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A Rapid, Small-Scale Method for Improving Fermentation Medium Performance

A thesis submitted in partial fulfilment
of the requirements for the degree of

Master of Science (Technology)
in Materials and Process Engineering

by

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Hamilton, New Zealand 2007

ABSTRACT

Cell biomass and chemicals (e.g. bioactive compounds) can be produced by fermentation. Optimising a fermentation system involves optimizing many variables such as determining the effect of inoculum quality and media components, and selecting the most appropriate fermenter design and operating conditions (such as agitation aeration and fermentation mode). Identifying the optimal media is very important because it can significantly affect product concentration, yield and productivity. However, the media contains many components so many trials need to be done, which makes the process laborious, expensive, open-ended, and often time-consuming. The data generated from the many trials can be difficult to analyse.

This study developed a rapid, inexpensive small-scale technique to identify how media components affected the growth of *Streptomyces hygroscopicus* and its production of a secondary metabolite, the anti-tumour agent rapamycin. A method was developed using microtitre plates to screen the effect of three concentrations of nine media components on cell growth and rapamycin production using the Box-Behnken experimental design. Firstly, the methodology for microtitre plates was developed, which involved characterizing the physical parameters of a fermentation system, identifying the incubation time to minimize evaporation, modifying the assay method to deal with the small sample volumes, and developing an alternative method to determinate the rapamycin concentration that was cheaper than the HPLC method. Data from shake flasks trials (the normal screening method) were used to validate the microtitre method and to assess the latter's usefulness in predicting scale-up effects.

Six media components - sodium chloride (NaCl), di-potassium orthophosphate (K_2HPO_4), l-aspartic acid, l-arginine, l-histidine and salt (formula 1) solution - significantly affected culture growth and/or rapamycin concentration. The regression tree method was used to indicate the importance and critical concentration range of each factor. The Pearson's product-moment value indicated a good correlation between data from microtitre plates and shake flasks (cell growth: $r=0.75$ $p=0.016$ $n=8$; rapamycin concentration $r=0.92$ $p=0.08$ $n=6$).

The speed of the microtitre plate and shake methods were compared by assessing the total cycle time and the time required for various stages in the method. Performance of each method was assessed as cost of media and equipment. Using microtitre plates to screen and optimise media in terms of biomass and secondary metabolite production is faster and cheaper than using shake flasks. Labour efficiency for the numerous, repetitive, small-scale experiments was substantially increased. Trials could be run without well-to-well cross contamination.

The “regression tree” statistics methodology successfully showed the effect of input variables on target variables and identified effective medium component concentrations and any interactions. It is recommended that the microtitre plate procedure developed in this research may be applied to any study investigating the optimum media composition for growing other *Streptomyces spp.* strains, in screening studies when searching for new bioactive molecules, or for characterizing natural or recombinant/mutated micro-organisms.

ACKNOWLEDGMENTS

It gives me great pleasure to acknowledge the many people who have assisted me throughout this work. I would like to thank my supervisors Professor Janis Swan and Sarah Reader for their assistance and guidance over the course of this project.

I would like to thank Donal Krouse and Dawn Scott for providing the impetus for the work I've done. Without their encouragement and support I would not have achieved the work presented in this thesis.

For their support and insight over the length of this work and more, I would like to thank my colleagues Jason Ryan, Dr Max Kennedy, Dr Tony Woolhouse, Dr Sarah Underwood, Dr Simon Hinkley, Kaylene Larking, Gary Bentley and my husband Zifeng Li and my son Paley Guangping Li.

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Chapter One: Introduction

1.1 Motivation

Large scale fermentations to produce microbial cells, and intra or extra-molecular components involve growing the appropriate micro-organism on a complex media, normally in submerged fermentation vessels. Medium composition can significantly affect product concentration, yield and volumetric fermentation productivity. Medium cost can substantially affect overall process economics, especially of commodity products. Therefore, designing a fermentation medium is critical when developing an industrial fermentation. The media can also affect the ease and cost of downstream product separation, such as separating and purifying a protein product from a medium containing proteins.

The complexity of medium composition studies derives from the fact that final yields are determined not only by the amount of basic nutrients such as carbon and nitrogen sources contained in the commercially available ingredients, but also by the proportions in which they are mixed and by their physical state. This means that there are many challenges associated with medium design. Media design involves investigating how a system works, and what happens when an ingredient or process condition is changed. It can be a laborious, expensive, open-ended, and often time-consuming process involving numerous experiments and trials. In industry, it often must be done frequently because new mutants and strains are continuously being introduced. Many constraints need to be considered during the design process, and the industrial scale must always be kept in mind when designing the medium (Kennedy & Krouse 1999).

Any fermentation involving biocatalysts, such as micro-organisms, plant cells, animal cells or enzymes, will result in transformation and production of biochemical substances. Fermenter or bioreactors should provide optimal growth conditions for micro-organisms to achieve conversion and/or production of biological products. Generally, only one specific form of life has to grow and produce the desired product. To prevent contamination by other micro-organisms, sterility of the reactors and media are an indispensable requirement. Cleaning is important to make sure that

impurities will not affect the bioconversion or spoil the final product. Homogeneous conditions for temperature, pH, dissolved oxygen, substrate, and product concentration have to be maintained in the reactors to ensure consistent processes and products. The bioprocess needs to be controlled and all available measurements should be logged to enable quality assurance. Safety regulations also have to be followed to prevent accidents and release of toxic products. Therefore, fermentations take time and consequently any experiments are going to take time to get the results.

Screening applications such as media optimization, searching for new bioactive molecules or characterizing recombinant/mutated micro-organisms, always involves analysing many medium compositions or cultures (Minas *et al.* 2000). Several different experimental designs may be used for a single investigation. For example, screening the main factors affecting the process may be done using the Plackett-Burman method initially, followed by optimization of critical medium components using the response surface methodology. Thus, a medium design campaign can involve testing hundreds of different media.

Another difficult aspect of the medium design process is recording and analysing the data produced. In reality, information generated from design experiments is often difficult to assess because of its sheer volume. After about 20 experiments with five variables, the researcher may find it very difficult to remember the trends in medium components, especially when more than one variable has been changed at a time. Therefore, data capture and data mining techniques are crucial (Kennedy & Krouse 1999).

The 96-well microtitre plate format is a small-scale process. It is also a standard format amenable to automation technology. It is widely applied in clinical and drug research for three purposes: production, storage and analysis. More common applications for microtitre plates include ELISA assays, serology assays, antibiotic susceptibility assay, DNA purification, PCR applications and high-throughput screening utilizing fluorescence (Wells 2003). During the past decade, the 96-well microtitre plate has been developed to improve productivity for industry microbiology. For example, recently-described cultivation systems use 96-well microtitre plate to grow cultures, to produce secondary metabolites, for culture

storage (Duetz *et al.* 2000), and to isolate the high-producing strains from many mutants (Xu *et al.* 2005).

This research was based on the premise that the 96-well microtitre plates could be used to screening and optimizing a cultivation media system for growing *Streptomyces hygroscopicus* and producing rapamycin. As a lot of data would be generated quickly, a statistical experimental design was used to manage the data. The aim of this research was to develop a rapid, small-scale method to improve fermentation medium performance. It involved screening the main factors that affect the target variables to identify the optimal medium components. Data from the experiment was captured by the statistical analysis termed regression trees. After identifying the optimal conditions, the trials would be scaled to shake flask to test the correlation between the micro and small-scale runs and to validate the robustness of the medium design. A guideline, based on a correlation analysis of results, was generated for possible use in other secondary metabolite production fermentation systems.

1.2 Objectives

The overall aims of this research were to develop a method using 96-well microtitre plates to screen and optimize media for growth of specific micro-organisms that would be cheaper, easier and quicker than the current methodology involving shake flasks. The data would be analysed to identify the importance of each input variable on the target response (biomass and secondary metabolite production) and to identify whether there were any interactions between medium components and their optimal concentration range. As a lot of data would be generated, a further aim was to develop a simple and visible way to deal with data collection and analysis. The specific objectives were:

- To characterise the fermentation system of *S. hygroscopicus* in the 96-well microtitre plate format.
- To investigate whether 96-well microtitre plates could be used to identify the effect of specific media components and their concentrations on biomass production and rapamycin concentration by *S. hygroscopicus*.
- To validate and scale up the medium optimization for rapamycin production from microtitre plate to a shake flask system.

- To use statistical analysis methods to collect and integrate data produced from this study.

1.3 Approach

The approach taken in this study was to use 96-well microtitre plates to screen and optimize selected, commercially available, medium components for their effect on *S. hygroscopicus* growth and rapamycin production. Data for optimal growth conditions obtained from microtitre plate experiments were used in shake flask trials to confirm the characterisation of the formulation of the medium.

Initial experiments on agitation rate, growth temperature and fermentation explored whether the 96-well microtitre plate format could be used for growing *S. hygroscopicus* and producing rapamycin. Measuring biomass with the spectrophotometry microplate reader and the rapamycin bioassay were validated.

Microtitre plates experiment were conducted to characterize the effect of nine media components (input variables) and their concentration on two target response variables, culture growth and secondary metabolite production, using a Box-Behnken design method. Culture growth and rapamycin concentration were assayed. The target results and correlations were generated between the medium components and their concentration. The culture growth and rapamycin concentrations were separately presented by a single regression tree.

Eight medium formulations were identified using change-one-at-one-time strategy and performed in shake flasks to validate the results obtained from the microtitre plate. The culture growth and rapamycin concentration were measured to generate a correlation between the microtitre plates and shake flasks to validate the microtitre plate methodology.

1.4 Overview of the Thesis

Chapter two reviews fermentation technology, media for fermentation, developing fermentation media, statistical experimental design and optimization, and metabolite production pathways, and provides the foundation on which the subsequent discussion is based. Specifically the chapter reviews the broad subjects of

fermentation technology and metabolite production pathways to provide information on submerged fermentation systems, secondary metabolite production and the inoculum. Additionally, this chapter reviews current use of the experimental design methods and medium optimization techniques in fermentation medium development, the different types of nutrient sources, and their mechanism of action. Finally, the technique for the microtitre plate and the statistical analysis methods for normal distribution, regression trees and Pearson production moment correlation coefficient in general and applications are discussed.

Materials, equipment and media, analytical methods, experimental designs, data statistical analysis and experimental procedure used in this research are described in chapter three.

Chapter four presents the experimental results and discusses the development of a rapid, small-scale, microtitre plate format for producing rapamycin, and how the methodology can be applied to the screening of medium components. Data were used to investigate the interaction between the medium components and their optimal concentration range. Several medium recipes were generated and data from the microtitre plate were compared with those from shake flasks. The correlation analysis and system validation are discussed. The speed, costs, and production performance are assessed.

Conclusions and recommendations for further work are summarised in chapter five.

Chapter Two: Literature Review

2.1 Fermentation Technology

2.1.1 Aspects of Submerged Culture

Fungi and bacteria are commonly used in submerged fermentation to produce a wide range of primary and secondary metabolites. Many other plant, insect and mammalian cell lines are also used to produce secondary metabolites, but these systems usually are not as effective as bacteria and fungi.

Three distinct phases of a micro-organism's growth can normally be distinguished in submerged culture fermentations: the lag, the linear (also known as log or exponential), and stationary phases (Fig. 2.1). The autolytic, or death phase, is not usually classed as a growth phase. The length of the lag phase, where cells adapt to the new environment and begin to grow depends on the micro-organism, initial cell concentration, environmental conditions and growth medium. In the linear phase (sometimes called the tropophase), there is rapid growth and the stationary phase (sometimes called the idiophase), there is no further net growth. Secondary metabolites such as antibiotics are usually produced in the stationary phase (Pelczar & Reid 1972; Giancarlo & Rolando 1993).

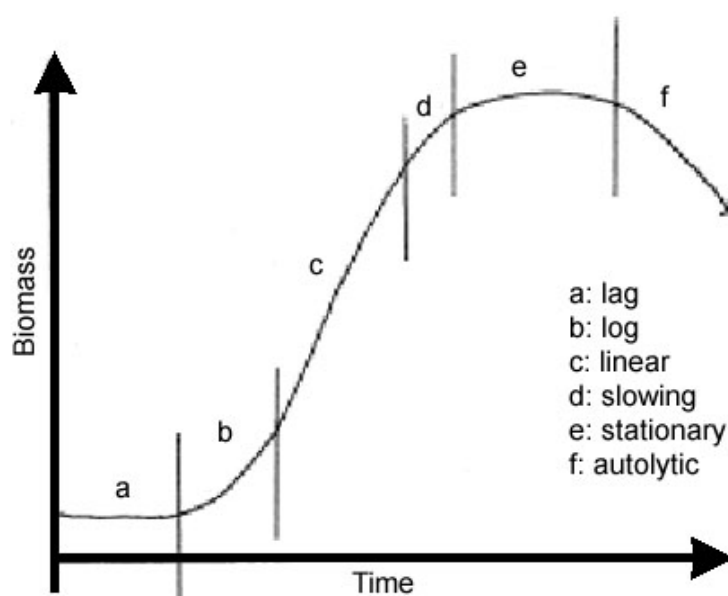


Figure 2.1 Typical growth phases for micro-organisms in submerged culture (Pelczar & Reid 1972)

Submerged cultures are widely used to produce many secondary metabolites because they allow filamentous fungi and bacteria to produce freely suspended mycelia and pellets, essential for secondary metabolite production (Pelczar & Reid 1972). Submerged culture fermentations require a stirred nutrient medium and, in the case of aerobic micro-organisms, a supply of oxygen.

The main focus of fermentation is to enhance the production of the desired product, whether it is a primary metabolite, secondary metabolite and/or biomass. Therefore optimising the fermentation is critical for ensuring the appropriate growth or production phase is extended.

Micro-organism morphology during the growth phase is influenced by strain, culture initiation method, growth medium and the hydrodynamic regime (Pelczar & Reid 1972; Casas *et al.* 2005). The morphology of filamentous bacteria and fungi in submerged cultures can vary from a network of freely dispersed mycelia to tightly packed, discrete pellets. This morphology can affect product yield. For instance, Casas *et al.* (2005) reported that lovastatin, a secondary metabolite produced by *Aspergillus terreus*, was significantly greater in tightly-packed pellets than in free mycelia.

Most bacterial and fungal fermentations have a high oxygen demand for biomass production and metabolite formation (Kalakoutskii & Agre 1976; Monnet *et al.* 1988; Casas *et al.* 2005). Media must contain at least a nitrogen and a carbon source to support microbial growth and metabolite production (Zhang *et al.* 2003). Rapidly growing filamentous fungi increase viscosity of submerged culture and concomitantly impeding secondary metabolites production (Casas *et al.* 2005).

2.1.2 Types of Bioreactors

Bioreactors for submerged microbial growth include shake flasks, mechanically stirred tanks, bubble column, airlift bioreactors, fluidized beds and packed bed bioreactors etc. Each has their own operating conditions.

The stirred tank (Fig. 2.2) is the most common type of bioreactor for large scale bacterial and fungal fermentations due to the relatively high oxygen transfer rate

(OTR) that can be obtained, its *in situ* sterilisation capability, and well-established scale-up relationships (Atkinson & Mavituna 1983; McNeil & Harvey 1990; Casas *et al.* 2005).

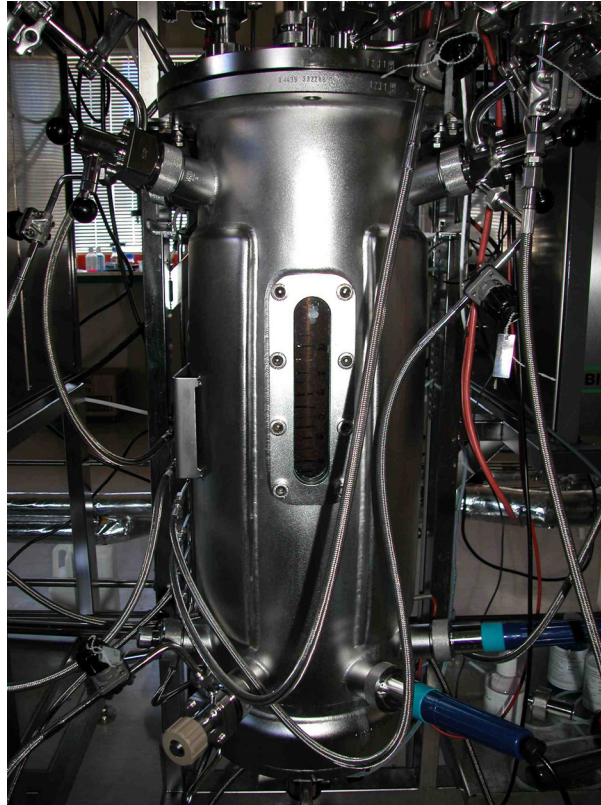


Figure 2.2 18-L working volume stirred tank bioreactor

The OTR is a very important aspect during the growth phase; if OTR is limited, productivity can be substantially reduced. Oxygen demand then decreases during the stationary phase (Atkinson & Mavituna 1983). The OTR in stirred tank bioreactors can be varied by varying agitation and airflow to ensure conditions are conducive for optimal growth and metabolite production. Micro-organisms with complex morphology, such as filamentous bacteria and fungi, usually have higher OTR requirements than simple single-celled bacteria. This oxygen can be distributed by using suitable agitation, airflow, baffles and impellor design (Atkinson & Mavituna 1983; Chen *et al.* 1999). Agitation in stirred bioreactors directly affects culture morphology of filamentous fungi and bacteria. Casas *et al.* (2005) reported that agitation speeds of $\sim 2.0 \text{ m s}^{-1}$ damaged *A. terreus* mycelia pellets, decreasing their size and lowering overall lovastatin production.

Shake flasks are used for media development, micro-organism strain selection, and small-scale fermentations. Examples include: culturing soil isolates in screening programs; comparing production capacity of mutant strains; assessing effect of media components on productivity; and providing inoculum for larger-scale fermentations (Giancarlo & Rolando 1993). Shake flasks vary greatly in size and form. Standard Erlenmeyer flasks, baffled shake flasks and Fernbach flasks are the most common types used for small scale microbial fermentations because they are readily available, their conical shape allows vigorous shaking without spillages, the small opening limits evaporation so slow-growing strains can be cultivated without excess moisture loss and the relatively large surface at the bottom allows a high surface-to-volume ratio (a main determinant of OTR) (Duetz & Witholt 2004). These features have helped Erlenmeyer flasks last the 20th century for generating cell mass, medium optimization and small-scale fermentation studies.

However, Erlenmeyer flasks are very inconvenient for handling and growing large numbers of aerobic strains or screening large numbers of medium components for new enzymes or secondary metabolites. Flasks cannot be handled mechanically (robotic handling) and there are limitations on miniaturisation/parallelisation. Surface-to-volume ratio decreases as flask volume increases, so oxygen transfer becomes limiting (Table 2.1). The OTR in flasks can be increased with baffles but baffles can produce more aerosols and more wall growth, and the hydromechanical stress can break the cells. Because of flask-to-flask variation, results from baffled flasks tend to be inconsistent (Duetz & Witholt 2004). Because OTR is an important factor in growth, flasks are normally used only for laboratory scale fermentations.

Table 2.1 Effect of fermenter design on typical oxygen transfer coefficient values (McNiel & Harvey 1990)

Fermenter	Oxygen transfer coefficient, K_La value (h^{-1})
Test-tube	20
Flat-bed bottle	50
Erlenmeyer flask	500
Baffled shake flask	1200
~10-L stirred bioreactor	3000-4000

Tubes have been used as alternatives to Erlenmeyer flasks. Their small size means many fermentations can be done simultaneously. However, tubes have other problems such as drying out, are not representative of stirred tanks, and can not be well aerated.

Another alternative for small-scale growth is the microtitre plate. The 96-well microtitre plate format is a small-scale, standard format amenable to automation and has been widely used in clinical and drug research for activities such as ELISA assays, DNA purification and high throughput screening drug discovery (Wells 2003). It has also been used for cultivating micro-organisms, producing secondary metabolites, culture storage (Minas *et al.* 2000; Duetz *et al.* 2000) and screening culture strains (Xu *et al.* 2005).

Duetz & Witholt (2004) investigated the OTR of differently sized square or round polypropylene wells in the horizontal plane on an orbital shaking. The OTR was measured enzymatically using glucose oxidase, horseradish peroxidase (HRP), and 2,2-azino-bis 3-ethylbenz-thiazoline-6-sulfonic acid (ABTS). The OTR for large square vessels (18 mm x 18 mm and 50 mm x 50 mm) was higher when the rotating 'throw' of the table was greater (Table 2.2). Square vessels had a regular shaking pattern, indicating they could be a viable, space-efficient, and alternative to baffled or unbaffled Erlenmeyer flasks.

Table 2.2 Effect of shaking diameter and vessel dimensions on OTRs for orbital shaking at 300 rpm and 25°C (Duetz & Witholt 2004)

Shape and size	Vessel volume (mL)	OTR (mmol O ₂ L ⁻¹)	OTR (mmol O ₂ L ⁻¹)
<i>Rotating throw</i>		<i>25 mm</i>	<i>50 mm</i>
Square wells			
4 mm x 4 mm	0.125	6.7	25.4 ± 5.9
8 mm x 8 mm	0.5	15.2 ± 2.4	33.6 ± 4.4
15 mm x 15 mm	2.5	39.3	50.5 ± 3.5
50 mm x 50 mm	19.5	47.7	58.1
Round wells			
6.6 mm diameter	0.26	5.0	16.4

2.1.3 Operating Modes for Submerged Bioreactors

Several techniques have been developed for growing micro-organisms in submerged culture. Batch and batch-fed have been used since antiquity and are still used for producing food products and secondary metabolites. In batch fermentations, all nutrients for growth and production are added during the initial setup. The micro-organism utilises all the nutrients until the fermentation is completed or harvested (McNeil & Harvey 1990; Casas *et al.* 2005).

In fed-batch fermentations, fresh medium containing specific nutrients are supplied in stages throughout the fermentation. Nutrient addition may be in response to specific events in the fermentation system such as adding a precursor for a particular metabolite during the production phase to enhance final product concentration. This system has greater control over the growth and production phase so final product concentration and/or yield is greater than achieved in a batch fermentation system while difficulties associated with obtaining the steady state required for continuous operation are minimised (Miller & Litsky 1976; McNeil & Harvey 1990).

Continuous culture techniques have been used successfully over the last 50 years and have been used to develop monitoring and control techniques (McNeil & Harvey 1990). Nutrient, organisms and metabolites are maintained at constant concentrations, known as a steady state. The main advantage of continuous systems is that the microbe's growth phase can be prolonged by continuous feeding of fresh nutrients and removal of spent medium and cells.

All fermentation systems require an inoculum, prepared either directly from a spore or vegetative cell bank or from streak plates or slants. Because industrial fermentation systems involve large volumes of medium, at least a two-stage inoculum is usually needed to produce sufficient biomass for large vessels. This is usually called the seed stage of the fermentation (Miller & Litsky 1976).

2.2 Fermentation Medium

2.2.1 General Review

Micro-organisms incorporate components from the fermentation medium into biomass and/or use them for metabolite biosynthesis. When a medium is used only for biomass production, biomass formation can be estimated by stoichiometry (Atkinson & Mavituna 1983). If, however, the micro-organism uses the medium components for producing metabolites and biomass, metabolite efficiency determines the concentrations of biomass and metabolite. The nutrient supplied to the micro-organism significantly affects metabolite concentration, both directly through metabolism and indirectly by influencing the specific metabolite production rate (Atkinson & Mavituna 1983). Secondary metabolite specific production rate can be influenced by catabolite regulation. Aharonwitz and Demain (1978) reported that cephalosporin production by *Streptomyces clavuligerus* is regulated by catabolic control. Cephalosporin specific production ($\text{g L}^{-1} \text{h}^{-1}$) was lower on glycerol and maltose carbon sources and higher on starch. Rapidly growing cultures usually have an adverse effect on secondary metabolite production, suggesting that the fermentation medium is critical for optimal metabolite production (Aharonwitz & Demain 1978).

2.2.2 Medium Components

Fermentation medium should allow a large biomass to be produced within the shortest time, and sustain the productive stationary phase for as long as possible. Medium composition is therefore the most important (and the most complicated) factor influencing production yields, particularly with high producing industrial strains (Giancarlo & Rolando 1993).

Fermentation medium for biomass and/or using metabolite biosynthesis must include sources of the following (Pirt 1975):

- The major elements carbon, hydrogen, oxygen and nitrogen
- The minor elements phosphate, potassium, sulphate and magnesium
- The trace elements iron, zinc, etc
- Vitamins and hormones.

Carbon sources provide both energy and the basic cellular building blocks for microbial activity. The carbon content of cells is high and carbon also plays a major role in energy production. Therefore, the carbon/energy component of the substrate is usually the major medium component. It frequently is the major cost of media. The most common carbon substrates used for antibiotic production are starch, oils, and various types of simple sugars, such as glucose (Pirt 1975; Giancarlo & Rolando 1993).

Nitrogen is a crucial component of proteins, nucleic acids, amino acids and enzymes for cell growth and function. Sources for industrial fermentations include proteins, ammonia and ammonium salts, urea, and nitrate salts (Bader 1988). When proteins are used, they must first be hydrolysed by extracellular proteolytic enzymes before they can be assimilated (Bader 1988). Proteins can supplement the carbon energy supply when other carbon sources are depleted. This releases ammonia, causing pH to become alkaline. Excessive ammonia levels can inhibit antibiotic production (Masuma *et al.* 1983).

Sources of phosphorus include inorganic phosphate and/or complex organic phosphates. Phosphate concentration is often critical, with only minor differences between concentrations that give satisfactory growth rates and those that inhibit antibiotic production (Giancarlo & Rolando 1993). Phosphorous is mainly incorporated into nucleic acids, phospholipids and cell wall polymers and is occasionally stored as polymeta-phosphate (Pirt 1975). The phosphorus requirement differs widely between cultures and fermentation conditions. Excess phosphate can strongly inhibit or repress antibiotic production (Bader 1988).

The medium must be balanced for major nutrients and for the mineral salt requirements. Minerals most often added are salts of sodium, potassium, calcium, and magnesium, which are added as counter ions of sulphate, phosphates, chlorides, etc. They also naturally occur in water and in other complex substrates.

Metal ions such as calcium, magnesium, manganese, iron, cobalt, copper, zinc and molybdenum may be important cofactors for high enzyme activity and/or production. They generally are required in very low concentrations (Bader 1988).

2.2.3 Complex Medium

Fermentation medium is a critical component of industrial fermentation and can directly affect the growth profile, metabolite production, productivity ($\text{g L}^{-1} \text{h}^{-1}$) and operations such as sterilisation, shear sensitivity of cells, nutrients requirements and broth viscosity (Zhang *et al.* 2003). The first stage of media development uses components known to assist microbial growth and metabolite production. Payne and Hagler (1983) observed that extracts from Brazilian peanuts known to contain aflatoxins produced from *Aspergillus parasiticus* induced aflatoxin production in submerged culture. Bacon *et al.* (1996) identified that *Fusarium sp.* produced fusaric acid in a mixed corn feed and successfully substituted corn feed with corn meal to produce fusaric acid in submerged culture.

Complex carbon and nitrogen sources, such as yeast extract and peptones are commonly used because they are inexpensive and provide a wide range of nutrients (Zhang *et al.* 2003). Yeast extract is produced by autolysing baker's or brewer's yeast at approximately 50°C. It is a rich source of various amino acids, peptides, water-soluble vitamins, trace elements and carbohydrates (Zakriskie *et al.* 1980; Zhang *et al.* 2003). Due to the poorly-controlled starting material and downstream processing, biomass and growth rates on yeast extract can vary by as much as 50% between different batches (Potvin *et al.* 1997).

Potvin *et al.* (1997) and Zhang *et al.* (2003) recommend that a representative sample of each raw material batch of a complex medium component be tested for several pre-determined parameters to minimise variability. Substitution methodology can be used for key carbon sources, nitrogen and trace minerals, based on standard statistical substitution models. It is possible to improve secondary metabolite production and consistency by screening common complex media components known to influence micro-organism growth or metabolite production (Potvin *et al.* 1997; Zhang *et al.* 2003).

2.2.4 Defined Medium

Chemically-defined media (also called synthetic media) are fully-characterised mixtures of carbon, nitrogen and trace metal components. They can enhance metabolite production through directed biosynthesis, improved process control,

fermentation consistency and enhanced product recovery (Basak & Majumdar 1973; Zhang *et al.* 1996; Zhang *et al.* 2003). Other benefits of defined medium included reduced foaming and viscosity, which improves ease and economics of product recovery (Zhang *et al.* 1996). Using defined medium for secondary metabolite production allows consistent microbial growth and metabolite production between batches, an essential requirement in large-scale industrial antibiotic production (Basak & Majumdar 1973; Zhang *et al.* 1996; Zhang *et al.* 2003). It has been successfully used for antibiotic production by a wide range of *Streptomyces* species.

Hajjaj *et al.* (2001) propose that using specifically-defined carbon and nitrogen is critical as the source of precursors and cofactors for synthesising secondary metabolites. Stringent starvation conditions promoted by selective nutrients can promote secondary metabolite production. Darken *et al.* (1960) reported that adding chloride ions in a synthetic medium for *Streptomyces aureofaciens* produced chlortetracycline whereas having no chloride produced tetracycline.

Defined carbon sources can give specific metabolic activity during growth and may exert complex regulations on gene expression and enzyme activity during antibiotic synthesis (Williams & Katz, 1977; Hajjaj *et al.* 2001). For example, including D-fructose in a synthetic medium for actinomycin D by *Streptomyces parvulus* decreased biomass production rate and ensured both carbon and energy were available during antibiotic production (Williams & Katz 1977).

Nitrogen in defined media may be a combination of organic (amino acid, amines, etc) and/or inorganic (nitrates, ammonia, nitrite, etc) sources. For most filamentous fungi, ammonium nitrate, sodium nitrate, and urea can be used for biomass formation and metabolite production (Hajjaj *et al.* 2001). Filamentous bacteria, however, utilise organic nitrogen sources more effectively than inorganic nutrients. For example, *Streptomyces griseus* cannot use nitrates as the sole nitrogen source (Williams & Katz, 1977; Zhang *et al.* 1996); organic acids, such as glutamic acid, histidine and proline produce three times more actinomycin D than inorganic nitrates, nitrites and ammonium salts (Williams & Katz 1977); and adding amino acids such as asparagine and proline stimulated biomass growth, respiration and

secondary metabolite production in some *Streptomyces spp.* and *Aspergillus spp.* (Payne & Halger 1983).

Basal trace metal solutions used for most chemically-defined media for *Streptomyces spp.* contains magnesium sulphate, calcium chloride, cobalt chloride, ferric sulphate, zinc sulphate, copper sulphate and di-potassium orthophosphate (Basak & Majumdar 1973; Williams & Katz 1977; Zhang *et al.* 1996; Hajjaj *et al.* 2001). Only low concentrations of the secondary metabolite, actinomycin D, are produced if a chemically-defined media lacked iron, magnesium and sometimes zinc ions (Williams & Katz 1977). Knowledge of the metabolic pathways for secondary and primary metabolites allows defined medium to be optimised (Williams & Katz 1977).

2.3 Developing Fermentation Medium

2.3.1 Medium Formulation

Designing a nutrient medium for growth and product formation is a key step in experimental or production runs. Chemical constituents of the medium must meet all elemental requirements for cell mass and products and must supply appropriate energy for synthesis and maintenance. Specific vitamins and trace minerals requirements must also be met. Any medium must contain, as a minimum, the correct proportions of the typical elemental composition of a microbial cell (Table 2.3).

Table 2.3 Typical elemental compositions for micro-organisms (Wang *et al.* 1979)

Element	Percent cell dry weight
Carbon	50
Nitrogen	7-12
Phosphorus	1-3
Sulphur	0.5-1.0
Magnesium	0.5

Although many micro-organisms can grow well on simple mineral salts media, some micro-organisms cannot synthesize all their own biochemical components and require one or more specific biochemical compounds. The most frequent

requirements are for vitamins and amino acids. Yeasts, for instance, often require biotin, thiamine, and riboflavin (Wang *et al.* 1979).

Micro-organisms can convert basic chemicals into complex molecules, but the second law of thermodynamics cannot be violated. The complex series of synthetic reactions performed in the cell requires energy, which usually comes from controlled oxidation of organic compounds. Thus, carbon compounds produce energy for biosynthesis as well as to meet the cell's elemental carbon requirement. Most heat is evolved during the terminal oxidation stages, where energy-carrying cofactors are used to reduce oxygen to water. To satisfy the cell's requirement for carbon and energy, sufficient carbon for biosynthesis and energy generation must be supplied (Wang *et al.* 1979).

Oxygen, the remaining nutrient is a sparsely soluble gas. Supplying oxygen involves mass transfer. Oxygen demand depends on the carbon source and utilisation efficiency (Wang *et al.* 1979).

Fermentation medium formulation has a large effect on fermentation processes. Fermenter design and process control have been widely studied to identify the optimal culture environment required for producing antibiotics. A fermentation protocol that initiates microbial growth to a sufficient density to facilitate producing large quantities of the antibiotic needs to be developed. However, cellular proliferation must not be too dense to prevent cells from obtaining vital nutrients and oxygen.

2.3.2 Media Development

There is a characteristic improvement pattern involved in developing a fermentation medium. The maximum (or minimum) values of various indicator variables such as volumetric productivity or medium cost per unit product plateau as the number of media tested increase. These relationships help indicate the number to be tested before a new optimisation strategy must be used (Kennedy *et al.* 1994).

As a very large number of fermentation substrates are available, open-ended studies require that many possibilities need to be investigated. Kennedy *et al.* (1994)

suggested medium design could be broken down into at least four stages. The first stage involves screening a large number of medium components to see which gives the best results. The process may be continued under the assumption that some ‘magic’ component may be found that drastically improves productivity. The second stage is to map the optimisation space with the aim of obtaining a complete picture of the effect of each component in the medium. The third stage involves identifying the region of optimum performance, which can be done using various mathematical tools. Stage four of the medium design process involves examining this optimal region.

Strategies for improving fermentation medium performance include: borrowing, component swapping, biological mimicry, one-at-a-time changes, statistical and mathematical techniques, experimental design and optimisation, artificial neural networks, fuzzy logic, genetic algorithms, continuous fermentation, pulsed batch and stoichiometric analysis (Kennedy & Krouse 1999). Each technique has advantages and disadvantages for particular situations, so there is no unique or optimum technique. However, it is advantageous to apply the techniques in a logical way and to combine them with good experimental design (Kennedy & Krouse 1999). The experimental design will specify the medium variants to test including replicates and how to arrange tests into an homogeneous ‘block’. Then a mathematical model is used with the data obtained to predict an improved medium composition. Often several iterations of the experimental design and optimisation are required, which involves many experiments (Kennedy *et al.* 1994).

2.3.3 Experimental Design

Experimental design or “design of experiments” is a body of knowledge, based on statistical and other scientific disciplines, for efficient and effective planning of experiments and for making sound inferences from the data. Through planned and balanced experimental runs, the effects of input variables and interactions on the response variables can be clearly seen. This gives an understanding of how a system works and what may happen if an ingredient or process condition is changed.

There are many experimental design strategies available, including full factorial, Plackett-Burmann, Hadamard, orthogonal arrays (OA), orthogonal Latin rectangles

and central composite (CCD). The full factorial approach examines every possible combination of independent variables and is useful for a small number of variables but impractical for larger studies because of the many experimental runs required. The Plackett-Burman design involves two-level partial factorials and reduces the experiments to a minimum by screening $N-1$ variables in N runs. It is often used when more than five independent variables are being investigated. The disadvantage of the Plackett-Burman design is that it only allows first order effects and ignores potential interactions. Compared with other medium design strategies, the Plackett-Burman design is simple and is often used to evaluate the important factors affecting culture requirements, biosynthesis production and extracting antimicrobials from fermentation broth.

Soliman *et al.* (2005) used the Plackett-Burman design to evaluate the effect of different culture conditions on polyglutamic acid (PGA) production by *Bacillus sp* SAB-26. Of the 15 variables examined, K_2HPO_4 , KH_2PO_4 , $(NH_4)_2SO_4$ and casein hydrolysate were the most significant. The optimised media produced 33.5 g L^{-1} PGA, which was over three times greater than the basal medium.

Bie *et al.* (2005) used the Plackett-Burman design to evaluate the importance of six factors, including methanol, ethanol, propanol, butanol, pH and time, for extracting an antimicrobial from *Bacillus sp* fmbJ. The most important components were methanol ($P < 0.0001$), ethanol ($P < 0.0001$), pH ($P < 0.0032$), and extraction time ($P < 0.0001$). Within the ranges tested, increasing methanol, ethanol, and time increased total extracted while increasing pH decreased extraction. The maximum prediction profile indicated that 50.21 mg of antimicrobial substance could be extracted from 100 mL ($P > 0.996$).

2.3.4 Optimization Techniques

Statistical optimization has become a common practice in biotechnology. Traditional methods involved changing one independent variable while others were fixed. This single-dimensional search is laborious, time-consuming, and cannot reach a true optimum because of interactions between variables. Response surface methodology (RSM) was first described by Box and Wilson (1951) as an experimental strategy for

obtaining optimum conditions of a multivariable system. It is widely applied in developing many food, fermentation and drug processes.

The key to RSM is representing the responses (e.g. product yield) as a surface. This optimisation method has several phases but the two main ones are following the path of steepest ascent and locating a stationary point (using canonical analysis). A path of steepest ascent is the direction where the surface rises most rapidly while a stationary point is a point of equilibrium such as a summit, saddle or ridge (Kennedy & Krouse 1999). Process optimization using RSM usually involves simultaneous testing of many factors in a limited number of experiments. Therefore, RSM takes less time and effort than the traditional, one-factor-at-a-time approach. This method quantifies possible interactions between factors, which is difficult to obtain using traditional optimization techniques. Detecting and quantifying interactions between various factors are of critical importance, especially for optimizing multivariate processes in biotechnology.

Roy *et al.* (2002) used RSM to estimate the values, associated *t*-values and significance level, and interactions for the effects of lactose concentration, cell concentration, reaction time, and temperature on galacto-oligosaccharide (OS) production by *Bifidobacterium infantis* RW-8120. Lactose had the greatest effect ($p < 0.01$) on OS production and yield. There was a small interaction between cell concentration and temperature but reaction time had no statistical effect.

Kiviharju *et al.* (2004) used composite face-centred (CCF) experimental designs to investigate the effect of environmental pH, temperature and dissolved oxygen (DO) when developing a complex medium for cultivating *Streptomyces peucetius* var. *caesius* N47 and to optimize aeration, pH, temperature and stirring rate for efficient ϵ -rhodomycinone production. Increasing the temperature increased both cell growth and glucose consumption. Best ϵ -rhodomycinone productivities were obtained around 30°C. Controlling DO increased all growth phase responses, but aeration in the production phase coupled with pH decreased ϵ -rhodomycinone production. In the non-aerated production phases, changing pH improved productivity. A higher pH and a lower temperature increased productivity. The data implied that dynamic

control strategies could increase overall process productivity of batch ϵ -rhodomycinone production.

Sharma and Satyanarayana (2006) optimised production of a highly alkaline and thermostable pectinase in submerged fermentation of *Bacillus pumilus*. The three fermentation variables (C:N ratio, K_2HPO_4 , and pH) identified by the Plackett-Burman design as most affecting pectinase production were further optimized using RSM and CCD. By optimising the variables, enzyme production increased more than 34-fold in shake flasks and more than 41-fold in a laboratory fermenter.

Delgado *et al.* (2005) found that bacteriocin production by *Lactobacillus pentosus* B96 can be expressed as a function of temperature and NaCl concentration. A two-level screening Plackett-Burman design was used to select the most important variable, then a CCD with three repetitions of pH, NaCl concentration, and temperature was done. Finally, using RSM that included the region of maximum accumulated bioactivity was done as a function of NaCl concentration and temperature. Bioactivity always increased during the exponential growth-phase. The culture grew best at about 30°C, neutral pH, and without NaCl. Environmental conditions for maximum bacteriocin activity could be expressed as a polynomial of temperature and NaCl concentration.

2.3.5 Box-Behnken Design

Multivariate optimization appears to be a more interesting and complete alternative, allowing maximum information to be obtained due to the possibility of evaluating interactions between variables. It also represents a more economical approach, as the number of experiments can be significantly reduced (Maranhao *et al.* 2007). One possibility is to use a Box-Behnken design, where the number of experiments is defined by the equation $N = k^2 + k + c_p$, where k represents the number of factors (parameters) involved in the study and c_p is the number of replicates of the central point (Maranhao *et al.* 2007). Box-Behnken design can be considered as a cube, consisting of a central point and the middle points of the edges (Fig. 2.3).

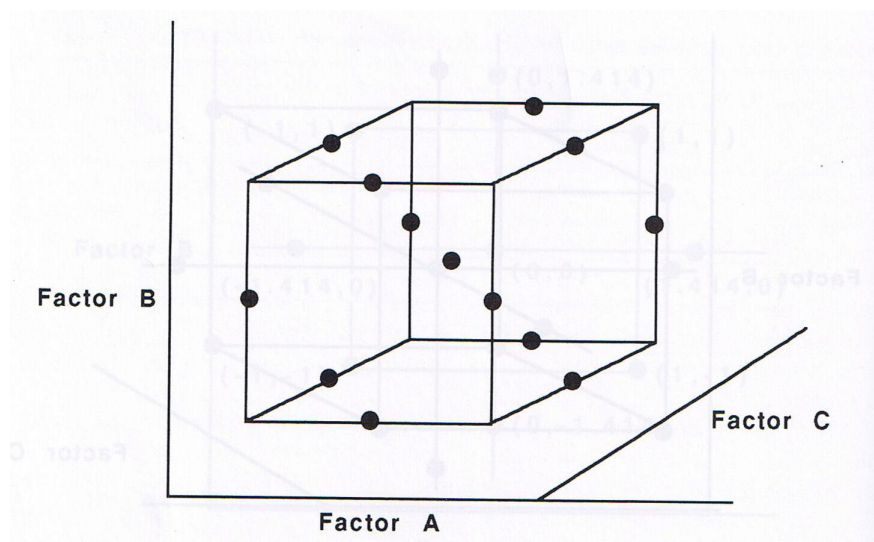


Figure 2.3 Box-Behnken design for a three-factor experiment (Armstrong & James 1996)

Values of the experimental points for this design are given in Table 2.4 (Armstrong & James 1996). A value of “+1” indicates that the corresponding factors is set to high level, a “-1” indicates the factor is set to its low level, and a “0” indicates the factor is set to its mid level.

Table 2.4 Box-Behnken design for a three-factor experiment (Armstrong & James 1996)

Factor A	Factor B	Factor C
0	-1	-1
-1	-1	0
0	-1	+1
+1	-1	0
-1	0	-1
-1	0	+1
+1	0	+1
+1	0	-1
0	0	0
-1	+1	0
0	+1	+1
+1	+1	0
0	+1	-1

The Box-Behnken design has become of increasing interest in areas such as analytical chemistry and optimization of process parameters. Applications included on-line pre-concentration systems (Souza *et al.* 2005) and capillary electrophoresis (Ragonese *et al.* 2002). Lotfy *et al.* (2007) used a Box-Behnken design to investigate the correlation between three variables (beet molasses, corn steep liquor and

inoculum concentration) and citric acid yield by a novel *Aspergillus niger* isolate. Estimated optimum media composition for citric acid production was 240.1 g L⁻¹ pre-treated beet molasses, 10.5 g L⁻¹ corn steep liquor, and 10⁸ spores mL⁻¹. This gave a citric acid yield of 87.81%, which was 14 times higher than the basal medium.

Abdel-Fattah *et al.* (2004) used a Box-Behnken design to investigate the correlation between three variables and uricase activity from *Pseudomonas aeruginosa*. The optimal combination, evaluated from the non-linear optimization algorithm of EXCEL-Solver, were pH 5.5, 0.001M CuSO₄ and 0.01M FeSO₄. The predicted optimum uricase activity of 7.1 UmL⁻¹ min⁻¹ was 16.5 times greater than from the basal medium.

2.4 Metabolite Production Pathway

2.4.1 Primary Metabolites

Primary metabolites include end-products of low molecular weight that can be building blocks for essential macromolecules or included into coenzymes. Intermediates in end-product biosynthetic pathways are also considered primary metabolites. Amino acids, purine and pyrimidine nucleotides, and the vitamins are the most important primary metabolites for industrial commercialisation. Other primary metabolites include intermediates of the major pathways of intermediary metabolism such as the Embden-Meyerhof pathway, the pentose phosphate pathway, and the tricarboxylic acid cycle. Under this interpretation, organic acids such as citric and fumaric acids are considered primary metabolites (Wang *et al.* 1979).

Cultures used to produce primary metabolites often undergo intensive development programmes to identify environmental and genetic modifications that decrease regulation and increase overproduction. Feedback regulation prevents oversynthesis of amino acids and nucleotides. Environmental manipulation can reduce the cell's ability to accumulate intracellular inhibitory and repressive end-products. Genetic manipulation can alter the enzyme or the rate of enzyme formation or decrease the sensitivity to feedback. For example, increasing bacterial membrane permeability can induce glutamic acid overproduction (Wang *et al.* 1979).

2.4.2 Secondary Metabolites

Secondary metabolites usually are mixtures of a closely-related chemical family, each being produced by a narrow taxonomic range of organisms. Production is easily lost by mutation (strain degeneration) (Wang *et al.* 1979). Although not required for growth, these metabolites may increase the organism's survival. Bacteria from the Actinomycetales group (commonly called Actinomycetes) and many fungi commonly produce secondary metabolites. These micro-organisms have filamentous growth and a complex morphology (Martin & Demain 1980; Guarro *et al.* 1999; Calvo *et al.* 2002).

Secondary metabolites can be classified into three broad categories: antibiotics pigments and toxins (Maggon *et al.* 1977; Calvo *et al.* 2002). After penicillin was identified and commercialised in 1930s, the first antibiotic produced by the filamentous bacterium *Streptomyces* spp was actinomycin in 1940. A successive range of similar antibiotics were identified in the following decades (Baltz 2005).

The effect of precursors in biosynthesis of secondary metabolites is very important. Occasionally, a precursor that increases secondary metabolite production is found or the precursor may direct formation of one specific product. In many fermentations, however, precursors show no activity because their synthesis is not rate-limiting. These effects are usually due to interactions of these compounds with regulatory mechanisms in the fermentation micro-organism.

Secondary metabolites are usually not produced during the rapid growth phase (linear phase or trophophase) but during the subsequent stationary phase (idiophase) (Fig. 2.4). Thus, they have also been termed “idiolites”. The delay in secondary metabolite formation is one of the major mechanisms that prevent ‘suicide’ in antibiotic-producing micro-organisms (Demain 1974a).

At the beginning of the trophophase, most micro-organisms are sensitive to their own antibiotic. However, during the idiophase, they become physiologically resistant to the antibiotic they are producing. Product formation usually starts when some nutrient such as a readily utilized sugar, nitrogen or phosphate is exhausted from the medium. These limitations may cause an inducer of secondary metabolic enzymes to

accumulate or release the genes of secondary metabolism from catabolite repression (Wang *et al.* 1979).

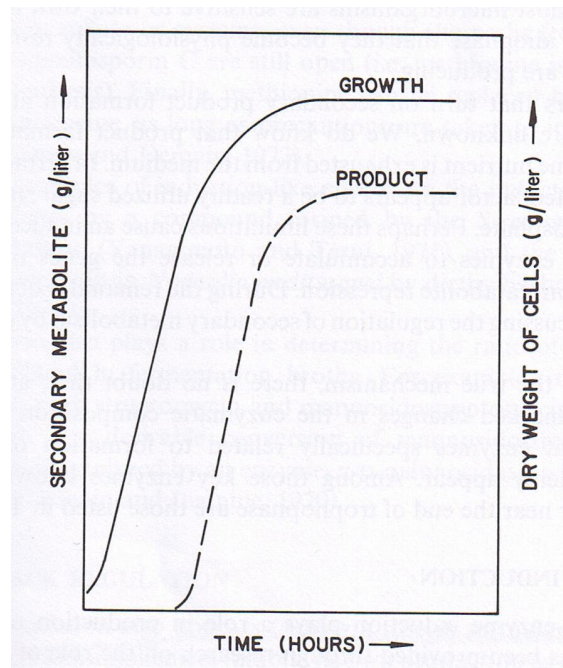


Figure 2.4 Cell growth and secondary metabolite production (Wang *et al.* 1979)

At the end of trophophase, marked changes in enzymatic composition occur and enzymes specifically related to forming secondary products suddenly appear. The role of enzyme induction in producing secondary metabolites was obtained by researching the role of tryptophan, a stimulator of alkaloid biosynthesis in *Claviceps* (Bu'Lock & Barr 1968). A similar induction effect was seen in methionine stimulation of cephalosporin C biosynthesis in *Cephalosporium acremonium* (Demain 1974b). Enzyme induction also affects the ratio of closely related antibiotics found in fermentation broths. For example, streptomycin and mannosidostreptomycin are produced concurrently in streptomycin fermentations. The desirable conversion of mannosidostreptomycin into streptomycin is catalysed by an enzyme, α -D-mannosidase, which is induced by mannan (Demain & Inamine 1970).

Feed-back regulation also affects secondary metabolism. For example, the amino acids tyrosine, tryptophan, and phenylalanine inhibit candicidin production by *S. griseus* (Liu *et al.* 1972). These amino acids are end products of a branched pathway leading also to *p*-aminobenzoic acid, a precursor of candicidin. Negative feedback regulation of an enzyme in the early common pathway of the amino acid would tend

to inhibit candicidin formation. Another example where feedback regulation controls secondary metabolites formation involves the feedback inhibition and repression of phosphatases by phosphate (Wang *et al.* 1979). At least three phosphatases are involved in the formation of the streptidine moiety of streptomycin biosynthesis. The enzyme that converts the final intermediate, streptomycin phosphate, to streptomycin is markedly inhibited by phosphate. Thus, streptomycin fermentations with high phosphate concentrations produce mainly phosphorylated streptomycin, which is biologically inactive as an antibiotic (Miller & Walker 1970).

2.4.3 Inoculum in Secondary Metabolites

Two development phases are normally recognized in microbial cultures producing secondary metabolites (Fig. 2.4): a vegetative phase (trophophase) with vigorous growth and negligible antibiotic production; and a fermentative phase (idiophase) where the culture is stationary and the antibiotic production is initiated. There are several good reasons to carry as much of the trophophase as possible in a separate vessel from the productive phase (Giancarlo & Rolando 1993). Experience has demonstrated that final antibiotic yields are influenced by size and metabolic state of the biomass used to initiate the fermentation stage. The importance of inoculum preparation has long been recognised (Orlova & Prokofieva-Belgovskaya 1961; Meyreth & McIntosh 1963) but there is very little published information.

Inoculum 'quality' is often stated to be important in determining performance of a final stage fermentation, although how this is best assessed is open to question. Many industrial processes use carbon dioxide evolution rate (CER) to determine the time to transfer fermenter-grown inoculum to the final stage production fermenter (Buckland *et al.* 1985). Other measures of inoculum quality include cell motility in *Clostridium acetobutylicum* fermentations (Gutierrez & Maddox 1987) and levels of specific intracellular enzymes in *Saccharomyces cerevisiae* fermentations (Millan *et al.* 1991). Smith and Calam (1980) suggested aldolase; glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase concentrations could be a rapid way to assess inoculum quality in *Penicillium spp.* fermentations.

Differences in culture morphology of mycelial organisms and inoculum size can play a key role in determining final stage morphology, and hence metabolite production

(Braun & Vecht-Lifschitz 1991). Inoculum morphology is not generally used to indicate inoculum quality although assessing morphology is implicit in the use of 'expert' visual examination to determine transfer times in some fermentations (Webb & Kamat 1993).

Seed medium composition was important factor in a titre development program for avermectin production by *Streptomyces avermitilis* (Nallin-Omstead *et al.* 1989). A combination of improved cultures and alternative seed media increased avermectin production 10-fold. Similarly, enhanced cephalosporin production was achieved by manipulating seed medium in *Cephalosporium acremonium* fermentations (Kozina *et al.* 1982).

If multiple seed stages are required to build up inoculum volume, a defined passage number is particularly important. Increased passage number adversely affected penicillin production by *Penicillium chrysogenum*, novobiocin production by *Streptomyces niveus* (Parton & Willis 1990) and tobramycin production by *Streptomyces cremeus* (Motkova *et al.* 1981). The detrimental effect of serial passages could be reduced by manipulating the seed medium and incubation conditions when producing milbemycin by *S. hygroscopicus* (Warr *et al.* 1996).

El-Enshasy *et al.* (2000) reported that production natamycin by *Streptomyces natalensis* is highly dependent on the oxygen level in the cultivation medium and the type of inoculum. Increasing the shaking speed and decreasing the medium volume improved both the volumetric and specific natamycin production. Decreasing the DO in the cultivation medium by adding a soluble biopolymer (alginate) significantly decreased natamycin yield without affecting cell growth.

Minimising contamination and process economics are other reasons to investigate the inoculum process (Giancarlo & Rolando 1993). If the final stage involves using a large inoculum, the effect of a few contaminant cells will be negligible. However, developing the vegetative culture in smaller vessels is convenient and reduces the capital cost of a fermentation plant and decreases other costs such as energy for aeration and agitation.

2.4.4 *Streptomyces hygroscopicus* Growth Conditions

Actinomycetes are gram-positive, commercially important bacteria. They produce approximately two-thirds of all known bioactive compounds. Many have clinical uses due to their activity against different organisms as antibacterial (macrolides, avermectins), anti-tumour (angucyclines, anthrax-cyclines, aureolic acid group) and immunosuppressant (rapamycin, FK506) activity. Most of these clinically useful pharmaceuticals are polyketides (Shapiro 1988; O’Cleirigh *et al.* 2005).

Streptomyces hygroscopicus from the *Actinomycetes* group, is a gram-positive, obligate aerobic bacterium that shares the environment with other bacteria and filamentous fungi, producing useful enzymes and secondary metabolites. This microbe forms either filamentous or densely aggregated mycelia, which may fragment or coalesce into pellets during fermentation (Chen *et al.* 1999).

Most *Actinomycetes* fermentations produce dense mycelia. Secondary metabolite formation (including antibiotics) is usually favoured by suboptimal growth conditions. Nutrient transfer problems can restrict culture metabolism, which is especially critical when the rheological properties of the culture shift from Newtonian to non-Newtonian during longer fermentation processes (Shapiro 1988). When developing a microbial fermentation medium strategy, it is necessary to maintain the medium components at an optimal level and to induce the micro-organism to switch from primary to secondary metabolism to produce high yields of the desired antibiotic. *Streptomyces spp.* are of biological interest for many reasons and the differentiation of *Streptomyces* can serve as a model system for industry microbiology study.

Rapamycin is produced by a complex medium usually containing glycerol, peptone, yeast extract, sodium chloride, lysine, phosphate salts and low concentrations of trace salts. *Streptomyces hygroscopicus* is grown in aerated, stirred, submerged culture at temperatures between 25 and 28°C and pH between 6.2 and 6.8. Additional carbon and lysine feeding is sometimes used during the trophophase to enhance rapamycin production by providing biosynthetic precursors. Improved rapamycin production has also been observed when DO is controlled above 30% air saturation (Chen *et al.* 1999). Kojima *et al.* (1995) gave a defined medium for

producing rapamycin where complex carbon and nitrogen components were replaced with fructose, mannose, and lysine and ammonium sulphate. Adding ammonia sulphate decreased medium pH significantly, requiring higher MES buffer concentrations (Kojima *et al.* 1995; Lee *et al.* 1997).

Improving culture productivity can be achieved in several ways. Conventional approaches usually concentrate initially on optimising the final fermentation stage but understanding the seed stage can also improve final stage productivity. Warr *et al.* (1996) reported significantly greater milbemycin production by *S. hygroscopicus* by manipulating several factors in the seed stage fermentation. Reducing passage number, using juvenile seeds, and higher incubation temperatures generally increased mibemycin production of both *S. hygroscopicus* strains used in this study.

2.4.5 Rapamycin and the Rapamycin Biosynthetic Pathway

Rapamycin (Fig. 2.5) is a 31-membered nitrogen-containing macrocyclic triene polyketide produced by *S. hygroscopicus*. It was first identified in a soil sample from Easter Island in 1975 (Sehgal *et al.* 1975; Vezina *et al.* 1975; Khaw *et al.* 1988; Ritacco *et al.* 2005). While originally discovered as a very active antifungal agent against *Candida* species (common human pathogenic yeasts) (Vezina *et al.* 1975), rapamycin has since been developed as an immunosuppressant (Sehgal *et al.* 1994). Its immunosuppressant activity results from inhibiting T and B cell proliferation and through the same mechanisms it also blocks cancer cell proliferation (Law 2005).

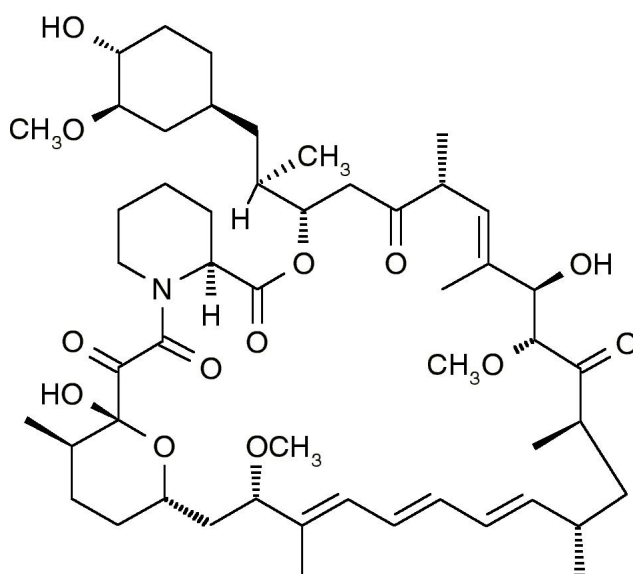


Figure 2.5 Structure of rapamycin (Ritacco *et al.* 2005)

Early preclinical and clinical studies indicate that rapamycin also possesses anti-tumour activity, with good activity against mammary, colon, and brain tumour model systems and against transplanted tumours (Eng *et al.* 1984). It appears to inhibit tumour growth by halting tumour cell proliferation, inducing tumour cell apoptosis, and suppressing tumour angiogenesis (Law 2005). Rapamycin derivatives are effective anti-tumour agents, both alone and when combined with other modes of therapy. These rapamycin actions are mediated through specific inhibition of the mTOR protein kinase, which serves as part of an evolutionarily conserved signalling pathway controlling the cell cycle in response to changing nutrient levels. The mTOR signalling network contains several tumour suppressor genes including *PTEN*, *LKB1*, *TSC1*, and *TSC2* and several proto-oncogenes including *PI3K*, *Akt*, and *eIF4E*, thus mTOR is an ideal target for anti-cancer agents such as rapamycin (Law 2005).

Reynolds and Demain (1997) reviewed rapamycin biosynthesis (Fig. 2.6). The precursors are acetate, propionate, shikimate, L-methionine, and L-lysine (via pipecolate). Rapamycin biosynthesis starts with a moiety derived from shikimate. Seven acetate and seven propionate units then participate to build up a polyketide backbone in a head-to-tail fashion. Finally, pipecolate attaches to the polyketide chain, followed by ring closure via lactone formation. Three methyl groups are transferred from methionine via S-adenosyl methionine to form the three methoxy groups (Reynolds & Demain 1997).

Rapamycin biosynthetic genes were found in a 110-kbp DNA region that encodes three polyketide synthase clusters and 24 additional open reading frames. A protein encoded by *rapP* is enzymatically similar to the pipecolate-incorporating enzyme in the immunomycin-producing *S. hygroscopicus* var *ascomyceticus*. Product of *rapM* and *rapQ* and those of *rapJ* and *rapN* are structurally homologous to methyltransferases and cytochrome P-450 enzymes, respectively (Paiva *et al.* 1993; Kojima & Demain 1998; Ritacco *et al.* 2005).

The enzyme lysine cyclodeaminase catalyses cyclization and conversion of L-lysine to L-pipecolate, which is then incorporated into rapamycin before final closure of the macrocyclic ring. A pipecolate-incorporating enzyme is thought to be responsible

for catalysing *L*-pipecolate incorporation and the ring closure. Other studies have shown that *L*-proline can be substituted for *L*-pipecolate, indicating that the pipecolate-incorporating enzyme allows some substitution (Ritacco *et al.* 2005).

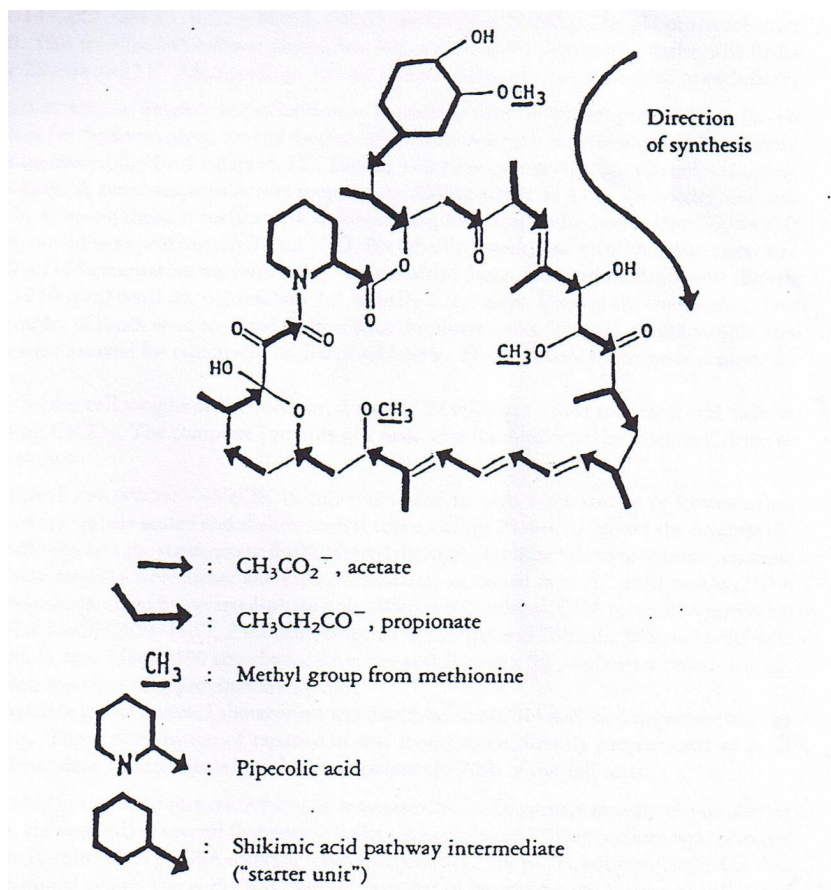


Figure 2.6 Proposed schemes for biosynthesis of rapamycin (Paiva *et al.* 1991)

2.5 Microtitre Plate

2.5.1 Introduction

Microtitre plate (also called a "shallow well" plate) usually describes the original design with well volumes of about 0.35 mL. It now refers to the common 8-row by 12-column format of interconnected wells in a single base plate. Samples are contained in a compact space and each well is uniquely identified by column number and row letter, e.g., A1, A2, A3, etc (Fig. 2.7). This compact format replaces the use of individual tubes for sample preparation (Wells 2003).

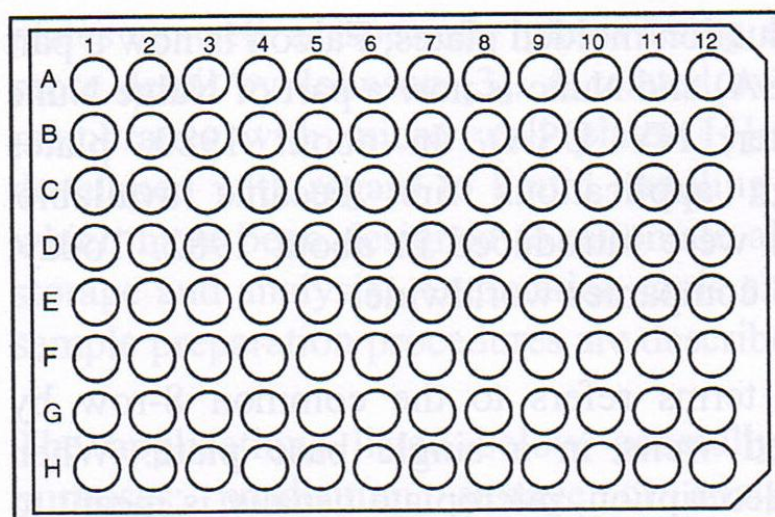


Figure 2.7 A microtitre of 8-rows by 12-columns. Each well is uniquely identified by column number and row letter (Wells 2003)

The 96-well plate has been used in many areas of pharmaceutical research (e.g., high throughput screening). They are more commonly available in the larger well volume (0.35 mL), but alternatives include those with a 0.12-mL well volume in a 384-well plate and about 0.012 mL in a 1536-well plate. The plates are made from polystyrene or polypropylene, a solvent resistance polymer that allows contact with common organic solvents. Filtration and solid-phase extraction were introduced in the 96-well plate format in flow-through processes. Microtitre plate architecture offers increased proficiency and productivity for sample preparation processes and analyses. It is easy to label, seal and manipulate. When the microtitre is combined with multiple-probe workstations, dramatically greater pipetting throughput is achieved via parallel sample processing (Wells 2003).

2.5.2 Microtitre Plate Fundamentals

- **Well shape and well bottom shape**

The 96-well microtitre plate design has evolved in response to new applications. There are now many varieties in well shape and well bottom. Most plates are molded as round or square wells. Four main well bottom geometries are available: flat, rounded, conical and rounded corners. Maximum light transmission occurs through a flat surface so flat-bottom plates are used if optical measurements are involved. Rounded bottoms give efficient mixing and approximate the profile of the

bottom of a test tube. Conical profiles are good for pellet small particles and retrieving small sample volumes efficiently. Rounded corners of a flat bottom plate improve washing and mixing performance as well as allow light transmission. Wicking is a problem with square wells and causes well-to-well contamination or “crosstalk”. Vortex mixing in square wells is not ideal so round wells are preferred to ensure thorough mixing.

- **Microtitre plate surfaces**

The top surface of the original microtitre plate design originally was completely flat. Improvements include having a raised flat surface around and between the wells that do not extend to the plate edge or having cavities between the wells so splashing will not fall into another well, reducing the potential for cross-contamination (Wells 2003). An improved top surface for ELISA analysis is achieved by using materials with different degrees of hydrophilicity. The PolySorp surface is hydrophobic for binding biomolecules that have hydrophobic properties; MediSorp is slightly hydrophilic and binds proteins with a mixed hydrophilic/hydrophobic composition, which balances high protein absorption for coating low assay not-specific binding; MixSorp is more hydrophilic and optimized for binding immunoglobulin and a wide range of other proteins; whilst MultiSorp is the most hydrophilic surface and binds biomolecules with a hydrophilic composition such as a glycoproteins and glycans (Cole-Parmer 2007).

- **Shallow-well and deep-well plates**

Typically shallow-well plates hold 0.3 - 0.4 mL, depending on well geometry. Almost all shallow-well plates are made of polystyrene, which is incompatible with the organic solvents commonly used in pharmaceutical bioanalysis. Polypropylene plates have the necessary chemical resistance.

Well geometry and the potential for cross-contamination are very important in a shallow-well plate, especially when used inside a vacuum manifold. The tips of a filtration or extraction plate occupy some of the available well volume. If they are placed too low and become submerged, bubbling can occur during vacuum elution, which can give well-to-well contamination. If the elute solution needs to be evaporated and then reconstituted before analysis, there must be sufficient space

above the liquid level to allow a mild to vigorous vortex action when heated nitrogen gas is introduced. An understanding of these limitations allows shallow-well plates containing very small elution volumes to be used (Wells 2003).

Deep-well plates made of polypropylene are used most often in bioanalysis as both sample containers and elute collection devices. The importance of the deep-well as a standard format is firmly supported through all stages of the analytical process from sample preparation through to analysis. The variation in deep-well plates is generally in well volume (0.35, 1 or 2 mL). The chimney well design with raised rims is generally preferred as they provide tighter sealing, especially with the use of heat sealable film (Wells 2003).

2.5.3 Applications

The microtitre plate is generally used for one of three purposes: production, storage or analysis. Many applications have used the 96-well plate format and an entire industry has been built around manufacturing the traditional 8-row by 12-column plastic collection plates as well as specialty plates with coated wells.

Some of most common applications in clinical and drug research include (Wells 2003):

- Clinical testing – ELISA assay, serology assays and antibiotic susceptibility assays
- Basic research – immunology, screening monoclonal antibodies, tissue culture of various normal mammalian cells and tumour cells, producing or screening ordered array genome libraries, and determination of protein and nucleic acid concentrations
- Molecular biology – DNA purification, PCR applications, and binding peptides, nucleic acids and proteins
- Drug discovery – high throughput screening using fluorescence, luminescence and other detection endpoints

Miniaturized growth systems for heterogeneous culture collection reduce demands for incubation space and medium and also allow parallel handling of large numbers of strains. Duetz *et al.* (2000) used microtitre plate for intense aeration, growth,

storage and replication of bacterial strains. They achieved oxygen transfer rates in deep-well microtitre plates as high as 38 mm O₂ L⁻¹ h⁻¹ in 0.5-mL volume or 18 mm O₂ L⁻¹ h⁻¹ in 1-mL of culture volume. *Pseudomonas putida* yields of 9 g L⁻¹ were obtained on glucose minimal medium. Other aerobic *Pseudomonas* and *Rhodococcus* strains were also evaluated.

By developing a replication system, simultaneous and reproducible sampling of 96 frozen glycerol stock cultures could be done while the remaining culture volume was kept frozen for long-term storage. Cross-contamination and excessive evaporation during vigorous aeration were adequately prevented by using a sandwich cover of spongy silicone and cotton wool on top of the microtitre plates. When screening a 1500 bacterial strain collection for the presence of specific enzyme activities in microtitre plate format, the time needed to get to the stage of washed cell suspensions was generally 7 to 8 days and involved approximately 20 working hours, including medium preparation and dispensing. This improved the system and overcame major time limit factors (Duetz *et al.* 2000; Duetz *et al.* 2001; Duetz *et al.* 2004).

Minas *et al.* (2000) used the microtitre-based cultivation system for growing mycelium-forming *Streptomyces* strains to produce secondary metabolites. The strains were grown in 1-mL liquid micro-cultures in square deep-well microtitre plates. Biomass yields were similar or better than in large-scale shake flasks or bioreactors. The increased yield in micro-culture was due to the heavy growth on the walls of the wells above the liquid phase. This wall growth was also observed in the bioreactor. Although there was well-to-well and plate-to-plate variation in secondary metabolite production, this variation was less than in shake flasks or the bioreactors. Despite repetitive sampling and extensive growth on the well walls, no cross contamination occurred. Cultivating in microwells allows many replications to be done, making it easy to identify differences due to culture-to-culture variations and factors such as a physiological condition. The system was also successfully used for storing *Streptomyces* stock culture at -20°C, directly prepared from cultures grown on agar in the microtitre plate (Minas *et al.* 2000). Cultures were retrieved by replicating aliquots from the frozen spore suspensions.

Xu *et al.* (2005) rapidly screened more than 7000 isolates of rapamycin-producing strains of *S. hygroscopicus* by duplicating cultures on the surfaces of agar-solidified in 96-wells microtitre plates. They found 10 high-yielding strains, one of which developed a mutant that produced 420 mg/L rapamycin. This was double that of the parent strain used in the submerged fermentation process. The advantages of the high-throughput method (HTS) they developed were high throughput and ease of isolating high producing strains. Within three months, three rounds of HTS can be performed and more than 7000 isolates evaluated. Expensive and tedious HPLC analysis can be minimized. The new HTS procedure is versatile for screening high-producing strains for antibiotic production and may be applied to other screening systems after improvement.

2.6 Statistical Analysis

Researchers have many methods to may obtain data. Using an appropriate experimental design will ensure strict control over the treatments used. Proper experimental designs are usually the subject matter of more advanced tests, since they often involve sophisticated statistical procedures. For a study to be useful, data needs to be examined statistically so decisions can be made before next trial. A logical method has to be used to analyse the data from a statically experimental design (Berenson & Levine 1989).

2.6.1 The Normal Distribution

The normal distribution (bell-shaped curve) is a theoretical function commonly used in inferential statistics as an approximation to sampling distributions (Distribution Fitting 2007) and generally provides a good model for a random variable that has a strong tendency to take a central value. Positive and negative deviations from this central value are equally likely, with the frequency of deviations falling rapidly as the deviation increases.

The normal distribution represents one of the empirically verified elementary “truths about the general nature of reality” and its status can be compared to the one of fundamental laws of natural sciences (Elementary Concepts 2007). The normal distribution is often used to describe random variables in a hypothesis testing. If the sample size is large enough and results of such replications are “normal distributed”,

we can precisely calculate the probability of obtaining “by chance” outcomes representing various levels of deviation from the hypothetical samples mean. If such a calculated probability is so low that it meets the previously accepted criterion of statistical significance, consequently, we can conclude that the result gives a better approximation of what is going on (Elementary Concepts 2007).

The normal distribution is defined by only two parameters; the mean and the standard deviation (Berenson & Levine 1989).

$$\mu_x = \frac{\sum_{i=1}^n X_i}{n}$$

Where μ_x = sample arithmetic mean;
 n = sample size
 X_i = i th observation of the random variable X

$$\sigma_x = \sqrt{\frac{\sum_{i=1}^n (X_i - \mu_x)^2}{n-1}}$$

Where σ_x = sample standard deviation

A characteristic property of the normal distribution is that 68% of all the observations fall within a range ± 1 standard deviation from the mean, and 95% of the scores fall within ± 2 standard deviations. In other words, values with a standardized value of less than -2 or more than +2 have a relative frequency of 5% or less. The Standardized value expresses difference of an individual value from the mean, divided by the standard deviation (Elementary Concepts 2007).

$$Z = \frac{X - \mu_x}{\sigma_x}$$

Where Z = Standardized value

2.6.2 Regression Trees

Regression trees are analytic procedures used to determine the relationship between a set of dependent variables (response variables) and one or more independent variables (input or predictor variables). The dependent variable is the one whose values you want to predict, based on values of the independent variables. The

purpose of analyses via tree-building algorithms is to determine a set of *if-then* logical (spilt) conditions that allow accurate prediction of cases. The classic computational algorithms for regression trees (*C&RT*) were popularized by Breiman *et al.* (1984).

In general, the social and natural sciences regression procedures are widely used in research. Regression trees allow the researcher to ask and hopefully answer the general question “what is the best predictor of ...”. For example, an educational researcher might want to learn what the best predictors of success in high-school are and psychologists may want to determine which personality variable best predicts social adjustment (MARSpline 2007).

The regression tree method represents a very general and powerful machine learning algorithm and has several advantages over alternative techniques. In most cases, interpreting results summarized in a tree is very simple. This simplicity is useful not only rapid classification of new observations, but can also give a much simpler “model” for explaining why observations are predicted in a particular manner. This technique is particularly well suited for data mining. Because the tree method is nonparametric and nonlinear; the final results can be summarized in a series of logical tree nodes. Therefore, data analyses by tree methods can often reveal simple relationships between just a few variables that could have easily gone unnoticed using other analytic techniques (C&RT 2007).

Regression tree analysis (Fig. 2.8) builds binary trees, i.e., partitions data into samples at each split node. Initially all records used to determine the tree structures are in one big box. The algorithm then separates data using every possible binary split on every field. The algorithm chooses the split so the sum of squared deviations from the mean in the separate parts is minimized. This process is then applied to each of the new branches. The process continues until each node reaches a user-specified minimum node size and becomes a terminal node (Fielding 1999).

When using a regression tree to predict the value of the target variable, the mean value of the target variable of rows falling in a terminal (leaf) node is the estimated value. For example, the target variable of a regression tree in the Figure 2.8 is

“Median value”. This example shows that if the value of the predictor variable “Num. rooms” is greater than 6.941, the estimated (average) value of the target variable is 37.238 and that if the value for Num. rooms is less than or equal to 6.941, the average value of the target variable is 19.934 (DTREG 2006).

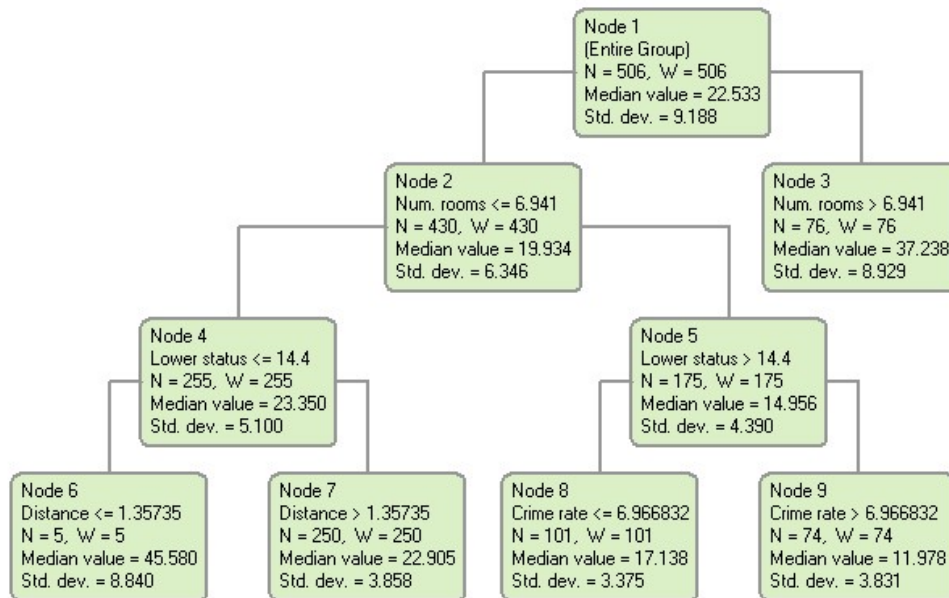


Figure 2.8 Example of a regression tree (DTREG 2006)

2.6.3 Pearson Product Moment Correlation Coefficient

The Pearson product moment correlation coefficient is the most widely-used measure of correlation or association. The “product moment” indicates that the coefficient is calculated by summing the products of the deviations from the mean (Mnstate 2006). The Pearson product moment correlation coefficient ‘cor (x, y)’ often is used to test for association between paired samples and has a distribution with ‘length (x)–2’ degrees of freedom if samples have independent normal distributions. If there are at least four complete pairs of observation, an asymptotic confidence interval is given based on Fisher’s Z transform. The correlation coefficient r is the sum of the product of the Z-scores for the two variables divided by the number of scores.

$$r = \frac{\sum Z_x Z_y}{n}$$

If the X-score and Y-score are substituted in formulas for the Z-scores, the following formula is obtained for the Pearson Product Moment Correlation Coefficient, which is used as a definitional formula (Mnstate 2006).

$$r = \frac{\sum (X - \mu_x)(Y - \mu_y)}{n\sigma_x\sigma_y}$$

Where $(X - \mu_x)$ = the deviation of a subject's X score from the mean of X
 $(Y - \mu_y)$ = the deviation of a subject's Y score from the mean of Y
 n = the number of samples
 σ_x = the standard deviation of X
 σ_y = the standard deviation of Y

The correlation coefficient r can vary from -1 (perfect negative correlation), through 0 (no correlation) to 1 (perfect positive correlation). The higher the value, the greater the likelihood that X and Y are correlated. A satisfactory r value depends on the number of samples (n); the purpose of the results is to be used for, slope, and scatter (Armstrong and James 1996).

2.7 Aims of the Research and Development

Biochemicals such as antibiotics and anti-tumour agents can be produced by micro-organisms. The aim of commercial production is to identify a high-producing strain and to then identify the best growth conditions for producing large quantities economically. This is a long, tedious and expensive process because many growth conditions and media components need to be investigated. Growth in microwells creates the opportunity of investigating many factors simultaneously using only small volumes, thereby reducing costs and the time required to identify the optimal conditions. The aims of this thesis are to:

- Identify the major media constituents in seed stage that affect growth of *S. hygroscopicus* and production of rapamycin
- Use a decision tree to screen the media
- Investigate/develop a method for rapid, small screening by using 96-well microtitre plate
- Investigate the economics of using microtitre plate for determining the optimum growth and production conditions for a microbe

- Compare aspects such as speed to obtain data, resource required, overall costs and performance quality of different methods for growth of *S. hygroscopicus* and production of rapamycin.

Chapter Three: Material and Methods

3.1 Materials, Equipments and Media

- **Micro-organisms**

Streptomyces hygroscopicus, designated IRL362B, was obtained by mutating the wild type (ATCC 29253). Stock cultures were stored in 1-mL aliquots in 10% glycerol solutions and held at -80°C.

Candida albicans, designated IRL 344, was obtained from ATCC 10231. Stock cultures were stored in 1-mL aliquots in 10% glycerol solutions and held at -80°C.

- **Cultivation medium**

All chemicals, other than complex medium components, were reagent grade and were purchased from either BDH or SIGMA. D-mannose was purchased from Pfanstiehl Laboratories Ltd. Agar, glycerol, Hy-soy, malt extract and yeast extract were purchased from DIFCO.

The production medium (SYLGG rapamycin production medium, Appendix 1.1) was developed by IRL Biopharm Ltd. Components were mixed well in 1 L purified water, adjusted to pH 6.0 with 4 M H₂SO₄ and then autoclaved for 15 min at 121°C.

The seed medium was developed by IRL Biopharm Ltd. in 2006 to replace Tomato Juice medium (Zhu 2006, unpublished). It contains 20 g fructose, 5 g mannose, 3 g yeast extract, 1.5 g l-aspartic acid, 0.5 g l-arginine, 0.5 g l-histidine, 5 g sodium chloride, 2 g di-potassium orthophosphate and 20 mL trace salts solution (MgSO₄, FeSO₄, ZnSO₄, CuSO₄, MnSO₄ and CoCl₂) in 1 L purified water. All ingredients were mixed well and autoclaved for 15 min at 121°C.

- **Culture conditions – microtitre plates**

One vial of IRL 362B culture was manually agitated with 10 mL of sterilised purified water. In seed stage, 20 µL of culture suspension was added to 200 µL sterilised experimental design seed medium (Appendix 3) in each well of a microtitre plate. The microtitre plates were incubated at 28°C for three days on a rotary shaker incubator (110 rpm, 26-mm throw). In production stage, 20-µL seed broths after

three days grown, were taken from each well and transferred to the corresponding well of aseptic microtitre plates containing 200 μ L of sterile SYLGG production medium (Appendix 1.1). These production plates were then incubated at 28°C for a further three days on a rotary shaker incubator (110 rpm, 26-mm throw).

- **Culture conditions – shake flasks**

In seed stage, each seed medium flask containing 50 mL of validating and scale-up study seed media (Table 3.3) was inoculated with 200- μ L of *S. hygroscopicus* IRL 362B stock culture and incubated at 28°C for three days on a rotary shaker (210 rpm). In production stage, two production flasks with 50 mL SYLGG production medium were inoculated with 5 mL (10% v/v) study seed medium broth from the corresponding flask and incubated for seven days at 28°C on a rotary shaker (250 rpm).

- **Microtitre plates and shake flasks**

The following equipment was used for small-scale growth:

- Nunc TM 96-well, flat-bottomed, sterile, microtitre plates (Nunc, No. EW-01928-04; VWR, Bridgeport, NJ, USA).
- Spectrophotometry microplate reader (Benchmark Model 170-6850) and its accompanying software, Microplate Manager, version 5.1, from Bio-Rad Laboratories (Richmond, NC, USA).
- Kimax #26500 250-mL conical flat, unbaffled Erlenmeyer flasks (Kimble, USA).
- Shaker incubator (Model Multitron 2; Infors, Bottmingen, Switzerland).

3.2 Analytical Methods

- **Cell mass by microplate reader**

The optical density (OD) at $\lambda=590$ nm of each well in the covered microtitre plates was monitored over three days using a plate reader. The Benchmark reader uses Beer's law to calculate absorbance of each well. This Law states that absorbance is equal to the \log_{10} the ratio of the baseline measurement (I_0) to the sample measurement (I).

The Benchmark plate reader was turned on and allowed to warm up. The wavelength was set to 590 nm, the microtitre plates were placed in the reader, the

cover was closed and the reading taken. The data file, with an appropriate name, was then saved in microplate manager before being exported to Excel format.

The calibration curve was obtained by measuring the OD of a range of dilutions from a sample of known cell mass, which had been measured gravimetrically.

- **Cell mass by PCV**

The pack cell volume (PCV) of samples from the seed stage in shake flasks was determined by putting a 10-mL broth sample into 15-mL graduated tubes and centrifuging at 2576 g for 5 min. The PCV was the pellet volume expressed as a percentage (%) of measured total volume in the tube.

- **Cell mass by DCW**

The dry cell weight (DCW) of a sample from the production stage in shake flasks was determined by placing a 10-mL sample of broth into a pre-weighed 15-mL graduated tube and centrifuging at 2576 g for 5 min. The supernatant was decanted the pellet was then mixed with 10 mL of purified water, vortex mixed and re-centrifuging at 2576 g for 5 min. After removing the supernatant, the cells and tube were dried in a 70°C oven for two days or to constant dry weight. Tubes were placed in a desiccator for at least 1 h before reweighing. Results were expressed as gram dry weight per litre broth.

- **Rapamycin concentration by bioassay**

An inhibition assay using *C. albicans* was used to determined rapamycin in the microtitre wells. A 5-μL aliquot from each well after three days incubation was placed on a lawn of *C. albicans* grown on malt yeast (MY) agar plates (Appendix 1.3) and incubated at 28°C for 20-24 h. Rapamycin concentration was recorded as the area of clear zone on the plate.

A rapamycin standard curve study was obtained by adding 10 mg rapamycin (>98% by HPLC; IRL Biopharm Ltd) to 1 mL analysis grade methanol to give a stock solution. Aliquots of stock solution (0.5 mL) were added to 4.5 mL SYLGG production medium (Appendix 1.1), then 5 μL of a dilution series (10^{-1} to 10^{-4}) were spotted on MY agar plates with a *C. albicans* lawn. Plates were incubated at 28°C

for 20-24 h. The area of each clear zone was determined ($\text{mm}^2 \times 10^2$). The inhibition area was plotted against the rapamycin concentration.

- **Rapamycin concentration by HPLC**

Rapamycin concentration in samples from shake flasks was analysed using a Waters HPLC with an Agilnet 1100 series DAD wavelength detector at 280 nm. The isocratic analysis used 80/20% methanol/water (v/v) over 20 min at 1 mL/min on a Phenomenex Hypersil C8 250 x 4.6mm column with a C8 AJO-4290 guard column at room temperature. The standard curve was generated by injecting a known amount of pure rapamycin (>98% by HPLC; IRL Biopharm Ltd).

Samples (3 mL) of fermentation broth were taken at known times and homogenised with 3 mL ethanol (1000 rpm 1 min in a Ultra-Turrex T-25, IKA, Staufen, Germany), then centrifuged at 2576 g for 5 min (Biofuge pico, Heraeus, Buckinghamshire, UK). A 1-mL aliquot was removed and duplicate 10- μL samples analysed by HPLC.

3.3 Screening Study

- **Choice of factors**

Previous work (Zhu 2006) on seed medium identified nine factors that influenced *S. hygroscopicus* growth and rapamycin production. A Box-Behnken design was constructed for three levels of the nine medium components: (A) fructose, (B) mannose, (C) yeast extract, (D) l-aspartic acid, (E) l-arginine, (F) l-histidine, (G) dipotassium orthophosphate (K_2HPO_4), (H) sodium chloride (NaCl), and (I) salt 1 solution (Table 3.1). Rapamycin concentration and culture biomass were determined as target response variables in this experiment design.

The Box-Behnken design incorporated ten factors at three levels to be examined in 170 runs using two blocks (Appendix 2). The design assigned variables as factor A to factor I, with factor J as the “dummy variable” for estimating experimental error. In the experimental layout, variables were assigned to columns in the design matrix, where “+1” represents the upper level, “0” the middle value and “-1” the lower level of each variable. For example, the -1 value of factor A (fructose) is 10 gL^{-1} , the 0 value is 20 gL^{-1} and the +1 value is 40 gL^{-1} .

Table 3.1 Factors and limits for seed media study (g L⁻¹)

	Factor	Upper	Middle	Low
A	Fructose	40	20	10
B	Mannose	10	5	0
C	Yeast extract	6	3	1.5
D	L-aspartic acid	3	1.5	0
E	L-arginine	1	0.5	0
F	L-histidine	1	0.5	0
G	K ₂ HPO ₄	4	2	0
H	NaCl	10	5	0
I	Salt 1 solution	40 mL	20 mL	5 mL
J	Dummy	0	0	0

- **Microtitre plate preparation**

Two microtitre plates with round wells and flat bottoms were used as two blocks in the Box-Behnken experimental design for both seed and production fermentations. An example of the layout for plate 1 is given in Figure 3.1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	T	C	T	T	C	T	T	T	T	T	T	T
B	C	T	T	C	T	C	T	C	C	T	T	T
C	T	T	T	C	T	C	C	T	C	T	T	T
D	T	T	T	T	C	T	T	T	T	T	T	T
E	T	T	T	T	T	T	T	T	T	T	T	T
F	T	T	T	T	T	T	T	T	T	C	C	C
G	T	T	T	T	T	T	T	T	C	T	T	T
H	T	T	T	T	T	T	T	T	T	T	T	T

Figure 3.1 Actual plate 1 layout, where C = control medium and T = test medium

Typical assay volume is 100–200 µL per well, with a minimum of 40 µL required for good mixing. Plates were covered with lids and sealed with parafilm during all experiments. Control medium was randomly distributed in each block (i.e. per microtitre plate). Sixteen wells in each microtitre plate contained control medium and 80 wells contained experimental medium.

- **Media preparation**

To prepare seed medium, a stock solution was made for each ingredient (Table 3.2). The required amount of each component was dispensed into a 50-mL test tube labelled for the well and plate (e.g. plate 1, H11 or plate 2, C8). After mixing well, the total volume was made to 20 mL with purified water then autoclaved at 121°C for 15 minutes. A 0.2-mL aliquot was dispensed into each well in the microtitre plate. The test and control media experimental design is in Appendix 3.

Table 3.2 Medium stock solution (g mL⁻¹)

Medium	Conc.	Upper (mL)	Middle (mL)	Lower (mL)
Fructose	0.2	4	2	1
Mannose	0.1	2	1	0
Yeast extract	0.03	4	2	1
l-aspartic acid	0.03	2	1	0
l-arginine	0.01	2	1	0
l-histidine	0.01	2	1	0
K ₂ HPO ₄	0.04	2	1	0
NaCl	0.1	2	1	0
Salt 1 solution	As formula	0.8	0.4	0.1

To prepare the production stage, 500 mL of production medium (SYLGG, Appendix 1.1) was prepared in a 1-L bottle and autoclaved at 121°C for 15 minutes. A 0.2-mL aliquot was dispensed into each well of the microtitre plates.

3.4 Validating and Scaling-Up Study

- **Choice of factors**

Preliminary studies indicated l-aspartic acid, l-arginine, l-histidine, di-potassium orthophosphate (K₂HPO₄), sodium chloride (NaCl), and salt 1 solution in the seed media affected *S. hygroscopicus* growth in the seed stage and rapamycin production in the production stage. Shake flask cultures were used to determine the effect of input variables on rapamycin concentration and biomass and compared with data from the preliminary microtitre plate study. A change-one-at-one-time strategy (Kennedy & Krouse 1999) was constructed to explore interactions between the medium variables. The concentration of other media constituents was kept constant throughout the investigations.

- **Media preparation**

To prepare the seed stage, 50 mL of prepared study seed medium (Table 3.3) was dispensed into 250-mL Erlenmeyer flasks. Each flask was covered with Bioshield and cheesecloth and then sterilized at 121°C for 15 minutes.

To prepare the production stage, 50 mL of SYLGG medium (Appendix 1.1) was dispensed into 250-mL Erlenmeyer flasks. Each flask was covered with Bioshield and cheesecloth and then sterilized at 121°C for 15 minutes.

Table 3.3 Shake flasks study seed medium preparation (g L⁻¹)

Medium	M411	M412	N413	M414	M415	M416	M417	M418
Fructose	20	20	20	20	20	20	20	20
Mannose	5	5	5	5	5	5	5	5
Yeast extract	3	3	3	3	3	3	3	3
L-aspartic acid	1.5	1.5	1.5	3	0	1.5	1.5	1.5
L-arginine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
L-Histidine	0.5	0.5	0.5	0.5	0.5	0	0.5	0.5
NaCl	0	2	2	2	2	2	2	5
K ₂ HPO ₄	2	0	2	2	4	2	2	2
Salt 1 solution	20	20	40	20	20	20	20	20

3.5 Statistical Analysis

- **Normal distribution**

The normal distribution was used to test the probability from individual well to well in microtitre plates.

- **Regression trees**

Data obtained from the Box-Behnken experimental design were analysed using the regression trees Statistics software (2005) with an “rpart” package. This performs recursive partitioning and allocates response variables into the tree structure to correlate the response variable and the independent variables. A tree is produced as the dependent variable is split into subsets using the “explanatory” variables. Each leaf is an irreducible subset for which the average and number (i.e. n) are given. The main variables, together with the splitting criterion are given at nodes of the tree.

- **Pearson product moment correlation coefficient**

The “cor.test” in Statistics software (2005) was used to test the association of cell growth in the seed stage and rapamycin concentration in the production stage between shake flasks and microtitre plates.

3.6 Experimental Procedure

- **Preliminary study - microtitre plates**

The preliminary study involved characterizing the fermentation system for microtitre plates, determining the optimum growth time, identifying a suitable organic solvent to extract rapamycin from the broth and running a rapamycin bioassay standard curve study.

Firstly, growth in the microtitre plates had to be shown to be similar to the standard process in shake flasks. A vial of IRL 362B stock culture was transferred into 10 mL of sterilised purified water and manually agitated to ensure suspension. Then, 1-mL aliquots of culture suspension were used to make 10^{-1} and 10^{-2} dilutions in 9 mL BYM broth (Appendix 1.2). A multiple autopipette was used to dispense 200- μ L aliquots of BYM broth (Appendix 1.2) into the A1 to A12 and E1 to E12 wells in the microtitre plate (blanks), 200- μ L aliquots of culture suspension into the B1 to B12 and F1 to F12 wells, 200- μ L of 10^{-1} culture suspension into the C1 to C12 and G1 to G12 wells, and 200- μ L of 10^{-2} culture suspension into the D1 to D 12 and H1 to H 12 wells. Duplicate blank and dilutions were repeated in a second microtitre plate. The plates were covered with lid and sealed with parafilm and incubated in a rotary shaker for three days (28°C, 110 rpm). Results were analysed by the normal distribution method (section 3.5).

Cultivation time had to be shorter to prevent significant evaporation. To determine maximum available cultivation time, two microtitre plates were prepared by placing 200- μ L of sterile purified water in each well. The plates were then incubated at 28°C and 110 rpm, and the volume of water was measured daily over four days.

Rapamycin is substantially insoluble in water but can dissolve in chloroform (5 mg mL⁻¹), methanol (25 mg mL⁻¹), and DMSO (25 mg mL⁻¹) and is soluble in ethanol, ether, acetone and N,N-dimethylformamide (Sigma-Aldrich Production information

2006). Some organic solvents are toxic to bacterial growth in a bioassay so the most suitable solvent for rapamycin needed to be identified. The effect of acetone, chloroform, ethanol and methanol were evaluated using an inhibition test with a lawn of *C. albicans* on MY agar plates. A 0.1-mL aliquot of *C. albicans* stock culture was spread over the plate and left on a sterile work bench for 5 to 10 min to dry. Then, 5- μ L and 20- μ L aliquots of each solvent were spotted on the plate. Plates were incubated at 28°C for up to three days. The effect of each solvent was evaluated by observing if the lawn had been killed.

A stock solution of 98% pure rapamycin was made to 1 mg mL⁻¹ in methanol. A dilutions series was made by adding 0.5 mL of rapamycin stock solution to 4.5 mL of SYLGG medium, and then 0.5 mL of this was added to a further 4.5 mL of SYLGG medium. The inhibition test was set up using a lawn of *C. albicans* on a MY agar plate. A 0.1-mL stock culture of *C. albicans* was spread over the MY agar plates and left on the bench for 5 to 10 min to dry. Then, 5- μ L, 10- μ L, 20- μ L and 50- μ L aliquots of each dilution series were spotted on the plates, which were then incubated at 28°C. The areas of the clear zone were recorded at 24, 48 and 72 h.

- **Medium screening and optimizing study - microtitre plates**

Seed stage control medium, seed stage experimental design medium and SYLGG production medium were prepared (section 3.3). To produce seed stage microtitre plates, 20 μ L of culture suspension from an IRL 362B stock culture was added to 10 mL sterilised purified water, then 20 μ L of this suspension was inoculated into 200 μ L of sterilized experimental design or control seed medium in each well of the microtitre plate. For production stage trials, production microtitre plates were prepared by transferring 20 μ L (10% v/v) of seed culture from each well of the seed microtitre plates to 200 μ L of sterilized SYLGG production medium in corresponding well of a production microtitre plate. Microtitre plates were covered with a lid and sealed with parafilm before being incubated for three days at 28°C on a rotary shaker (110 rpm).

The biomass in the seed stage and rapamycin concentration in the production stage was determined (section 3.2). Regression tree analysis (section 3.5) was used to

investigate the effect of media components on culture growth and rapamycin production.

- **Validating and scale-up study - shake flasks**

Seed stage control medium, seed study medium and SYLGG production medium were prepared (section 3.4). Inoculating 0.2 mL of culture suspension from IRL 362B stock culture to a 250-mL shake flask containing 50 mL sterilized seed study or control medium produced seed flasks. Production flasks were prepared by transferring 5 mL (10% v/v) of seed culture to a 250-mL shake flask containing 50 mL of sterilized SYLGG production medium. Duplicate flasks were sealed with two layers of cheesecloth and one layer of Bioshield. Seed stage flasks were incubated for three days at 28°C on a rotary shaker (210 rpm) and production stage flasks were incubated for seven days 28°C on a rotary shaker (250 rpm).

At the end of the incubation, biomass in both the seed stage and the production stage and rapamycin concentration in the production stage were determined (section 3.2). Biomass and rapamycin data were analysed to validate the microtitre plate method (section 3.5).

Chapter Four: Results and Discussion

4.1 Preliminary Study - Microtitre Plates

4.1.1 Characterizing Physical Parameters of the Fermentation System

Antibiotic production by filamentous micro-organisms is usually under aerobic conditions (Calvo *et al.* 2002). Therefore, two major requirements for a satisfactory production are sufficient oxygen and a suitable temperature.

The increase in biomass in the microtitre plates was measured as increase in absorbance at 590 nm using the plate reader, where:

$$\Delta A = \text{absorbance at 590 nm} - \text{blank absorbance (average of 24) at 590 nm}$$

The 96-well plate had 24 wells with BYM broth (blanks) and 72 wells with various dilutions (10^0 to 10^{-2}) of *S. hygroscopicus* (Fig. 4.1).



Figure 4.1 Growth of *S. hygroscopicus* on BYM broth in microtitre plates after two days

If growth in the wells follows a normal statistical distribution, 95% of the growth ΔA values will be due to the effect of factor X and only 5% should lie outside this range. To investigate this particular value, the following steps were used:

1. The value of X is unknown but the standardized value Z can be determined because it is a particular area under the normal curve. For 95% probability, the

Z value = ± 1.96 . After determining Z, the transformation formula can be used to determine X.

$$Z = \frac{X - \mu_x}{\sigma_x}$$

Where μ_x is the mean of all results and σ_x is the standard deviation of all results.

2. μ_x , the mean of 72 samples, is:

$$\mu_x = \frac{\sum_{i=1}^{72} X_i}{72} = 0.1903$$

3. σ_x , standard deviation of 72 samples, is:

$$\sigma_x = \sqrt{\frac{\sum_{i=1}^{72} (X_i - \mu_x)^2}{72}}$$

4. Calculate the 95% of confidence intervals of X_i . If Z follows the standard normal distribution, the 95% of confidence will be [-1.96, 1.96] or:

$$0.95 = P(-1.96 \leq Z \leq +1.96)$$

$$0.95 = P\left(-1.96 \leq \frac{X - \mu_x}{\sigma_x} \leq +1.96\right)$$

$$0.95 = P\left(-1.96 \leq \frac{X - 0.1903}{0.067} \leq +1.96\right)$$

$$0.95 = P(0.06 \leq X \leq 0.32)$$

This gives the specific range of ΔA values associated with a 95% probability, with a lower limit of 0.06 and higher limit of 0.32. Of the 72 ΔA results from the wells (Table 4.1), only four (bolded, being 0.331, 0.346, 0.350, 0.358) are above the 95% probability maximum of 0.32 and none are less than the minimum value of 0.06. This analysis shows that the ΔA values from individual wells in the microtitre plates were acceptable.

The increase in absorbance (ΔA) of inoculated wells after two days (Table 4.1) indicated that 28°C and 110 rpm supported *S. hygroscopicus* growth, with submerged

medium under uniform conditions. There was a direct relationship between absorbance and inoculum concentration. These conditions were therefore used for the growth trials.

Table 4.1 Increase (ΔA) in 590 nm absorbance when growing *S. hygroscopicus* in microtitre plates

Dil ⁿ		1	2	3	4	5	6	7	8	9	10	11	12
10 ⁰	B	0.223	0.217	0.271	0.163	0.214	0.235	0.346	0.157	0.310	0.358	0.226	0.229
10 ⁻¹	C	0.131	0.126	0.100	0.156	0.179	0.190	0.136	0.155	0.152	0.134	0.121	0.177
10 ⁻²	D	0.087	0.110	0.134	0.136	0.139	0.146	0.128	0.127	0.146	0.130	0.151	0.087
10 ⁰	F	0.146	0.223	0.350	0.285	0.160	0.286	0.168	0.307	0.270	0.331	0.311	0.282
10 ⁻¹	G	0.186	0.192	0.162	0.215	0.219	0.190	0.192	0.202	0.214	0.251	0.254	0.185
10 ⁻²	H	0.118	0.139	0.102	0.144	0.128	0.207	0.105	0.130	0.209	0.199	0.219	0.198

4.1.2 Determining Optimum Cultivation Time

The main hurdle to using microtitre plates for aerobic microbial growth is preventing cross-contamination and excessive evaporation during incubation. Rapamycin productions by *S. hygroscopicus* grown in shake flasks were normally incubated at 28° for seven days. To determine optimal cultivation for rapamycin production and culture growth in microtitre plates, information on incubation time and volume of water in the well is required.

Volume of water in two microtitre plates was measured daily (Figure 4.2). After three days, 20% of the water in the wells had evaporated and after four days, 25% had evaporated. Further trials were done over three days so concentration of media would not affect rapamycin production. To further reduce evaporation, the 96-well microtitre plates were covered with parafilm, a flexible, mouldable, self-sealing barrier. Parafilm is an ideal for the scientific laboratory because it is a thermoplastic, self-sealing film. It holds moisture loss to a minimum and offers excellent protection for the content of broth tubes, flasks and Petri dishes. It has unique permeability properties, very good water vapour transport properties and is resistant to many common reagents (Parafilm M 2007).

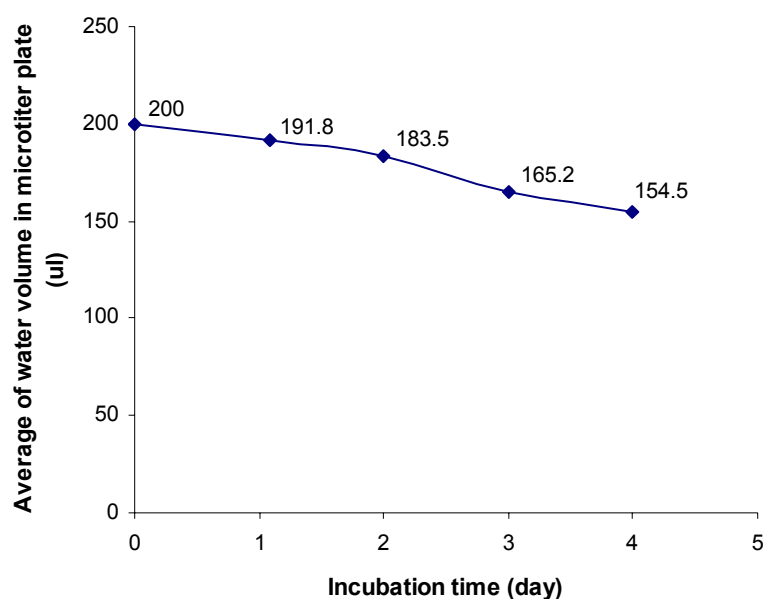


Figure 4.2 Evaporation from microtitre plates during incubation

4.1.3 Organic Solvent for Extracting Rapamycin

The toxicity of four organic solvents on *C. albicans* (indicator organism in the rapamycin bioassay), was investigated. Methanol gave the lowest response, acetone and ethanol had a slight positive response and chloroform gave the highest response (Table 4.2). Therefore, methanol was selected for the bioassay to ensure any response was due to rapamycin rather than solvent.

Table 4.2 Effect of solvent on MY agar inhibition of *C. albicans* growth

Solvent	Observation	Comment
Acetone	Very slight zone	Slight positive
Chloroform	Clear zone	Positive
Ethanol	Very slight zone	Slight positive
Methanol	No zone	Negative

4.1.4 Bioassay Study

A standard curve was required to convert the area of the inhibition zone (mm^2) to rapamycin concentration ($\mu\text{g}/\mu\text{L}$). After 24 hours, there were sharply defined inhibition zones for *C. albicans* (Fig. 4.3). The area of the ring was calculated as πr^2 , where r is radius of the zone (average of four diameter measurements divided by 2, $\pi = 3.14$). Results were presented in $\text{mm}^2 \times 10^2$.



Figure 4.3 Example of rapamycin inhibitory zone against *C. albicans*.

There was a direct relationship between the area of the inhibition zone and the rapamycin concentration (Fig. 4.4). Data were means of duplicate samples. The high correlation ($r=0.99$) indicated that the inhibition zone could be used to indicate rapamycin concentration as $\mu\text{g}/\mu\text{L}$.

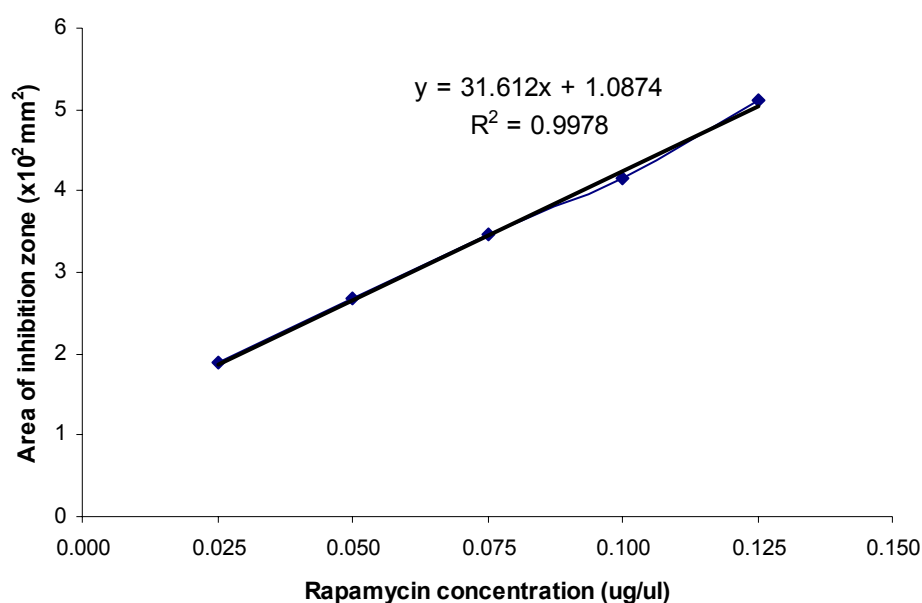


Figure 4.4 Rapamycin concentration standard curve

4.2 Medium Screening and Optimizing Study - Microtitre Plates

4.2.1 Medium Screening and Optimization

After the small-scale, rapid, high-capacity procedure for microtitre plates had been developed, it could be used to screen and optimize seed medium in a secondary

metabolite production process. The effect of three levels of nine medium components in seed media followed by fermentation in SYLGG production medium on biomass generation (seed stage) and rapamycin production (production stage) was determined. A total of 192 different seed medium recipes were investigated in microtitre wells using the Box-Behnken experimental design, with two microtitre plate blocks (Appendix 2).

Two response variables were measured. For the seed stage, biomass generation was determined by optical density (OD) at 590 nm and expressed as change absorbance values ΔA . Rapamycin concentration in the production stage was determined by bioassay and expressed as mg/L (Appendix 4).

4.2.2 Cell Growth

Experimental conditions and cell growth were expressed as a regression tree (Fig. 4.5). The effect estimates the significance of input variables and describes interactions between the variables by the length of the corresponding line segment and the sequence of levels. Absolute values of observed effects are directly plotted on normal probability paper.

Sodium chloride (NaCl), l-arginine, l-aspartic acid and l-histidine have the greatest effect on cell growth. Data were first split (partitioned) into cases with values for the plate above or below 1.5 (Fig. 4.5). Two microtitre plates (plate 1 and plate 2) were used in this experiment. Data from plate 1 are on the left side of the first level and from plate 2 are on the right side. Some data are missing from plate 1 because the plate reader gave unreliable results for the blank broth. Consequently, the value 0.109 on the left side is the average of ΔA values at 590 nm from 48 rather than 96 data points.

The right side of the tree for plate 2 is next split on whether the NaCl value was ≥ 2.5 g/L. When NaCl concentration is < 2.5 g/L, a terminal node is reached when average OD_{590} is 0.376 ($n=15$), which represents the highest biomass value obtained. For NaCl concentrations > 2.5 g/L, the regression tree is split by the variable l-arginine at 0.75 g/L. If l-arginine is > 0.75 g/L, the OD_{590} is 0.321 ($n=14$).

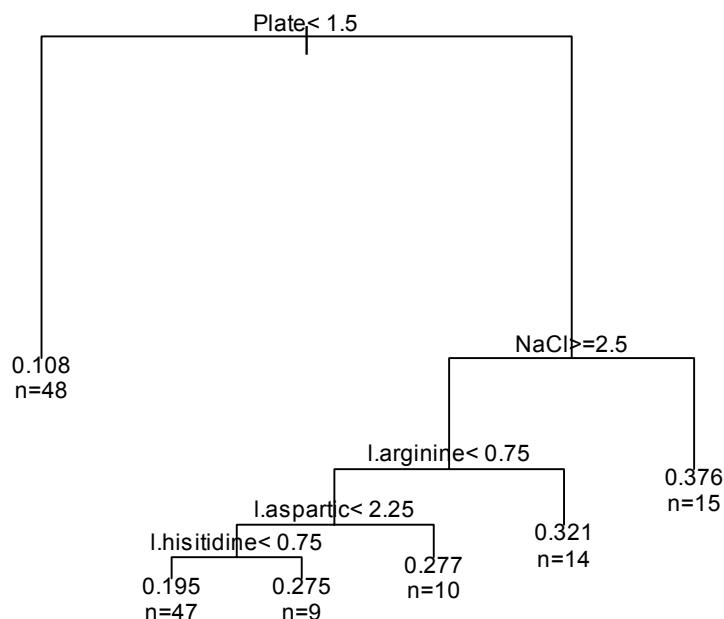


Figure 4.5 The effect of media components on cell growth, presented as a regression tree

After the branch for l-arginine < 0.75 g/L, the regression tree was split by the value for l-aspartic acid at < 2.25 g/L. At high l-aspartic acid concentrations (> 2.25 g/L) the average was 0.277 ($n=10$). For l-aspartic acid < 2.25 g/L, the regression tree is split by the value for l-histidine < 0.75 g/L. There were 47 data, with an average of 0.195, for low l-histidine concentrations (< 0.75 g/L) and 9 data, with an average of 0.275, for high l-histidine concentrations (> 0.75 g/L).

4.2.3 Rapamycin Concentration

The effect of media components on rapamycin concentration were analysed using the regression tree method. Data were plotted on normal probability paper (Fig. 4.6). The most important relevant components for rapamycin concentration were dipotassium orthophosphate (K_2HPO_4), l-aspartic acid, l-histidine and salt 1 solution. The carbon source (fructose and mannose) and yeast extract (one of the nitrogen sources) did not have much effect. The best path starts at a K_2HPO_4 , which then splits at a first and second node before continuing to l-aspartic acid, l-histidine and salt 1 solution at the third, fourth and fifth nodes.

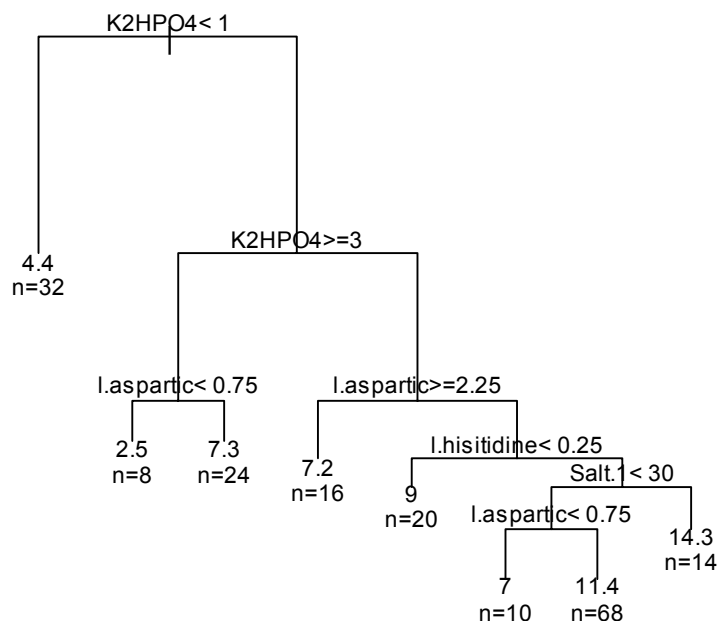


Figure 4.6 The effect of media components on rapamycin concentration, presented as a regression tree

A value of $K_2HPO_4 < 1$ g/L was chosen for the first split variable in the regression tree. For $K_2HPO_4 \geq 1$ g/L ($n=192$), then K_2HPO_4 is again chosen as the second split point. Values of $K_2HPO_4 < 1$ g/L are on the left side and have an average rapamycin concentration of 4.4 mg/L ($n=32$). When $K_2HPO_4 \geq 3$ g/L ($n=160$), then l-aspartic acid is the divider for both sides of the tree. However, $K_2HPO_4 \leq 3$ g/L and l-aspartic acid ≥ 2.25 g/L appear to be a critical node, while l-aspartic acid < 0.75 g/L is another node when $K_2HPO_4 \geq 3$ g/L. At the node $K_2HPO_4 \geq 3$ g/L and l-aspartic acid < 0.75 g/L, average rapamycin concentration is 2.5 mg/L ($n=8$). For the node l-aspartic acid > 0.75 g/L, average rapamycin concentration is 7.3 mg/L ($n=24$).

The best l-aspartic acid concentration for rapamycin concentration is between 0.75 and 2.25 g/L. When K_2HPO_4 is < 3 g/L and with a high l-aspartic concentration, average rapamycin concentration is 7.2 mg/L ($n=16$). This is similar to the value when 0.75 g/L l-aspartic acid is in the medium (7.3 mg/L, $n=24$).

Based on optimum l-aspartic acid concentration, increased rapamycin concentration depends on two factors – the concentration of l-histidine and salt 1 solution. At low l-histidine concentrations (i.e. l-histidine < 0.25 g/L), rapamycin concentration is 9

mg/L (n=20). However, if l-histidine concentration is >0.25 g/L and salt 1 solution is >30 mL/L, average rapamycin concentration is 14.3 mg/L (n=14), which is triple that obtained with <1 g/L K₂HPO₄ and double that when l-aspartic acid is used.

At low salt 1 concentrations (i.e. <30 mL/L), l-aspartic acid affected rapamycin concentration. For l-aspartic acid concentration <0.75 g/L, rapamycin concentration was only 7 mg/L (n=10) but if l-aspartic acid concentration is >0.75 g/L, rapamycin concentration increased to 11.4 mg/L (n=68).

4.2.4 Media Components on Cell Growth and Rapamycin Concentration

The regression tree analyses clearly present data from medium optimization studies in microtitre plates and allow key variables for cell growth and rapamycin concentration to be identified, as well as identifying any interactions between variables. It is a simple method to visualise the data.

- **Carbon source**

Fructose and mannose were included as carbon sources in the trials, based on defined media for rapamycin reported in the literature (Kojima *et al.* 1995). Three levels of fructose and mannose were investigated. Fructose had an upper limit of 40 g/L, a medium limit of 20 g/L, and a low limit of 10 g/L respectively. The values for mannose were 10 g/L, 5 g/L, and 0 g/L respectively.

Fructose and mannose did not appear in the regression tree analysis for cell growth and rapamycin concentration, indicating that these carbon sources or the concentrations used (upper limit of 50 and lower limit of 10 g/L) did not appear to influence cell growth or rapamycin concentration. This is surprising so it is recommended that a wider concentration range be investigated to determine the limits for carbon.

- **Yeast extract**

Using yeast extract as a nitrogen source in the seed medium had been investigated in a previous study (Zhu 2006). Results from that trial demonstrated that this component gave higher growth rates and favourable levels of rapamycin concentration by *S. hygroscopicus*.

The regression tree analysis for cell growth and rapamycin concentration indicated that including yeast extract (1 g/L to 6 g/L) in the media did not influence growth or rapamycin concentration. Instead, adding various pure amino acids or NaCl had more effect than yeast extract.

- **Amino acids**

The chemical composition of culture medium, particularly nitrogen concentration, gives physiological control and regulation of microbial metabolism. Rapamycin is a triene macrolide containing nitrogen. The immediate precursor of the nitrogen-containing ring is pipercolic acid (Paiva *et al.* 1993). Adding l-lysine to the production media increased rapamycin concentration by *S. hygroscopicus* by 150% (Cheng *et al.* 1995) but the effect of adding l-arginine, l-aspartic acid and l-histidine on performance of *S. hygroscopicus* in seed medium is unknown. The main effects on cell growth occurred when NaCl concentrations were greater than 2.5 g/L. Having a high NaCl chloride concentration and lower concentrations of the three amino acids gave poor cell growth, with the absorbance values (ΔA) of 0.195 (n=47).

The regression tree analysis indicated interactions between the amino acids affected cell growth. At the lower l-arginine concentration (<0.75 g/L), adding either l-aspartic acid or l-histidine increased absorbance value to 0.277 (n=10) or 0.275, (n = 9) respectively.

At a constant K₂HPO₄ concentration, l-aspartic acid significantly affected rapamycin concentration (measured by bioassay), l-histidine had less effect and l-arginine had no effect. The optimal l-aspartic acid concentration is between 0.75 and 2.25 g/L (Fig. 4.7) and the highest rapamycin concentration is obtained when the medium contained 1.5 g/L l-aspartic acid and 1 to 3 g/L K₂HPO₄.

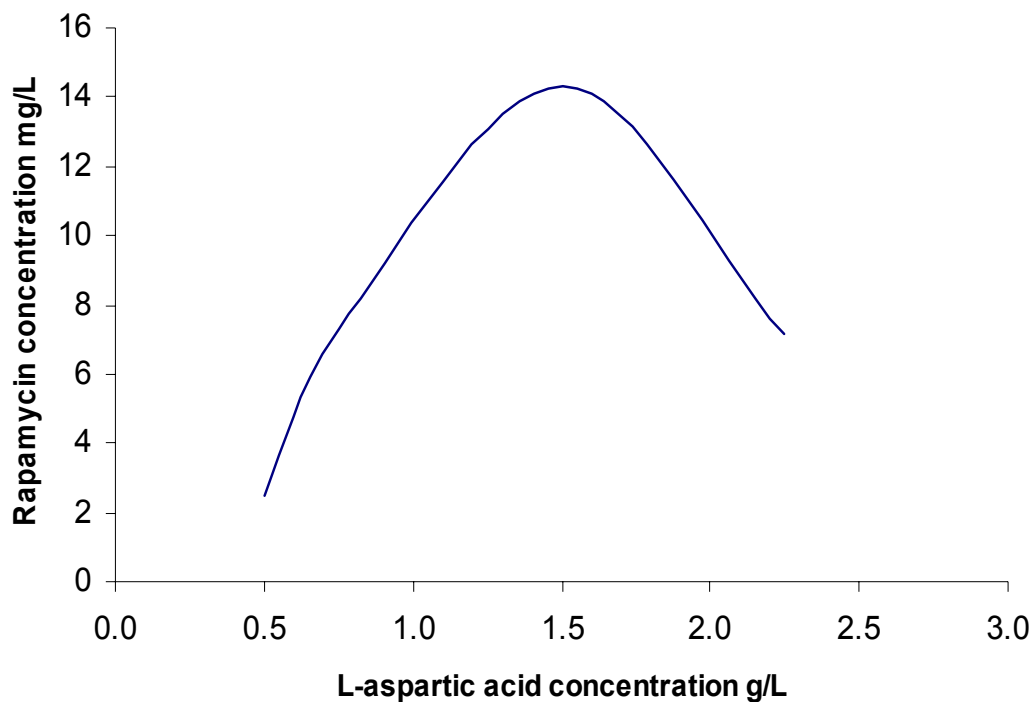


Figure 4.7 Effect of l-aspartic acid concentration on rapamycin concentration ($K_2HPO_4 = 2$ g/L)

- **Sodium chloride (NaCl)**

Using NaCl in the seed medium significantly affected *S. hygroscopicus* growth. At the low NaCl concentration (≤ 2.5 g/L), which is half the concentration in the current medium, biomass increased (absorbance of 0.376; $n=15$). However, the media also contained 1 to 6 g/L yeast extract, which has 11.5 % w/w NaCl (Appendix 5). The salt in the yeast extract would increase the NaCl in the medium. This, in turn, may inhibit cell growth and rapamycin concentration. Thus, the positive response at no or low (e.g. 1 g/L) added NaCl does not mean that the medium should not contain NaCl or that the culture does not require it. Instead, the NaCl the culture requires may be coming from ingredients such as yeast extract.

The regression tree analysis shows that higher NaCl levels (e.g. >2.5 g/L) gave interactions with other components, especially amino acids.

- **Di-potassium orthophosphate (K_2HPO_4)**

The regression tree analysis indicated that K_2HPO_4 concentration had a significant effect on rapamycin concentration. If K_2HPO_4 concentration was less than 1 g/L, rapamycin concentration was only 4.4 mg/L ($n=32$) but if K_2HPO_4 concentration was

increased to 3 g/L, rapamycin concentration increased to 9.0 mg/L (n=20), depending on amino acid concentration, or 14.3 mg/L (n=14), depending on trace mineral elements (salt 1) solution. Overall, the optimal concentration range for K_2HPO_4 is between 1 and 3 g/L (Fig. 4.8).

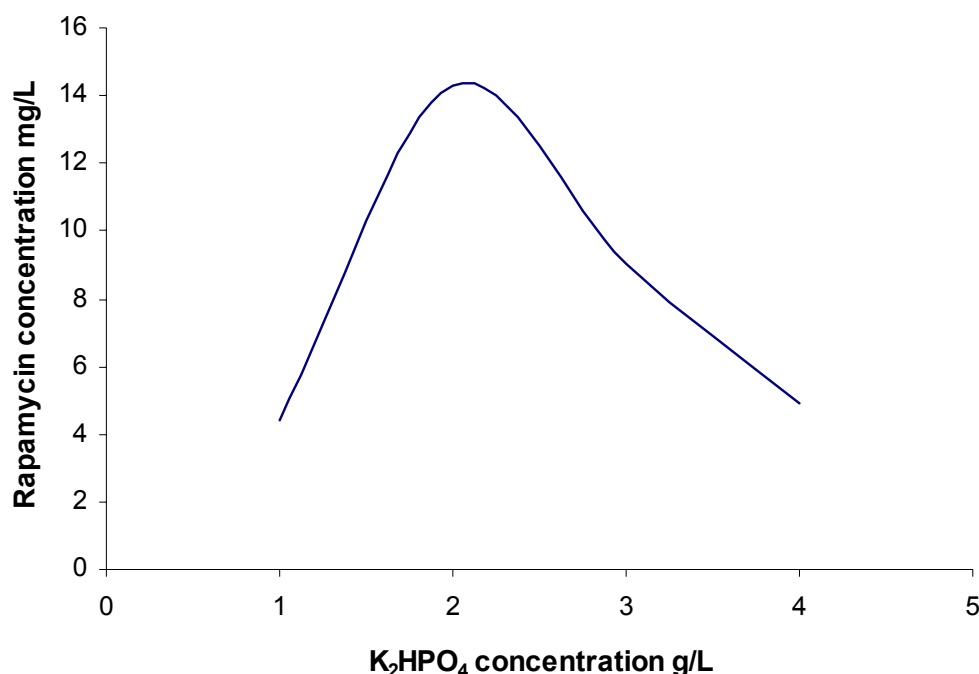


Figure 4.8 Effect of K_2HPO_4 concentration on rapamycin concentration at constant carbon and nitrogen concentration

- **Mineral trace elements (salt 1 solution)**

Published studies show that metal ions such as Mg^{2+} , Fe^{2+} , Mn^{2+} and Cu^{2+} appear to be most important in rapamycin biosynthesis (Cheng *et al.* 1995a; Gesheva *et al.* 2005). The highest rapamycin concentrations achieved in the current experiments occurred when the medium containing the following mineral salt solution: $MgSO_4$ (28.9 g/L), $MnSO_4$ (0.1 g/L), $FeSO_4$ (0.5 g/L), $CuSO_4$ (0.05 g/L), $ZnSO_4$ (0.5 g/L) and $CoCl_2$ (0.04 g/L). The data indicate that adding more mineral salts (>30 mL/L) gave 14.3 mg/L of rapamycin (n=14). If less than 30 mL/L salt solution was added, the rapamycin concentration was less than 7 mg/L if less than 0.75 g/L l-aspartic acid was added (n=10) and 11.4 mg/L if more than 0.75 g/L l-aspartic acid was added (n=68).

4.3 Validating and Scale-Up Study - Shake Flasks

4.3.1 Seed Stage Medium Verification

The microtitre plate experiments showed that the seed stage media components most affecting rapamycin concentration were K_2HPO_4 , l-aspartic acid, l-histidine and salt 1 solution while the media components most affecting cell growth were NaCl, l-arginine, l-aspartic acid and l-histidine. To validate that the effects on cell growth and rapamycin concentration in the microtitre plate method were due to variations in the seed media, shake flask experiments were done using eight different seed medium recipes (Table 3.3) selected from 170 media. Some of the recipes had been developed for high growth and concentration (medium M411) while other recipes were selected to confirm low growth and concentration (medium M415). Because previous trials had shown that fructose, mannose, yeast extract and l-arginine had little effect on cell growth and rapamycin concentration, their concentrations were fixed. However, NaCl, K_2HPO_4 , l-aspartic acid, l-histidine and salt 1 solution had significant effects on both production and cell growth so using different concentrations of these components will help determine their effect on cell growth and rapamycin concentration (Table 4.3).

Table 4.3 Shake flasks seed media – validating and scale-up study

Medium	Comment
M411	NaCl <2.5 g/L
M412	K_2HPO_4 <1 g/L
M413	Salt solution >30 mL/L
M414	l-aspartic acid >2.25 g/L
M415	l-aspartic acid <0.75 g/L, K_2HPO_4 >3 g/L
M416	l-histidine <0.25 g/L
M417	0.75 g/L < l-aspartic acid <2.25 g/L
M418	Control medium

Trials were run in duplicate using M418 as the control. Seed stage cultivation in eight different seed media was followed by fermentation in SYLGG production medium. This study allowed the variability of the process optimization in the region of maximum or minimum observed in the microtitre plate trials to be estimated and hence is indicates process reproducibility and robustness.

4.3.2 Effects on Biomass Generation in Seed Stage

Biomass generation in the seed stage from eight different seed media in the shake flasks after three days incubation was determined by packed cell volume (PCV). The PCV of *S. hygroscopicus* grown on seed media M414 (0.77%), M416 (0.91%), and M417 (0.61%) were similar to the that (0.89%) on control medium M418 (Fig. 4.9). The 1.5% biomass produced on medium M413 was nearly double the control medium. The highest biomass occurred when the culture was grown on medium M411 (4.17%) and M412 (3.48%). These values were four times greater than on the control medium. The PCV for growth on M415 (0.21%) was much lower the others.

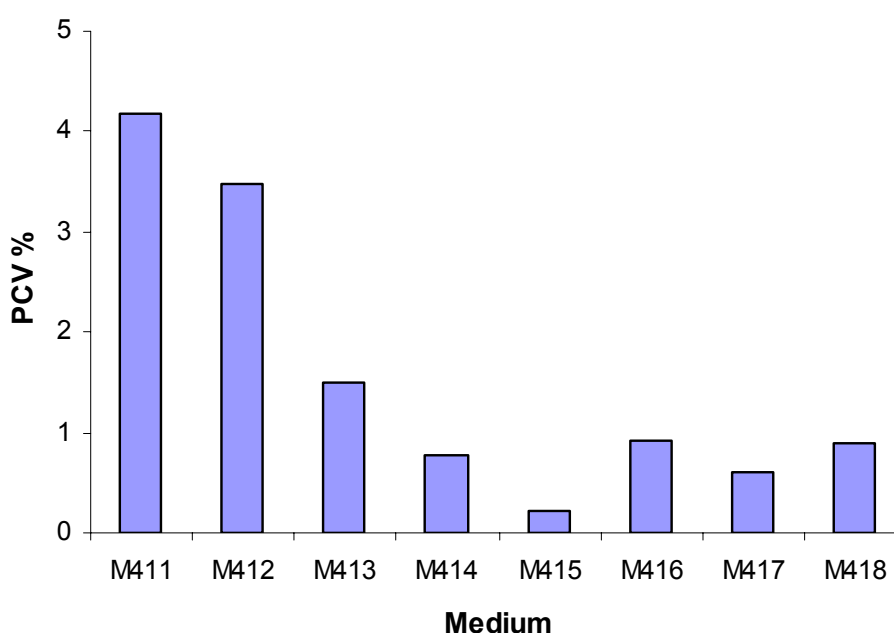


Figure 4.9 Effect of eight seed media on biomass generation in shake flasks after three days (seed media composition is in Table 3.3).

Thus, growing *S. hygroscopicus* on M411, M412 and M13 improved biomass yield whilst growth was low on M415. These data confirm that NaCl (M411), l-aspartic acid (M415), and l-histidine (M416) significantly affect growth and supports the data obtained using the microtitre plate procedure.

4.3.3 Comparison of Methods

As well as measuring the economic and operational performance of the microtitre plate procedure, we need to compare performance against goals set for this study and contrast these with the shake flask procedure to identify gaps in production

reliability. Evaluating effectiveness of the medium optimization procedure in microtitre plates with cell growth in seed stage using shake flasks could do this.

To compare seed stage cell growth in microtitre plate and shake flasks, a calibration curve was required to convert OD 590 nm used to measure cell mass in the microtitre procedure to the PCV% used in the shake flask procedure. This calibration curve was created by measuring OD from samples of known cell mass, which had been measured by PCV. There was a direct relationship between PCV and absorbance at OD 590 nm (Fig. 4.10). The high correlation ($r=0.98$) indicates that ΔA at $\lambda = 590$ can be used to indicate PCV as %.

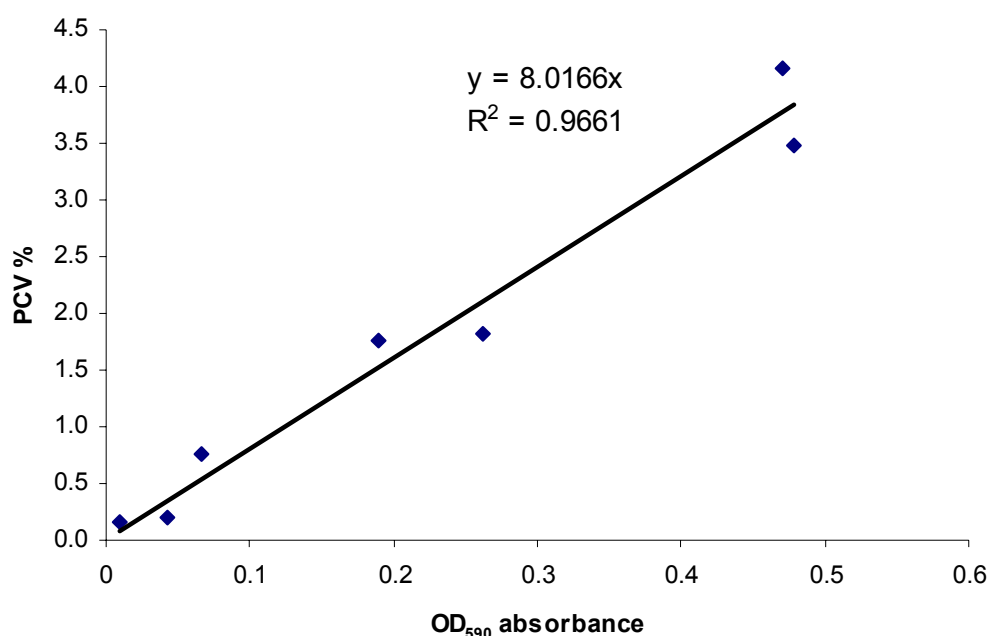


Figure 4.10 Correlation between OD₅₉₀ absorbance and PVC

The effect of media on growth in microtitre plates (as OD₅₉₀ absorbance, converted to PVC %) and shake flask growth (as PCV %) are summarized in Table 4.4. Media 411 gave higher biomass for both procedures (3.02 in microtitre plates; 4.17 in shake flasks), Media 412 and 413 gave moderate biomass (2.57 in microtitre plates; 3.48 and 1.5 respectively in shake flasks) and Media 416 gave low growth (1.56 in microtitre plates and 0.91 in shake flasks). Media 414, 415, 417 and 418 give similar growth (PVC of 2.20 – 2.22) in microtitre plates but variable, lower growth (PVC of 0.21 - 0.89) in shake flasks.

Table 4.4 Summary of the effect of media composition on growth in microtitre plates and shake flasks

Medium	Microtitre plate PCV%	Shake flask PCV%
M411	3.02	4.17
M412	2.57	3.48
M413	2.57	1.5
M414	2.22	0.77
M415	2.20	0.21
M416	1.56	0.91
M417	2.20	0.61
M418	2.20	0.89

There is a good correlation between data obtained from the microtitre plate procedure (OD₅₉₀ converted to PVC) and data from shake flasks (Fig. 4.11).

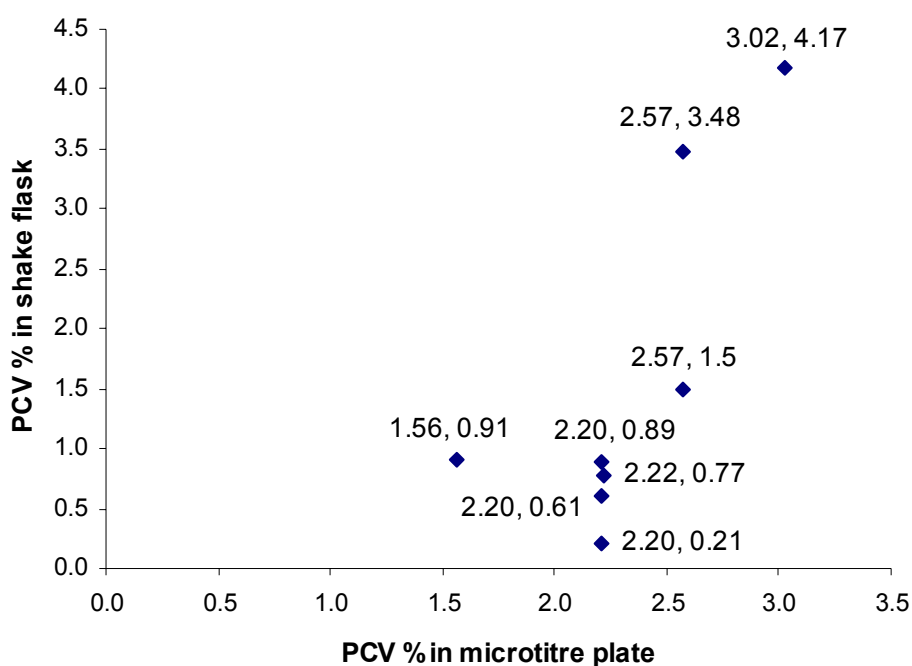


Figure 4.11 Comparison of cell growth data from microtitre plates and shake flasks ($r=0.75$; $n=8$)

Medium 411 has no added NaCl, medium 418 has the highest added NaCl (5 g/L), and the other media contained 2 g/L NaCl. Growing inoculum on seed medium 411 (NaCl = 0 g/L) improved biomass generation whilst biomass was lower if grown on medium 416 (histidine = 0 g/L). In summary, the data confirm that NaCl was a major factor for *S. hygroscopicus* growth in the seed stage. The difference in cell

growth on other media (at constant NaCl concentration), show that K_2HPO_4 , l-aspartic acid and l-histidine concentrations in seed stage media can affect cell growth.

The Pearson's product moment correlation coefficient tests the association between paired samples. The Pearson's product moment correlation coefficient between paired data from the microtitre plate and the shake flask cell growth for eight different seed media, calculated using the "cor.test" (Statistics software 2005), was 0.75, indicating a positive correlation between the two procedures (Table 4.5). The *p*-value of 0.016 for no association versus a positive association indicates a strong association.

Table 4.5 Calculation of correlation coefficient on cell growth

```
cor.test(Shake.Flask, Microtitre, alternative="greater")
Pearson's product-moment correlation
Data: pcv shake flask and pcv microtitre plate
t = 2.7875, df = 6, p-value = 0.01584
Alternative hypothesis: true correlation is greater than 0
95% confidence interval:
0.2355671 1.0000000
sample estimates:
Cor = 0.751771
```

Overall, the data indicated that the microtitre procedure was really good, especially for quickly screening many variables at many levels.

4.3.4 Effects on Rapamycin Concentration

Another goal of this study was to measure amount of rapamycin produced in the microtitre procedure with that produced in shake flasks, and to identify if there are any gaps in production reliability. This was done by evaluating the effectiveness of the medium optimization procedure in microtitre plates with rapamycin concentration in shake flasks. Average concentration in microtitre plates was significantly lower than in shake flasks, most probably because of the shorter incubation time (and therefore different cell mass production) and different analysis method. The microtitre plates were only incubated for three days to limit the effect of evaporation whereas shake flasks were incubated for seven days. However,

results for rapamycin production provided useful information on the inter-relationship between microtitre plates and shake flasks (Table 4.6).

Table 4.6 Summary of the effect of media composition on rapamycin concentration in microtitre plates and shake flasks

Medium	Shake flask (mg/L)	Microtitre plate (mg/L)
M411	135.6	14.3
M412	82.8	4.4
M413	88.9	14.3
M414	97.1	7.2
M415	30.3	2.5
M416	6.1	9
M417	83.1	7
M418	114.7	11.4

There was a direct correlation between rapamycin production in microtitre plates and shake flasks, irrespective of the seed media used (Fig. 4.12). The regression line goes through the origin and has a regression coefficient r of 0.54 ($n=8$).

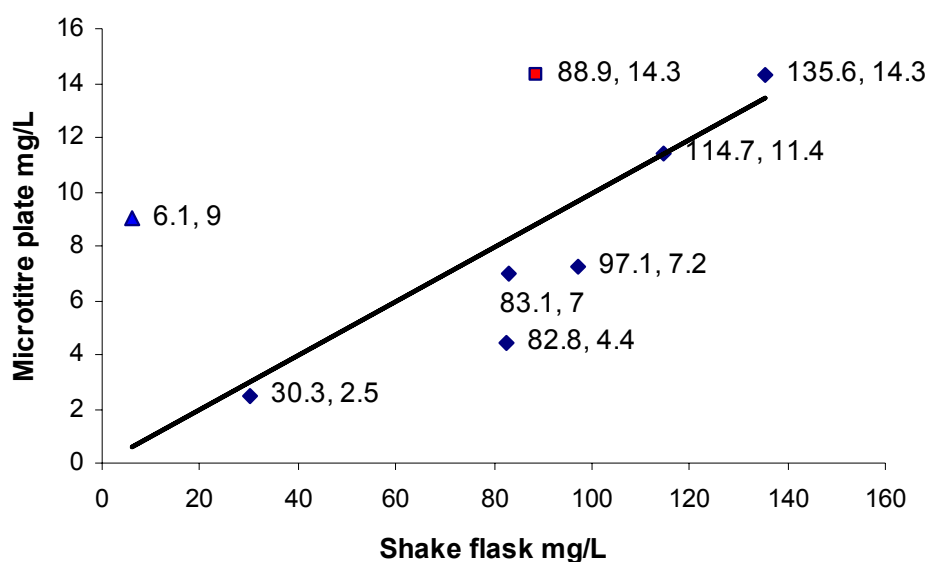


Figure 4.12 Comparison of rapamycin concentration data from microtitre plates and shakes flasks ($r=0.54$; $n=8$)

The calculated Pearson's product moment correlation coefficient for association between the paired data from microtitre plates and shake flasks for the eight different

media was 0.54, indicating a positive correlation (Table 4.7). The *p*-value (0.08) also indicates a strong positive association.

Table 4.7 Calculation of correlation coefficient on rapamycin concentration

```
cor.test(Shake.Flask, Microtitre, alternative="greater")
Pearson's product-moment correlation
Data: Shake flask and microtitre
t = 1.574, df = 6, p-value = 0.08327
Alternative hypothesis: true correlation is greater than 0
95% confidence interval:
-0.1298553 1.0000000
sample estimates:
Cor = 0.5406
```

Rapamycin concentration on Media M415 was low for both the microtitre plate and shake flask procedures. This media had the lowest l-aspartic acid level (0 g/L) and highest K₂HPO₄ level (4 g/L). After seven days incubation in shake flasks, the DCW was 16.77 g/L and the rapamycin was 30.3 mg/L rapamycin. After three days growth, only 2.5 mg/L of rapamycin had been produced in the microtitre plates, which was the lowest concentration recorded. These data indicate that either the high K₂HPO₄ concentration is inhibiting growth and/or rapamycin production or that the secondary metabolite requires l-aspartic acid.

Media M411 and M418 gave higher product concentration in both the microtitre plate and the shake flask procedures. The highest rapamycin concentration in microtitre plates (14.3 mg/L) and shake flasks (135.6 mg/L) occurred when the media had no added NaCl (M411). Rapamycin production in shake flasks with M418 (control medium) corresponded well with data obtained from microtitre plates, being 114.7 mg/L and 11.4 mg/L respectively

Data in Figure 4.12 have a lot of scatter due to two outliers. Rapamycin concentration on Media M416 and M413 when the culture was grown in microtitre plates was very different than production in shake flasks. If the two outliers are removed from the dataset, the correlation improves substantially (*r*=0.92; *n*=6). The lowest rapamycin concentration in shake flasks was 6.1 mg/L on M416. However, 9 mg/L was produced in microtitre plates, which was a moderate value. This media

has no l-histidine. These results indicate that l-histidine is a variable amino acid in the seed media, and its effect on *S. hygroscopicus* growth and rapamycin production may be affected by the growth the procedure or incubation time. Data obtained from both growth procedures indicate that l-histidine concentration must be 0.25 to 0.5 g/L for effective rapamycin production.

The second outlier occurred for media M413, which has the highest salt 1 solution (40 ml/L). High (14.3 mg/L) rapamycin concentration occurs in the microtitre plate procedure but only moderate concentration (88.9 mg/L) in shake flasks. These values indicate that the concentration of salt 1 solution affects rapamycin concentration. The effect of trace mineral elements may be sensitive to production scale.

These results illustrate the usefulness of the microtitre plate method. It gave comparable results to the shake flask procedure. The recommendation is to continue developing this method so it is the standard procedure for rapid and efficient screening fermentation media.

4.4 Influence of Inoculum

Inoculum ‘quality’ is often regarded as an influential factor in performance of final production stage fermentation. However, methods to assess “quality” are open to question. Extensive efforts have been made to improve rapamycin concentration through mutagenesis of *S. hygroscopicus* (Cheng *et al.* 2001; Xu *et al.* 2005) and optimising fermentation conditions using new process control strategies (Chen *et al.* 1999). However, very little has been published on the effects of cultivation conditions on rapamycin concentration, specifically in relation to the seed stage inoculum medium.

In the present work, the effects of variations in seed medium components and their concentrations on rapamycin concentration with *S. hygroscopicus* by fermentation in SYLGG production medium were determined. Maximum rapamycin concentration (135.6 mg/L) was obtained in M411, which had no added NaCl (Fig. 4.13). Significantly less rapamycin (6.1 mg/L) occurred in medium M416, which had no added l-histidine. Rapamycin concentration in medium M415, which had no added

l-aspartic acid but a higher K_2HPO_4 concentration was also low (30.3 mg/L). The NaCl concentration significantly affected rapamycin concentration; Media M411, M417 and M418 had 0, 2 and 5 g/L NaCl respectively and produced 135.6, 83.1 and 114.7 mg/L of rapamycin respectively. M412, M413, M414 and M417 contained the same concentration of NaCl (2 g/L) but differing concentrations of K_2HPO_4 , salt 1 solution, and l-aspartic acid.

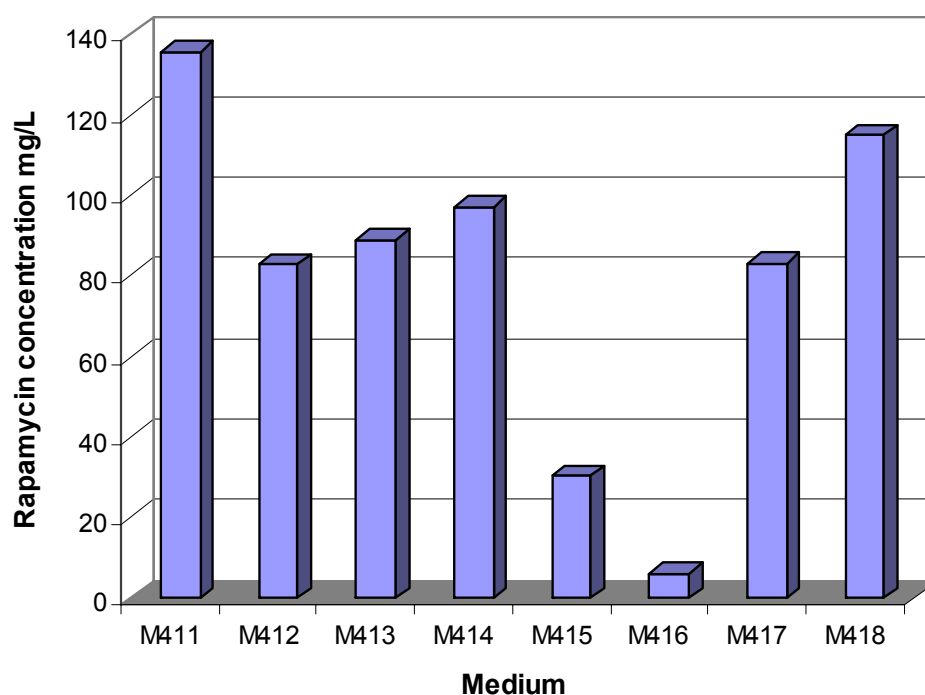


Figure 4.13 Effect of seed media composition on rapamycin concentration by *S. hygroscopicus* after seven days fermentation in SYLGG production medium (seed media composition in Table 3.3)

These results demonstrated that components in preceding seed stage medium affect rapamycin concentration in the final product fermentation. Results from this study suggest that rapamycin concentration by *S. hygroscopicus* tends to be primarily influenced by NaCl, l-histidine, K_2HPO_4 and l-aspartic acid. Reducing the NaCl concentration, then selecting an appropriate concentration range for K_2HPO_4 , l-aspartic acid and l-histidine in the seed stage medium generally increases rapamycin concentration in the production stage.

4.5 Effects on Specific Productivity

In secondary metabolite production processes, maximum cell biomass and secondary metabolite production are not correlated but depend on various other factors such as

medium composition, fermentation conditions and incubation time. Thus, a higher biomass may not necessarily give higher concentrations or productivity of the desired second metabolite.

Specific productivity (i.e. units product per unit time or per unit biomass per unit time) can indicate the effect of fermentation processes, culture growth and product yields. This study did not investigate the effect of inoculum size on rapamycin concentration; instead, seed culture inoculation was kept at the 10% v/v as determined in previous trials. Rapamycin concentration was measured at a given time (7 days). Specific productivity was based on the increase in DCW between inoculation (time = 0) and harvest (time = 7 days or 168 hours in shake flask).

$$\text{Specific productivity at 168 h} = \frac{\text{rapamycin titre at 168 hours}}{\text{change in DCW between 0 and 168 hours}}$$

$$= \frac{R}{D_h - D_0} = \frac{\text{mg} / \text{L}}{\text{g} / \text{L}} = \text{mg rapamycin} / \text{g DCW}$$

$$\text{Specific productivity mg rapa} / \text{g dcw} / \text{h} = \frac{\text{specific productivity at 168 hours}}{168 \text{ hours}}$$

The effect of seed media composition followed by fermentation in SYLGG production medium for seven days on DCW, rapamycin concentration (by HPLC) and rapamycin specific productivity by *S. hygroscopicus* in shake flasks are summarised in Table 4.8. The highest specific productivity after seven days occurred on Medium 411 (Fig. 4.14). This value was 15 times greater than on M416, four times higher than on M415 and almost twice that of M413, M414, M417 and M418 (control medium). The NaCl concentration in the seed medium significantly affects rapamycin concentration. The initial study in microtitre plates showed the higher rapamycin production by *S. hygroscopicus* occurred if the optimized seed medium contained less than 2 g/L NaCl, or even no added NaCl. Data from shake flasks confirmed this observation, and also indicate that the microtitre plate procedure gave indicative results.

Table 4.8 Effect of seed media on DCW, rapamycin concentration and rapamycin specific productivity by *S. hygroscopicus* after seven days fermentation (control media = M418)

	Media							
	M411	M412	M413	M414	M415	M416	M417	M418
DCW, g/L (production stage)	18.46	14.48	29.07	24.01	16.77	13.8	22.77	27.94
Rapamycin, mg/L	135.6	82.8	88.9	97.1	30.3	6.1	83.1	114.7
Spec. productivity, mg/g DCW-h	0.044	0.034	0.018	0.024	0.011	0.003	0.022	0.024

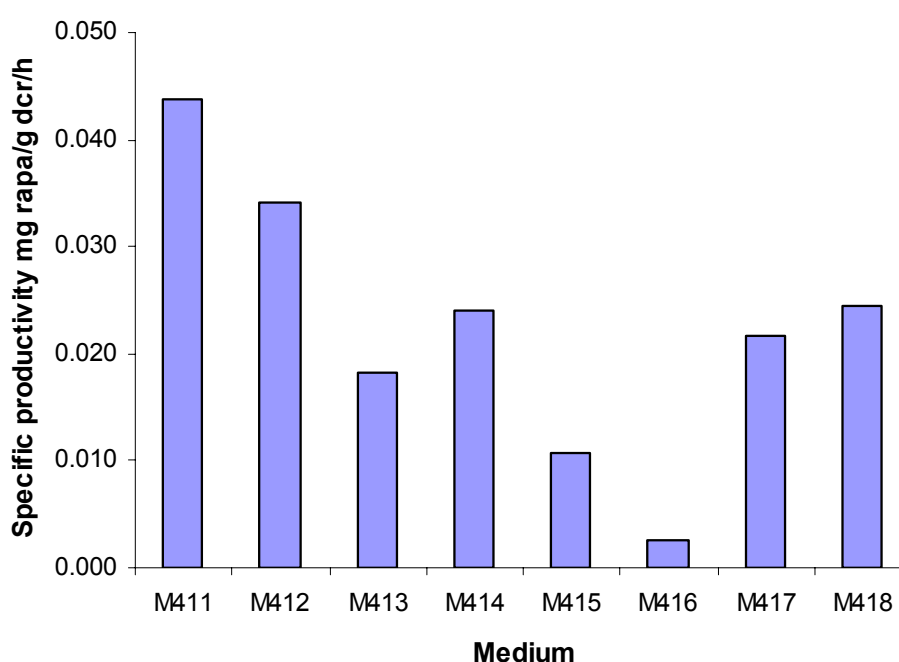


Figure 4.14 Effect of seed media composition on rapamycin specific productivity in shake flasks after seven days fermentation in SYLGG production medium (seed media composition in Table 3.3)

The data indicate that optimized seed medium for producing rapamycin with *S. hygroscopicus* is (g/L): fructose 20, mannose 5, yeast extract 3, l-aspartic acid monosodium 1.5, l-arginine 0.5, l-histidine 0.5, K₂HPO₄ 2 and 30 mL of salt 1 solution. Specific rapamycin productivity increased from 0.024 in the original media to 0.044 mg rapamycin/g DCW-h.

4.6 Assessing the Microtitre Plate Method

When comparing methodology, factors such as precision, accuracy, time, materials, labour and equipment need to be considered. Identifying factors that affect microbial growth and productivity involves many trials and therefore tends to be costly. Any method that reduces time, labour, and/or materials and equipment without sacrificing accuracy and precision will speed developments. The microtitre plate method developed in this study was assessed on two factors: speed and product performance. Speed can be measured by total cycle time (from culture inoculation to product concentration analysis) and time taken for the various stages (medium preparation, medium sterilization, culture inoculation, product analysis and equipment cleaning). Product performance can be measured as total cost, including unit cost, raw material cost and development cost by stage, and product quality.

4.6.1 Speed Analysis

To compare the time taken to carry out the microtitre plate and the shake flask methods, the time characteristics were ranked on a 5-point scale (Table 4.9). Five process stages considered: medium preparation, medium sterilization, culture inoculation, samples analysis, and equipment cleaning. The time spent measuring each unit was based on the time used to analyse one sample in both the microtitre plate and the shake flasks trials. These measurements were used to illustrate the efficiency with reference to the medium development processes. Lower total scores would indicate a rapid process with high efficiency.

Table 4.9 Time score criteria for various operations in the procedure

Attribute	Minimal (<1 min)	Less (1 to 5 min)	Moderate (5 to 15 min)	More (15 to 30 in)	Extensive (>30 min)
Medium preparation	1	2	3	4	5
Medium sterilization	1	2	3	4	5
Culture inoculation	1	2	3	4	5
Sample analysis	1	2	3	4	5
Equipment cleaning	1	2	3	4	5

Scores for the various process stages in the microtitre and shake flask methods were analyzed using the criteria developed and then totalled to give the overall score for each method (Table 4.10).

Table 4.10 Time scores for microtitre plate and shake flask procedures for investigative cell culture

Attribute	Microtitre plate		Shake flask	
	Score	Notes	Score	Notes
Medium preparation	3	Using stock solution	5	
Medium sterilization	4	1 autoclave run	4	1 autoclave run
Culture inoculation	1	Using multi-pipette	2	
Sample analysis	2	Bioassay	4	HPLC assay
Equipment cleaning	0	No cleaning required	3	Washing
Total	10		18	

The time total score for the microtitre plate procedure was 10 (maximum of 25), compared with 18 for the shake flask procedure. This score indicates that the microtitre plate method is much more efficient and saves time in the medium preparation, culture inoculation, sample analysis and equipment cleaning steps.

4.6.2 Costs Analysis

As well as scoring the time attributes, the cost of equipment and operational costs must also be considered when comparing overall performance of the two methodologies. Relative costs, based on catalogue prices for raw materials, glassware and equipment were calculated (Table 4.11). It is assumed that rapamycin in the microtitre plate method is analysed by bioassay and whilst rapamycin in shake flasks is analysed by HPLC. A Box-Behnken experimental design to investigate ten factors, each at three levels was used for both methods.

The capital and material costs (excluding labour costs) are much higher for shake flasks than for microtitre plates. The major cost in shake flasks is the cost associated with the glassware, which is 100-fold higher than for the microtitre plates. However, shake flasks are reusable after cleaning, so replacement costs are only associated with breakages. On the other hand, microtitre plates are disposed at the end of each run (which has an associated cost) and new ones are needed for the next run.

Table 4.11 Comparison of processing costs for using microtitre plates and shake flasks for fermentation runs

Unit	Process steps	Shake flask	Microtitre plate
Experiment scope	Number of factors tested simultaneously	10	10
	Number of medium levels tested simultaneously	3	3
	Number of samples for the Box-Behnken experimental design	170	170
Glassware	Number of containers required	170 x 250-mL Kimax Erlenmeyer flasks 26500	2 x Nunc™ 96 well microplates EW-01928-04
	Material capital costs	US\$ 691.20	US\$ 6.48
	Material replacement costs	US\$ 6.91 (1% for breakage)	US\$ 6.48
Shaker	Number of shakers required	4 x Infors Multitition shaker	1 x Infors Multitition shaker
	Capital cost of shaker	4 x US\$24,932.00 = US\$99,728.00	1 x US\$24,932.00 = US\$24,932.00
Raw material	Seed medium required	8500 mL	50 mL
	Seed medium costs	8.5L x US\$5.07/L = US\$43.10	0.05 L x US\$5.07/L = US\$0.25
	Production medium required	8500 mL	50 mL
	Production medium cost	8.5 L x US\$7.90/L = US\$67.15	0.05 L x US\$7.90/L = US\$0.40
Production analysis	Analysis method	HPLC	Bioassay
	Sample preparation	Required	nil
	Reference material	Column, stationary phase, reagents and hardware	<i>C. albicans</i> and MY agar (2 L)
	Analysis costs	US\$9,811.90	<i>C. albicans</i>: US\$ 69 MY: 2 L x US\$5.97/L = US\$11.94
Cycle time	Medium preparation	7 days	3 days
	Seed stage incubation	3 days	3 days
	Production stage incubation	7 days	3 days
	Analysis	8 days	2 days
	Total	25 days	11 days

A Multitition shaker holds a maximum of 48 flasks, so four shakers are required if a trial with 170 flasks is run in a single batch. The alternative is to divide the run into batches, but this extends testing time and also introduces variability between batches. Buying four shakers considerably increases equipment cost. However, running the

trial in four batches increases labour cost. Only one shaker is required for the microtitre plate method.

Raw material costs for the microtitre plate method are much lower than for shake flasks because the former method uses small wells (200 μ l working volume in 400 μ l wells). Catalogue prices (Appendix 6) indicate that seed medium costs US\$5.07/L, and production medium costs US\$7.90/L. The microtitre plate process requires only US\$0.25 of seed medium and US\$0.40 of production medium, making it much more economic than the shake flask method.

If a bioassay rather than HPLC is used to measure rapamycin, costs decrease significantly. The total cost for a HPLC, assuming an HPLC system with columns, stationary phase, reagents and hardware, is US\$ 9811.90 whereas the total cost for the bioassay, including micro-organism culture and media raw material is only US\$80.94. Labour cost for both methods was estimated from the labour used in the current study, and costed at US\$30/h (US\$210/day). Because media preparation and samples analysis contribute a lot of time to shake flask procedure, labour costs are more than twice (US\$5,250.00) that for the microtitre plate method (US\$2,310.00).

The advantages of the microtitre plate method developed in this study are the speed of doing the tests (three days to investigate the effect of 10 factors at three levels) and the ease of isolating media that will produce high rapamycin concentrations. Within 11 days, one complete trial using the microtitre plate method, from medium preparation to complete bioassay, can be performed with one shaker. Glassware and raw material costs are lower and the expensive and tedious HPLC analysis can be replaced by an easier and cheaper bioassay analysis. Thus the microtitre plate procedure is versatile for screening high-producing medium for secondary metabolite production. Major opportunities may exist for using this methodology to test the effect of a large number of factors within a short time when optimising the growth of completely new strains or reassessing current strains.

Chapter Five: Conclusions and Recommendations

Screening trials to optimise fermentation media are labour and time intensive. After identifying a suitable micro-organism, many variables including fermentation conditions, nutrient requirements, and media composition need to be optimised to obtain high productivity. A new method was developed and used to identify how to increase productivity of the rapamycin-producing micro-organism *S. hygroscopicus*.

5.1 Conclusions

- **Seed stage medium**

The preceding seed stage media significantly influences production of the secondary metabolite rapamycin by *S. hygroscopicus* in final stage fermentations. Reducing NaCl concentration, then selecting an appropriate concentration range for K₂HPO₄, l-aspartic acid and l-histidine in the seed stage medium tends to increase biomass production in the seed stage and rapamycin concentration in the production stage.

- **Medium optimisation**

Regression tree analysis and validation experiments indicated that the most effective semi-defined seed medium for fermentation of *S. hygroscopicus* to produce rapamycin contained (in g/L): fructose 20, mannose 5, yeast extract 3, l-aspartic acid 1.5, l-arginine 0.5, l-histidine 0.5, K₂HPO₄ 2 and 30 mL salt solution.

- **Regression trees**

Using regression trees to present data from medium optimization studies in microtitre plates allowed the key variables affecting culture growth and rapamycin production to be identified, as well as any interaction between variables. The regression tree is a simple way to visualise the data.

- **Microtitre plate procedure**

- ❖ A microtitre plate method was developed to investigate the effects of media components and their concentrations on cell growth and rapamycin concentration. The microtitre plates were used to investigate both the seed growth stage and the production stage.

- ❖ The test involved transferring 20- μ L aliquots of inoculum to study seed media in wells of a microtitre plate, and incubating the plates for the required temperature and time on a rotary shaker. Then 20- μ L of culture media was transferred to production media in wells of a microtitre plate and incubated.
- ❖ The microtitre plate procedure developed was done at 110 rpm and 28°C in a rotary shaker using 0.2 mL broth in 0.4-mL wells. The *S. hygroscopicus* growths with submerged medium under uniform conditions from individual well to well in microtitre plate were acceptable. Data had a normal distribution with corresponding 95% probability of variation.
- ❖ Broth volume in the microtitre plates decreased with incubation time due to evaporation, especially in wells on the edge of the plate. Production fermentation time had to be shortened, which increased the variability of the final results. Evaporative losses could be reduced by wrapping the microtitre plates in parafilm. Optimum culture time to reduce excess evaporation was three days, which would affect growth, product concentration, nutrient limitation, bioassay analysis and productivity.
- ❖ A bioassay was used to determine rapamycin concentration in the microtitre plates, which means expensive and tedious HPLC analyses can be reduced to the minimum. There was a direct relationship between the area of inhibition zone in the bioassay and rapamycin concentration.
- ❖ There was a direct correlation between the effect of seed media composition on cell growth in the seed stage and rapamycin concentration in production stage obtained by microtitre plate and shake flask procedures. The correlation coefficient of $r=0.75$ ($n=8$) and $p=0.016$ calculated using Pearson's product moment indicated a positive association between cell growth from the two procedures and the correlation coefficient $r=0.92$ ($n=6$) and $p=0.08$ showed a positive correlation between rapamycin production in microtitre plates and shake flasks on the same media.

- ❖ The microtitre plate technique developed was simple, effective, and efficient. This method saves a lot of time in medium preparation, culture inoculation, sample analysis and equipment cleaning steps.
- ❖ Using microtitre plates for screening studies can markedly reduce costs in terms of media used, labour, assay costs and equipment capital. These help reduce the overall cost involved in media design studies.
- ❖ The microtitre plate procedure could be used as a high-throughput screening technique for optimising media for producing secondary metabolites.

5.2 Recommendations

- **Scale up**

Biomass and rapamycin production in microtitre plates and shake flasks were positively correlated. The best medium identified from shake flask trials will normally also give good results in larger bioreactors such as stirred tanks. To maximise the advantages of doing screening trials in microtitre plates, the effect of scaling from plates to bioreactors should be determined.

- **Microtitre plate applications**

Producing rapamycin using *S. hygroscopicus* in submerged fermentation is currently commercialised. The aim of developing the microtitre plate procedure was to have a quick way to identify an optimal seed medium. The microtitre plate procedure allows a large number of repetitive, rapid, small-scale, low-cost experiments to be carried out without cross-contamination. It also gave comparable results to the shake flask procedure. It is recommended that the procedure be trailed for other media composition optimisation studies and could be further developing, so it is the standard procedure for rapid and efficient screening fermentation media.

- **Medium component – yeast extract**

Yeast extract is a common key component in complex media for industrial fermentations and was the only complex raw material used in the optimal seed medium trials. However, due to lot-to-lot variation, which affects fermentation, further work should be done to identify a chemically-defined, yeast extract substitute.

- **Microtitre plate procedure improvement**

Oxygen transfer, which is affected by factors such as agitation rate, is important in large-scale microbial production whether the product is biomass, or primary or secondary metabolites. The microtitre plate procedure developed was done at 110 rpm and 28°C in a rotary shaker using 0.2 mL broth in 0.4-mL volume well. The broth volume in the microtitre plates decreases with incubation time, especially in wells on the edge of the plate. Ways to obtain optimal oxygen transfer whilst limiting evaporation should be investigated.

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APPENDICES

Appendix 1 Media Formulation

1.1 SYLGG Rapamycin Production Medium

Glucose	10 g/L
Glycerol	30 g/L
Yeast extract	6.5 g/L
Hy soy	10 g/L
L-lysine	6.5 g/L
K ₂ HPO ₄	1.14 g/L
KH ₂ PO ₄	0.7 g/L
NaCl	5 g/L
FeSO ₄	0.01 g/L
Distilled water	1.0 L
pH = 6.0	

1.2 BYM Broth

Yeast extract	3 g/L
Malt extract	3 g/L
Hy soy	5 g/L
Glucose	10 g/L
Distilled water	1.0 L
pH = 6.2	

1.3 Malt Yeast Extract Agar

Malt extract	30 g/L
Yeast extract	5 g/L
Agar	15 g/L
Distilled water	1.0 L
pH = 5.4	

Appendix 2 Box-Behnken Design Matrix

Source: Ronald, B. Crosier, 15 June 1993; revised 23 May 2001
<http://lib.stat.cmu.edu/designs/rbc>

Box-Behnken design for 10 factors: 170 runs, 3 levels, 2 blocks. Last column is block number.

0	1	0	0	0	-1	-1	0	0	-1	1
0	-1	0	0	0	1	-1	0	0	-1	1
0	-1	0	0	0	-1	1	0	0	-1	1
0	1	0	0	0	1	1	0	0	-1	1
0	-1	0	0	0	-1	-1	0	0	1	1
0	1	0	0	0	1	-1	0	0	1	1
0	1	0	0	0	-1	1	0	0	1	1
0	-1	0	0	0	1	1	0	0	1	1
1	-1	0	0	-1	0	0	0	0	-1	1
-1	1	0	0	-1	0	0	0	0	-1	1
-1	-1	0	0	1	0	0	0	0	-1	1
1	1	0	0	1	0	0	0	0	-1	1
-1	-1	0	0	-1	0	0	0	0	1	1
1	1	0	0	-1	0	0	0	0	1	1
1	-1	0	0	1	0	0	0	0	1	1
-1	1	0	0	1	0	0	0	0	1	1
0	1	-1	0	0	0	-1	-1	0	0	1
0	-1	1	0	0	0	-1	-1	0	0	1
0	-1	-1	0	0	0	1	-1	0	0	1
0	1	1	0	0	0	1	-1	0	0	1
0	-1	-1	0	0	0	-1	1	0	0	1
0	1	1	0	0	0	-1	1	0	0	1
0	1	-1	0	0	0	1	1	0	0	1
0	-1	1	0	0	0	1	1	0	0	1
0	1	0	-1	0	-1	0	0	-1	0	1
0	-1	0	1	0	-1	0	0	-1	0	1
0	-1	0	-1	0	1	0	0	-1	0	1
0	1	0	1	0	1	0	0	-1	0	1
0	-1	0	-1	0	-1	0	0	1	0	1
0	1	0	1	0	-1	0	0	1	0	1
0	1	0	-1	0	1	0	0	1	0	1
0	-1	0	1	0	1	0	0	1	0	1
1	0	0	0	0	0	0	-1	-1	-1	1
-1	0	0	0	0	0	0	1	-1	-1	1
-1	0	0	0	0	0	0	-1	1	-1	1
1	0	0	0	0	0	0	1	1	-1	1
-1	0	0	0	0	0	0	-1	-1	1	1
1	0	0	0	0	0	0	1	-1	1	1

1	0	0	0	0	0	0	-1	1	1	1
-1	0	0	0	0	0	0	1	1	1	1
0	0	1	-1	-1	0	0	0	0	-1	1
0	0	-1	1	-1	0	0	0	0	-1	1
0	0	-1	-1	1	0	0	0	0	-1	1
0	0	1	1	1	0	0	0	0	-1	1
0	0	-1	-1	-1	0	0	0	0	1	1
0	0	1	1	-1	0	0	0	0	1	1
0	0	1	-1	1	0	0	0	0	1	1
0	0	-1	1	1	0	0	0	0	1	1
1	0	0	-1	0	0	-1	-1	0	0	1
-1	0	0	1	0	0	-1	-1	0	0	1
-1	0	0	-1	0	0	1	-1	0	0	1
1	0	0	1	0	0	1	-1	0	0	1
-1	0	0	-1	0	0	-1	1	0	0	1
1	0	0	1	0	0	-1	1	0	0	1
1	0	0	-1	0	0	1	1	0	0	1
-1	0	0	1	0	0	1	1	0	0	1
0	0	1	0	-1	0	-1	0	-1	0	1
0	0	-1	0	1	0	-1	0	-1	0	1
0	0	-1	0	-1	0	1	0	-1	0	1
0	0	1	0	1	0	1	0	-1	0	1
0	0	-1	0	-1	0	-1	0	1	0	1
0	0	1	0	1	0	-1	0	1	0	1
0	0	1	0	-1	0	1	0	1	0	1
0	0	-1	0	1	0	1	0	1	0	1
1	0	-1	0	0	-1	0	0	-1	0	1
-1	0	1	0	0	-1	0	0	-1	0	1
-1	0	-1	0	0	1	0	0	-1	0	1
1	0	1	0	0	1	0	0	-1	0	1
-1	0	-1	0	0	-1	0	0	1	0	1
1	0	1	0	0	-1	0	0	1	0	1
1	0	-1	0	0	1	0	0	1	0	1
-1	0	1	0	0	1	0	0	1	0	1
0	0	0	1	-1	-1	0	-1	0	0	1
0	0	0	-1	1	-1	0	-1	0	0	1
0	0	0	-1	-1	1	0	-1	0	0	1
0	0	0	1	1	1	0	-1	0	0	1
0	0	0	-1	-1	-1	0	1	0	0	1
0	0	0	1	1	-1	0	1	0	0	1
0	0	0	1	-1	1	0	1	0	0	1
0	0	0	-1	1	1	0	1	0	0	1
0	0	0	0	0	0	0	0	0	0	1
0	0	0	0	0	0	0	0	0	0	1
0	0	0	0	0	0	0	0	0	0	1
0	0	0	0	0	0	0	0	0	0	1

0	0	0	0	0	0	0	0	0	0	1
0	-1	0	0	0	-1	-1	0	0	-1	2
0	1	0	0	0	1	-1	0	0	-1	2
0	1	0	0	0	-1	1	0	0	-1	2
0	-1	0	0	0	1	1	0	0	-1	2
0	1	0	0	0	-1	-1	0	0	1	2
0	-1	0	0	0	1	-1	0	0	1	2
0	-1	0	0	0	-1	1	0	0	1	2
0	1	0	0	0	1	1	0	0	1	2
-1	-1	0	0	-1	0	0	0	0	-1	2
1	1	0	0	-1	0	0	0	0	-1	2
1	-1	0	0	1	0	0	0	0	-1	2
-1	1	0	0	1	0	0	0	0	-1	2
1	-1	0	0	-1	0	0	0	0	1	2
-1	1	0	0	-1	0	0	0	0	1	2
-1	-1	0	0	1	0	0	0	0	1	2
1	1	0	0	1	0	0	0	0	1	2
0	-1	-1	0	0	0	-1	-1	0	0	2
0	1	1	0	0	0	-1	-1	0	0	2
0	1	-1	0	0	0	1	-1	0	0	2
0	-1	1	0	0	0	1	-1	0	0	2
0	1	-1	0	0	0	-1	1	0	0	2
0	-1	1	0	0	0	-1	1	0	0	2
0	-1	-1	0	0	0	1	1	0	0	2
0	1	1	0	0	0	1	1	0	0	2
0	-1	0	-1	0	-1	0	0	-1	0	2
0	1	0	1	0	-1	0	0	-1	0	2
0	1	0	-1	0	1	0	0	-1	0	2
0	-1	0	1	0	1	0	0	-1	0	2
0	1	0	-1	0	-1	0	0	1	0	2
0	-1	0	1	0	-1	0	0	1	0	2
0	-1	0	-1	0	1	0	0	1	0	2
0	1	0	1	0	1	0	0	1	0	2
-1	0	0	0	0	0	0	-1	-1	-1	2
1	0	0	0	0	0	0	1	-1	-1	2
1	0	0	0	0	0	0	-1	1	-1	2
-1	0	0	0	0	0	0	1	1	-1	2
1	0	0	0	0	0	0	-1	-1	1	2
-1	0	0	0	0	0	0	1	-1	1	2
-1	0	0	0	0	0	0	-1	1	1	2
1	0	0	0	0	0	0	1	1	1	2
0	0	-1	-1	-1	0	0	0	0	-1	2
0	0	1	1	-1	0	0	0	0	-1	2
0	0	1	-1	1	0	0	0	0	-1	2
0	0	-1	1	1	0	0	0	0	-1	2
0	0	1	-1	-1	0	0	0	0	1	2

0	0	-1	1	-1	0	0	0	0	1	2
0	0	-1	-1	1	0	0	0	0	1	2
0	0	1	1	1	0	0	0	0	1	2
-1	0	0	-1	0	0	-1	-1	0	0	2
1	0	0	1	0	0	-1	-1	0	0	2
1	0	0	-1	0	0	1	-1	0	0	2
-1	0	0	1	0	0	1	-1	0	0	2
1	0	0	-1	0	0	-1	1	0	0	2
-1	0	0	1	0	0	-1	1	0	0	2
-1	0	0	-1	0	0	1	1	0	0	2
1	0	0	1	0	0	1	1	0	0	2
0	0	-1	0	-1	0	-1	0	-1	0	2
0	0	1	0	1	0	-1	0	-1	0	2
0	0	1	0	-1	0	1	0	-1	0	2
0	0	-1	0	1	0	1	0	-1	0	2
0	0	1	0	-1	0	-1	0	1	0	2
0	0	-1	0	1	0	-1	0	1	0	2
0	0	-1	0	-1	0	1	0	1	0	2
0	0	1	0	1	0	1	0	1	0	2
-1	0	-1	0	0	-1	0	0	-1	0	2
1	0	1	0	0	-1	0	0	-1	0	2
1	0	-1	0	0	1	0	0	-1	0	2
-1	0	1	0	0	1	0	0	-1	0	2
1	0	-1	0	0	-1	0	0	1	0	2
-1	0	1	0	0	-1	0	0	1	0	2
-1	0	-1	0	0	1	0	0	1	0	2
1	0	1	0	0	1	0	0	1	0	2
0	0	0	-1	-1	-1	0	-1	0	0	2
0	0	0	1	1	-1	0	-1	0	0	2
0	0	0	1	-1	1	0	-1	0	0	2
0	0	0	-1	1	1	0	-1	0	0	2
0	0	0	1	-1	-1	0	1	0	0	2
0	0	0	-1	1	-1	0	1	0	0	2
0	0	0	-1	-1	1	0	1	0	0	2
0	0	0	1	1	1	0	1	0	0	2
0	0	0	0	0	0	0	0	0	0	2
0	0	0	0	0	0	0	0	0	0	2
0	0	0	0	0	0	0	0	0	0	2
0	0	0	0	0	0	0	0	0	0	2
0	0	0	0	0	0	0	0	0	0	2
0	0	0	0	0	0	0	0	0	0	2

Appendix 3 Microtitre Plate Medium Preparation Actual Layout

Fructose	Monnose	Yeast	L-aspartic	L-arginine	L-histidine	NaCl	K ₂ HPO ₄	Salt 1	Dummy	Plate	Well
20	10	3	1.5	0.5	0	0	2	20	-1	1	B11
20	0	3	1.5	0.5	1	0	2	20	-1	1	H11
20	0	3	1.5	0.5	0	10	2	20	-1	1	E8
20	10	3	1.5	0.5	1	10	2	20	-1	1	F6
20	0	3	1.5	0.5	0	0	2	20	1	1	A11
20	10	3	1.5	0.5	1	0	2	20	1	1	D9
20	10	3	1.5	0.5	0	10	2	20	1	1	D6
20	0	3	1.5	0.5	1	10	2	20	1	1	E11
40	0	3	1.5	0	0.5	5	2	20	-1	1	F2
10	10	3	1.5	0	0.5	5	2	20	-1	1	E4
10	0	3	1.5	1	0.5	5	2	20	-1	1	H8
40	10	3	1.5	1	0.5	5	2	20	-1	1	G4
10	0	3	1.5	0	0.5	5	2	20	1	1	G7
40	10	3	1.5	0	0.5	5	2	20	1	1	B3
40	0	3	1.5	1	0.5	5	2	20	1	1	A8
10	10	3	1.5	1	0.5	5	2	20	1	1	H6
20	10	1	1.5	0.5	0.5	0	0	20	0	1	D8
20	0	6	1.5	0.5	0.5	0	0	20	0	1	B12
20	0	1	1.5	0.5	0.5	10	0	20	0	1	D4
20	10	6	1.5	0.5	0.5	10	0	20	0	1	H4
20	0	1	1.5	0.5	0.5	0	4	20	0	1	G6
20	10	6	1.5	0.5	0.5	0	4	20	0	1	A10
20	10	1	1.5	0.5	0.5	10	4	20	0	1	D11
20	0	6	1.5	0.5	0.5	10	4	20	0	1	C8
20	10	3	0	0.5	0	5	2	5	0	1	F4
20	0	3	3	0.5	0	5	2	5	0	1	F3
20	0	3	0	0.5	1	5	2	5	0	1	C12
20	10	3	3	0.5	1	5	2	5	0	1	G1
20	0	3	0	0.5	0	5	2	40	0	1	C1
20	10	3	3	0.5	0	5	2	40	0	1	H9
20	10	3	0	0.5	1	5	2	40	0	1	H1
20	0	3	3	0.5	1	5	2	40	0	1	E6
40	5	3	1.5	0.5	0.5	5	0	5	-1	1	C11
10	5	3	1.5	0.5	0.5	5	4	5	-1	1	E3
10	5	3	1.5	0.5	0.5	5	0	40	-1	1	D1
40	5	3	1.5	0.5	0.5	5	4	40	-1	1	G10
10	5	3	1.5	0.5	0.5	5	0	5	1	1	E7
40	5	3	1.5	0.5	0.5	5	4	5	1	1	H3
40	5	3	1.5	0.5	0.5	5	0	40	1	1	B7
10	5	3	1.5	0.5	0.5	5	4	40	1	1	H5
20	5	6	0	0	0.5	5	2	20	-1	1	E2
20	5	1	3	0	0.5	5	2	20	-1	1	H10
20	5	1	0	1	0.5	5	2	20	-1	1	B5
20	5	6	3	1	0.5	5	2	20	-1	1	F5
20	5	1	0	0	0.5	5	2	20	1	1	E12
20	5	6	3	0	0.5	5	2	20	1	1	H12

20	5	6	0	1	0.5	5	2	20	1	1	G11
20	5	1	3	1	0.5	5	2	20	1	1	H7
40	5	3	0	0.5	0.5	0	0	20	0	1	E10
10	5	3	3	0.5	0.5	0	0	20	0	1	A6
10	5	3	0	0.5	0.5	10	0	20	0	1	G5
40	5	3	3	0.5	0.5	10	0	20	0	1	G12
10	5	3	0	0.5	0.5	0	4	20	0	1	D12
40	5	3	3	0.5	0.5	0	4	20	0	1	E1
40	5	3	0	0.5	0.5	10	4	20	0	1	B2
10	5	3	3	0.5	0.5	10	4	20	0	1	C5
20	5	6	1.5	0	0.5	0	2	5	0	1	F8
20	5	1	1.5	1	0.5	0	2	5	0	1	A7
20	5	1	1.5	0	0.5	10	2	5	0	1	G8
20	5	6	1.5	1	0.5	10	2	5	0	1	F9
20	5	1	1.5	0	0.5	0	2	40	0	1	D10
20	5	6	1.5	1	0.5	0	2	40	0	1	B10
20	5	6	1.5	0	0.5	10	2	40	0	1	G3
20	5	1	1.5	1	0.5	10	2	40	0	1	A3
40	5	1	1.5	0.5	0	5	2	5	0	1	E9
10	5	6	1.5	0.5	0	5	2	5	0	1	F1
10	5	1	1.5	0.5	1	5	2	5	0	1	F7
40	5	6	1.5	0.5	1	5	2	5	0	1	C10
10	5	1	1.5	0.5	0	5	2	40	0	1	C3
40	5	6	1.5	0.5	0	5	2	40	0	1	G2
40	5	1	1.5	0.5	1	5	2	40	0	1	H2
10	5	6	1.5	0.5	1	5	2	40	0	1	A2
20	5	3	3	0	0	5	0	20	0	1	C2
20	5	3	0	1	0	5	0	20	0	1	A9
20	5	3	0	0	1	5	0	20	0	1	E5
20	5	3	3	1	1	5	0	20	0	1	A12
20	5	3	0	0	0	5	4	20	0	1	D2
20	5	3	3	1	0	5	4	20	0	1	D3
20	5	3	3	0	1	5	4	20	0	1	A4
20	5	3	0	1	1	5	4	20	0	1	D7
20	5	3	1.5	0.5	0.5	5	2	20	0	1	B6
20	5	3	1.5	0.5	0.5	5	2	20	0	1	C4
20	5	3	1.5	0.5	0.5	5	2	20	0	1	F11
20	5	3	1.5	0.5	0.5	5	2	20	0	1	A5
20	5	3	1.5	0.5	0.5	5	2	20	0	1	C9
20	0	3	1.5	0.5	0	0	2	20	-1	2	D1
20	10	3	1.5	0.5	1	0	2	20	-1	2	D12
20	10	3	1.5	0.5	0	10	2	20	-1	2	B6
20	0	3	1.5	0.5	1	10	2	20	-1	2	H6
20	10	3	1.5	0.5	0	0	2	20	1	2	A12
20	0	3	1.5	0.5	1	0	2	20	1	2	A3
20	0	3	1.5	0.5	0	10	2	20	1	2	D5
20	10	3	1.5	0.5	1	10	2	20	1	2	F7
10	0	3	1.5	0	0.5	5	2	20	-1	2	D11
40	10	3	1.5	0	0.5	5	2	20	-1	2	D7
40	0	3	1.5	1	0.5	5	2	20	-1	2	H2
10	10	3	1.5	1	0.5	5	2	20	-1	2	G10
40	0	3	1.5	0	0.5	5	2	20	1	2	C2
10	10	3	1.5	0	0.5	5	2	20	1	2	H4

10	0	3	1.5	1	0.5	5	2	20	1	2	B2
40	10	3	1.5	1	0.5	5	2	20	1	2	C10
20	0	1	1.5	0.5	0.5	0	0	20	0	2	F12
20	10	6	1.5	0.5	0.5	0	0	20	0	2	A11
20	10	1	1.5	0.5	0.5	10	0	20	0	2	F1
20	0	6	1.5	0.5	0.5	10	0	20	0	2	D2
20	10	1	1.5	0.5	0.5	0	4	20	0	2	C8
20	0	6	1.5	0.5	0.5	0	4	20	0	2	A7
20	0	1	1.5	0.5	0.5	10	4	20	0	2	A1
20	10	6	1.5	0.5	0.5	10	4	20	0	2	H1
20	0	3	0	0.5	0	5	2	5	0	2	G8
20	10	3	3	0.5	0	5	2	5	0	2	G9
20	10	3	0	0.5	1	5	2	5	0	2	B4
20	0	3	3	0.5	1	5	2	5	0	2	A5
20	10	3	0	0.5	0	5	2	40	0	2	C5
20	0	3	3	0.5	0	5	2	40	0	2	A2
20	0	3	0	0.5	1	5	2	40	0	2	G6
20	10	3	3	0.5	1	5	2	40	0	2	C4
10	5	3	1.5	0.5	0.5	5	0	5	-1	2	F10
40	5	3	1.5	0.5	0.5	5	4	5	-1	2	F4
40	5	3	1.5	0.5	0.5	5	0	40	-1	2	C3
10	5	3	1.5	0.5	0.5	5	4	40	-1	2	G2
40	5	3	1.5	0.5	0.5	5	0	5	1	2	H9
10	5	3	1.5	0.5	0.5	5	4	5	1	2	B12
10	5	3	1.5	0.5	0.5	5	0	40	1	2	A10
40	5	3	1.5	0.5	0.5	5	4	40	1	2	F9
20	5	1	0	0	0.5	5	2	20	-1	2	H3
20	5	6	3	0	0.5	5	2	20	-1	2	B10
20	5	6	0	1	0.5	5	2	20	-1	2	D9
20	5	1	3	1	0.5	5	2	20	-1	2	G3
20	5	6	0	0	0.5	5	2	20	1	2	E2
20	5	1	3	0	0.5	5	2	20	1	2	E11
20	5	1	0	1	0.5	5	2	20	1	2	C9
20	5	6	3	1	0.5	5	2	20	1	2	B1
10	5	3	0	0.5	0.5	0	0	20	0	2	E9
40	5	3	3	0.5	0.5	0	0	20	0	2	A9
40	5	3	0	0.5	0.5	10	0	20	0	2	E8
10	5	3	3	0.5	0.5	10	0	20	0	2	A8
40	5	3	0	0.5	0.5	0	4	20	0	2	B9
10	5	3	3	0.5	0.5	0	4	20	0	2	D4
10	5	3	0	0.5	0.5	10	4	20	0	2	G5
40	5	3	3	0.5	0.5	10	4	20	0	2	B11
20	5	1	1.5	0	0.5	0	2	5	0	2	H11
20	5	6	1.5	1	0.5	0	2	5	0	2	C11
20	5	6	1.5	0	0.5	10	2	5	0	2	G11
20	5	1	1.5	1	0.5	10	2	5	0	2	H12
20	5	6	1.5	0	0.5	0	2	40	0	2	C12
20	5	1	1.5	1	0.5	0	2	40	0	2	C1
20	5	1	1.5	0	0.5	10	2	40	0	2	G1
20	5	6	1.5	1	0.5	10	2	40	0	2	G12
10	5	1	1.5	0.5	0	5	2	5	0	2	F5
40	5	6	1.5	0.5	0	5	2	5	0	2	G7
40	5	1	1.5	0.5	1	5	2	5	0	2	F8
10	5	6	1.5	0.5	1	5	2	5	0	2	H7

40	5	1	1.5	0.5	0	5	2	40	0	2	A6
10	5	6	1.5	0.5	0	5	2	40	0	2	C6
10	5	1	1.5	0.5	1	5	2	40	0	2	H10
40	5	6	1.5	0.5	1	5	2	40	0	2	D10
20	5	3	0	0	0	5	0	20	0	2	A4
20	5	3	3	1	0	5	0	20	0	2	F3
20	5	3	3	0	1	5	0	20	0	2	F2
20	5	3	0	1	1	5	0	20	0	2	E12
20	5	3	3	0	0	5	4	20	0	2	H8
20	5	3	0	1	0	5	4	20	0	2	C7
20	5	3	0	0	1	5	4	20	0	2	G4
20	5	3	3	1	1	5	4	20	0	2	B3
20	5	3	1.5	0.5	0.5	5	2	20	0	2	E6
20	5	3	1.5	0.5	0.5	5	2	20	0	2	E7
20	5	3	1.5	0.5	0.5	5	2	20	0	2	D3
20	5	3	1.5	0.5	0.5	5	2	20	0	2	D8
20	5	3	1.5	0.5	0.5	5	2	20	0	2	E5

Appendix 4 Microtitre Plate Result Layout

Plate	Well code	Total C g/L	Total N g/L	C:N Ratio	Total Cl g/L	Total P g/L	Rapa mg/L	Biomass OD ₅₉₀
1	B11	12.623	0.508	24.829	0.046	0.356	0	0.000
1	H11	8.971	0.709	12.656	0.215	0.356	10	0.000
1	E8	8.627	0.508	16.969	6.116	0.356	5	0.000
1	F6	12.967	0.709	18.294	6.285	0.356	10	0.073
1	A11	8.627	0.508	16.969	0.046	0.356	5	0.000
1	D9	12.967	0.709	18.294	0.215	0.356	25	0.000
1	D6	12.623	0.508	24.829	6.116	0.356	10	0.061
1	E11	8.971	0.709	12.656	6.285	0.356	0	0.000
1	F2	16.620	0.476	34.938	3.166	0.356	10	0.084
1	E4	8.628	0.476	18.138	3.166	0.356	10	0.110
1	H8	4.974	0.742	6.708	3.166	0.356	10	0.000
1	G4	20.958	0.742	28.264	3.166	0.356	25	0.219
1	G7	4.632	0.476	9.737	3.166	0.356	5	0.000
1	B3	20.616	0.476	43.338	3.166	0.356	25	0.106
1	A8	16.962	0.742	22.875	3.166	0.356	25	0.000
1	H6	8.970	0.742	12.097	3.166	0.356	10	0.059
1	D8	12.795	0.489	26.187	0.108	0	0	0.000
1	B12	8.799	0.849	10.369	0.176	0	0	0.000
1	D4	8.799	0.489	18.009	6.178	0	10	0.049
1	H4	12.795	0.849	15.078	6.246	0	10	0.120
1	G6	8.799	0.489	18.009	0.108	0.712	10	0.220
1	A10	12.795	0.849	15.078	0.176	0.712	10	0.000
1	D11	12.795	0.489	26.187	6.178	0.712	5	0.000
1	C8	8.799	0.849	10.369	6.246	0.712	5	0.000
1	F4	12.159	0.373	32.606	3.081	0.356	25	0.119
1	F3	9.091	0.644	14.121	3.081	0.356	10	0.099
1	C12	8.506	0.573	14.838	3.25	0.356	10	0.000
1	G1	13.431	0.844	15.910	3.25	0.356	25	0.088
1	C1	8.163	0.373	21.890	3.081	0.356	10	0.151
1	H9	13.087	0.644	20.328	3.081	0.356	10	0.000
1	H1	12.502	0.573	21.808	3.25	0.356	25	0.028
1	E6	9.435	0.844	11.176	3.25	0.356	10	0.207
1	C11	18.789	0.609	30.872	3.166	0	0	0.000
1	E3	6.801	0.609	11.175	3.166	0.712	10	0.099
1	D1	6.801	0.609	11.175	3.166	0	10	0.084
1	G10	18.789	0.609	30.872	3.166	0.712	5	0.000
1	E7	6.801	0.609	11.175	3.166	0	5	0.000
1	H3	18.789	0.609	30.872	3.166	0.712	5	0.047
1	B7	18.789	0.609	30.872	3.166	0	0	0.000
1	H5	6.801	0.609	11.175	3.166	0.712	10	0.023
1	E2	10.162	0.580	17.514	3.211	0.356	10	0.102
1	H10	11.090	0.491	22.583	3.143	0.356	0	0.000
1	B5	10.504	0.486	21.612	3.143	0.356	0	0.267
1	F5	11.432	1.117	10.235	3.211	0.356	10	0.033
1	E12	10.162	0.220	46.148	3.143	0.356	10	0.000
1	H12	11.090	0.851	13.031	3.211	0.356	0	0.000
1	G11	10.504	0.846	12.415	3.211	0.356	5	0.000
1	H7	11.432	0.757	15.104	3.143	0.356	5	0.000
1	E10	18.325	0.473	38.733	0.131	0	0	0.000

1	A6	7.265	0.744	9.765	0.131	0	10	0.062
1	G5	6.337	0.473	13.394	6.201	0	5	0.054
1	G12	19.253	0.744	25.878	6.201	0	10	0.000
1	D12	6.337	0.473	13.394	0.131	0.712	0	0.000
1	E1	19.253	0.744	25.878	0.131	0.712	0	0.140
1	B2	18.325	0.473	38.733	6.201	0.712	5	0.063
1	C5	7.265	0.744	9.765	6.201	0.712	5	0.091
1	F8	10.626	0.716	14.847	0.176	0.356	0	0.000
1	A7	10.968	0.622	17.647	0.108	0.356	10	0.000
1	G8	10.626	0.356	29.874	6.178	0.356	0	0.000
1	F9	10.968	0.982	11.175	6.246	0.356	10	0.000
1	D10	10.626	0.356	29.874	0.108	0.356	5	0.000
1	B10	10.968	0.982	11.175	0.176	0.356	0	0.000
1	G3	10.626	0.716	14.847	6.246	0.356	25	0.099
1	A3	10.968	0.622	17.647	6.178	0.356	25	0.050
1	E9	18.617	0.388	47.933	3.058	0.356	5	0.000
1	F1	6.629	0.748	8.858	3.126	0.356	10	0.139
1	F7	6.973	0.589	11.842	3.227	0.356	10	0.000
1	C10	18.961	0.949	19.984	3.295	0.356	5	0.000
1	C3	6.629	0.388	17.068	3.058	0.356	25	0.230
1	G2	18.617	0.748	24.876	3.126	0.356	10	0.081
1	H2	18.961	0.589	32.202	3.227	0.356	25	0.142
1	A2	6.973	0.949	7.349	3.295	0.356	25	0.116
1	C2	10.919	0.511	21.371	3.081	0	0	0.068
1	A9	10.332	0.506	20.426	3.081	0	5	0.000
1	E5	10.334	0.440	23.464	3.25	0	0	0.096
1	A12	11.604	0.977	11.876	3.25	0	10	0.000
1	D2	9.990	0.240	41.625	3.081	0.712	0	0.075
1	D3	11.260	0.777	14.497	3.081	0.712	10	0.069
1	A4	11.262	0.711	15.833	3.25	0.712	10	0.327
1	D7	10.675	0.706	15.116	3.25	0.712	5	0.000
1	B6	10.797	0.609	17.741	3.166	0.356	10	0.081
1	C4	10.797	0.609	17.741	3.166	0.356	10	0.094
1	F11	10.797	0.609	17.741	3.166	0.356	10	0.000
1	A5	10.797	0.609	17.741	3.166	0.356	25	0.062
1	C9	10.797	0.609	17.741	3.166	0.356	10	0.000
1	C6	10.797	0.609	17.741	3.166	0.356	10	0.128
1	B4	10.797	0.609	17.741	3.166	0.356	10	0.177
1	G9	10.797	0.609	17.741	3.166	0.356	10	0.000
1	B1	10.797	0.609	17.741	3.166	0.356	10	0.130
1	B8	10.797	0.609	17.741	3.166	0.356	10	0.000
1	D5	10.797	0.609	17.741	3.166	0.356	10	0.069
1	C7	10.797	0.609	17.741	3.166	0.356	10	0.000
1	F12	10.797	0.609	17.741	3.166	0.356	25	0.000
1	A1	10.797	0.609	17.741	3.166	0.356	10	0.116
1	B9	10.797	0.609	17.741	3.166	0.356	10	0.000
1	F10	10.797	0.609	17.741	3.166	0.356	5	0.000
2	D1	8.627	0.508	16.969	0.046	0.356	0	0.207
2	D12	12.967	0.709	18.294	0.215	0.356	10	0.460
2	B6	12.623	0.508	24.829	6.116	0.356	0	0.058
2	H6	8.971	0.709	12.656	6.285	0.356	10	0.277
2	A12	12.623	0.508	24.829	0.046	0.356	25	0.453
2	A3	8.971	0.709	12.656	0.215	0.356	25	0.399
2	D5	8.627	0.508	16.969	6.116	0.356	0	0.093

2	F7	12.967	0.709	18.294	6.285	0.356	10	0.203
2	D11	4.632	0.476	9.737	3.166	0.356	0	0.397
2	D7	20.616	0.476	43.338	3.166	0.356	0	0.347
2	H2	16.962	0.742	22.875	3.166	0.356	10	0.379
2	G10	8.970	0.742	12.097	3.166	0.356	10	0.406
2	C2	16.620	0.476	34.938	3.166	0.356	25	0.162
2	H4	8.628	0.476	18.138	3.166	0.356	25	0.113
2	B2	4.974	0.742	6.708	3.166	0.356	10	0.154
2	C10	20.958	0.742	28.264	3.166	0.356	5	0.334
2	F12	8.799	0.489	18.009	0.108	0	0	0.329
2	A11	12.795	0.849	15.078	0.176	0	5	0.383
2	F1	12.795	0.489	26.187	6.178	0	0	0.033
2	D2	8.799	0.849	10.369	6.246	0	0	0.090
2	C8	12.795	0.489	26.187	0.108	0.712	5	0.335
2	A7	8.799	0.849	10.369	0.176	0.712	10	0.489
2	A1	8.799	0.489	18.009	6.178	0.712	10	0.003
2	H1	12.795	0.849	15.078	6.246	0.712	10	0.363
2	G8	8.163	0.373	21.890	3.081	0.356	10	0.259
2	G9	13.087	0.644	20.328	3.081	0.356	0	0.391
2	B4	12.502	0.573	21.808	3.25	0.356	5	0.152
2	A5	9.435	0.844	11.176	3.25	0.356	5	0.179
2	C5	12.159	0.373	32.606	3.081	0.356	0	0.105
2	A2	9.091	0.644	14.121	3.081	0.356	10	0.328
2	G6	8.506	0.573	14.838	3.25	0.356	5	0.125
2	C4	13.431	0.844	15.910	3.25	0.356	10	0.196
2	F10	6.801	0.609	11.175	3.166	0	0	0.228
2	F4	18.789	0.609	30.872	3.166	0.712	10	0.081
2	C3	18.789	0.609	30.872	3.166	0	10	0.098
2	G2	6.801	0.609	11.175	3.166	0.712	5	0.101
2	H9	18.789	0.609	30.872	3.166	0	0	0.293
2	B12	6.801	0.609	11.175	3.166	0.712	10	0.304
2	A10	6.801	0.609	11.175	3.166	0	5	0.318
2	F9	18.789	0.609	30.872	3.166	0.712	0	0.224
2	H3	10.162	0.220	46.148	3.143	0.356	10	0.092
2	B10	11.090	0.851	13.031	3.211	0.356	5	0.327
2	D9	10.504	0.846	12.415	3.211	0.356	10	0.447
2	G3	11.432	0.757	15.104	3.143	0.356	10	0.096
2	E2	10.162	0.580	17.514	3.211	0.356	5	0.290
2	E11	11.090	0.491	22.583	3.143	0.356	0	0.239
2	C9	10.504	0.486	21.612	3.143	0.356	5	0.353
2	B1	11.432	1.117	10.235	3.211	0.356	5	0.374
2	E9	6.337	0.473	13.394	0.131	0	5	0.256
2	A9	19.253	0.744	25.878	0.131	0	10	0.518
2	E8	18.325	0.473	38.733	6.201	0	0	0.239
2	A8	7.265	0.744	9.765	6.201	0	5	0.461
2	B9	18.325	0.473	38.733	0.131	0.712	0	0.000
2	D4	7.265	0.744	9.765	0.131	0.712	0	0.105
2	G5	6.337	0.473	13.394	6.201	0.712	10	0.115
2	B11	19.253	0.744	25.878	6.201	0.712	0	0.227
2	H11	10.626	0.356	29.874	0.108	0.356	25	0.377
2	C11	10.968	0.982	11.175	0.176	0.356	10	0.317
2	G11	10.626	0.716	14.847	6.246	0.356	5	0.329
2	H12	10.968	0.622	17.647	6.178	0.356	25	0.649
2	C12	10.626	0.716	14.847	0.176	0.356	10	0.492

2	C1	10.968	0.622	17.647	0.108	0.356	10	0.526
2	G1	10.626	0.356	29.874	6.178	0.356	10	0.213
2	G12	10.968	0.982	11.175	6.246	0.356	25	0.399
2	F5	6.629	0.388	17.068	3.058	0.356	10	0.120
2	G7	18.617	0.748	24.876	3.126	0.356	25	0.221
2	F8	18.961	0.589	32.202	3.227	0.356	5	0.341
2	H7	6.973	0.949	7.349	3.295	0.356	25	0.343
2	A6	18.617	0.388	47.933	3.058	0.356	5	0.165
2	C6	6.629	0.748	8.858	3.126	0.356	0	0.094
2	H10	6.973	0.589	11.842	3.227	0.356	10	0.502
2	D10	18.961	0.949	19.984	3.295	0.356	0	0.346
2	A4	9.990	0.240	41.625	3.081	0	10	0.192
2	F3	11.260	0.777	14.497	3.081	0	5	0.061
2	F2	11.262	0.711	15.833	3.25	0	0	0.104
2	E12	10.675	0.706	15.116	3.25	0	10	0.228
2	H8	10.919	0.511	21.371	3.081	0.712	5	0.315
2	C7	10.332	0.506	20.426	3.081	0.712	0	0.438
2	G4	10.334	0.440	23.464	3.25	0.712	0	0.182
2	B3	11.604	0.977	11.876	3.25	0.712	25	0.179
2	E6	10.797	0.609	17.741	3.166	0.356	10	0.225
2	E7	10.797	0.609	17.741	3.166	0.356	5	0.264
2	D3	10.797	0.609	17.741	3.166	0.356	10	0.092
2	D8	10.797	0.609	17.741	3.166	0.356	10	0.313
2	E5	10.797	0.609	17.741	3.166	0.356	10	0.083
2	F6	10.797	0.609	17.741	3.166	0.356	0	0.111
2	E1	10.797	0.609	17.741	3.166	0.356	10	0.133
2	E3	10.797	0.609	17.741	3.166	0.356	25	0.177
2	E10	10.797	0.609	17.741	3.166	0.356	5	0.253
2	F11	10.797	0.609	17.741	3.166	0.356	10	0.301
2	B5	10.797	0.609	17.741	3.166	0.356	0	0.128
2	E4	10.797	0.609	17.741	3.166	0.356	25	0.150
2	B7	10.797	0.609	17.741	3.166	0.356	10	0.484
2	D6	10.797	0.609	17.741	3.166	0.356	0	0.079
2	H5	10.797	0.609	17.741	3.166	0.356	0	0.153
2	B8	10.797	0.609	17.741	3.166	0.356	25	0.456

Appendix 5 Composition of Yeast Extract

Source: Solomons, G.L. 1969. *Materials and Methods in Fermentation*. Academic Press, London and New York

Composition of yeast extract (light grade) (Courtesy of English Grains Ltd)

Constituent	% w/w
Moisture	29.5
Total nitrogen	7.0
Amino nitrogen (Van Slyke)	3.6
Ash	19.0
Lipid	Trace
Carbohydrate (by difference)	7.7
Chloride (by NaCl)	11.5
Phosphorus (as P ₂ O ₅)	4.1
Sodium (by Na ₂ O)	7.5
Potassium (as K ₂ O)	3.8
pH of 5% solution	5.5
Amino acids	3.5
Lysine	
Tryptophan	0.6
Phenylalanine	2.1
Methionine	0.9
Threonine	2.5
Leucine	3.5
Isoleucine	2.8
Valine	2.6

Appendix 6 Chemicals Prices List

Sources: Lancaster Research Chemicals 2004 – 2005
Lancaster Synthesis Ltd, Newgate, White Lund, Morecambe,
Lancashire LA3 3BN, England
2007 DIFCO & BBL price list www.vgdusa.com/DIFCO_media.htm

Description	Sale unit	Price USD/unit
Agar	500 g	102.40
L-arginine	500 g	63.40
L-aspartic	500 g	24.80
CoCl ₂	250 g	29.10
CuSO ₄	500 g	16.10
FeSO ₄	500 g	18.30
D-Fructose	1 kg	39.60
Glucose	2.5 kg	47.90
Glycerol	500 g	82.05
L-histidine	250 g	55.60
Hy soy	500 g	61.50
KH ₂ PO ₄	500 g	15.00
K ₂ HPO ₄	1 kg	33.50
L-lysine	1 kg	55.10
Malt extract	500 g	34.80
Mannose	100 g	52.50
MgSO ₄	500 g	14.50
MnSO ₄	500 g	34.30
NaCl	5 kg	24.50
Yeast extract	500 g	81.10
ZnSO ₄	500 g	13.50