements of *B. subtilis natto* is fundamental in developing an ideal fermentation process to selectively promote the production of the biologically efficacious isomer while reducing the formation of *cis* MK-7.

A DOE strategy was employed to optimise the value of significant fermentation parameters, specifically the inoculum size, fermentation temperature, agitation speed, and fermentation period, to increase the concentration of the all-*trans* isomer and diminish the amount of the *cis* isomer obtained from fermentation.

The outcomes of this study contribute to the subsequent chapters (Chapters 6–8) and, along with the findings from the previous chapter (Chapter 4), form the basis of an optimal fermentation process that is then explored in a broader setting in later sections with respect to the overall aims of this research.

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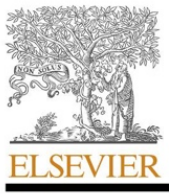

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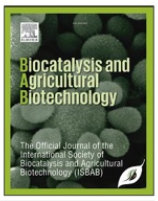

# The impact of key fermentation parameters on the production of the all-*trans* isomer of menaquinone-7

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ABSTRACT

Menaquinone-7 (MK-7) offers many health benefits and is the most superior form of vitamin K2 from a health and nutritional perspective. MK-7 can exist as geometric isomers; however, only the all-*trans* isomer is bioactive. Consequently, the therapeutic value of MK-7 products is solely determined by the proportion of the all-*trans* isomer. The fermentation-based synthesis of MK-7 is preferred, and the resulting product comprises both the all-*trans* and *cis* forms of the vitamin. The fermentation conditions influence the isomer profile. Hence, the objective of this investigation was to assess the significant fermentation parameters to enhance the concentration of the all-*trans* isomer and diminish the production of *cis* MK-7. Accordingly, a central composite face-centred (CCF) design was used to optimise the inoculum size, fermentation temperature, agitation speed, and fermentation time. The optimum conditions comprised an inoculum size of 2% (*v/v*), a fermentation temperature of 40 °C, an agitation speed of 200 rpm, and a fermentation period of 7 days. This resulted in an all-*trans* MK-7 concentration of 53.292 mg/L and a *cis* isomer concentration of 1.222 mg/L. This study was the first to evaluate the impact of essential fermentation parameters on the MK-7 isomer profile to boost the production of bioactive MK-7 and reduce the concentration of the ineffectual isomer. The ideal fermentation conditions determined from this investigation present a valuable opportunity to develop a fermentation process that selectively favours the production of all-*trans* MK-7, which is commercially attractive, as it will refine the production process and decrease the concomitant expenses.

## 1. Introduction

Vitamin K collectively refers to a group of fat-soluble compounds that contain a common 2-methyl-1,4-naphthoquinone moiety but differ in the structure of a lateral isoprenoid chain at the 3-position (Beulens et al., 2013). The nature and properties of the different forms of vitamin K are determined by the length and degree of saturation of the isoprenoid side chain (Beulens et al., 2013; Sitkowski et al., 2018).

Vitamin K1 (phylloquinone), vitamin K2 (menaquinones), and vitamin K3 (menadione) are the prevalent forms of vitamin K, and only phylloquinone (PK) and menaquinones (MK) are of relevance to human health and nutrition (Azuma and Inoue, 2019). PK is a single compound comprising a side chain of one unsaturated and three saturated isoprenoid residues and functions as an electron carrier in the chloroplasts of photosynthetic plants and algae (Beulens et al., 2013; Ren et al., 2019). Thus, PK can be easily obtained from a range of green vegetables, plant-based oils, and products derived from such vegetable oils and is the predominant dietary form

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of vitamin K (Basset et al., 2017; Berenjian et al., 2015; Beulens et al., 2013; Booth, 2012; Szterk et al., 2018a). In contrast, MK encompass a series of structures with side chains of different lengths and degree of saturation, which is described by the notation MK-*n*, where *n* is usually between four and thirteen and represents the number of unsaturated isoprenoid units in a chain (Beulens et al., 2013; Szterk et al., 2018b). MK are primarily of microbial origin and are not as readily available in the diet, as they occur at low concentrations in selected fermented, dairy, and animal goods (Berenjian et al., 2013; Beulens et al., 2013; Booth, 2012).

Lately, vitamin K, particularly vitamin K2, has gained extensive interest in the scientific community due to its numerous health benefits. Although all subtypes of vitamin K are involved in haemostasis, several studies have established the valuable role of MK in the prevention of osteoporosis and cardiovascular diseases (CVDs) (Azuma and Inoue, 2019; Berenjian et al., 2015; Beulens et al., 2013; Ferland, 2012; Sato et al., 2020; Scheiber et al., 2015; Shea and Holden, 2012; Shearer and Newman, 2008; Vermeer and Schurgers, 2000; Vik, 2020). Additionally, vitamin K intake has been linked to various other health advantages, including the prevention of cancer, the suppression of Parkinson's disease, assisting the functional recovery of the liver, decreasing the risk of several health conditions, and improving the outcomes associated with coronavirus disease 2019 (COVID-19) (Anastasi et al., 2020; Berenjian and Sarabadani, 2020; Dofferhoff et al., 2020; Fusaro et al., 2020; Halder et al., 2019; Janssen et al., 2021; Juanola-Falgarona et al., 2014; Karamzad et al., 2020; Ren et al., 2019; Sato et al., 2020; Schwalfenberg, 2017; Tarkesh et al., 2020; Xv et al., 2018).

Of the different types of vitamin K, MK-7 is the most exceptional and provides significant health gains, owing to its longer half-life and greater extrahepatic availability (Akbulut et al., 2020; Brugè et al., 2011; Schurgers et al., 2007; Vik, 2020). However, it is difficult to obtain adequate levels of MK-7 from regular dietary sources, as it exists in small quantities in limited food products; consequently, satisfying the recommended daily intake requirements would entail the consumption of impractically large quantities of MK-7-rich foods (Berenjian et al., 2012b, 2013). Therefore, the development of nutritional supplements and functional food products to accompany natural food sources and improve the dietary intake of MK-7 has become progressively widespread.

It is important to appreciate that MK-7 molecules exist as geometric isomers, consisting of the biologically significant all-*trans* isomer and various *cis* forms of the vitamin, which are inactive (Sitkowski et al., 2018; Szterk et al., 2018a, 2018b). The bioactivity of MK-7 is determined by the shape of the molecule, and this is governed by the configuration of the double bonds in its isoprenoid chain (Lowenthal and Rivera, 1979; Sitkowski et al., 2018). In the all-*trans* isomer, all seven double bonds in the side chain have the *trans* arrangement, resulting in a linear organisation (Fig. 1) (Lal and Berenjian, 2020). In contrast, the presence of one or more double bonds in the *cis* conformation creates a bend in the isoprenoid side chain, giving rise to a non-linear molecular structure (Fig. 1) (Lal and Berenjian, 2020). Depending on the number of *cis* double bonds and their position in the isoprenoid side chain, in addition to the *trans* bonds, various *cis/trans* isomers, with different combinations of double bonds in the *cis* and *trans* configurations, may potentially be achieved. However, the stability of the diverse molecular shapes resulting from the different *cis* and *trans* bond arrangements is likely to vary, as some conformations may be less energetically favourable and, thus, their occurrence in nature is more improbable than other structures with greater stability.

The existence of MK-7 isomers has only been acknowledged in very few investigations, which have only considered the isomer composition of MK-7-containing consumer end products, particularly dietary supplements and formulations of different origins (Bus et al., 2022; Orlando et al., 2019; Sitkowski et al., 2018; Szterk et al., 2018a, 2018b). Szterk et al. (2018a), Szterk et al. (2018b), and Sitkowski et al. (2018) have recognised five *cis/trans* MK-7 isomers in addition to the all-*trans* form, and Orlando et al. (2019) have demonstrated the presence of various unidentified peaks (postulated to represent the *cis/trans* isomers of MK-7) in different preparations. More recently, Bus et al. (2022) have isolated and established the chemical structure of thirteen new MK-7 isomers and verified the identity of three previously known MK-7 compounds, namely all-*trans* MK-7 ((*E6,ω*)-MK-7) and two *cis* isomers ((*Z,E5,ω*)-MK-7 and (*E,Z3,E2,ω*)-MK-7), from a synthetic mixture of MK-7. Although the presence of *cis* MK-7 isomers in nutritional supplements and other preparations has been established (Bus et al., 2022; Orlando et al., 2019; Sitkowski et al., 2018; Szterk et al., 2018a, 2018b), there are limited reports in the literature exploring the production of MK-7 isomers through fermentation.

It is widely assumed that bacterial fermentation exclusively produces the naturally occurring all-*trans* isomer, and studies specifically focusing on the production of MK-7 isomers from microbial fermentation under different conditions are lacking. Consequently, the isomer profile of fermented samples has not been extensively explored, and our previous study (Lal et al., 2022) was the first to elucidate the presence of a single *cis* MK-7 isomer in fermented samples obtained under the investigated conditions. Observation of *cis*

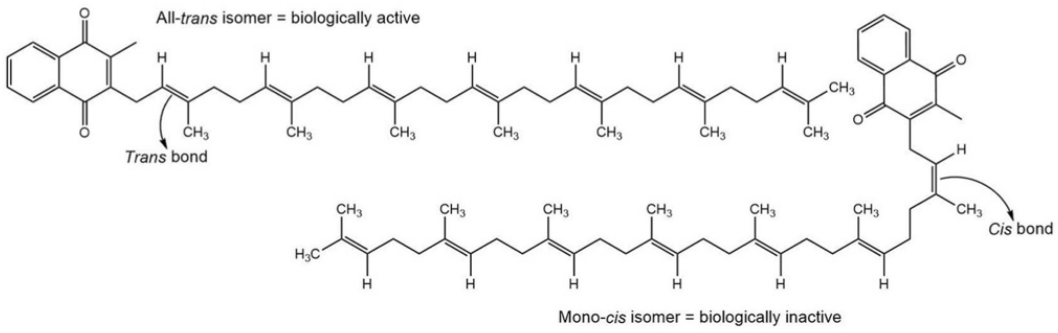


**Fig. 1.** Double bond configurations in the all-*trans* and *cis* isomers of MK-7 (adapted from Lal et al. (2022)).

MK-7 in fermented samples was an interesting phenomenon, as it has been reported that MK-7 produced from bacterial fermentation only occurs in the all-*trans* configuration. However, it has been suggested that exposure to certain conditions may promote the isomerisation of the all-*trans* isomer to *cis* MK-7. Hence, while the bacterium synthesises the all-*trans* isomer intracellularly, secretion into the fermentation broth subjects all-*trans* MK-7 to the extracellular environment, which can induce its transformation to the *cis* isomer. Since the nature of the fermentation broth is determined by and varies with the fermentation conditions, key aspects of the fermentation process, including the media composition and the value of important fermentation parameters, such as the inoculum size, fermentation temperature, agitation speed, and length of fermentation, play a significant role in determining the characteristics of the extracellular environment and, therefore, the MK-7 isomer profile resulting from fermentation.

The isomer composition of MK-7 products is noteworthy, as only all-*trans* MK-7 is bioactive, and it has been recognised that the *cis* isomers of vitamin K merely possess 1% of the biological activity of the all-*trans* form (Knauer et al., 1975; Lowenthal and Rivera, 1979; Matschiner and Bell, 1972; Sitkowski et al., 2018). Moreover, a recent comparison of the bioactivity and carboxylative efficacy of *cis* and *trans* MK-7 has revealed that while not completely ineffective, the *cis* forms of MK-7 have significantly reduced carboxylative capacity, compromising their biological function relative to the all-*trans* isomer (Cirilli et al., 2022).

MK-7 can be produced from chemical reaction schemes or naturally by fermentation with various microbial strains, and the isomer composition of the resulting MK-7 is influenced by the methods used for its synthesis and purification of the post-reaction mixture (Sitkowski et al., 2018; Szterk et al., 2018a, 2018b). Essentially, fermentation-based synthesis is preferred from a consumer's point of view, as it is considered a superior natural and organic alternative to synthetic formulations. In addition, natural synthesis methods are a more sustainable approach for large-scale MK-7 production, and microbial fermentation can help meet both the market demand and sustainable development goals (Kang et al., 2022).

Various aspects of MK-7 fermentation have been comprehensively explored to improve the production of the vitamin in different contexts, including liquid-state fermentation (LSF) (Berenjian et al., 2011a, 2011b, 2012a, 2013; Luo et al., 2016; Puri et al., 2015; Song et al., 2014), solid-state fermentation (SSF) (Lal et al., 2019; Mahanama et al., 2011a, 2011b, 2012a, 2012b; Mahanma et al., 2011; Singh et al., 2015), and biofilm reactors (Mahdinia et al., 2018a, 2018b, 2018c, 2018d, 2019a, 2019b). Several wild-type and engineered bacterial strains, both Gram-positive and Gram-negative, are suitable for the production of MK-7 via fermentation, but members of the *Bacillus* species, namely *Bacillus subtilis natto*, *Bacillus licheniformis*, and *Bacillus amylolyquifaciens*, are the most prominent (Kang et al., 2022; Mahdinia et al., 2018a; Zhang et al., 2021). *B. subtilis natto* is regarded as the ideal strain for the industrial production of MK-7, as it is generally recognised as safe (GRAS) and enables a high MK-7 yield; hence, it is commonly employed for the production of fermented MK-7 supplements and functional food products (Berenjian et al., 2012a, 2015; Luo et al., 2016; Mahdinia et al., 2019a, 2019b; Puri et al., 2015). In addition to the microbial strain, crucial fermentation conditions, such as the inoculum loading, fermentation temperature, agitation speed, and length of fermentation, are instrumental in determining the efficacy of the fermentation process and the resulting MK-7 concentration.

The use of design of experiments (DOE) to optimise the important fermentation parameters is advantageous, as it allows both the individual and interactive effects of the different factors to be evaluated to determine the optimum conditions for a specific fermentation process, which is distinct from the conventional method where the different factors are varied and analysed independently. It is essential to note that while optimisation of the significant aspects of MK-7 fermentation to increase the process productivity has been the focus of many investigations (Berenjian et al., 2012a, 2013; Luo et al., 2016; Mahanama et al., 2011a, 2011b, 2012a; Mahanma et al., 2011; Mahdinia et al., 2018c, 2018d; Novin et al., 2020; Sato et al., 2001; Singh et al., 2015; Wu and Ahn, 2011), the aim has been to enhance MK-7 production holistically, and the isomer composition resulting from fermentation under the conditions examined has not been considered. Knowledge of the MK-7 isomer profile attained from fermentation is beneficial for the development of efficacious MK-7 enriched foods and dietary supplements that predominantly contain the bioactive isomer, as the other isomeric forms of MK-7 are effectively impurities that lack therapeutic value.

Therefore, the objective of this study was to build on our previous investigation (Lal et al., 2022) and evaluate key fermentation parameters to maximise the concentration of the bioactive all-*trans* isomer and minimise the production of *cis* MK-7. To accomplish this, a CCF design was employed to assess and optimise the inoculum size, fermentation temperature, agitation speed, and fermentation time. This study will be a valuable step forward in developing a commercially appealing industrial fermentation process that favours the synthesis of the all-*trans* MK-7 isomer, which will streamline the production process and reduce the associated costs. Furthermore, the findings of this investigation will be an important advancement in increasing the availability of biologically active fermented MK-7 nutritional supplements and functional foods, which will help improve the dietary intake of MK-7 and provide consumers with better health outcomes.

## 2. Materials and methods

### 2.1. Chemicals and materials

The all-*trans* MK-7 analytical standard (98.1% purity) was purchased from ChromaDex (Los Angeles, CA, USA). Glucose was acquired from Ajax Finechem Pty Ltd (Taren Point, NSW, Australia). Yeast extract and tryptone were obtained from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Glycerol, soy peptone, methanol, 2-propanol, and *n*-hexane were purchased from Merck Millipore (Burlington, MA, USA). NaCl was acquired from a domestic supplier, and $CaCl_2$ was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Nutrient agar plates were purchased from Fort Richard Laboratories (Auckland, New Zealand).

### 2.2. Microorganism and inoculum preparation

The *B. subtilis natto* strain was isolated from a commercially available natto product and prepared as described previously (Berenjian et al., 2011b). Accordingly, the cells were cultivated in a liquid culture medium containing 1% (*w/v*) tryptone, 0.5% (*w/v*) yeast extract, and 1% (*w/v*) NaCl before streaking on nutrient agar plates. The plates were incubated at 37 °C for 48 h. Afterwards, the cells were scraped off the plates and suspended in a sterilised saline solution (0.9% (*w/v*) NaCl). The suspension was then placed in a water bath at 80 °C for 30 min to inactivate the vegetative cells and induce the production of spores before centrifuging (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 3000 rpm for 10 min to remove the cell debris. The resulting spore suspension ($4.8 \times 10^6$ CFU/mL) was used as the inoculum for the fermentation experiments.

### 2.3. Experimental design and statistical analysis

The effect of significant fermentation parameters, specifically the inoculum size, fermentation temperature, agitation speed, and length of fermentation, on the production of MK-7 isomers was evaluated and optimised. The inoculum size was examined between 2-10% (*v/v*), the fermentation temperature was investigated in the range of 35–45 °C, the agitation speed was explored between 100-200 rpm, and the length of fermentation was considered in the span of 4–10 days. The factors were studied at three levels (high, intermediate, and low), and the upper and lower limits of the investigated range for each factor were derived from the literature (Benedetti et al., 2010; Berenjian et al., 2011b, 2012a; Luo et al., 2016; Mahdinia et al., 2018c, 2018d; Sato et al., 2001; Singh et al., 2015; Wu and Ahn, 2011) and our preliminary experiments.

A CCF design was employed to optimise the selected variables, and the results were analysed using response surface methodology (RSM). The experimental values were scaled factors, and the response was described by a quadratic equation. The MODDE 13 software (Sartorius, Gottingen, Germany) was used to create the design matrices, develop a model, and determine the optimum level of the factors to achieve the highest concentration of all-*trans* MK-7 and reduce the concentration of the *cis* isomer. The experimental data was then used to generate a second-order polynomial regression model (Eq. (1)) for each response.

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j \quad (1)$$

Where $Y$ represents the all-*trans* or *cis* MK-7 isomer concentration; $b_0$ is a constant term; $b_i$, $b_{ii}$, and $b_{ij}$ are the coefficients of the linear, quadratic, and synergistic effects, respectively; and $X_i$ and $X_j$ correspond to the significant factors. The quality of the fit for the developed regression models was expressed via the $R^2$ value, and the analysis of variance (ANOVA) test was used to determine the statistical significance, which was accepted at $P < 0.1$.

### 2.4. Fermentation procedure

The fermentation media employed in this investigation consisted of 1% (*w/v*) glucose, 2% (*w/v*) yeast extract, 2% (*w/v*) soy peptone, 2% (*w/v*) tryptone, and 0.1% (*w/v*) $CaCl_2$ and was derived from our preceding study (Lal et al., 2022). This was determined to be the optimal media composition to enhance the concentration of the all-*trans* isomer and decrease the production of *cis* MK-7. The media for each sample was prepared individually in McCartney bottles and sterilised at 121 °C for 20 min using an autoclave (TOMY SX-700E, Tokyo, Japan). Each sample was then inoculated with the *B. subtilis natto* spore suspension and fermented under aerobic conditions according to the DOE plan, which specified the inoculum size, fermentation temperature, agitation speed, and length of fermentation for each sample. A small fermentation volume (6 mL) was used to enable the extraction of the whole sample. This allowed all of the MK-7 produced during fermentation to be analysed and eliminated any errors associated with sampling from the fermentation media.

### 2.5. MK-7 extraction

Prior to analysis, MK-7 was extracted from the samples using a mixture of 2-propanol and *n*-hexane in the ratio of 1:2 (*v/v*) and a liquid-to-organic ratio of 1:4 (*v/v*) (Berenjian et al., 2011b). The mixture was vigorously shaken for 2 min using a vortex mixer and centrifuged (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 3000 rpm for 10 min to separate the two phases. Since MK-7 is a lipid-soluble vitamin, it preferentially dissolves in *n*-hexane, which is a non-polar solvent. Thus, the upper hexane layer was separated from the aqueous phase and evaporated under a vacuum to recover the extracted MK-7.

### 2.6. MK-7 analysis

MK-7 analysis was carried out using the method outlined in our previous investigation (Lal et al., 2022). Briefly, a Dionex high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with four pumps (P680), an automated sample injector (ASI-100), a thermostatted column compartment (TCC-100), and a photodiode array UV detector (UVD340U) was used to determine the MK-7 isomer concentration in the fermented samples. A COSMOSIL Cholester packed column (100 mm × 2 mm × 2.5 μm; Nacalai Tesque Inc., Kyoto, Japan), which was operated at 40 °C, was employed to separate the compounds. Methanol was used as the mobile phase at a flow rate of 0.2 mL/min (isocratic elution). The analytical wavelength, injection volume, autosampler temperature, and run-time were 248 nm, 10 μL, 10 °C, and 30 min, respectively. Data collection was carried out using the Chromeleon 7 software (Thermo Fisher Scientific, Waltham, MA, USA), and a relative retention time (RRT) of 1.12 allowed the identification of the *cis* MK-7 isomer.

Liquid chromatography-mass spectrometry (LC–MS) techniques were implemented to verify the presence and confirm the chromatographic retention times of the all-*trans* and *cis* MK-7 isomers. The LC–MS system consisted of a Dionex Ultimate 3000 ultra-high-

performance liquid chromatography (UHPLC) instrument and a QExactive mass spectrometer with a HESI II source (Thermo Fisher Scientific, Waltham, MA, USA). The Thermo XCalibur 4.3 software (Thermo Fisher Scientific, Waltham, MA, USA) was employed to control the system, and the Chromeleon 7.3 software (Thermo Fisher Scientific, Waltham, MA, USA) was used for data handling. The chromatographic conditions outlined above were applied to separate the compounds by liquid chromatography; however, the injection volume was altered to 5 μL, and the run-time was extended to 37 min to adapt to the requirements of the LC–MS platform. Data collection was carried out in the positive ionisation mode with an MS1 scan range of 150–1000 $m/z$, a resolution of 70,000, an AGC target of $3 \times 10^6$, and a maximum injection time of 200 ms. The Thermo FreeStyle 1.6 software (Thermo Fisher Scientific, Waltham, MA, USA) was used to analyse the mass spectrometry (MS) data.

A calibration curve was constructed based on the peak area corresponding to known concentrations of the MK-7 analytical standard and used to infer the MK-7 concentration of the fermented samples. The resulting MK-7 calibration curve was linear between 0.1 mg/L and 50 mg/L ($R^2 = 0.99$).

## 3. Results and discussion

### 3.1. Fermentation experiments and statistical analysis

The inoculum size, fermentation temperature, agitation speed, and fermentation time were optimised using a CCF design and RSM to determine the ideal values of these fermentation parameters to increase the concentration of all-*trans* MK-7 and reduce the production of the *cis* isomer. The CCF design matrix and isomer concentrations for each sample are outlined in Table 1, and the statistical analysis for the optimisation study is provided in Table 2. Polynomial regression models, including only the significant model terms, were generated to determine the concentration of the all-*trans* (Eq. (2)) and *cis* (Eq. (3)) MK-7 isomers depending on the inoculum size, temperature, agitation speed, and length of fermentation.

$$Y_1 = 36.287 - 5.736\,X_2 - 10.033\,X_4^2 - 9.453\,X_2X_3 \tag{2}$$

$$Y_2 = 1.037 - 0.178\,X_2 - 0.296\,X_2X_3 - 0.289\,X_2X_4 \tag{3}$$

Where $Y_1$ represents the all-*trans* isomer concentration; $Y_2$ corresponds to the concentration of *cis* MK-7; and $X_1$, $X_2$, $X_3$, and $X_4$ refer to the inoculum size, fermentation temperature, agitation speed, and fermentation time, respectively.

The linear term for temperature ($X_2$) and interactive term for temperature and agitation ($X_2X_3$) were particularly significant for both responses. This implies that the fermentation temperature is directly related to the MK-7 isomer concentration, and the fermentation temperature and agitation speed have a combined effect on the isomer concentration. The fermentation temperature plays a crucial role in bacterial growth and metabolism, as it influences the microbial growth rate and enzyme activity (Weissbrodt et al., 2020). The preferred temperature for each bacterial strain encompasses a narrow range of values. Generally, the rate of microbial

**Table 1**
Experimental design and responses from the optimisation study.

| Sample | Inoculum size (% (*v*/*v*)) | Temperature (C) | Agitation speed (rpm) | Fermentation time (days) | All-*trans* MK-7 concentration (mg/L) | *Cis* MK-7 concentration (mg/L) |
|---|---|---|---|---|---|---|
| 1 | 2 | 35 | 100 | 4 | 24.606 | 0.627 |
| 2 | 10 | 35 | 100 | 4 | 22.596 | 0.621 |
| 3 | 2 | 45 | 100 | 4 | 11.411 | 0.528 |
| 4 | 10 | 45 | 100 | 4 | 23.313 | 0.692 |
| 5 | 2 | 35 | 200 | 4 | 34.96 | 0.837 |
| 6 | 10 | 35 | 200 | 4 | 35.637 | 0.942 |
| 7 | 2 | 45 | 200 | 4 | 17.047 | 1.024 |
| 8 | 10 | 45 | 200 | 4 | 10.119 | 0.696 |
| 9 | 2 | 35 | 100 | 10 | 24.038 | 0.860 |
| 10 | 10 | 35 | 100 | 10 | 23.032 | 0.998 |
| 11 | 2 | 45 | 100 | 10 | 29.649 | 0.834 |
| 12 | 10 | 45 | 100 | 10 | 33.93 | 1.022 |
| 13 | 2 | 35 | 200 | 10 | 41.729 | 1.819 |
| 14 | 10 | 35 | 200 | 10 | 43.775 | 1.901 |
| 15 | 2 | 45 | 200 | 10 | 2.528 | 0.000 |
| 16 | 10 | 45 | 200 | 10 | 3.940 | 0.000 |
| 17 | 2 | 40 | 150 | 7 | 46.121 | 1.190 |
| 18 | 10 | 40 | 150 | 7 | 42.202 | 1.125 |
| 19 | 6 | 35 | 150 | 7 | 36.459 | 1.255 |
| 20 | 6 | 45 | 150 | 7 | 26.896 | 0.865 |
| 21 | 6 | 40 | 100 | 7 | 31.795 | 1.262 |
| 22 | 6 | 40 | 200 | 7 | 48.237 | 1.039 |
| 23 | 6 | 40 | 150 | 4 | 32.814 | 1.832 |
| 24 | 6 | 40 | 150 | 10 | 24.69 | 1.029 |
| 25 | 6 | 40 | 150 | 7 | 29.772 | 0.832 |
| 26 | 6 | 40 | 150 | 7 | 31.116 | 0.856 |
| 27 | 6 | 40 | 150 | 7 | 32.987 | 1.013 |

**Table 2**
Statistical analysis of the optimisation study.

| **All-*trans* MK-7 concentration** | | | |
|---|---|---|---|
| **Term** | **Coefficient** | **Standard Error** | ***P*-value** |
| Constant | 36.287 | 2.443 | 0.000 |
| $X_1$ | −1.016 | 1.682 | 0.558 |
| $X_2$ | −5.736 | 1.682 | 0.006 |
| $X_3$ | −0.619 | 1.682 | 0.720 |
| $X_4$ | −0.552 | 1.682 | 0.749 |
| $X_1^2$ | 5.376 | 4.133 | 0.220 |
| $X_2^2$ | −7.108 | 4.133 | 0.113 |
| $X_3^2$ | 1.231 | 4.133 | 0.771 |
| $X_4^2$ | −10.033 | 4.133 | 0.034 |
| $X_1X_2$ | −0.862 | 1.800 | 0.641 |
| $X_1X_3$ | 0.549 | 1.800 | 0.766 |
| $X_1X_4$ | 1.740 | 1.800 | 0.354 |
| $X_2X_3$ | −9.453 | 1.800 | 0.000 |
| $X_2X_4$ | −1.960 | 1.800 | 0.299 |
| $X_3X_4$ | −0.610 | 1.800 | 0.741 |
| ***Cis* MK-7 concentration** | | | |
| **Term** | **Coefficient** | **Standard Error** | ***P*-value** |
| Constant | 1.037 | 0.109 | 0.000 |
| $X_1$ | −0.040 | 0.070 | 0.585 |
| $X_2$ | −0.178 | 0.070 | 0.030 |
| $X_3$ | −0.010 | 0.070 | 0.892 |
| $X_4$ | 0.038 | 0.075 | 0.620 |
| $X_1^2$ | 0.053 | 0.182 | 0.778 |
| $X_2^2$ | −0.045 | 0.182 | 0.810 |
| $X_3^2$ | 0.046 | 0.182 | 0.807 |
| $X_4^2$ | −0.182 | 0.223 | 0.432 |
| $X_1X_2$ | −0.080 | 0.075 | 0.310 |
| $X_1X_3$ | 0.023 | 0.075 | 0.767 |
| $X_1X_4$ | 0.091 | 0.075 | 0.252 |
| $X_2X_3$ | −0.296 | 0.075 | 0.003 |
| $X_2X_4$ | −0.289 | 0.075 | 0.003 |
| $X_3X_4$ | −0.002 | 0.075 | 0.978 |

$X_1$ = inoculum size; $X_2$ = temperature; $X_3$ = agitation speed; $X_4$ = fermentation time; $R^2$ = 0.8; significance accepted at $P < 0.1$.
$X_1$ = inoculum size; $X_2$ = temperature; $X_3$ = agitation speed; $X_4$ = fermentation time; $R^2$ = 0.817; significance accepted at $P < 0.1$.

growth and enzyme activity increase with a rise in the fermentation temperature and reach a maximum at the optimum temperature. Any further increase in the temperature decreases the microbial growth rate and enzyme activity due to the thermal denaturation of proteins and enzymes that facilitate microbial growth and vital metabolic processes. Therefore, since MK-7 production is partly growth-associated (Berenjian et al., 2011b, 2013; Lal et al., 2019; Novin et al., 2020; Ranmadugala et al., 2017; Song et al., 2014), the fermentation temperature has a substantial contribution to the MK-7 yield. The agitation speed also affects microbial growth and MK-7 biosynthesis, as it impacts the homogeneity of the fermentation broth, cellular oxygen and nutrient availability, and rate of mass transfer. The quadratic term for time ($X_4^2$) and interactive term for temperature and time ($X_2X_4$) were also significant for the all-*trans* and *cis* isomer concentrations, respectively. This suggests that the fermentation time has a notable effect on the isomer concentration. The length of fermentation is closely associated with the microbial growth profile (Lal et al., 2022); thus, the MK-7 concentration that is attained varies depending on the stage of growth that the microbial culture is in at the conclusion of fermentation.

The results from the ANOVA analysis (Table 3) established that the proposed models were coherent with the experimental observations, as the standard deviation (SD) of the regression was considerably greater than the SD of the residuals for both the all-*trans* and *cis* MK-7 isomer concentrations. This is further illustrated by the significant *P*-value (0.006 and 0.035) and high *F*-value (4.799 and 3.200) for each response. An $R^2$ value of 0.859 for the all-*trans* MK-7 response and 0.817 for the *cis* MK-7 response indicate a good model fit. The observed versus predicted plot for each response, which compares the actual experimental observations with those predicted by the statistical model, is also provided in Fig. 2. It can be seen that for both responses, the data points fit relatively close to the line with only a small amount of scatter. This supports the conclusions inferred from the ANOVA analysis and implies that the model developed for each response is appropriate.

**Table 3**
ANOVA for the quadratic models.

| **All-*trans* MK-7 concentration** | | | | | | |
|---|---|---|---|---|---|---|
| **Source of variation** | **DF** | **SS** | **MS** | ***F*-value** | ***P*-value** | **SD** |
| Total corrected | 25 | 3425.112 | 137.004 | | | 11.705 |
| Regression | 14 | 2943.271 | 210.234 | 4.799 | 0.006 | 14.499 |
| Residual | 11 | 481.841 | 43.804 | | | 6.618 |
| ***Cis* MK-7 concentration** | | | | | | |
| **Source of variation** | **DF** | **SS** | **MS** | ***F*-value** | ***P*-value** | **SD** |
| Total corrected | 24 | 4.176 | 0.174 | | | 0.417 |
| Regression | 14 | 3.413 | 0.244 | 3.200 | 0.035 | 0.494 |
| Residual | 10 | 0.762 | 0.076 | | | 0.276 |

DF: degree of freedom; SS: sum of squares; MS: mean sum of squares; SD: standard deviation.

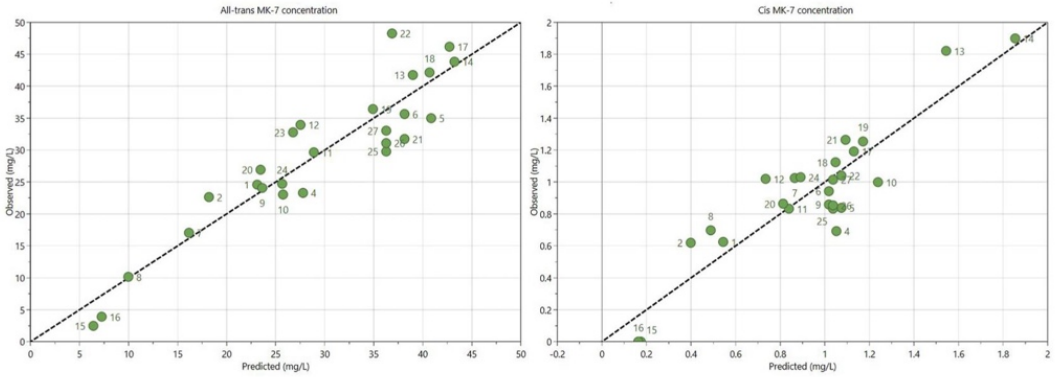


**Fig. 2.** Observed versus predicted plot for the all-*trans* and *cis* isomer concentrations.

Six response surface plots were generated for the all-*trans* and *cis* MK-7 isomer concentrations to display the interactions between each pair of fermentation conditions when the level of the remaining factors is set at their intermediate value. The contour plots are provided in Fig. 3 for the all-*trans* MK-7 response and Fig. 4 for the *cis* MK-7 response. The response surface plots portray a complex picture, as the concentration of both isomers is interdependent and is synonymously determined by the investigated factors. Overall, the contour plots demonstrate that a small inoculum size, moderate temperature, high agitation speed, and an intermediate fermentation time best satisfy the aim of this study, which was to maximise the production of the all-*trans* isomer and minimise the concentration of *cis* MK-7.

### *3.2. Optimum fermentation conditions*

It is necessary to understand that despite being the inactive form of MK-7, production of the *cis* isomer during fermentation is unlikely to be eliminated, as it is synthesised in small quantities alongside the all-*trans* isomer. Nonetheless, it is possible to optimise the fermentation conditions to favour the production of the bioactive isomer and reduce the relative concentration of *cis* MK-7.

The optimum fermentation parameters comprised an inoculum size of 2% (*v/v*), a temperature of 40 °C, an agitation speed of 200 rpm, and a fermentation period of 7 days. The resulting all-*trans* and *cis* isomer concentrations (mean ± SD) from triplicate samples were 53.292 ± 1.291 mg/L and 1.222 ± 0.050 mg/L, respectively. Moreover, compared to fermentation using only the optimal media (Lal et al., 2022), employing the optimised fermentation conditions resulted in an approximately 46.5% increase in the concentration of the biologically active isomer, while the *cis* isomer concentration was comparable. This clearly demonstrates the vital contribution of the fermentation environment in determining the yield of the intended product.

It has generally been established that a small inoculum size is appropriate for MK-7 biosynthesis. Higher inoculum levels have an adverse effect on MK-7 production, which is likely attributed to a decrease in oxygen availability and the depletion of essential nutrients required for the production of microbial metabolites, such as MK-7, in the presence of a large biomass concentration (Mahanama et al., 2011b; Zhao et al., 2021). Consequently, an inoculum size of 2% (*v/v*) has been frequently used in MK-7 fermentation studies (Berenjian et al., 2011a, 2011b, 2012a, 2012b; Ma et al., 2019).

*B. subtilis natto* can tolerate a wide range of temperatures, typically between 28-45 °C (Berenjian et al., 2015; Ren et al., 2019), but our results demonstrate that 40 °C is the optimum temperature for high MK-7 production, which is comparable to prior studies (Berenjian et al., 2011a, 2011b, 2012a, 2012b, 2015; Ma et al., 2019; Mahanma et al., 2011; Ren et al., 2019; Takenaka et al., 2002; Wu and Ahn, 2011). *B. subtilis natto* is a mesophilic microorganism, and 40 °C is the optimum temperature to promote high enzyme activity in mesophiles (da Rosa et al., 2019). Therefore, a fermentation temperature of 40 °C facilitates critical metabolic processes in *B. subtilis natto* and enhances MK-7 biosynthesis.

While various agitation speeds have been considered in the context of MK-7 fermentation, higher agitation speeds tend to be preferable (Berenjian et al., 2013; Ma et al., 2019; Novin et al., 2020), as greater agitation rates increase the homogeneity of the fer-

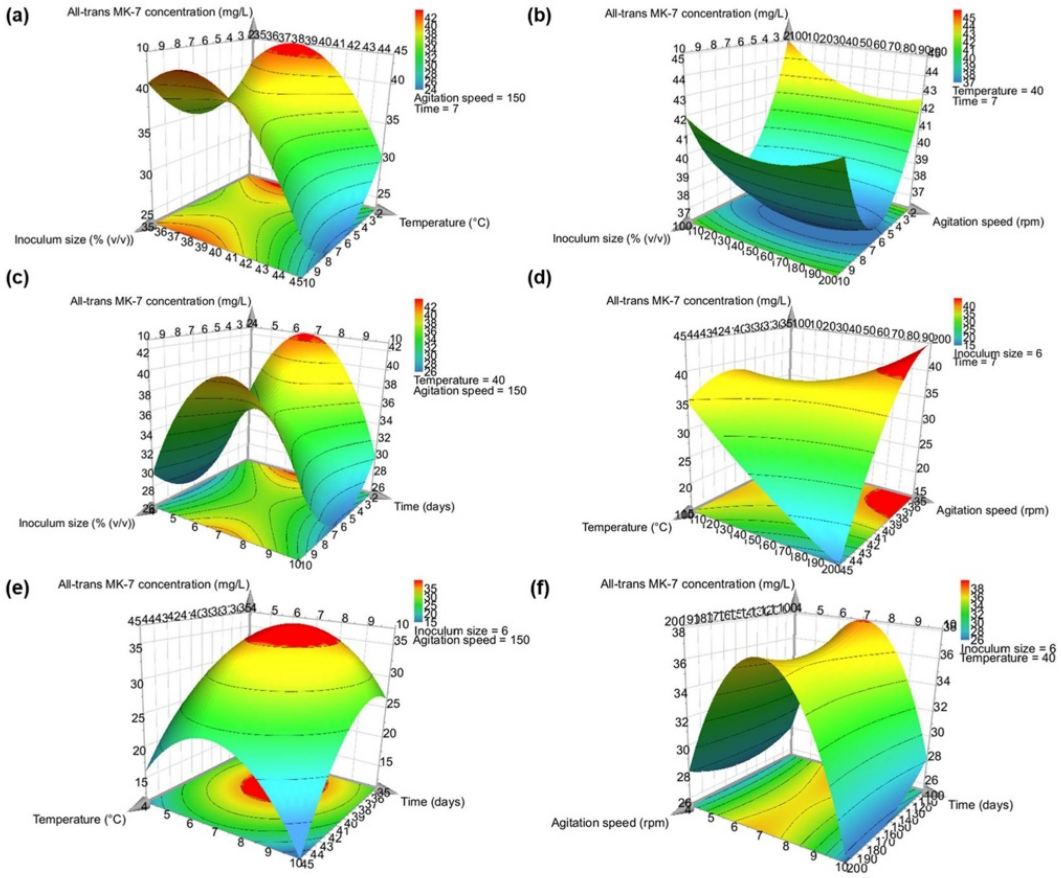


**Fig. 3.** Response surface plots (a–f) illustrating the pairwise effect of key fermentation parameters on the all-*trans* MK-7 isomer concentration.

mentation broth, enhance the rate of mass transfer, and improve the availability of oxygen and nutrients to cells. Although an agitation speed of 200 rpm was determined to be the optimum in this investigation, the experimental observations suggest that higher agitation rates may be beneficial to promote a greater all-*trans* MK-7 yield.

Additionally, the length of fermentation significantly influences the MK-7 concentration that is achieved, as it largely depends on the stage of microbial growth. MK-7 is synthesised intracellularly, and during fermentation, it is secreted from the cell as a soluble complex with an acidic binding factor (Chatake et al., 2018; Ikeda and Doi, 1990; Ma et al., 2022; Yanagisawa and Sumi, 2005). A comparison of MK-7 production with the microbial growth profile has revealed that MK-7 synthesis gradually increases as the bacterial culture proceeds from the lag to the exponential growth stage, and a notable increase in the MK-7 concentration occurs during the stationary phase when the intracellular MK-7 is released into the fermentation broth due to cell lysis (Lal et al., 2022; Sato et al., 2001; Song et al., 2014; Xu and Zhang, 2017). Our previous investigation (Lal et al., 2022) determined that the bacterial culture enters the stationary phase from day 4 onwards, and a fermentation time of 7 days potentially corresponds to the end of the stationary growth period; therefore, maximal MK-7 release is likely to occur at this stage. It is advantageous to terminate fermentation before the onset of the death phase, as exposure to proteases and other cellular components that are released during cell lysis may degrade the MK-7 present in the extracellular environment and decrease its concentration over an extended timeframe. Consequently, MK-7 fermentation is usually carried out for 6–7 days (Benedetti et al., 2010; Berenjian et al., 2011b; Gao et al., 2020; Mahanama et al., 2012b; Sato et al., 2001; Tani and Taguchi, 1989; Xu and Zhang, 2017), which correlates with the late stationary phase.

A desirability study was also performed to visualise how variation in the fermentation parameters, namely the inoculum size, fermentation temperature, agitation speed, and fermentation time, impact the resulting all-*trans* and *cis* MK-7 isomer concentrations, and this is depicted in Fig. 5.

It is essential to note that excluding our preceding investigation (Lal et al., 2022), prior research focusing on the production of MK-7 isomers through fermentation by analysing the isomer composition of fermented samples is scarce. Hence, the absence of existing studies on this subject limits the ability to make comparisons with directly applicable literature. However, it is possible to draw parallels with the findings of previous MK-7 fermentation studies of relevance, as although they have not examined the production of MK-7 isomers, they have considered MK-7 synthesis in a wide range of fermentation-based scenarios. The fundamental outcomes of which are, to a large extent, likely to be pertinent to the production of MK-7 isomers from fermentation employing different conditions. Furthermore, the lack of comparable studies on this topic demonstrates the novelty of our research, and the findings of this investigation

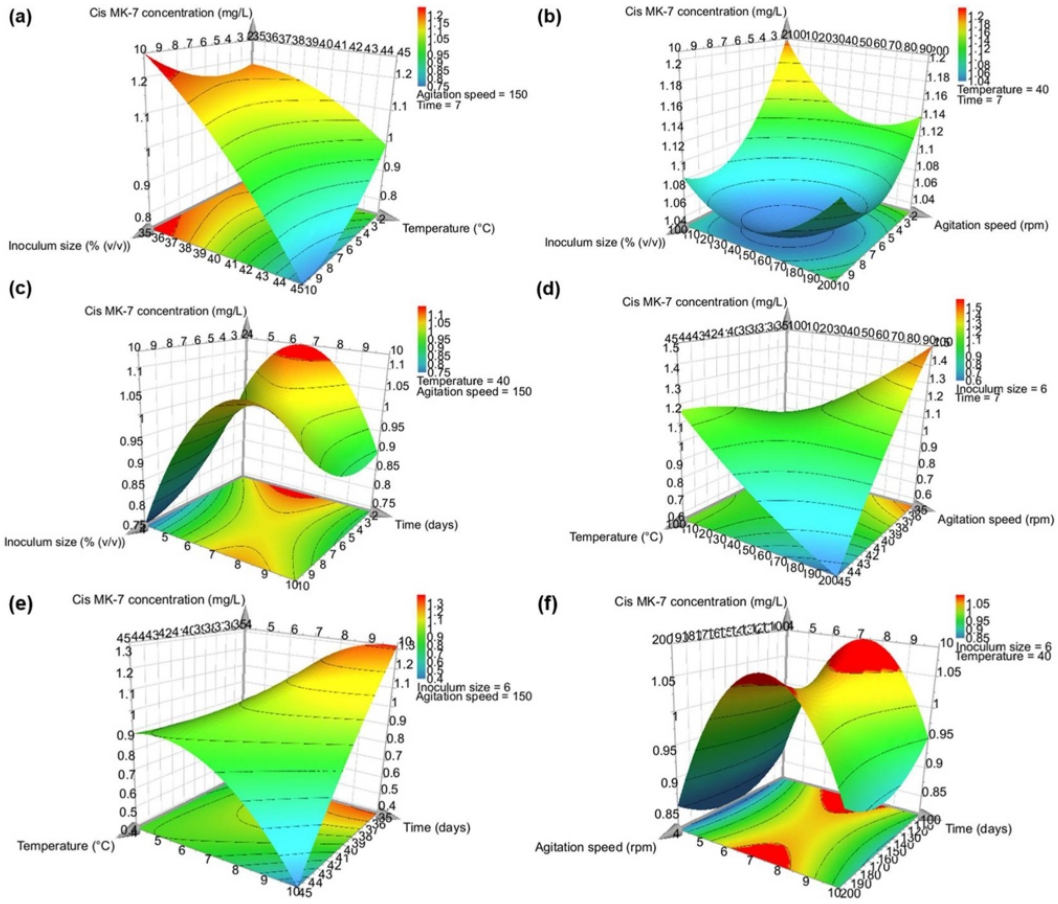


**Fig. 4.** Response surface plots (a–f) illustrating the pairwise effect of key fermentation parameters on the *cis* MK-7 isomer concentration.

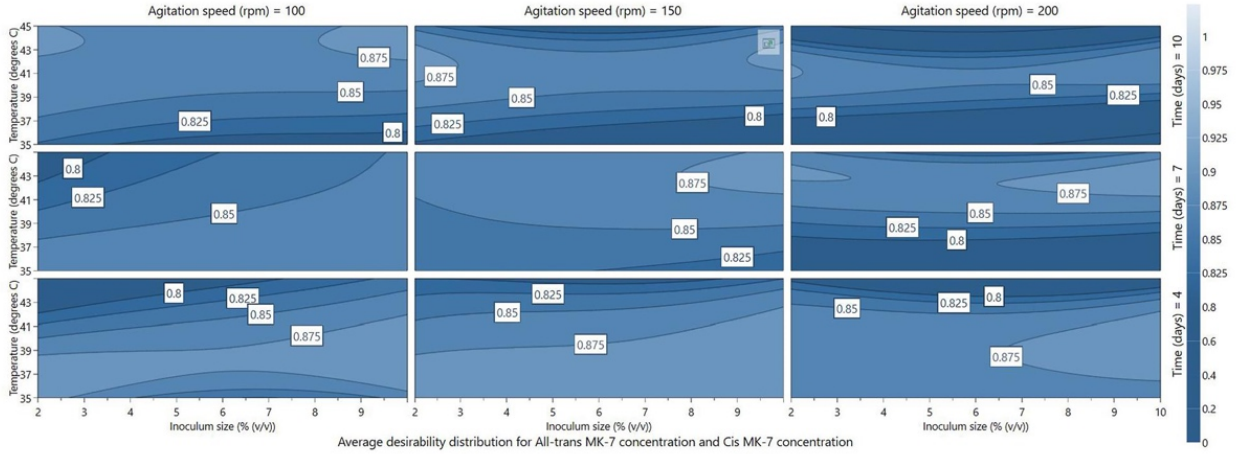


**Fig. 5.** Desirability chart for both responses.

will contribute new knowledge and improve the current understanding of the MK-7 isomer profile resulting from fermentation under various conditions.

## 4. Conclusions

This study was the first to evaluate the impact of key fermentation parameters on the MK-7 isomer profile to enhance the concentration of the bioactive all-*trans* MK-7 isomer and reduce the production of the inefficacious *cis* isomer. The inoculum size, fermenta-

tion temperature, agitation speed, and fermentation time were evaluated and optimised using a CCF design. An inoculum size of 2% (*v/v*), a temperature of 40 °C, an agitation speed of 200 rpm, and a fermentation period of 7 days were determined to be the optimum conditions, which resulted in an all-*trans* isomer concentration of 53.292 mg/L and a *cis* isomer concentration of 1.222 mg/L. MK-7 production employing optimised fermentation conditions is a favourable alternative to conventional fermentation processes that do not specifically target the production of the bioactive isomer, as it will necessitate fewer downstream purification steps and increase the productivity of the fermentation system, which is commercially promising.

## Compliance with ethical standards

This article does not contain studies involving human or animal participants.

## Declaration of competing interest

The authors have no conflict of interest to declare.

## Data availability

The data used to support the findings of this study are included within the article.

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# 6

# The Effect of Iron Oxide Nanoparticles on the Menaquinone-7 Isomer Composition and Synthesis of the Biologically Significant All-*Trans* Isomer

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By

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# Chapter 6 – The Effect of Iron Oxide Nanoparticles on the Menquinone-7 Isomer Composition and Synthesis of the Biologically Significant All-*Trans* Isomer

This chapter centres on investigating nanobiotechnological approaches to overcome the primary challenges associated with the fermentation and downstream processing of MK-7. The low fermentation yield and many complex downstream processing steps raise the cost of MK-7 production and increase the price of the fermented product, which, consequently, cannot be invariably afforded by all populations. Bacterial cell immobilisation with IONs holds great promise for enhancing the MK-7 concentration and yield achieved from fermentation, and the magnetic properties of IONs can be utilised to aid cell separation for process intensification. Furthermore, given the differing biological significance of MK-7 isomers, the value of IONs for this purpose is only justified if all-*trans* MK-7 is attained almost exclusively or in the greatest fraction.

Naked (uncoated) IONs were synthesised from the co-precipitation of Fe (II) and Fe (III) ions and characterised using various methods to assess the structure and properties of the prepared IONs and their interaction with *B. subtilis natto* cells. The impact of fermentation with immobilised bacterial cells on microbial growth and the MK-7 isomer yield was also studied. The optimum naked ION concentration, which resulted in the greatest all-*trans* MK-7 yield, was additionally examined in a monitoring study to explore its effect on the bacterial growth, MK-7 isomer, and pH profiles.

This chapter acts as a foundation for the successive chapter (Chapter 7), which considers amine-functionalised IONs to further develop the use of nanobiotechnological strategies to improve the fermentation-based synthesis of MK-7.

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# The Effect of Iron Oxide Nanoparticles on the Menaquinone-7 Isomer Composition and Synthesis of the Biologically Significant All-*Trans* Isomer

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**Abstract:** Menaquinone-7 (MK-7) is the most therapeutically valuable K vitamin owing to its excellent bioavailability. MK-7 occurs as geometric isomers, and only all-*trans* MK-7 is bioactive. The fermentation-based synthesis of MK-7 entails various challenges, primarily the low fermentation yield and numerous downstream processing steps. This raises the cost of production and translates to an expensive final product that is not widely accessible. Iron oxide nanoparticles (IONPs) can potentially overcome these obstacles due to their ability to enhance fermentation productivity and enable process intensification. Nevertheless, utilisation of IONPs in this regard is only beneficial if the biologically active isomer is achieved in the greatest proportion, the investigation of which constituted the objective of this study. IONPs ($Fe_3O_4$) with an average size of 11 nm were synthesised and characterised using different analytical techniques, and their effect on isomer production and bacterial growth was assessed. The optimum IONP concentration (300 µg/mL) improved the process output and resulted in a 1.6-fold increase in the all-*trans* isomer yield compared to the control. This investigation was the first to evaluate the role of IONPs in the synthesis of MK-7 isomers, and its outcomes will assist the development of an efficient fermentation system that favours the production of bioactive MK-7.

**Keywords:** menaquinone-7 isomers; biological significance; iron oxide nanoparticles; bacterial cell immobilisation; fermentation



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## 1. Introduction

The vitamin K group comprises a series of lipid-soluble molecules that share the same basic structure, consisting of a 2-methyl-1,4-naphthoquinone component, but vary in the arrangement of an isoprenoid side chain at the 3-position [1]. The characteristics of the various forms of vitamin K are governed by the length and degree of unsaturation of its isoprenoid chain [2]. Of the different types of vitamin K, only vitamin K1 (phylloquinone) and vitamin K2 (menaquinones) are nutritionally relevant to humans [3]. Phylloquinone (PK) is an individual compound and is abundantly available from a range of photosynthetic plants, vegetable oils, and their derivatives [2,4–6]. In comparison, menaquinones (MK) are a collection of molecules that have isoprenoid chains of various lengths and the degree of unsaturation, which is represented by the general description MK-*n*, where *n* is typically between four and thirteen and signifies the number of unsaturated isoprenoid units in the side chain [2,7,8]. MK are predominantly from microbial sources and exist in low quantities in certain fermented, dairy, and animal products [2,5,8–10].

While all K vitamins participate in haemostasis, studies have revealed that the health benefits of vitamin K, especially MK, transcend the activation of hepatic coagulation factors.

Specifically, vitamin K consumption has been linked to a decreased risk of cardiovascular diseases (CVDs) and osteoporosis, as well as many other health gains, such as improving the outcomes of coronavirus disease 2019 (COVID-19) and reducing the likelihood of cancer, Parkinson's disease, immune disorders, type 2 diabetes mellitus, neurological disease, obesity, and chronic kidney disease [2,3,11–28].

MK-7 is the superior subtype of vitamin K and offers the most significant health advantages due to its longer plasma half-life and exceptional extrahepatic availability [25,29]. However, it is not prevalent in the diet and occurs at low concentrations in certain food items [30,31]. Consequently, the formulation of nutritional supplements and functional foods to accompany natural sources and satisfy daily intake requirements has become increasingly popular.

MK-7 exists as geometric isomers, and solely all-*trans* MK-7 is bioactive. The *cis* forms of the vitamin have comparatively little or no biological significance, and it has been ascertained that *cis* MK-7 has considerably compromised carboxylative capacity and biological function compared to the all-*trans* isomer [6,8,32,33]. The bioactivity of MK-7 is related to the configuration of double bonds in its isoprenoid chain, which influences the shape of the molecule and its ability to interact with subcellular structures [32,34]. All double bonds in the isoprenoid chain of all-*trans* MK-7 have the *trans* arrangement, which gives rise to a linear organisation (Figure 1), whereas the occurrence of one or more unsaturated bonds in the *cis* format distorts the structure of the side chain, resulting in a non-linear shape (Figure 1).

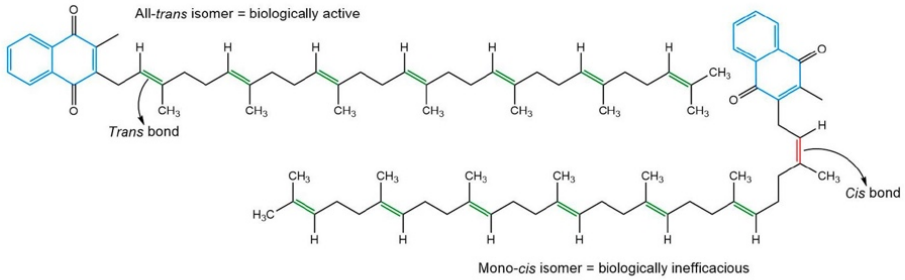


**Figure 1.** Comparison of the chemical structure and bond arrangement of MK-7 isomers.

MK-7 can be synthesised via chemical methods or from fermentation with several bacterial strains, and the production process and techniques employed to purify the post-reaction mixture impact the isomer profile of MK-7 [6–8,32,35–40]. From a consumer's perspective, fermentation-based synthesis is more favourable, as it is a natural and organic substitute for synthetic preparations. However, various challenges are linked to the natural synthesis of MK-7, the fundamental issue being the low fermentation yield [41,42]. Additionally, the large number of tedious unit operations involved in the downstream processing of the vitamin increases production expenses and, consequently, the price of the final product, thereby reducing its accessibility [41,43].

Numerous investigations have attempted to improve the MK-7 concentration and yield obtained from fermentation by optimising different aspects of the fermentation process, namely the fermentation method, media composition, and operating conditions [30,44–55]. Other approaches, including the genetic modification of microbial strains, the use of surfactants to increase the bacterial membrane permeability, and the application of various treatments to enhance MK-7 extraction from the culture medium, have also been explored to boost the synthesis and concentration of MK-7 achieved from fermentation [56]. Although these advancements have provided significant insights and increased the production of the vitamin, there is still scope for further improvement. All but our previous studies [47,57] have not accounted for the synthesis of MK-7 isomers, consideration of which is essential given their differing bioactivity. Moreover, optimising features of the fermentation process itself only enhances MK-7 production and does little to streamline the fermentation system

(process intensification). Hence, there is a need for novel approaches to improve the fermentation yield and/or reduce the number of unit operations involved and ensure that the biologically significant all-*trans* isomer is produced almost exclusively or in the greatest proportion. In this respect, the application of nanomaterials (NMs) in MK-7 fermentation is a promising and innovative technique that can potentially address the challenges associated with MK-7 production.

NMs are materials that have structural elements less than 1 µm (1000 nm) in at least one dimension [58,59]. The nanoscale size of NMs offers a large surface-area-to-volume ratio and confers unique properties not observed in the corresponding bulk material, making them suitable for various purposes [58,60–63]. Of the several classes of NMs, nanoparticles (NPs) can be used to address the major issues accompanying the fermentation-based synthesis of MK-7. NPs can be employed to enhance the productivity of the process by increasing the metabolic efficiency of the cells and/or simplifying the downstream processing steps (process intensification) through bacterial cell immobilisation. Iron-based NPs, specifically IONPs, have the ability to boost the MK-7 yield and improve fermentation productivity [41–43,64]. IONPs also exhibit superparamagnetism, which can allow cell separation using an external magnetic field to prospectively overcome the downstream limitations of industrial MK-7 fermentation [43].

Several studies have examined the potential for bacterial cell immobilisation with IONPs to enhance MK-7 production and aid cell recovery for process intensification [41–43,64]. In these investigations, *Bacillus subtilis natto* cells were decorated with IONPs to evaluate the effect of cell immobilisation on bacterial growth, MK-7 production, and the opportunity for in situ product recovery and cell recycling. These studies demonstrated that bacterial immobilisation with IONPs enhances the production and yield of MK-7 relative to free-cell fermentation. Ebrahiminezhad and co-workers also utilised the superparamagnetic nature of IONPs to run consecutive recycle batches, and it was noted that the capture efficiency and MK-7 production were not greatly compromised [41,43]. Magnetic separation technology is scalable and can be incorporated into a recycle loop in a bioreactor to recover bacterial cells during fermentation and enable process intensification [43]. Integrating product formation and in situ cell recovery has many advantages, including cell reusability, simple equipment requirements, and low energy consumption [43].

Despite the extensive interest in the application of IONPs in MK-7 fermentation, their effect on the production of MK-7 isomers has not been explored. Although bacterial cell immobilisation with IONPs can increase the concentration and yield of MK-7, the resulting isomer composition is yet to be elucidated. Considering that solely all-*trans* MK-7 is biologically effective, using IONPs to enhance MK-7 production and enable process intensification is only valuable if the all-*trans* isomer is attained in the greatest proportion.

Therefore, this investigation aimed to assess the influence of bacterial cell immobilisation with IONPs on the MK-7 isomer profile obtained from fermentation. Accordingly, IONPs were synthesised and characterised, and their effect on microbial growth and the production and yield of all-*trans* and *cis* MK-7 was evaluated. The findings of this study will broaden the current knowledge and understanding of the role of IONPs in MK-7 fermentation and facilitate the development of an innovative large-scale fermentation process that selectively promotes the synthesis of the biologically significant isomer. This, together with process intensification achieved through magnetic separation technology, has the potential to streamline the production system and decrease related expenses. Enhancing the efficiency of the fermentation process will help reduce the price and increase the availability of fermented bioactive MK-7 dietary supplements and functional foods. This is likely to alleviate the low dietary intake of MK-7, provide consumers with greater health benefits, and aid the prevention of and improve the outcomes associated with globally relevant diseases.

## 2. Materials and Methods

### *2.1. Chemicals and Materials*

The reference standard for all-*trans* MK-7 was acquired from ChromaDex (Los Angeles, CA, USA). Glucose and $FeSO_4 \cdot 7H_2O$ were purchased from Ajax Finechem Pty Ltd. (Taren Point, NSW, Australia), and tryptone and yeast extract were supplied by Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Soy peptone, ethanol, methanol, 2-propanol, *n*-hexane, and $NH_4OH$ (32%) were obtained from Merck Millipore (Burlington, MA, USA). NaCl was purchased locally, and $CaCl_2$, $FeCl_3 \cdot 6H_2O$, glutaraldehyde (25%), and sodium cacodylate were acquired from Sigma-Aldrich Co. (St. Louis, MO, USA). Nutrient agar plates were obtained from Fort Richard Laboratories (Auckland, New Zealand).

### *2.2. Microorganism and Inoculum Preparation*

*B. subtilis natto* was selected as the ideal microbial strain for this investigation, as it has been commonly employed in MK-7 fermentation, including studies considering NPs [41–43]. It is also deemed the most suitable for industrial MK-7 production and is preferentially used to manufacture MK-7 products, as it is generally recognised as safe (GRAS) and results in a high MK-7 yield [48,65]. A spore suspension was prepared according to the procedure outlined by Berenjian et al. [45]. The cells were cultured in an aqueous medium comprising yeast extract, tryptone, and NaCl before streaking on agar plates, which were then incubated for 48 h at 37 °C. Following incubation, the bacterial cells were scraped off the plates and immersed in a sterile saline solution (0.9% (*w/v*) NaCl). The bacterial suspension was then kept in a water bath set at 80 °C for 30 min to deactivate the vegetative cells and promote the production of spores. Afterwards, the cell debris was removed by centrifuging (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) the mixture at 3000 rpm for 10 min. The resulting *B. subtilis natto* spore suspension served as the inoculum for the fermentation studies.

### *2.3. NP Synthesis and Characterisation*

#### 2.3.1. Synthesis of $Fe_3O_4$ NPs

Naked (uncoated) $Fe_3O_4$ NPs were synthesised from the co-precipitation of $Fe^{2+}$ and $Fe^{3+}$ ions with an alkali ($NH_4OH$) in an inert atmosphere, as described by Ranmadugala et al. [42]. Accordingly, 0.74 g of $FeSO_4 \cdot 7H_2O$ and 1.17 g of $FeCl_3 \cdot 6H_2O$ were dissolved in distilled water (50 mL), and the solution was briskly stirred at 70 °C under nitrogen to prevent oxidation. After 1 h, 5 mL of $NH_4OH$ solution was added to the reaction mixture. The solution was further stirred for 1 h until the IONPs precipitated. The magnetic particles were separated using a permanent magnet, and the resulting black precipitate was washed with hot distilled water to remove impurities and dried overnight (24 h) in an oven (Contherm Thermotec 2000, Contherm Scientific Ltd., Wellington, New Zealand) at 50 °C. Approximately 0.75 g of naked IONPs were obtained from the reaction.

#### 2.3.2. Characterisation

The morphology and size of the IONPs were ascertained by transmission electron microscopy (TEM; Philips, CM 10, Philips Electron Optics, Eindhoven, The Netherlands). For the TEM procedure, a drop of the NP dispersion (in distilled water) was placed on a carbon-coated copper grid, and images were captured at HT 100 kV. Fourier-transformed infrared (FTIR) spectroscopy (Bruker VERTEX 70 FTIR spectrometer, Bruker, Kassel, Germany), in the range of 4000–400 $cm^{-1}$, was used to characterise the NPs and determine the presence of key chemical bonds and functional groups. For the FTIR analysis, a pellet with a NP-to-KBr ratio of 1% was prepared and placed in a hydraulic press for 10 min to form a compact disc, and the samples were analysed at room temperature. The crystal structure of the NPs was established by X-ray powder diffraction (XRD; Siemens D5000, Munich, Germany) with an exploration range (2θ) between 20° and 90° and a step size of 0.0530° at 45 kV, 40 mA, and ambient temperature.

2.3.3. Sample Preparation for SEM

Scanning electron microscopy (SEM; Hitachi Regulus SU8230 FE-SEM, Tokyo, Japan) was employed to visualise the surface structure of the NPs and the interactions between the IONPs and the bacterial cells. Cell fixation of the free and immobilised bacterial cells was carried out using an approach similar to that employed by Ebrahiminezhad et al. [43]. A small drop of the sample was placed on a glass coverslip and heat-fixed by passing through the flame of a Bunsen burner three times. The bacterial cells were fixed with 2.5% (*v/v*) glutaraldehyde in 0.1 M sodium cacodylate buffer for 45 min and rinsed with saline (0.9% (*w/v*) NaCl) for 15 min. Cell dehydration was conducted by placing the coverslip in a graded series of ethanol (30, 50, 70, 80, 90, and 95%) for 10 min each. The dehydrated sample was kept in absolute ethanol for 20 min and subjected to critical point drying (Polaron E3000, Quorum Technologies, East Sussex, UK). Prior to the SEM analysis, the dried bacterial samples and the IONP powder were mounted on an aluminium stub and coated with platinum. SEM images of the pure IONPs, untreated bacterial cells, and cells immobilised with IONPs were taken at 3 kV.

### *2.4. Bacterial Cell Immobilisation and Fermentation*

The fermentation media, containing glucose (1% (*w/v*)), yeast extract (2% (*w/v*)), soy peptone (2% (*w/v*)), tryptone (2% (*w/v*)), and $CaCl_2$ (0.1% (*w/v*)) [47], was prepared and sterilised at 121 °C in an autoclave (TOMY SX-700E, Tokyo, Japan) for 20 min. The samples were then inoculated with 2% (*v/v*) of the microbial spore suspension. A stock solution of the IONPs was prepared using sterilised distilled water (0.01 g/mL), and different concentrations (0–600 µg/mL) of the NP stock solution were added to the samples. It is recognised that naked IONPs with bare surfaces tend to be susceptible to agglomeration owing to their high surface energy and the presence of strong magnetic and other attractive forces between particles [66]. Hence, before the IONP stock solution was added to the samples and used to coat the bacterial cells, it was thoroughly sonicated to fully disperse the NPs and prevent them from aggregating. The samples were prepared in triplicate (3 samples for each NP concentration that was investigated) and fermented for 7 days at 40 °C and 200 rpm under aerobic conditions to allow the NPs to interact with and adhere to the bacterial cell surface. The inoculum volume and operating conditions were derived from our previous study [57].

### *2.5. MK-7 Extraction*

The fermented MK-7 was extracted using 2-propanol and *n*-hexane, which was combined in the ratio of 1:2 (*v*/*v*) with a liquid-to-organic ratio of 1:4 (*v*/*v*) [45]. The mixture was vortexed for 2 min, and the two phases were separated by centrifugation (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 3000 rpm for 10 min. The top layer of liquid was then removed and evaporated under a vacuum to obtain the MK-7.

### *2.6. MK-7 Analysis*

MK-7 was analysed using the approach proposed in our earlier study [47]. High-performance liquid chromatography (HPLC) was employed to evaluate the MK-7 isomer concentration of the fermented samples. A Dionex HPLC system (Thermo Fisher Scientific, Waltham, MA, USA), consisting of a thermostatted column compartment, an automated sample injector, a photodiode array UV detector, and four pumps, was used for the analysis. The compounds were separated at 40 °C with a reversed-phase column (COSMOSIL Cholester, 100 mm × 2 mm × 2.5 µm, Nacalai Tesque Inc., Kyoto, Japan). Pure methanol comprised the mobile phase and had a flow rate of 0.2 mL/min (isocratic elution). The autosampler temperature, run time, injection volume, and analytical wavelength were 10 °C, 30 min, 10 µL, and 248 nm, respectively. Data were collected using the Chromeleon 7 application (Thermo Fisher Scientific, Waltham, MA, USA). An MK-7 standard curve, which was linear between 0.1 mg/L and 50 mg/L ($R^2$ = 0.99), was implemented to estimate

the isomer concentration, and a relative retention time (RRT) of approximately 1.12 enabled the identification of *cis* MK-7.

The presence of all-*trans* and *cis* MK-7 isomers and their chromatographic retention times were verified using liquid chromatography-mass spectrometry (LC–MS), as described in our previous investigation [47]. The chromatograms and mass spectrometry (MS) data for the reference standard and an experimental sample are provided in the supplementary materials (Figures S1 and S2). The LC–MS apparatus included a Dionex Ultimate 3000 ultra-high-performance liquid chromatography (UHPLC) system and a QExactive mass spectrometer with a HESI II source (Thermo Fisher Scientific, Waltham, MA, USA). The Thermo XCalibur 4.3 platform (Thermo Fisher Scientific, Waltham, MA, USA) was used to control the equipment, and data handling was carried out with the Chromeleon 7.3 program (Thermo Fisher Scientific, Waltham, MA, USA). The compounds were separated using liquid chromatography and the aforementioned chromatographic conditions. However, the run-time and injection volume were modified to 37 min and 5 µL, respectively. Data were obtained in the positive ionisation mode with an AGC target of $3 \times 10^6$, a MS1 scan range of 150–1000 *m/z*, and a maximum injection time of 200 ms. The MS data were analysed with the Thermo FreeStyle 1.6 package (Thermo Fisher Scientific, Waltham, MA, USA).

### *2.7. Cell Density and pH Measurements*

Cell density measurements were used to approximate bacterial growth, which was evaluated with a UV–vis spectrophotometer (Shimadzu UV-1900, Kyoto, Japan). The samples were diluted with distilled water, and the optical density (OD) was assessed at a wavelength of 600 nm. A handheld meter (CyberScan pH 100, Eutech Instruments, Paisley, UK) was used to measure the pH of the samples.

### *2.8. Statistical Methods*

Statistical significance was evaluated using analysis of variance (ANOVA), and the mean values of different groups were compared using a two-sample *t*-test. Significance was accepted at $p < 0.05$, and the data were described as the mean ± standard error (SE) of three replicates.

## 3. Results and Discussion

### *3.1. Synthesis and Characterisation of IONPs*

IONPs were synthesised in an aqueous medium from the co-precipitation of Fe (II) and Fe (III) ions. The addition of $NH_4OH$ to the reaction mixture resulted in a rapid colour change from reddish brown to black, denoting the formation of iron oxide cores in the solution. Extension of the reaction with continued stirring enabled the production of magnetic $Fe_3O_4$ NPs, as described in Equation (1).

$$\text{Fe (II)} + 2\text{Fe (III)} + 8\,\text{OH} \rightarrow \text{Fe}_3\text{O}_4 + 4\,\text{H}_2\text{O} \quad (1)$$

The SEM image (Figure 2) and TEM micrograph (Figure 3) of the IONPs illustrate that the NPs have a spherical shape. IONPs with a relatively uniform size distribution between 7 and 20 nm and an average size of 11 nm were synthesised from three reproducible batches, and the sizes achieved are comparable with previous studies [43,67].

The FTIR spectrum of the synthesised NPs is presented in Figure 4 and shows the characteristic Fe-O peaks at approximately 617 $cm^{-1}$ and 430 $cm^{-1}$. During the synthesis of IONPs via the co-precipitation method, the surface of the $Fe_3O_4$ NPs is altered by OH groups from the liquid medium due to the coordination of unsaturated surface iron atoms with water molecules and $OH^-$ ions [43]. These OH groups also absorb infrared waves, and this can be visualised at approximately 3449 $cm^{-1}$ (stretching point) and 1980 $cm^{-1}$ (deforming point) [43].

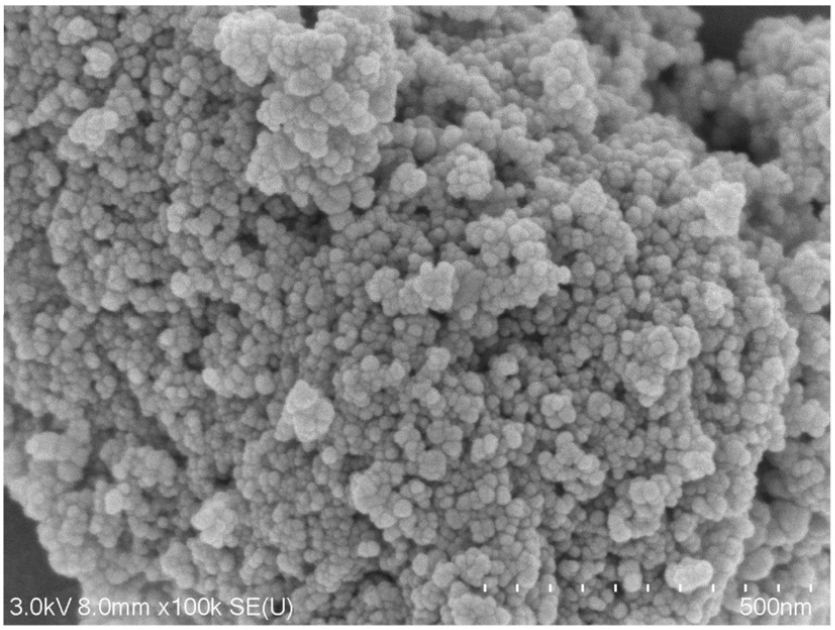


**Figure 2.** SEM image of the surface structure of the prepared IONPs.

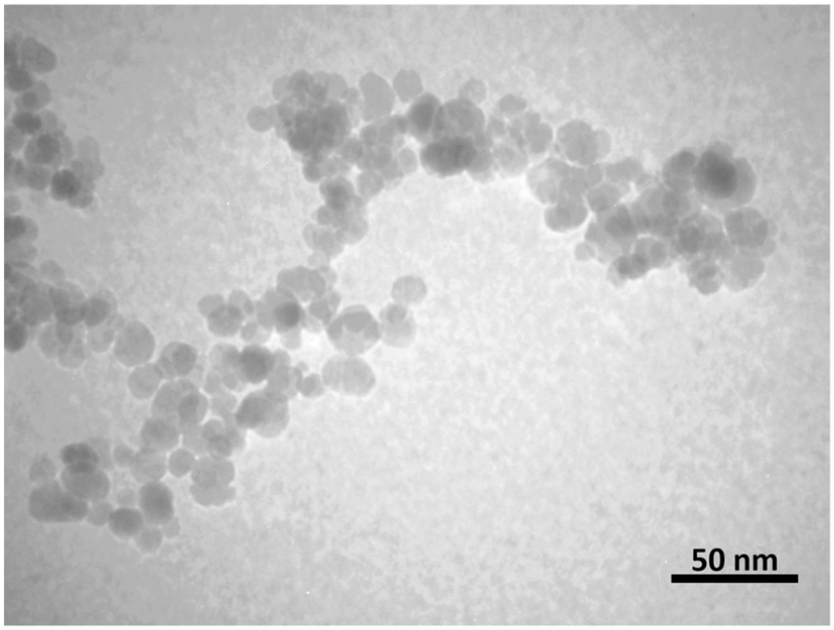


**Figure 3.** TEM micrograph of the IONPs.

The XRD pattern obtained for the fabricated IONPs displays distinctive intensity peaks at 2θ degrees of 30°, 35.5°, 43°, 53°, 57°, and 63°, which correspond to (220), (311), (400), (422), (511), and (440) Bragg reflections, respectively (Figure 5). These characteristic peaks denote the crystalline structure of magnetite and verify the formation of $Fe_3O_4$ NPs [43].

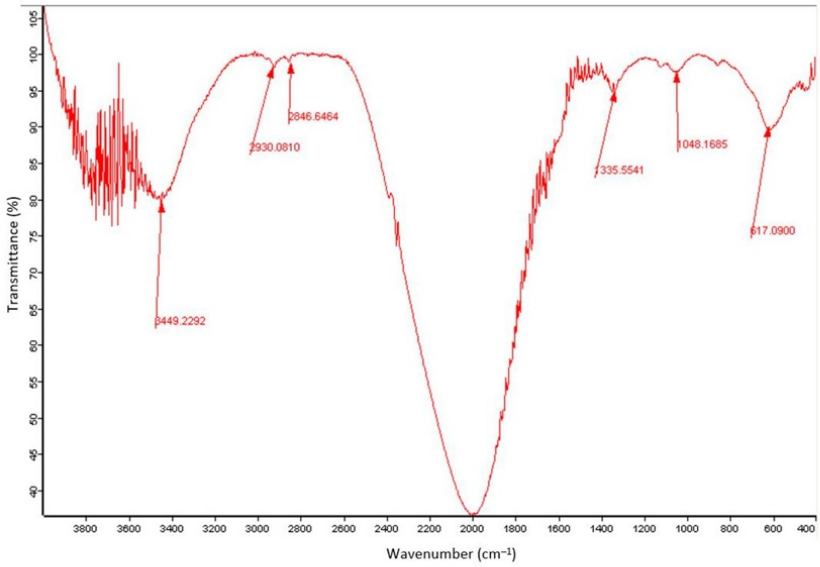


**Figure 4.** FTIR spectrum of the synthesised IONPs.

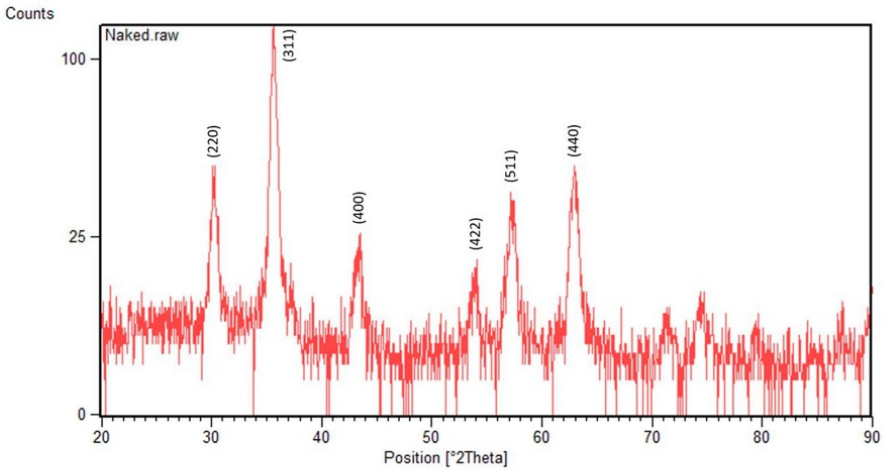


**Figure 5.** XRD pattern of the IONPs.

### *3.2. Interaction of IONPs with the Bacterial Cell Surface*

SEM was used to view the interactions between the IONPs and bacterial cells. Figure 6 illustrates the successful decoration of *B. subtilis natto* cells with the IONPs compared to the free-floating cells. IONPs have a small size and large surface-area-to-volume ratio, which facilitates their attachment to the bacterial cell surface via various non-specific interactions, such as hydrogen bonds, Van der Waals forces, electrostatic forces, and hydrophobic interactions [43]. Due to the non-specific nature of these interactions, the IONPs randomly attach to the bacterial cell surface. Since the interaction is not uniform among all cells, some bacterial cells tend to be more heavily decorated than others. The immobilisation of bacterial cells with magnetic IONPs is a unique feature that provides an opportunity to reduce the number of downstream processing steps (process intensification) through cell separation using an external magnetic field. The use of magnetic cell separation will decrease the complexity of the overall fermentation system and allow the separated cells to be reused in successive fermentation batches, thus reducing production expenses. However,

to enable bacterial cell recycling, it is vital to ensure that immobilisation with magnetic IONPs does not significantly impact cell viability.

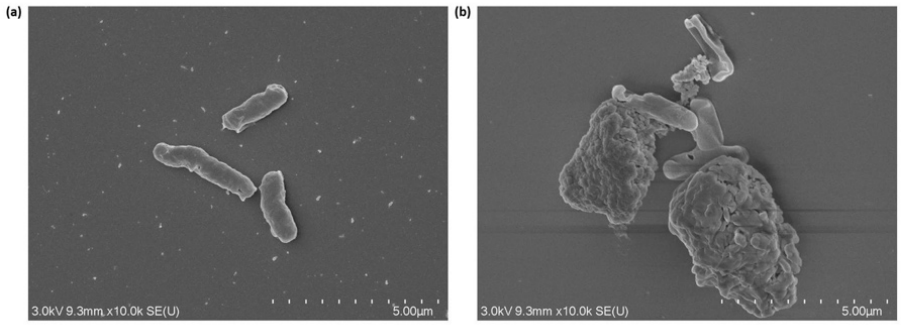


**Figure 6.** SEM images of the (**a**) free bacterial cells and (**b**) bacterial cells decorated with IONPs.

### *3.3. The Impact of IONPs on Microbial Growth*

The effect of bacterial cell immobilisation with IONPs on microbial growth was assessed (Figure 7). Bacterial growth declined with an increase in the concentration of IONPs from 0 to 300 µg/mL, and the minimum cell density was obtained at 300 µg/mL. A further increase in the IONP concentration from 300 to 600 µg/mL resulted in a rise in the OD. Despite the variation in the cell density measurements that were observed, the ANOVA results indicate that there is no statistically significant difference ($p$ = 0.075) in the OD between the different IONP concentration groups. Hence, it is evident that immobilisation of *B. subtilis natto* cells with IONPs has little effect on microbial growth.

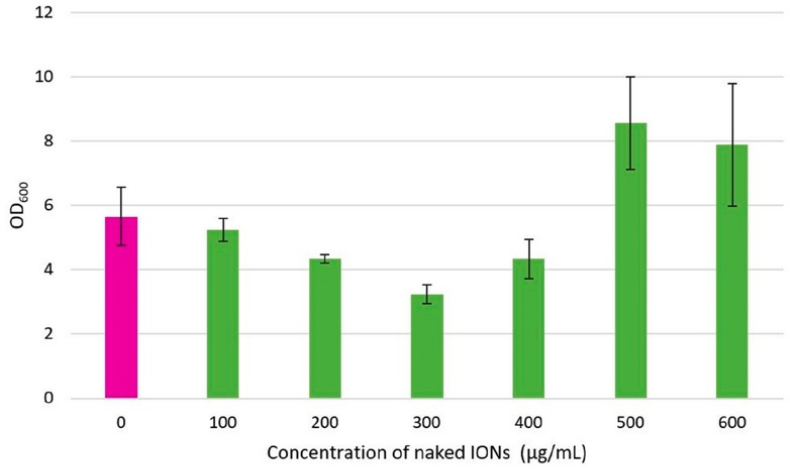


**Figure 7.** Bacterial growth in the presence and absence of IONPs.

Bacterial cells tend to exhibit variable responses to magnetic cell immobilisation with IONPs. The specific nature of the interaction between IONPs and microbial cells depends on many aspects, such as the bacterial species, the properties of the IONPs, and the culture conditions [68,69]. Possible outcomes include changes in cell growth (may result in either enhanced growth or growth inhibition), alteration of cell morphology, induced gene expression, altered membrane permeability (increased membrane conductance and facilitation of mass transfer), penetration of IONPs into the bacterial cell membrane (membrane damage and cell inactivation), and the generation of reactive oxygen species (ROS) (can lead to cell membrane disruption, DNA damage, lipid peroxidation, mitochondrial damage, and oxidation of cellular components) [69,70]. It has been noted that the effect of IONPs differs

between Gram-positive and Gram-negative bacterial strains, most likely due to the differences in their cell wall structure, cellular composition, and metabolic characteristics [70]. Gabrielyan et al. [70] compared the impact of IONPs on the growth profile of *Enterococcus hirae* and *Escherichia coli*, which can be considered model organisms for Gram-positive and Gram-negative bacterial strains, respectively. The authors determined that IONPs had a concentration-dependent inhibitory effect on the growth of *E. coli*, whereas for *E. hirae*, growth stimulation or inhibition was observed depending on the NP concentration, and it was established that $Fe_3O_4$ NPs do not exhibit significant antibacterial activity against Gram-positive bacteria. These findings are largely comparable with the present study, as *B. subtilis natto* is also a Gram-positive bacterium, and stimulation or inhibition of bacterial growth (denoted by the OD measurements) was noted for different concentrations of IONPs. However, holistically, there was no significant difference in the OD between the various NP concentration groups, implying that the IONPs did not show substantial antibacterial activity against *B. subtilis natto*. Ebrahiminezhad et al. [43] evaluated the effect of IONPs on the growth profile of *B. subtilis natto* in particular, and it was determined that in comparison to the free-floating cells, approximately 5% growth inhibition occurred for the magnetically immobilised bacteria at the end of fermentation. Furthermore, the final cell density attained for untreated bacteria was approximately 10% greater than that for the cells coated with IONPs, which suggests that in this investigation, magnetic immobilisation with IONPs had an unfavourable effect on the growth of *B. subtilis natto*. These outcomes contrast the present study and can likely be attributed to the different NP concentrations that were explored. An IONP concentration of 0–600 μg/mL was examined in the current study, whereas Ebrahiminezhad et al. [43] focused on a lower range of concentrations (0–150 μg/mL). It is also interesting to note that the NP concentrations explored by Ebrahiminezhad et al. [43] fall within the band of concentrations in the present investigation for which a decrease in the OD was observed (0–300 μg/mL). Therefore, the findings of these two studies can be deemed consistent when considering the same range of concentrations, and it appears that the effect of magnetic cell immobilisation with IONPs on the growth profile of *B. subtilis natto* is indeed concentration-dependent.

### 3.4. The Influence of Bacterial Cell Immobilisation with IONPs on the MK-7 Isomer Yield

The impact of bacterial cell immobilisation with IONPs on the productivity of the fermentation process was determined by measuring the MK-7 isomer yield. Figures 8 and 9 depict the effect of the various concentrations of IONPs on the yield of all-*trans* and *cis* MK-7, respectively. The yield of both isomers followed a similar bell-shaped pattern. A small yield was obtained for low (0–200 μg/mL) and high (400–600 μg/mL) IONP concentrations, and the greatest yield was achieved at an IONP concentration of 300 μg/mL.

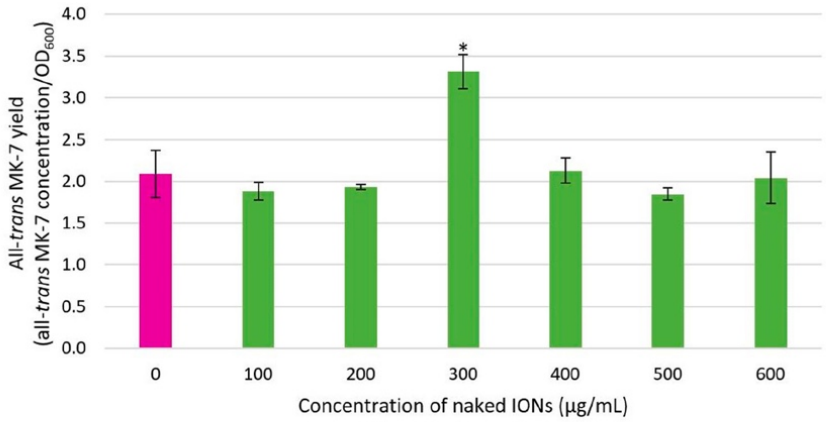


**Figure 8.** The impact of bacterial cell immobilisation with IONPs on the yield of the all-*trans* MK-7 isomer, where * indicates a significantly different all-*trans* MK-7 yield compared to the control ($p < 0.05$).

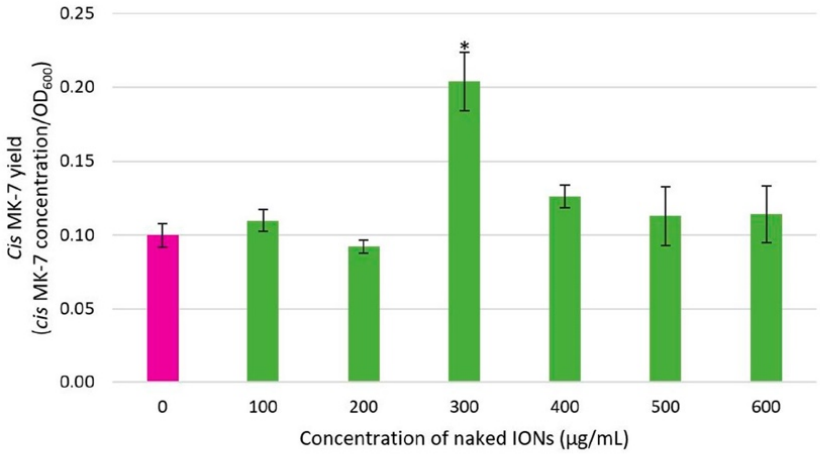


**Figure 9.** The impact of bacterial cell immobilisation with IONPs on the yield of the *cis* MK-7 isomer, where * indicates a significantly different *cis* MK-7 yield compared to the control ($p < 0.05$).

The ANOVA assessment established a statistically significant difference in the all-*trans* and *cis* MK-7 isomer yield between the various IONP concentration groups ($p = 0.008$ for all-*trans* MK-7 and $p = 0.007$ for *cis* MK-7). The yield of both isomers for each IONP concentration was also compared with the control (0 µg/mL). It was determined that the all-*trans* and *cis* MK-7 isomer yield for only an IONP concentration of 300 µg/mL was significantly different from the control ($p = 0.047$ for the all-*trans* isomer and $p = 0.015$ for the *cis* isomer). The all-*trans* and *cis* MK-7 yield was also compared for different pairs of IONP concentrations. From this analysis, it was ascertained that there is a statistically significant difference in the all-*trans* MK-7 concentration between the 300 µg/mL group and all other NP concentrations ($p = 0.008$ for 100 µg/mL and 300 µg/mL, $p = 0.006$ for 200 µg/mL and 300 µg/mL, $p = 0.020$ for 300 µg/mL and 400 µg/mL, $p = 0.006$ for 300 µg/mL and 500 µg/mL, and $p = 0.048$ for 300 µg/mL and 600 µg/mL), while the difference for the remaining groups was insignificant ($p > 0.05$). In contrast, for the *cis* isomer, the 100 µg/mL and 300 µg/mL ($p = 0.021$), 200 µg/mL and 300 µg/mL ($p = 0.010$), 200 µg/mL and 400 µg/mL ($p = 0.037$), and 300 µg/mL and 400 µg/mL ($p = 0.039$) IONP concentration groups were significantly different, and all other groups were comparable ($p > 0.05$).

Since only all-*trans* MK-7 is biologically important, it is desirable to exclusively enhance the yield of this isomer from fermentation; however, the yield of all-*trans* MK-7 is positively correlated with the yield of the biologically inefficacious *cis* isomer. Thus, a higher yield of all-*trans* MK-7 is associated with a greater *cis* MK-7 yield. Although the yield of both isomers was notably greater for the optimum IONP concentration (300 µg/mL), the proportion of the total yield for each isomer was similar to that for the other IONP concentration groups, including the control. This implies that despite the considerable difference in the isomer yield between an IONP concentration of 300 µg/mL and the remaining IONP concentration groups, the fraction of the total yield for each isomer did not vary appreciably, which is beneficial. Therefore, 300 µg/mL can be regarded as the optimum IONP concentration, as it significantly enhances the yield of bioactive MK-7 without increasing the proportional yield of the undesirable isomer. This is advantageous, as the *cis* isomers are comparatively redundant with respect to their carboxylative potential, and the therapeutic benefits of MK-7 nutritional supplements are only determined by the quantity of all-*trans* MK-7. Furthermore, fermentation processes that are not optimised to selectively promote the production of all-*trans* MK-7 would necessitate additional downstream purification steps to remove appreciable amounts of the *cis* isomer, which is unfavourable, as it will increase the manufacturing costs for biologically effective MK-7 products.

Although all-*trans* MK-7 production at an IONP concentration of 300 µg/mL was comparable to the control, the OD was lower. This suggests that magnetic IONPs at the optimum concentration slightly inhibited bacterial growth. However, the all-*trans* isomer yield at an IONP concentration of 300 µg/mL was 58.61% greater than the free cells, which suggests that magnetically immobilised cells have superior metabolic efficiency. It has been proposed that the non-specific interactions between IONPs and the bacterial cell surface promote disorganisation of the lipid packing and increase the permeability of the cell membrane [42,68]. Hence, while the presence of IONPs slightly decreased bacterial growth at the optimum IONP concentration, the improved metabolic efficiency of the cells facilitated mass transfer and enabled greater MK-7 secretion into the fermentation medium, resulting in a higher yield of the biologically efficacious isomer and enhancing the productivity of the entire fermentation system.

### *3.5. Monitoring Study in the Presence of the Optimum IONP Concentration*

Figure 10 illustrates the changes in the MK-7 isomer profile, microbial growth, and pH over a time-course fermentation study employing the optimal IONP concentration (300 µg/mL).

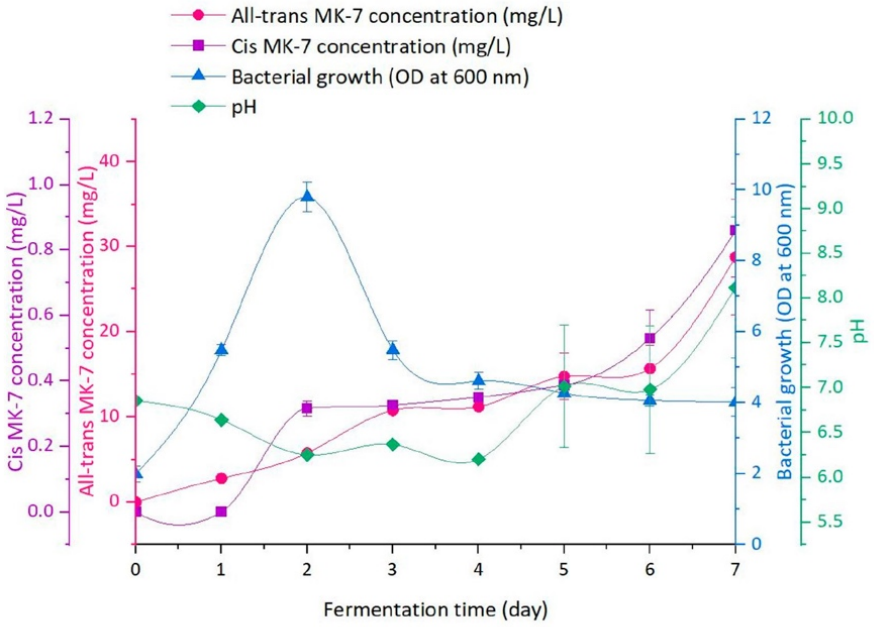


**Figure 10.** Variation in the MK-7 isomer profile, bacterial growth, and pH in the presence of the optimal IONP concentration over a time-course fermentation study (the error bars represent the SE calculated from three replicate samples for each response).

The variation in the OD during fermentation reflects the typical bacterial growth curve depicting the lag, exponential, and stationary phases of growth, which are three of the four characteristic stages of the cell growth profile. The OD gradually increased from 1.99 to 5.48 between days 0 and 1, after which it quickly rose to a maximum of 9.81 on day 2 of fermentation. The OD then declined to 5.49 on day 3 and plateaued for the remainder of the fermentation period, reaching a final value of 4.01 on day 7. The final stage of microbial growth was not observed during the investigated timeframe, as fermentation was terminated before the death phase to avoid degradation of MK-7 in the fermentation broth due to exposure to proteases and other cellular contents released upon cell lysis.

MK-7 isomer production mirrored the bacterial growth profile, which is consistent with accounts from previous studies [42,45,47,71,72]. Synthesis of all-*trans* MK-7 commenced at

the start of fermentation and increased steadily until day 3. As the bacterial culture entered the stationary phase of growth, a notable increase in the production of the all-*trans* isomer occurred, particularly from day 6 onwards, reaching a final concentration of 28.78 mg/L at the conclusion of fermentation. In contrast, synthesis of the *cis* isomer began on day 2 and remained relatively steady (0.32–0.39 mg/L) until day 5, following which it increased more rapidly to a concentration of 0.86 mg/L on day 7. Essentially, 9.69% of the total all-*trans* and 0% of the total *cis* MK-7 was synthesised during the lag stage, 10.33% of the total all-*trans* and 36.79% of the total *cis* MK-7 was detected during the exponential period, and 79.98% of the total all-*trans* and 63.21% of the total *cis* MK-7 was produced over the remainder of the fermentation time (stationary phase). It is evident that very little or no MK-7 is observed during the lag phase, a small amount of both isomers is synthesised during the exponential growth stage, and the majority of all-*trans* and *cis* MK-7 is noted during the stationary phase of growth. These observations comply with prior studies [47,73–75] and indicate that MK-7 is a mixed metabolite, as its synthesis is partially growth-associated.

The pH of the medium fluctuated over the fermentation period and increased from an initial value of 6.86 to 8.12 on day 7. While the pH increased overall, it first decreased to a minimum of 6.20 on day 4 before rising to the final value during the latter part of the process. The changes in the medium pH can be correlated with the variation in the OD and, thus, the phases of microbial growth. It is apparent that the pH slowly decreases with an increase in the OD during the initial stages of bacterial growth and only begins to rise once the OD levels off during the stationary phase. Moreover, a rapid increase in the pH of the medium occurred on the final day of fermentation (between days 6 and 7), corresponding to the sharp rise in the MK-7 isomer concentration. Similar trends have been observed in previous investigations and can be ascribed to the metabolic activities of *B. subtilis natto* [42,45,47,72,75].

It is worth mentioning that the overall trends in the bacterial growth, MK-7, and pH profiles are comparable to our initial investigation [47], which presented the findings of a monitoring study in the absence of IONPs using the same bacterial strain and fermentation media. Although the value of key fermentation parameters differed slightly between the two reports, the fundamental outcomes of both studies are congruent and suggest that bacterial cell immobilisation with IONPs does not adversely affect the growth and metabolic characteristics of *B. subtilis natto* and the fermentation process as a whole. The only substantial dissimilarity between the two studies is the length of each phase in the bacterial growth curve. In the present investigation, each phase of microbial growth occurred a day earlier than in our previous study, which had a flow-on effect on the timing of MK-7 isomer production. There are two probable explanations for this observation. Firstly, since the duration of fermentation differed for each analysis (6 days in our prior study as opposed to 7 days in the present investigation), it may have impacted the start and length of the individual stages of bacterial growth. Alternatively, and more likely, the presence of IONPs in the fermentation media and their interaction with the microbial cells in the current study may have altered the permeability of the bacterial cell membrane, facilitating mass transfer and improving MK-7 secretion into the fermentation broth. This would have served to enhance the metabolic efficiency of the cells, resulting in faster growth, thereby reducing the length of the lag and exponential stages of the microbial growth curve.

While this study has considered the effect of uncoated IONPs on bacterial growth and the production of MK-7 isomers, it is important to appreciate that relative to naked IONPs, coated IONPs are more stable and less susceptible to agglomeration. Surface functionalisation with different coating materials can also enable the synthesis of a vast range of biocompatible IONPs that can be customised for different applications. Therefore, it would be valuable to investigate and compare the impact of naked and coated IONPs on *B. subtilis natto* cells and MK-7 isomer production in the future.

## 4. Conclusions

This study presents a novel insight into the role of IONPs in the production of MK-7 isomers. Naked $Fe_3O_4$ NPs were synthesised and characterised, and their effect on the MK-7 isomer profile and bacterial growth was evaluated. The results demonstrated that although bacterial cell immobilisation with IONPs inhibited bacterial growth at most of the concentrations explored, it enhanced the metabolic efficiency of the cells. An IONP concentration of 300 μg/mL was the optimum, resulting in a 1.6-fold rise in the all-*trans* MK-7 yield compared to the uncoated bacteria. The conclusions drawn from this investigation are a valuable step forward in establishing innovative production techniques that target the synthesis of the biologically important all-*trans* isomer and overcome the challenges in MK-7 fermentation. This will reduce production-related expenses and decrease the price of fermented bioactive MK-7 products. The improved accessibility of which will notably benefit the health and well-being of consumers and reduce the burden of globally significant diseases.

**Supplementary Materials:** The chromatograms and MS data for the reference standard (Figure S1) and an experimental sample (Figure S2) can be downloaded at: https://www.mdpi.com/article/10.3390/nano13121825/s1.

**Author Contributions:** Conceptualisation, A.B. and M.S.; methodology, N.L., M.S. and A.B.; validation, N.L.; formal analysis, N.L., M.S., A.E. and A.B.; investigation, N.L.; data curation, N.L., M.S. and A.B.; writing—original draft preparation, N.L.; writing—review and editing, N.L., M.S. and A.B.; visualisation, N.L. and A.E.; supervision, A.B. and M.S. All authors have read and agreed to the published version of the manuscript.

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*nanomaterials*

MDPI

*Supplementary Materials*

# The Effect of Iron Oxide Nanoparticles on the Menaquinone-7 Isomer Composition and Synthesis of the Biologically Significant All-*Trans* Isomer

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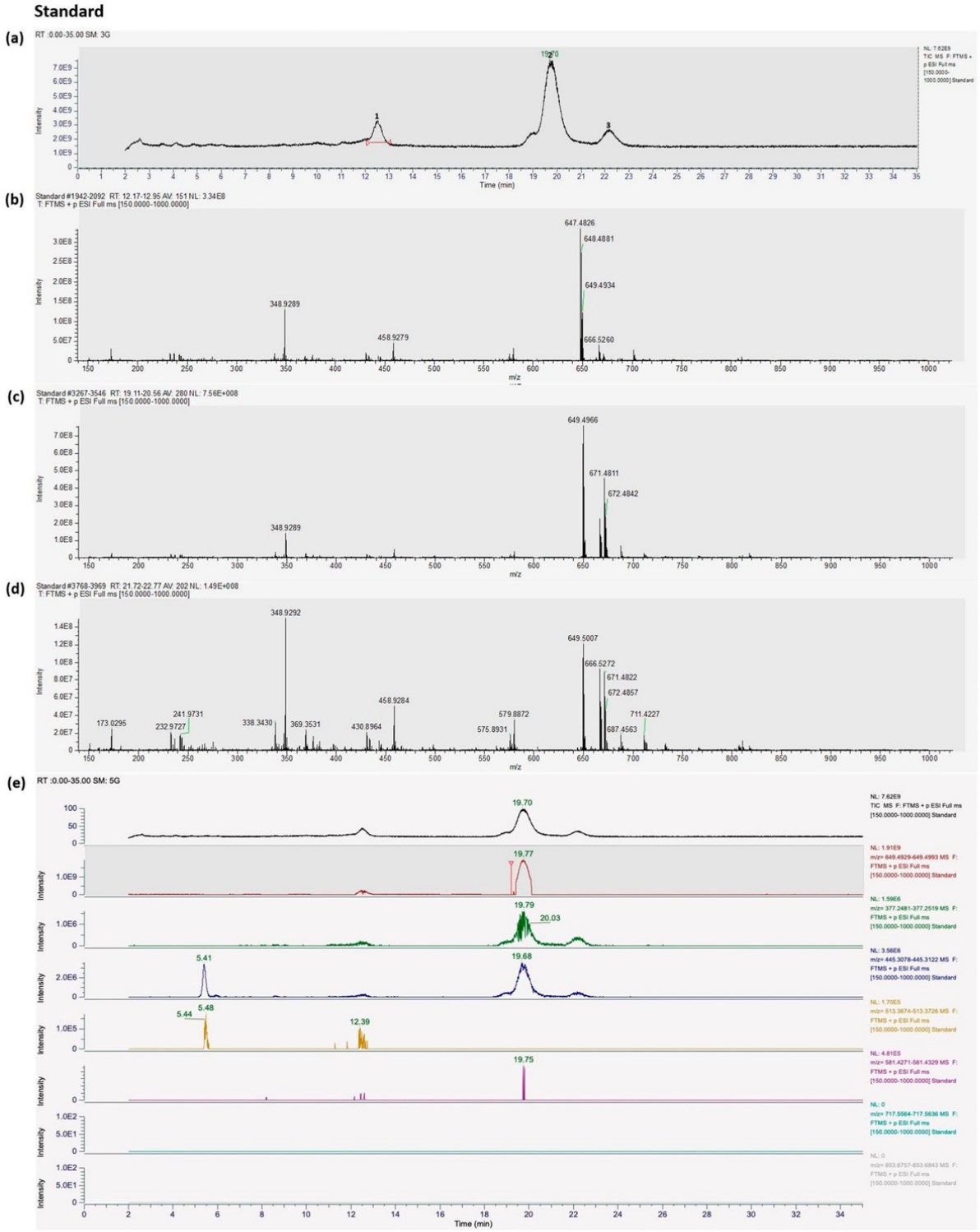


**Figure S1.** (**a**) Liquid chromatography chromatogram, (**b**) MS data for peak 1, (**c**) MS data for peak 2, (**d**) MS data for peak 3, and (**e**) extracted ion chromatogram for the all-*trans* MK-7 reference standard.

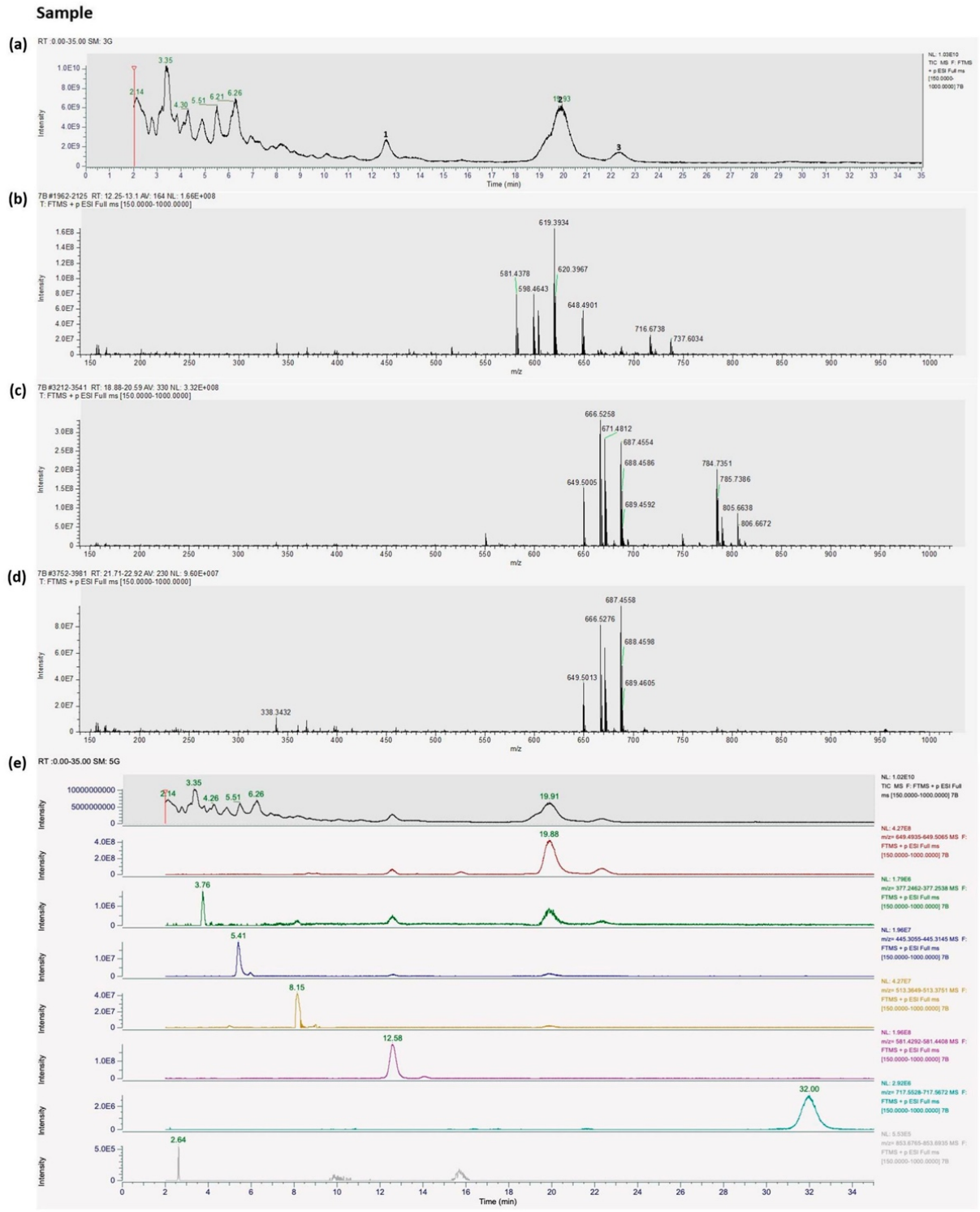


**Figure S2.** (**a**) Liquid chromatography chromatogram, (**b**) MS data for peak 1, (**c**) MS data for peak 2, (**d**) MS data for peak 3, and (**e**) extracted ion chromatogram for an experimental sample.

# 7

# The Impact of Amine-Functionalised Iron Oxide Nanoparticles on the Menaquinone-7 Isomer Profile and Production of the Bioactive Isomer

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By

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# Chapter 7 – The Impact of Amine-Functionalised Iron Oxide Nanoparticles on the Menquinone-7 Isomer Profile and Production of the Bioactive Isomer

This chapter expands on the preceding chapter (Chapter 6) and continues to explore the potential of nanobiotechnological strategies to enhance the production and yield of the all-*trans* isomer and address the underlying challenges of MK-7 fermentation. Although the findings of Chapter 6 indicate that naked IONs positively influence the fermentation yield of bioactive MK-7, their properties are inferior to IONs coated with biocompatible materials, such as amino acids. Amino acid coatings, for example, APTES and L-Lys, can mitigate the unfavourable characteristics of naked IONs, increase the stability and biocompatibility of IONs, and promote beneficial interactions with *B. subtilis natto* cells. Moreover, certain amino acid coatings can reduce biofilm formation and offer additional advantages for MK-7 fermentation on a large scale. Hence, IONs with biocompatible coatings are superior and more apt to improve the production and yield of the biologically effective MK-7 isomer and overcome the difficulties in industrial MK-7 fermentation.

Accordingly, two amine-functionalised IONs, namely IONs@APTES and L-Lys@IONs, were synthesised, and several techniques were employed to characterise the coated IONs and visualise their association with the microbial cells. The effect of fermentation with bacterial cells immobilised with each type of biocompatible amino acid-coated ION on microbial growth and the production and yield of MK-7 isomers was also evaluated. The optimal concentration of the amine-functionalised ION that resulted in the most favourable outcomes with regard to the investigated parameters was subsequently assessed in a monitoring study to examine the trends in bacterial growth, isomer production, and pH over the fermentation period.

The outcomes of this chapter conclude the aims set out in the previous chapter (Chapter 6) and demonstrate the value of nanobiotechnological methods in enhancing the production and yield of bioactive MK-7 and providing an opportunity to streamline the overall fermentation system through process intensification.

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ORIGINAL PAPER

# The Impact of Amine-Functionalised Iron Oxide Nanoparticles on the Menaquinone-7 Isomer Profile and Production of the Bioactive Isomer

Neha Lal[1] · Mostafa Seifan[1] · Alireza Ebrahiminezhad[2] · Aydin Berenjian[1,3]

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**Abstract**
The K family of vitamins includes a collection of molecules with different pharmacokinetic characteristics. Menaquinone-7 (MK-7) has the finest properties and is the most therapeutically beneficial due to its long plasma half-life and outstanding extrahepatic bioavailability. MK-7 exhibits *cis–trans* isomerism, and merely the all-*trans* form is biologically efficacious. Therefore, the remedial value of MK-7 end products is exclusively governed by the quantity of all-*trans* MK-7. Consumers favour fermentation for the production of MK-7; however, it involves several challenges. The low MK-7 yield and extensive downstream processing requirements increase production costs, resulting in an expensive final product that is not universally available. Bacterial cell immobilisation with iron oxide nanoparticles (IONs) can potentially address the limitations of MK-7 fermentation. Uncoated IONs tend to have low stability and can adversely affect cell viability; thus, amine-functionalised IONs, owing to their increased physicochemical stability and biocompatibility, are a favourable alternative. Nonetheless, employing biocompatible IONs for this purpose is only advantageous if the bioactive MK-7 isomer is obtained in the most significant fraction, exploring which formed the aim of this investigation. Two amine-functionalised IONs, namely 3-aminopropyltriethoxysilane (APTES)-coated IONs (IONs@APTES) and L-Lysine (L-Lys)-coated IONs (L-Lys@IONs), were synthesised and characterised, and their impact on various parameters was evaluated. IONs@APTES were superior, and the optimal concentration (300 µg/mL) increased all-*trans* MK-7 production and improved its yield relative to the untreated cells by 2.3- and 3.1-fold, respectively. The outcomes of this study present an opportunity to develop an innovative and effective fermentation method that enhances the production of bioactive MK-7.

**Keywords** Menaquinone-7 isomers · Biocompatible iron oxide nanoparticles · 3-Aminopropyltriethoxysilane · L-Lysine · Bacterial cell immobilisation · Fermentation

## Introduction

The vitamin K series encompasses a range of structurally similar fat-soluble molecules. Vitamin K1 or phylloquinone (PK) and vitamin K2 or menaquinones (MKs) are the prevalent types of dietary vitamin K that have nutritional value for humans [1]. Both PK and MKs contain a 2-methyl-1,4-naphthoquinone constituent, but the structure of the isoprenoid group at the 3-position is unique for each isoform [2]. PK is an individual molecule that is ubiquitous in the chloroplasts of plants and algae that carry out photosynthesis [3, 4]. Hence, it can be readily obtained from numerous dietary sources, including green vegetables (e.g. spinach, broccoli, and iceberg lettuce), plant oils (e.g. olive, cottonseed, canola, and soybean), and products derived from vegetable oils (e.g. salad dressings, margarines, and spreads) [5, 6]. In contrast, MKs consist of a group of molecules with distinct side chains, which can be described by the script MK-*n* (*n* is commonly between four and thirteen and symbolises the number of repeating isoprene units) [7, 8]. MKs are mostly of microbial origin and function as electron acceptors in the electron transport system; thus, they are found in specific

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dairy, animal, and fermented goods but at low concentrations [6, 8, 9].

All vitamin K subtypes are essential cofactors for the γ -glutamyl carboxylase (GGCX) enzyme and play a role in the activation of both hepatic and extrahepatic vitamin K-dependent proteins (VKDPs) [10]. VKDPs participate in several key metabolic pathways, such as the blood coagulation cascade, increasing bone mineralisation, and preventing arterial calcification, which are the primary health gains linked to the sufficient intake of vitamin K [8, 10–12]. Additionally, recent research has revealed that vitamin K consumption also contributes to reducing the risk of various other illnesses and globally relevant diseases, including type 2 diabetes mellitus, Parkinson's disease, cancer, neurological illnesses, immune disorders, chronic kidney disease, and obesity, and improving the recovery and outcomes of coronavirus disease 2019 (COVID-19) [1, 13–22].

As a result of its long plasma half-life and excellent extrahepatic bioavailability, MK-7 has the greatest efficacy and superior therapeutic value among all K vitamers [23, 24]. However, the dietary sources of MK-7 are limited, and it occurs in small amounts in foods that interest mainstream consumers [25, 26]. This has heightened the demand for MK-7 dietary supplements and nutraceuticals with widespread appeal to accompany natural foods and satisfy the daily intake needs of all populations.

It must be appreciated that, like many natural molecules, MK-7 exhibits geometric isomerism. The all-*trans* isomer is biologically significant, and the *cis* isomers have negligible bioactivity [8, 27, 28]. The all-*trans* isomer has a linear organisation (Fig. 1) due to the *trans* bond arrangement. In contrast, the *cis* isomers, which contain one or more *cis* double bonds, have a non-linear shape (Fig. 1). The biological activity of MK-7 molecules is dictated by the double bond configuration in the isoprenoid side chain, as it impacts their shape and ability to associate with subcellular structures and perform their biological function [29, 30]. It has been established that the *cis* isomer has reduced carboxylative capacity and biological significance in comparison to all-*trans* MK-7 [31]. Therefore, consideration of the amount of the different geometric isomers in MK-7 consumer products is vital, as only the all-*trans* isomer is biologically effective.

Natural fermentation or chemical reaction methods can be used to synthesise MK-7, and the manufacturing process and techniques employed to purify the crude reaction mixture influence the isomer profile of the MK-7 product [28, 32–36]. Although chemical approaches tend to be cheaper, natural synthesis has greater acceptance among consumers. Moreover, fermentation-based production using microorganisms for the industrial synthesis of MK-7 ensures environmental sustainability and, hence, can fulfil the desire of consumers while supporting sustainable development initiatives [37]. Nevertheless, MK-7 production by fermentation is accompanied by many challenges, the major concerns being the low yield of the vitamin and the need for several complex steps and unit operations to satisfy its extensive downstream processing requirements [38, 39]. These drawbacks increase the production cost and result in an expensive final product that is not widely accessible to consumers.

Many studies have aimed to boost the fermentation yield and MK-7 concentration by exploring and optimising various facets of the fermentation process, such as the media composition, fermentation strategy (using liquid-state fermentation (LSF), solid-state fermentation (SSF), or biofilm reactors), operating conditions, and cell treatment methods [40–48]. The outcomes of prior investigations and their associated developments have contributed significantly to the comprehension of the different features of MK-7 fermentation and enhanced the production of this essential K vitamin. However, further opportunities for improvement still exist, and all except our prior studies [41, 47] have not considered the occurrence of MK-7 isomers, accounting for which is paramount in view of their differing bioactivity. Additionally, optimising aspects inherent to the fermentation process

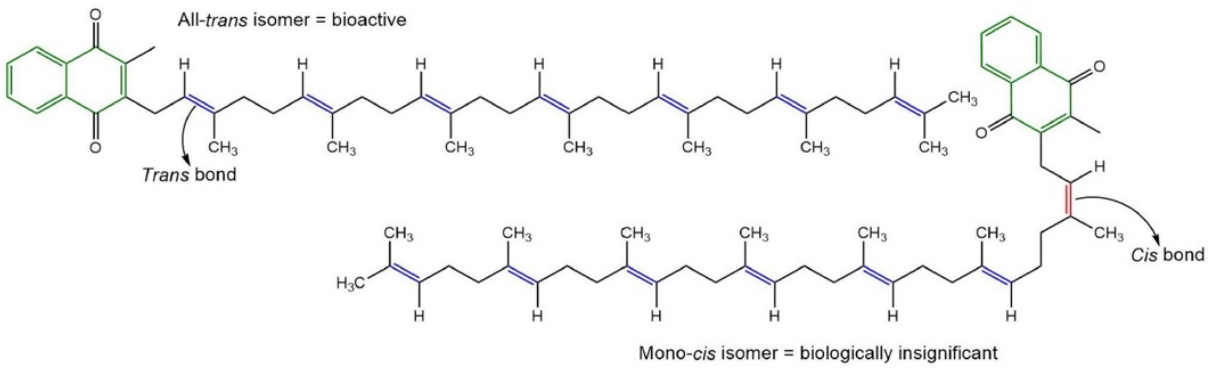


**Fig. 1** The double bond arrangement and resulting molecular structure of the all-*trans* and *cis* geometric isomers of MK-7

alone only has the capacity to increase the production of MK-7 and provides minimal scope to refine the overall fermentation scheme through process intensification. Consequently, there is a demand for new methods to enhance the MK-7 yield achieved from fermentation and/or streamline the production system by decreasing the number of complex unit operations required, in addition to ensuring that the bioactive MK-7 isomer is obtained entirely or in the largest proportion. In this regard, nanomaterials (NMs) show great promise in addressing the limitations of MK-7 fermentation.

NMs contain structural components between 1 and 100 nm in size in at least one aspect [49]. The nanoscale structure increases the surface area-to-volume ratio and provides NMs with unique biological and physicochemical characteristics, which are absent in the equivalent macroscopic material, making them ideal for many novel applications [49–52]. Among the various types of NMs, nanoparticles (NPs) are pertinent to MK-7 fermentation and have the ability to overcome the primary challenges linked to the fermentation-based production of the vitamin. IONs are particularly relevant to MK-7 fermentation and have been employed to immobilise bacterial cells to increase the MK-7 yield and enhance the process output by improving the metabolic productivity of the cells [38, 39, 53]. Furthermore, IONs display superparamagnetism, which can be manipulated to allow cell separation and dispersion with an external magnetic field [38]. This will decrease the number of downstream purification steps and create an opportunity for process intensification. Thus, it is apparent that the use of IONs for bacterial cell immobilisation is an innovative approach to address the obstacles in large-scale MK-7 production.

Previous investigations [38, 39, 53] have explored the ability of bacterial cell immobilisation with IONs to increase MK-7 synthesis and enable process intensification using magnetic separation technology to facilitate cell recovery. In these studies, magnetic IONs were employed to immobilise *Bacillus subtilis natto* cells, and the surface of the cells was decorated through several non-specific bonds. The effect of cell immobilisation on bacterial growth and MK-7 production was also assessed, along with the prospect for in-place product removal and cell reutilisation. It was established that immobilisation with IONs improves both the concentration and yield of MK-7 relative to fermentation with free microbial cells. Moreover, the superparamagnetic nature of IONs allows the separation of bacterial cells using an externally applied magnetic field with a high capture efficiency that is not significantly compromised over successive cycles.

In light of the differing efficacy of MK-7 isomers, using IONs to increase the productivity of MK-7 fermentation and aid process intensification is only advantageous if the all-*trans* isomer is synthesised almost exclusively or in the largest quantity. It is important to understand that all earlier studies have examined MK-7 production without considering the isomer composition obtained from fermentation. Our previous investigation was the first to assess the impact of uncoated IONs on microbial growth and the MK-7 isomer concentration and yield achieved from fermentation [54]. Immobilisation of *B. subtilis natto* cells improved the efficiency of the fermentation process, and a 1.6-fold greater yield of the bioactive isomer was attained at the optimal NP concentration compared to the free cells. However, it has been observed that although naked IONs positively affect MK-7 production, they exhibit low physicochemical stability and toxic effects on microbial cells, inhibiting bacterial growth. This is disadvantageous from the perspective of cell recycling using magnetic separation, as it is essential to preserve the viability and metabolic efficiency of bacterial cells to ensure the effectiveness of this technique.

Therefore, biocompatible coatings like amino acids could eliminate such unfavourable properties. Amino acids are an ideal coating material due to their biocompatibility, chemical stability, and surface activity. Many amino acids are also suitable for human consumption and have been used as biocompatible coatings or constituents in dietary supplements. Thus, amino acid coatings can mitigate the toxic effects of IONs in food-related applications. L-Lys is especially desirable, as it does not have a detrimental impact on the fundamental properties of IONs [39]. It also adds amine functional groups to the structure of NPs, which improves their interaction with negatively charged regions of the bacterial cell membrane and, consequently, increases the potential for surface associations [39]. Another suitable amine-functionalised coating is APTES, which confers the same properties as L-Lys but prevents oxidation of NPs and preserves their crystalline structure [55]. Furthermore, IONs@APTES significantly decrease biofilm formation without impairing cell growth and viability [53]. This is beneficial for industrial MK-7 production, as the formation of biofilms is one of the dominant issues in large-scale fermentation since it leads to many process and operational problems that decrease the performance of the system, reduce the yield and quality of the target product, and increase the process and equipment-related costs. Overall, previous studies suggest that IONs, particularly IONs with biocompatible coatings, are a valuable tool to enhance the production and yield of the vitamin and overcome the challenges accompanying large-scale MK-7 fermentation.

Hence, this study focused on assessing the effect of amine-functionalised IONs on the MK-7 isomer composition obtained from fermentation. Two types of biocompatible NPs, specifically IONs@APTES and L-Lys@IONs, were prepared and analysed using various methods, and the impact of each on microbial growth and MK-7 isomers were evaluated. The conclusions drawn from this investigation will expand the existing knowledge of the ability of biocompatible amino acid-coated IONs to boost the productivity

of MK-7 fermentation. This, in addition to the potential for process intensification with the assistance of magnetic separation technology, will likely enable the evolution of a streamlined industrial fermentation process that selectively targets the production of the all-*trans* isomer. A more efficient fermentation system will possibly entail lower production costs and improve the affordability and accessibility of biologically efficacious fermented MK-7 nutraceuticals, functional products, and dietary supplements.

## Materials and Methods

### Chemicals and Materials

The all-*trans* MK-7 (98.1%) analytical standard was obtained from ChromaDex (Los Angeles, CA, USA). $FeSO_4 \cdot 7H_2O$ and glucose were supplied by Ajax Finechem Pty Ltd (Taren Point, NSW, Australia), and tryptone and yeast extract were acquired from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Methanol, ethanol, *n*-hexane, 2-propanol, $NH_4OH$, and soy peptone were procured from Merck Millipore (Burlington, MA, USA). $CaCl_2$, $FeCl_3 \cdot 6H_2O$, glutaraldehyde, sodium cacodylate, APTES, and L-Lys were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). NaCl was obtained from a domestic source, and nutrient (BHI) agar plates were acquired from Fort Richard Laboratories (Auckland, New Zealand).

### Microorganism and Inoculum Preparation

*B. subtilis natto* is often utilised in MK-7 fermentation experiments, as well as those involving NPs [38, 39, 53, 54]. Additionally, *B. subtilis natto* enables a high fermentation yield and is classed as generally recognised as safe (GRAS); thus, it is considered suitable for large-scale MK-7 production and is preferably employed for manufacturing MK-7 products [42, 56]. Therefore, it was deemed the most appropriate microbial strain for this investigation. The microbial spore solution was produced using the approach implemented by Berenjian et al. [40]. The *B. subtilis natto* cells were grown in a liquid mixture of NaCl, yeast extract, and tryptone and plated on BHI agar plates. The plates were stored in an incubator at 37 °C for 48 h. The bacterial growth was scraped off the agar plates and submersed in a sterile saline solution. The microbial suspension was then placed in an 80 °C water bath for 30 min to inactivate the growing cells and induce the generation of spores. Centrifugation (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 3000 rpm for 10 min was used to isolate the cell debris. The consequent microbial spore suspension acted as the inoculum for the NP fermentation procedures.

### NP Synthesis and Characterisation

#### Synthesis of Uncoated IONs

Uncoated IONs ($Fe_3O_4$) were produced in an inert atmosphere using the co-precipitation technique outlined by Ebrahiminezhad et al. [38]. $FeCl_3 \cdot 6H_2O$ (1.17 g) and $FeSO_4 \cdot 7H_2O$ (0.74 g) were combined in 50 mL of distilled water, and the solution was briskly agitated for 1 h at 70 °C in a nitrogen atmosphere to avoid oxidation. $NH_4OH$ (5 mL) was rapidly added to the solution, which was then stirred for a further 1 h until precipitation occurred. After the reaction, the magnetic particles were separated with a permanent magnet. The IONs were washed several times with boiled distilled water to eliminate unwanted compounds before drying for 24 h at 50 °C in an oven (Contherm Thermotec 2000, Contherm Scientific Ltd, Wellington, New Zealand).

#### Synthesis of IONs@APTES

APTES coating was carried out using the methodology proposed by Ebrahiminezhad et al. [55]. Uncoated IONs (0.7 g) were added to a 1:1 (*v/v*) solution of ethanol and distilled water (25 mL) and sonicated (Qsonica-Q800R, Newtown, CT, USA) while kept in an ice bath for 2 min to achieve a homogenous mixture. Afterwards, 2.8 mL of APTES solution was injected into the mixture under nitrogen and vigorously stirred for 2 h at 40 °C. The coated IONs were then collected with a permanent magnet. A mixture of ethanol and distilled water was used to wash the magnetic particles to exclude contaminants before drying for 24 h at 50 °C in an oven (Contherm Thermotec 2000, Contherm Scientific Ltd, Wellington, New Zealand).

#### Synthesis of L-Lys@IONs

The procedure employed by Ebrahiminezhad et al. [39] was used to synthesise the L-Lys@IONs. Accordingly, $FeSO_4 \cdot 7H_2O$ (0.74 g), $FeCl_3 \cdot 6H_2O$ (1.17 g), and L-Lys (1.6 g) were combined in 50 mL of distilled water, and the solution was robustly mixed at 70 °C under nitrogen for 1 h. $NH_4OH$ (5 mL) was successively added to the reaction mixture, which was agitated for another 1.5 h until precipitation occurred. The particles were then magnetically separated, washed with boiled distilled water to extract pollutants, and dried for 24 h at 50 °C in an oven (Contherm Thermotec 2000, Contherm Scientific Ltd, Wellington, New Zealand).

### Characterisation

The size and morphology of the synthesised NPs were determined by transmission electron microscopy (TEM; Philips, CM 10, Philips Electron Optics, Eindhoven, The Netherlands). A NP dispersion was prepared in distilled water for the TEM analysis, and a drop of the solution was put on a copper grid coated with carbon. All TEM images were taken at HT 100 kV. The identity of important chemical bonds and functional groups was established using Fourier-transformed infrared (FTIR) spectroscopy (Bruker VERTEX 70 FTIR spectrometer, Bruker, Kassel, Germany) in the span of 4000–400 $cm^{-1}$. Before the FTIR procedure, a pellet with a NP-to-KBr ratio of 1% was made and subjected to a hydraulic press for 10 min to create a solid disc. All samples were assessed at room temperature by the FTIR instrument. X-ray powder diffraction (XRD; Siemens D5000, Munich, Germany) was used to evaluate the crystalline structure of the coated NPs. The dried powder was packed on a zero-background silicon holder, and the excess was removed using a brush and straight edge. The analysis was conducted at ambient temperature, 40 mA, and 45 kV, with a step size of 0.0530° and an exploration range (2θ) within 20° and 90°.

### Cell Fixation and Scanning Electron Microscopy (SEM) Analysis

SEM (Hitachi Regulus SU8230 FE-SEM, Tokyo, Japan) was used to visualise the surface structure of the coated NPs and their interaction with the bacterial cells. The bacterial cell samples were prepared following a similar technique to Ebrahiminezhad et al. [38]. A 10 µL drop of the sample was put on a coverslip before passing it through the flame of a Bunsen burner to heat-fix the bacterial smear. The sample was then chemically fixed for 45 min by placing the coverslip in 2.5% (*v/v*) glutaraldehyde in 0.1 M sodium cacodylate buffer before rinsing with saline for 15 min. The coverslip was then kept in a series of ethanol concentrations (30, 50, 70, 80, 90, and 95%) for 10 min each to dehydrate the bacterial cells. Afterwards, the sample was stored for 20 min in pure ethanol and dried using a critical point dryer (Polaron E3000, Quorum Technologies, East Sussex, England, UK). The dried microbial samples and pure ION powders were coated with platinum after mounting them on an aluminium stub. SEM images of IONs, free-floating cells, and bacterial cells immobilised with coated IONs were captured at 3 kV.

### Bacterial Cell Immobilisation and Fermentation

The fermentation media, comprising 1% (*w/v*) glucose, 2% (*w/v*) yeast extract, 2% (*w/v*) soy peptone, 2% (*w/v*) tryptone, and 0.1% (*w/v*) $CaCl_2$ [41], was made and autoclaved (TOMY SX-700E, Tokyo, Japan) at 121 °C for 20 min. Following sterilisation, the samples were inoculated with 2% (*v/v*) of the bacterial spore suspension. Stock solutions of IONs@APTES and L-Lys@IONs (0.01 g/mL) were individually prepared with sterilised distilled water and added to the samples at various concentrations (0–600 µg/mL). The samples for each type of coated NP were prepared separately, and three replicate samples for each concentration were considered. All samples were fermented aerobically at 200 rpm and 40°C for 7 days to enable the coated NPs to attach to the surface of the microbial cells. The fermentation parameters were selected from our earlier investigation [47].

### MK-7 Extraction

Preceding analysis, MK-7 was removed from the fermented samples, as summarised by Berenjian et al. [40]. A solution of *n*-hexane and 2-propanol was added to the samples in a ratio of 1:2 (*v/v*) while maintaining a liquid-to-organic ratio of 1:4 (*v/v*) before thoroughly mixing for 2 min with a vortex mixer. The different layers of liquid were split by centrifuging (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) the mixture for 10 min at 3000 rpm. The top layer was separated from the remaining liquid and evaporated to isolate the extracted MK-7.

### MK-7 Analysis

MK-7 analysis was performed employing the methodology discussed in our previous report [41]. The MK-7 isomer concentration was assessed with a Dionex high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific, Waltham, MA, USA), which consisted of a photodiode array UV detector, an automated sample injector, four pumps, and a thermostatted column compartment. Separation was conducted at 40 °C using a COSMOSIL Cholester packed (reversed-phase) column (100 mm × 2 mm × 2.5 µm; Nacalai Tesque Inc., Kyoto, Japan). The compounds were eluted isocratically with methanol (mobile phase) at a flow rate of 0.2 mL/min. The injection volume was 10 µL, the autosampler temperature was 10 °C, the analytical wavelength was 248 nm, and the run-time was 30 min. Data were collected with the Chromeleon 7 program (Thermo Fisher Scientific, Waltham, MA, USA), and the *cis* MK-7 isomer was ascertained by a relative retention time (RRT) of 1.12. The MK-7 concentration of the samples was deduced from a linear MK-7 standard curve ($R^2 = 0.99$), which was developed by measuring the area of peaks corresponding to known concentrations of the reference standard.

Liquid chromatography–mass spectrometry (LC–MS) was utilised to validate the identity of the MK-7 isomers and confirm their retention times, as outlined in our earlier study [41]. The LC–MS setup contained a Dionex Ultimate 3000 ultra-high-performance liquid chromatography (UHPLC)

apparatus and a QExactive mass spectrometer with a HESI II source (Thermo Fisher Scientific, Waltham, MA, USA). The equipment was controlled with the Thermo XCalibur 4.3 platform (Thermo Fisher Scientific, Waltham, MA, USA), and data handling was accomplished using the Chromeleon 7.3 package (Thermo Fisher Scientific, Waltham, MA, USA). The previously described chromatography conditions were employed to separate the MK-7 isomers using liquid chromatography; however, an injection volume and a run-time of 5 µL and 37 min, respectively, were used to suit the needs of the LC–MS equipment. Data were acquired in the positive ionisation mode with a maximum injection time of 200 ms, a resolution of 70,000, an AGC target of $3 \times 10^6$, and a MS1 scan range between 150 and 1000 *m/z*. The mass spectrometry (MS) data were evaluated with the Thermo FreeStyle 1.6 application (Thermo Fisher Scientific, Waltham, MA, USA).

### Cell Density and pH Measurements

Bacterial growth was estimated from measurements of the cell density. The optical density (OD) of the samples was measured using a UV–vis spectrophotometer (Shimadzu UV-1900, Kyoto, Japan) at an analytical wavelength of 600 nm after suitable dilution with distilled water. The pH of the samples was assessed using a laboratory pH meter (CyberScan pH 100, Eutech Instruments, Paisley, UK).

### Statistical Methods

Analysis of variance (ANOVA) was used to determine the statistical significance, and the mean values of different groups were compared with a two-sample *t*-test. All data were described as the mean ± standard error (SE) of triplicate samples, and statistical significance was accepted at $p < 0.05$.

## Results and Discussion

### Synthesis and Characterisation of Biocompatible Amine-Functionalised IONs

The co-precipitation method was used to synthesise biocompatible amino acid-coated IONs (IONs@APTES and L-Lys@IONs). The SEM images (Fig. 2) and TEM micrographs (Fig. 3) show that both types of amine-functionalised IONs have a spherical morphology. The NPs also have a reasonably uniform size distribution ranging from 7–20 nm for the IONs@APTES and 4–10 nm for the L-Lys@IONs, with an average particle size of 11 nm and 7 nm, respectively.

The FTIR spectra of the coated IONs are provided in Fig. 4 and illustrate the distinguishing Fe–O peaks at around 608 $cm^{-1}$ and 420 $cm^{-1}$ for the IONs@APTES and 612 $cm^{-1}$ and 425 $cm^{-1}$ for the L-Lys@IONs. The peak at approximately 1081 $cm^{-1}$ signifies the stretching vibration of the Si–O bond in the IONs@APTES [53]. The successful coating of IONs with APTES is also indicated by the peaks at about 2850 $cm^{-1}$ and 2900 $cm^{-1}$, which depict the presence of aliphatic -$CH_2$ groups and can be ascribed to the symmetric and asymmetric –C–H stretching vibrations, respectively [53]. For the L-Lys@IONs, C–O and C=O stretching vibrations can be visualised at approximately 1440 $cm^{-1}$ and 1630 $cm^{-1}$, respectively, and the peak at roughly 2925 $cm^{-1}$ denotes the overlap between the C-H and N–H stretching vibrations with the O–H stretching vibration [57]. In addition, during the production of IONs using the co-precipitation technique, the unsaturated iron atoms at the surface interact with water molecules and $OH^-$ ions in the

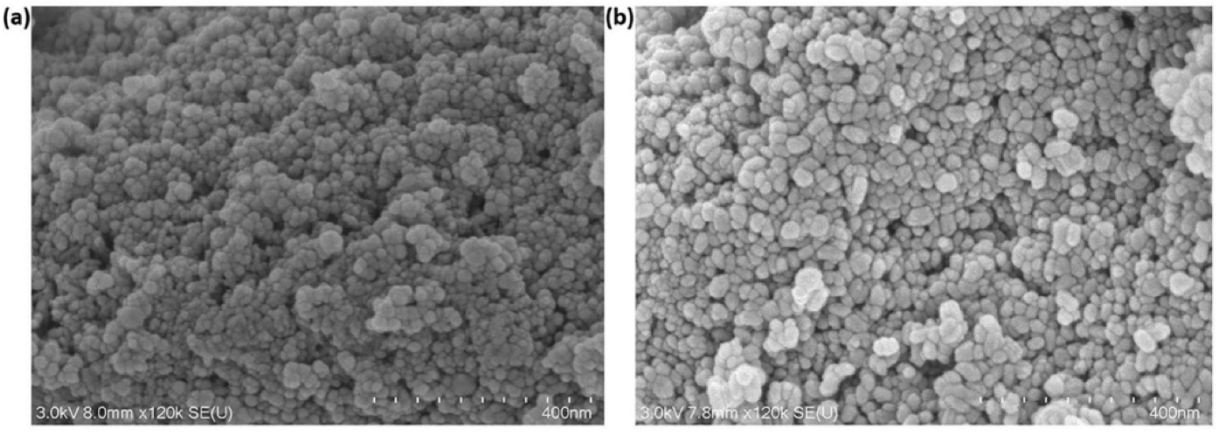


**Fig. 2** SEM image of the exterior surface structure of the **a** IONs@APTES and **b** L-Lys@IONs

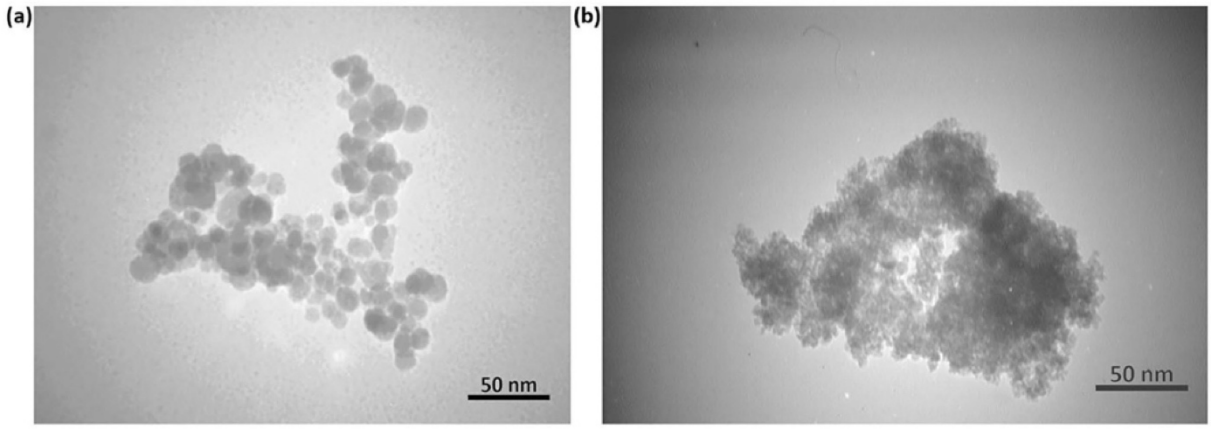


**Fig. 3** TEM micrograph of the **a** IONs@APTES and **b** L-Lys@IONs

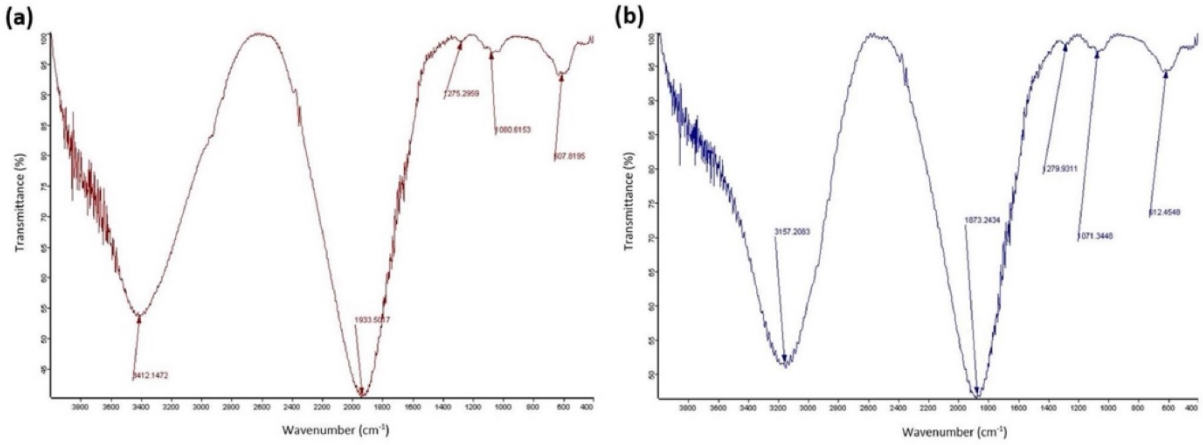


**Fig. 4** FTIR spectrum of the **a** IONs@APTES and **b** L-Lys@IONs

aqueous medium and modify the surface of the NPs [38, 57]. This is represented by the peaks at about 3412 $cm^{-1}$ and 1934 $cm^{-1}$ for the IONs@APTES and 3157 $cm^{-1}$ and 1873 $cm^{-1}$ for the L-Lys@IONs, which correspond to the O–H bending and stretching vibrations, respectively.

The XRD patterns for the amine-functionalised IONs are presented in Fig. 5, and the intensity peaks obtained from the analysis are comparable for the two kinds of coated IONs. The XRD pattern for the IONs@APTES shows definite intensity peaks at 2θ degrees of 30°, 35.5°, 43°, 53°, 57°, and 63°, which represent (220), (311), (400), (422), (511), and (440) Bragg reflections, respectively. Similarly, the XRD pattern for the L-Lys@IONs displays characteristic intensity peaks at 2θ degrees of 30°, 35.5°, 43°, 57°, and 63°, which denote (220), (311), (400), (511), and (440) Bragg reflections, respectively. These distinctive peaks correspond to the crystal structure of magnetite and confirm the production of IONs [39, 53]. The sharper peaks for the IONs@APTES suggest that they have greater crystallinity than the L-Lys@IONs synthesised in this study. However, since the XRD analysis resulted in equivalent peaks for both types of coated IONs, it indicates that amine-functionalisation with biocompatible amino acid coatings does not significantly impact the crystal structure.

## Interaction of Coated IONs with the Bacterial Cell Surface

Interactions between the biocompatible IONs and microbial cells were viewed using SEM. The effective decoration and immobilisation of *B. subtilis natto* cells with IONs@APTES and L-Lys@IONs in comparison to the free bacterial cells are depicted in Fig. 6. Both types of amine-functionalised IONs are many orders of magnitude

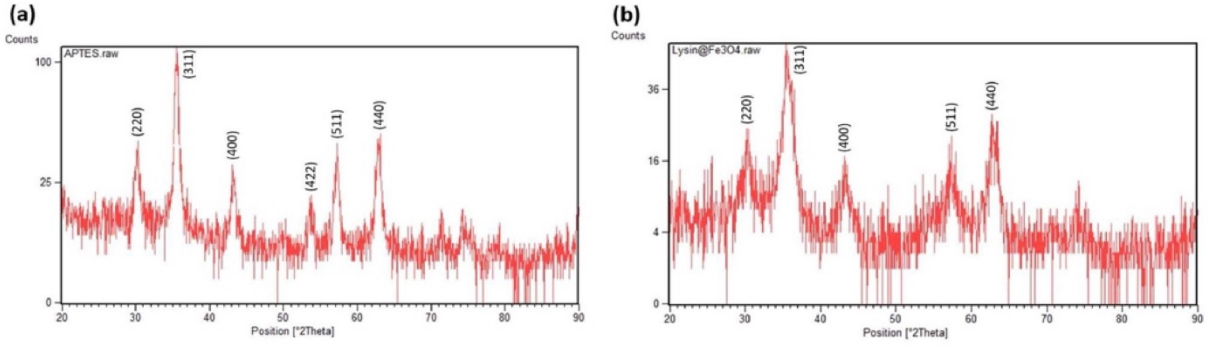


**Fig. 5** XRD pattern of the **a** IONs@APTES and **b** L-Lys@IONs

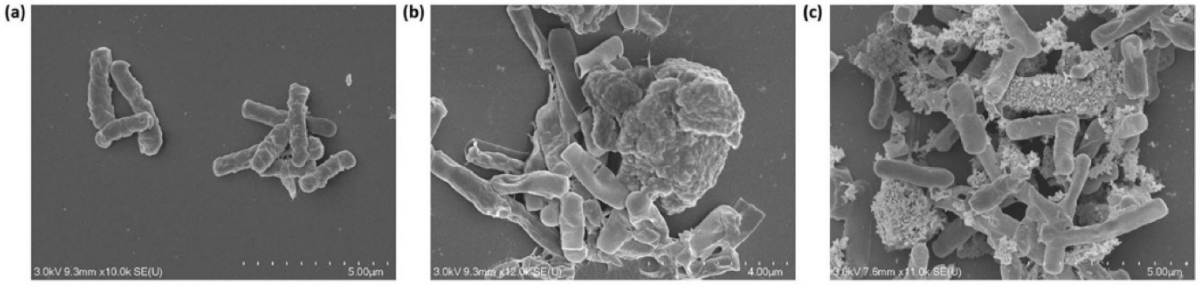


**Fig. 6** SEM image of the **a** untreated bacterial cells, **b** bacterial cells immobilised with IONs@APTES, and **c** bacterial cells immobilised with L-Lys@IONs

smaller than the microbial cells. The small particle size and large surface-area-to-volume ratio of the coated IONs and the presence of positively charged amine groups allow them to attach to the surface of the bacterial cells through several bonding interactions, including hydrogen bonds, hydrophobic interactions, electrostatic attractions, and Van der Waals forces [39, 53]. As the interactions between the NPs and microbial cells are non-specific, the IONs arbitrarily attach to their surface. Since the interaction between all cells is not consistent, some bacteria are more extensively decorated than others.

Decoration of microbial cells with biocompatible magnetic IONs is advantageous, as it can enable cell removal with an external magnetic field, which creates an opportunity for process intensification by reducing the number of complex downstream processing steps required for the isolation and purification of MK-7. This will streamline the production process and permit the separated bacterial cells to be re-employed in subsequent fermentation batches, decreasing production costs. Nonetheless, to allow the cells to be recycled, it is vital to preserve their growth and metabolic characteristics and ensure that immobilisation with amine-functionalised magnetic IONs has no undesirable effects on cell viability.

## The Influence of Bacterial Cell Immobilisation with Amine-Functionalised IONs on Microbial Growth

The effect of microbial cell immobilisation with IONs@APTES and L-Lys@IONs on bacterial growth was considered, and it can be seen that the impact on cell growth differs between the two types of amino acid coatings (Fig. 7).

For the IONs@APTES, the OD for the control (6.39), 400 µg/mL (6.72), and 600 µg/mL (7.21) groups is comparable. The OD attained for an IONs@APTES concentration of 100 µg/mL (7.91) and 200 µg/mL (8.88) is relatively similar and noticeably higher than the other groups, whereas that for the 300 µg/mL (4.96) and 500 µg/mL (4.97) groups is alike and considerably lower than all other concentration groups. In contrast, for the L-Lys@IONs, the OD obtained for the majority of the investigated concentrations (control, 100 µg/mL, 200 µg/mL, 300 µg/mL, and 400 µg/mL) is fairly consistent (between 5.97 and 6.87), while that for the 500 µg/mL and 600 µg/mL groups is appreciably greater than

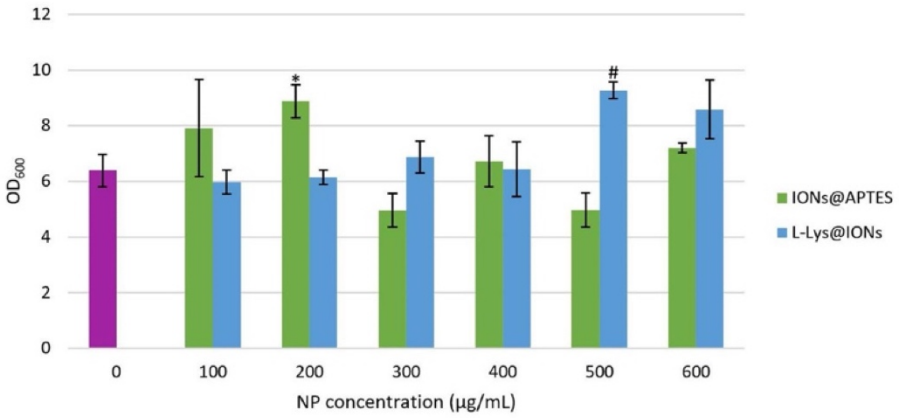


**Fig. 7** The impact of bacterial cell immobilisation with coated IONs on microbial growth, where * and # indicate a significantly different OD compared to the control ($p < 0.05$) for the IONs@APTES and L-Lys@IONs, respectively

the other L-Lys@IONs concentration groups. Interestingly, the OD achieved at an IONs@APTES concentration of 100 µg/mL and 200 µg/mL and a L-Lys@IONs concentration of 500 µg/mL and 600 µg/mL is almost equivalent.

The ANOVA assessment demonstrated that the difference in the OD between all ION concentration groups is not statistically significant for the IONs@APTES ($p = 0.106$) but is statistically significant for the L-Lys@IONs ($p = 0.038$). The OD achieved at each NP concentration for both forms of amino acid coatings was also compared with the control using a *t*-test. It was determined that the OD obtained for an IONs@APTES and L-Lys@IONs concentration of 200 µg/mL and 500 µg/mL, respectively, were appreciably higher than the untreated cells ($p = 0.018$ for the IONs@APTES and $p = 0.004$ for the L-Lys@IONs). The ANOVA results also established a statistically significant difference ($p = 0.028$) in the OD measurements between the different concentrations of IONs@APTES and L-Lys@IONs when considered together, implying that the nature of the amino acid coating influences bacterial growth.

The response of microbial cells to immobilisation with magnetic IONs is often variable and tends to differ among Gram-positive and Gram-negative bacterial strains, possibly as a result of the dissimilarities in their cell wall structure, metabolic characteristics, and cellular composition [58]. The exact nature of the interaction between IONs and bacterial cells is determined by several factors, such as the characteristics of the IONs, bacterial species, and culture conditions [59, 60]. Possible outcomes encompass alteration in cell growth, changes in cell shape and morphology, variation in membrane permeability, induced gene expression, permeation of IONs into the microbial cell membrane, and the production of reactive oxygen species (ROS) [58, 60]. It has been observed that IONs do not demonstrate substantial antibacterial activity against Gram-positive bacteria, and stimulation or inhibition of growth occurs depending on the concentration of NPs [58]. Furthermore, in our previous study [54], *B. subtilis natto* (Gram-positive) growth was either stimulated or inhibited at different concentrations of uncoated IONs; however, an insignificant difference in the OD measurements between the various NP concentration groups suggested that IONs have negligible antibacterial activity against *B. subtilis natto*.

Overall, the OD values attained in the present investigation for both types of amine-functionalised IONs are substantially greater than that achieved in our earlier study [54], which explored uncoated IONs in a similar context. Ebrahiminezhad et al. [39] considered the influence of L-Lys@IONs on microbial growth, and it was determined that while bacterial immobilisation reduced cell growth by approximately 16% at the end of fermentation, the difference in the OD between the NP concentrations assessed was not significant. However, Ebrahiminezhad et al. [39] examined a lower band of L-Lys@IONs concentrations (0–150 µg/mL) and evaluated bacterial growth using the plate count method over 5 days of fermentation. Despite these minor differences, the findings of this study are essentially similar to the current investigation, as it was determined that L-Lys@IONs do not have a significant negative impact on bacterial growth. It is also worth mentioning that the NP concentrations explored by Ebrahiminezhad et al. [39] are within the range of concentrations in this study for which a decline in the OD occurred (0–200 µg/mL). On the other hand, Ranmadugala et al. [53] examined the effect of different concentrations of IONs@APTES (0–700 µg/mL) on *B. subtilis* growth. It was seen that the cell density increased with an increase in the IONs@APTES concentration up to 500 µg/mL, where it reached a maximum, and a decrease in the OD was seen at higher concentrations (600 and 700 µg/mL). A similar trend in the OD measurements was observed in the present investigation, except the maximum cell density was obtained at a lower IONs@APTES concentration (200 µg/mL). This disparity may be assigned to the different

fermentation media and operating conditions employed in each study.

Moreover, it is intriguing that despite the higher cell densities attained from fermentation with the IONs@APTES in comparison to the naked IONs synthesised in our earlier investigation [54], the size of both types of NPs is the same (7–20 nm, with an average particle size of 11 nm). It is likely that the biocompatible amino acid (APTES) coating on the surface of the IONs@APTES decreases the toxicity of these NPs and results in more favourable interactions with *B. subtilis natto* cells. The L-Lys@IONs have a much smaller particle size (4–10 nm, with an average particle size of 7 nm) than the IONs@APTES and the naked IONs from our initial investigation (7–20 nm, with an average particle size of 11 nm) [54]. Certain amino acids in the reaction mixture can inhibit the growth of magnetite NPs during synthesis, resulting in smaller-sized particles, and this effect is more profound for L-Lys [57]. It has been suggested that the size of IONs influences their antimicrobial properties and toxicity towards microbial cells [59]. Smaller particles tend to have greater antimicrobial capacity, as they can easily penetrate the bacterial cell membrane relative to larger particles due to their greater surface-area-to-volume ratio, which substantially enhances their interaction with bacterial cells [59, 61, 62]. Penetration of IONs into the microbial cell can lead to membrane damage and cell inactivation through various mechanisms, primarily oxidative stress mediated by the generation of ROS (can cause DNA damage, mitochondrial impairment, lipid peroxidation, cell membrane disruption, and the oxidation of biological macromolecules), the release of metal ions (can react with the bacterial membrane and other cellular components), and the physical disruption of the cell membrane and cellular transport processes [59, 62, 63]. Nevertheless, although the L-Lys@IONs are considerably smaller than the other two types of IONs, they did not negatively affect the cell density. Also, the OD values for the L-Lys@IONs were noticeably higher than those for the uncoated IONs. This demonstrates the value of the L-Lys coating, as it reduces the antimicrobial characteristics of the NPs and significantly enhances their biocompatibility, mitigating the adverse consequences of their small particle size.

Collectively, the outcomes of the current investigation and prior studies imply that biocompatible amino acid coatings promote beneficial interactions with bacterial cells, thus reducing harmful effects on cell growth and viability. Therefore, immobilisation of *B. subtilis natto* cells with biocompatible amine-functionalised IONs does not have a negative impact on bacterial growth. Preserving the growth and metabolism of the bacteria is advantageous to enhance MK-7 synthesis and the yield of the vitamin.

## The Effect of Biocompatible IONs on the Production of All-*Trans* and *Cis* MK-7 Isomers

The influence of bacterial cell immobilisation with different concentrations of biocompatible IONs on MK-7 isomer production was assessed, and the results are outlined in Fig. 8. For the APTES-coated NPs, the concentration of the all-*trans* isomer increased with an increase in the IONs@APTES concentration to a maximum of 36.57 mg/L at a NP concentration of 200 µg/mL, and a marginally lower concentration (32.51 mg/L) was attained at an IONs@APTES concentration of 300 µg/mL. The all-*trans* MK-7 concentration was appreciably lower for the 400 and 500 µg/mL groups, and a slightly greater all-*trans* isomer concentration (26.24 mg/L) was obtained at the highest NP concentration. A similar trend was observed for the *cis* MK-7 concentration, except the maximum *cis* concentration was achieved at an IONs@APTES concentration of 300 µg/mL. Conversely, for the L-Lys-functionalised IONs, a comparable all-*trans* isomer concentration was obtained at a NP concentration of 0–300 µg/mL. Considerably greater all-*trans* MK-7 production was observed at higher concentrations of L-Lys@IONs (400–600 µg/mL), almost twice that attained at the lower concentrations. The greatest all-*trans* MK-7 concentration (32.08 mg/L) occurred at a L-Lys@IONs concentration of 400 µg/mL. Production of the *cis* isomer also followed a similar pattern, but the difference in the *cis* MK-7 concentration achieved between the low and high NP concentration groups was not as substantial.

The ANOVA evaluation indicated a statistically significant difference in all-*trans* MK-7 production between the different concentration groups for both kinds of amine-functionalised IONs ($p=0.007$ for the IONs@APTES and $p=0.032$ for the L-Lys@IONs). The ANOVA results also determined a statistically insignificant difference in the *cis* isomer concentration between the various IONs@APTES concentration groups ($p=0.205$), while that for the L-Lys@IONs was significant ($p=0.003$). However, it is important to appreciate that the production of both isomers in the presence of IONs@APTES was greater than the control, whereas the MK-7 isomer concentration achieved at specific concentrations of the L-Lys@IONs was less than the control. A *t*-test comparison of each NP concentration group with the control for both types of coated IONs revealed that relative to the untreated cells, all-*trans* isomer production was significantly greater for an IONs@APTES concentration of 100 µg/mL, 200 µg/mL, 300 µg/mL, and 600 µg/mL and a L-Lys@IONs concentration of 400 µg/mL and 500 µg/mL. In contrast, the *cis* MK-7 concentration was significantly higher than the control for an IONs@APTES concentration of 100 µg/mL, 200 µg/mL, 300 µg/mL, 500 µg/mL, and 600 µg/mL and a L-Lys@IONs concentration of 400 µg/mL

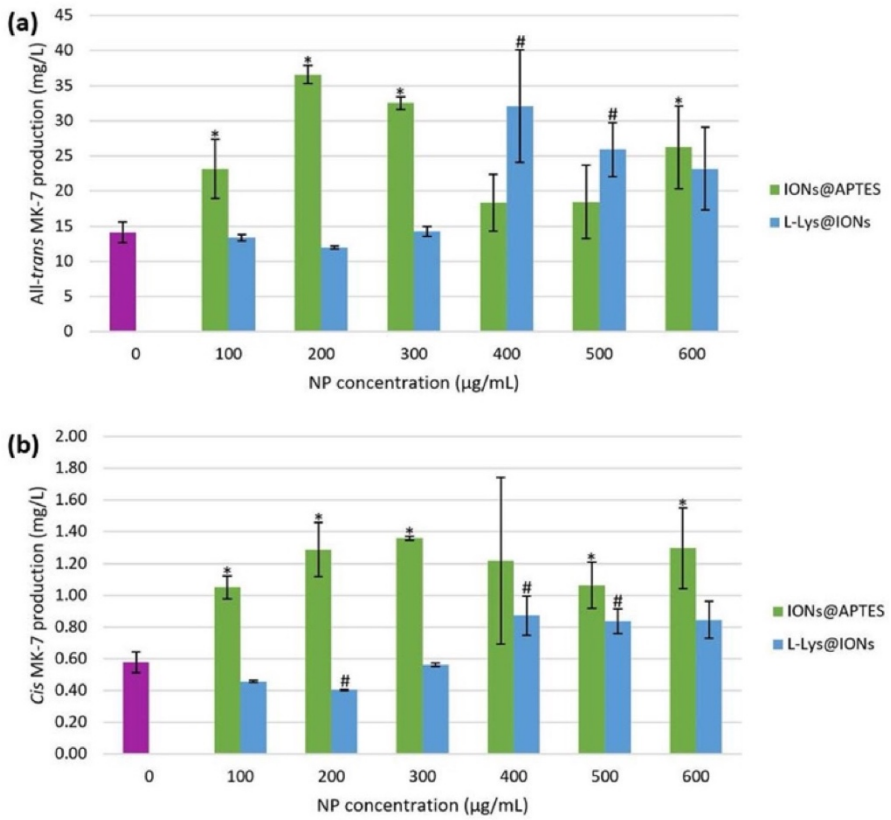


**Fig. 8** Isomer production in the presence of various concentrations of amine-functionalised IONs, where * and # indicate a significantly different **a** all-*trans* MK-7 and **b** *cis* MK-7 concentration in comparison to the control ($p < 0.05$) for the IONs@APTES and L-Lys@IONs, respectively

and 500 µg/mL, while that for a L-Lys@IONs concentration of 200 µg/mL was significantly lower than the free cells.

These observations are supported by the findings of Ranmadugala et al. [53] and Ebrahiminezhad et al. [39], who have explored MK-7 production during fermentation with *B. subtilis natto* cells immobilised with IONs@APTES and L-Lys@IONs, respectively. The MK-7 concentration achieved was greater than the control for all IONs@APTES concentrations (0–700 µg/mL) considered by Ranmadugala et al. [53], whereas Ebrahiminezhad et al. [39] obtained a slightly lower MK-7 concentration for the immobilised bacteria (0–150 µg/mL) compared to the untreated cells on each day of fermentation. In addition, Ranmadugala et al. [53] noted significantly greater MK-7 production than the control for an IONs@APTES concentration of 200 µg/mL and 300 µg/mL, which is consistent with this investigation. The MK-7 concentration attained at these IONs@APTES concentrations (around 30 mg/L) is also similar to the all-*trans* isomer concentration obtained in this study for the same IONs@APTES concentrations (36.57 mg/L for 200 µg/mL and 32.51 mg/L for 300 µg/mL). Ebrahiminezhad et al. [39] achieved a final MK-7 concentration of 10.80 mg/L, 11.57 mg/L, and 11.56 mg/L for a L-Lys@IONs concentration of 50 µg/mL, 100 µg/mL, and 150 µg/mL, respectively, and all of these were marginally lower than the control (11.80 mg/L). These outcomes support the results of the current investigation for the same range of L-Lys@IONs concentrations (0–200 µg/mL). In this study, an all-*trans* MK-7 concentration of 14.13 mg/L was obtained for the free-floating cells, which was slightly higher than the cells immobilised with 100 µg/mL (13.33 mg/L) and 200 µg/mL (11.96 mg/L) of L-Lys@IONs.

Holistically, the experimental findings suggest noticeable variation in the concentration of all-*trans* MK-7 but not the *cis* isomer between all IONs@APTES concentrations examined. On the contrary, there is a considerable difference in the production of both isomers between all investigated concentrations of L-Lys@IONs. However, when evaluating the effect of bacterial cell immobilisation on the MK-7 isomer concentration by comparing the untreated cells with those immobilised with various concentrations of amine-functionalised IONs, it is apparent that certain concentrations of L-Lys@IONs impair all-*trans* MK-7 production, which is undesirable. Therefore, IONs@APTES are preferable, as production of the bioactive isomer was enhanced in comparison to the control (free cells) at all NP concentrations explored.

## The Impact of Coated IONs on the MK-7 Isomer Yield

The effect of bacterial cell immobilisation with amine-functionalised IONs on fermentation productivity was evaluated by determining the isomer yield. Figure 9 illustrates the effect of the different concentrations of IONs@APTES and L-Lys@IONs on the all-*trans* and *cis* MK-7 yield. The yield of the two isomers for both types of coated IONs depicted a bell-shaped pattern. For the IONs@APTES, the isomer yield was modest for low (0–200 µg/mL) and high (400–600 µg/mL) ION concentrations, and the maximum yield was obtained at a concentration of 300 µg/mL. A similar trend was noted for the L-Lys@IONs, except the greatest yield of both isomers occurred at a concentration of 400 µg/mL.

From the ANOVA test, it was established that a statistically significant difference exists in the all-*trans* and *cis* MK-7 yield between all NP concentrations that were considered for both kinds of coated IONs (all-*trans* isomer: $p = 0.001$ for the IONs@APTES and $p = 0.001$ for the L-Lys@IONs; *cis* isomer: $p = 0.029$ for the IONs@APTES and $p = 0.014$ for the L-Lys@IONs). A *t*-test comparison of the various NP concentrations with the control for each type of amine-functionalised ION indicated that the yield of the all-*trans* MK-7 isomer was significantly higher than the untreated cells for an IONs@APTES concentration of 200 µg/mL, 300 µg/mL, and 500 µg/mL and an L-Lys@IONs concentration of 400 µg/mL. In addition, the *cis* MK-7 yield was significantly higher than the free cells for an IONs@APTES concentration of 300 µg/mL, 500 µg/mL, and 600 µg/mL, whereas, for the L-Lys@IONs, the difference in the *cis* isomer yield between the control and the remaining concentration groups was insignificant.

It is evident that generally, the yield of the bioactive isomer was higher for the IONs@APTES compared to the L-Lys@IONs. The maximum all-*trans* MK-7 yield obtained for the IONs@APTES (300 µg/mL) was 3.1-fold more than the untreated cells (0 µg/mL) and 1.4-fold greater than the highest all-*trans* isomer yield for the L-Lys@IONs (400 µg/mL). Ranmadugala et al. [53] also noted a significantly higher MK-7 yield at an IONs@APTES concentration of 200 µg/mL and 300 µg/mL; however, in contrast to our findings, the maximum yield occurred at 200 µg/mL. Ebrahiminezhad et al. [39] also observed a significantly greater MK-7 yield from fermentation with bacterial cells immobilised with L-Lys@IONs relative to the control. Furthermore, the yield of the biologically effective isomer achieved in the current investigation with 300 µg/mL of IONs@APTES and 400 µg/mL of L-Lys@IONs was 2.1- and 1.4-fold higher than that obtained for

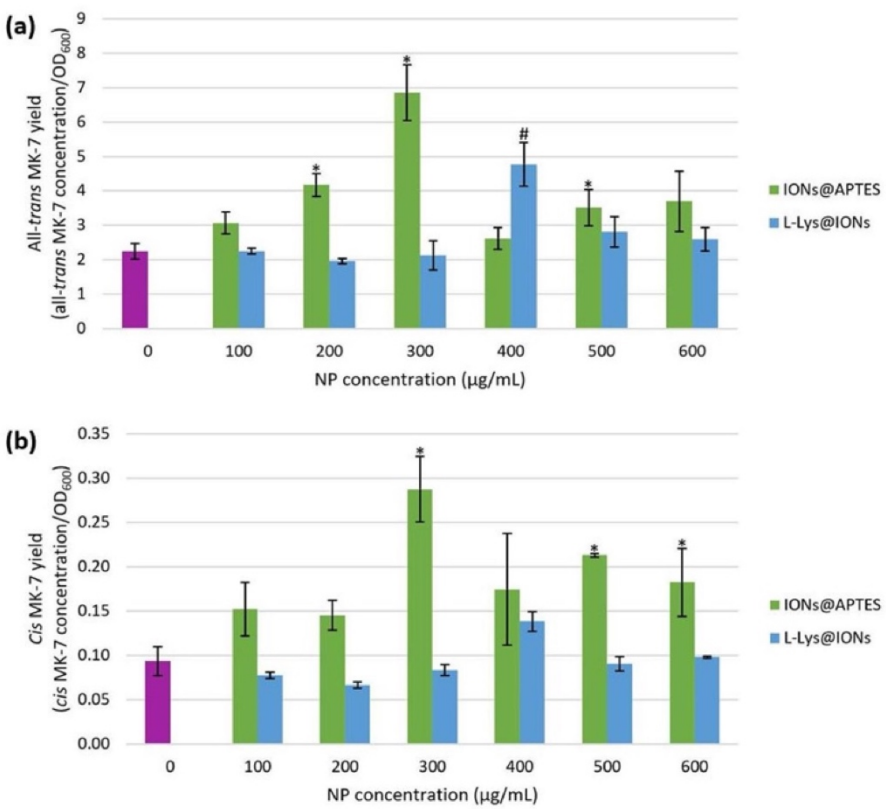


**Fig. 9** The influence of bacterial cell immobilisation with biocompatible IONs on the isomer yield, where * and # indicate a significantly different **a** all-*trans* MK-7 and **b** *cis* MK-7 yield relative to the control ($p < 0.05$) for the IONs@APTES and L-Lys@IONs, respectively

the optimum concentration of naked IONs (300 µg/mL) in our previous study [54], respectively.

It has been proposed that microbial cells immobilised with IONs have better metabolic efficiency due to the non-specific bonding associations between the NPs and the bacterial cell surface. These interactions disrupt the lipid layer of the bacterial cell membrane and increase its permeability, aiding mass transfer and enhancing the secretion of all-*trans* MK-7 into the fermentation broth [53, 59]. This improves the yield of the bioactive isomer and boosts the fermentation productivity. The superior all-*trans* isomer yield resulting from bacterial cell immobilisation with amine-functionalised IONs compared to uncoated IONs can be credited to the biocompatible APTES and L-Lys coatings, which enhance the production of biologically effective MK-7 and have a positive influence on cell growth and metabolism.

Moreover, it is worthwhile to recognise that both the production and yield of bioactive MK-7 have a direct relationship with the production and yield of the *cis* isomer. Accordingly, greater production and yield of the all-*trans* isomer correlates with higher production and yield of the *cis* isomer. While the yield of all-*trans* and *cis* MK-7 was considerably greater for the optimal concentration of IONs@APTES (300 µg/mL) and L-Lys@IONs (400 µg/mL), the fraction of the overall yield for each geometric isomer was comparable to the remaining concentration groups and the untreated cells for each type of coated ION. This indicates that although there is a noticeable difference in the isomer yield between the optimum concentration of IONs@APTES and L-Lys@IONs and the other concentration groups for both amine-functionalised IONs, the portion of the total yield for each isomer did not differ significantly. Therefore, bacterial cell immobilisation with the ideal concentration of IONs@APTES and L-Lys@IONs significantly improves the fermentation yield of the desirable isomer relative to the free cells without enhancing the proportional yield of redundant by-products, which is beneficial.

## Monitoring Study Employing the Optimal IONs@APTES Concentration

Of the two biocompatible amine-functionalised IONs that were evaluated, IONs@APTES were superior, as immobilisation of *B. subtilis natto* cells with IONs@APTES improved both the production and yield of the biologically significant isomer. Additionally, IONs@APTES have the ability to decrease biofilm formation, a prevalent issue in industrial fermentation, without any detrimental impact on cell growth and viability [64]. A comparable concentration of the all-*trans* isomer was obtained at an IONs@APTES concentration of 200 µg/mL and 300 µg/mL; however, the yield of all-*trans* MK-7 was approximately 64% greater for an IONs@APTES concentration of 300 µg/mL. Consequently, 300 µg/mL was determined to be the optimal IONs@APTES concentration to increase the production of bioactive MK-7, preserve microbial growth and metabolism, reduce biofilm formation, lessen production expenses, and enhance the efficiency of the fermentation process.

Figure 10 demonstrates the variation in all-*trans* and *cis* MK-7 production, bacterial growth, and pH during a

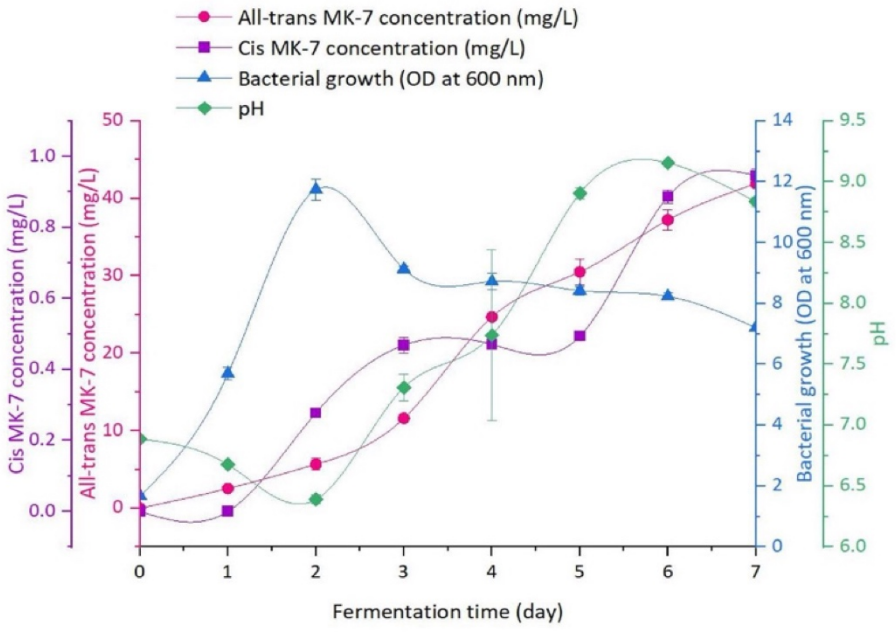


**Fig. 10** Trends in the all-*trans* and *cis* MK-7 isomer profile, microbial growth, and pH during a monitoring study utilising the optimal IONs@APTES concentration (the error bars represent the SE calculated from triplicate samples for each response)

monitoring analysis using the optimum concentration of IONs@APTES. The trend in the OD illustrates the characteristic microbial growth profile, comprising all four stages of bacterial growth (lag, exponential, stationary, and death). The cell density progressively rose from an initial OD of 1.68 to 5.69 on day 1 before rapidly escalating to a peak value of 11.74 on day 2 of fermentation. The OD subsequently dropped to 9.13 on day 3 and levelled off (8.73–8.24) until day 6, attaining a final value of 7.21 at the conclusion of fermentation.

Production of the all-*trans* and *cis* isomer corresponded to the microbial growth curve, and the rate at which this occurred varied depending on the phase of bacterial growth. Synthesis of all-*trans* MK-7 began from day 0 and gradually increased from 0 to 11.62 mg/L until the stationary phase (day 3). The bulk of the all-*trans* isomer was produced during the stationary period (days 3 to 6), and its concentration continued to increase through to the beginning of the death phase (from day 6), reaching a value of 41.93 mg/L on day 7 of the monitoring study. In contrast, production of the *cis* isomer was first noted on day 2 (0.28 mg/L), and its concentration remained reasonably constant (0.47–0.49 mg/L) between days 3 and 5. The *cis* MK-7 concentration then rapidly increased to 0.89 mg/L on day 6, and a final concentration of 0.95 mg/L was achieved on the last day of fermentation. It appears that a minimal amount of both isomers (approximately 6.06% and 0% of the total all-*trans* and *cis* MK-7, respectively) is synthesised during the lag phase, a slightly greater concentration (around 7.50% and 29.35% of the total all-*trans* and *cis* MK-7, respectively) is observed during the exponential growth stage, and the most significant quantity (roughly 75.23% and 64.38% of the total all-*trans* and *cis* MK-7, respectively) is produced during the stationary phase. A small fraction of each isomer (about 11.21% and 6.28% of the total all-*trans* and *cis* MK-7, respectively) was also noticed during the death phase, which likely commenced on day 6. It is valuable to mention that, unlike this study, the death segment did not occur during the monitoring analyses conducted in our preceding investigations [41, 54], which employed similar fermentation conditions and also considered bacterial cell immobilisation with uncoated IONs [54]. In these studies, fermentation was usually concluded before the death stage to prevent the MK-7 in the fermentation broth from being degraded due to contact with proteases and other cellular constituents released during cell rupture. In the current investigation, it is likely that immobilisation with IONs@APTES enhanced the growth and metabolism of *B. subtilis natto,* decreasing the length of each stage of microbial growth. Thus, the death phase began earlier than our previous studies and was observed during the monitoring period. Overall, the trends and observations from this investigation are consistent with prior accounts and demonstrate that MK-7 is a mixed metabolite, as its production partially depends on bacterial growth [10, 41, 54, 65–67].

The pH of the medium varied over the time-course study, increasing from a starting value of 6.89 to 8.84 at the completion of the monitoring investigation. The rise in the medium pH during the fermentation process can be ascribed to proteolysis and the consequent release of ammonia, which occurs when the bacterium utilises proteins as a source of energy [40, 67, 68]. Despite the overall increase in the pH, it first declined to the lowest value of 6.39 on day 2 and increased to a maximum of 9.16 on day 6 before decreasing to the final value on day 7. The fluctuation in the pH can be associated with the changes in the OD and, hence, the stages of the bacterial growth curve. It is evident that the pH gradually decreased with an increase in the OD between days 0 and 2, and the lowest pH (6.39) occurred when the peak OD (11.74) was attained on day 2. The pH then increased during the stationary growth phase to the highest value (9.16) on day 6 and decreased to the final value between days 6 and 7, corresponding to the decline in the OD (death stage). The change in the pH profile and its correlation with the OD is accordant with previous reports and can be attributed to the metabolic activities of *B. subtilis natto* during the different stages of microbial growth [40, 41, 54, 67].

Ranmadugala et al. [53] also carried out a time-course fermentation study to evaluate the impact of IONs@APTES on MK-7 production, bacterial growth, and pH. While the IONs@APTES concentration differed (200 µg/mL) from the current investigation (300 µg/mL), the general trends were similar. The final MK-7 concentration achieved by Ranmadugala et al. [53] (37.36 mg/L) was slightly lower than the all-*trans* isomer concentration obtained in the present study (41.93 mg/L), indicating the excellent ability of an IONs@APTES concentration of 300 µg/mL to boost the concentration of the biologically effective MK-7 isomer. The maximum cell density reported by Ranmadugala et al. [53] (42.93) was considerably greater than that in the current investigation (11.74). This disparity may be assigned to the different IONs@APTES concentrations examined (200 µg/mL versus 300 µg/mL) and the interval over which measurements were taken during the fermentation period (every 12 h for 120 h versus every day for 7 days). Furthermore, in this study, a higher OD was achieved at the conclusion of fermentation for an IONs@APTES concentration of 200 µg/mL (8.88) than 300 µg/mL (4.96). Thus, a peak OD similar to Ranmadugala et al. [53] may have been obtained when employing an IONs@APTES concentration of 200 µg/mL for the monitoring study. However, 300 µg/mL was determined to be the optimal IONs@APTES concentration in the present investigation, as it resulted in a superior yield of the all-*trans* isomer.

In addition, the findings of this study are comparable to our earlier investigation [54], which examined uncoated IONs in a similar context, except a higher peak OD and final all-*trans* MK-7 concentration were attained from fermentation using *B. subtilis natto* cells immobilised with IONs@APTES. In particular, the maximum OD and concentration of the bioactive isomer obtained from fermentation with IONs@APTES were 19.71% and 45.66% greater than that achieved in the presence of naked IONs, respectively.

Collectively, these observations indicate that bacterial cell immobilisation with IONs containing a biocompatible APTES coating has a favourable impact on the growth and metabolism of *B. subtilis natto* and serves to enhance the metabolic efficiency of the cells, thereby increasing the concentration of the bioactive isomer and the productivity of the fermentation process.

## Conclusions

This investigation was the first to explore the effect of bacterial cell immobilisation with biocompatible amine-functionalised IONs on the concentration and yield of the all-*trans* and *cis* isomers of MK-7 achieved from fermentation. IONs@APTES and L-Lys@IONs were synthesised and characterised, and the IONs@APTES resulted in better outcomes. Immobilisation of *B. subtilis natto* cells with the optimal IONs@APTES concentration (300 µg/mL) enhanced both the production and yield of the biologically functional isomer relative to the untreated cells by 2.3- and 3.1-fold, respectively. The results of this study offer new perspectives for developing novel production methods tailored to favour the synthesis of the bioactive isomer and address the challenges of MK-7 fermentation. These advancements have the ability to streamline the fermentation system and lower manufacturing costs. This will increase the affordability of biologically efficacious MK-7 consumer end products, and their improved availability will help boost the dietary intake of all-*trans* MK-7, providing consumers with numerous health gains and easing the socioeconomic effects of an ageing global population.

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**Author Contributions** AB and MS conceptualised this investigation. NL, MS, and AB developed the associated methodology. The investigation and characterisation studies were carried out by NL and AE, respectively. Formal analysis was conducted by NL, MS, AE, and AB, and NL and AE visualised the data. NL validated the results, and the data were curated by NL, MS, and AB. NL prepared the original draft, and NL, MS, and AB reviewed and edited the paper. All authors have read and approved the manuscript.

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**Data Availability** All relevant data that support the findings of this research are included in this article.

## Declarations

**Competing Interests** All authors declare that they have no conflict of interest.

**Ethical Approval** This article does not contain studies involving human or animal participants.



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# 8

# Fermentation of Menaquinone-7: The Influence of Environmental Factors and Storage Conditions on the Isomer Profile

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By

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# Chapter 8 – Fermentation of Menquinone-7: The Influence of Environmental Factors and Storage Conditions on the Isomer Profile

This chapter extends from the fermentation-based production of MK-7 to analyse the quality of the final fermented product with respect to its isomer profile and quantity of the bioactive isomer. While it is important to ensure that the fermentation process itself is optimised to favour the production of the all-*trans* isomer, its concentration must also be maintained over the shelf life of fermented MK-7 dietary supplements and fortified or functional foods to guarantee that the finished product retains biological efficacy and is able to elicit its associated health benefits. Fermented MK-7 products are potentially exposed to several environments and subjected to various conditions during manufacture, transportation, and storage before they reach the consumer and during consumption. Therefore, it is essential to consider the effect of common environmental factors and storage conditions likely to be encountered by fermented MK-7 end products on the isomer composition and stability of all-*trans* MK-7.

Exposure to key environmental and storage conditions, primarily atmospheric oxygen, different temperatures, and light, were first evaluated in a short-term investigation to assess the influence of these factors on the all-*trans* and *cis* MK-7 isomer concentrations over a short timeframe and determine the optimal storage environment. The conditions that resulted in the least deterioration of all-*trans* MK-7 in the initial study were further examined over a prolonged period to explore their effect on the stability of the biologically significant isomer during long-term storage.

This chapter and its findings conclude the overall study and offer unique insights into the factors that have a detrimental impact on the stability and concentration of the all-*trans* isomer in finished fermented products and, ultimately, influence the quality, biological efficacy, and therapeutic value of MK-7 dietary supplements and fortified or functional foods that reach the consumer.

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# Fermentation of Menaquinone-7: The Influence of Environmental Factors and Storage Conditions on the Isomer Profile

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**Abstract:** Menaquinone-7 (MK-7) provides significant health gains due to its excellent pharmacokinetic properties. However, MK-7 occurs at low concentrations in mainstream foods, heightening the demand for nutritional supplements. MK-7 exists as geometric isomers, and only all-*trans* MK-7 is bioactive. Exposure to certain environments impacts the isomer profile. Knowledge of these factors and their influence on the isomer composition is important, as the efficacy of fermented MK-7 end products is solely determined by the all-*trans* isomer. This investigation aimed to evaluate the short- and long-term effect of atmospheric oxygen, common temperatures, and light on the isomer profile. From the short-term study, it was ascertained that MK-7 is moderately heat-stable but extremely light-sensitive. The stability of all-*trans* MK-7 was then examined during 8 weeks of storage at a low temperature with minimal oxygen exposure in the absence of light. Negligible change in the all-*trans* MK-7 concentration occurred, suggesting it is reasonably stable during prolonged storage in this environment. These findings will aid the development of optimal storage conditions to preserve bioactive MK-7 in fermented nutritional supplements, the large-scale availability and consumption of which will help compensate for the dietary deficit of this essential vitamin and provide consumers with better health outcomes.

**Keywords:** menaquinone-7 isomer profile; bioactivity; fermentation; environmental factors; storage conditions



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## 1. Introduction

The vitamin K family consists of a set of fat-soluble vitamins, namely vitamin K1 (phylloquinone), vitamin K2 (menaquinones), and vitamin K3 (menadione). The various K vitamers are structurally similar, as they all contain a 2-methyl-1,4-naphthoquinone group [1]. However, they differ in the nature of an isoprenoid side chain at the 3-position, the length and degree of unsaturation of which confers unique properties to each kind of vitamin K [2]. Phylloquinone (PK) and menaquinones (MK) are the natural forms of the vitamin and play an essential role in human health and nutrition [3]. PK has one unsaturated and three saturated isoprenoid units in its side chain. It is ubiquitous within the chloroplasts of photosynthetic plants and algae, where it functions as an electron carrier during photosynthesis [2,4]. Therefore, PK can be consumed from a selection of everyday foods, including leafy greens, vegetable oils, and products resulting from such plant oils, and is the most abundant type of dietary vitamin K [4,5]. Conversely, MK are a series of compounds with isoprenoid chains of various lengths and degrees of unsaturation. The structure of the side chain can be represented by the format MK-*n*, where *n* is generally between four and thirteen and signifies the number of unsaturated isoprenoid residues in the chain [2]. MK are typically of microbial origin and are present in small quantities in specific animal, dairy, and fermented goods [2,5–8].

It is well-known that all vitamin K subtypes perform essential functions in the coagulation cascade and haemostasis. However, recent research has uncovered numerous other

roles and health gains of vitamin K. The intake of vitamin K2, in particular, has been related to the maintenance of bone and cardiovascular health, the suppression of neurological conditions, the prevention of cancer, aiding the functional recovery of the liver, reducing the likelihood of many health disorders, and decreasing the morbidity and mortality linked to coronavirus disease 2019 (COVID-19) [2,9–17].

MK-7 is the most notable vitamin K2 isoform due to its superior physicochemical characteristics and long plasma half-life (72 h), which enhance its extrahepatic bioavailability and therapeutic value [17–19]. Despite the significant health gains associated with MK-7, obtaining sufficient levels of the vitamin from regular food products is challenging for most consumers, as it is present in insufficient concentrations in limited foods. Natto, a Japanese fermented soybean containing nearly 800–1000 µg of MK-7 per 100 g of natto, is the richest source of MK-7 [6,8,20]. However, owing to its strong flavour and pungent aroma, most individuals perceive natto as unappetising and, hence, it tends to be a niche product that is not universally consumed by all populations. The MK-7 concentration in other dietary sources that appeal to mainstream consumers is inadequate. Consequently, meeting the daily intake requirements without the aid of nutritional supplements is not achievable for most populations, as it would require the consumption of unfeasibly large amounts of MK-7-containing foods [6]. This has increased the demand and created a lucrative market for MK-7 dietary supplements and enriched foods to complement natural sources, and the availability of such products has become progressively widespread [21].

It must be acknowledged that MK-7 demonstrates *cis*-*trans* isomerism, a feature that is common to most biomolecules. The all-*trans* isomer is the naturally occurring and bioactive form of the vitamin, whereas the various *cis* isomers are biologically ineffectual [7,22,23]. The bioactivity of MK-7 is related to its shape and structure, which depend on the arrangement of unsaturated bonds in the side chain. All-*trans* MK-7 has a straight isoprenoid chain (Figure 1), as all double bonds have the *trans* organisation [24]. In contrast, one or more unsaturated bonds in the *cis* conformation deform the linear molecular structure (Figure 1), and different numbers and combinations of *cis* double bonds can give rise to several *cis* MK-7 isomers [24]. The shape of MK-7 molecules determines their capacity to engage with subcellular components, and the non-linear configuration of the *cis* isomers impairs their ability to perform their biological role [25]. The *cis* isomers of vitamin K only sustain 1% of the biological significance of the all-*trans* form [26–28]. More recently, it has been established that *cis* MK-7 isomers have considerably diminished carboxylative potential and compromised bioactivity compared to all-*trans* MK-7 [25]. It is anticipated that the presence of *cis* MK-7 does not diminish the activity of the all-*trans* isomer. When the geometric isomers of the vitamin co-exist in a formulation, it is unlikely for interaction with the *cis* isomer to change the structure and bond arrangement of all-*trans* MK-7. As a result, the shape and, thus, the biological function of the all-*trans* isomer is expected to be unaffected. However, the remedial value of the preparation is only determined by the quantity of the biologically important isomer. Therefore, while the presence of *cis* MK-7 does not explicitly impact the activity of the all-*trans* isomer, its presence in the formulation is essentially an impurity, which decreases the biological function and therapeutic efficacy of all-*trans* MK-7 end products. In this regard, the isomer profile of MK-7 functional foods and dietary supplements is noteworthy, as their effectiveness is fundamentally governed by the proportion of the all-*trans* isomer.

MK-7 can be synthesised via chemical methods or microbial fermentation. The isomer composition of the MK-7 product is determined by various factors, primarily the manufacturing process and the techniques used to purify the post-reaction mixture [7,23]. Although chemical approaches are likely to be cost-effective, fermentation is more favourable from the perspective of both consumers and the environment. This is predominantly due to the recent consumer market trend promoting natural alternatives over synthetic formulations and the call for sustainable production methodologies. Fermentation is not only a natural approach for the synthesis of MK-7, but it is also more eco-friendly for the industrial

production of the vitamin. Hence, microbial fermentation can fulfil both consumer demand and environmental sustainability goals [29].

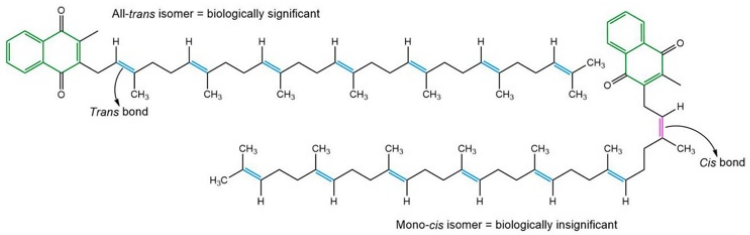


**Figure 1.** Chemical structure and bond organisation of MK-7 isomers.

The MK-7 isomer composition achieved from fermentation has not been widely examined, and it is generally believed that MK-7 resulting from fermentation-based synthesis exclusively occurs in the all-*trans* conformation. However, it has been proposed that exposure to particular factors may induce the transformation of the all-*trans* isomer to *cis* MK-7, and our previous studies were the first to ascertain the existence of *cis* MK-7 in fermented samples [30,31]. The occurrence of a *cis* isomer in samples obtained from fermentation implies that although the bacterium produces the naturally occurring all-*trans* isomer intracellularly, secretion into the fermentation broth exposes all-*trans* MK-7 to the extracellular milieu, which can promote its isomerisation to the *cis* form. It is important to appreciate that the exact structural identity (the number and location of *cis* double bonds in the isoprenoid side chain) of the *cis* MK-7 isomer produced from fermentation under the investigated conditions cannot be established in the absence of nuclear magnetic resonance (NMR) spectroscopy methods. It has been suggested that all-*trans* MK-7 is most likely to isomerise to the mono-*cis* isomer under certain conditions [32]. Therefore, it is anticipated that the *cis* MK-7 observed is a mono-*cis* isomer, which contains a single *cis* double bond in its isoprenoid chain. Nevertheless, complete structure determination is not essential when considering the bioactivity of fermented MK-7 end products, as all *cis* isomers, irrespective of the number and location of *cis* bonds in their isoprenoid side chain, have significantly reduced biological efficacy compared to the all-*trans* isomer.

Although our prior investigations have focused on optimising the extracellular environment, specifically the fermentation media [30] and key fermentation parameters [31], to increase the synthesis of the all-*trans* isomer and reduce the production of *cis* MK-7 during fermentation, it is crucial to guarantee that the quantity of the bioactive isomer is maintained in the final product. Hence, not only is it necessary to achieve a high concentration of all-*trans* MK-7 from fermentation, but its quantity must also be preserved in dietary supplements and functional foods to develop effective fermented MK-7 consumer end products. It has been postulated that exposure to light (especially ultraviolet (UV) light), atmospheric oxygen, and elevated temperatures may encourage the geometric isomerisation of isoprenoid residues in the side chain of MK-7 and result in the formation of *cis* isomers [7,23,25,33,34]. However, this aspect has not been explicitly explored, particularly in the context of MK-7 isomers produced from fermentation. The effect of typical environmental and storage conditions is worthy of consideration from the perspective of fermented MK-7 dietary supplements and fortified or functional foods, as these consumer end products are likely to be subjected to such factors during their manufacture, use, and shelf life, which will influence their effectiveness and therapeutic value.

Therefore, the objective of this study was to assess the effect of various storage conditions on the isomer profile of fermented MK-7 from the perspective of MK-7 end products. Accordingly, factors representing possible conditions and environments that MK-7 dietary supplements and fortified or functional foods may be subjected to during their production, consumption, and general shelf life were selected and examined. These include exposure to atmospheric oxygen, different temperatures, and light. All factors were initially investigated over a short interval, and the conditions that resulted in the least isomerisation

and/or degradation of all-*trans* MK-7 were further evaluated to explore the stability of the all-*trans* isomer in an ideal storage environment over an extended timeframe. The outcomes of this study will offer valuable insights for the development of optimum storage conditions to preserve the quantity of the all-*trans* isomer in fermented MK-7 end products. This will likely be an important progression in improving the accessibility of bioactive fermented MK-7 nutritional supplements and functional foods, as the *cis* isomers of the vitamin are effectively contaminants that have little therapeutic value. The widespread availability and consumption of such products by diverse populations will help boost the dietary intake of MK-7 and offer more significant health benefits to consumers.

## 2. Materials and Methods

### 2.1. Chemicals and Materials

The all-*trans* MK-7 reference standard (98.1% purity) was acquired from ChromaDex (Los Angeles, CA, USA). Glucose was obtained from Ajax Finechem Pty Ltd. (Taren Point, NSW, Australia). Yeast extract and tryptone were supplied by Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Soy peptone, methanol, 2-propanol, and *n*-hexane were obtained from Merck Millipore (Burlington, MA, USA). NaCl was acquired from a local supplier, and $CaCl_2$ was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Nutrient agar plates were procured from Fort Richard Laboratories (Auckland, New Zealand). All media components were microbiology grade, and all solvents were analytical grade.

### 2.2. Microorganism and Inoculum Preparation

*Bacillus subtilis natto* was selected for the fermentation experiments since it results in a high MK-7 yield and is considered the most suitable strain for industrial MK-7 production. Furthermore, there are no safety issues accompanying the *B. subtilis natto* strain, and it is generally recognised as safe (GRAS). Consequently, it is ideal for synthesising fermented MK-7 end products expected for human consumption. The procedure outlined by Berenjian et al. [20] was used to prepare the *B. subtilis natto* strain. The microbial cells were grown in an aqueous culture medium comprising yeast extract, tryptone, and NaCl and streaked on nutrient agar plates, which were incubated for 48 h at 37 °C. After incubation, the cells were removed from the plates and submerged in a sterile NaCl solution. The mixture was then put in a water bath for 30 min at 80 °C to inactivate the vegetative cells and stimulate the production of spores prior to centrifuging (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 3000 rpm for 10 min to remove the cell debris. The resulting bacterial spore suspension functioned as the inoculum for the fermentation studies.

### 2.3. Fermentation Procedure

MK-7 was synthesised from fermentation using the optimal media and conditions determined from our previous investigations [30,31] to enable maximal all-*trans* and minimal *cis* MK-7 isomer production. The media, containing 1% (*w*/*v*) glucose, 2% (*w*/*v*) yeast extract, 2% (*w*/*v*) soy peptone, 2% (*w*/*v*) tryptone, and 0.1% (*w*/*v*) $CaCl_2$ [30], was prepared in bulk to maintain consistency and sterilised at 121 °C for 20 min by autoclaving (TOMY SX-700E, Tokyo, Japan). Afterwards, the samples were inoculated with 2% (*v*/*v*) of the *B. subtilis natto* spore suspension and fermented in individual McCartney bottles under aerobic conditions at 40 °C and 200 rpm for 7 days [31].

### 2.4. MK-7 Extraction

Following fermentation, MK-7 was extracted from the samples with 2-propanol and *n*-hexane, which were mixed in a ratio of 1:2 (*v*/*v*), and the liquid-to-organic ratio was 1:4 (*v*/*v*) [20]. The solution was vortexed for 2 min, and phase separation was achieved by centrifugation (laboratory centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 3000 rpm for 10 min. Due to the fat-soluble nature of MK-7, it favourably

dissolves in non-polar solvents, such as *n*-hexane. Thus, the hexane layer was isolated from the mixture and evaporated in another set of McCartney bottles under a vacuum to obtain the extracted MK-7.

### 2.5. Exposure Studies

The extracted MK-7 samples (contained in transparent McCartney bottles) were then exposed to various environmental and storage conditions to explore the short- and long-term impact of these factors on the MK-7 isomer composition. Different temperature (low (4 °C), ambient (20 °C), and high (100 °C)), light (no light/dark, ambient light, and UV light), and oxygen (exposed to atmospheric oxygen and not exposed to atmospheric oxygen) conditions were selected to simulate likely storage environments for fermented MK-7 consumer end products, such as MK-7-enriched fortified or functional foods and dietary supplements. Possible conditions that fermented MK-7 could be exposed to during the manufacture of these products were also considered.

The effect of short-term exposure to the different temperature, light, and oxygen conditions on the isomer profile of fermented MK-7 was initially assessed. As a part of this process, samples were subjected to the factors outlined in Table 1 for 0, 3, 6, and 9 days to investigate the variation in the isomer composition over a brief timeframe for all conditions.

**Table 1.** Environmental and storage conditions for the short-term exposure study.

| Sample | Conditions |
|---|---|
| 1 | Low temperature (4 °C) and exposed to atmospheric oxygen = stored in the fridge with the lid off |
| 2 | Low temperature (4 °C) and not exposed to atmospheric oxygen = stored in the fridge with the lid on (purged with nitrogen) |
| 3 | High temperature (100 °C) and exposed to atmospheric oxygen = stored in the oven with the lid off |
| 4 | High temperature (100 °C) and not exposed to atmospheric oxygen = stored in the oven with the lid on (purged with nitrogen) |
| 5 | No light and exposed to atmospheric oxygen = stored in the dark with the lid off at ambient temperature (by default) |
| 6 | No light and not exposed to atmospheric oxygen = stored in the dark with the lid on (purged with nitrogen) at ambient temperature (by default) |
| 7 | Ambient light and exposed to atmospheric oxygen = stored in ambient light (lamp) with the lid off at ambient temperature (by default) |
| 8 | Ambient light and not exposed to atmospheric oxygen = stored in ambient light (lamp) with the lid on (purged with nitrogen) at ambient temperature (by default) |
| 9 | UV light and exposed to atmospheric oxygen = stored in UV light (lamp) with the lid off at ambient temperature (by default) |
| 10 | UV light and not exposed to atmospheric oxygen = stored in UV light (lamp) with the lid on (purged with nitrogen) at ambient temperature (by default) |

The optimum storage conditions, which resulted in the least deterioration of all-*trans* MK-7, determined from the short-term investigation, were further analysed in a monitoring study to explore the stability of the all-*trans* isomer and variation in the isomer profile over an extended period. Accordingly, samples were prepared in triplicates and stored at a low temperature (4 °C) with minimal oxygen exposure in the absence of light for 8 weeks. The MK-7 isomer composition was analysed after 0, 1, 2, 3, 4, 5, 6, 7, and 8 weeks of storage.

### 2.6. MK-7 Analysis

At the conclusion of the exposure period, the MK-7 isomer composition of all samples was analysed, as discussed in our earlier study [30]. The all-*trans* and *cis* MK-7 concentrations were determined using a Dionex high-performance liquid chromatography (HPLC) instrument (Thermo Fisher Scientific, Waltham, MA, USA) composed of four P680 pumps, an ASI-100 automated sample injector, a TCC-100 thermostatted column compartment,

and a UVD340U photodiode array UV detector. A packed column (COSMOSIL Cholester, 100 mm × 2 mm × 2.5 µm; Nacalai Tesque Inc., Kyoto, Japan) was used to separate the compounds at 40 °C. Pure methanol constituted the mobile phase, and the compounds were eluted isocratically at a flow rate of 0.2 mL/min. The run-time, analytical wavelength, autosampler temperature, and injection volume were 30 min, 248 nm, 10 °C, and 10 µL, respectively. The Chromeleon 7 program (Thermo Fisher Scientific, Waltham, MA, USA) was used for data collection, and a relative retention time (RRT) of 1.12 was used to ascertain the *cis* isomer.

Liquid chromatography–mass spectrometry (LC–MS) methods were employed to verify the identity and corroborate the retention times of all-*trans* and *cis* MK-7, using the approach described in our previous investigation [30]. The LC–MS platform comprised a Dionex Ultimate 3000 ultra-high-performance liquid chromatography (UHPLC) system and a QExactive mass spectrometer with a HESI II source (Thermo Fisher Scientific, Waltham, MA, USA). The Thermo XCalibur 4.3 package (Thermo Fisher Scientific, Waltham, MA, USA) was used to operate the equipment, and data were obtained using the Chromeleon 7.3 application (Thermo Fisher Scientific, Waltham, MA, USA). The conditions summarised above were implemented for liquid chromatography; however, the injection volume was modified to 5 µL, and the run-time was increased to 37 min to suit the LC–MS system. Data were collected in the positive ionisation mode with a resolution of 70,000, a MS1 scan range of 150–1000 $m/z$, a maximum injection time of 200 ms, and an AGC target of $3 \times 10^6$. The Thermo FreeStyle 1.6 software (Thermo Fisher Scientific, Waltham, MA, USA) was utilised to evaluate the mass spectrometry (MS) data.

The MK-7 concentration of the samples was determined using a calibration curve (linear between 0.1 mg/L and 50 mg/L ($R^2 = 0.99$)), which was created with reference to the peak area corresponding to known concentrations of the analytical standard.

### 2.7. Statistical Methods

Statistical significance was determined by analysis of variance (ANOVA), and a two-sample *t*-test was used to compare the mean values of different groups. The data were reported as the mean ± standard deviation (SD) of three replicates, and significance was accepted at $p < 0.05$.

## 3. Results and Discussion

The various factors explored were selected to represent the likely storage environments for fermented MK-7 supplements and fortified or functional foods, together with potential conditions to which fermented MK-7 may be exposed during the manufacture of such products. For example, fermented MK-7-enriched dairy goods or supplements requiring cold temperature storage will probably be stored in the fridge at a low temperature (4 °C). In comparison, most other supplements and MK-7-enriched foods are likely to be stored and consumed at ambient temperature (20 °C). The packaging material and design for MK-7 products can influence the amount of light that MK-7 supplements and functional foods are exposed to, as dark, opaque, and transparent materials all permit the passage of variable amounts of light. Additionally, exposure to atmospheric oxygen is inevitable in the case of all MK-7 end products. High temperature (100 °C) conditions and exposure to UV light are likely to represent conditions that fermented MK-7 may be exposed to during the production, transportation, and storage of certain MK-7-enriched supplements and fortified or functional foods. All variables were first considered over a short timeframe. The optimal conditions that promoted minimal degradation and/or isomerisation of bioactive MK-7 were further explored to evaluate the stability of all-*trans* MK-7 over a longer period.

### 3.1. Effect of Environmental Factors and Storage Conditions on the MK-7 Isomer Composition

#### 3.1.1. Light

The MK-7 isomer profile resulting from short-term storage in the dark at room temperature is outlined in Figure 2. Approximately 61% and 39% of the all-*trans* isomer and

87% and 67% of the *cis* isomer remained in the presence and absence of oxygen after 9 days of exposure. In addition, there is also no statistically significant difference in the all-*trans* and *cis* MK-7 isomer concentrations for the dark samples between days 3, 6, and 9 when assessing the presence and absence of oxygen independently ($p = 0.652$ and $p = 0.115$ for all-*trans* MK-7 in the presence and absence of oxygen and $p = 0.785$ and $p = 0.797$ for *cis* MK-7 in the presence and absence of oxygen). These findings indicate that no considerable reduction in the all-*trans* and *cis* MK-7 concentration occurs during short-term storage in the dark at ambient temperature, both with and without exposure to atmospheric oxygen.

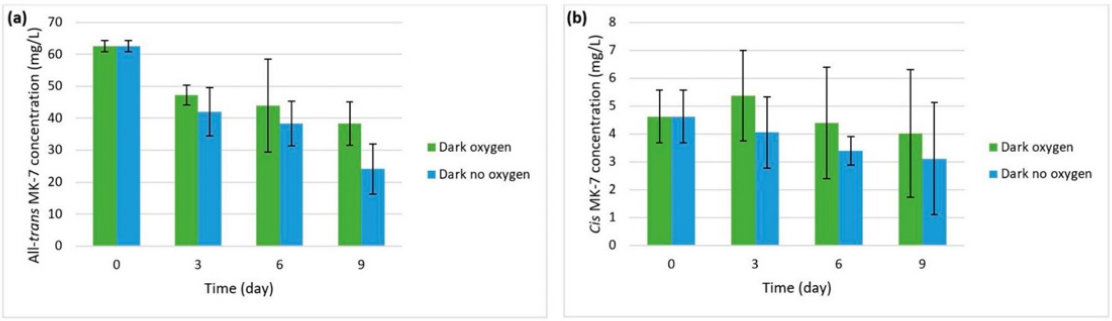


**Figure 2.** MK-7 isomer composition following short-term storage in the dark at ambient temperature in the presence and absence of oxygen for (**a**) all-*trans* MK-7 and (**b**) *cis* MK-7.

Figure 3 illustrates the effect of ambient light at room temperature on the all-*trans* and *cis* MK-7 concentration, and it is evident that exposure to ambient light has a detrimental impact on the isomer concentration. Over 99% of all-*trans* and 100% of *cis* MK-7 was degraded to undetectable levels within 3 days of exposure to ambient light with and without oxygen, implying that contact with oxygen does not have a noticeable effect on MK-7 stability in the presence of ambient light. The influence of UV light exposure on the MK-7 isomer concentration is displayed in Figure 4. It is apparent that MK-7 is very unstable in UV light, as both all-*trans* and *cis* MK-7 were not detected over the entire exposure period, regardless of the presence or absence of atmospheric oxygen.

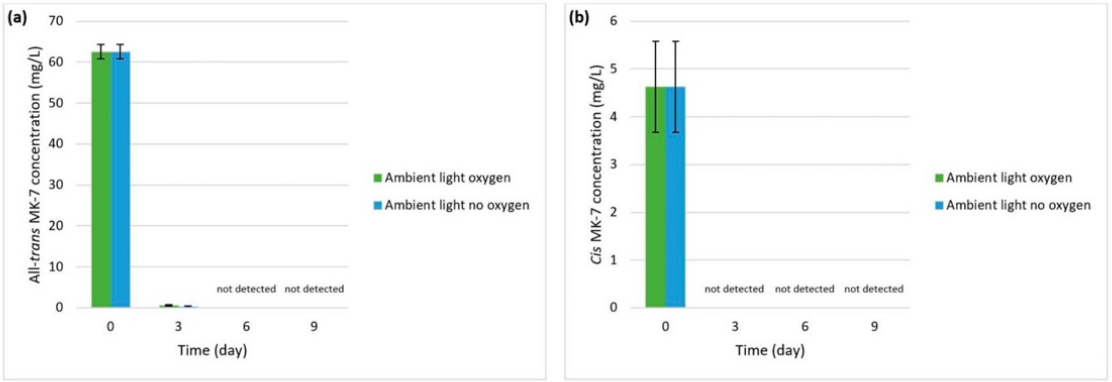


**Figure 3.** MK-7 isomer profile resulting from short-term exposure to ambient light at room temperature in the presence and absence of oxygen for (**a**) all-*trans* MK-7 and (**b**) *cis* MK-7.

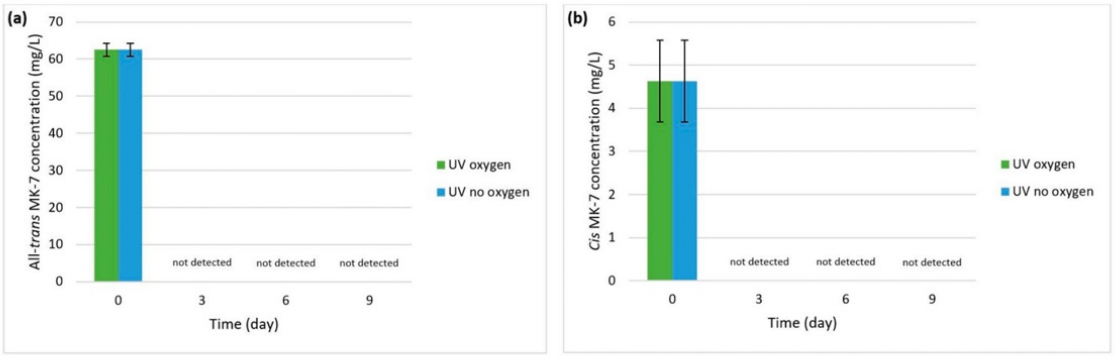


**Figure 4.** MK-7 isomer composition obtained from short-term exposure to UV light at ambient temperature in the presence and absence of oxygen for (**a**) all-*trans* MK-7 and (**b**) *cis* MK-7.

Fat-soluble vitamins, including vitamin K, tend to be light-sensitive and are degraded by exposure to various forms of light, such as ambient light, daylight, and UV light [35–38]. It has been suggested that light exposure may promote photoisomerisation, which refers to the conversion of one isomer of a molecule to another by light. Transformation of all-*trans* MK-7 to one or more *cis* forms of the vitamin may potentially occur due to light; however, there are no specific reports in the literature [7,23,34]. Geometric isomerisation of all-*trans* MK-7 as a result of exposure to ambient or UV light was not observed in the current study. Instead, the concentration of all-*trans* and *cis* MK-7 was quickly reduced to undetectable levels in the presence of both types of light. It is also worth noting that the degree of photosensitivity of all-*trans* MK-7 might vary depending on the intensity and wavelength of light. Additionally, only particular intensities or wavelengths of light may induce the isomerisation of all-*trans* MK-7. Therefore, it could be that the light sources used in this investigation were not of the appropriate intensity or wavelength to promote this effect.

While it has been established that K vitamins are destroyed by light, limited research has been conducted to explore the stability of MK in the presence of different light sources. Moreover, studies explicitly considering MK-7 and its isomers in this context are absent. Ferland and Sadowski [39] assessed the PK content of several vegetable oils and evaluated the effect of light (daylight and fluorescent light) exposure on the stability of vitamin K1 in rapeseed and safflower oils over 22 days. It was determined that after only 2 days of exposure, the PK content of rapeseed and safflower oils was reduced by 46% and 59%, respectively, in the presence of fluorescent light and by 87% and 94%, respectively, when exposed to daylight. The effect of the type of storage container was also examined for rapeseed oil. It was ascertained that after 36 h of daylight and fluorescent light exposure, the PK content decreased by 93% and 44% for oil stored in clear bottles, respectively. In contrast, storage in amber bottles did not have a significant impact. Despite consideration of different types of light and vitamin K compounds, the results of this research are similar to the present study and demonstrate that vitamin K forms are highly vulnerable to light.

Collectively, these observations illustrate the destructive effect of various light sources on MK-7 and emphasise the importance of using dark or amber bottles and opaque packaging materials for the storage of fermented all-*trans* MK-7 dietary supplements and fortified or functional foods to preserve the quantity of the vitamin over its shelf life. UV exposure must also be avoided and is unsuitable for the manufacture of all-*trans* MK-7-containing supplements, milk, and other products that may require UV treatment as a means of processing or sterilisation. Strategies such as encapsulation, especially for dietary supplements, can also be implemented to further protect the all-*trans* isomer from exposure to light. Furthermore, it would be worthwhile to increase the awareness of consumers and state that exposure to light should be avoided on the product packaging to ensure the optimal performance of fermented all-*trans* MK-7 nutraceuticals.

### 3.1.2. Temperature

The impact of low (4 °C) and high (100 °C) temperatures on the MK-7 isomer composition was investigated in the presence and absence of oxygen. The results are depicted in Figures 5 and 6 for storage in the fridge (4 °C) and oven (100 °C), respectively. Around 68% and 61% of all-*trans* MK-7 and 79% and 61% of *cis* MK-7 remained in the presence and absence of oxygen at the end of the exposure period under low temperature conditions. There is also no statistically significant difference in the all-*trans* and *cis* MK-7 concentration for the fridge samples between days 3, 6, and 9 when considering the presence and absence of oxygen separately ($p = 0.997$ and $p = 0.944$ for all-*trans* MK-7 in the presence and absence of oxygen and $p = 0.749$ and $p = 0.098$ for *cis* MK-7 in the presence and absence of oxygen). This indicates that there is no notable decrease in the all-*trans* and *cis* isomer concentration over short-term exposure to low temperature conditions, both with and without contact with atmospheric oxygen. In comparison, approximately 17% and 33% of the all-*trans* isomer and 43% and 43% of *cis* MK-7 remained in the presence and absence of oxygen after 9 days of storage at a high temperature. Furthermore, there is no statistically significant difference in the all-*trans* and *cis* isomer concentrations for the oven samples between days 3, 6, and 9 when individually examining the effect of atmospheric oxygen ($p = 0.062$ and $p = 0.488$ for all-*trans* MK-7 in the presence and absence of oxygen and $p = 0.830$ and $p = 0.689$ for *cis* MK-7 in the presence and absence of oxygen). However, there is a statistically significant difference in the all-*trans* isomer concentration for the oven samples in the presence of oxygen between days 3 and 9 ($p = 0.002$). These observations suggest that although there is no appreciable difference in the isomer concentrations with and without contact with atmospheric oxygen between all three days of exposure holistically, there is a substantial decrease in the all-*trans* MK-7 concentration in the presence of oxygen between days 3 and 9. Thus, oxygen seems to accelerate the decomposition of the biologically active isomer at high temperatures. Although the effect of heat treatment and oxygen exposure has not been previously investigated for MK-7 isomers, it has been observed that during heating, oxygen increases the rate of degradation of all-*trans*-β-carotene, a precursor of vitamin A, which, similar to MK-7, is a lipid-soluble vitamin [40].

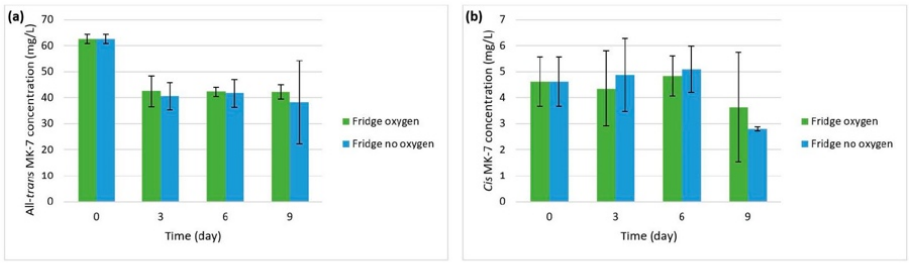


**Figure 5.** MK-7 isomer profile arising from short-term storage in the fridge at a low temperature in the presence and absence of oxygen for (**a**) all-*trans* MK-7 and (**b**) *cis* MK-7.

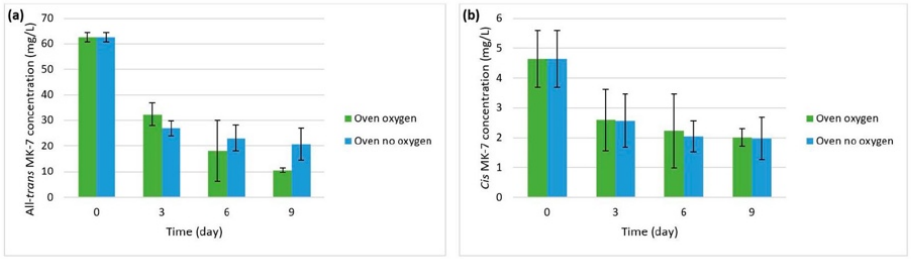


**Figure 6.** MK-7 isomer composition occurring from short-term storage in the oven at a high temperature in the presence and absence of oxygen for (**a**) all-*trans* MK-7 and (**b**) *cis* MK-7.

The stability of vitamin K compounds is only slightly affected by heat exposure; hence, they are regarded as fairly heat-stable [35,36,41]. While there are no prior studies assessing the influence of heat on the stability of MK-7 isomers specifically, Ferland and Sadowski [39] have investigated the thermal stability of vitamin K1 in different vegetable oils at temperatures between 185 and 190 °C over 20 and 40 min. A slight loss of PK was observed, and approximately 7% and 11% of the original vitamin K was lost over 20 and 40 min of exposure, respectively. The findings of this research are largely comparable with the present study. Despite the differences in the investigated K vitamers, temperatures, and exposure times between the two studies, they both demonstrate a moderate loss of vitamin K upon heating, suggesting that MK-7 is relatively stable when exposed to reasonably high temperatures over a short period.

These observations imply that thermal sterilisation and manufacturing processes involving high temperatures, such as milling and drying for the synthesis of MK-7 dietary supplements and extrusion cooking for the production of cereals and other foods fortified with MK-7, may not reduce the MK-7 content significantly. Although such technological processes could require greater temperatures than that investigated in the current study (100 °C), the exposure times are expected to be much shorter (over a few minutes or hours rather than for 3, 6, or 9 days), which will likely mitigate the detrimental effect of higher temperatures. Additionally, encapsulation methods could be used to protect all-*trans* MK-7 in processes involving extreme temperatures and/or long handling times.

It is also evident that storage of MK-7 at ambient temperature is acceptable. This is demonstrated by the relatively high MK-7 content remaining after 9 days for the samples that were stored in the dark at room temperature (Figure 2). Moreover, there is no statistically significant difference in the all-*trans* and *cis* MK-7 concentrations between the dark and fridge conditions in the presence and absence of oxygen following short-term exposure ($p = 0.498$ for all-*trans* MK-7 and $p = 0.832$ for *cis* MK-7 from the overall ANOVA analysis of the dark and fridge groups). These outcomes also indicate that the negligible MK-7 isomer concentrations observed for both the ambient and UV light samples were due to exposure to the different light conditions and did not result from storage at room temperature.

The amount of light exposure was similar between the samples kept in the dark, fridge, and oven, as closure of the fridge and oven doors also eliminated light exposure in the low and high temperature conditions. This allows the effect of temperature to be meaningfully assessed between these groups. The ANOVA results indicate that there is a statistically significant difference in the all-*trans* isomer concentration between the dark, fridge, and oven conditions in the presence and absence of oxygen over the entire exposure period ($p = 2.201 \times 10^{-4}$). However, there is no statistically significant difference in the *cis* MK-7 concentration between these three groups in the presence and absence of oxygen over the investigated timeframe ($p = 0.149$). This suggests that while exposure to high temperatures negatively impacts the concentration of the bioactive isomer, it does not considerably affect the concentration of the biologically insignificant isomer.

#### 3.1.3. Atmospheric Oxygen

Vitamin K is slowly affected by exposure to atmospheric oxygen [35,41]. It has been proposed that contact with atmospheric oxygen during the storage of MK-7 dietary supplements may lead to autoxidation processes, which adversely impact the concentration of the all-*trans* isomer and promote its isomerisation to *cis* MK-7 [7,23,34]. Nevertheless, this phenomenon has not been explicitly assessed, particularly for MK-7 isomers produced from fermentation.

The experimental observations from the present investigation suggest that contact with atmospheric oxygen does not have a substantial negative effect on the stability of MK-7 over a short period. The statistical analysis for the dark, fridge, and oven conditions (Table 2) indicates that there is no statistically significant difference ($p > 0.05$) in the all-*trans* and *cis* MK-7 concentrations between the samples that were and were not in contact with atmospheric oxygen for each day of the exposure period. For the ambient and UV light

conditions, all-*trans* MK-7 was only noted in the presence and absence of oxygen for the ambient light samples on day 3 of exposure, and there was no statistically significant difference in the all-*trans* isomer concentration between these two groups ($p$ = 0.109). Furthermore, since all-*trans* and *cis* MK-7 were essentially undetectable following short-term exposure to ambient and UV light, it is evident that both isomers are rapidly degraded due to the different light exposures, irrespective of contact with atmospheric oxygen.

**Table 2.** Comparison of the MK-7 isomer concentrations between the oxygen and no oxygen samples on each day of the exposure period for the dark, oven, and fridge conditions using a two-sample *t*-test.

| **DARK** | | **FRIDGE** | | **OVEN** | |
|---|---|---|---|---|---|
| **All-*trans* MK-7 concentration** | | **All-*trans* MK-7 concentration** | | **All-*trans* MK-7 concentration** | |
| Groups compared | *p*-value | Groups compared | *p*-value | Groups compared | *p*-value |
| Day 3 oxygen and no oxygen | 0.416 | Day 3 oxygen and no oxygen | 0.754 | Day 3 oxygen and no oxygen | 0.212 |
| Day 6 oxygen and no oxygen | 0.653 | Day 6 oxygen and no oxygen | 0.893 | Day 6 oxygen and no oxygen | 0.610 |
| Day 9 oxygen and no oxygen | 0.125 | Day 9 oxygen and no oxygen | 0.748 | Day 9 oxygen and no oxygen | 0.085 |
| ***Cis* MK-7 concentration** | | ***Cis* MK-7 concentration** | | ***Cis* MK-7 concentration** | |
| Groups compared | *p*-value | Groups compared | *p*-value | Groups compared | *p*-value |
| Day 3 oxygen and no oxygen | 0.416 | Day 3 oxygen and no oxygen | 0.736 | Day 3 oxygen and no oxygen | 0.984 |
| Day 6 oxygen and no oxygen | 0.454 | Day 6 oxygen and no oxygen | 0.772 | Day 6 oxygen and no oxygen | 0.852 |
| Day 9 oxygen and no oxygen | 0.699 | Day 9 oxygen and no oxygen | 0.603 | Day 9 oxygen and no oxygen | 0.960 |

Therefore, oxygen exposure is unlikely to significantly influence the all-*trans* MK-7 concentration of products that are consumed quickly and have a short shelf life, such as fortified or functional dairy products containing bioactive MK-7. However, it may adversely affect the all-*trans* MK-7 content of dietary supplements and products that are consumed over a longer period and have an extended shelf life. Encapsulation may be an effective technique to minimise the oxygen exposure of MK-7 contained in dietary supplements. Including labels on products to lessen their contact with oxygen (by closing the bottle lid or securing the packaging material) is also advisable for MK-7-enriched goods with a long shelf life.

#### 3.1.4. Geometric Isomerisation of All-*trans* MK-7

It is interesting to notice that although it has been proposed that vitamin K, particularly all-*trans* MK-7, is susceptible to isomerisation upon exposure to various conditions, such as light, atmospheric oxygen, and elevated temperatures [7,23,34], conversion of the all-*trans* isomer to the *cis* form was not observed in the current study (only degradation of the vitamin and reduction in the concentration of both isomers was noted). A decrease in the concentration of the biologically effective isomer and a concurrent increase in the concentration of the *cis* isomer over time would denote the geometric isomerisation of all-*trans* MK-7. The *cis* MK-7 isomer concentration fluctuated considerably over the different days of exposure. While a slight increase in the concentration of *cis* MK-7 was observed for the fridge and dark storage conditions, there is no statistically significant difference in the *cis* isomer concentration in the presence and absence of oxygen between days 0 and 9 for the fridge and dark samples ($p$ = 0.850). Moreover, a general downward trend in the *cis* isomer concentration can be noted over the entire exposure period for not only the fridge and dark samples but for all investigated storage conditions. This indicates a gradual decline, rather than an increase, in the *cis* isomer concentration over the 9-day storage period. Hence, it can be concluded that geometric isomerisation of all-*trans* MK-7 did not occur during the short-term exposure study. A potential explanation for the lack of isomerisation observed in this investigation could be that the conditions explored were reasonably mild and may not be sufficient to stimulate the isomerisation of all-*trans*

MK-7 over the timeframe explored. However, it may be possible for isomerisation to occur following longer periods of exposure to the same conditions. Alternatively, different conditions and/or harsher and more extreme environments may be required to promote the geometric isomerisation of the all-*trans* isomer.

### 3.2. Stability of All-trans MK-7 in an Optimal Storage Environment

A monitoring study was conducted to assess the stability of all-*trans* MK-7 and variation in the isomer composition over an extended timeframe during storage at a low temperature (4 °C) with minimal oxygen exposure in the absence of light.

In the short-term exposure study, the smallest decline in the concentration of the bioactive isomer was observed for the samples stored in the fridge at a low temperature and in the dark at ambient conditions. As previously outlined, the results obtained for the dark and fridge samples were comparable over a short span (no statistically significant difference in the MK-7 isomer concentration existed between these two groups). However, it is recognised that heat has a negative impact on the stability of MK-7 over a longer interval. Therefore, it was decided to consider storage at low rather than ambient temperature conditions over an extended period. The samples were also stored in dark/opaque bottles to further eliminate any light exposure, as it was determined from the short-term study that light has a detrimental effect on the stability of MK-7. In addition, no statistically significant difference in the MK-7 isomer concentration was noted between the samples that were and were not in contact with atmospheric oxygen during the short-term exposure investigation. Although oxygen is known to slowly impact the stability of MK-7, the effect of which is only likely to be observed after a prolonged period, the samples for the long-term study were just stored with the lid on (to decrease oxygen exposure) and not purged with nitrogen. This was done to simulate the potential environmental conditions that bioactive fermented MK-7 consumer end products will likely be subjected to during their manufacture, consumption, and overall shelf life, as, in reality, it would not be feasible to avoid exposure to atmospheric oxygen completely.

The samples were kept in an optimum storage environment for an extended timeframe. Figure 7 illustrates the variation in the all-*trans* and *cis* isomer concentrations over the long-term storage investigation. There was no appreciable change in the concentration of both isomers during 8 weeks of storage at a low temperature in the dark with minimal oxygen exposure. This implies that all-*trans* MK-7 is reasonably stable in this environment and is not susceptible to geometric isomerisation under optimal conditions.

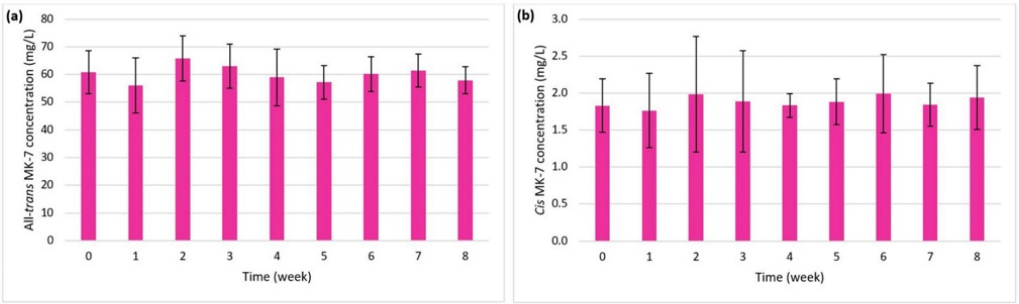


**Figure 7.** Variation in the isomer concentration over 8 weeks of storage at a low temperature with minimal oxygen exposure in the absence of light for (**a**) all-*trans* MK-7 and (**b**) *cis* MK-7.

These observations are supported by the ANOVA assessment, which indicates no statistically significant difference in the all-*trans* and *cis* isomer concentrations between the different weeks of exposure ($p$ = 0.951 for all-*trans* MK-7 and $p$ = 1.00 for *cis* MK-7). Additionally, a comparison of the MK-7 isomer concentration for every week of storage (weeks 1–8) with the control (week 0) via a *t*-test revealed that both the all-*trans* and *cis*

MK-7 concentration for each week of storage did not differ significantly from that of the control ($p > 0.05$).

Overall, the results of the long-term monitoring study demonstrate that low temperature conditions, reduced oxygen exposure, and the absence of light constitute the ideal storage environment for fermented MK-7. These conditions prevent the deterioration and preserve the concentration of the bioactive isomer, thereby retaining the therapeutic value of fermented MK-7 products.

### *3.3. Study Limitations*

The findings of this investigation offer key insights into the effect of typical environmental and storage conditions that fermented MK-7 supplements and fortified or functional foods are likely to be subjected to on the MK-7 isomer profile and have shed light on the stability of the all-*trans* isomer over an extended period in an optimal storage environment. The ideal conditions to preserve the concentration of fermented all-*trans* MK-7 constituted the absence of light, low temperatures, and minimal oxygen exposure. Therefore, it is proposed that, where appropriate, fermented MK-7 products are packaged in dark/opaque bottles or materials and stored with the lid on or packaging tightly secured in the fridge at low temperature conditions (around 4 °C) to ensure that they retain their biological efficacy.

Although the impact of these factors on the isomer composition has been considered from the perspective of fermented MK-7 consumer end products, they have been examined in isolation. Thus, the experimental observations are restricted, and the conclusions drawn may differ slightly when the fermented MK-7 is actually formulated into supplements and fortified or functional foods.

It has been established that MK-7 is liable to degradation during storage, and the rate at which this occurs is accelerated in certain environments. Whilst exposure to specific storage conditions has been investigated in this study, it only covers a subset of the many factors that may be encountered during the manufacture and overall shelf life of a particular product. In addition, different preparations of bioactive fermented MK-7 may be exposed to unique storage environments depending on the characteristics of the final product (tablets, capsules, or fortified/functional foods).

For instance, fermented all-*trans* MK-7 formulated into tablets or capsules is likely to be exposed to various excipient compounds and active ingredients (in the case of multi-nutrient supplements), such as magnesium oxide (MgO), calcium carbonate ($CaCO_3$), calcium citrate ($Ca_3(C_6H_5O_7)_2$), cellulose, gelatine, and other vitamins and minerals. Certain compounds, including MgO, may also promote alkalinisation, and since MK-7 is vulnerable to alkaline conditions, such additives can create an unfavourable milieu that may enhance its deterioration. Hence, the inclusion of additional compounds and ingredients and their different combinations can create and expose the vitamin to different environments, which can affect the isomer profile and stability of all-*trans* MK-7 in different preparations. In contrast, fermented bioactive MK-7-enriched fortified or functional foods will likely be subjected to a different set of environmental factors specific to the selected food matrix and its storage requirements. For example, fresh dairy products require refrigeration at low temperatures and have a relatively short shelf life compared to cereals and other dry goods, commonly stored at ambient conditions over a longer period. Therefore, the isomer composition and stability of all-*trans* MK-7 will likely vary with the nature of the end product. Consequently, future research efforts need to be directed towards exploring and comprehensively understanding the effect of different environmental factors and storage conditions on the isomer composition and stability of all-*trans* MK-7 in context rather than independently of the desired application.

While prior studies have examined the stability of commercially available MK-7 dietary supplements and similar preparations, there have been no attempts thus far to explore the stability and isomer profile of fermented MK-7 in different types of formulated products. Furthermore, since the therapeutic benefits of fermented MK-7 nutritional supplements and fortified or functional foods solely result from the quantity of all-*trans* MK-7, it is essential

to ensure that they contain the bioactive isomer almost exclusively or in the most significant proportion following their manufacture, during their consumption, and throughout their overall shelf life. Therefore, in subsequent investigations, it would be advantageous to consider formulating fermented all-*trans* MK-7 into various consumer end products, such as tablets, capsules, and fortified or functional foods, to develop a deeper understanding of the effect of different production processes, preparations, and foods matrices on the MK-7 isomer profile.

In future work, it would also be valuable to carry out shelf life or degradation studies to explore both the short- and long-term stability of fermented all-*trans* MK-7 when formulated in various dietary supplement preparations and fortified or functional foods. This will allow the impact of a range of environmental and storage conditions, including product-specific features and those relating to the proposed packaging materials and design, on the quantity of bioactive MK-7 and the isomer composition resulting from different end uses to be elucidated. Factors often governed by commercial pressures also contribute to the overall shelf life of a product, and these include the time it takes for it to reach the consumer, the range of temperatures and climates that it is likely to be subjected to between production and consumption, and the rate at which it is expected to be consumed. Thus, such aspects also need to be considered in future research when assessing the stability of fermented all-*trans* MK-7 over its shelf life after it has been formulated into a diverse range of consumer products. Additionally, it is vital to ensure that a therapeutically significant concentration of the vitamin remains in the product at the end of its shelf life after taking into account the impact of the many factors that contribute to its holistic storage environment.

Essentially, the outcomes of appropriate shelf life and degradation studies examining the stability of fermented all-*trans* MK-7 in different product formulations will aid the estimation of a realistic shelf life and initial content (overage) of the vitamin in various end products in the future. This will also inform decisions regarding the product packaging and its recommended storage conditions to preserve the quantity of the bioactive isomer in specific applications.

## 4. Conclusions

This investigation presents unique insights into the impact of various environmental factors and storage conditions that fermented MK-7 consumer end products, such as dietary supplements and fortified or functional foods, may be subjected to during their production, consumption, and overall shelf life. The selected parameters included exposure to atmospheric oxygen, different temperature conditions, and light. These factors were first considered over a short interval to determine the optimal storage conditions. Essentially, there appeared to be no discernible differences in the degradation profiles of all-*trans* and *cis* MK-7 under the studied conditions, as, despite minor dissimilarities (possibly due to experimental variation), the overall trends in the reduction of both isomers with exposure to the various factors were comparable. Storage in the absence of light at a low temperature with minimal oxygen exposure preserved the quantity of the all-*trans* isomer to the greatest extent and was the ideal storage environment for fermented MK-7. The stability of the biologically significant MK-7 isomer under the optimal conditions was then evaluated over an extended period, and negligible change in the concentration of all-*trans* MK-7 occurred after 8 weeks of storage. This implies that the all-*trans* isomer is reasonably stable and not prone to substantial degradation during long-term storage in this environment. The findings of this study are significant, as they will facilitate the development of suitable storage conditions to maintain the concentration of the all-*trans* isomer in fermented MK-7 end products. The results will also aid the estimation of suitable overage levels of the vitamin in different products to account for its deterioration during storage, which will ensure that the amount of the biologically important isomer remaining at the end of a product's shelf life is not below the required or stated quantity. Collectively, this will be a significant advancement in improving the availability of bioactive fermented MK-7 nutritional supplements and fortified or functional foods, as the *cis* isomers

have considerably compromised biological function and therapeutic value. The broad consumption of such efficacious products by a range of populations will boost the dietary intake of MK-7 and help decrease the risk and progression of several age-related disorders and diseases of global relevance.

**Author Contributions:** Conceptualisation, A.B. and M.S.; methodology, N.L., M.S. and A.B.; validation, N.L.; formal analysis, N.L.; investigation, N.L.; data curation, N.L., M.S. and A.B.; writing—original draft preparation, N.L.; writing—review and editing, N.L., M.S. and A.B.; supervision, A.B. and M.S. All authors have read and agreed to the published version of the manuscript.

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# 9

# Conclusions and Recommendations

# Chapter 9 – Conclusions and Recommendations

## 9.1 Overview

The primary focus of this research was to evaluate the MK-7 isomer composition of fermented samples resulting from various synthesis conditions to determine the optimal fermentation procedure for selectively enhancing the production of the biologically significant all-*trans* isomer.

Essentially, by systematically investigating the fundamental aspects of a typical fermentation process from its preliminary stages to the final consumer product, this study endeavoured to develop a targeted fermentation method tailored to improve the synthesis and maintain the concentration of bioactive all-*trans* MK-7 while minimising the production of the ineffectual *cis* isomer. Key features of upstream fermentation, specifically the media composition and important fermentation parameters, were initially examined before considering the isomer profile and production of all-*trans* MK-7 from a broader perspective. In this respect, the potential of nanobiotechnological approaches to address the major challenges of MK-7 fermentation and provide an opportunity to simplify the downstream processing of the vitamin through process intensification was explored. Factors influencing the isomer profile of fermented MK-7 preparations were also assessed.

This chapter summarises the notable outcomes of this investigation and proposes directions for future research based on the knowledge gained from this study.

## 9.2 Key findings

Prior to this study, limited information was available on the topic of MK-7 isomers, especially regarding the type and proportion of MK-7 isomers achieved from fermentation under different synthesis conditions. The majority of previous investigations centred around evaluating the MK-7 isomer composition of dietary supplements and similar formulations, and the isomer profile of fermented samples remained to be elucidated. Therefore, a structured approach was employed to examine the pivotal aspects of a standard fermentation process, and the study was also extended to consider strategies for the large-scale production and storage of the vitamin.

It has been reported that MK-7 produced from microbial synthesis only occurs in the all-*trans* configuration, implying that *cis* isomers cannot arise from fermentation. However, variable amounts of *cis* MK-7 were observed in fermented samples obtained from the different conditions explored in this investigation. This leads to the notion that while bacterial fermentation results in the synthesis of the all-*trans* isomer intracellularly, secretion of the vitamin into the fermentation broth subjects it to the extracellular environment, promoting its isomerisation to the *cis* isomer. Thus, the biologically inferior *cis* isomer is produced in minor amounts parallel to all-*trans* MK-

7, and solely attaining the bioactive MK-7 isomer from fermentation is largely inevitable. Nevertheless, it is possible to maximise the concentration of the all-*trans* isomer and minimise the production of *cis* MK-7 to achieve an acceptable compromise. Since the nature of the fermentation broth is governed by and varies with the fermentation conditions, fundamental aspects of the fermentation process, including the media composition and the value of key fermentation parameters, such as the inoculum size, fermentation temperature, agitation speed, and length of fermentation, play a significant role in determining the characteristics of the extracellular environment and, consequently, the MK-7 isomer profile obtained from fermentation.

The fermentation media is an important facet of any fermentation process, as every microorganism has specific growth and metabolic requirements. The selection of nutrients and their respective concentrations directly affect the productivity of the fermentation system and the yield of the product of interest and, hence, must be customised to satisfy microbial demands and achieve the desired outcomes in each context. Therefore, as outlined in Chapter 4, numerous carbon, nitrogen, and salt sources were first screened to determine the media components that have a significant effect on MK-7 isomer production. The concentration of the identified nutrients was then optimised to design the optimum fermentation media to enhance the synthesis of the all-*trans* isomer and diminish the production of *cis* MK-7. The optimal media contained 1% (*w/v*) glucose, 2% (*w/v*) yeast extract, 2% (*w/v*) soy peptone, 2% (*w/v*) tryptone, and 0.1% (*w/v*) $CaCl_2$, which resulted in an all-*trans* isomer concentration of 36.37 mg/L and a *cis* isomer concentration of 1.23 mg/L. Moreover, it was ascertained that only a single *cis* isomer is produced from fermentation under the conditions employed. It was also observed that variation in the composition of the fermentation media resulted in different concentrations of the *cis* isomer, indicating that different combinations of nutrients and their corresponding quantities in the fermentation media create a unique extracellular environment in the fermentation broth, which likely influences the degree of isomerisation of the all-*trans* isomer to *cis* MK-7.

The fermentation conditions are also an integral aspect of upstream fermentation, as they impact microbial growth and metabolism and, ultimately, the efficacy of the fermentation process. Thus, the nature of the fermentation environment must be adapted to suit microbial needs and favour the production of the desired product. Accordingly, key fermentation parameters, namely the inoculum size, fermentation temperature, agitation speed, and length of fermentation, were optimised to establish the ideal fermentation conditions to maximise the all-*trans* isomer concentration and decrease the formation of the insignificant *cis* isomer (Chapter 5). The optimal fermentation conditions comprised an inoculum size of 2% (*v/v*), a fermentation temperature of 40 °C, an agitation speed of 200 rpm, and a fermentation period of 7 days, which resulted in an all-*trans* and *cis* isomer concentration of 53.29 mg/L and 1.22 mg/L, respectively. Relative to fermentation using only the optimal media, applying the optimised fermentation conditions resulted in an approximately 46.5% increase in the concentration of the biologically active isomer,

while the *cis* isomer concentration was comparable. This clearly demonstrates the vital contribution of the fermentation conditions in determining the concentration of the preferred product.

A nanobiotechnological approach was then implemented in Chapters 6 and 7 to overcome the obstacles in MK-7 fermentation, primarily the low fermentation yield and extensive downstream processing requirements, and simultaneously boost the synthesis of the biologically efficacious isomer and decrease the concentration of *cis* MK-7. In this respect, magnetic IONs were incorporated into the fermentation process, and the effect of bacterial cell immobilisation with biocompatible IONs on microbial growth and MK-7 isomer production was explored. Naked IONs, IONs@APTES, and L-Lys@IONs were synthesised, characterised, and used to immobilise *B. subtilis natto* cells for the fermentation studies. It was ascertained that IONs@APTES were the most effective in improving the concentration of all-*trans* MK-7 and reducing the synthesis of the *cis* isomer. The optimum naked ION concentration was 300 µg/mL, and it increased the process productivity and enabled a maximum all-*trans* isomer concentration of 28.78 mg/L and a 1.6-fold greater all-*trans* MK-7 yield than the free-floating cells. Compared to the naked IONs, the amine-functionalised IONs had excellent properties and resulted in more favourable outcomes. The optimum concentration of IONs@APTES and L-Lys@IONs was 300 µg/mL and 400 µg/mL, and the highest all-*trans* MK-7 concentration achieved was 41.93 mg/L and 32.08 mg/L, respectively. Furthermore, the yield of the all-*trans* isomer, relative to the control, was enhanced at the optimal concentration by 3.1-fold for IONs@APTES (300 µg/mL) and 2.1-fold for L-Lys@IONs (400 µg/mL). This suggests that both types of coated IONs, especially IONs@APTES, have immense potential as a novel approach to increase the fermentation yield of biologically significant MK-7. In addition, the superparamagnetic properties of IONs can facilitate magnetic cell removal, which could streamline the downstream processing of the vitamin and enhance the efficiency of the overall fermentation system. Improved fermentation productivity will likely reduce the price and increase the accessibility of fermented bioactive MK-7 products.

Since the quality and effectiveness of fermented MK-7 products are predominantly dictated by the concentration of the biologically effectual all-*trans* isomer, it is essential to consider the impact of environmental factors and storage conditions on their isomer profile. Accordingly, the influence of common environmental conditions, such as exposure to light, atmospheric oxygen, and various temperatures, on the isomer composition of fermented MK-7 was explored in Chapter 8 to preserve the concentration of the all-*trans* isomer and prevent its transformation to *cis* MK-7. The selected factors were initially examined in a short-term study, and it was determined that the vitamin is reasonably heat-stable but extremely sensitive to light. Long-term storage at a low temperature with reduced oxygen exposure in the absence of light resulted in an insignificant change in the concentration of the all-*trans* isomer and was, thus, the optimal environment for the prolonged storage of fermented all-*trans* MK-7. These findings will

help establish optimal storage conditions to conserve the quantity of the bioactive isomer in fermented MK-7 preparations.

Overall, this research has significantly contributed to the existing knowledge regarding the production of MK-7 isomers from fermentation in different contexts and provides a basis for further exploration of this topic.

## 9.3 Prospects for future research

While this study has elucidated the ideal fermentation procedure to favour the production of the biologically significant all-*trans* isomer and minimise the synthesis of the ineffective *cis* isomer in various contexts, a great deal is yet to be explored and ascertained. Aspects relating to the microbial strain, process scale, and development of fermented all-*trans* MK-7-enriched foods and dietary supplements are potential areas that deserve further attention, and the following sections outline possible directions for future research in these respects.

### 9.3.1 Microbial strain

Only a single strain of *B. subtilis natto* isolated from a commercial natto product was considered in this study, and all inferences drawn were from fermentation processes employing this type of *B. subtilis natto*. It would be advantageous to screen other variants of *B. subtilis natto* isolated from different varieties and brands of natto products available on the market or from culture collection banks to determine the influence of different *B. subtilis natto* strains on the MK-7 isomer profile obtained from fermentation. This is particularly important as different subtypes of the same bacterium tend to have unique characteristics, and it may be possible for other strains of *B. subtilis natto* to produce a greater concentration of the all-*trans* isomer than the variant investigated in this study.

Genetic manipulation strategies to engineer *B. subtilis natto* strains with a high all-*trans* MK-7 production capacity could also be explored. However, their potential for the production of food supplements or functional foods is likely to be limited due to safety concerns and consumer resistance to the use of genetically modified organisms (GMOs) for such applications.

Essentially, examining other suitable forms of *B. subtilis natto* will likely provide an opportunity for the findings of this investigation to be extended to other strains of the bacterium and open up new possibilities to improve the production of bioactive MK-7 and further optimise the isomer profile resulting from fermentation.

### 9.3.2 Process scale

#### 9.3.2.1 Potential issues associated with increasing the scale of fermentation

Although this study has primarily assessed the production of MK-7 isomers on a small scale, it has drawn attention to some of the difficulties associated with enhancing the concentration of all-*trans* MK-7 on a larger scale. Most of the fermentation experiments (Chapters

4, 5, and 8) were carried out in McCartney bottles, and only fermentation involving bacterial cells immobilised with IONs (Chapters 6 and 7) was conducted in small shake flasks. A lower concentration of the all-*trans* isomer was obtained for the control samples in Chapters 6 and 7 (less than 15 mg/L) compared to the optimum sample in Chapter 2 (53.29 mg/L). The media composition and fermentation conditions (inoculum size, temperature, agitation speed, and duration of fermentation) were identical, and only the fermentation volume differed between the two sets of experiments (6 mL for Chapter 2 and approximately 20 mL for Chapters 6 and 7). Hence, it is evident that even a slight increase in the volume and, consequently, the scale of the fermentation process has a notable effect on the concentration of the desired product. These outcomes emphasise that the fermentation volume is a key determinant of the all-*trans* isomer concentration achieved on a larger scale and present basic insight into likely challenges accompanying the scale-up of fermentation processes targeting the production of biologically active MK-7.

The drop in the all-*trans* MK-7 concentration with an increase in the fermentation volume can be attributed to the scale-up phenomenon, as the expansion of the scale of a fermentation process significantly impacts the culture conditions and, ultimately, the productivity of the overall process. The performance of a fermentation system and its inherent dynamics are influenced by many physical, biochemical, and process factors, the majority of which are likely to vary with an increase in the fermentation volume and the geometry of the culture vessel [1]. Even minor increases in the fermentation volume and changes in the vessel characteristics, such as progressing from a small McCartney bottle to a larger shake flask or from a shake flask to a laboratory-scale fermenter, can greatly alter the fermentation environment and reduce the product yield, as it is not feasible to precisely replicate the fermentation conditions when increasing the scale of the process.

Therefore, since the present study suggests that MK-7 isomer production and the all-*trans* and *cis* isomer concentrations attained are sensitive to changes in the process scale, further research and experimentation on different fermentation strategies to boost all-*trans* MK-7 production on a larger laboratory scale are necessary to enable the large-scale production of bioactive MK-7 by microbial fermentation.

#### 9.3.2.2 Scale-up fermentation studies

Despite the value of small-scale experiments in facilitating the development of a targeted fermentation process to favour the production of the biologically significant MK-7 isomer, it is imperative to increase the experimental scale and consider a greater fermentation volume to gain an understanding of the effect of scale-up on the isomer profile and all-*trans* MK-7 concentration and realise industrial-scale production. Essential parameters inherent to large-scale fermentation processes, especially the agitation speed, rate of aeration, and pH, must also be adapted to meet the goals of a particular fermentation process on a larger scale.

Consequently, evaluating MK-7 isomer production in a benchtop fermenter is an important direction for future research. Optimisation of factors such as the agitation speed, aeration rate, and pH on a larger laboratory scale while employing the fermentation media and conditions previously optimised on a small scale (Chapters 1 and 2) will lay the foundation for the development of a large-scale fermentation process that selectively enhances the concentration of all-*trans* MK-7 and reduces the production of the *cis* isomer.

In this regard, the agitation speed and rate of aeration can initially be investigated and optimised in a benchtop bioreactor in a free pH environment to maximise the concentration of the all-*trans* isomer and minimise the concentration of *cis* MK-7. The ideal agitation and aeration levels without pH control can then be used to examine the effect of different pH environments (acidic, neutral, and basic) on isomer production. This will allow the isomer concentrations achieved from fermentation at different pH values to be compared with those obtained from fermentation under uncontrolled conditions to ascertain the optimal pH environment (acidic, neutral, basic, or uncontrolled) to favour the production of bioactive MK-7 on a laboratory scale. These experimental findings could subsequently be extended to explore and optimise the process in larger-sized fermenters, as it is expected that there will be difficulties associated with selectively increasing the concentration of the all-*trans* isomer at progressively greater fermentation volumes.

#### 9.3.2.3 Fermentation strategies to enhance all-*trans* MK-7 production on a larger scale

Previous studies have investigated and demonstrated the success of various approaches, including adding key nutrients at specific time points or intervals and methods to decrease foam generation, to obtain high concentrations of MK-7 from fermentation in benchtop bioreactors [2-4]. However, it must be noted that these analyses solely focused on improving MK-7 production on a laboratory scale as a whole and did not account for the occurrence of MK-7 isomers. Thus, it is necessary to delve into such fermentation techniques and consider their impact on the isomer profile to determine their value in increasing the concentration of all-*trans* MK-7 on a laboratory scale.

Possible strategies that have the potential to enhance the concentration of the bioactive MK-7 isomer and minimise the concentration of *cis* MK-7 achieved in larger-scale fermentation processes are discussed below. It would be valuable to examine these methods in greater depth in future research to assess their suitability in this context and determine the ideal strategy to obtain a high concentration of the all-*trans* isomer when increasing the fermentation scale.

##### *9.3.2.3.1 Fed-batch nutrient addition*

Fed-batch fermentation techniques allow high biomass densities and are advantageous for processes where the synthesis of the desired product is positively correlated with microbial

growth, as is the case for MK-7 production. Different feeding regimes can be employed to add either a fixed or variable volume of fresh medium or nutrients continuously, at different rates, in pulses, or at specific time points or intervals over the course of fermentation. The nutrient concentrations(s) and feeding strategy can also be tailored to satisfy the aims and requirements of each fermentation process. Accordingly, different nutrient feeding schemes could be considered to improve the production of all-*trans* MK-7 using a fed-batch approach in a benchtop fermenter.

#### *9.3.2.3.1.1 Glucose*

In this study, glucose, the primary carbon source, was present in the media at its optimal concentration from the start of the process and allowed to deplete in accordance with bacterial utilisation during fermentation. Since bacteria use carbon sources for growth, metabolism, and MK-7 synthesis, the glucose concentration in the media decreases as fermentation proceeds, consequently leading to the exhaustion of glucose in the fermentation media and impeding MK-7 productivity after a certain timeframe. If glucose is added to the fermentation media at particular time points or intervals or when its concentration drops below a specified value, it will likely sustain microbial growth, metabolism, and MK-7 production over a longer period. This may also result in a different MK-7 isomer profile at the end of fermentation and possibly boost the final concentration of the all-*trans* isomer. Hence, it would be beneficial to develop fed-batch strategies for the addition of glucose during fermentation in a benchtop bioreactor. Different feeding regimes, glucose concentrations, and supplementation rates can be explored and optimised to selectively increase the production of the biologically effective MK-7 isomer on a larger laboratory scale.

#### *9.3.2.3.1.2 Glycerol*

Fed-batch glycerol addition may also be advantageous to improve the yield of all-*trans* MK-7. Although glycerol was determined to have an insignificant effect on MK-7 isomer production on a small scale, it aids MK-7 biosynthesis and secretion in *B. subtilis natto* and increases MK-7 productivity on a per-cell basis [5-7]. Thus, glycerol is often reported as the most effective carbon source in MK-7 fermentation [2, 4, 8, 9]. As the characteristics and dynamics of the fermentation system change with the process scale, incorporating glycerol using a fed-batch approach may benefit all-*trans* MK-7 production in a laboratory- or large-scale fermentation process. Therefore, subsequent studies could explore the effect of fed-batch glycerol addition on the MK-7 isomer composition and production of the biologically efficacious isomer in a benchtop fermenter. Various glycerol concentrations and different schemes and rates of glycerol addition can be examined to maximise the concentration of all-*trans* MK-7 and reduce the production of the *cis* isomer.

### *9.3.2.3.2 Foam reduction*

Due to the high foaming tendency of solutions containing biomaterials, foaming is an inherent issue in most fermentation processes, especially on a larger scale [10]. Foam refers to the dispersion of gas bubbles in a continuous liquid phase and is located above the fermentation broth [11, 12]. Several factors contribute to foaming during fermentation, such as cell growth, metabolite formation, temperature, and pH, as well as the medium composition (concentration of sugars, proteins, and salts), viscosity and rheological properties of the broth, agitation speed, aeration rate, vessel geometry, and presence of surface-active substances and other compounds [11-13]. It has been noted that a high protein content in the fermentation broth promotes foaming during fermentation, and high agitation and aeration rates also favour foam production [10-12]. Furthermore, the bacterial growth phase influences foam generation. It tends to be more pronounced during the early stages of fermentation, most likely due to the presence of proteins and other foaming-forming agents in the initial fermentation medium, and the latter phases of the process, possibly owing to protein release during cell lysis or other properties of the bacterial culture [11].

Unrestrained foaming during fermentation is undesirable, as it decreases the fermentation productivity and may result in sterility and containment issues due to loss of the fermentation broth, cells, and product from the air outlet and seepage into bearings and other attachments [10, 13]. However, a certain degree of foam production improves the mass transfer characteristics of the fermentation broth, as the presence of gas bubbles increases the surface area available for mass transfer [11, 12]. Thus, to enhance product formation and ensure the optimal performance of the fermentation system, a compromise must be achieved between the two conditions to permit controlled foam generation and repress excessive foaming during fermentation.

While various methods have been proposed to control foaming during laboratory- or large-scale fermentation in a bioreactor, they have not been examined from the perspective of MK-7 isomers. Potential approaches are outlined below, and investigating the impact of such foam reduction strategies on the production of MK-7 isomers in the future will benefit the development of an effective fermentation process that selectively enhances the concentration of bioactive MK-7 on a large scale.

#### *9.3.2.3.2.1 Antifoaming agents*

The use of surface-active chemical agents (antifoams) is the most common approach to restrict foaming during laboratory- and large-scale fermentation processes [10]. Many classes of antifoaming agents exist, including alcohols, esters, oils, fatty acids and their derivatives, silicones, and sulfonates [10-12]. Generally, the properties of a chemical antifoaming agent should enable it to successfully regulate foam production and be appropriate for the fermentation organism and end application of the fermentation product [11, 14]. Additionally, the choice of antifoam must be non-toxic to humans and the environment, have minimal impact on oxygen and mass transfer, be stable during sterilisation, and not interfere with the desired product and the

methods used for its downstream isolation and purification [11]. Consequently, antifoam selection usually depends on its compatibility with the fermentation microorganism, its suitability for the fermentation system, and the nature and intended use of the final product.

Of the numerous antifoams available, silicone-based antifoams are the most effective for foam control in bacterial fermentations [11]. Moreover, fermentation products with food-related applications necessitate food grade antifoaming agents, and many chemical antifoams, including silicone-based products, are available for such microbial fermentations [14]. However, the main drawback of chemical antifoams is their potential negative impact on oxygen and mass transfer in the fermentation broth, as the addition of antifoaming agents can reduce gas holdup and the air/solution interfacial area, which decreases the volumetric mass transfer coefficient ($k_La$) and impairs the availability of oxygen to the bacterial cells, hampering the process productivity [10-12, 14].

It has been reported that adding large amounts of silicone-based antifoaming agents to control foaming during fermentation impedes MK-7 production, possibly due to their adverse effect on oxygen transfer and solubility in the fermentation broth [2]. Hence, optimising the concentration and amount of antifoam added to the fermentation system to regulate foaming is necessary. Alternatively, although silicone-based antifoams are best suited to bacterial fermentations, it may be worthwhile to trial other types of antifoaming agents that are not silicone-based, such as polyalkylene glycol-based antifoams and those derived from fatty acids/esters, polyesters, and oils.

It is essential to appreciate that the foam formation profile is specific for each fermentation process. The nature and extent of foaming for a fermentation system targeting the production of the bioactive MK-7 isomer will likely be unique, developing an understanding of which is important, as it will influence the choice of antifoam and the concentration and amount required to achieve adequate foam control. Therefore, with respect to MK-7 isomer production, once the ideal agitation speed, aeration rate, and pH conditions have been determined on a laboratory scale, it would be advantageous to study the foaming characteristics of the fermentation broth in a laboratory-scale bioreactor to gain insight into the foam generation profile for the optimal process parameters to enhance the production of all-*trans* MK-7 on a larger scale. Chemical antifoams of different compositions can then be screened to determine the most suitable antifoaming agent. Subsequently, observation of foam evolution over the fermentation process employing the optimal antifoaming agent can guide the development of the most effective foam control strategy and can help determine the appropriate time(s) and condition(s) for antifoam addition (for example, antifoam can be automatically dispensed when the foam height reaches a pre-decided level), as well as the concentration(s) and amounts(s) in which it is added to the fermentation broth to restrict foaming without impairing the productivity of the fermentation process.

#### *9.3.2.3.2.2 Alternative methods for foam control and medium reformulation*

While chemical antifoams are the most feasible and effective approach to regulate foaming during fermentation, it is recognised that antifoaming agents can decrease the fermentation productivity, largely due to the reduction in oxygen and mass transfer in the fermentation broth upon antifoam addition. Thus, it is valuable to consider alternative options to decrease foaming that do not negatively impact the characteristics of the process and can be used in conjunction with antifoams to avoid using only chemical agents as the primary method of foam control.

Various mechanical and physical foam disruption techniques, including mechanical foam breakers (revolving disks, impellers, stirrers, injectors, ejectors, and orifices) and ultrasound, thermal, and electrical treatments, have been used to mitigate foaming during fermentation; however, they are often not as effectual as chemical agents, entail high power consumption, and may adversely affect cell viability [11-13, 15]. Nevertheless, combining mechanical devices and chemical antifoaming agents can lessen antifoam addition by 33-50% and may be a viable compromise [10, 11]. Reformulation of the fermentation medium is another approach that can be employed to alleviate excessive foaming during fermentation, and it has been successfully applied in MK-7 fermentation to reduce antifoam addition [2].

The optimal fermentation media determined from this study consists of several complex protein sources (yeast extract, soy peptone, and tryptone), which increase the protein content of the media and are likely to encourage foam formation during all-*trans* MK-7 production on a larger scale. Higher agitation speeds were also favourable for improving the production of the all-*trans* isomer in the small-scale experiments, and high rates of agitation and aeration during fermentation in a bioreactor are known to enhance MK-7 biosynthesis. These findings are expected to apply to the production of all-*trans* MK-7, and high agitation and aeration conditions, especially coupled with the large amount of protein in the optimal media, will possibly heighten foam generation.

Since it is desirable to minimise antifoam addition to the fermentation system, it may be advantageous to consider reformulation of the fermentation medium in future studies to lessen the extent of foaming, mainly at the start of fermentation, by decreasing the initial protein content of the media. This can be achieved by reducing the amount of protein-rich nutrients that are added to the preliminary fermentation media, and the remaining quantity required to satisfy the substrate demand and achieve optimal all-*trans* MK-7 productivity can be supplemented in the fermentation broth at specified intervals or during one or more stages of the fermentation process. This will likely diminish the requirement for antifoam addition and improve the yield of the bioactive isomer.

### 9.3.3 Development of fermented all-*trans* MK-7-enriched foods

The isomer profile of fermented MK-7-enriched fortified or functional foods has not yet been considered, and investigating the isomer profile of such products is worthy of attention, as

they hold great promise to help increase the widespread dietary intake of bioactive MK-7. While MK-7 nutritional supplements are currently the most popular and accessible form of MK-7 supplementation, they only reach selected groups of individuals who have exposure and are able to afford dietary supplements. In contrast, enriching commonly consumed food items with all-*trans* MK-7 will allow the development of biologically effective fermented MK-7-rich fortified or functional food products that can be made available to a diverse range of consumers at a reasonably low price. Broad consumption of such products by large populations is likely to improve the vitamin K status of individuals and provide greater health benefits relative to nutritional supplements in the form of tablets or capsules.

#### 9.3.3.1 Possible matrices for fortified or functional foods

Foods that are widely accessible and can be easily incorporated into the dietary routine of different populations are ideal matrices for the development of fermented all-*trans* MK-7-enriched fortified or functional foods, as they have the potential to reach nearly all individuals universally. The suitability of a specific food matrix depends on many factors, including the intended population, the types of production processes likely to be involved, and the desired health outcomes. Hence, it would be valuable to first screen a diverse range of food sources and assess their ability to act as an appropriate matrix for fortification and the development of functional goods in different contexts.

Generally, foods can either be fortified with fermented all-*trans* MK-7 or used as a substrate that can be fermented to create a bioactive MK-7-enriched functional product. Items such as cereals, dairy goods, fats, oils, beverages, and condiments are possible foods that can serve as favourable matrices for the production of fermented biologically significant MK-7-containing fortified or functional foods.

Dairy products, in particular, have immense potential for the development of fermented bioactive MK-7 fortified or functional foods, and this has been exemplified in previous studies considering the enrichment of milk, yoghurt, and other dairy goods with MK-7 to achieve various health outcomes [16-22]. Furthermore, dairy products are a good source of Ca, and supplementation with all-*trans* MK-7 will enhance their nutritive value and help improve bone health, decrease the incidence of fractures, and reduce the progression of osteoporosis. Therefore, in forthcoming studies, it is of benefit to investigate the fortification of dairy items with fermented all-*trans* MK-7 or the fermentation of suitable dairy products with *B. subtilis natto* or other MK-7-producing microorganisms commonly used for the manufacture of dairy goods, such as lactic acid bacteria, to produce fermented bioactive MK-7-enriched functional foods with probiotic properties. However, for such nutritionally enhanced products to have high therapeutic value, they must contain the biologically significant all-*trans* isomer in the greatest proportion. Consequently, fermentation strategies should focus on improving the concentration of the all-*trans* isomer and reducing the production of *cis* MK-7.

Other edible and food grade substrates, especially those with a high existing nutrient profile, could also be assessed to develop fermented bioactive MK-7-enriched fortified or functional foods in a similar manner. Moreover, this will give a good indication of the effect that assorted food matrices have on the fermentation yield of all-*trans* MK-7 and their influence on the quantity of the bioactive isomer during the short- or long-term storage of different types of fortified or functional products.

#### 9.3.3.2 The use of NPs to increase all-*trans* MK-7 production for fermented functional foods

This investigation considered the use of biocompatible magnetic IONs for bacterial cell immobilisation, which has the potential to increase the yield of the all-*trans* isomer and address the major challenges associated with large-scale MK-7 fermentation. However, these magnetic IONs are not appropriate for developing fermented bioactive MK-7-enriched functional foods, as they are not safe for human consumption. Generally, when NPs are used to produce and enhance the MK-7 concentration of fermented functional foods, they often remain in the final product and are not removed, unlike magnetic IONs. Thus, NPs must be non-toxic and coated with biocompatible food grade materials to be suitable for inclusion in fermented functional foods.

Iron-based NPs are particularly valuable for the development of MK-7-rich fermented functional foods, as they have the ability to boost the MK-7 concentration and can serve as a source of iron. Iron supplementation, in addition to MK-7, through the consumption of such fermented functional foods can help increase the dietary intake of iron and reduce the incidence of iron deficiency anaemia, a health condition affecting a substantial proportion of the population worldwide.

Previously, biocompatible XG-coated FeOOH NPs have been used for bacterial cell immobilisation to enhance the MK-7 yield of a fermented functional dairy product (milk) [23]. Although using XG-coated FeOOH NPs increased the MK-7 concentration in the fermented milk product, the study evaluated MK-7 production holistically without accounting for the proportion of the bioactive all-*trans* isomer achieved. This is important, as the therapeutic value of fermented MK-7 functional foods stems from their content of the all-*trans* isomer. As a result, exploring the potential for biocompatible XG-coated FeOOH NPs to enhance the all-*trans* MK-7 concentration of fermented MK-7 functional foods would be beneficial.

Other types of NPs with nutritional benefits, including ZnO (source of Zn) and ferric pyrophosphate ($FePO_4$) and various $Fe_2O_3$ compounds (sources of iron), have also been explored for the development of food products, and they can be evaluated for their suitability to increase the concentration of the bioactive MK-7 isomer for the development of fermented functional foods.

Furthermore, other biocompatible polysaccharide coatings with favourable properties, such as acacia gum, guar gum, carrageenan, pectin, alginates, inulin, and dextran, could be studied

in combination with FeOOH or other nutritionally relevant NPs to boost the all-*trans* MK-7 profile of fermented functional foods. These biocompatible polysaccharides, along with XG, are commonly used as thickeners, emulsifiers, and stabilisers in the food industry and are considered safe for human consumption and the development of food products.

In this regard, there is a substantial domain for further research into different types and combinations of nutritionally valuable NPs and biocompatible coatings that can be used to fabricate non-toxic NPs to enhance the all-*trans* MK-7 content of dairy-based and other fermented functional foods. However, many factors, such as the safety and properties of the NPs used, the concentration of NPs in foods, the digestion and clearance of NPs following consumption, and the potential for the accumulation of NPs in the body, must be investigated for the various types and combinations of NPs and biocompatible coatings for food-related applications. Additionally, finished products will likely be subjected to several regulatory criteria before they are deemed safe and acceptable for human consumption.

#### 9.3.3.3 Shelf life studies of fermented all-*trans* MK-7 after formulation into various consumer products

It has been acknowledged that MK-7 is liable to degradation during storage, and the rate at which this occurs is accelerated in certain environments. While exposure to specific storage conditions has been considered in this study, it only covers a subset of the many factors encountered during the manufacture and overall shelf life of a particular product. In addition, different preparations of fermented bioactive MK-7 may be subjected to unique storage environments depending on the characteristics of the final product (tablets, capsules, or fortified/functional foods).

For instance, fermented all-*trans* MK-7 formulated into tablets and capsules is likely to be exposed to several excipient compounds and active ingredients (in the case of multi-nutrient supplements), such as MgO, $CaCO_3$, calcium citrate ($Ca_3(C_6H_5O_7)_2$), cellulose, gelatine, and other vitamins and minerals. Certain compounds, including MgO, may also promote alkalinisation, and since MK-7 is vulnerable to alkaline conditions, such additives can create an unfavourable milieu that enhance its deterioration. Hence, the inclusion of supplementary compounds and ingredients and their various combinations can create and expose the vitamin to distinct environments, which can affect the isomer profile and stability of all-*trans* MK-7 in different preparations. In contrast, fermented bioactive MK-7-enriched fortified or functional foods will likely be subjected to a different set of environmental factors specific to the selected food matrix and its storage requirements. For example, fresh dairy products require refrigeration at low temperatures and have a fairly short shelf life in comparison to cereals and other dry goods, commonly stored at ambient conditions over a longer period.

Consequently, the isomer composition and stability of all-*trans* MK-7 will likely vary with the nature of the end product, and in order to comprehensively understand the effect of

various environmental factors and storage conditions on the isomer profile and stability of all-*trans* MK-7, it is imperative to examine these aspects in context. Although prior studies have assessed the quality of commercially available MK-7 dietary supplements and similar preparations, there have been no attempts thus far to explore the isomer composition and stability of fermented all-*trans* MK-7 in different formulated products.

Therefore, in future research, it would be advantageous to formulate fermented all-*trans* MK-7 into various consumer end products, such as tablets, capsules, and fortified or functional foods, to develop a deeper understanding of the effect of different production processes, preparations, and foods matrices on the MK-7 isomer profile. It would also be valuable to carry out shelf life or degradation studies to explore both the short- and long-term stability of fermented all-*trans* MK-7 when formulated into assorted dietary supplement preparations and fortified or functional foods to elucidate the impact of a range of environmental factors and storage conditions, including product-specific features and those relating to the proposed packaging materials and design, on the quantity of bioactive MK-7 and the isomer composition resulting from different end uses.

### 9.3.4 Concluding remarks

Essentially, there is broad scope and ample opportunity for additional investigation regarding the production of MK-7 isomers during fermentation. Research with a greater emphasis on further improving the concentration of the all-*trans* isomer, particularly on a larger scale, evaluating the isomer profile and stability of fermented all-*trans* MK-7 in different formulations, and assessing the shelf life of possible end applications is warranted. This will likely enable the commercial production of fermented bioactive MK-7 dietary supplements and fortified or functional foods.

Although the findings of this research are not yet ready to be implemented on an industrial scale to enable commercial production, food companies could apply the fermentation process developed in this study in their research to create a MK-7-enriched product. Dairy companies, in particular, would be a good start, as previous research considering the development of MK-7-enriched functional dairy products has provided promising results. Furthermore, dairy products have a rich existing nutrient profile that could complement the function of MK-7 in the body. Research could focus on using a current dairy good, such as milk or yoghurt, as a fermentation substrate to synthesise a bioactive MK-7-enriched product by optimising and enhancing the synthesis of all-*trans* MK-7 in each food matrix. Alternatively, companies could use the synthesis procedure developed in this study to produce fermented all-*trans* MK-7 to add to or fortify different dairy goods and create a functional dairy product rich in bioactive MK-7. Subsequently, the sensory characteristics of such functional products can be investigated to assess important features relating to consumer acceptance, including appearance, taste, texture, and aroma. If these trials are successful, it will likely reduce the time required for bioactive MK-7 products to become

available on the market and increase the accessibility of consumers to effective and therapeutically significant all-*trans* MK-7-enriched products in the future.

## 9.4 References


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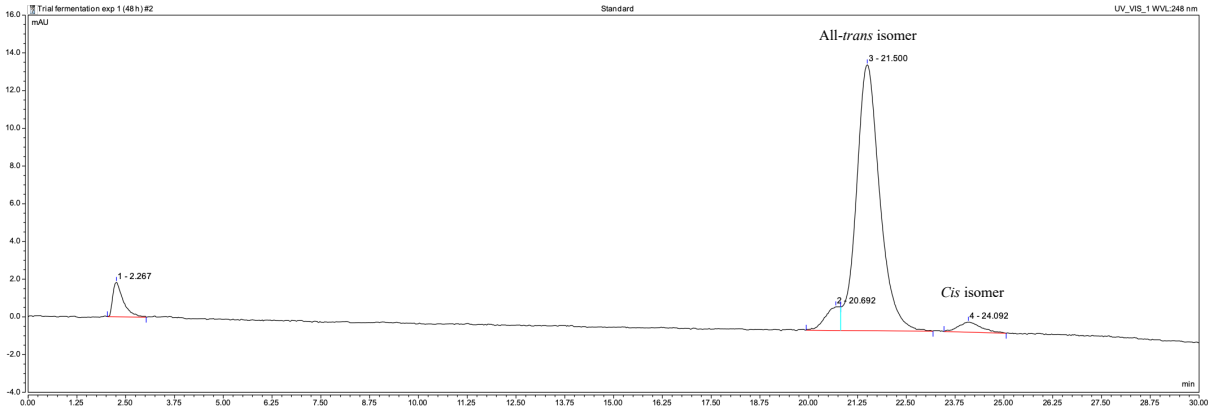
# Appendices

# Appendix A – Preliminary Results

## Experiment 1

### Standard

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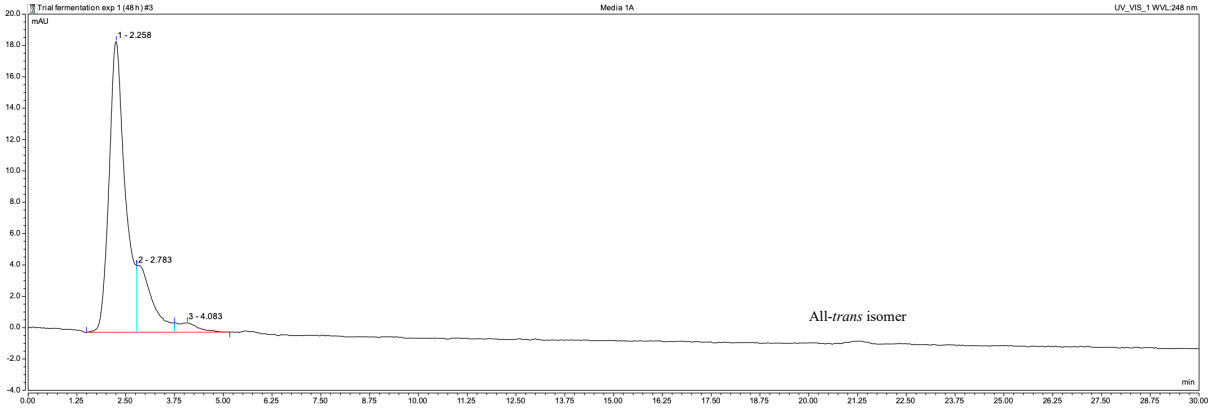


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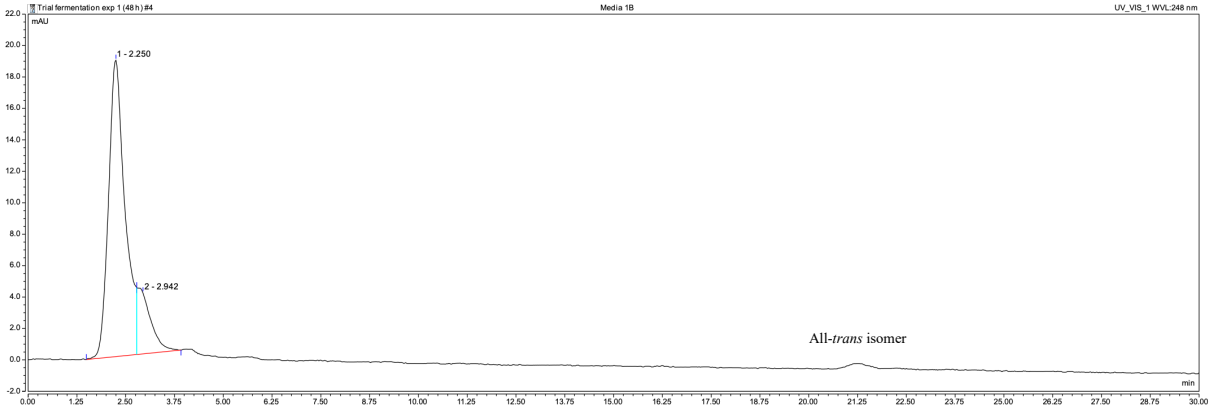


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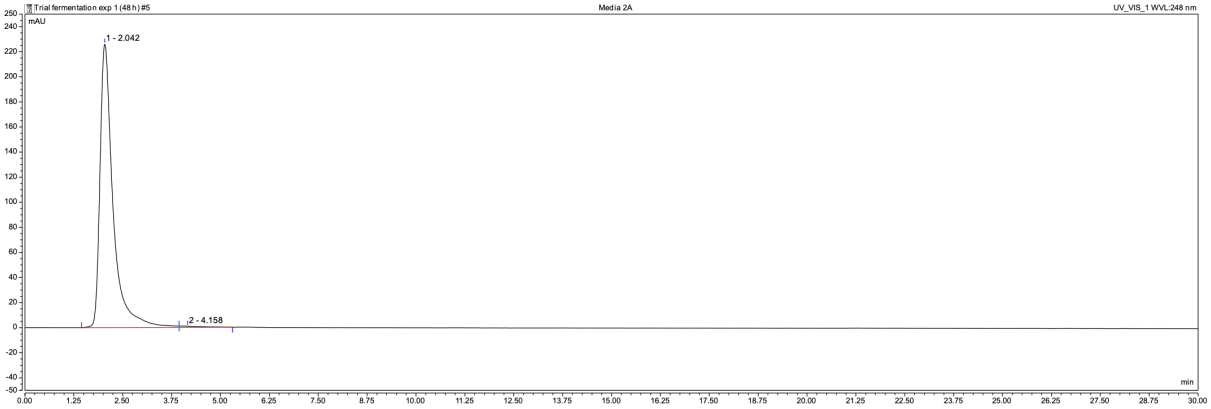


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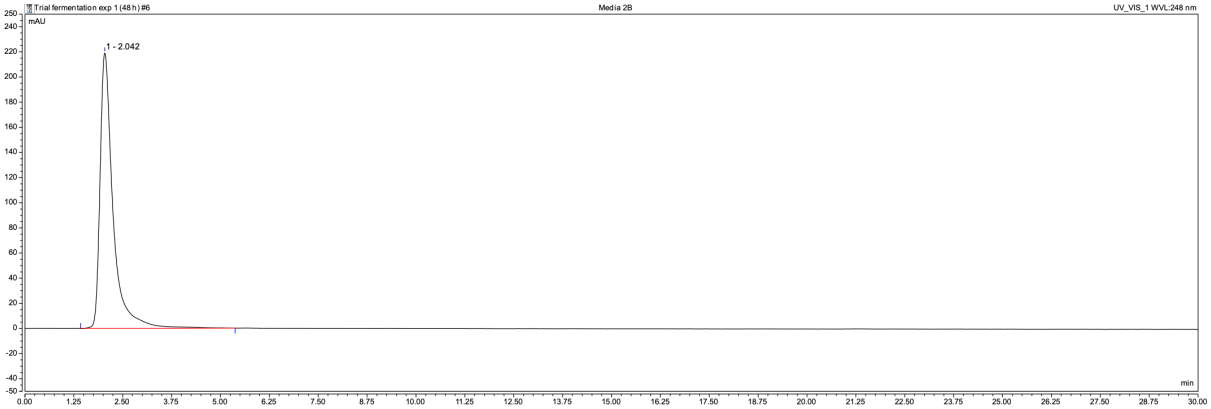


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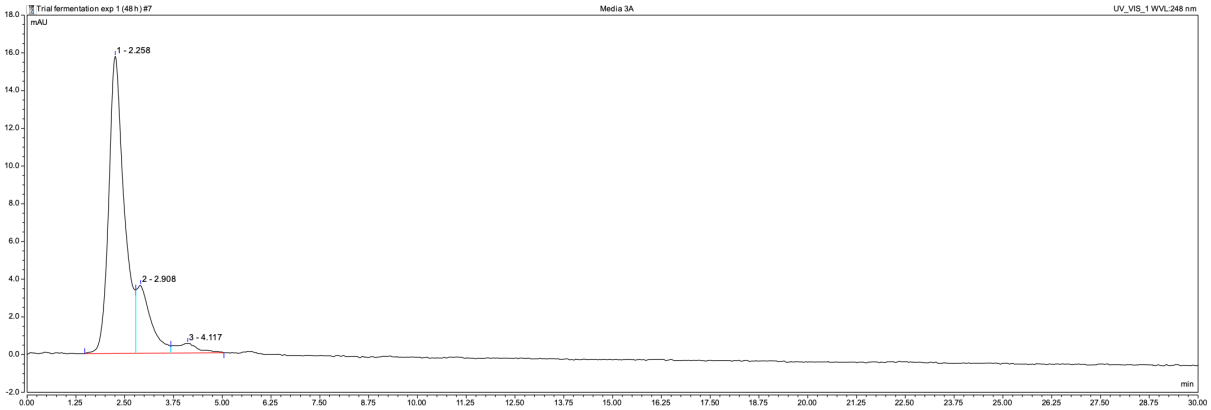


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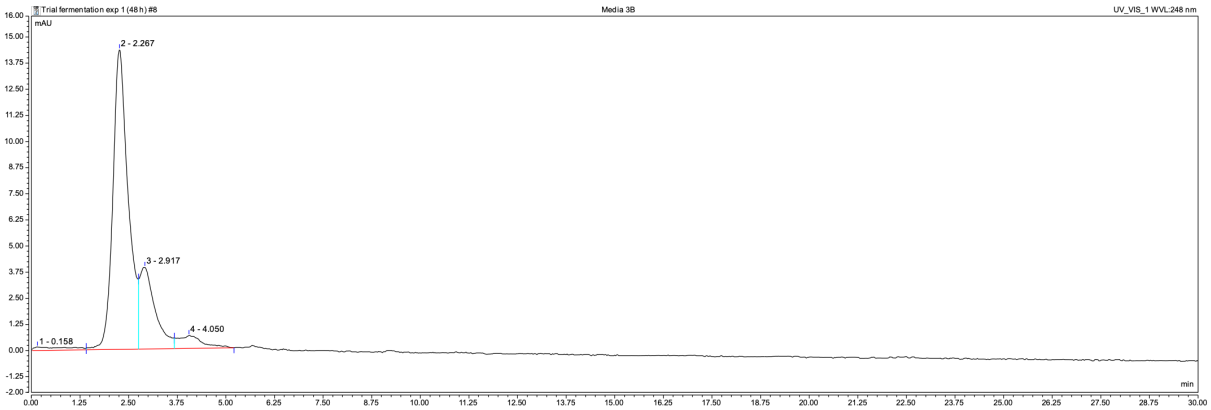

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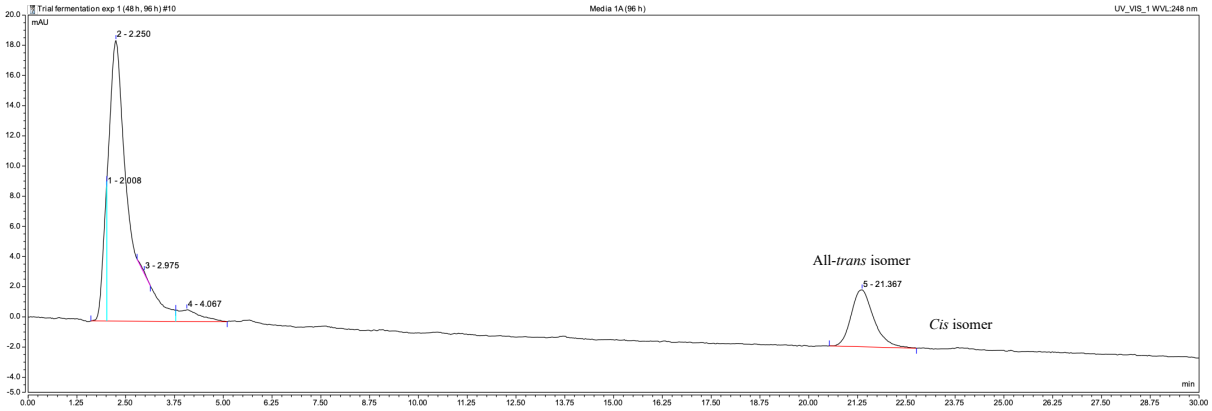


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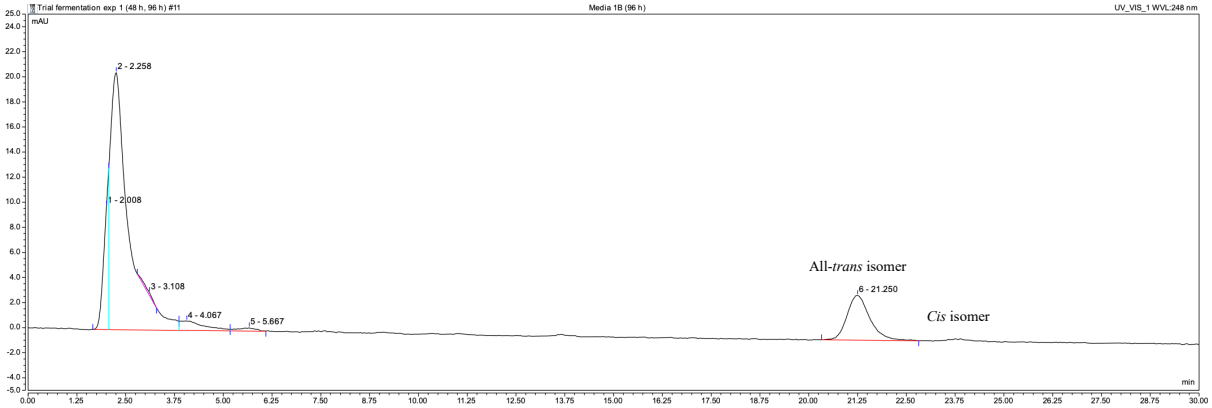


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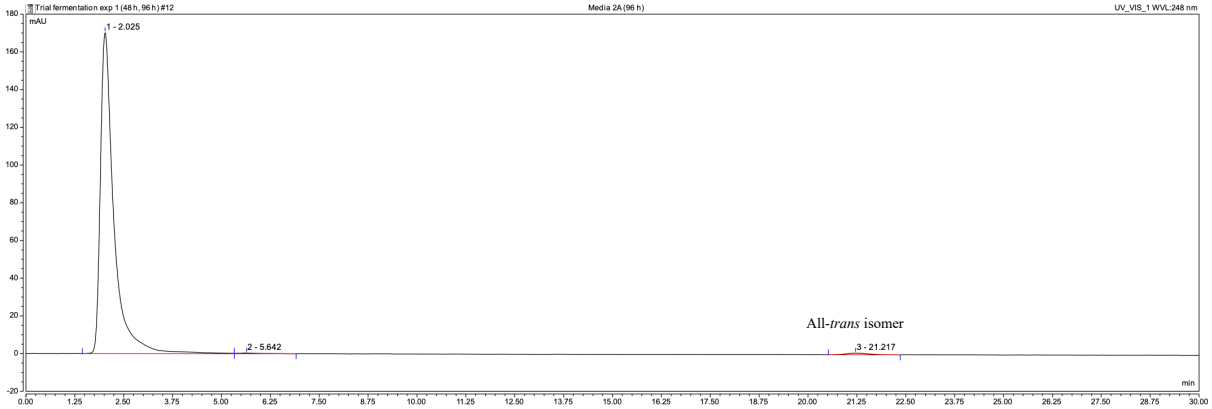


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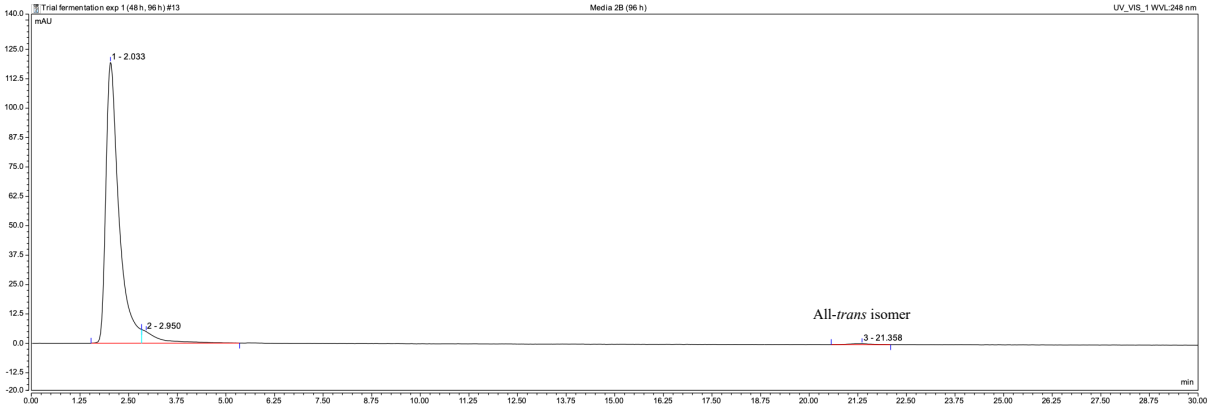


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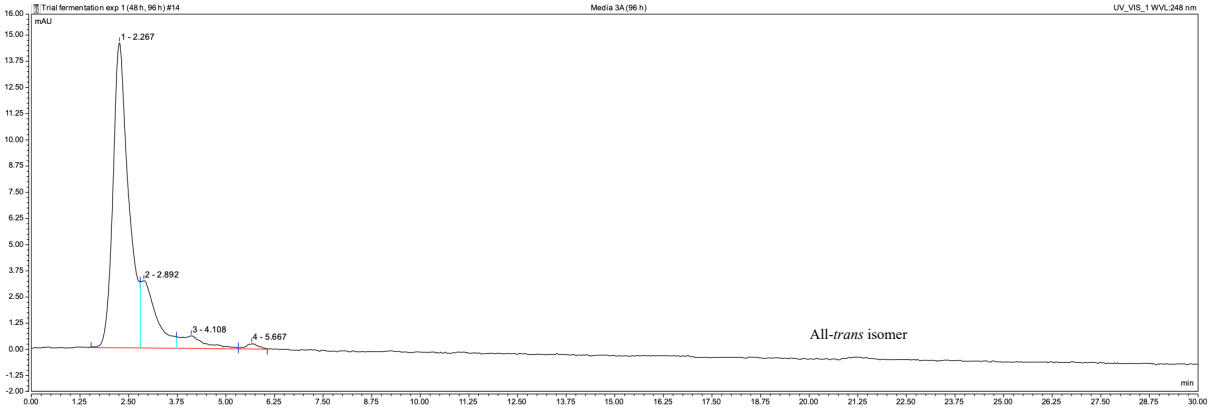


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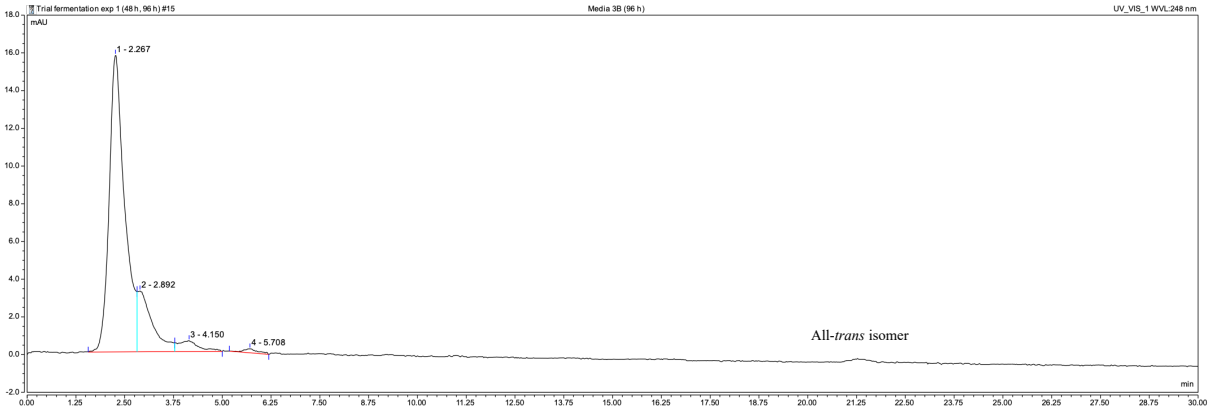


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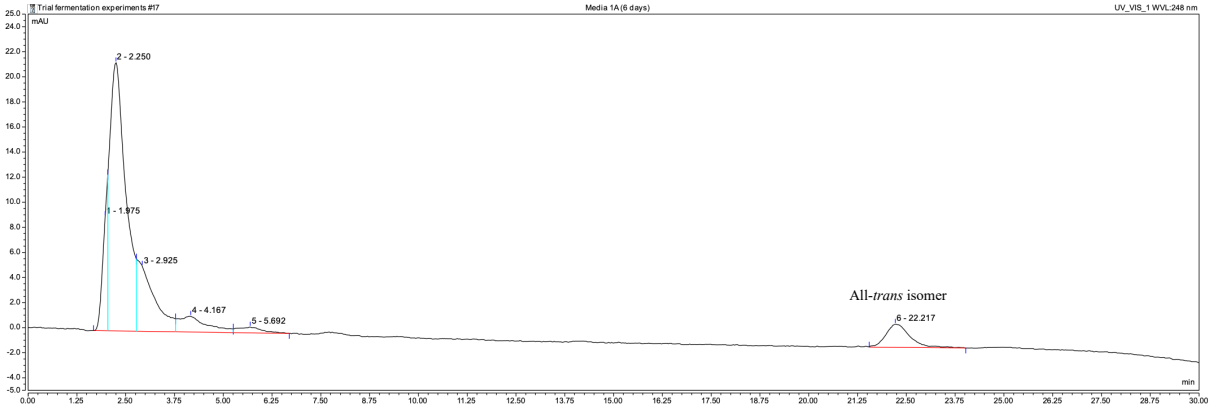


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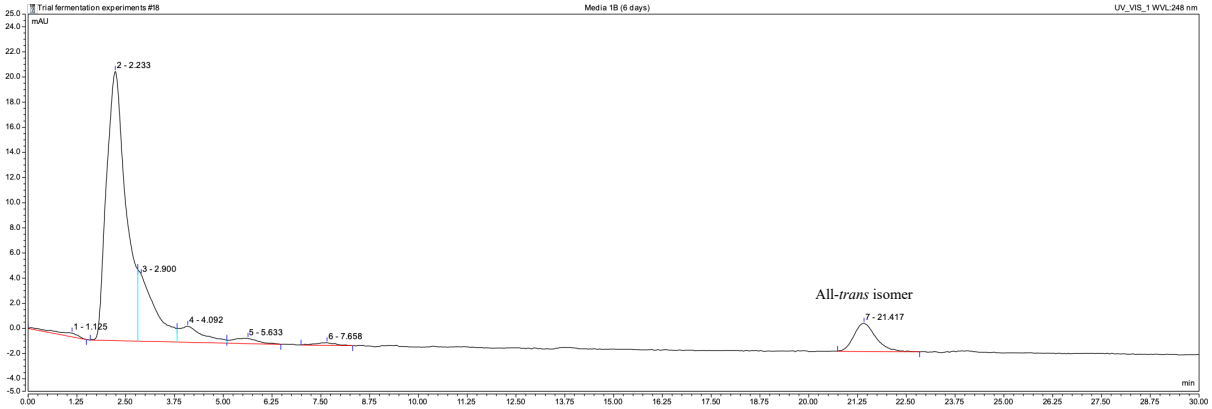


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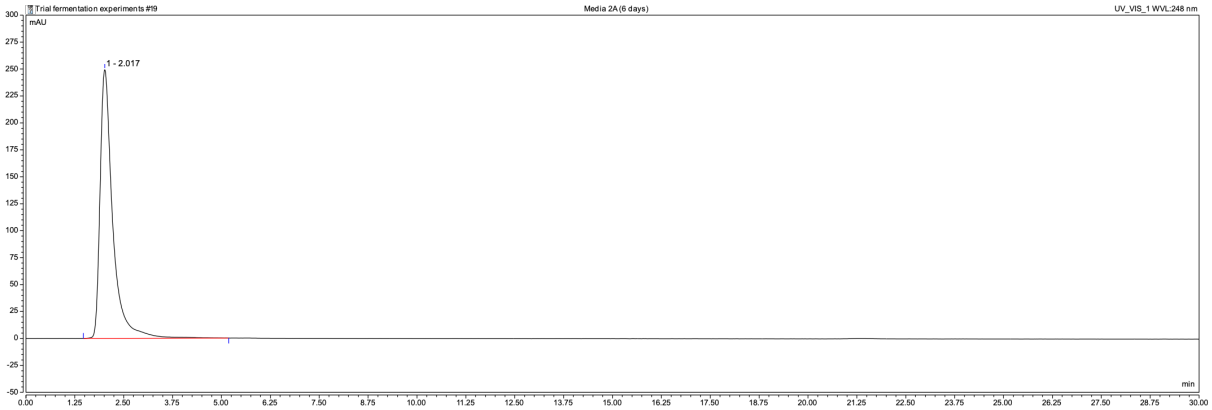


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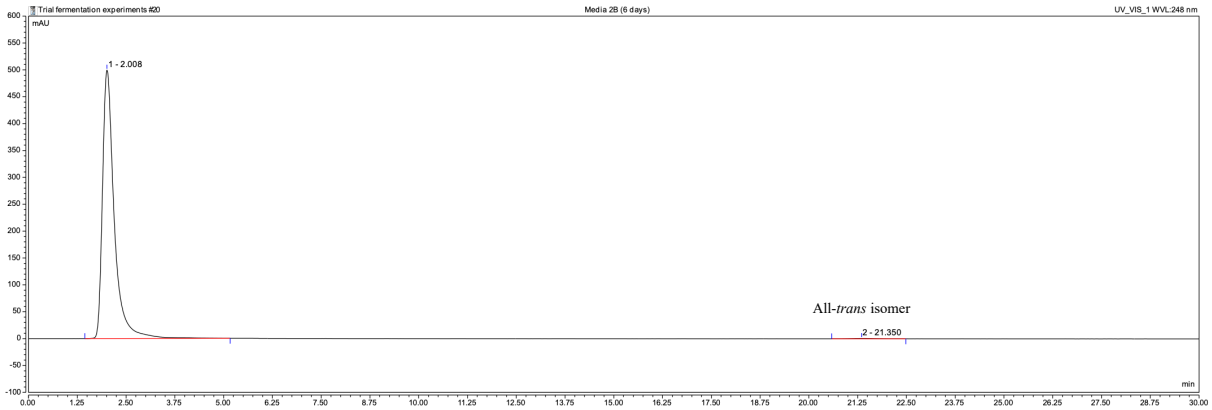


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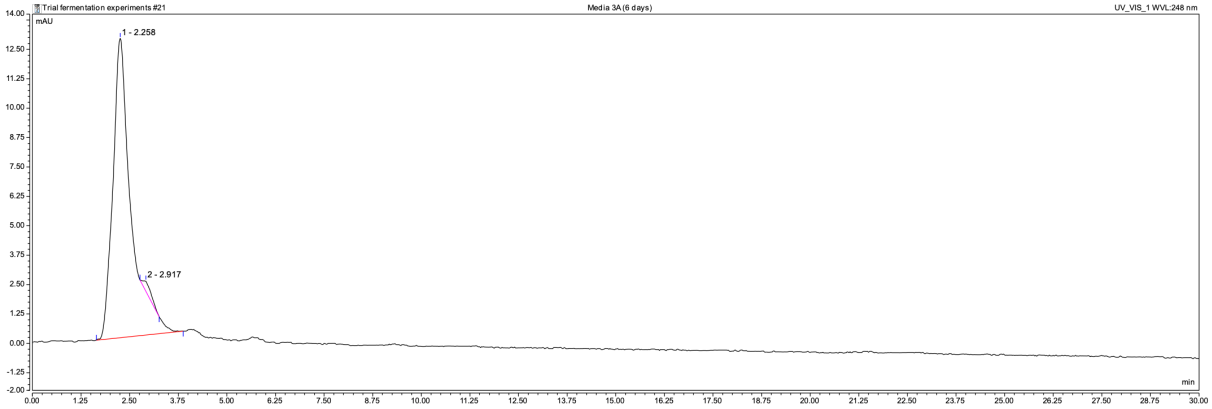


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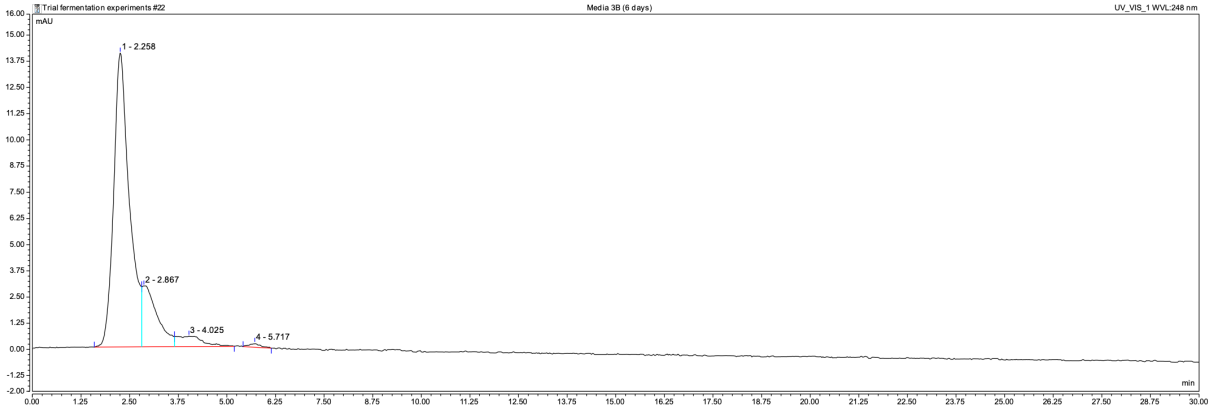


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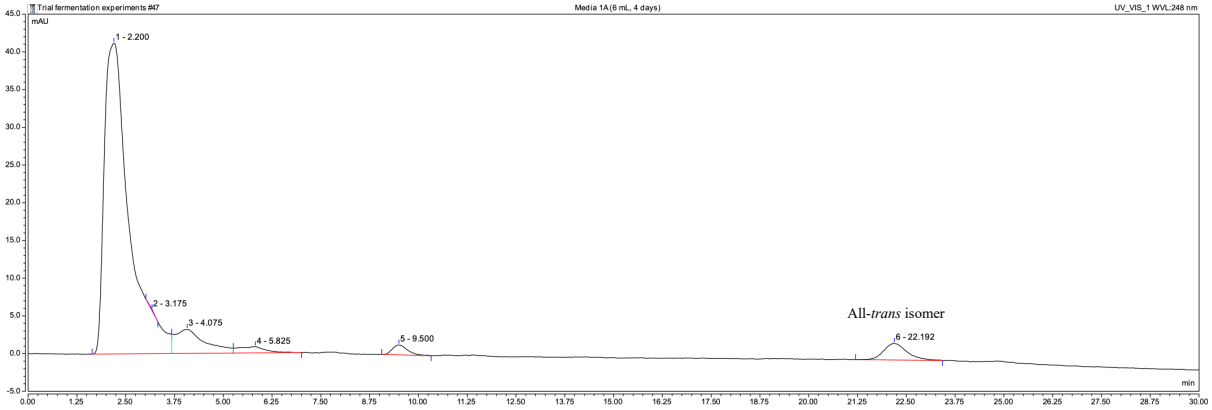


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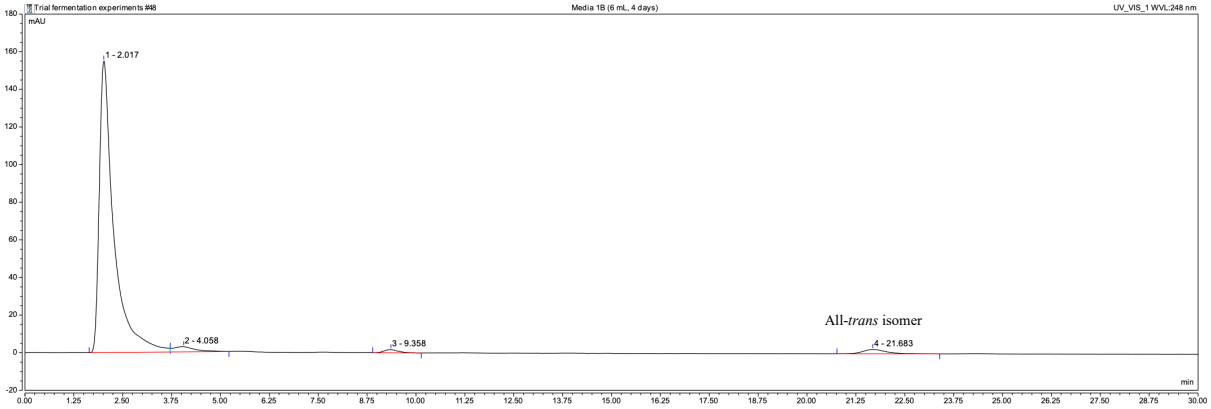


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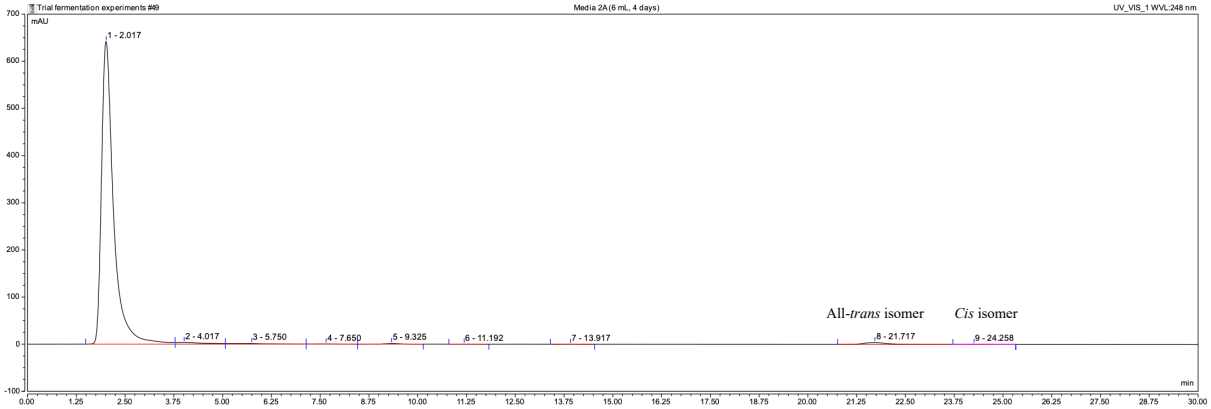


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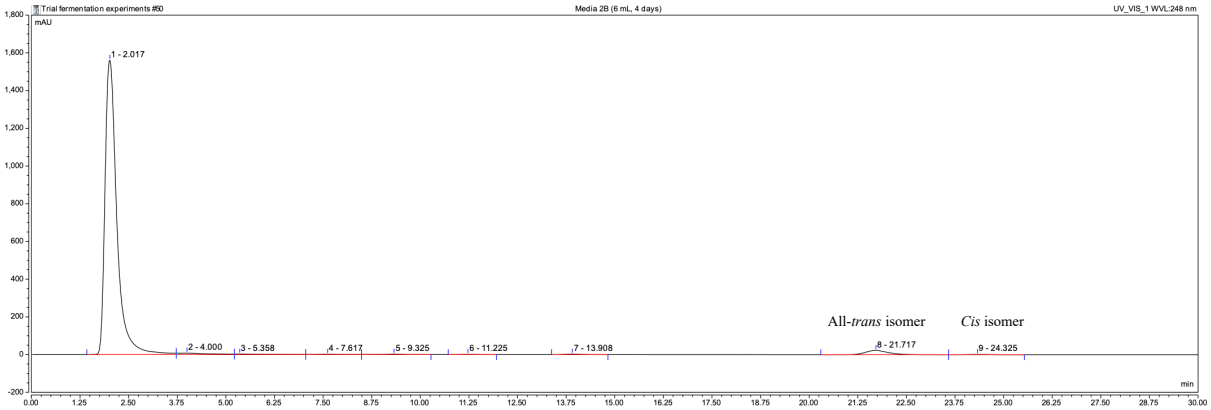


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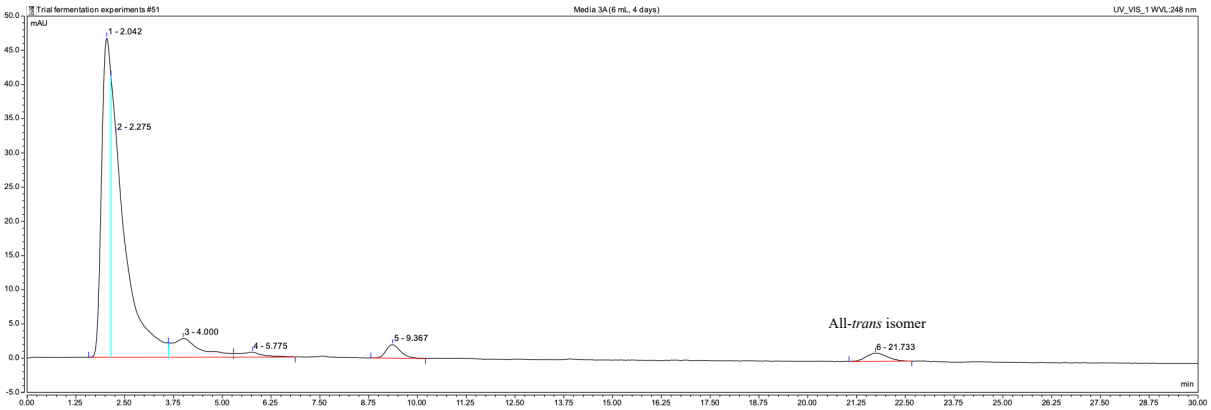


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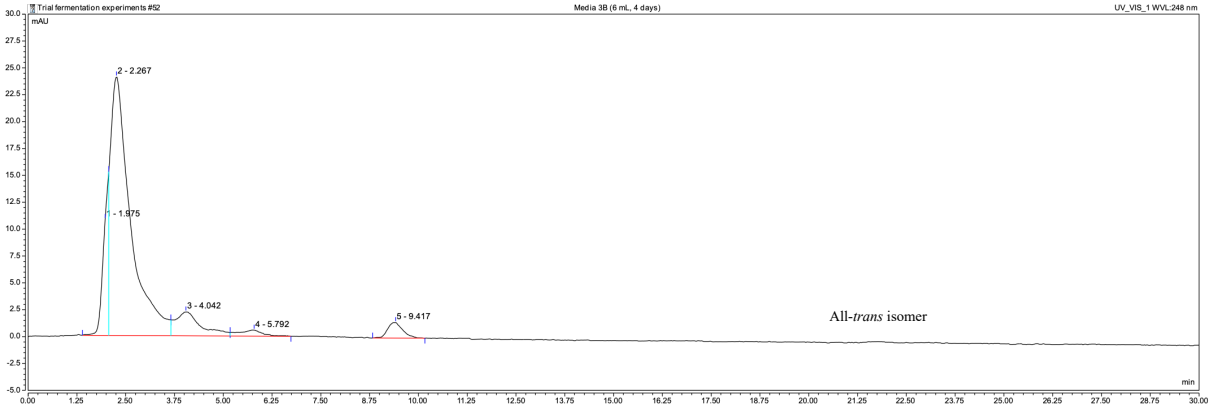


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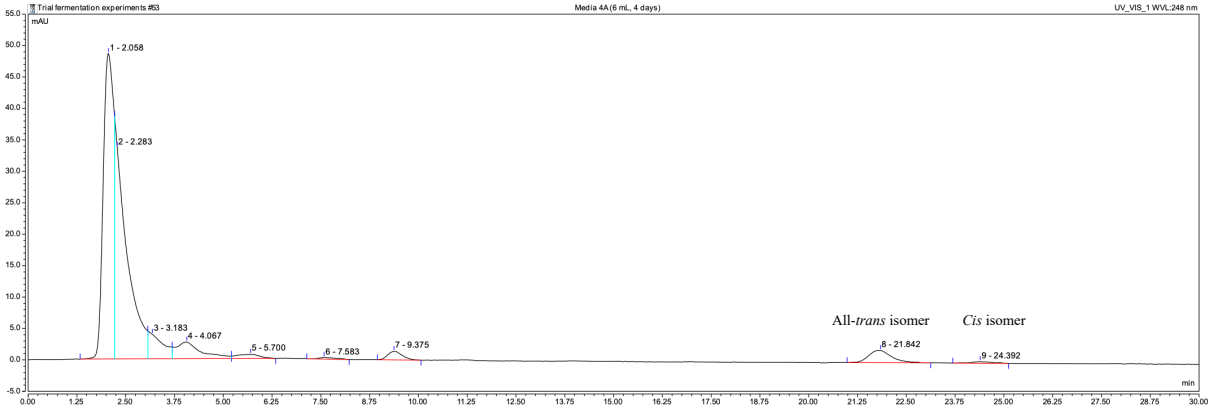


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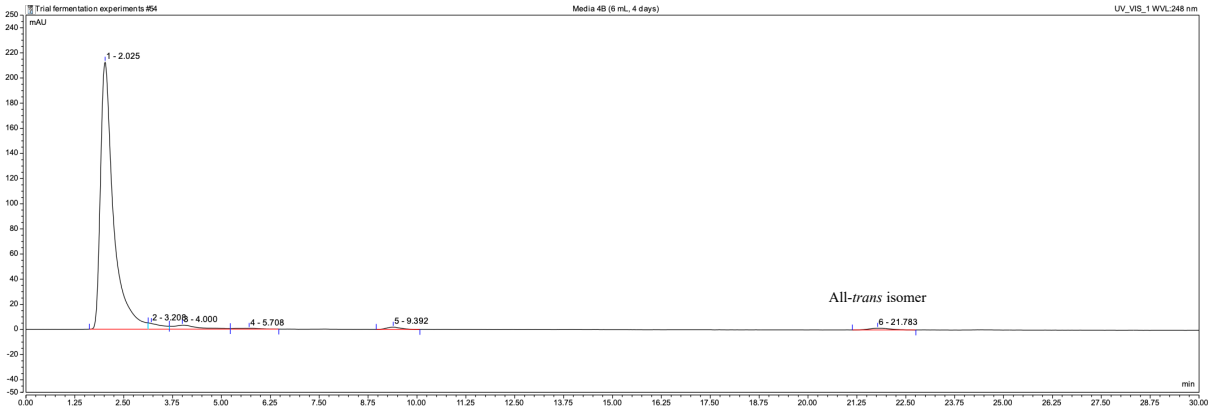
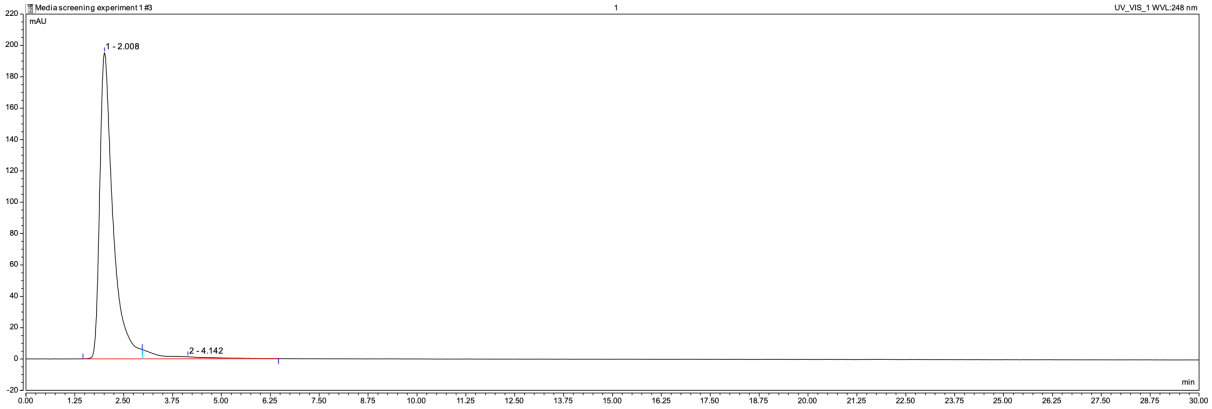



## <u>Experiment 5</u>

## Sample 1

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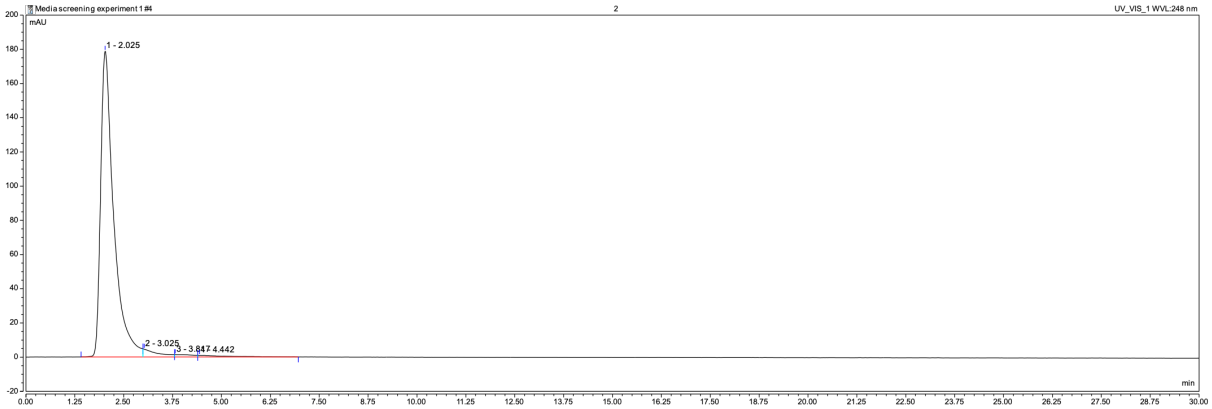


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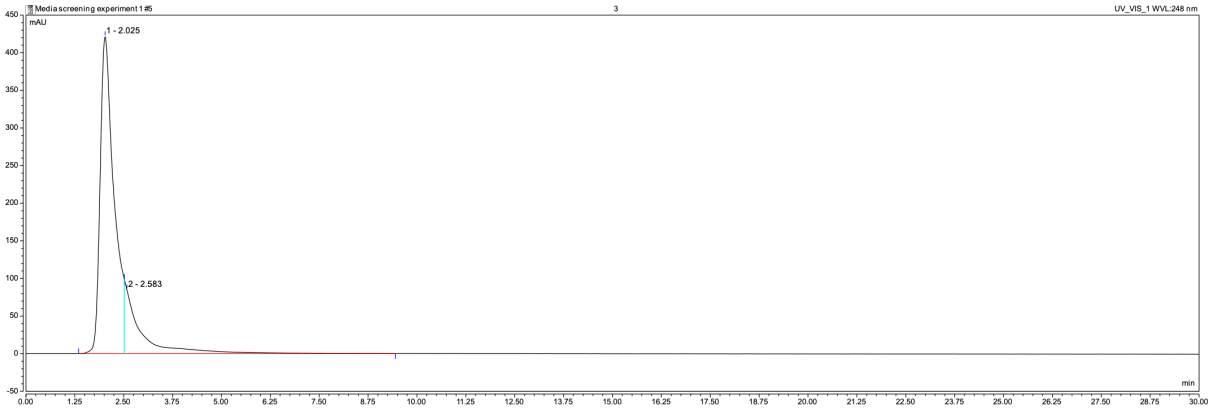


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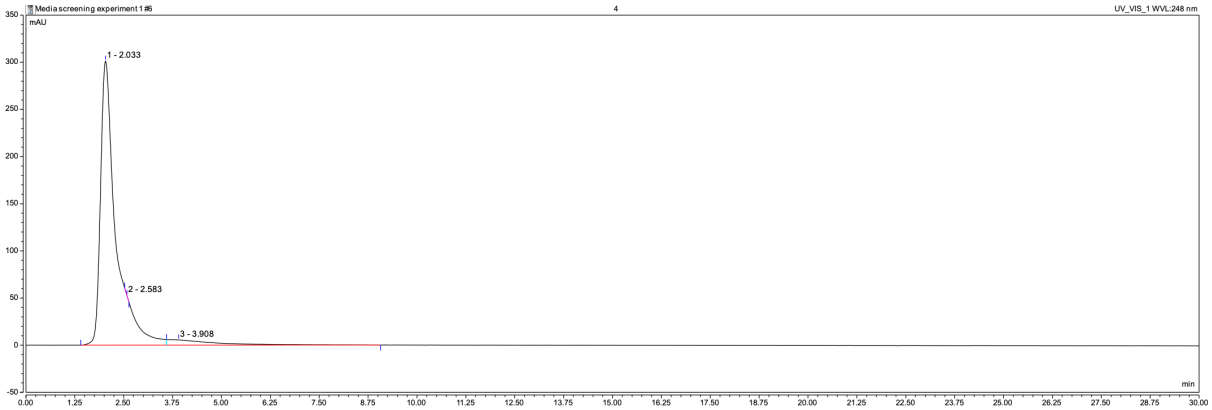


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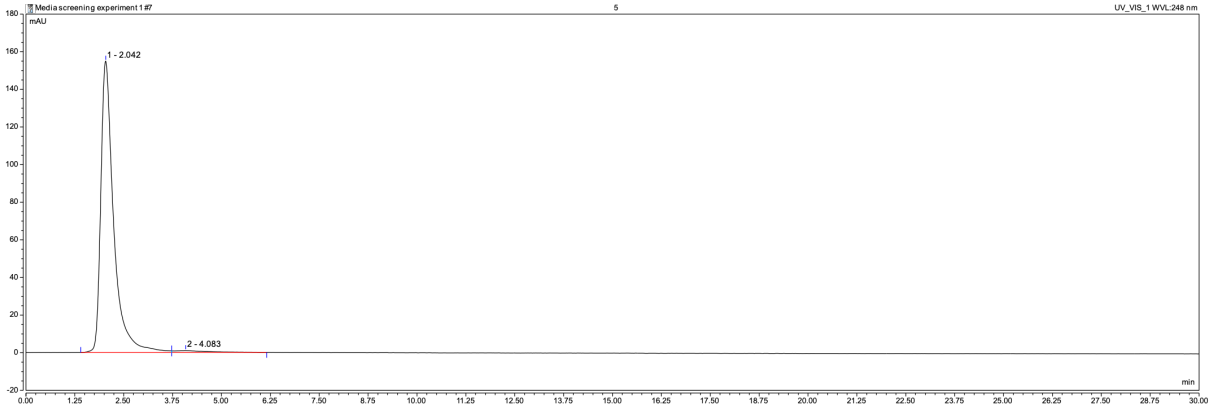


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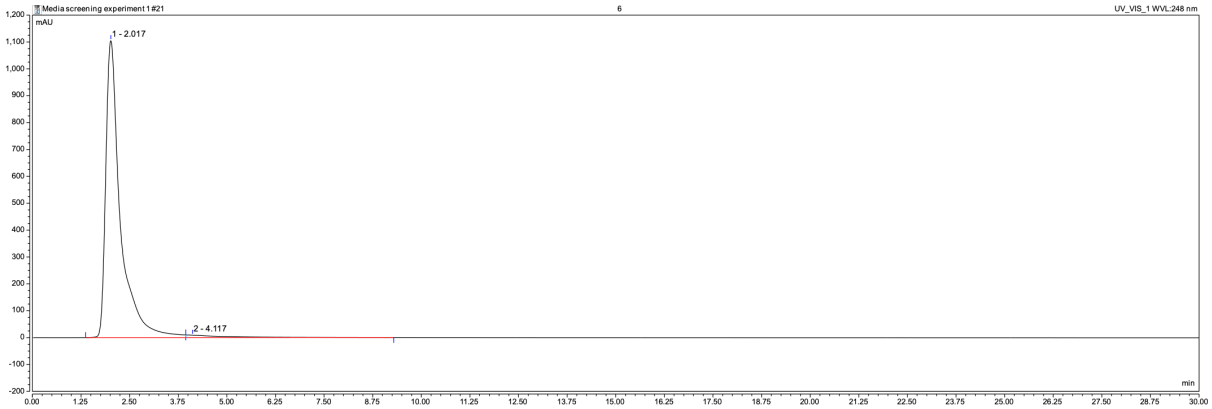


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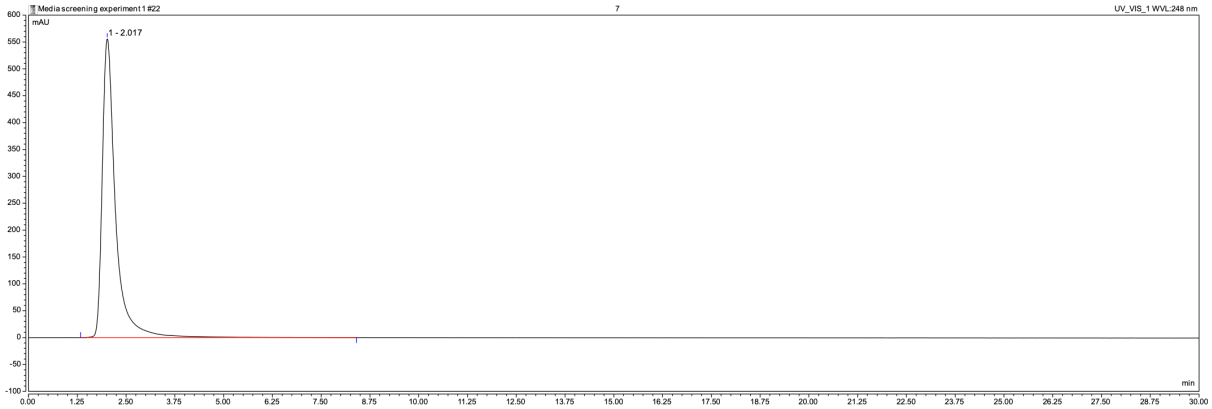


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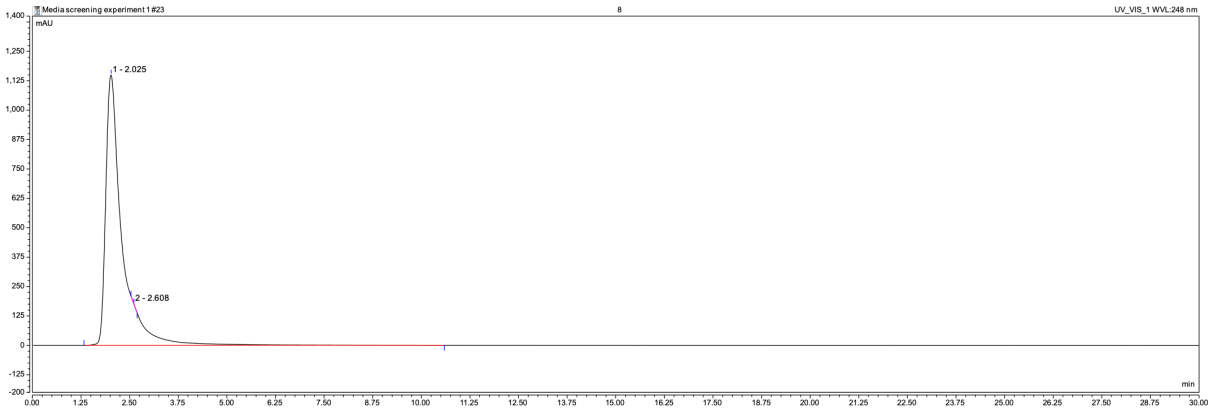


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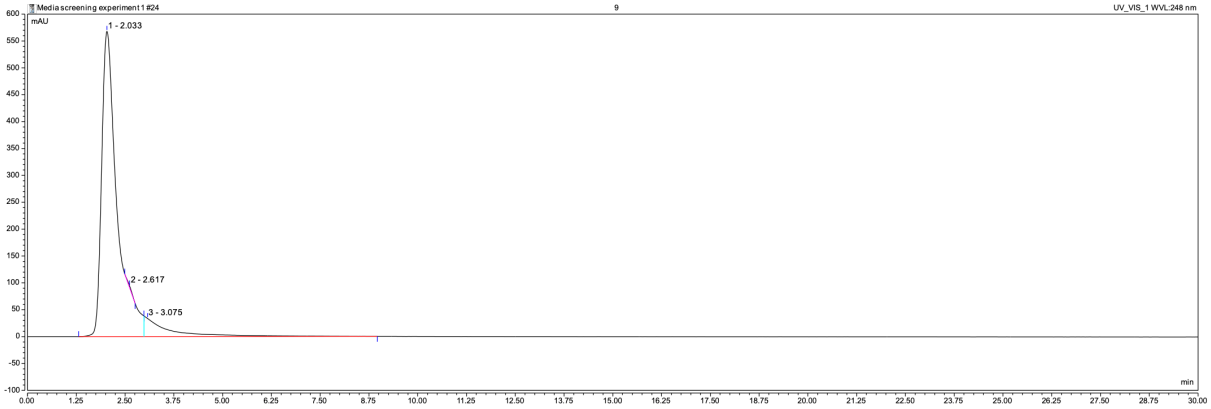


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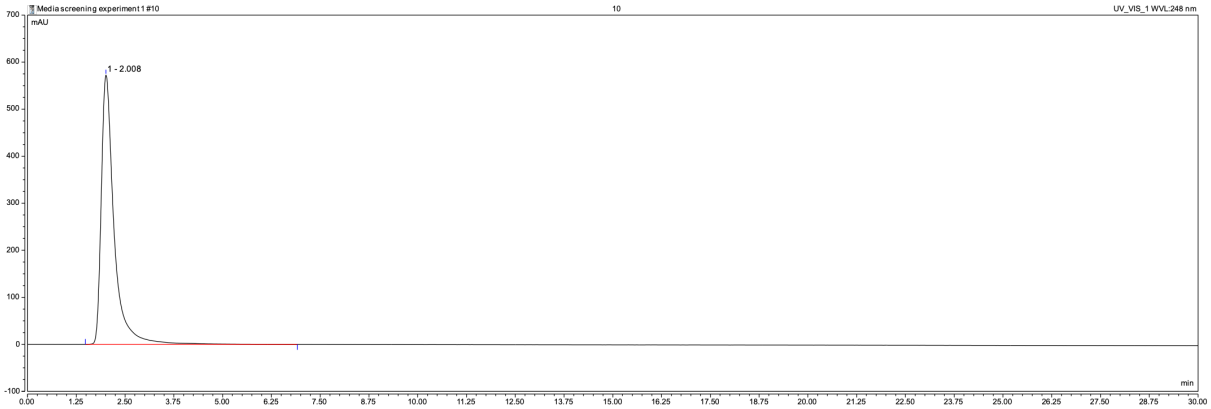


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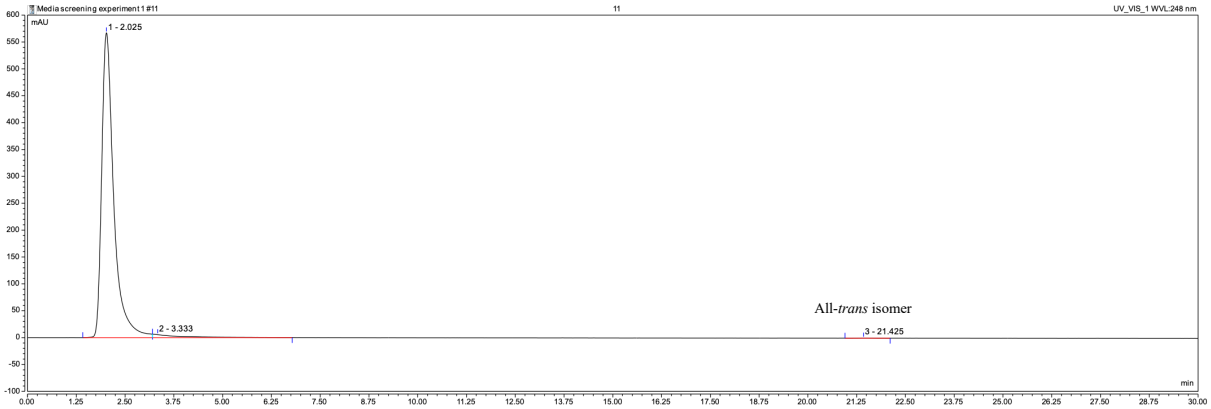


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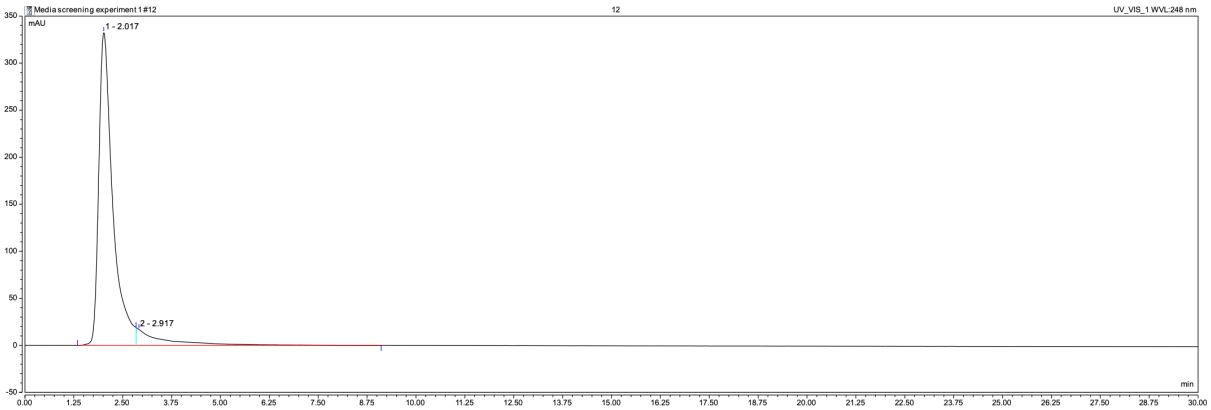


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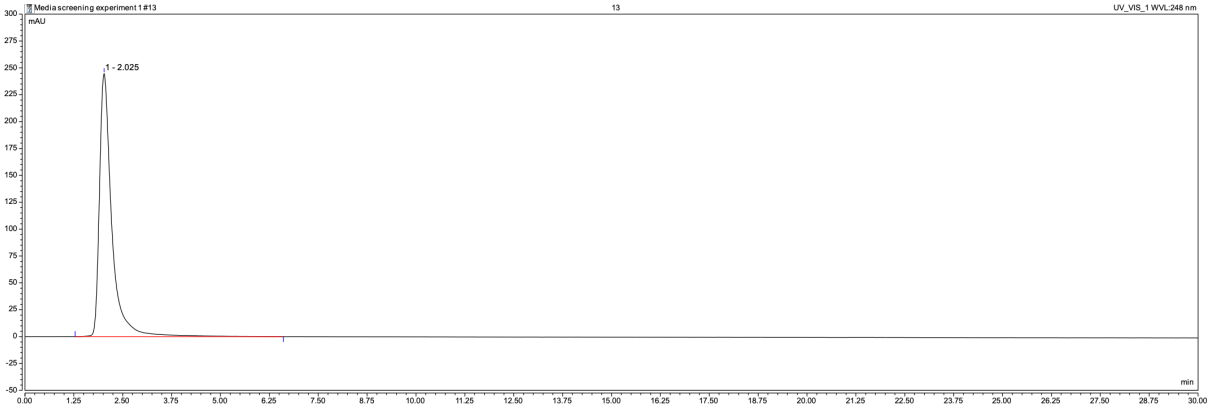


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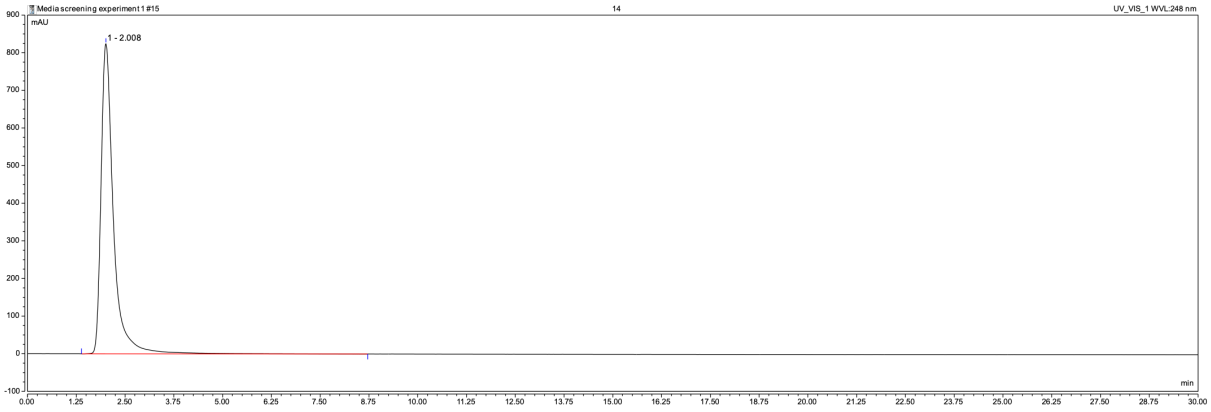


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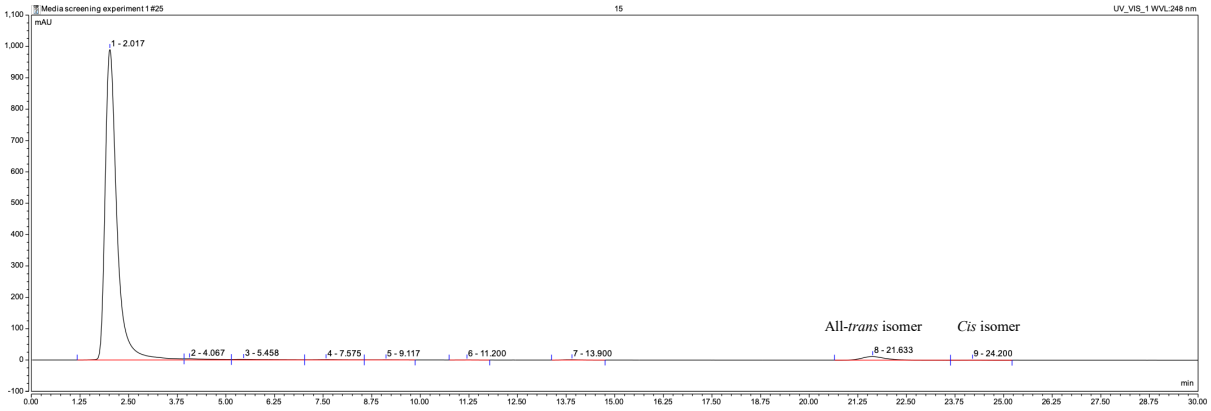


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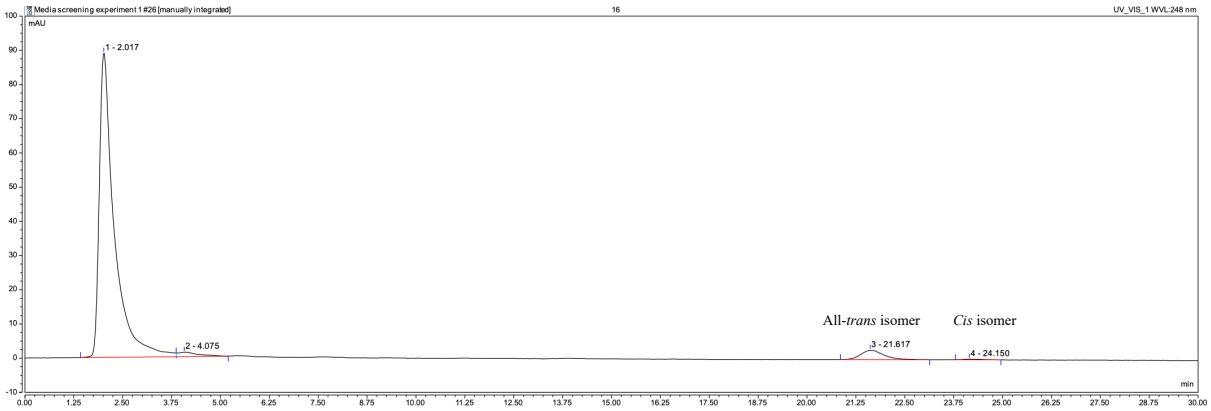


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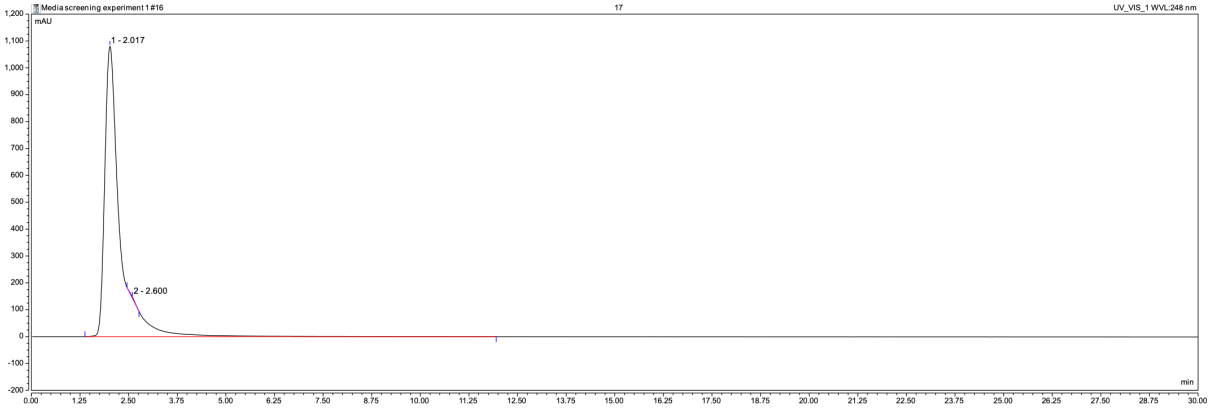


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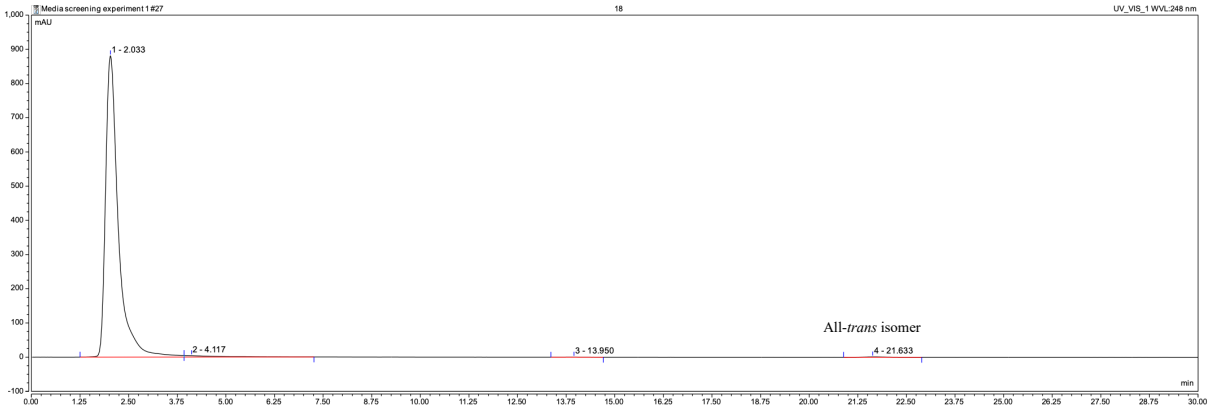


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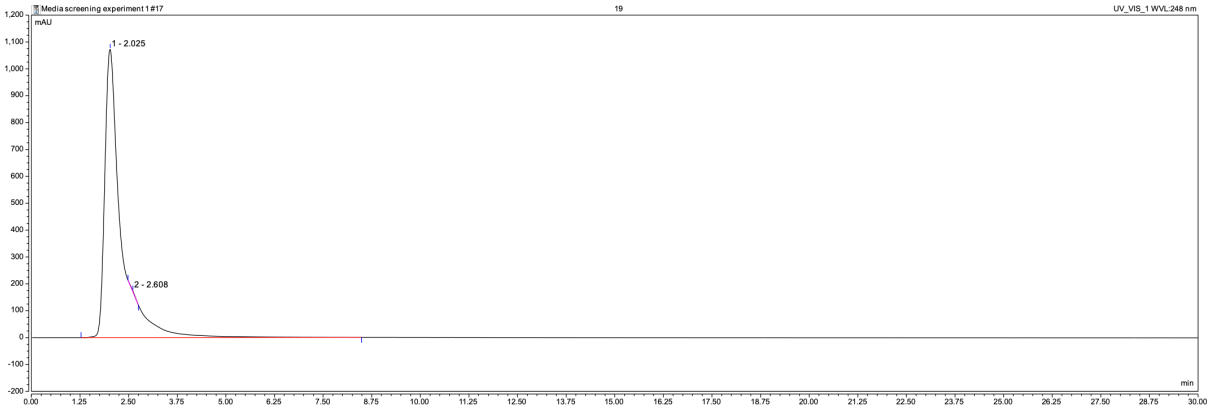
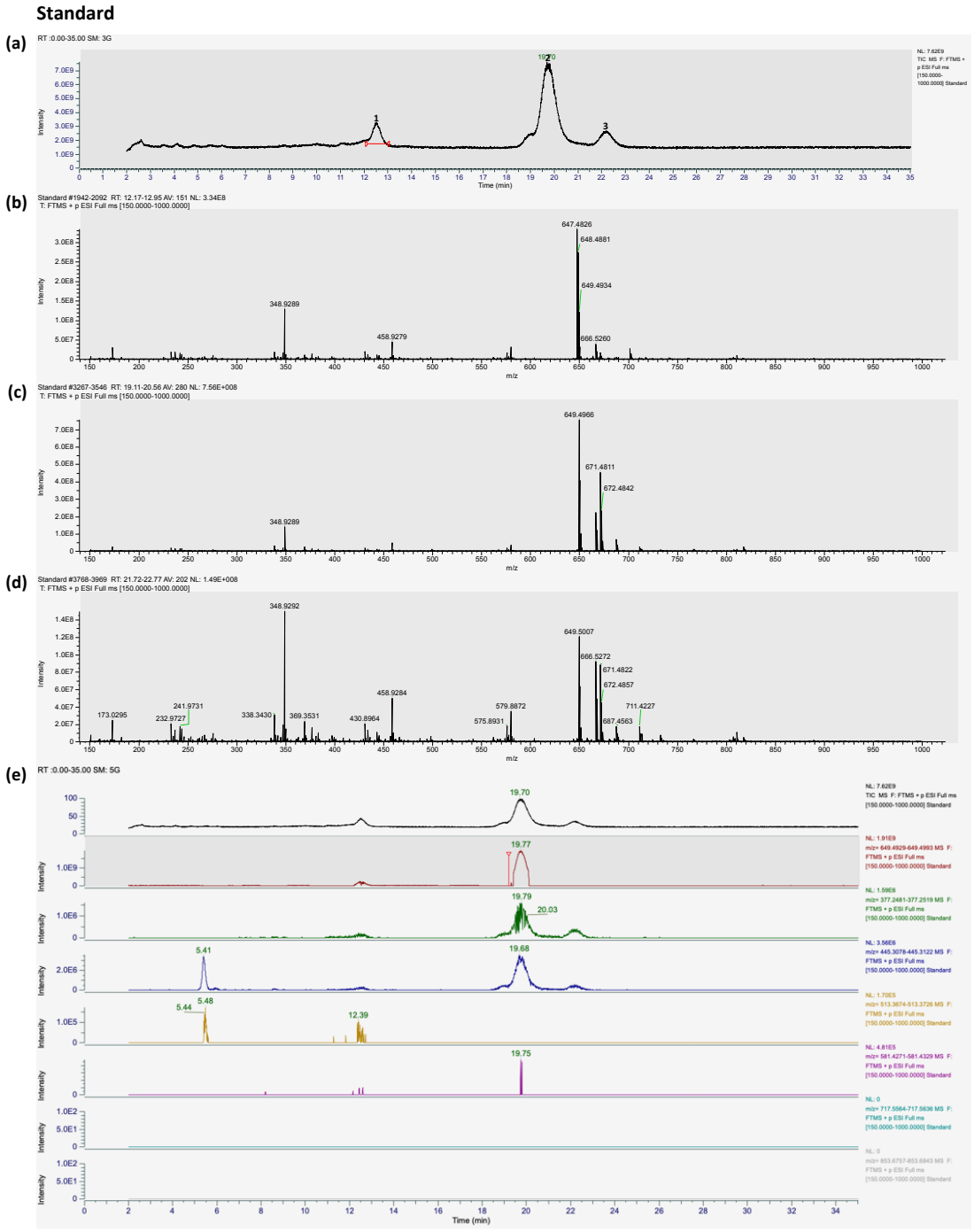
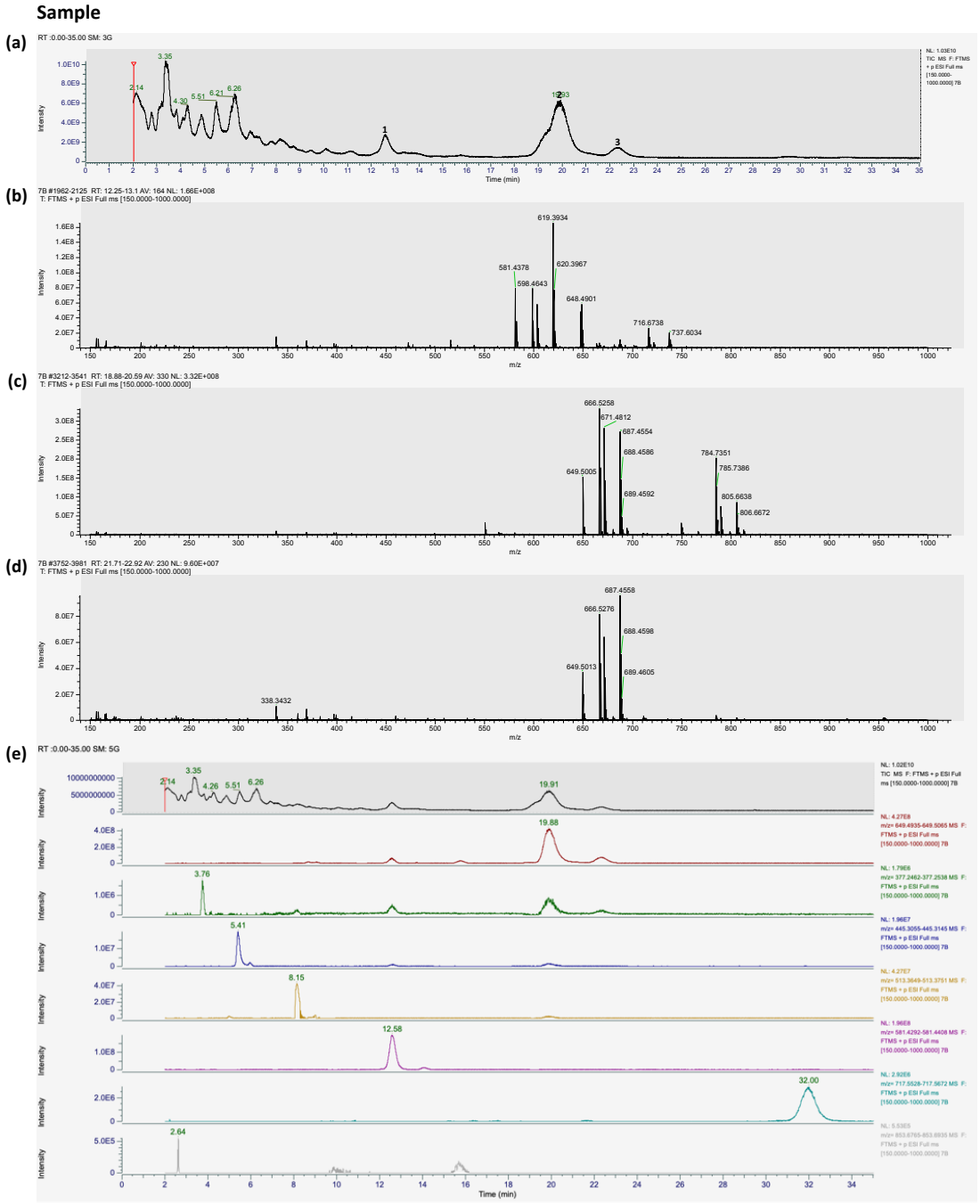


# Appendix B – LC-MS Data




(a) LC chromatogram, (b) MS data for peak 1, (c) MS data for peak 2, (d) MS data for peak 3, and (e) extracted ion chromatograms for the all-*trans* MK-7 reference standard




(a) LC chromatogram, (b) MS data for peak 1, (c) MS data for peak 2, (d) MS data for peak 3, and (e) extracted ion chromatograms for an experimental sample

# Appendix C – Co-Authorship Forms

The co-authorship forms detailing the contribution of all co-authors to each publication included in this thesis are provided on the following pages.

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| Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.<br><br>Chapter 4: "Optimisation of the Fermentation Media to Enhance the Production of the Bioactive Isomer of Vitamin Menaquinone-7"<br><br>This journal article has been published in Bioprocess and Biosystems Engineering. | |
| Nature of contribution by PhD candidate | Methodology, investigation, formal analysis, validation, visualisation, data curation, writing–original draft preparation, writing–review and editing |
| Extent of contribution by PhD candidate (%) | 90 |

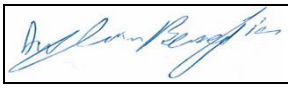
## CO-AUTHORS

| Name | Nature of Contribution |
|---|---|
| Mostafa Seifan | Conceptualisation, methodology, data curation, writing–review and editing, supervision |
| Aydin Berenjian | Conceptualisation, methodology, data curation, writing–review and editing, supervision |
| | |
| | |
| | |
| | |

## Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and

| Name | Signature | Date |
|---|---|---|
| Mostafa Seifan |  | 8 July 2023 |
| Aydin Berenjian |  | 8 July 2023 |
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| Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.<br><br>Chapter 5: "The Impact of Key Fermentation Parameters on the Production of the All-*Trans* Isomer of Menaquinone-7"<br><br>This journal article has been published in Biocatalysis and Agricultural Biotechnology. |

| | |
|---|---|
| Nature of contribution by PhD candidate | Methodology, investigation, formal analysis, validation, visualisation, data curation, writing–original draft preparation, writing–review and editing |
| Extent of contribution by PhD candidate (%) | 90 |

## CO-AUTHORS

| Name | Nature of Contribution |
|---|---|
| Mostafa Seifan | Conceptualisation, methodology, data curation, writing–review and editing, supervision |
| Aydin Berenjian | Conceptualisation, methodology, data curation, writing–review and editing, supervision |
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| Name | Signature | Date |
|---|---|---|
| Mostafa Seifan | Mostafa | 8 July 2023 |
| Aydin Berenjian | | 8 July 2023 |
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|---|---|
| Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.<br><br>Chapter 6: "The Effect of Iron Oxide Nanoparticles on the Menaquinone-7 Isomer Composition and Synthesis of the Biologically Significant All-*Trans* Isomer"<br><br>This journal article has been published in Nanomaterials. | |
| Nature of contribution by PhD candidate | Methodology, investigation, formal analysis, validation, visualisation, data curation, writing–original draft preparation, writing–review and editing |
| Extent of contribution by PhD candidate (%) | 90 |

## CO-AUTHORS

| Name | Nature of Contribution |
|---|---|
| Mostafa Seifan | Conceptualisation, methodology, formal analysis, data curation, writing–review and editing, supervision |
| Alireza Ebrahiminezhad | Formal analysis, visualisation |
| Aydin Berenjian | Conceptualisation, methodology, formal analysis, data curation, writing–review and editing, supervision |
| | |
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| Name | Signature | Date |
|---|---|---|
| Mostafa Seifan | Mostafa | 8 July 2023 |
| Alireza Ebrahiminezhad | alireza | 8 July 2023 |
| Aydin Berenjian | | 8 July 2023 |
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| Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.<br><br>Chapter 7: "The Impact of Amine-Functionalised Iron Oxide Nanoparticles on the Menaquinone-7 Isomer Profile and Production of the Bioactive Isomer"<br><br>This journal article has been published in Molecular Biotechnology. |

| | |
|---|---|
| Nature of contribution by PhD candidate | Methodology, investigation, formal analysis, validation, visualisation, data curation, writing–original draft preparation, writing–review and editing |
| Extent of contribution by PhD candidate (%) | 90 |

## CO-AUTHORS

| Name | Nature of Contribution |
|---|---|
| Mostafa Seifan | Conceptualisation, methodology, formal analysis, data curation, writing–review and editing, supervision |
| Alireza Ebrahiminezhad | Characterisation studies, formal analysis, visualisation |
| Aydin Berenjian | Conceptualisation, methodology, formal analysis, data curation, writing–review and editing, supervision |
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|---|---|---|
| Mostafa Seifan | Mostafa | 8 July 2023 |
| Alireza Ebrahiminezhad | alireza | 8 July 2023 |
| Aydin Berenjian | | 8 July 2023 |
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|---|---|
| Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.<br><br>Chapter 8: "Fermentation of Menaquinone-7: The Influence of Environmental Factors and Storage Conditions on the Isomer Profile"<br><br>This journal article has been published in Processes. | |
| Nature of contribution by PhD candidate | Methodology, investigation, formal analysis, validation, visualisation, data curation, writing–original draft preparation, writing–review and editing |
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