Bioconversion of Glycerol to Dihydroxyacetone by immobilized *Gluconacetobacter xylinus* cells

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ABSTRACT

Dihydroxyacetone, (DHA) is one of the primary ingredients used in tanning products by the cosmetic industry. DHA reacts with the free amino acids on the epidermis to form a tan-like appearance. DHA is also a building block for lactic acid; that is, a common ingredient in the food industry. In the pharmaceutical industry, DHA is used as a precursor for 1, 2-propylene glycerol and methotrexate.

Due to the Food and Drug Administration's strict regulations, DHA is produced via microbial synthesis. This is because the chemical synthesis involves toxic chemical reactions with epichlorohydrin. This poses problems with a chemical widely used for human application. Currently, the industrial process of DHA is carried out under microbial synthesis of glycerol. The strain, *Gluconobacter oxydans* produces high DHA yields although its productivity is low. As a result, its high cost of production is reflected in its price.

Glycerol is the only organic compound that can be converted into DHA. The conversion requires oxidation of the secondary hydroxyl group by glycerol dehydrogenase (GlyDH). In this study, DHA was converted from glycerol using *Gluconacetobacter xylinus* (*G.xylinus)* in a large-scale reactor. Previously the strain, *G.xylinus*, has been shown to produce high DHA yields under immobilization.

In this study, *G.xylinus* was immobilized inside two carrier materials- calcium alginate and chitosan-coated alginate beads. A series of investigations were carried out to determine the DHA conversion efficiency using the mentioned carriers. The conversion efficiency of alginate immobilized cells was investigated under varying initial glycerol concentrations of: 1%, 2%, 4% and 7% (w/v) and aeration rates of: 0.3, 0.6 and 1.0 vvm. This study found that the optimal glycerol concentration was at 2% (w/v) and the optimal aeration rate was at 0.3 vvm. The DHA conversion efficiency of chitosan-alginate immobilized cells was also tested under the same aeration rate previously mentioned. The investigation found that chitosan coating provided greater stability to the alginate matrix with increased aeration rate. The optimal aeration rate was found at 1.0 vvm.

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NOMENCLATURE

Abbreviation	Description	Unit
$\mathbf{r_p}$	Productivity	$gL^{-1}h^{-1}$
$\mathbf{r_{pmax}}$	Maximal productivity	$gL^{-1}h^{-1}$
$\mathbf{q_p}$	Specific product formation rate	$gg^{-1}\ h^{-1}$
$\mathbf{r_s}$	Substrate consumption rate	$gL^{-1}h^{-1}$
$\mathbf{q_s}$	Specific substrate consumption rate	$gg^{-1}\ h^{-1}$
$\mathbf{Y_{p/s}}$	Yield coefficient	gg^{-1}

1 INTRODUCTION

In recent years, there has been an influx of glycerol on the market as a result of a booming biodiesel industry. The biodiesel industry produces glycerol as a by-product at 10% (w/w) the weight of biodiesel.

Bio-diesel is a promising source of alternative fuel and has been viewed with enthusiasm. (Yang et al., 2012). The mounting interest may be driven by: rising fuel prices, climate change, a decrease in reserves, and global population increase (Encyclopedia, 2012; Leoneti et al., 2012; Yang, et al., 2012). In 2005, global biodiesel production was estimated at 3.8 million tonnes and by 2020, it is expected to reach over 8 billion tonnes (Thurmond, 2008). That is, 800 million tonnes of glycerol will hit the market. In its raw form, glycerol contains several impurities that make its disposal costly and difficult (Anand et al., 2010). As a result, the price of glycerol is forecasted to fall in the coming years, making it an ideal raw material for industrial processes (da Silva et al., 2009).

Glycerol is arguably one of the most common ingredients used in industrial processes. Its tri-hydric nature gives it a hygroscopic characteristic, allowing it to be used in a variety of consumer-related products. Also, its highly functionalised groups allows it to form a large number of value-added derivatives, namely; 1,3-propanediol, dihydroxyacetone (DHA), ethanol, succinate and citric acid (da Silva, et al., 2009). As the production of biodiesel continues to grow, more research into producing value-added products from glycerol is imperative to utilise glycerol and to reduce the its environmental impact..

DHA is a value-added derivative of glycerol and a primary ingredient in the cosmetics industry (Petersen et al., 2004). DHA can be produced via two routes; chemical synthesis or microbial synthesis. Microbial synthesis is the preferred route because it has greater specificity and does not involve toxic chemical treatments often associated with chemical synthesis.

Since its approval in the 1970s by the US Food and Drug Administration (FDA), DHA has been a valuable chemical in the cosmetic industry due to is pigmentation properties. The pigmentation gives a tan-like appearance and is a result of glycation reactions taking place on the epidermis (Petersen, et al., 2004). The pigmentation cannot be washed off, but fades as the upper layers of the skin are shed. As a result, application of tanning products (and consequently, DHA) is currently the most popular way of gaining a tan-like appearance as it carries less health risks than exposure to the sun's UV rays (Hu et al., 2011; Stasiak-Różańska et al., 2011a).

The value of DHA is not limited to cosmetics. It is also used as a precursor for fine chemicals and pharmaceutical product. It is a safe therapeutic option for recalcitrant vitiligo (Liu et al., 2013) and has been involved in dietary supplementation for enhanced endurance capacity (Stanko et al., 1990). DHA is also a building block for 1,2-propylene glycerol (Hekmat et al., 2003a), lactic acid (Bicker et al., 2005) and methotexrate, a chemical used in the treatment of cancer patients (Gatgens et al., 2007; Stasiak-Różańska, et al., 2011a),

DHA is a physiological product of the body and utilized in the metabolic pathway for the synthesis of energy. Research into possible derivatives that can stem from an important intermediary product in the glycolytic pathway is limited. This is mainly due to its high market value. The current industrial production of DHA is carried out via microbial conversion of glycerol using Gluconobacter oxydans. This conversion yields high DHA, however productivity rates are low. As a result, the production costs are high and this is reflected in its market value.

With the influx of glycerol on the market and its downward spiralling costs, now is an opportune time to undertake research on improving the production of DHA from glycerol.

2 LITERATURE REVIEW

2.1 Production, properties and uses of Dihydroxyacetone

2.1.1 Production

DHA can be produced via two different routes: (1) chemical synthesis and (2) microbial synthesis.

2.1.1.1 Chemical synthesis

The chemical synthesis of DHA occurs through catalytic oxidation of glycerol or condensation of formaldehyde with calcium carbonate (Stasiak & Blazejak, 2009). Chemical conversion can be rate controlled and the occurrence of contamination is very low. However, this often leads to the formation of other by-products that are toxic and difficult to remove. Therefore, microbial synthesis is generally preferred.

2.1.1.2 Microbial synthesis

DHA can be obtained from glycerol via microbial fermentation by a few specific acetic acid bacteria (AAB). Microbial fermentation is usually preferred over chemical synthesis because it gives greater specificity and requires less chemical treatments making it an ideal process for products used for human application and consumption (Stasiak & Blazejak, 2009).

DHA is currently obtained in the industrial scale via incomplete microbial fermentation of glycerol by *Gluconobacter oxydans* (Bauer *et al.*, 2005; Claret *et al.*, 1994; Gatgens *et al.*, 2007; Hekmat *et al.*, 2003a). The bioconversion involves oxidising the secondary hydroxyl group to produce DHA as seen in Figure 2.1. The industrial conversion takes place in a conventional batch reactor at a productivity rate of 2.9 g L^{-1} h^{-1} and achieving DHA concentrations of ~60gL^{-1} with a yield of ~90% after 32 hours (Rodrigues *et al.*, 2011; Yang *et al.*, 2012; Zheng *et al.*, 2012). Although the yield is high the productivity rate is considered low and should be improved to decrease the production cost of DHA.

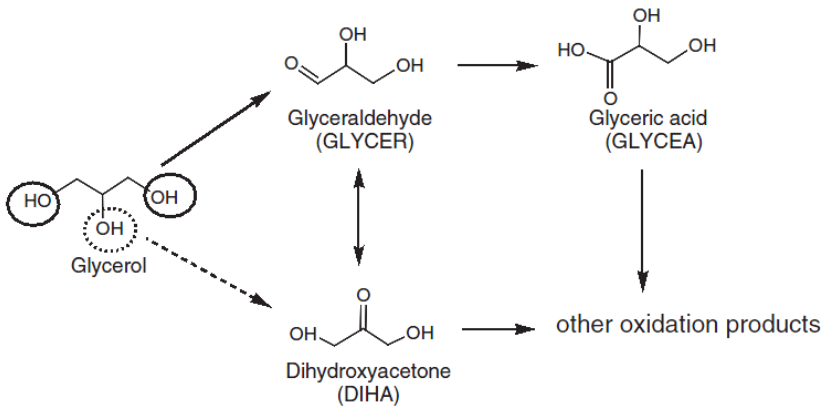


Figure 2.1 Reaction pathway for glycerol; oxidation of primary hydroxyl groups produce glyceraldehyde; oxidation of secondary hydroxyl group produces DHA (Rodrigues, *et al.*, 2011).

2.1.2 The use of Acetic acid bacteria in DHA conversion

AAB is valued in biological processes due to its rapid and incomplete oxidation of a variety of sugars, alcohols, aldehyde and ketones and the subsequent excretion of oxidative products into the medium (Deppenmeier *et al.*, 2002; Yamada & Yukphan, 2008).

The species of *Gluconobacter oxydans (G.oxydans)* has been studied extensively for the conversion of glycerol to DHA; due to its high conversion rate and yield (Bories *et al.*, 1991; Gatgens, *et al.*, 2007; Hekmat, *et al.*, 2003a; Hu *et al.*, 2009). The oxidative reaction is catalysed by the enzyme glycerol dehydrogenase (GlyDH, EC 1.1.1.6) and coupled by the respiratory chain located inside the cytoplasmic membrane (Deppenmeier, *et al.*, 2002). There has been extensive studies on the optimisation of various parameters such as varying glycerol concentrations (Claret *et al.*, 1992a), immobilization method (Adlercreutz *et al.*, 1985; Wei *et al.*, 2007), introduction of oxygen vectors (Adlercreutz, *et al.*, 1985; Flickinger & Perlman, 1977; Holst *et al.*, 1982) and modifying reactor vessels as well as genetic modification of the specie (Gatgens, *et al.*, 2007; Li *et al.*, 2010a; Ma *et al.*, 2010).

There are other AAB capable of oxidising glycerol to DHA. Past research has found that specie of *Acetobacter suboxydans* (Nabe *et al.*, 1979) and *Gluconobacter melanogenus* (Flickinger & Perlman, 1977) are capable of this

conversion. However, the specie showed low oxidising activity and therefore this study has not been progressed.

A study using *Gluconacetobacter xylinus (G.xylinus)* by Nabe, *et al.* (1979) achieved DHA yield ~0.8 and high glycerol oxidising activity. Since then, only one other study by Stasiak-Różańska *et al.* (2011a) has been carried out using the same strain that reported DHA yields greater than 0.9. The cells of *G.xylinus* are characterised for their synthesis of bacterial cellulose (Stasiak & Blazejak, 2009; Yamada & Yukphan, 2008). Less known is its capability of producing DHA from glycerol. The positive results obtained by Nabe, *et al.* (1979) and Stasiak-Różańska, *et al.* (2011a) on DHA conversion using *G.xylinus* cells provides the impetus for further research.

2.1.2.1 Using Gluconacetobacter xylinus for DHA conversion

The cells of *G.xylinus* are non-motile, gram-negative obligate aerobes with optimal growth temperature of 30 ^{o}C and pH ranges from 5.4 to 6.3 (Stasiak & Blazejak, 2009; Yamada & Yukphan, 2008). The morphology of growing cells takes a form of ellipsoidal to elongated bacilli with a slightly bent and/or straight configuration. These may grow individually, in pairs or in short chains in the range of 0.6-1.2 x 1.0-3.0 μm (Stasiak & Blazejak, 2009). Like *G.oxydans*, *G.xylinus* is also equipped with membrane bound glycerol dehydrogenase that is responsible for oxidation of glycerol to DHA (Lidia & Stanisław, 2012; Stasiak-Różańska, *et al.*, 2011a; Yamada *et al.*, 1982).

2.1.2.2 Activity of glycerol dehydrogenase

Any bacterium capable of converting glycerol to DHA is equipped with glycerol dehydrogenase enzyme (GlyDH) and its ability to quantify DHA is dependent on the quantity of ubiquinone-10 (Gatgens, *et al.*, 2007; Lu *et al.*, 2012a; Stasiak-Różańska, *et al.*, 2011a). Table 2.1 shows both genus of *Gluconobacter, Gluconacetobacter* possess ubiquinone-10.

The ubiquinone-10 is member of pyrroloquinoline quinone (PQQ), a cofactor responsible for continuous redox cycling in GlyDH (Matsushita *et al.*, 2002). PQQ has an orthoquinone structure, as seen in Appendix 2, and is deeply embedded within the centre of the GlyDH structure. This cofactor is

responsible for its oxidoreduction activity. The active centre of GlyDH is located in the periplasmic space along with the respiratory system. This gives a periplasmic oxidase system where the transport of substrate and product from the cytoplasm is unnecessary and DHA conversion is carried out without NADH mediation (Claret, *et al.*, 1994; Matsushita, *et al.*, 2002; Stasiak-Różańska, *et al.*, 2011a).

Table 2.1 Differentiation of Acetobacter, Gluconacetobacter and Gluconobacter genus belonging to α-subclass of the Proteobactera.

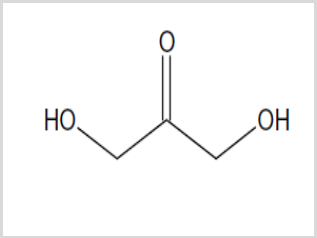
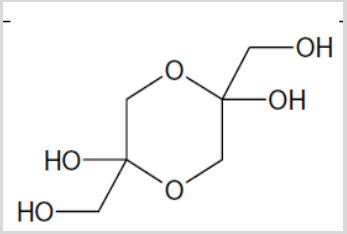
Genus	Flagella type	Oxidation of acetic acid to CO_2 and H_2O	Oxidation of lactate to CO_2 and H_2O	Ubiquinone type	Reference
Acetobacter	Peritrichous	+	+	Q9	(Yamada & Yukphan, 2008)
Gluconacetobacter	Peritrichous	+	+	Q10	(Deppenmeier, *et al.*, 2002)
Gluconobacter	Polar	-	-	Q10	(De Muynck *et al.*, 2007)

Both genus of *Gluconacetobacter* and *Gluconobacter* possess the Ubiquinone-10 responsible for the quantified production of DHA. This study aims to supplement the research of *G.xylinus* for the conversion of glycerol to DHA.

2.1.3 Properties

Dihydroxyacetone (DHA, 1,3-Dihydroxypropan-2-one, Glycerone) is a ketotriose that is prepared via microbial fermentation of glycerol by species of the *Acetobacter* family (Stasiak & Blazejak, 2009). DHA exists naturally as a monomer in plants but is normally purchased as a dimer in the form of a white, crystalline powder. Both forms do not possess a chiral centre and are soluble in alcohol, ether and acetone (Table 2.2).

Table 2.2 Properties of DHA in monomer and dimer forms (Slepokura & Lis, 2004).

	Monomer (wet basis)	**Dimer (dry basis)**
Empirical formula	$C_3H_8O_3$	$C_6H_{12}O_6$
Molecular weight ($gmol^{-1}$)	90.08	180.16
Appearance	Colourless liquid	White, crystalline powder
Molecular structure		

2.1.3.1 Biochemistry of DHA in cells

The biochemistry of DHA in living cells is briefly reviewed. It is through metabolic pathway that the principal of DHA conversion from glycerol is assimilated.

DHA is a physiological product of the cells involved in the intermediary process of the metabolic pathway. DHA is utilized by the glycolytic pathway for the generation of ATP. Figure 2.2 below, shows that the first stage of glycolysis begins when glucose enters the cell and is phosphorylated by ATP to glucose 6-phopshate (1) then isomerised to fructose 6-phosphate (2). This process leads to phosphorylation into compound fructose 1,6-biphosphate (3) that is then isomerised to dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (5) (Berg *et al.*, 2002; Berg *et al.*, 2012; Petersen *et al.*, 2004). The five steps involved in the breakdown of glucose to DHAP require an expenditure of 2 ATP.

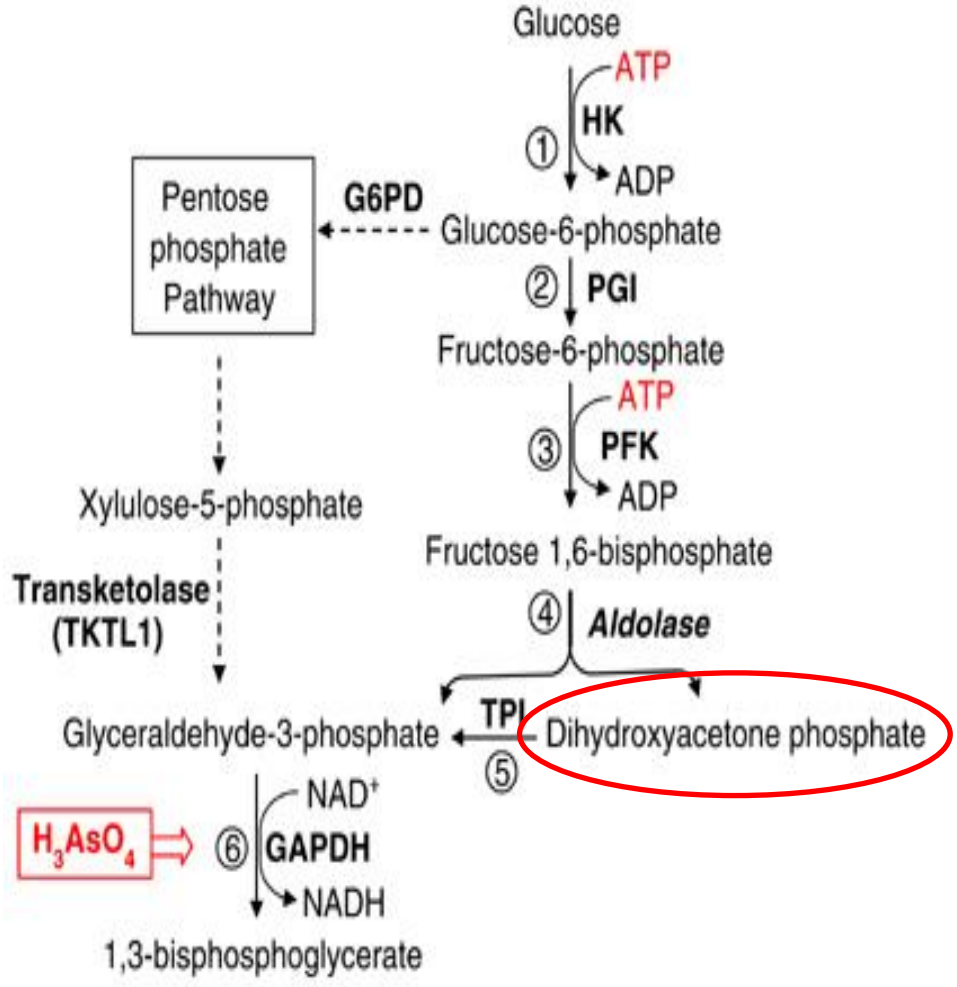


Figure 2.2 Glycolytic pathway and its metabolic interconnection with the pentose phosphate pathway. The solid arrows indicate glycolytic reactions, whereas the dashed arrows show the pentose phosphate pathway. The red eclipse is the Dihydroxyacetone phosphate (DHAP) involved in the intermediary step of the glycolytic pathway (Pelicano *et al.*, 2006)

In the absence of glucose, lipids (triglycerides) stored within the body are hydrolysed into glycerol and fatty acids. These elements undergo a series of conversions before entering the glycolytic pathway. Figure 2.3 below, shows that glycerol is phosphorylated to glycerol-3-phosphate by glycerol kinase and then further oxidised to DHAP by glycerol-3-phophate dehydrogenase; localised inside the mitochondria. The glycerol degradation pathway requires only two conversion steps and the expenditure of 1 ATP to produce DHAP.

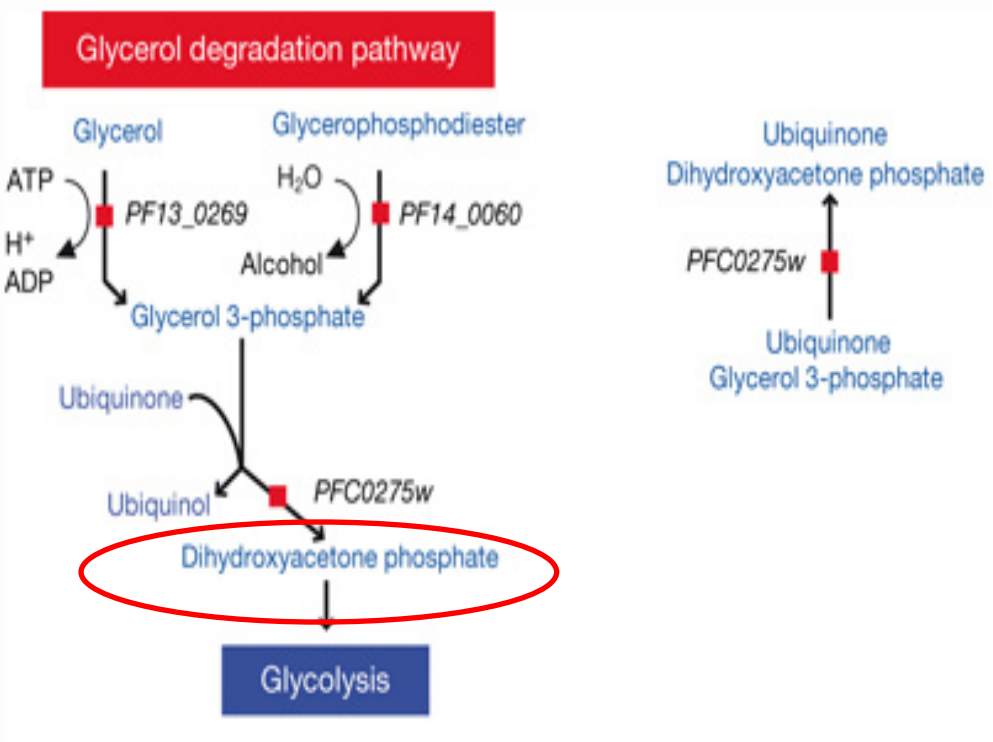


Figure 2.3 Glycerol degradation pathway used by cells in absence of glucose. There are much less steps required to reach DHAP compared to glucose (Daily *et al.*, 2007).

2.1.4 Uses of DHA

DHA was originally used as an oral treatment for patients with glycogen storage disease (Wittgenstein and Berry 1960). American researcher, Eva Wittgenstein discovered the phenomenon of skin colouration with DHA when she discovered that patients, who had received the treatment, showed brown-coloured spots from contact with the skin (Wittgenstein & Berry, 1960). In the 1970s, DHA was approved by the US Food and Drug Administration (FDA) as a colour additive for drugs and cosmetics (Petersen, *et al.*, 2004). To date, it is the only active ingredient approved by the FDA for use in sunless tanning products (Petersen, *et al.*, 2004). DHA also used as an intermediate for the synthesis of various organic chemicals, surfactants and fine chemicals (Hu, *et al.*, 2009; Pagliaro *et al.*, 2007).

2.1.4.1 DHA as a tanning agent

Due to its pigmentation properties, DHA is widely used as a primary ingredient in tanning products. The chemical staining is the result of glycation reaction between DHA and the free amino acids found on the epidermis (Petersen, *et al.*, 2004). The pigmentation cannot be washed off, but fades as the upper

layers of the epidermis are shed. As a result, application of DHA is currently the most popular way of gaining a tan-like appearance as it carries less health risks than exposure to the sun's UV rays (Hu *et al.*, 2011; Stasiak-Różańska, *et al.*, 2011a).

2.1.4.2 Other uses of DHA

DHA is a precursor for fine chemicals and pharmaceutics. It is a safe therapeutic option for recalcitrant vitiligo (Liu *et al.*, 2013) and has been involved in dietary supplementation for enhanced endurance capacity (Stanko *et al.*, 1990). It is also a building block for 1,2-propylene glycerol (Hekmat, *et al.*, 2003a), lactic acid (Bicker *et al.*, 2005) and methotrexate - a chemical used in the treatment of cancer patients (Gatgens, *et al.*, 2007; Stasiak-Różańska, *et al.*, 2011a).

2.2 Growth media and conditions

Fermentation media greatly affects the metabolic activity and metabolite synthesis of any microorganism. DHA is a primary metabolite, which means that it's production rates are dependent on biomass formation (Demain & Sanchez, 2007). Culture environments should aim to decrease biomass regulation and increase metabolite production to avoid substrate limitation (Zhu, 2007). Media formulations usually contain the correct proportions of the typical elements that make up the microbial cell

2.2.1 Carbon source

Carbon source is one of the major components in media preparation. It is an essential building block for cellular structure and energy metabolism of microorganisms. It may also be the substrate for product conversion. Glycerol is capable of participating in both roles and is the only substrate that can be converted to DHA (Hu, et al., 2009).

The presence of glycerol, as the sole carbon source leads to biomass formation and DHA production; resulting in the quick depletion of available substrate. The presence of two carbon sources; one for cell growth, as well as glycerol for

DHA production, can affect cell growth and ability GlyDH. Once study, using glycerol and mannitol as carbon sources in media, found that glycerol was still the most favourable carbon source for biomass formation (Hu, et al., 2009).

Glycerol, as the sole carbon source in media formulation, has to be optimised to increase DHA production and regulate biomass formation. The Newtonian properties meant that high glycerol concentration would increase viscosity and also affect mass transfer. A media formulation for the conversion of glycerol to DHA using cells of *G.oxydans* had initial glycerol concentration ranging from 31 gL^{-1} to 129 gL^{-1} (Claret, *et al.*, 1992a). The study discovered that; as the concentration of glycerol increased, the growth rate of cells and DHA formation rate decreased. Similar findings were also made in Ma, *et al.* (2010), which used initial glycerol concentrations ranging between 50 gL^{-1} to 150 gL^{-1}. This study also found decreased DHA conversion rates, as well as increased fermentation time, corresponded with the increase in glycerol concentration. The aforementioned studies also discovered that biomass concentrations, at the end of the fermentation period, were very similar across all of the initial glycerol concentrations. These findings lead to a conclusion shared by other studies that increased glycerol concentrations inhibit the activity of GlyDH in *G.oxydans* (Hekmat, *et al.*, 2003a; Tkac *et al.*, 2001; Zheng, *et al.*, 2012).

There has been limited research into the effects of glycerol concentrations on *G.xylinus* for the production of DHA. Glycerol concentrations between 2% (w/v) and 5% (w/v) have been studied by Nabe, *et al.* (1979) and Stasiak-Różańska, *et al.* (2011a). Stasiak-Różańska, *et al.* (2011a) reported an increase in fermentation period when initial glycerol concentration was increased from 2% (w/v) to 5% (w/v). The study speculates substrate inhibition however there are no available data on the kinetics of substrate inhibition of *G.xylinus* to support this.

2.2.2 Nitrogen source

Nitrogen is an essential building block for proteins, amino acids, nucleic acids and enzymes that are required for cell maintenance and metabolite synthesis. The concentration in media does not vary and usually ranges between 0.5% (w/v) and 1% (w/v), depending on the media requirements. Nitrogen sources

commonly used for DHA conversion from glycerol substrate containing cells of *G.oxydans* include: ammonium sulphate (Gatgens, *et al.*, 2007; Hekmat, *et al.*, 2003a; Li, *et al.*, 2010a), yeast extract (Claret, *et al.*, 1992a; Gatgens, *et al.*, 2007; Hekmat, *et al.*, 2003a; Hu, *et al.*, 2009; Li, *et al.*, 2010a; Tkac, *et al.*, 2001) and peptone (Hu, *et al.*, 2009; Li, *et al.*, 2010a). Nitrogen sources in media containing *G.xylinus* cells for the same conversion process include: ammonium sulphate (Stasiak-Różańska, *et al.*, 2011a), yeast extract (Nabe, *et al.*, 1979; Stasiak-Różańska, *et al.*, 2011a), corn steep liquor and diammonium fumarate (Nabe, *et al.*, 1979).

2.2.3 Phosphorous and mineral salts

Inorganic phosphate or complex organic phosphates are important for protein and phospholipid formation and are both used in the production of DHA from glycerol containing cells of *G.oxydans* (Gatgens, *et al.*, 2007; Hekmat, *et al.*, 2003a; Hu, *et al.*, 2009; Li, *et al.*, 2010a). Mineral salts are added as counter ions of sulphates and phosphates but can also be key co-factors for high enzyme activity. Small quantities of magnesium (Gatgens, *et al.*, 2007; Hekmat, *et al.*, 2003a; Hu, *et al.*, 2009; Li, *et al.*, 2010a) calcium (Hu, *et al.*, 2009; Li, *et al.*, 2010a) and iron (Gatgens, *et al.*, 2007; Hekmat, *et al.*, 2003a) were used by *G.oxydans* in the conversion of glycerol to DHA.

There is limited information on glycerol media for *G.xylinus.* This research will formulate a media from previous research on DHA production and media used for bacterial cellulose production.

2.2.4 Oxygen supply

Oxygen supply is crucial to the production of DHA. It is the final electron acceptor in GlyDH for the synthesis of DHA. Increasing aeration rates close to saturation point in a media containing freely suspended cells can induce high biomass growth (Stanbury *et al.*, 2000). This causes substrate limitation problems for glycerol media because the carbon source fuels biomass formation and is the substrate for DHA conversion. Studies have been carried out to regulate the formation of biomass from *G.oxydans* under increased aeration rates for DHA formation. This has been achived through immobilization of *G.oxydans* cells (Wei, *et al.*, 2007), co-immobilising cells of

G.oxydans with O_2 producing bacteria (Adlercreutz *et al.*, 1982), substituting O_2 transport with p-benzoquinone (Adlercreutz, *et al.*, 1985) and introducing hydrogen peroxide as an oxygen vector (Holst, *et al.*, 1982). To date, no studies have measured the effect of varying aeration rates on *G.xylinus* production of DHA.

2.2.5 pH

The pH of the reaction medium has a pronounced effect on the enzyme activity and cell kinetics (Riet & Tramper, 1991). The glycerol molecule has three highly functionalized groups and preferential oxidation of the secondary hydroxyl group must be carried out to produce DHA. A study carried out by Ameyama *et al.* (1985a) discovered that the membrane bound GlyDH directly catalysed DHA production in a weakly acidic environment, ranging between pH 4.0-6.0. This finding is also supported by all of the studies on DHA production.

2.2.6 Temperature

Temperature affects the rate of individual reactions within a cell from cellular growth rate to enzymatic reactions. The cells of *G.oxydans* and *G.xylinus* are mesophiles and operate at temperatures ranging between 20 ^{o}C and 40 ^{o}C (Stasiak & Blazejak, 2009). According to Arrhenius' rule on activation energy, optimal temperatures can increase reaction rates (Riet & Tramper, 1991; Sinclair *et al.*, 1987). The enzymatic reactions inside a cell offer a narrow gap of temperature changes that can lead to enzyme denaturation (Riet & Tramper, 1991). Investigations using cells of *G.oxydans* on DHA production were carried out at temperatures ranging between 28 ^{o}C and 30 ^{o}C (Adlercreutz, *et al.*, 1985; Claret, *et al.*, 1992a; Gatgens, *et al.*, 2007; Hekmat, *et al.*, 2003a; Hu, *et al.*, 2009; Li, *et al.*, 2010a; Wei, *et al.*, 2007). Studies using *G.xylinus* cells for DHA conversion were carried out at temperatures of 28 ^{o}C (Stasiak-Różańska, *et al.*, 2011a) and 30 ^{o}C (Nabe, *et al.*, 1979). This leads the investigation to speculate optimal activity of GlyDH ranges between 28 ^{o}C and 30 ^{o}C.

2.3 Glycerol

In recent years, there has been an influx of glycerol on the market (Thurmond, 2008). As a result, the price of glycerol is forecasted to fall in the coming years, making it an ideal raw material for industrial processes (da Silva *et al.*, 2009). Glycerol is commonly used as an additive in the food, cosmetics, pharmaceutics and materials industry. Glycerol an also be used as raw material for fine chemicals and value added products (Britannica Encyclopedia, 2012).

Glycerol (1,2,3-Propanetriol, Glycerine, Trihydroxypropane) is abundant in nature in the form of triacylglycerols. Due to its hygroscopic nature and highly functionalised hydroxyl groups, it can be used to make derivatives of higher value. Glycerol is the only organic compound that can be converted into DHA (Hu, *et al.*, 2009).

2.3.1 Production of glycerol

In 1948, glycerol was produced via chemical synthesis from epicholorhydrin; a derivative of propylene and fossil fuels (Appleby, 2005). The market was supplemented by glycerol obtained as a by-product of fatty alcohol reactions and soap production (Appleby, 2005; Behr *et al.*, 2008). The glycerol market followed a basis supply/demand curve and as a result, there was little fluctuation in price (Appleby, 2005).

In recent years, the growth of the biodiesel industry has led to changes in the glycerol market. Glycerol is a by-product of biodiesel production; at 10% (w/w) of the amount of biodiesel produced (Ayoub & Abdullah, 2012; Knothe, 2005). For example, 500 tonnes of each biodiesel produced creates 50 tonnes of glycerol. Bio-diesel is a promising source of alternative fuel and has been viewed with enthusiasm. (Yang, *et al.*, 2012). The recent spike in demand for glycerol may be driven by: rising fuel prices, climate change, a decrease in reserves, and global population increase (Encyclopedia, 2012; Leoneti *et al.*, 2012; Yang, *et al.*, 2012).

The growth of the biodiesel industry has had a marked effect on other producers. Figure 2.4 illustrates the biodiesel industry's effect on other glycerol producers.

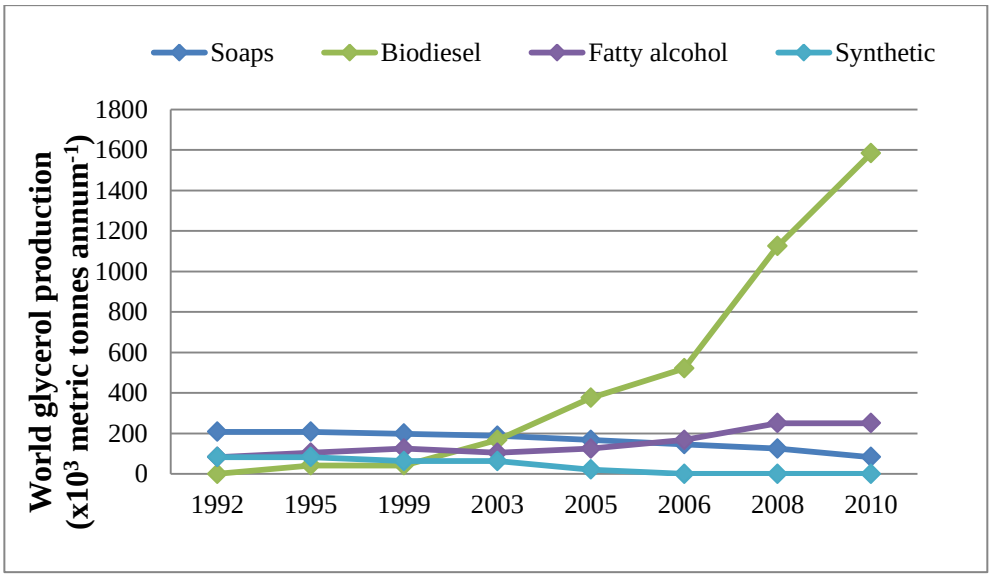


Figure 2.4 Contributing processes to the global glycerol market between 1992 – 2010. Graph is extracted from data found in Appendix 1.

In 2005, global biodiesel production was estimated at 3.8 million tonnes and by 2020, it is expected to reach over 8 billion tonnes. That is, 800 million tonnes of glycerol will on the market be produced (Thurmond, 2008). Considering the little amount of glycerol that could be absorbed by the pharmaceutical and other industries, this boom in the glycerol production would pose a serious environmental problem by way of its disposal. The price of glycerol is expected to decrease; making it an ideal feedstock material for industrial processes. Therefore, the potential uses of glycerol as a raw material for the production of useful compounds must be explored.

2.3.2 Uses of glycerol and its derivatives

Glycerol is arguably one of the most common ingredients used in industrial processes. Its tri-hydric nature gives it a hygroscopic characteristic allowing it to be used in a variety of consumer-related products. Glycerol's highly functionalised groups allows it to form a large number of derivatives, namely; 1,3-propanediol, DHA, ethanol, succinate and citric acid. (da Silva, et al., 2009). Research into value added products is necessary to address an expected increase in glycerol production.

2.4 Immobilization

Immobilization provides a solution to many of the problems associated with bioprocess technology and drug delivery. Immobilization refers to the technology of trapping a material (solid/liquid/gas) within or throughout a semi-permeable and biodegradable matrix (Vidhyalakshmi *et al.*, 2009). The matrix is generally sourced from polymeric materials such as κ-carrageenan, alginates, cellulose acetate and chitosan. Furthermore, the carrier selection is determined by the biological material and its environmental conditions (Anal & Singh, 2007).

The productivity of immobilized cells is generally as high; if not higher than, the corresponding free cell fermentations. This is due to the increased stability offered from the microenvironment of the matrix (Phillips & Poon, 2011). Bioprocess technology often faces slow productivity rates due to external factors such as pH and temperature fluctuations, as well as, substrate and/or product inhibition. When the cells are held within the semipermeable matrix, the effect of these external factors are greatly reduced (Anwar *et al.*, 2009). Another advantage is the cost effectiveness of downstream processing, as immobilized cells are easier to separate and can be recycled.

Previous research on the immobilization of *G.oxydans* in polyvinyl alcohol (PVA), potassium pectate and alginate reported increased tolerance for temperature and pH change, increased cell viability, as well as, increased tolerance to substrate inhibition (Adlercreutz, *et al.*, 1985; Tkac, *et al.*, 2001; Wei, *et al.*, 2007).

The *G.xylinus* cells immobilized in polyvinyl alcohol (PVA) showed yields greater than 0.80 and optimal pH tolerance ranged between 4.0 to 5.5 as opposed to the optimal pH of 5.5 using freely suspended cells (Nabe, *et al.*, 1979). Another study immobilized cells and cellular fractions *G.xylinus* in calcium alginate (Stasiak-Różańska, *et al.*, 2011a). The high DHA yield of 0.98 through the intact *G.xylinus* cells leads this investigation to pursue immobilization using this polymeric material.

2.4.1 Immobilization of cells in alginate beads

Alginate is a common biopolymer used in carrier systems and has been used by the food and the pharmaceutics industry for over 65 years (Anal & Singh, 2007). Alginate is typically obtained from algae; in the form of brown seaweed - *Laminaria hyperborea*, *Ascophyllum nodosum* and *Macrocystis Pyrifera*. These sources produce alginate as a phycocolloid (starch like chemical). This can be obtained from the chemical treatments used to remove heavy metals, proteins and other carbohydrates, before processed into powder form (Encyclopedia, 2012; Goh *et al.*, 2012).

2.4.1.1 Structure of alginate beads

Alginate is founded on two building blocks; α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues. These may conform up to three different polymer configurations, held together by glycosidic linkages in randomised order (Figure 2.5) (Goh, *et al.*, 2012). In the presence of divalent cations (Ca^{2+} or Mg^{2+}) alginate form covalent bonds that lead to a rigid gel formation (Anal & Singh, 2007). The surface polymerisation occurs instantaneously. With prolonged exposure divalent ions permeate through the matrix to solidify the structure. The randomised order of the different configurations provides a porous structure and is an ideal method of encapsulation for reactions with high oxygen requirements.

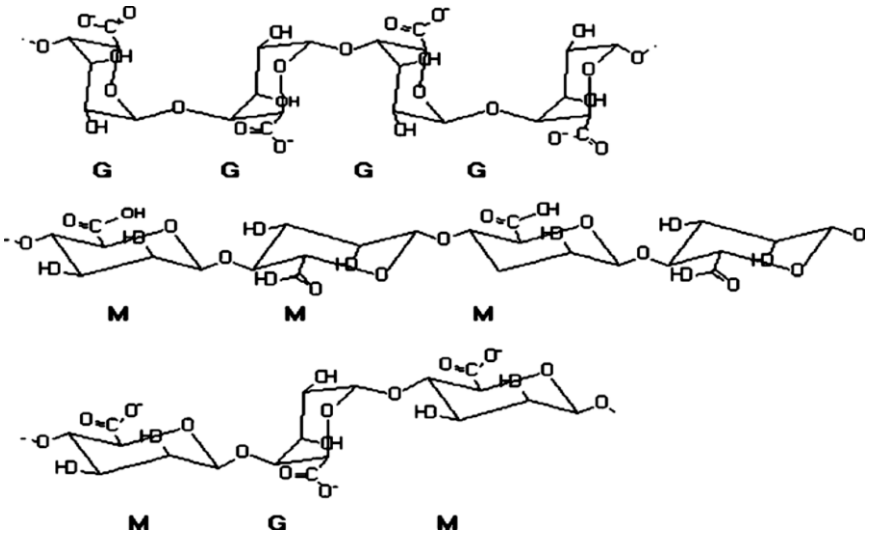


Figure 2.5 Glycosidic linkages; G-G, M-M and M-G block (Goh, *et al.*, 2012).

2.4.1.2 Optimisation of alginate bead formation

The performance of alginate beads depends on sodium alginate concentration, calcium chloride ($CaCl_2$) concentration, cell density and particle diameter (Adlercreutz, *et al.*, 1985; Anal & Singh, 2007).

Low sodium alginate concentration leads to a weak matrix and the leaking of essential metabolites. On the other hand, a high concentration of sodium alginate may lead to increased mass and oxygen transfer limitations (Willaert, 2007). A study by Anwar, *et al.* (2009) discovered that sodium alginate concentrations between 2% and 3% are suitable for most types of bioconversion. Other studies also support this finding: (Anal & Singh, 2007; Stasiak-Różańska, *et al.*, 2011a).

Blandino *et al.* (1999) investigated the effect of $CaCl_2$ concentration on bead structure. This study discovered that concentrations greater than 0.3 M $CaCl_2$ resulted in thicker membrane formation and subsequently greater mass transfer limitation. Calcium chloride concentrations, commonly used by other studies, typically vary between 0.1 M and 0.3 M (Adlercreutz, *et al.*, 1985; Jobanputra *et al.*, 2011; Stasiak-Różańska, *et al.*, 2011a).

The cell density of the immobilized bead is an important factor to consider as cells retain the ability to form biomass. One the one hand, a high cell density may lead to quick bead rupture. Conversely, low cell density may limit the rate of conversion. A study by (Adlercreutz, *et al.*, 1985) investigated the performance of cell densities and particle diameter on DHA conversion. The study reported that increased cell densities ranging between 4 gL^{-1} and 28gL^{-1} resulted in decreased activity. The increased cell densities acted as a diffusion barrier for DHA transport into the external medium. This led to an increase in DHA concentration within the alginate bead, causing GlyDH deactivation (Bauer, *et al.*, 2005).

The diameter of an immobilized cell is also an important factor in mass transfer and stability. According to Fick's law of diffusion, mass transfer limitation increases when the thickness of a barrier is increased (Seader & Henley, 2006). A study by Adlercreutz, *et al.* (1985) on alginate immobilized *G.oxydans*

observed increased particle diameter between 2.0 mm and 4.0 mm resulted in decreased DHA production. This may be due to decreased oxygen transfer, resulting in decreased GlyDH activity. Increasing the oxygen rate and/or impeller speed can decrease oxygen limitation (Karimi *et al.*, 2013; Martín *et al.*, 2008; Özbek & Gayik, 2001).

2.4.2 Chitosan coating for alginate reinforcement

Chitosan is a biodegradable, natural polymer that is commonly used for cell immobilization. Its structure, as seen in Figure 2.6, comprises of copolymers of glucosamine and *N*-acetyl-glucosamine and the free amine groups allow it to chelate with metal ions (Sinha *et al.*, 2004).

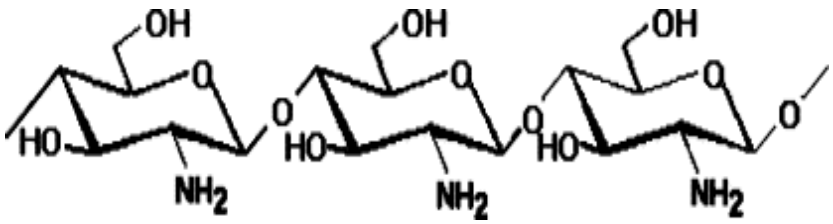


Figure 2.6 Structural configuration of chitosan (Sinha, *et al.*, 2004).

Due to its porous and non-toxic properties, chitosan is commonly used in the pharmaceutics industry for drug delivery (Croisier & Jérôme, 2013). Research into drug delivery has led to incorporating chitosan with alginate to increase the physical strength of the bead (Hari *et al.*, 1996; Kim *et al.*, 2008). When chitosan coats the surface of the bead, it forms cross links on the surface as well as within it. This increases the tensile strength but also maintains porosity. The alginate matrix is delicate and prone to shear damage and swelling. This investigation with coat the alginate immobilised cells with chitosan to improve its stability.

2.5 Bioreactor and mode of operation

The microbial synthesis of DHA is carried out inside a bioreactor - an apparatus used to carry out microbial conversion of substrates under sterile conditions. It operates under different modes: batch, fed-batch or continuous, depending on the kinetics parameters of the organism.

2.5.1 Bioreactors used for DHA production

The industrial production of DHA is carried out inside a stirred tank reactor, using freely suspended cells of *G.oxydans* (Hekmat, *et al.*, 2003a). Low productivity rates and the effects of inhibition have led to studies on alternative reactor designs. Some studies incorporate immobilization with alternative reactors to reduce the effect of substrate and product inhibition.

Hekmat, *et al.* (2003a) combined the design of a stirred tank reactor with a trickle bed reactor that contained cells immobilized inside a hydrophilised Ralu-ring carrier material. The design led an increase in production. However, problems with the trickle bed reactors include: large concentration gradients and limited oxygen throughout the column (Willaert, 2007). Another study by Tkac, *et al.* (2001) produced DHA inside an airlift reactor using cells immobilized in pectate. Air lift reactors lack moving parts; making them ideal for processes using immobilized cells. This is because air life reactors cause less shear. However, air lift reactors also use compressed air for mixing, which can be expensive on an industrial scale. They are also used for media with low viscosity, and are thus not ideal for media with high glycerol concentrations. Another study, using a bubble column, found increased DHA production rates with increased aeration rate. However excessive foaming was also recorded (Adlercreutz, *et al.*, 1985). Bubble columns are simple in design and have high gas transfer rates that often lead to excessive foaming. Although there a various types of designs, stirred tank reactors are still popular among researchers for DHA production (Bories, *et al.*, 1991; Claret, *et al.*, 1992a; Li, *et al.*, 2010a; Wei, *et al.*, 2007). Their popularity is attributed to good oxygen transfer and variable mixing intensity, which are both ideal for high viscosity media (Willaert, 2007)

Previous research using immobilized cells of *G.xylinus* in the production of DHA have only been carried out in shake flask experiments (Nabe, *et al.*, 1979; Stasiak-Różańska, *et al.*, 2011a). One of the objectives of this investigation is to produce DHA as efficiently as possible, using a large-scale reactor. As previously mentioned, DHA conversion is an oxygenic reaction; thus good oxygen transfer is crucial. Due to the concentration gradients and limited

oxygen, trickled bed reactors and packed bed reactors will not be used. The lack of mechanical parts in an air lift reactor and bubble column are useful for maintaining the stability of an immobilized carrier. However, a large amount of compressed air is required to achieve homogeneity within the media. This contributes to high power consumption in industrial scale. For this reason, those reactors will not be used. Stirred tank reactors are known to cause shear damage to sensitive matrices. However, they are ideal for increased viscosity and also allow good oxygen transfer and variable mixing. Accordingly, a stirred tank reactor was the chosen apparatus for DHA production in this research.

2.5.1.1 Stirred tank reactor

Stirred tank reactor is used industrially for the production of DHA and is the most common type of bioreactor for aerobic processes. Its useful attributes include: relatively high oxygen transfer rate, *in situ* sterilisation capability and well established scale-up relations (Zhu, 2007).

The size of bioreactors vary from shake flasks (≤ 500 mL) to large-scale (3-L) and industrial scale (>500-L). Shake flasks are used for media development, micro-organism strain selection and small-scale fermentations (Zhu, 2007). large-scale fermenters are used in media development, seed cultures and for investigating the effect of parameters on product rates. Industrial scale bioreactors are used for mass production and also in the food, beverage and pharmaceutical industry. Both large-scale and industrial scale bioreactors can be made of glass, stainless steel or a durable plastic, as seen in Figure 2.7.

(a) **(b)**

Figure 2.7 Stirred tank bioreactor vessels; (a) 5-L glass vessel, (b) 500-L stainless steel vessel.

The above vessel has various components including: an impeller, baffles (to break up laminar flow), an air sparger, a heat exchanger and a heating rod. It also has sensors for pH, temperature and dissolved oxygen. Together, these parts maintain a controlled, homogeneous and sterile environment.

2.5.2 Mode of operations

Batch, fed-batch and continuous mode operations are three techniques used in submerged fermentation (Zhu, 2007). Studies into DHA conversion have been carried out under batch (Flickinger & Perlman, 1977; Nabe, *et al.*, 1979; Stasiak-Różańska, *et al.*, 2011a) and fed-batch (Bauer, *et al.*, 2005; Claret, *et al.*, 1992a; Hekmat, *et al.*, 2003a) mode.

A fed-batch mode is often preferred when there is substrate inhibition. Substrate inhibition has been reported for *G.oxydans* in the production of DHA. No studies have reported the kinetics on substrate inhibition for *G.xylinus*. For this reason, a batch mode operation was used in this investigation.

2.5.2.1 Batch mode operation for DHA production

A batch fermentation does not add or withdraw from the culture broth after inoculum; with the exception of air, antifoam and alkaline/acid for pH

adjustment (Cinar, 2003). The growth and formation of products within the vessel under batch mode may be modelled from the growth curve, as seen in Figure 2.8. The growth curve models the dynamic reactions with distinct phases: (i) lag phase, (ii) exponential phase, (iii) stationary phase and (iv) death phase.

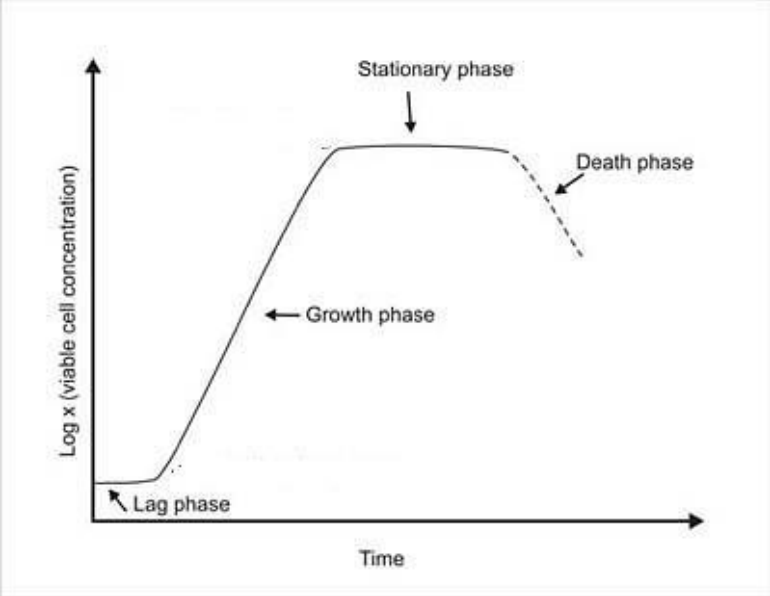


Figure 2.8 Growth curve observed from a conventional batch operation (Cinar, 2003).

These growth phases are important factors that aid in decision making when dealing with large-scale and industrial processes.

i. The lag phase (or initial phase) is the period of adaptation of cells to the new environment and minimal cell growth is observed. Extended lag phases are undesirable and should be avoided.
ii. The growth phase (exponential phase) is the logarithmic growth phase and cells are dividing at a constant rate. During this time nutrient availability is in excess and microorganisms are capable of producing synthetic precursors that aid in the synthesis of metabolites. Free cell inoculums are usually transferred during this phase to decrease the lag phase of the media (Cinar, 2003).
iii. The stationary phase occurs when the ratio of dividing and dying cells is held in equilibrium. This may be a result of

one or more of the following factors: substrate depletion, accumulation of toxic by-products, and possible contamination. Cells for immobilization are ideally harvested during this phase to decrease the chances of bursting the immobilization carrier (Nabe, *et al.*, 1979).

iv. Death phase, otherwise known as the decline phase occurs when the rate of dying cells is greater than the rate of dividing cells.

In a general fermentation process, the growth curve is constructed to optimize process. The growth curves help identify optimal time for inoculum transfer to reduce lag, optimal time for harvesting to reduce time and energy waste as well as a basis for improving process. Figure 2.9 shows the stages involved in large-scale or industrial scale fermentation.

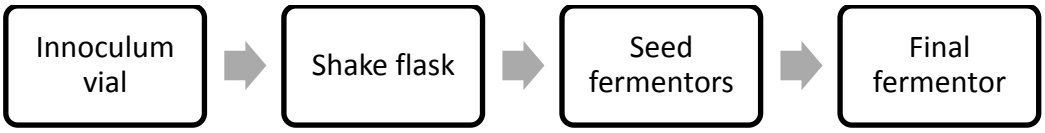


Figure 2.9 Stages of batch fermentation begins with an inoculated media in a shake flask that is subsequently fed into a larger fermenter until it reaches the product fermenter.

Growth curves should be modelled for shake flasks and large-scale fermenters. Reproducibility between the both is nearly impossible due to the dynamic nature of microorganisms and various parameters that can affect large-scale reactors (i.e. impeller speed, DO etc…). The growth curves and kinetics obtained from a large-scale reactor can be quantified to industrial scale processes using mathematical models and estimating trajectories found in batch fermentation books (Cinar, 2003; Stanbury, *et al.*, 2000).

2.6 Research objectives

The objective of this research was to produce DHA from glycerol via microbial oxidation using immobilized *G.xylinus* cells. The booming biodiesel industry is expected to result in an influx of glycerol into the market. This will lead to a downward spiralling of its cost, making it an attractive raw material for industrial processes. However, glycerol available in the market is expected to exceed consumption. This will result in a serious environmental problem by way of its disposal. Due to this, there is a need for research on converting glycerol into high-value products and DHA is one of them.

Currently DHA is produced by the microbial oxidation of glycerol using the strain *G.oxydans*. Due to high production costs, DHA is not considered a 'commodity' in the market. One of the ways to decrease the production cost is to increase DHA productivity in submerged fermentations using glycerol as a carbon source. Since the 1960s there have been studies on improvements to DHA production using *G.oxydans*. However, there are not many investigations on the production of DHA using *G.xylinus* strain.

The main aim of this research is to investigate the possibility of enhancing DHA productivity using *G.xylinus* cells immobilized in alginate. Immobilization provides the advantage of decreased production costs because of separation techniques such as centrifugation and filtration are not necessary to remove the bacteria in the culture medium. The production period can also be extended by immobilization. Shake flask as well as large-scale fermentations will be carried out to investigate various parameters on DHA production by immobilized *G.xylinus* cells.

3 MATERIALS AND METHODS

3.1 Microorganism and maintenance

Gluconacetobacter xylinus DSM46604 was obtained from Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures. Cells were maintained on agar slants of the following composition advised from the source (w/v): 2% agar, 5% glucose, 0.5% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.3% KH_2PO_4 and 0.05% $MgSO_4$; pH 6.8. The slants were incubated at 30 ^oC for 7 days and stored at -20°C. Cultures were periodically transferred to freshly prepared agar slants to maintain high activity.

3.2 Inoculum development

The screening media was obtained from previous research reported in literature characterised for the growth of *G.xylinus.* The media by Stasiak-Różańska *et al.* (2011b) of the following composition (w/v); 2.0% glycerol, 0.5% yeast extract at pH 5.0; characterised growth of *G.xylinus* for DHA production. The media by Iguchi *et al.* (2000) used the following composition (w/v); 5.0%sucrose, 0.5% $(NH_4)_2SO_4$, 0.5% yeast extract, 0.3% KH_2PO_4, 0.05% $MgSO_4$ at pH 6.8 to characterise the production of microbial cellulose using the mentioned specie. The original carbon sources were substituted for glycerol at 1:1 (v/v) ratio and the pH of the latter was reduced to 6.0 from 6.8. The media were modified to contain glycerol as the sole carbon source.

3.3 Chemicals

Glycerol, glucose, ammonium sulphate ((NH4)2SO4), and sodium dihydrogen orthophosphate (KH2PO4) were purchased from UNIVAR LTD. Yeast extract was purchased from Oxoid Microbiology Products LTD, DHA from SIGMA Ltd and magnesium sulphate ($MgSO_4$) from May & Baker Ltd. Alginate for immobilization was purchased from AJAX Finechem and chitosan from Aldrich LTD. All other chemicals used were of analytical grade. Solutions and media were prepared using distilled water.

3.4 Equipment

The following equipment were used in the experiment;

- Autoclave: TOMY SX-700E
- Incubator shaker: New Brunswick INNOVA 4200
- Fermentor: INCELTECH LH series 210 (5-L)
- Peristaltic pump: Watson Marlow 505U
- Centrifuge: SIGMA 6K-15
- Refractometer: RHW-25ATC
- Dryer: Contherm Thermotec 2000 oven
- Spectrophotometer: Shimadzu Pharma Spec UV- 1700
- Laminar hood: Heraeus KS12

3.5 Cultivation medium and growth

Three 250 mL Erlenmeyer shake flask were prepared with 100 mL of sterilised media of the following composition; 2% glycerol, 0.5% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.3% KH_2PO_4 and 0.05% $MgSO_4$. The initial pH was adjusted to 6.0 with the addition of 6M NaOH solution before autoclaving. The flasks were then incubated at 30 ^oC for 60 h at 150 rpm. Fifteen millilitres aliquots were taken at 24 h intervals for analysis.

The seed inoculum containing the same volume and composition of media were incubated under the same conditions as above for a day (exponential phase) then transferred to the production media of the same composition at 10% (v/v).

3.6 Large-scale cultivation

Large-scale cultivations were carried out with a working volume of 3 L in a 5-L stirred tank bioreactor, as seen in the schematic diagram below.

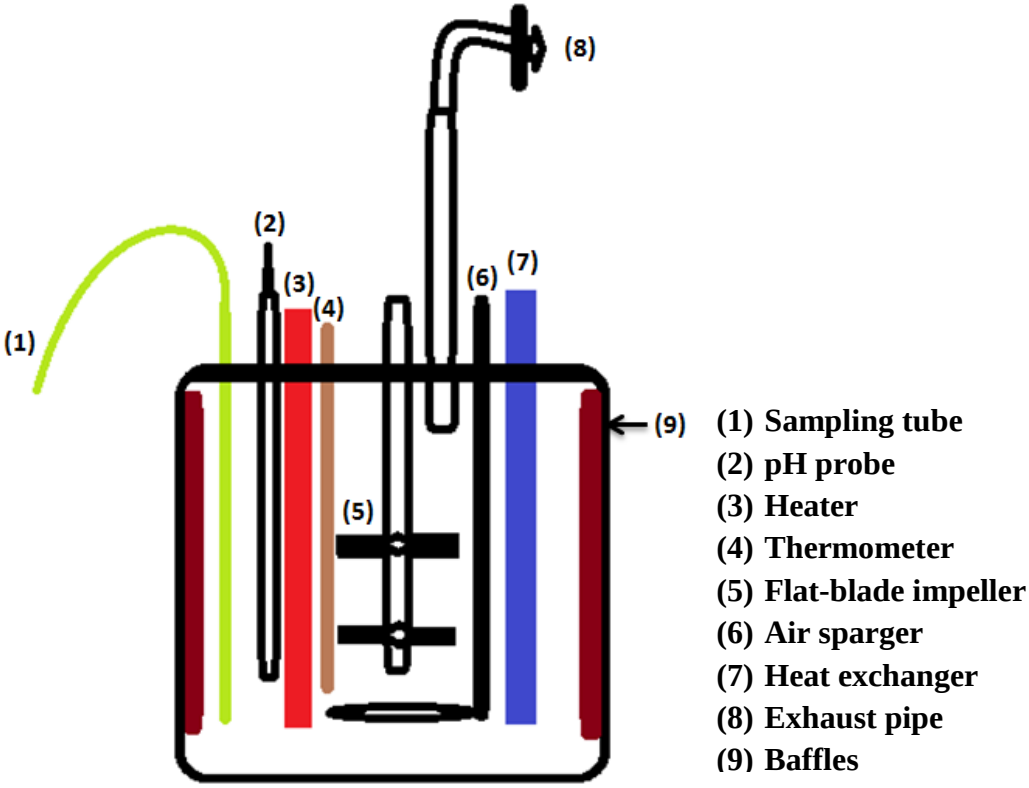


Figure 3.1Schematic diagram of the 5 L bioreactor used in this investigation.

The bioreactor was equipped with two flat blade impellers for agitation and a ring sparger for aeration. The bioreactor had sensors for control of temperature, pH and agitation rate. The aeration rate was controlled by a rotameter. The cultivations were carried out at 30 °C and pH was controlled at 6.0 by addition of 6M HCl or NaOH. The agitation rate was controlled at 150 rpm for free cell production and 100 rpm for immobilized cells to decrease the shear stress on the beads containing immobilized cells. The effect of glycerol concentration, effect of immobilization material, and effect of aeration rate on DHA production were investigated.

3.7 Immobilization

G.xylinus cells were immobilized in an alginate matrix, as well as a chitosan-alginate layered matrix.

3.7.1 Production of *G.xylinus* cells for immobilization

G.xylinus cells were cultivated in the fermenter as described above and harvested via centrifuging at 4000 g for 20 min and washed with distilled water to remove residual medium components prior to immobilization. If cells were

not immobilized immediately after processing, it was stored in sterile aqueous glycerol solution of 3% (w/v) concentration at -20°C to prevent cell autolysis.

3.7.2 Immobilization of cells in alginate beads

Approximately 5.0 ± 0.5 g cells were weighed out from the harvested cells described above, washed with sterile distilled water and re-centrifuged to remove trace contaminants. The cells were then suspended in 150 mL of sterile distilled water in a Duran laboratory glass bottle. One-hundred and fifty millilitre of sodium alginate solution 4% (w/v) was prepared with sterile distilled water and the solution was gently mixed for well over an hour to obtain homogeneity and eradicate air bubbles that may get trapped in the beads. When this has been accomplished the alginate solution was added to the solution of suspended cells at 1:1(v/v) to obtain a final alginate concentration of 2% (w/v). One litre solution of sterile 0.2M calcium chloride ($CaCl_2$) was prepared in a 2-L glass bottle (with stirring to prevent bead aggregation) and cooled to room temperature. The solutions and pump were arranged in an automated set up shown in Figure 3.2.

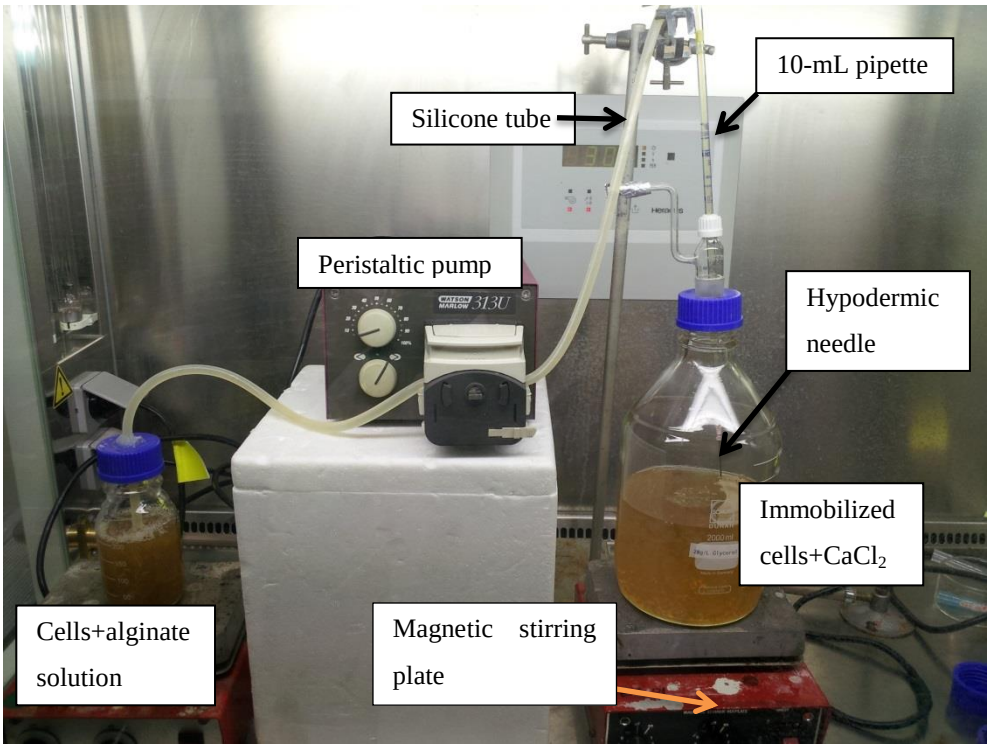


Figure 3.2 Automated set-up in the laminar hood for cell immobilization.

A total of 300 mL alginate/cell solution with a cell concentration of ~17 gL^{-1} formed beads of uniform size and shape. The set-up was designed to extrude immobilized cells of 2 mm diameter bead through 18 gauge hypodermic needle in a sterile environment.

3.7.3 Immobilization of cells in chitosan-coated alginate beads

Low molecular weight chitosan 0.4% (w/v) was dissolved in acidified water containing 0.4% (v/v) glacial acetic acid. After dissolution, 1M NaOH was added to adjust the pH to 5.7 and the solution drained using a sterilised Buchner filter to remove gelatinous filaments. Fully formed alginate beads prepared above were immersed in chitosan solution for about 40 min in a rotary shaker. Chitosan-coated alginate beads were then washed with sterile distilled water and used. If the beads were not immediately required it was stored in sterile peptone at 4°C until needed (Kim, et al., 2008). All the operations were carried out in the laminar flow under aseptic conditions.

3.8 Analytical methods

3.8.1 Biomass

The biomass was determined gravimetrically by dry weight measurement. Known volume of culture samples were centrifuged at 4000 g for 20 min in pre-weighed graduated tubes. The addition of glass beads in shake flasks experiments suppressed the formation of cellulose (Stasiak-Różańska, *et al.*, 2011b) so the residue left upon centrifugation is assumed to be entirely biomass. The tubes were dried at 80 °C until constant weight was achieved and the cell concentration was expressed as gram dry cell weight per litre media (g DCW L^{-1}). The supernatant was used for the determination of glycerol and DHA concentrations.

3.8.2 Dihydroxyacetone

Dihydroxyacetone (DHA) was assayed by the colorimetric method (Stasiak-Różańska, *et al.*, 2011b). DHA reduces 3,5-dinitrosalicylic acid (DNS) and the resultant compound display an orange red colour, the intensity of which depends on the concentration of DHA.

The DNS solution was added to the DHA sample at 1:1 (v/v) ratio and incubated at 100 ^{o}C for 10 min. The incubation time was kept constant through every assay as the colour intensifies with prolonged heating. When the sample has cooled the absorbance is measured at an optical density (OD) of 550 nm. The concentration of DHA was obtained from a calibration graph, as seen in Appendix 3. The calibration curve was constructed with analytical grade DHA as the standard.

3.8.2.1 DHA formation kinetics

The kinetics of product formation was calculated to better understand the dynamics and efficiency of the reaction taking place in the reactor. Yield, product formation rate, maximal productivity and specific product formation rate collectively make up the kinetics of DHA production.

The yield coefficient of DHA, $Y_{p/s}$ was calculated as the final concentration of DHA produced, p_f with reference to the amount of glycerol consumed in the reaction;

$$Y_{p/s} = \frac{p_f}{(s_i - s_f)}; \ where\ s_i \gg s_f \tag{3-1}$$

where, p_f was the final DHA concentration at the end of the fermentation period, s_i was the initial glycerol concentration and s_f the final glycerol concentration at the end of the fermentation period.

The product formation rate (g DHA $L^{-1}h^{-1}$), r_p is a measure of the reactor efficiency and is defined by the following equation;

$$r_p = \frac{p_f}{t_f - t_o}; \quad where\ t_o = 0 \tag{3-2}$$

where t_f was the time (h) at the end of the fermentation period.

The maximal volumetric productivity (g DHA $L^{-1}h^{-1}$), r_{pmax} was achieved when nutrients were available in excess and was calculated from the exponential phase of the production curve from the following equation;

$$r_{pmax} = \frac{p_2 - p_1}{t_2 - t_1}; \quad where\ 0 < p_1 < p_2\ ;\ 0 < t_1 \ll t_2 \tag{3-3}$$

where p_2 was the DHA concentration at the end of the exponential phase and p_1 at the beginning of the exponential phase. The times t_2 and t_1 were the respective times of exponential phase.

The specific product formation rate, q_p (g product g substrate $^{-1}h^{-1}$), defined the efficiency of DHA production from the glycerol substrate. In a batch process q_p is measured as an instantaneous value from the exponential phase of growth only and is not constant during batch fermentation. This investigation was more interested in the specific product formation from inoculation to the end of the exponential phase rather than the instantaneous value. The q_p value was calculated from the following equation

$$q_p = \frac{1}{p}\frac{\Delta p}{\Delta t} \tag{3-4}$$

where p was the DHA concentration at the end of the exponential phase, Δp was the change in DHA concentration a (≈ 0) to the end of the exponential phase and Δt was the respective time (h) .

3.8.3 Glycerol

Glycerol concentration was measured using the refractive index method (Stasiak-Różańska, *et al.*, 2011b) which measures the change in angle of light between two mediums. The application follows Snell's Law which defines the refractive index as a constant ratio of the sine of the angle of incidence to the sine of the angle of refraction.

Using a pipette, a few drops of sample with a temperature of 20 ^{o}C was placed on the prism and the lid was sealed to create a vacuum. The handheld device was pointed towards a light source and the refractive index was noted. The refractive index was converted to glycerol concentration using the standard graph, as seen in Appendix 3 DHA calibration graph carried out with spectrophotometer at 550 optical density, OD550.

. The calibration curve was prepared using analytical grade glycerol.

3.8.3.1 Substrate kinetics

Glycerol consumption rate and rate of substrate uptake are important parameters that were considered in the reaction.

Volumetric substrate consumption rate (g glycerol $L^{-1}h^{-1}$), r_s was measured as the glycerol consumption rate over the course of the fermentation and was defined by the equation;

$$r_s = \frac{s_i - s_f}{t_f - t_o}, \ \ where\ t_o = 0 \tag{3-5}$$

where *s* is the glycerol concentrations; *i* denotes the initial concentration and *f* the final concentration at the end of the fermentation period; t is the fermentation time (h) where *f* denotes the time at end of fermentation period and *o* is the beginning when time is 0 h.

The specific rate of substrate uptake (g substrate g $product^{-1}$ h^{-1}), q_s defines the amount of glycerol substrate used to produce a specific product, DHA, and was expressed by the following equation;

$$q_s = \frac{q_p}{Y_{p/s}} \tag{3-6}$$

where q_p is the rate of DHA formation (h^{-1}) and $Y_{p/s}$ is the yield coefficient previously described.

3.9 Shake flask experiments

Three sets of 100 mL of media in 250-mL shake flasks were prepared and the initial pH was adjusted to 6.0 with the addition of 2M NaOH. The flasks were sterilised at 121 ^{o}C for 15 min in an autoclave then allowed to cool to 30 ^{o}C before inoculation. In free cell shake flask experiments, approximately 20 glass beads of 1.0 mm diameter were added to prevent the formation of microbial cellulose (Stasiak-Różańska, *et al.*, 2011b). Approximately 3.00± 0.25 g of immobilized cells were added to each shake flask that were then

incubated at 30 ^{o}C and 150 rpm in an orbital shaker. Culture samples were withdrawn at regular intervals for analysis.

The shake flasks prepared for inoculum was incubated for a day prior to transfer to the production media. The production media was inoculated at 10% (v/v) seed media (Riet & Tramper, 1991; Stanbury *et al.*, 2010).

3.10 Large-scale experiments

Large-scale experiments were carried out using 3 L media of varying glycerol concentration, aeration rates and immobilization material to investigate the effects of these parameters on DHA production in a 5-L table-top fermenter. For fermentations using free cells, the fermenter was inoculated with 10% (v/v) seed media, which had been grown as described previously. For fermentations using immobilized cells, 10% (w/v) immobilized beads were aseptically added to the fermenter. The fermentation was carried out 30^{o}C and the pH was controlled at 6.0 by the addition of 6M HCl or NaOH.

3.10.1 Fermentations using varying glycerol concentration

One of the ways to increase DHA production by *G.xylinus* cells is to process high concentrations of glycerol. However, the primary effect of increased glycerol concentration is increased viscosity and density of the medium surrounding the beads which introduces mass-transfer limitation and homogeneity problems. The effect of glycerol concentration on DHA production was investigated by varying the concentration (w/v) of glycerol (1%, 2%, 4% and 7%) in the fermentation medium carried out in a 5-L fermenter as described above. Samples were withdrawn at regular intervals and analysed for DHA.

3.10.2 Fermentations using varying aeration rates

The effect of aeration rates on DHA production were compared within the alginate beads and chitosan-coated alginate beads. In order to investigate this effect the fermentations were carried out in the 5-L fermenter using 3 L medium as described above at aeration rates of 0.3 vvm, 0.6 vvm and 1.0 vvm. Culture samples were withdrawn at regular intervals for analysis.

3.10.3 Effect of immobilization material on bead stability and DHA production

The effect of immobilization material was investigated by assessing the instantaneous conversion rate (%), C_R of DHA throughout the cultivation period under varying aeration rates. The conversion rate was directly correlated to the instantaneous yield and has no relation to the product formation rate. It was calculated from data collected under Section 3.10.2 using the following equation;

$$C_R\,\% = \frac{dp/_{dt}}{ds/_{dt}} \times 100 \tag{3-7}$$

where $dp/_{dt}$ is the change in DHA concentration in one sample interval and $ds/_{dt}$ is the change in glycerol concentration over the same time interval.

4 RESULTS AND DISCUSSION

4.1 Media development

The preliminary media described in Section 3.2 was modified from its original version by substituting the carbon source for glycerol. The media by Stasiak-Różańska, *et al.* (2011a) had ethanol substituted for glycerol at 1:1 (w/v) ratio but only little growth was observed from the shake flask experiment. The media composed of only two components to provide carbon and nitrogen source was missing micronutrients essential for cell growth and development (Stanbury, *et al.*, 2010). The media used by Iguchi, *et al.* (2000) had sucrose substituted for glycerol at the same ratio as above and at 0.2:1 (w/v). The pH of the media was decreased from the original level of 6.8 to 6.0 to promote the activity of membrane bound GlyDH (Ameyama, *et al.*, 1985a; Li, *et al.*, 2010a), the enzyme responsible for catalysing DHA conversion from glycerol. The lower glycerol concentration media had a faster biomass and DHA formation rate. This led to the production media of the following composition (w/v); 2% glycerol, 0.5% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.3% KH_2PO_4 and 0.05% $MgSO_4$; pH 6.0, which has not been used in the study of DHA conversion from glycerol.

4.2 Shake flask experiments

4.2.1 Free cells

Shake flask fermentations using *G.xylinus* cells were carried out as described in Section 3.9. During the incubation period of 60 h, the biomass formation, glycerol utilisation and DHA production were monitored. The results are shown in Figure 4.1.

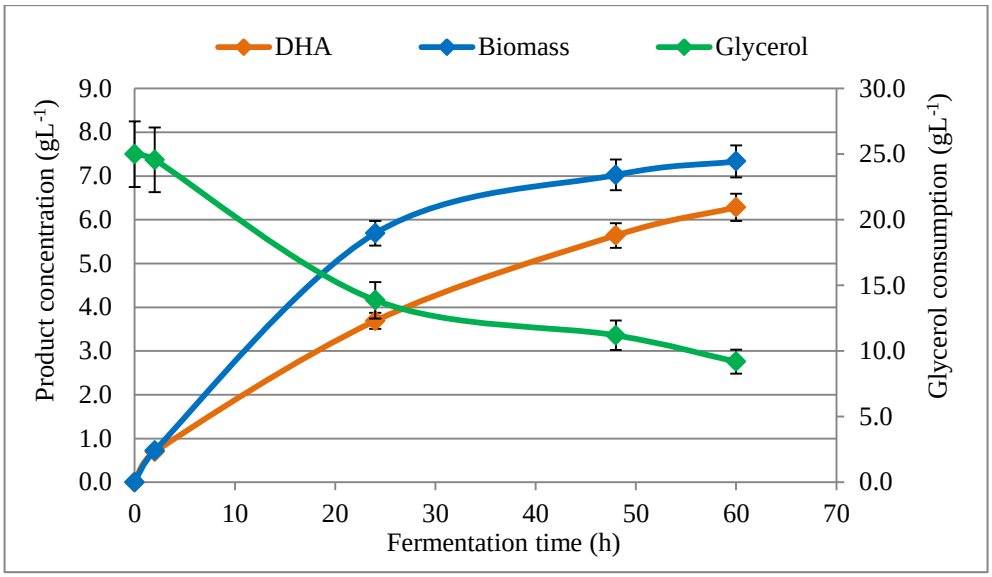


Figure 4.1 Time course of biomass formation, glycerol utilisation, and DHA production by free *G.xylinus* cells in shake flasks

The culture did not exhibit any lag phase and at the end of the fermentation period a biomass concentration of 7.3 gL^{-1} was obtained. The DHA concentration and productivity obtained in shake flasks were 6.3 gL^{-1} and 0.11 $gL^{-1}h^{-1}$, respectively.

4.2.2 Alginate immobilized cells

Shake flask fermentations using immobilized *G.xylinus* cells was carried out under the same conditions as the free cells. Figure 4.1 shows the DHA production and glycerol consumption during the course of fermentation.

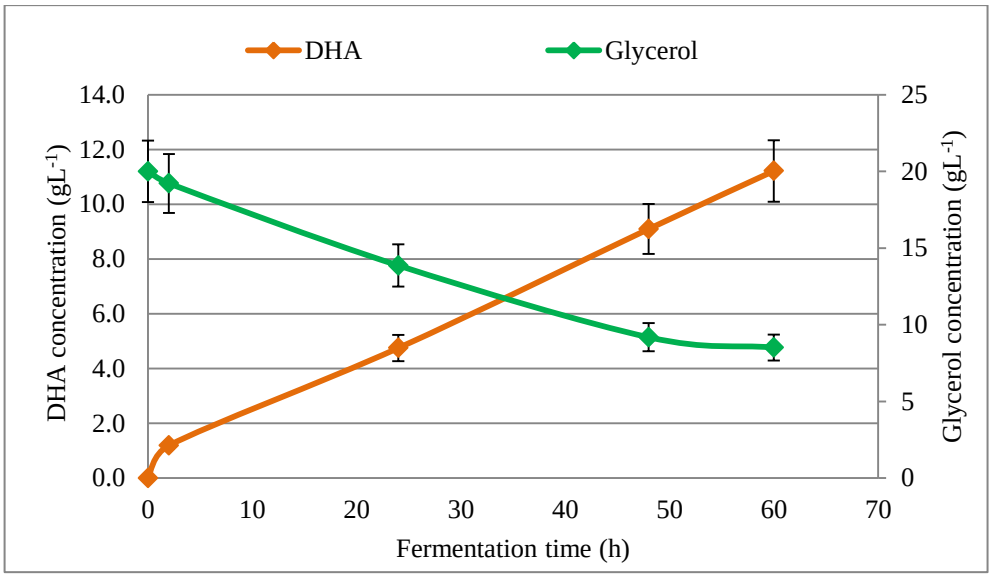


Figure 4.2 Time course of glycerol utilisation and DHA production by alginate immobilized *G.xylinus* cells in shake flasks.

As seen in the above figure, the production of DHA increased with fermentation time. The DHA concentration and the productivity obtained were 11.2 gL^{-1} and 0.19 $gL^{-1}h^{-1}$, respectively, which is a 43% increase from DHA produced by free cells.

4.3 Large-scale experiments

Large-scale experiments were carried out using free cells or alginate immobilized cells or chitosan-coated alginate immobilized cells in a 5-L fermenter containing 3 L medium. The large-scale fermenter containing free *G.xylinus* cells was inoculated with 10% (v/v) of its volume. The inoculum was grown in a shake flask under the same conditions carried out in the previous investigation (Subsection 4.2.1). It was transferred during the exponential phase of growth in shake flask, as seen in Figure 4.1. For fermentations using immobilized cells, immobilized cells were added at 10% (w/v) of the glycerol media. The DHA production and glycerol consumption were monitored from the time of inoculation and throughout the cultivation period at increments of 24 h. The cultivation time for every experiment varied depending on when the product formation reached stationary phase.

4.3.1 Free cells

The production of DHA in large-scale fermentations using free *G.xylinus* cells was carried out for 168 h (7 days). The time course of biomass formation, glycerol utilisation and DHA production is shown in Figure 4.3.

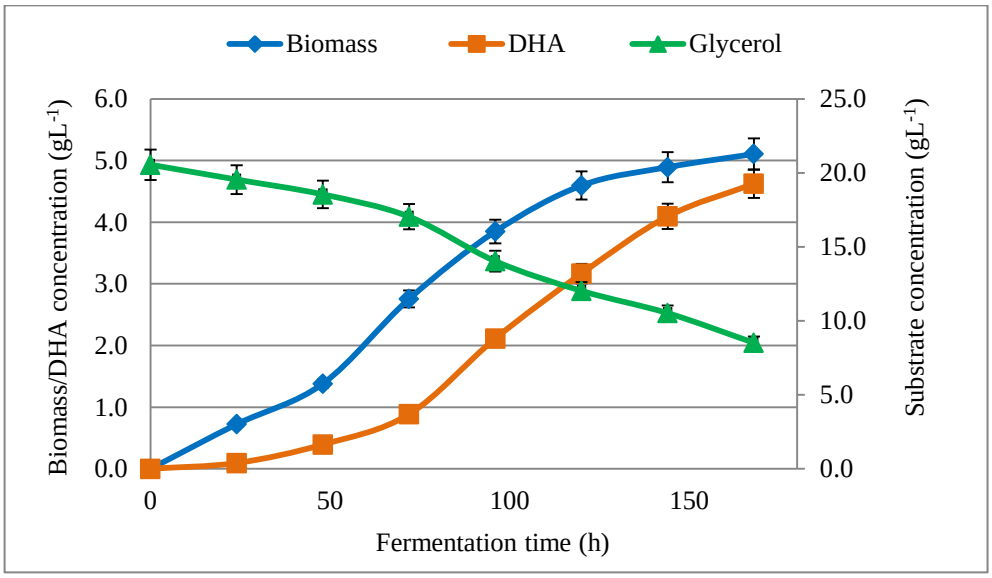


Figure 4.3 Time course of biomass formation, glycerol utilisation, and DHA production by free *G.xylinus* cells in 5-L bioreactor.

As seen from Figure 4.3, the lag phase of biomass formation lasted for 48 h. Biomass formation and productivity at the end of fermentation was 5.2 gL^{-1} and 0.04 $gL^{-1}h^{-1}$, respectively. The yield coefficient of biomass formation from glycerol consumption was 0.42.

The production of DHA increased during the course of the fermentation period. At the end of fermentation, the DHA concentration and the productivity obtained were 4.7 gL^{-1} and 0.03 $gL^{-1}h^{-1}$, respectively. The yield coefficient of DHA from glycerol consumption was 0.38. This is approximately 10% decrease from the yield coefficient of biomass.

4.3.2 Alginate immobilized cells

Two sets of experiments were conducted using alginate immobilized *G.xylinus* cells; (1) varying glycerol concentration to determine optimal concentration and (2) varying aeration rates to determine optimal aeration rate.

G.xylinus cells for immobilization were harvested from the large-scale reactor during the stationary phase (168 h) of the reaction, as seen in Figure 4.3. A previous study found cells of *Acetobacter xylinum A-9* performed more efficiently at converting glycerol to DHA when immobilized during the stationary phase (Nabe, Izuo et al. 1979). This is due to decreased biomass growth which reduces the probability of the beads bursting. As well, there is also maximal formation of GlyDH, the enzyme responsible for glycerol conversion to DHA (Ameyama *et al.*, 1985b). This allows for efficient bioconversion to take place.

Following the procedure outlined in Section 3.7.2, immobilized beads of *G.xylinus* cells were formed under aseptic conditions. Three-hundred millilitre of alginate-cell solution with a cell density of 17.0±0.5 gL^{-1} cells, formed beads of 2.0±0.2 mm in diameter as seen in Figure 4.4.

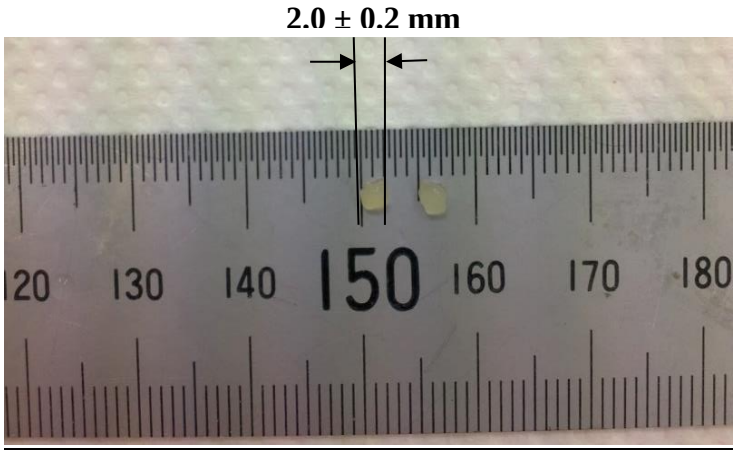


Figure 4.4 Size of alginate beads containing *G.xylinus* cells.

An average diameter of 2.0 mm was used by Adlercreutz, *et al.* (1985) for the immobilization of *G.oxydans* in the conversion of glycerol to DHA. The cell concentration was within the range (4-30 gL^{-1}) reported on the immobilization of *G.oxydans* in the conversion of DHA from glycerol (Holst, *et al.*, 1982; Tkac, *et al.*, 2001; Wei, *et al.*, 2007). A study by Adlercreutz and Mattiasson (1984) observed that an increase in cell density in alginate beads resulted in decreased GlyDH activity. The increased cell density acts as an obstruction

barrier for product diffusivity and results in an accumulation of product leading to cell deactivation.

4.3.2.1 Effect of glycerol concentration on DHA production

The study of varying initial glycerol concentrations on DHA production has been widely reported for *G.oxydans* (Claret *et al.*, 1992b; Hekmat *et al.*, 2003b; Li *et al.*, 2010b; Lu *et al.*, 2012b) and little is known of its effect on *G.xylinus*.

This investigation varied the initial glycerol concentration between 1% (w/v) and 7% (w/v). It aims to investigate the effect that these concentration ranges have on DHA productivity using immobilized *G.xylinus* cells.

Figure 4.5 illustrates the DHA production under various initial glycerol concentrations. The fermentation time (h) for maximal DHA production increased by 24 h increments; from 144 h for 1% (w/v) glycerol to 216 h for 7% (w/v) glycerol.

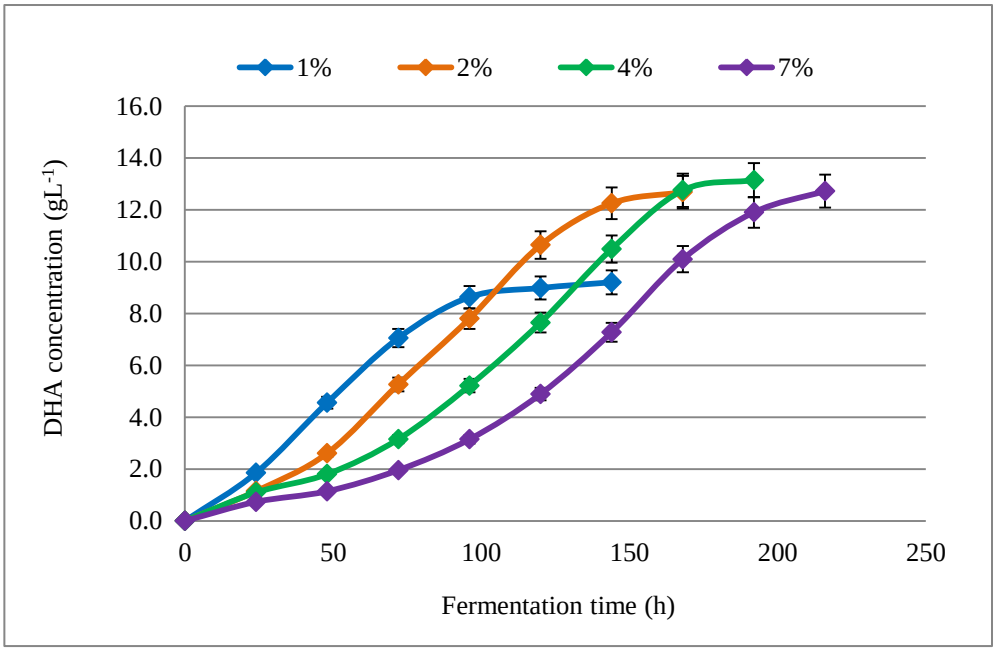


Figure 4.5 Effect of varying glycerol concentrations (% w/v) on DHA production by alginate immobilized *G.xylinus* cells.

Figure 4.5 shows DHA production was increased when the initial glycerol concentration was increased from 1% (w/v) and 2% (w/v). At 1% (w/v) initial glycerol concentration, the final DHA concentration and productivity obtained were 9.2 gL^{-1} and 0.07 $gL^{-1}h^{-1}$, respectively. At 2% (w/v) initial glycerol, DHA

production increased considerably and the final DHA concentration and productivity obtained were 12.7 gL^{-1} and 0.09 $gL^{-1}h^{-1}$, respectively. The DHA production did not increase further, by any significant means, for initial glycerol of 4% (w/v) and 7% (w/v). At initial glycerol of 4% (w/v), only a marginal, 8% increase in final DHA concentration was observed. The productivity dropped by 11% to 0.08 $gL^{-1}h^{-1}$. A similar trend was also observed at initial glycerol of 7% (w/v); the final DHA concentration of 12.7 gL^{-1} was lower than that of 4% (w/v) glycerol and DHA productivity was reduced by 22% to 0.06 $gL^{-1}h^{-1}$.

The effect of initial glycerol concentration on DHA productivity and glycerol consumption rate is illustrated in Figure 4.6.

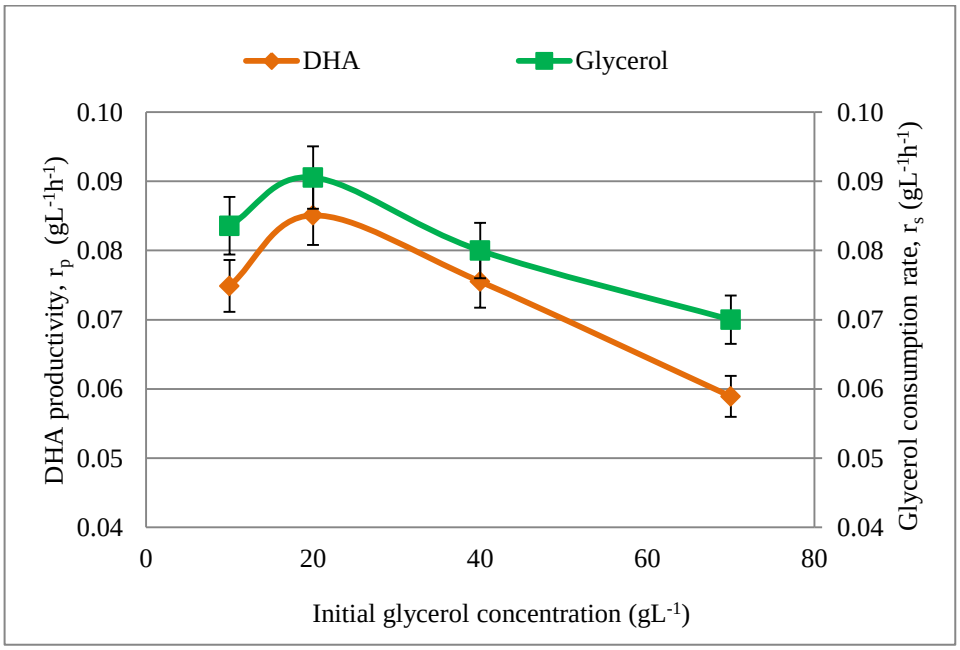


Figure 4.6 DHA productivity and glycerol consumption rate under varying initial glycerol concentrations.

The increased efficiency of DHA production at 2% (w/v) initial glycerol and the decreased trend following that may be due to media viscosity, substrate inhibition or an accumulation of DHA and lactic acid within the alginate bead (Flickinger & Perlman, 1977). When the initial glycerol concentration was increased from 1% (w/v) to 2% (w/v), the rates of DHA production and substrate consumption increased. This same trend was also observed in the kinetic parameters found in Appendix 5, increased in proportion. The DHA

production was greater due to increased availability of glycerol substrate in the media. At low initial glycerol concentrations, substrate inhibition does not exist. Furthermore, the viscosity of the medium on account of lower glycerol concentration did not impose any mass transfer limitation.

The declining trend observed in Figure 4.6 shows that an increase in initial glycerol concentration to 4% (w/v) resulted in an 11% drop in DHA formation rate. At an initial glycerol of 7% (w/v), the volumetric DHA productivity (r_p) decreased by another 22%.

When the initial glycerol concentration increased, its rheological properties, mainly viscosity of the media, increases in a linear manner (Malkin *et al.*, 2006). According to the laws of fluid dynamics, the boundary layer surrounding the surface of the immobilized carrier is where the effects of viscosity are significant (Kundu *et al.*, 2012). The effect of viscosity on DHA production is noted from the increased lag phase, as seen in Figure 4.5. The reduced activity may be due to slower diffusion of substrate into the alginate bead. As a result, there was an increase in time to yield a specified DHA concentration; for example to produce 5 gL^{-1} of DHA cultivation had to be carried out for 48 h at 1% (w/v), 72 h at 2% (w/v), 96 h at 4% (w/v) and 120 h at 7% (w/v). The *G.xylinus* cells are obligate aerobes and the alginate matrix surrounding them also imposes mass transfer limitations. According to Fick's law of diffusivity; mass transfer limitation is increased (Seader & Henley, 2006). The oxygen limitation can also lead to the build-up of lactic acid in the bead leading to cell deactivation (Flickinger & Perlman, 1977). The presence of substrate inhibition is also possible and has reported over a range of studies using *G.oxydans* for the conversion of DHA from glycerol (Claret, *et al.*, 1992b; Hekmat, *et al.*, 2003b; Li, *et al.*, 2010b; Lu, *et al.*, 2012b).

DHA yield (g DHA/g glycerol) remained greater than 0.9 at 1% (w/v) and 2% (w/v) initial glycerol and at 7% (w/v) initial glycerol a 15% reduction in yield was observed. As the initial glycerol concentration increased the fermentation time was also increased and at 7% (w/v) glycerol, fermentation lasted for 216 h (9 days). The drop in yield may be due to the break down in alginate bead, releasing alginate that has antibacterial properties (Pielesz *et al.*, 2011).

4.3.2.2 Effect of varying aeration rate on DHA production using alginate immobilized *cells*

The effect of aeration rate on DHA production by alginate immobilized cells was monitored over a period of 7 days (168 h) and the results are shown in Figure 4.7. The aeration rate, measured as volume of air per volume of media per time (vvm) was set at 0.3 vvm, 0.6 vvm and 1.0 vvm.

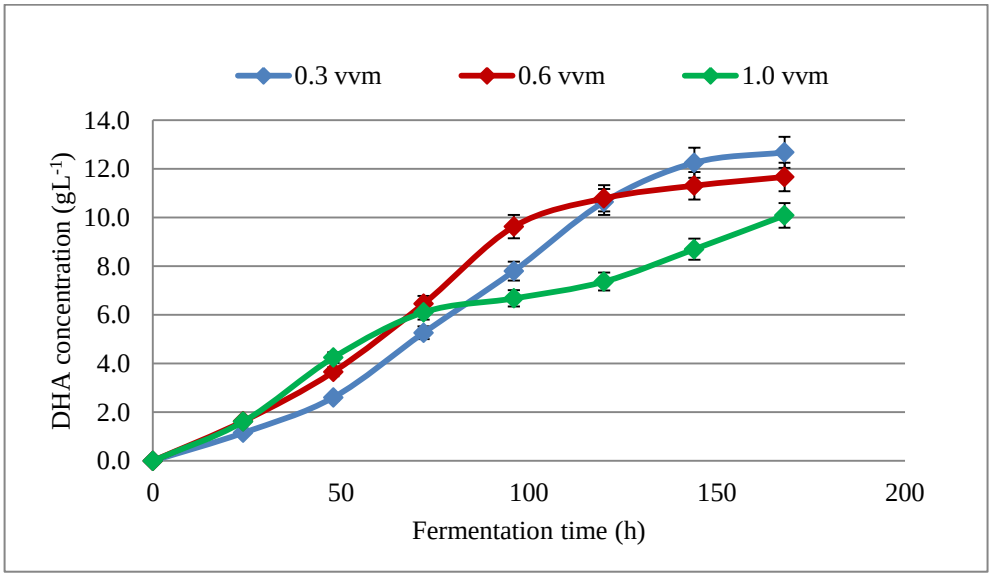


Figure 4.7 Time course of DHA production by alginate immobilized *G.xylinus* cells for varying aeration rates.

Figure 4.7 shows DHA production decreased with increasing aeration rate. The final DHA concentration and productivity of the alginate immobilized culture grown at the aeration rate of 0.3 vvm were 12.7 gL^{-1} and 0.09 $gL^{-1}h^{-1}$, respectively. A further increase in aeration rate to 0.6 vvm resulted in 11.7 gL^{-1} and 0.08 $gL^{-1}h^{-1}$ for these respective parameters. The culture grown at aeration rate of 1.0 vvm, the final DHA concentration and productivity reached 11.1 gL^{-1} and 0.07 $gL^{-1}h^{-1}$, respectively.

The conversion of glycerol to DHA requires oxygen as the final electron acceptor in the metabolic process (Li, *et al.*, 2010b; Stasiak-Różańska, *et al.*, 2011a). The alginate matrix surrounding the *G.xylinus* cells increase oxygen limitation. Therefore, increased aeration rates were expected to increase DHA productivity. However, this was not observed in the investigation. Rather, the

productivity of DHA decreased. To investigate this further, kinetic parameters listed in Appendix 6 were analysed.

The effect of aeration rate on DHA productivity, r_p and glycerol consumption rate, r_s is illustrated in Figure 4.8.

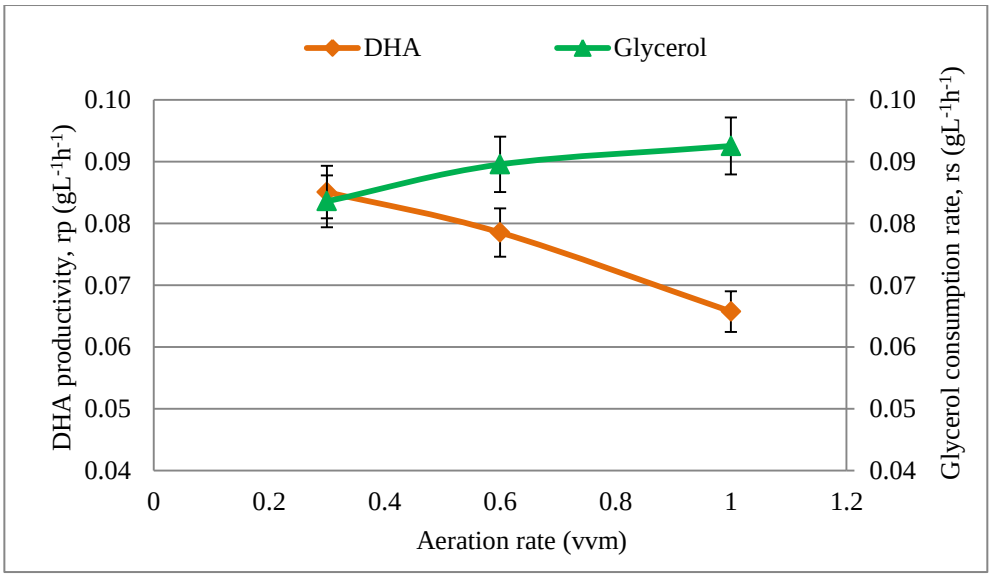


Figure 4.8 Effect of aeration rates on DHA productivity and glycerol consumption rates by alginate immobilized *G.xylinus* cells.

Figure 4.8 shows that increased aeration rate resulted in a decline of DHA production and an increase in glycerol consumption. When the aeration rate for the culture broth increased from 0.3 vvm to 0.6 vvm, a reduction of 8% in DHA productivity was observed. At the aeration rate of 1.0 vvm, the reduction increased further by 16%. The rate of glycerol uptake increased by 7% when aeration rate was increased from 0.3 vvm to 0.6 vvm and an additional 3% increase was observed at 1.0 vvm.

It is evident that glycerol was being utilised by *G.xylinus* cells for the formation of biomass. The product formation curve, as seen on Figure 4.7, illustrates the extended deceleration phase when aeration rate was increased. A longer deceleration phase is due to less affinity for product formation, as a result of greater affinity for another product within in media (Stanbury, *et al.*, 2010).

The consumption of glycerol for biomass production happened as a result of alginate beads bursting. The increase in aeration rate resulted in increased oxygen availability to *G.xylinus* cells within the alginate matrix. The cells surrounding the edges of the bead had greater supply of oxygen than cells located in the centre of the matrix. As a result, the cells on the edges of the bead form biomass at an accelerated rate. This led to the rupture of the alginate beads and proliferation of biomass in the reactor.

4.3.3 Chitosan-coated alginate immobilized cells

An investigation on the effect of aeration rate on DHA production was conducted using chitosan-coated alginate immobilized *G.xylinus* cells. Cultivation conditions were identical to the conditions used in alginate investigations in Section 4.3.2.2.

In order to enhance the stability of alginate beads, fully formed alginate beads containing *G.xylinus* cells were coated with low molecular weight chitosan. The low molecular weight enhances the tensile properties by increasing fibre content. This method has also been studied in the past for various applications such as for immobilising probiotic bacteria (Rokka & Rantamäki, 2010) and for drug delivery applications (Matricardi *et al.*, 2008).

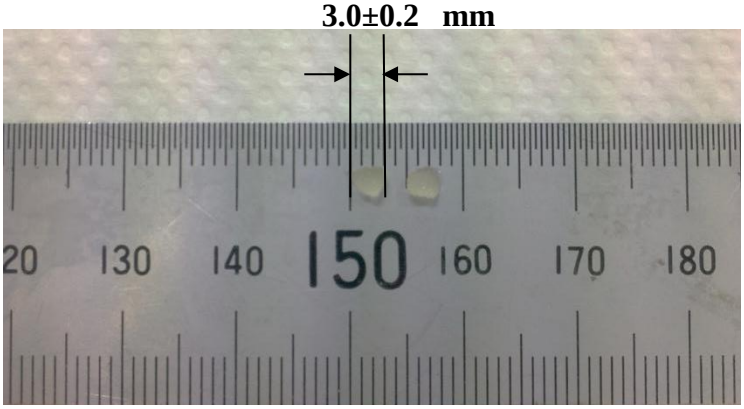


Figure 4.9 Size of chitosan-coated alginate immobilized cell.

Chitosan coating solution was prepared following the procedure outlined in Section 3.7.3. Fully formed beads of alginate containing cells were prepared following the procedure in 3.7.2. The chitosan coating was added to alginate

beads under sterile conditions. The resulting beads increased the diameter by approximately 1.0 mm, as seen in Figure 4.9. The stability was further examined in large-scale fermentations.

4.3.3.1 Effect of aeration rate on DHA production by chitosan-coated alginate immobilized *cells*

The effect of aeration rate on DHA production using chitosan-coated alginate immobilized cells was monitored over a period of 7 days (168 h). The culture was aerated at 0.3 vvm, 0.6 vvm and 1.0 vvm. The results are shown in Figure 4.10.

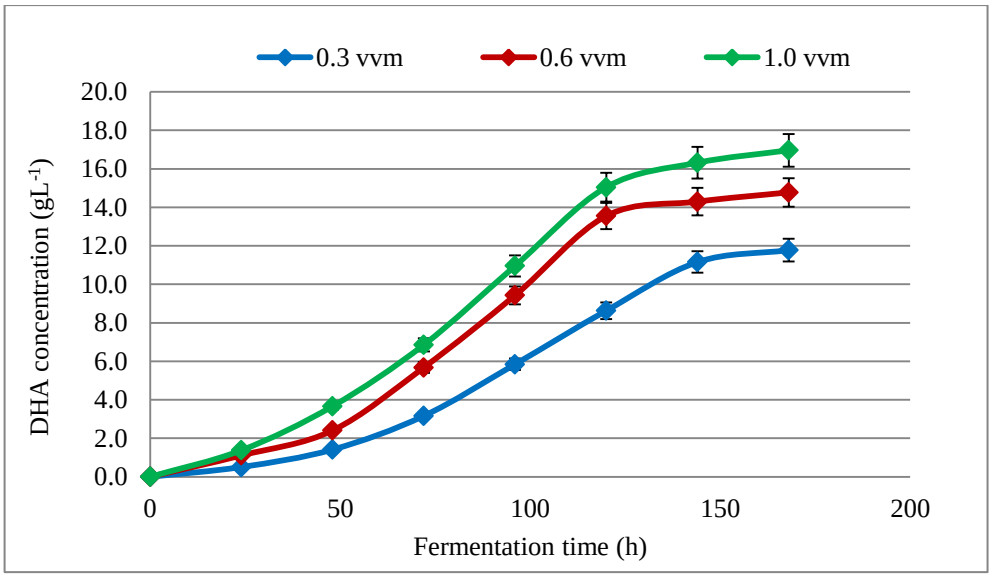


Figure 4.10 Time course of DHA production by chitosan-coated alginate immobilized *G.xylinus* cells for varying aeration rates.

The time curve of DHA production under varying aeration rates is comparable to any bacterial primary metabolite synthesis in a batch process. The final DHA concentration and productivity of the culture grown at the aeration rate of 0.3 vvm were 11.9 gL^{-1} and 0.07 $gL^{-1}h^{-1}$, respectively. A further increase in aeration rate to 0.6 vvm produced 14.8 gL^{-1} and 0.10 $gL^{-1}h^{-1}$ for the respective parameters. The culture grown at aeration rate of 1.0 vvm resulted in a final DHA concentration of 17.0 gL^{-1} and productivity of 0.11 $gL^{-1}h^{-1}$.

As previously mentioned, the conversion of glycerol to DHA is an aerobic process. Figure 4.10 shows the expected trend; that is, an increase in DHA productivity and final product concentration with increased aeration rate. This

trend was also observed in kinetic parameters of DHA production and glycerol consumption, as shown in Appendix 7. Figure 4.11 illustrates the effect of aeration rate on productivity and substrate consumption.

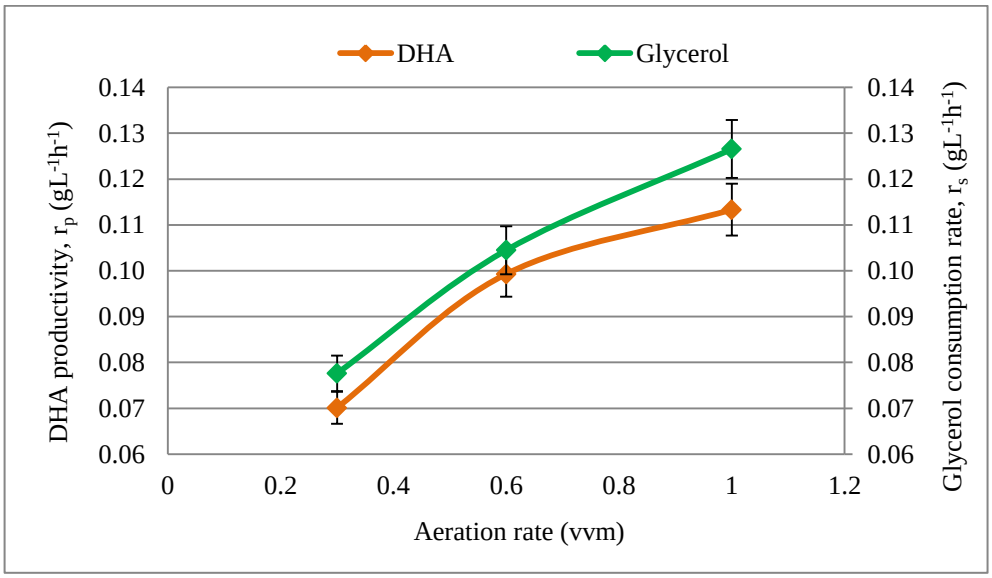


Figure 4.11 Effect of aeration rate on DHA productivity and glycerol consumption rate by chitosan-coated alginate immobilized *G.xylinus* cells.

Figure 4.11 above shows that the rate of DHA formation and glycerol consumption increased with aeration rate. The shift in aeration rate from 0.3 vvm to 0.6 vvm increased the rate of DHA formation and glycerol consumption by 31% and 20% respectively. A further shift from 0.6 vvm to 1.0 vvm increased the respective parameters by 31% and 25%. The DHA yield (gg^{-1}) was not affected by aeration rate and only decreased by 6% at 1.0 vvm from 0.3 vvm.

The chitosan-coated alginate immobilized cells maintained high DHA activity with increased aeration rate. The chitosan layer added 1.0 mm to the diameter of the alginate bead. This increased matrix stability and as well as mass transfer limitations. The effect of mass transfer limitations was evident at an aeration rate of 0.3 vvm. The significant decrease in DHA production and substrate consumption was due to low O_2, which decreased the activity of GlyDH. The low O_2 may also lead to a build-up of lactic acid from the breakdown of pyruvate within the cell (Flickinger & Perlman, 1977). Also, the increased diameter from the chitosan coating would have decreased the external diffusion

rate. This would have led to increased concentration in DHA and acidic products causing deactivation of cellular activity.

When the aeration rate was increased to 0.6 vvm and 1.0 vvm, the effect of mass transfer limitation decreased. This can be seen from the increase in DHA productivity, shown in Figure 4.11. The increase in aeration rate resulted in increased oxygen transfer and diffusion of substrate into the matrix, allowing for increased GlyDH activity (Karimi, *et al.*, 2013; Martín, *et al.*, 2008). As a result, DHA productivities and yield obtained at the end of fermentation period were high. Furthermore, the exponential phase stopped after approximately 75% of the initial glycerol had been converted to DHA. The high yields indicate that glycerol was still present in the media. This, the decreased growth may have been due to the loss in the oxidative ability of GlyDH. (Yamada, *et al.*, 1982).

4.3.4 Comparison between free cells and alginate immobilized cells

The production of DHA in shake flasks and large-scale experiments was compared between free cells and alginate immobilized cells of *G.xylinus*. The production of DHA in 100 mL of culture broth was carried out in a 250-mL shake flask. The formation rate of DHA is illustrated below in Figure 4.12

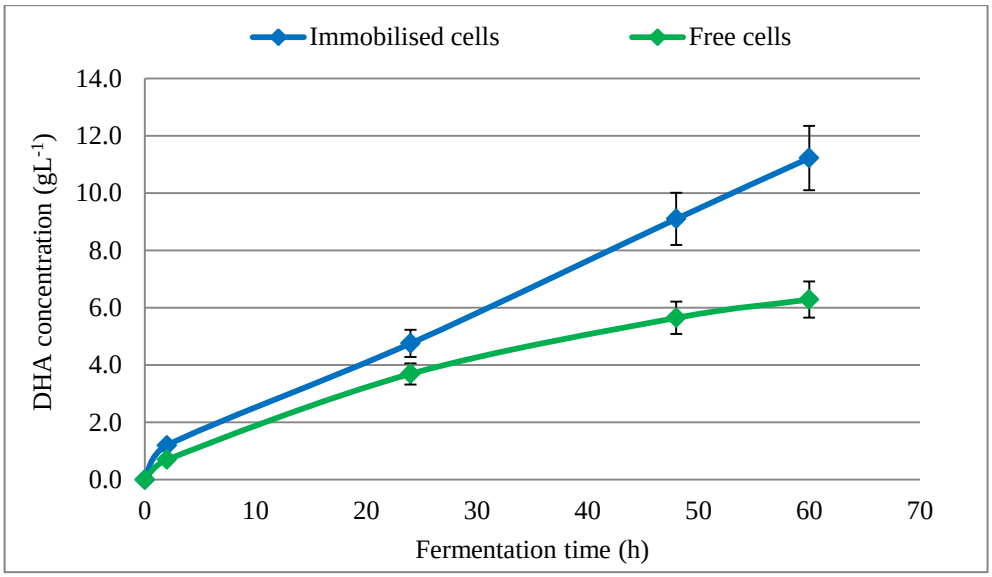


Figure 4.12 DHA production in shake flasks using free cells and alginate immobilized *G.xylinus* cells.

Figure 4.12 shows that alginate immobilized *G.xylinus* cells were more efficient at producing DHA than free cells when carried out inside shake flasks. The DHA concentration measured at the end of the fermentation period and the productivity of immobilized cells were 11.2 gL^{-1} and 0.19 $gL^{-1}h^{-1}$, respectively. Both values were 44% greater than the respective parameters achieved using free cells of *G.xylinus*.

DHA production increased in a linear pattern with immobilized cells as opposed to the lagging increase observed using freely suspended cells. The freely suspended cells use glycerol for biomass and DHA formation. As a result, the substrate limitation is immediate. The alginate beads did not show disintegration within the 60 h of incubation because of the low shear environment in shake flasks.

A similar trend in the efficiency of DHA production was also observed in large-scale investigations. A comparison between alginate immobilized *G.xylinus* cells and free cells on the production of DHA in 3 L of media is illustrated below, in Figure 4.13.

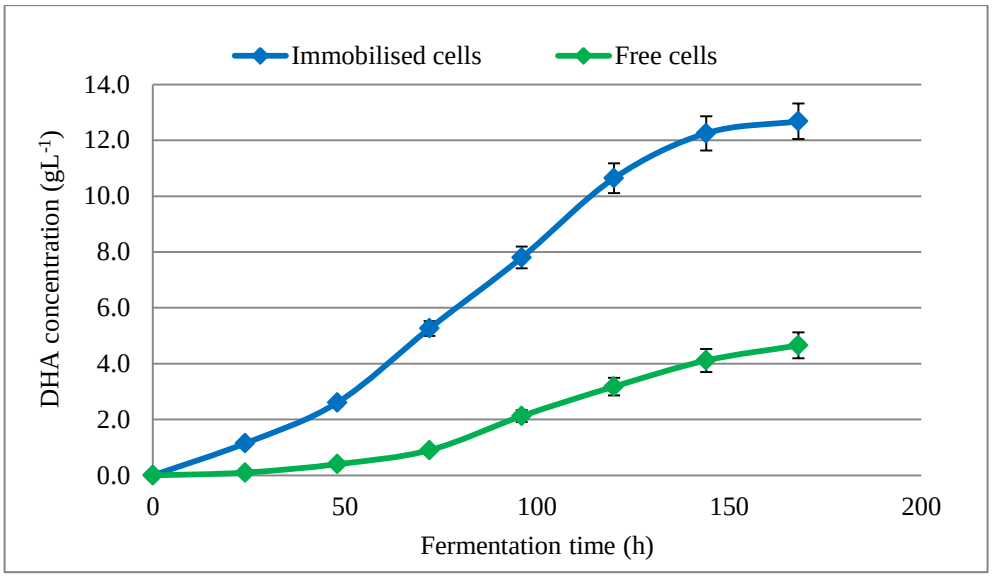


Figure 4.13 DHA production in large-scale fermenter using free cells and alginate immobilized *G.xylinus* cells.

Figure 4.13 shows that alginate immobilized cells achieved higher DHA productivity than free cells investigation. This was also the trend observed in shake flasks experiment previously discussed.

In the large-scale experiment, alginate immobilized cells achieved final DHA concentration and productivity of 12.7 gL^{-1} and 0.09 $gL^{-1}h^{-1}$, respectively. That is 61% greater than the respective parameters achieved using free *G.xylinus* cells.

Immobilized *G.xylinus* cells significantly increased DHA yield from glycerol substrate. When immobilized, the DHA yield coefficient obtained from shake flask and large-scale investigations increased by approximately 60%. One of the reasons is because *G.xylinus* cells have greater affinity for biomass formation. This is evident from the production time curve found in Figure 4.3 The biomass growth curve has a shorter deceleration phase compared to DHA product curve (Stanbury, *et al.*, 2000). By immobilising *G.xylinus* cells in the matrix, the rate of biomass By immobilising *G.xylinus* cells in the matrix, the rate of biomass formation is decreased from the physical stress imposed by the matrix (Willaert, 2007). Also, the immobilization of *G.xylinus* cells enhanced DHA production from creating a more stable environment for GlyDH. The activity of enzymes are susceptible to small changes in environmental conditions (Phillips & Poon, 2011).

4.3.5 Comparison of operational stability between alginate immobilized beads and chitosan-coated alginate immobilized beads under varying aeration rates.

The operational stability of alginate immobilized beads and chitosan-coated alginate immobilized beads under varying aeration rates were compared. The operational stability of the beads is directly correlated to the instantaneous yield of DHA. The instantaneous yield, % is calculated following equation (3-7).

4.3.5.1 Stability of beads at 0.3 vvm

The effect of 0.3 vvm aeration rate on stability of alginate beads and chitosan-coated alginate beads is shown in Figure 4.14.

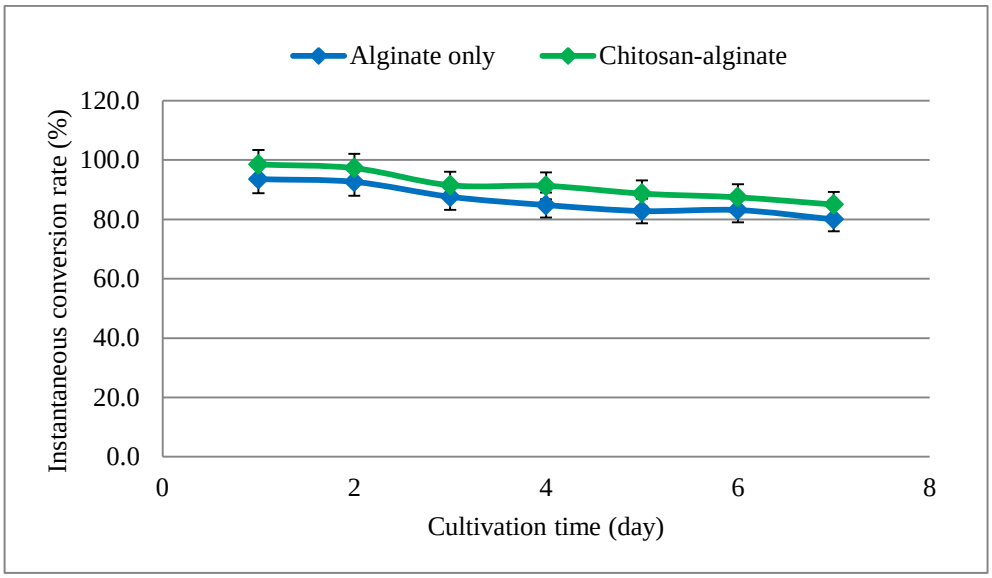


Figure 4.14 Stability of immobilized beads at aeration rate of 0.3 vvm expressed as the instantaneous conversion rate (%) as a function of time

Figure 4.14 above shows that the bead stability of both immobilization carriers did not differ significantly. On the final day of the cultivation period, the conversion rates remained greater than 80%. The instantaneous conversion rates remained relatively stable (compared to increased aeration rated). However, the alginate beads showed greater disintegration, thus there should have been increased biomass growth observed. This was not the case, which may have been due to cell death. The disintegration of beads released alginate that has antibacterial properties (Pielesz, *et al.*, 2011)

4.3.5.2 Stability of beads at 0.6 vvm

The effect of 0.6 vvm aeration rate on the stability of alginate beads and chitosan-coated alginate beads is shown in Figure 4.15

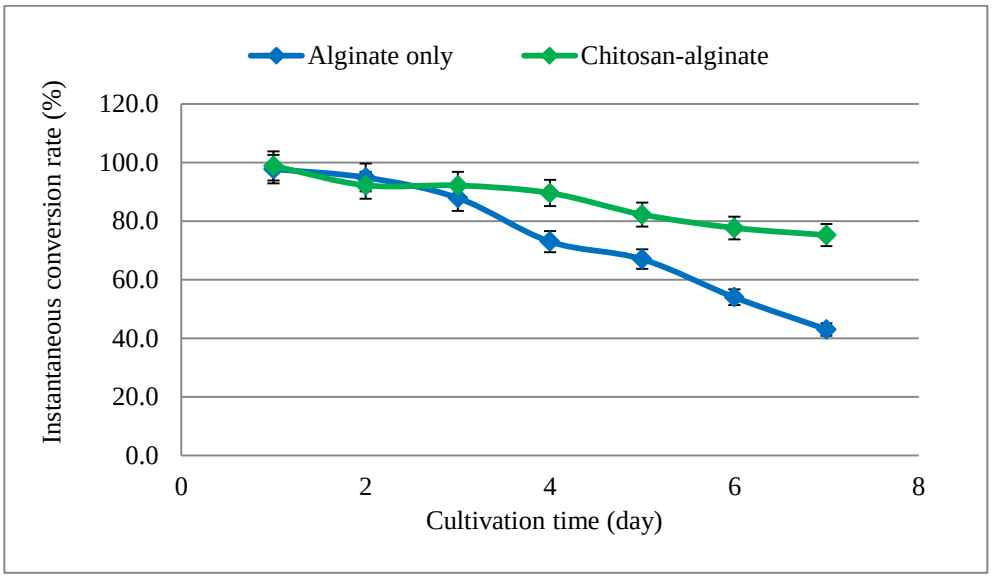


Figure 4.15 Stability of immobilized beads at aeration rate of 0.6 vvm expressed as the instantaneous conversion rate (%) as a function of time

Figure 4.14 shows that; during the course of cultivation, stability of alginate immobilized beads decreased significantly compared with chitosan-coated alginate beads. On day four of cultivation period - using alginate beads, the conversion rate had reached 73%. Referring to Figure 4.7, day four marked the end of the exponential phase. The operational stability of alginate beads continually decreased, and on day seven the alginate immobilized beads had a conversion rate of 43%. In contrast, the chitosan-coated alginate beads had a relatively stable decline in conversion rate and on the final day of cultivation, the operational stability was measured at 75%.

4.3.5.3 Stability of beads at 1.0 vvm

The effect of 1.0 vvm aeration rate on the stability of alginate beads and chitosan-coated alginate beads is shown in Figure 4.16 below.

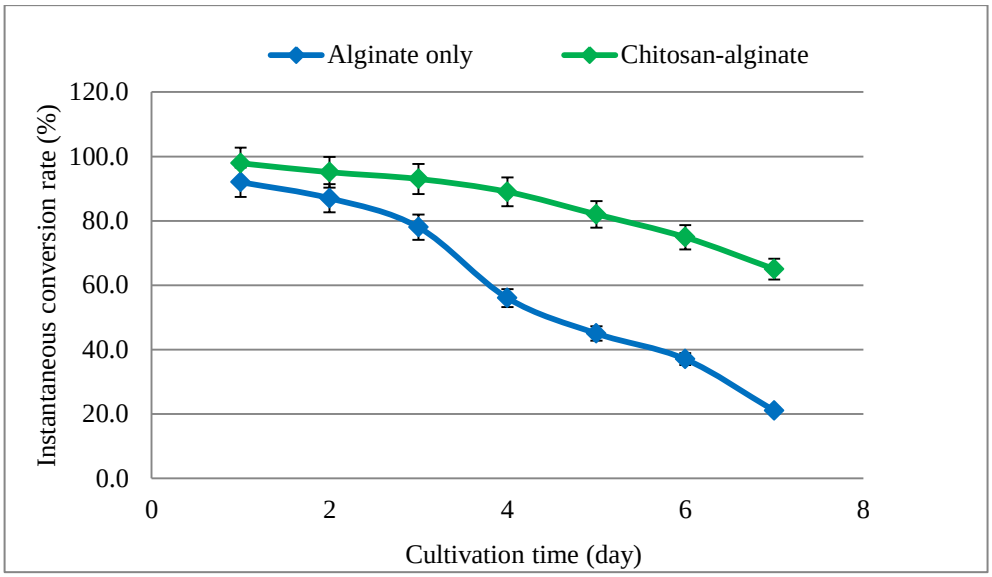


Figure 4.16 Stability of immobilized beads at aeration rate of 1.0 vvm expressed as the instantaneous conversion rate (%) as a function of time.

When the aeration rate was set at 1.0 vvm, the stability of alginate immobilized beads decreased significantly compared with the chitosan-coated alginate beads. The trend is similar to that shown in previous graph; at aeration rate of 0.6 vvm. However, the effect of 1.0 vvm on bead stability showed significant differences on day two and throughout the cultivation period. On day three of the cultivation, the conversion rate of alginate beads reached 78%. Referring to Figure 4.7, day three marked the end of the exponential phase. The operational stability of alginate beads continually decreased, and on day seven, the conversion rate had decreased to 21%. The chitosan-coated alginate beads had greater stability throughout the cultivation period. The conversion rate dropped below 80% on day six, which was also the approximate time DHA production entered the stationary phase, as shown in Figure 4.10. On the final day of cultivation, the operational stability of chitosan-coated alginate beads was measured at 70%.

The disintegration of the beads also led to an increase in the visual turbidity of the culture media. The correlation between the conversion rate and turbidity observed at aeration rate of 1.0 vvm is shown in Figure 4.17 and Figure 4.18.

Visual turbidity	CR%	Day	Description
	**92**	**1**	**No turbidity.**
	**87**	**2**	**Light turbidity.**
	**56**	**4**	**Moderate turbidity.** (half of the alginate beads from day 1 have disintegrated).
	**37**	**6**	**Heavy turbidity.** Complete disintegration of alginate immobilized beads

Figure 4.17 Turbidity change observed during cultivation using alginate encapsulated cells at 1.0 vvm. CR%= conversion rate.

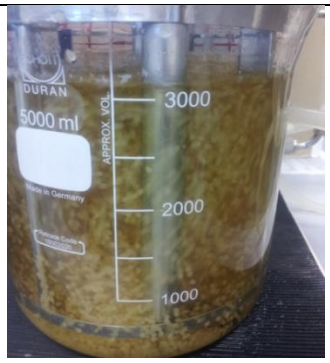
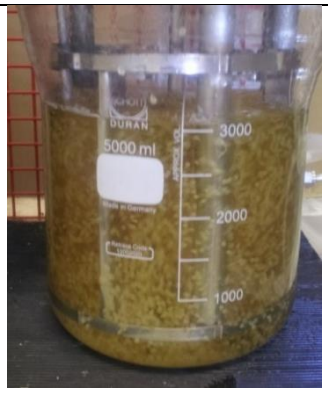
Visual turbidity	CR%	Day	Description
	**98**	**1**	**No turbidity.**
	**93**	**3**	**No turbidity.**
	**89**	**4**	**Light turbidity.**
	**65**	**7**	**Moderate turbidity.** Nearly 50% of beads disintegrated

Figure 4.18 Turbidity changes observed during cultivation period of DHA using chitosan-coated alginate encapsulated cells at 1.0 vvm; CR%= conversion rate.

A comparison between Figure 4.17 and Figure 4.18 illustrates that chitosan-coated alginate beads disintegrated slower than the alginate beads. On day two of cultivation, the media containing alginate immobilized cells showed light turbidity. On day three of cultivation, the media containing chitosan-alginate beads had no turbidity. The difference in turbidity is more pronounced at day five; alginate beads show 'half-life' and chitosan-alginate beads show light turbidity.

The disintegration of the alginate matrix increases under high aeration rates. This may be due to increased shear on the surface of the beads, as well as increased biomass growth. When cells are immobilized in an alginate bead, they are scattered within the matrix at random locations. When aeration rate is high, the *G.xylinus* cells located on the edges of the alginate bead increase DHA production as well as biomass formation. The biomass cells are larger than the porous matrix so remain entrapped. This eventually leads to a cell density high enough to rupture the alginate matrix. When this happens, biomass released to the media consumes available components for biomass formation and maintenance. DHA production is still carried out but at a slower rate.

This investigation has found that coating alginate beads with chitosan increased stability. The chitosan coating increased the tensile strength of alginate from increased fibrous connections at interface of the alginate bead. This serves to reinforce the matrix (Anal & Stevens, 2005; Knill *et al.*, 2004). This 'double-coating' does lead to increased mass transfer limitation. However, this investigation has found that increased aeration rates can overcome these limitations and maintain stability.

5 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The *G.xylinus* cells have a greater tendency for biomass formation than DHA production. This has been proven with the free cell experiments conducted in this investigation. Accordingly, high production of DHA from glycerol can be carried out by immobilization. Immobilization increased DHA production by imposing physical stress on the *G.xylinus* thereby reducing biomass formation. In addition, it creates a stable environment for GlyDH to carry out catalytic conversions of DHA. Nevertheless, immobilization has some disadvantages; namely, an increase in mass transfer limitations.

Alginate immobilized *G.xylinus* cells can produce DHA from the following initial glycerol concentrations (w/v); 1%, 2%, 4% and 7%. The highest DHA productivity was achieved using the glycerol concentration of 2% (w/v). The initial glycerol concentrations of 4% (w/v) and 7% (w/v) resulted in lower DHA production. Owing to its rheological properties, higher glycerol concentration resulted in an increase in media viscosity. According to the laws of fluid dynamics, the effects of viscosity are significant at the boundary layer of the immobilized carrier (Kundu, *et al.*, 2012). This also leads to an increase in the diffusion barrier for nutrient and product transfer. This would have led to an accumulation of DHA within the immobilized gel; thereby causing GlyDH to deactivate (Bauer, *et al.*, 2005). It is also possible that the increase in initial glycerol led to substrate inhibition - a common problem for DHA production using *G.oxdyans (Claret, et al., 1992a; Hekmat, et al., 2003a; Li, et al., 2010b; Lu, et al., 2012a).* However, no studies have measured the influence of the initial glycerol concentrations on the kinetics of *G.xylinus* cell in DHA production.

The aeration rates were varied at 0.3 vvm, 0.6 vvm and 1.0 vvm inside a large-scale reactor, This was used to determine the optimal aeration rate for DHA production. This process used the alginate immobilized cells and the chitosan-coated alginate immobilized *G.xylinus* cells. It was discovered that 0.3 vvm provides the optimal aeration rate for alginate immobilized cells. This aeration

rate had the highest DHA production at 0.085 $gL^{-1}h^{-1}$ and a yield of 0.94. The r_{pmax} at 0.3 vvm was 13% lower than the r_{pmax} achieved at the aeration rate of 0.6 vvm. However, when aeration rate was at 0.6 vvm, there was a 17% drop in DHA yield. An investigation using chitosan-coated alginate beads found that the r_p and r_{pmax} increased at higher aeration rates. The optimal aeration rate was found at 1.0 vvm. This aeration rate achieved the highest r_p and r_{pmax} values. At the end of the fermentation period, the DHA yield was measured at 0.88.

The choice of an immobilized carrier is a crucial factor in the performance of *G.xylinus* cells. Alginate structures are porous and allow good mass transfer. However, they are prone to shear damage (Wei, *et al.*, 2007). Chitosan is also porous like alginate and prone to shear damage. However, as a composite, chitosan acts as reinforcement for the alginate matrix. This method of reinforcement is used in the pharmaceutics industry to increase stability of beads for drug delivery (Anal & Stevens, 2005; Pasparakis & Bouropoulos, 2006).

This investigation found the stability of the alginate beads were significantly reduced with high aeration rates. This may be due to increased shear on the surface of the beads, as well as increased biomass growth. When cells are immobilized in an alginate bead, they are scattered within the matrix at random locations. Under high aeration rates, the *G.xylinus* cells located on the surface of the alginate bead increase DHA production as well as biomass formation. The biomass cells are larger than the diameter of the pores. As a result, they remain entrapped. This eventually leads to a cell density high enough to rupture the alginate matrix. When this happens, biomass released to the media consumes available components for biomass formation and maintenance. DHA production is still carried out but at a slower rate.

This investigation has found that the stability of the alginate beads was significantly increased when it was coated with chitosan. The chitosan coating increased cross linking at the surface of the alginate bead. This resulted in increased tensile strength of the alginate bead (Anal & Stevens, 2005; Knill, *et al.*, 2004). The ‘double-coating’ of polymer leads to increased mass transfer

limitation. However, this investigation has also found that increased aeration rate can overcome these limitations and maintain stability.

Overall, this research has shown that *G.xylinus* can produce high yields of DHA for a longer period of time if it remains immobilized. Increased oxygen can decrease mass transfer limitations encountered in calcium-coated alginate beads.

5.2 Recommendations

It is possible to increase the DHA production by processing high concentrations of glycerol. However, the increase in initial glycerol concentration in the media leads to increased viscosity. This increases mass transfer limitation as discussed above. One way to reduce the effect of viscosity is to carry out DHA production in a fed-batch modeThis strategy feeds a high concentration of media into the reactor at specific time intervals, which reduces the effect of high substrate concentrations on growth. This method can minimize the effect of viscosity on mass transfer limitations.

The research has found that as long as *G.xylinus* remains within the immobilized carrier, it can carry out DHA conversions more efficiently. The chitosan-coated alginate beads used in this experiment showed greater stability than alginate immobilized beads. However, mass transfer was a problem. The next step in this research may look at methods to increase oxygen transfer to the immobilized cells. This may be through introducing oxygen vectors such as hydrogen peroxide, or co-immobilizing *G.xylinus* with an O_2 producing bacteria.

Likewise, cell immobilization in chitosan-coated alginate is a fairly new concept. Further research is needed to improve the mechanical properties of the immobilization matrix. Work can look at alginate with other biodegradable polymers as well as optimising the thickness of coating layer for better bead stability.

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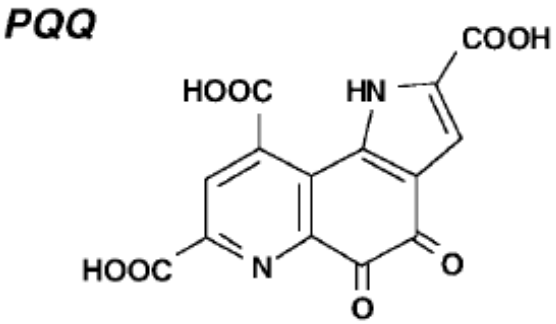
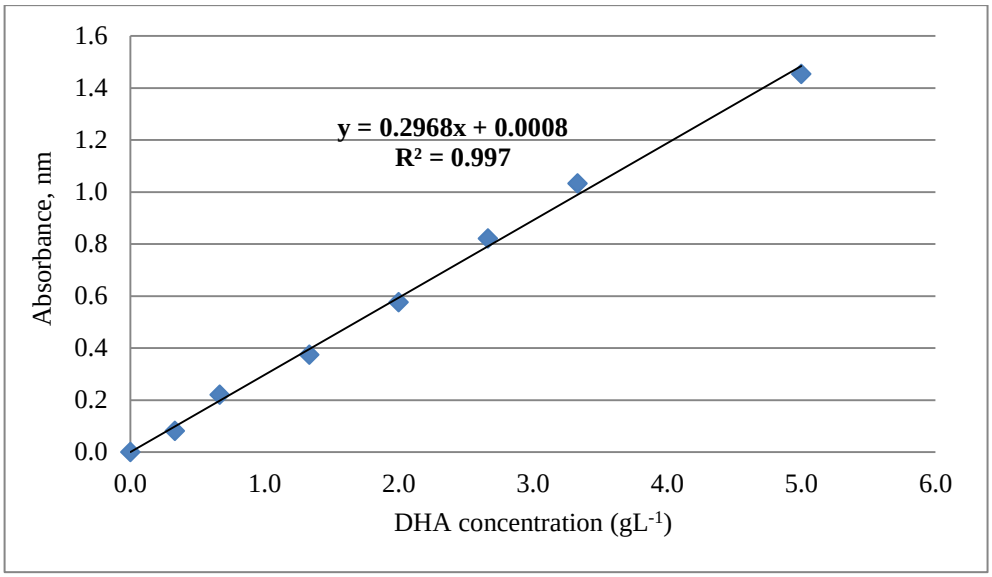
APPENDIX

Appendix 1 Global production of glycerol from various production processes between 1992-2010 (Ayoub & Abdullah, 2012).

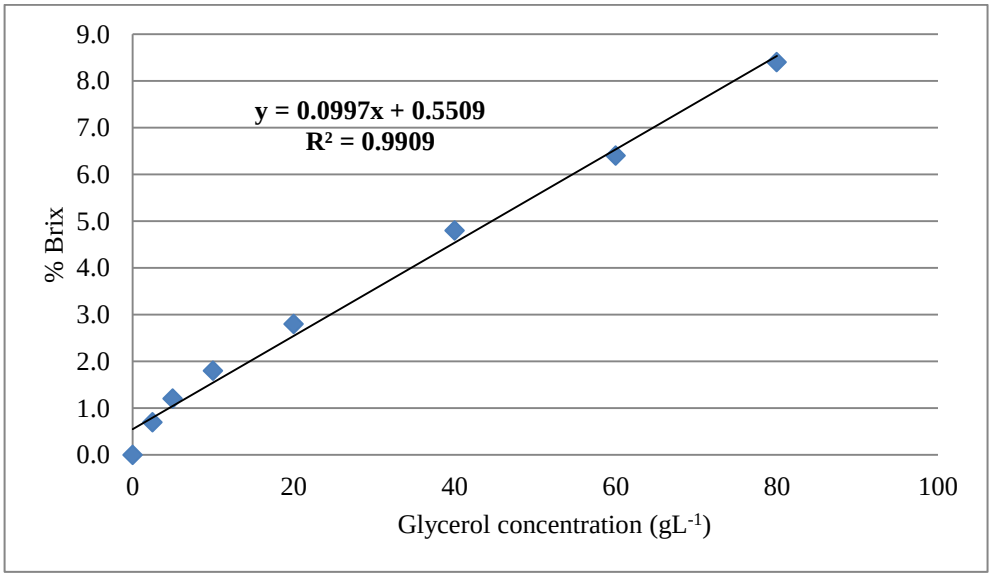
World glycerol production (x10^3 metric tons per annum)					
Year	**Soaps**	**Biodiesel**	**Fatty alcohol**	**Synthetic**	**Total Production**
1992	208	0	83	83	**374**
1995	208	42	104	83	**437**
1999	198	42	125	63	**428**
2003	188	167	104	63	**522**
2005	167	375	125	21	**688**
2006	146	521	167	0	**834**
2008	125	1125	250	0	**1500**
2010	83	1583	250	0	**1916**



Appendix 2 Chemical structure of amino acid-derived Pyrroloquinoline quinone (PQQ) cofactor. The cofactor is embedded in GlyDH enzyme and responsible to inducing DHA conversion from glycerol (Matsushita, et al., 2002).



Appendix 3 DHA calibration graph carried out with spectrophotometer at 550 optical density, OD_{550}.



Appendix 4 Glycerol calibration graph using a refractometer. Refractive index is measured in units of %Brix.

Appendix 5 Kinetics parameters of DHA production under varying initial glycerol concentration %(w/v) using alginate immobilized cells of *G.xylinus*.

		Glycerol concentration %(w/v)			
Parameters	**Units**	**1%**	**2%**	**4%**	**7%**
t	h	120	144	168	216
r_p	$gL^{-1}h^{-1}$	0.07	0.09	0.08	0.06
r_{pmax}	$gL^{-1}h^{-1}$	0.11	0.11	0.10	0.09
q_p	h^{-1}	0.01	0.01	0.01	0.01
r_s	$gL^{-1}h^{-1}$	0.08	0.09	0.08	0.07
q_s	h^{-1}	0.01	0.01	0.01	0.01
$Y_{p/s}$	gg^{-1}	0.96	0.94	0.89	0.81

Appendix 6 Kinetic parameters of DHA production under varying aeration rates (vvm) using alginate immobilized *G.xylinus* cells.

		Aeration rate (vvm)		
Parameters	**Unit**	**0.3**	**0.6**	**1.0**
t	h	168	168	168
r_p	$gL^{-1}h^{-1}$	0.09	0.08	0.07
r_{pmax}	$gL^{-1}h^{-1}$	0.11	0.12	0.09
q_p	h^{-1}	0.01	0.01	0.02
r_s	$gL^{-1}h^{-1}$	0.08	0.09	0.09
q_s	h^{-1}	0.01	0.01	0.03
$Y_{p/s}$	gg^{-1}	0.94	0.78	0.71

Appendix 7 Kinetic parameters of DHA production under varying aeration rates (vvm) using chitosan-coated alginate immobilized *G.xylinus* cells.

		Aeration rate (vvm)		
Parameters	**Unit**	**0.3**	**0.6**	**1.0**
t	h	168	168	168
r_p	$gL^{-1}h^{-1}$	0.07	0.10	0.11
r_{pmax}	gL-1h-1	0.10	0.15	0.17
q_p	h^{-1}	0.01	0.01	0.01
r_s	$gL^{-1}h^{-1}$	0.08	0.10	0.13
q_s	h^{-1}	0.01	0.01	0.01
$Y_{p/s}$	gg^{-1}	0.90	0.89	0.88

Appendix 8 Kinetic parameters of DHA production carried out in shake flasks of 100 mL culture using free cells and alginate immobilized cells of *G.xylinus*.

		Shake flasks	
Parameter	**Unit**	**Free**	**Immobilized**
t	h	60	60
r_p	$gL^{-1}h^{-1}$	0.10	0.19
r_{pmax}	$gL^{-1}h^{-1}$	0.14	0.17
q_p	h^{-1}	0.15	0.19
r_s	$gL^{-1}h^{-1}$	0.27	0.18
q_s	h^{-1}	0.42	0.18
$Y_{p/s}$	gg^{-1}	0.36	0.94

Appendix 9 Kinetic parameters of DHA production carried out in large-scale fermentation using free cells and alginate immobilized cells of *G.xylinus*.

		***G.xylinus* cells**	
Parameter	**Unit**	**Free**	**Immobilized**
t	h	168	168
r_p	$gL^{-1}h^{-1}$	0.03	0.09
r_{pmax}	$gL^{-1}h^{-1}$	0.04	0.11
q_p	h^{-1}	0.00	0.01
r_s	$gL^{-1}h^{-1}$	0.07	0.09
q_s	h^{-1}	0.01	0.01
$Y_{p/s}$	gg^{-1}	0.38	0.94