Bioconversion of Glycerol to Dihydroxyacetone by Immobilized *Gluconacetobacter Xylinus* Cells

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***Abstract*—In this study, *Gluconacetobacter xylinus* cells were immobilized in calcium alginate and chitosan-coated alginate beads. The immobilized cells were used in the conversion of glycerol to dihydroxyacetone (DHA) in a stirred-tank reactor. Fermentations using free cells and 2% (w/v) initial glycerol yielded 6.3 gL^{-1} DHA after 60 h. This corresponded to a productivity of 0.11 $gL^{-1}h^{-1}$. Using 2% (w/v) initial glycerol and 0.3 vvm air flow, *G.xylinus* cells immobilized in alginate beads gave a DHA concentration of 12.7 gL^{-1} and a productivity of 0.09 $gL^{-1}h^{-1}$. The final DHA concentration and productivity of *G.xylinus* cells immobilized in chitosan-coated alginate beads were 11.9 gL^{-1} and 0.07 $gL^{-1}h^{-1}$, respectively, at 0.3 vvm air flow. Final DHA concentration and productivity further increased to 17.0 gL^{-1} and 0.11 $gL^{-1}h^{-1}$ at 1.0 vvm airflow. Chitosan coating provided greater stability to the alginate beads with increased aeration rate.**

***Index Terms*—Dihydroxyacetone *gluconacetobacter xylinus* glycerol immobilization**.

I. Introduction

Dihydroxyacetone (DHA) is a value-added chemical commonly used in cosmetics as an artificial browning agent [1]. It also serves as a building block for several fine chemicals such as 1, 2-propylene glycerol and methotrexate [2]. It is produced by glycerol oxidation. In recent years, there has been an influx of glycerol in the market as a result of a booming biodiesel industry. The biodiesel industry produces crude glycerol as a by-product at a level of approximately 10% (w/w) of biodiesel manufactured by transesterification of oils with methanol [3]. In 2005, global biodiesel production was estimated at 3.8 million tonnes and by 2020, it is expected to reach over 8 billion tonnes [4]. That is, 800 million tonnes of glycerol will be generated. In its raw form, glycerol contains several impurities that make its disposal costly and difficult [5]. As a result, the price of glycerol is forecasted to fall in the coming years, making it an ideal raw material for industrial processes [6].

Currently, DHA is industrially produced via microbial conversion of glycerol via *Gluconobacter oxydans* [7]. Although the microbial oxidation process can provide high selectivity to DHA compared to chemical oxidation, it has some drawbacks such as low productivity and high production cost [8].

Manuscript received July 8, 2013; revised September 12, 2013. This work was carried out at the University of Waikato, Hamilton, NZ. Paper titles should be written in uppercase and lowercase letters, not all uppercase.

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G.oxydans has been studied extensively for the conversion of glycerol to DHA [9]-[13]. Oxidation of glycerol to DHA by *G.oxydans* is inhibited by high concentrations of both DHA and glycerol [14], [15]. Besides, the microbial oxidation of glycerol to DHA has a high oxygen requirement [15], [16]. Several studies have been carried out in the past to address these problems and to increase DHA yields. These include immobilization of *G.oxydans* cells in a carrier [17], [18], adding oxygen vectors to enhance oxygen availability [19], and genetic modification of the species [20]-[22].

The present study concerns the use of immobilized *Gluconacetobacter xylinus* (previously known as *Acetobacter xylinum*) cells for the conversion of glycerol to DHA. There has been two reports on the use of immobilized *G. xylinus* for the conversion of glycerol to DHA. In one study, the use of *G.xylinus* cells immobilized in polyvinyl alcohol (PVA) showed higher yields and greater pH tolerance [23]. Another study immobilized whole cells or cell preparations of *G. xylinus* on calcium alginate which also resulted in high DHA yield [24]. The higher DHA yields obtained in these investigations motivated this study to evaluate the ability of immobilized *G.xylinus cells* in converting glycerol to DHA in stirred tank reactors.

II. Materials and Methods

A. Microorganism and Maintenance

Gluconacetobacter xylinus DSM 46604 was obtained from Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures. Cells were maintained on agar slants of the following composition (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 ℃. Cultures were periodically transferred to freshly prepared agar slants to maintain high activity.

B. Cultivation

1) Shake flask experiments

Shake flask experiments were carried out using 100 mL medium in 250-mL Erlenmeyer flasks. The medium contained (w/v) 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 before autoclaving. The flasks were inoculated with the microorganism and incubated at 30 ^oC for 60 h at 150 rpm. Fifteen millilitre aliquots were taken at 24 h intervals for analysis.

For developing seed medium for large-scale experiments, glass beads (approximately 5.0 mm diameter) were added to

shake flasks, which suppressed the formation of bacterial cellulose [24].

2) Large-scale experiments

Batch fermentations were carried out using a working volume of 3 L in a 5-L fermenter (Inceltech LH Series 210) equipped with two flat blade turbine agitators. The media used was identical to that used for the shake flask experiments. The agitation was controlled at 150 rpm for free cell production for immobilization and at 100 rpm for immobilized cells to decrease the shear stress on the beads containing cells. The pH was controlled at 6.0 with the addition of 6M NaOH.

For immobilization, *G.xylinus* cells were harvested from the broth via centrifuging at 4000 g for 20 min. The cells were washed with sterile distilled water to remove residual medium components prior to immobilization.

For fermentations using free cells, the fermenter was inoculated with 10% (v/v) seed medium. For fermentations using immobilized cells, 10% (w/v) immobilized beads were aseptically added to the fermenter. The effect of glycerol concentration, effect of immobilization material, and effect of aeration rate on DHA production were investigated.

C. Immobilization

G.xylinus cells were immobilized in calcium alginate beads, as well as in chitosan-coated alginate beads. The whole procedure of immobilization was carried out under sterile conditions.

1) Calcium alginate

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 0.2 M calcium chloride ($CaCl_2$) was prepared in a 2-L glass bottle. A total of 300 mL alginate-cell solution with a cell concentration of ~17 gL^{-1} formed beads of approximately 2.0 ±0.2 mm in diameter.

2) 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.

D. Analytical Methods

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. Since the addition of glass beads in shake flasks suppressed the formation of cellulose [22], the residue left upon centrifugation is assumed to be entirely biomass. The tubes were dried at 80 ℃ until constant weight was achieved and the cell concentration was expressed as gram dry cell weight per litre (g DCW L^{-1}). The supernatant was used for the determination of glycerol and DHA concentrations.

2) Dihydroxyacetone

DHA was assayed by the colorimetric method [24]. 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 was measured in a spectrophotometer at 550 nm. The DHA concentration was determined using a calibration curve.

3) Glycerol

Glycerol concentration was measured using the refractive index method [24]. 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 glycerol concentration was determined using a calibration curve.

III. Results and Discussion

A. Shake Flask Fermentations Using Free Cells

Shake flask fermentations were carried out as previously described and the results are shown in Fig. 1. The culture did not exhibit any lag phase and a biomass concentration of 7. 3gL^{-1} was obtained after 60 h. The DHA concentration and productivity obtained were 6.3 gL^{-1} and 0.11 $gL^{-1}h^{-1}$, respectively.

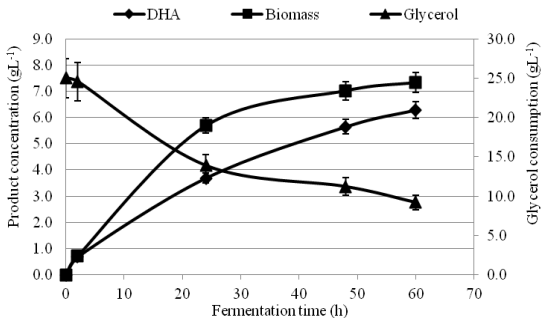

Fig. 1. Time course of biomass formation, glycerol utilisation and DHA production by free *G.xylinus* cells in shake flasks.

B. Fermentations Using Calcium Alginate Immobilized Cells

1) Varying glycerol concentration

Fig. 2 illustrates the DHA production under varying initial glycerol concentrations. The fermentation time 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. The DHA production was increased when the initial glycerol concentration was increased from 1% (w/v) and 2% (w/v). At 1% (w/v), 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.

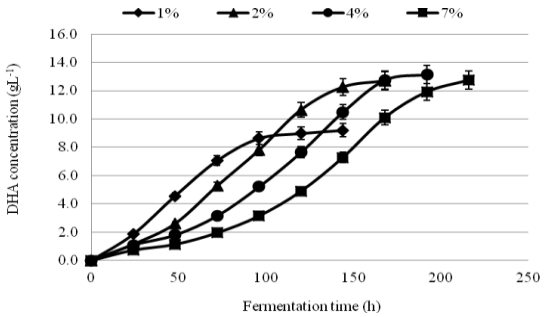

Fig. 2. Effect of varying glycerol concentrations (% w/v) on DHA production by alginate immobilized *G.xylinus* cells.

The DHA production did not increase further, by any significant means, for initial glycerol of 4% (w/v) and 7% (w/v). At 4% (w/v) glycerol, 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. 7gL^{-1} was lower than that of 4% (w/v) glycerol and DHA productivity was reduced by 22% to 0.06 $gL^{-1}h^{-1}$.

When the initial glycerol concentration in the medium increased, its rheological properties, mainly viscosity of the media, increases in a linear manner [25]. The reduced activity may be due to slower diffusion of substrate into the alginate bead. As a result, there was an increase in the fermentation 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).

2) Effect of aeration rate

The effect of aeration rate on DHA production by alginate immobilized cells was monitored over a period of 7 days for 0.3 vvm, 0.6 vvm and 1.0 vvm aeration rates and the results are shown in Fig. 3.

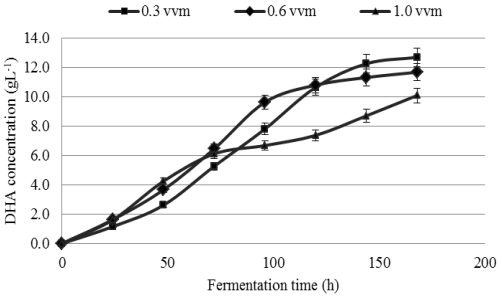

Fig. 3. Time course of DHA production by alginate immobilized *G.xylinus* cells under varying aeration rates.

Fig. 3 shows that for alginate immobilized cells, there was no significant difference in DHA production until about 75 h for all the three aeration rates investigated. However, after 75 h the DHA production rate decreased for 0.6 vvm and 1.0 vvm aeration rates. The final DHA concentration and productivity of the alginate immobilized cells grown at 0.3 vvm aeration rate were 12.7gL^{-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 11.1gL^{-1} and 0.07$gL^{-1}h^{-1}$, respectively.

G.xylinus cells are obligate aerobes and the quantity of DHA formed is directly related to the amount of oxygen available [26]. Under oxygen limited growth acid products continues to accumulate leading to cell deactivation [26]. The conversion of glycerol to DHA requires oxygen as the final electron acceptor in the metabolic process [22], [24]. The alginate matrix surrounding the *G.xylinus* cells increases mass transfer resistance and decreases oxygen availability to the cells. Therefore, increased aeration rates were expected to increase the dissolved oxygen available in the liquid phase and increase DHA productivity. However, this was not observed in the investigation. Rather, the productivity of DHA decreased.

The effects of aeration rate on volumetric DHA productivity (r_p) and glycerol consumption rate (r_s) are illustrated in Fig 4.

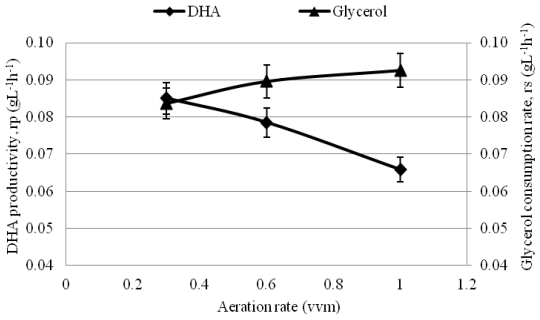

Fig. 4. Effect of aeration rates on DHA productivity and glycerol consumption rates by alginate immobilized *G.xylinus* cells.

Fig. 4 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 from Fig. 4 that glycerol was increasingly being utilised by *G.xylinus* cells for the formation of biomass at higher aeration rates. The product formation curve, as seen on Fig. 3, illustrates the extended deceleration phase when aeration rate was increased. This may be due to the build-up of DHA within the beads, which inhibited the cell growth and further product formation [14].

It was also noticed that in the case of alginate immobilized cells, the beads disintegrated slowly as the fermentation progressed. The increase in aeration rate resulted in increased oxygen availability to the cells within the alginate beads. 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 at the expense of higher glycerol consumption.

C. Fermentations Using Chitosan-Coated Alginate Immobilized Cells

In an attempt to enhance the stability, the alginate beads containing *G.xylinus* cells were coated with chitosan as described in the Materials and Methods section. The immobilized cells were then used in the oxidation of glycerol to DHA in the stirred tank reactor.

Effect of aeration rate

The effect of aeration rate on DHA production using chitosan-coated alginate immobilized cells was monitored over a period of 7 days. The culture was aerated at 0.3 vvm, 0.6 vvm and 1.0 vvm. The results are shown in Fig. 5.

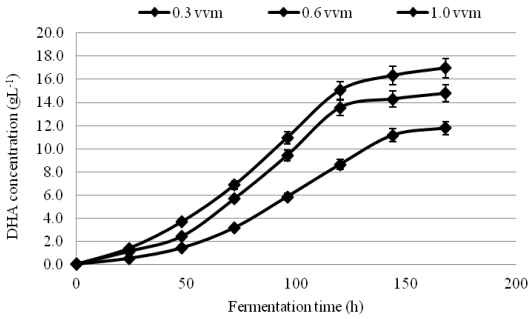

Fig. 5. Time course of DHA production by *G.xylinus* cells immobilized in chitosan-coated alginate beads under varying aeration rates.

The time course 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. Fig. 5 shows the expected trend; that is, an increase in DHA productivity and final product concentration with increased aeration rate.

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. Although this increased the stability of the beads, it added another resistance to mass transfer. The effect of mass transfer limitation 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 glycerol dehydrogenase (GlyDH) involved in the oxidation of glycerol in *G. xylinus*. The low O_2 may also lead to a build-up of acids from the breakdown of pyruvate within the cell [26]. In addition, the increased diameter from the chitosan coating would have decreased the resistance to diffusion of substrate and products. This would have led to increased concentration of DHA and acidic products causing deactivation of cellular activity.

As previously mentioned, the conversion of glycerol to DHA is an aerobic process. Fig. 5 shows the expected trend; that is, an increase in DHA productivity and final product concentration with increased aeration rate. Fig. 6 illustrates the effect of aeration rate on productivity and substrate consumption.

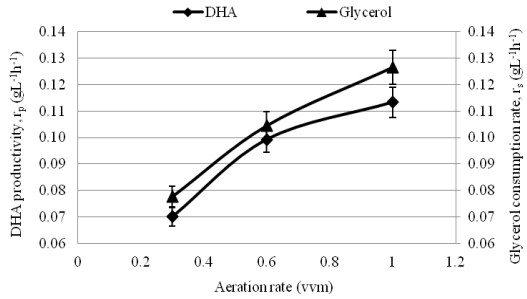

Fig. 6. Effect of aeration rate on DHA productivity and glycerol consumption rate by chitosan-coated alginate immobilized *G.xylinus* cells.

Fig. 6 shows 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 [15]. 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 Fig. 6. The increase in aeration rate resulted in increased oxygen transfer and diffusion of substrate into the matrix, allowing for increased GlyDH activity. 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. Thus, the decreased growth may have been due to the loss in the oxidative ability of GlyDH. [27].

IV. Conclusion

Alginate immobilized *G.xylinus* cells can produce DHA in stirred tank reactors. The highest DHA productivity was achieved using 2% (w/v) glycerol. The initial glycerol concentrations of 4% (w/v) and 7% (w/v) resulted in lower DHA production. Further studies have to be directed towards the influence of initial glycerol concentrations on the kinetics

of *G.xylinus* cell in DHA production.

G.xylinus cells were immobilized in alginate and chitosan-coated alginate beads under varying aeration rates: 0.3 vvm, 0.6 vvm and 1.0 vvm. It was found 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. At the end of the fermentation period, the DHA yield was measured at 0.88. Chitosan-coated alginate beads were found to be more stable than alginate beads under the reaction conditions.

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