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γ – Alumina Doped Alginate Gel for Cell Immobilization in Fermentation Processes

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γ-Alumina (γ-Al_2O_3) doped alginate gel (AEC) was developed as a cell carrier in fermentation processes of *Saccharomyces cerevisiae* M.30 for ethanol production and *Clostridium butyricum* DSM 5431 for 1,3-propanediol production. In a single batch system of ethanol fermentation, the final ethanol concentration of suspended cell (SC), immobilized cell on γ-Al_2O_3 (AC) and AEC cultures were 82.4, 77.1 and 74.6 g/l, respectively. In 4-cycle repeated batch fermentation, the AEC culture demonstrated a good potential of reusability. Its ethanol production and conversion yield of the 1st, 2nd and 3rd repeated batch were comparable to those of the SC and AC cultures with the immobilization yield of 86%. AEC was also found to be effective for the cell immobilization of *C. butyricum* with the immobilization yield of 83%. However, the strong inhibition effect of cell-γ-Al_2O_3 immobilization towards 1,3-propanediol production was observed. Moreover, 1, 3-propanediol fermentation stability in the SC, AC and AEC systems tended to be lowered during the repeated batch fermentation. Interfering of positive charge of γ-Al_2O_3 on the cell membrane was thought to be the cause of the inactivity of *C. butyricum* DSM 5431 in 1,3-propanediol production.

Keywords: γ-Alumina, alginate, *Clostridium butyricum*, immobilization, *Saccharomyces cerevisiae*

INTRODUCTION

Due to the world energy crisis, the potential for renewable energy and its by-product development is experiencing an increase in attention. The production of ethanol from renewable carbohydrate materials has become interesting worldwide (Bai et al. 2008; Yu et al., 2007). Sugar cane molasses is an abundant and low cost sustainable raw material in Thailand. It is a by-product from sugar industries that can be fermented by yeast to produce ethanol under anaerobic condition (Bai et al., 2008; Nguyen, 2008). On the other hand, the demand of biodiesel has been increasing from time to time which leads to glycerol surplus in the world market since glycerol is a main by-product of biodiesel production. The production of biodiesel fuel produces glycerol about 10% by weight (Eggersdorfer et al., 1992; Meesters et al., 1996). Therefore, it is essential to develop a technology to convert glycerol

into products of high value. Under anaerobic condition, *Clostridium butyricum* can ferment low grade glycerol and produce 1, 3 propanediol. 1, 3 Propanediol is a useful compound for polymer industries, especially for producing biodegradable polymers such as polytrimethylene terephthalate (PTT).

Immobilized cell technology has been suggested as an effective mean for improved fermentation. The immobilization of cells leads to a high productivity, and good operational stability. The main advantages in the use of immobilized cells in comparison with suspended cells are the retention in the reactor of higher concentrations of cells, protection of cells against toxic substances and elimination of costly processes of cell recovery and cell recycle. However, the major problems of immobilization are mass transfer limitation, gel degradation and cell detachment (Yu et al., 2007; Verbelen et al., 2006; Kourkoutasa et al., 2004). For improving the performance of immobilized cell carrier, γ-Al_2O_3 and calcium alginate were applied as materials for constructing immobilized cell carriers. γ-Al_2O_3 has been reported as a good support for cells because of the electrostatic attraction between γ-Al_2O_3 and cells (Kanellaki et al., 1989; Koutinas et al., 1988). Calcium alginate is the most wildly used material for entrapment because of its simplicity and non-toxic (Verbelen et al., 2006; Arasaratnam, 1994).

In this study, adsorption and entrapment techniques were used together for improving the drawback of cell immobilization. γ – Alumina doped alginate gel (AEC) was developed as a new type of cell carrier. The immobilization system of *Saccharomyces cerevisiae* M.30 for ethanol production and *Clostridium butylicum* DSM 5431 for 1, 3– propanediol production from glycerol were used to evaluate the performance of the new carrier. These systems were examined by a single batch and 4-cycle repeated batch. The activities of the immobilized cells were then compared to the systems of free cells and immobilized cells by adsorption on γ-Al_2O_3.

MATERIALS AND METHODS

Microorganism

S.cerevisiae M30 strain was kindly provided by Prof. Dr Savitree Limtong, from Department of Microbiology, Kasetsart University, Bangkok. The culture was stored in Potato Dextrose Agar (PDA) slant at 4 °C. *C. butyricum* DSM 5431, obtained from American Type Culture Collection (ATCC) BAA-557™. The culture was stored in Reinforced Clostridial Medium (RCM) at 4 °C

Pre-culture and immobilization

S. cerevisiae M30 was grown in a 500 ml Erlenmeyer flask containing 100 ml pre-culture medium. The composition of the pre-culture medium per liter was: 50 g sugar from palm sugar; 0.5 g $(NH_4)_2SO_4$; 0.1 g KH_2PO_4; 0.035 g $MgSO_4.7H_2O$. The medium was adjusted to pH of 5, and sterilized at 121 °C for 15 min. Cell cultivation was carried in an Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C for 20 hours.The late exponential phase cells were harvested by decantation to obtain stock cell suspension.

C. butyricum DSM 5431 was grown in a 500 ml Erlenmeyer flask containing 100 ml pre-culture medium. The composition of the pre-culture medium per liter was: 3.4 g K_2HPO_4; 1.3 g KH_2PO_4; 2 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4.7H_2O$; 0.02 g $CaCl_2.2H_2O$; 2 g $CaCO_3$; 1 g yeast extract; 20 g glycerol; 1 ml trace element solution; 2 ml Fe solution. The Fe solution per liter consisted of: 5 g $FeSO_4.7H_2O$; 4 ml HCl (37%). The trace element solution per liter consisted of: 70 mg $ZnCl_2$; 0.1 g $MnCl_2.4H_2O$; 60 mg H_3BO_3; 0.2 g $CoCl_2.2H_2O$; 20 mg $CuCl_2.2H_2O$; 25 mg $NiCl_2.6H_2O$; 35 mg $Na_2MoO_4.2H_2O$; 0.9 ml HCl (37%). Cell cultivation was carried in shaker at 100 rpm, 33°C for 20 hours.

Immobilization

Immobilization of cells was investigated using two general methods. The first was the adsorption of cells onto γ-Al_2O_3 powder. The second was entrapment of γ-Al_2O_3 -cells in calcium alginate matrix.

Sterilized γ-Al_2O_3 powder was immersed in pre-culture medium and incubated with cell culture for 20 hours to induce natural cells adhesion. γ-Al_2O_3 -cell mixture was added to 3% w/v sodium alginate solution to form an alginate- γ-Al_2O_3 -cell mixture with volumetric ratio of 1:1. The formation of AEC bead was initiated by adding the alginate- γ-Al_2O_3 -cell mixture drop wisely into 500ml 0.12M $CaCl_2$ using a syringe (1.2 mm diameter). AEC carriers with the diameter (Ø) of 3 mm were left to harden in $CaCl_2$ solution for 30 minutes and then rinsed 3 times with 0.9% w/v NaCl. Immobilization yield (Y_I, %) was calculated as follows:

$$Y_I = \frac{X_I}{X_T} \times 100$$

X_I = immobilized cell concentration, g/L

X_T = total cell concentration, g/L

Batch fermentation

For ethanol fermentation, the composition of the fermentation medium per liter was: 220 g reducing sugar from molasses; 0.5 g $(NH_4)_2SO_4$ at pH of 5. Suspended / immobilized cells were cultured in a 500 ml Erlenmeyer flask containing 250 ml fermentation medium. Batch fermentation was performed in the incubator shaker at 150 rpm, 33°C.

For 1,3-propanediol fermentation, The composition of the fermentation medium per liter was: 1.0 g K_2HPO_4; 0.5 g KH_2PO_4; 4 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4.7H_2O$; 0.02 g $CaCl_2.2H_2O$; 1 g yeast extract; 80 g glycerol; 1 ml trace element solution; 1 ml Fe solution at pH of 7. Suspended / immobilized cells were cultured in a 1-L glass fermenter (Biostat Q®, B Braun Biotech International, Germany) containing 600 ml fermentation medium and the system was purged under nitrogen at a rate of 0.1 vvm in order to promote anaerobic condition. The agitation speed was controlled at 100 rpm and the pH was adjusted to 7.0 by automatic addition of 4 M NaOH. The incubation temperature was 33°C.

Analytical methods

Ethanol concentration was determined using gas chromatography (GC-7AG, Shimadzu, Japan) (Phisalaphong et al., 2005). Residual sugars were measured using the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959). 1, 3-Propanediol assay was measured by HPLC equipped with refractive index Detector (LC-3A, Shimadzu, Japan) using a column (Lichrocart C18) with length of 250 mm, outer diameters of 4 mm. Operating condition was: 20 mM H_3PO_4 as a mobile phase, flow rate 1.2 ml/min, column temperature at room temperature. Free and immobilized cell concentrations were measured as cell dry weight. Samples of fermentation broth were centrifuged at 2000 rpm for 15 min and the cells were re-suspended in water for free cell determination. A known mass of cell carriers was dissolved in 0.05 M sodium citrate. The suspension was treated similarly as fermentation broth to obtain its corresponding immobilized cell concentration. Biomass concentrations were measured as optical density (UV-2450, Shimadzu, Japan) at 660 nm for *S. cerevisiae* M30 and 650 nm for *C. butyricum* DSM 5431 and converted to dry cell concentration on the basis of a corresponding standard curve.

RESULTS AND DISCUSSION

Ethanol fermentation

Ethanol fermentation was carried out with 220 g/l of initial sugar concentration from cane molasses as a carbon source for *S. cerevisiae* M30. The fermentations were performed using suspended cell (SC) culture, immobilized cells on γ-Al_2O_3 (AC) culture and γ-Al_2O_3 doped alginate gel (AEC) culture in a single batch for

60 hours and a 4-cycle repeated batch with the duration of each batch of 48 hours.

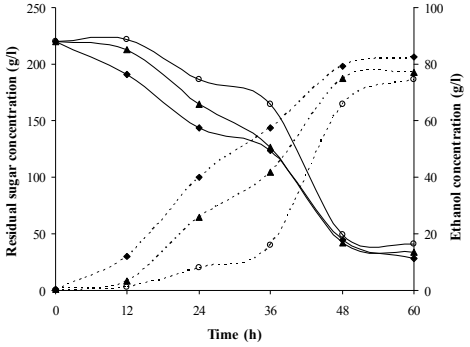

Figure 1 Time-course of single batch fermentation by *S. cerevisiae* M30 at 220 g/l of initial sugar concentration; residual sugar concentration (solid lines) and ethanol concentration (dash lines);◆, SC;▲, AC and ○, AEC.

The time-course of the single batch of ethanol fermentation is shown in Figure 1. The final total cell concentrations of AC culture at 4.3 g/l and AEC culture at 4.2 g/l were obtained (data not shown), which were slightly higher than that of SC culture (3.9 g/l). The immobilization yields of the AC and AEC cultures were 90 % and 86% respectively. It was demonstrated that γ-Al_2O_3 has a positive effect on the growth of *S. cerevisiae* M30. This is similar to the finding of Kanellaki et al. (1989) who reported that γ-Al_2O_3 was a good supporter of ethanol fermentation because of the electrostatic attraction between alumina particles and yeast cells. *S. cerevisiae* can be adsorbed on γ-Al_2O_3 in a wide pH range from 3.0 to 6.5 owing to the opposite electric charges (Kana et al., 1989). As shown in Figure 1. sugar concentration gradually decreased while ethanol concentration increased for duration of 60 hours. The final ethanol concentration of the SC, AC and AEC systems were 82.4, 77.1 and 74.6 g/l, respectively ($Y_{P/S}$ of 43%, 41% and 42%, respectively). During fermentation time from 12 to 36 hours, the ethanol concentration of the AEC system was lower than that of the AC system. This could be indicated that the mass transfer resistance of the AEC carrier did affect cell growth and product formation. However, the ethanol concentration of the AEC culture increased until it reached similar level of the AC culture at the end of the fermentation.

For the repeated batch mode, the results of the fermentation are summarized in Table 1. In the first batch, after 48 hours, ethanol concentration of the SC system was 77.1 g/l, whereas the final ethanol concentrations of immobilized cells (IC) in AC and AEC systems were 67.3 g/l and 69.7 g/l, respectively. In the second - the forth batch, all system exhibited ethanol production without any occurrence of the lag phase. In the fourth batch, the majority of sugar was consumed with the final ethanol concentrations of 70.9, 71.8 and 70.7 g/l for the SC, AC and AEC cultures, respectively. The final total cell concentrations of the AC culture (5.8 g/l, Y_I 90%) and the AEC culture (5.7 g/l, Y_I 86%) were higher than that of the SC culture (4.5 g/l). Increase in cell concentrations in the AC and AEC carriers was due to the growth of immobilized cells in the carriers during the fermentation. Instability of the SC culture in the repeated batch fermentation was observed from the comparison of its final ethanol concentration and $Y_{P/S}$ from batch to batch. The ethanol concentration of the SC system dropped from 77.1 g/l in the first batch to 72.9 and 70.9 g/l in the second and the fourth batch, respectively. This may be attributed to the negative effect of high ethanol concentration on cell activity and viability. The inhibition of ethanol and sugar especially at high concentrations on suspended cell activities and stability has been previously reported (Phisalaphong et al., 2007). The ethanol production of immobilized cells in the AC and AEC cultures were relatively stable; the ethanol yield factors ($Y_{P/S}$) were quite stable in all batches. The stability of the immobilized cell cultures was higher than that of the SC culture since the matrix of the cell carriers could protect yeast by fortification from toxins and inhibitor (Verbelen et al., 2006; Kourkoutasa et al., 2004; Phisalaphong et al., 2007).

Table 1 Yields and end products of the repeated batch ethanol fermentations (each batch 48 hours), using the cultures of suspended cell (SC) culture, immobilized cells on γ-Al_2O_3 (AC) culture and γ-Al_2O_3 doped alginate gel (AEC) culture.

Batch	P	X (g/l)		Y_I	Y_S	$Y_{P/S}$
	(g/l)	X_E	X_I	(g/g)	(g/g)	(g/g)
I						
SC	77.1	-	-	-	0.79	0.43
AC	67.3	-	-	-	0.78	0.39
AEC	69.7	-	-	-	0.78	0.40
II						
SC	72.9	-	-	-	0.80	0.40
AC	70.3	-	-	-	0.78	0.40
AEC	67.2	-	-	-	0.76	0.39
III						
SC	72.8	-	-	-	0.79	0.40
AC	69.5	-	-	-	0.77	0.40
AEC	71.8	-	-	-	0.79	0.40
IV						
SC	70.9	4.5	-	-	0.78	0.39
AC	71.8	0.6	5.3	0.90	0.78	0.41
AEC	70.7	0.8	5.0	0.86	0.78	0.40

Table 2 Yields and end products of the repeated batch 1,3-propanediol fermentation (each batch 24 hours), using the cultures of suspended cell (SC) culture, immobilized cells on γ-Al_2O_3 (AC) culture and γ-Al_2O_3 doped alginate gel (AEC) culture.

Batch	P	X (g/l)		Y_I	$Y_{P/S}$
	(g/l)	X_E	X_I	(g/g)	(g/g)
I					
SC	36.9	-	-	-	0.46
AC	14.8	-	-	-	0.19
AEC	11.4	-	-	-	0.14
II					
SC	32.7	-	-	-	0.41
AC	13.9	-	-	-	0.17
AEC	6.0	-	-	-	0.08
III					
SC	33.5	-	-	-	0.42
AC	11.6	-	-	-	0.14
AEC	4.5	-	-	-	0.06
IV					
SC	26.4	4.4	-	-	0.33
AC	12.5	1.1	4.6	0.80	0.16
AEC	3.3	0.8	3.9	0.83	0.04

1, 3-propanediol fermentation

The fermentations of *C. butyricum* DSM 5431 were carried out in the 1 L glass fermenter with 80 g/l initial glycerol concentration. The fermentations in this study were also performed using SC, AC and AEC cultures. These three cultures were examined by a single batch for 33 h and a 4-cycle repeated batch with the duration of each batch of 24 hours.

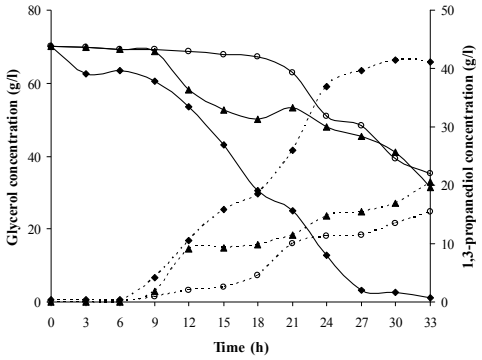

Figure 2 Time-course of single batch fermentation by *C.butyricum* DSM 5431 at 80 g/l of initial glycerol concentration; residual glycerol concentration (solid lines) and 1, 3-propanediol concentration (dash lines); ◆, SC; ▲, AC and ○, AEC

The time-course of the single batch of 1, 3-propanediol is shown in Figure 2. At the end of the fermentation, the total cell concentration of the AC culture (4.4 g/l) and AEC culture (3.6 g/l) (data not shown) were higher than that of the SC culture (3.1 g/l) and the immobilization yield of the AC and AEC cultures were 80 % and 83 % respectively. It was demonstrated that the cell carriers might promote the growth of *C. butyricum* DSM 5431. This result agreed with the previous ethanol fermentation study. However, the final 1, 3-propanediol concentrations of the SC, AC and AEC systems were 41.4, 20.6 and 15.5 g/l, respectively. The 1, 3-propanediol yields ($Y_{P/S}$) of AC culture (26 %) and AEC culture (19 %) were significantly lower than that of the SC culture (52 %). It was found that there was an occurrence of the inhibition effect on 1, 3-propanediol production by γ-Al_2O_3 based carrier. High positive charge density on the surface of the carrier (γ-Al_2O_3) was considered to be the possible negative impact on the cell activities. The adverse effects on cell membrane could affect its enzyme activities.

In 4-cycle repeated batch fermentation, the experimental results are shown in Table 2. The 1, 3-propanediol concentrations of all systems decreased from the first to the fourth batch. Instability of the SC culture was observed from the comparison of $Y_{P/S}$ in the first to the fourth batch (46 % to 33%). This can be attributed to the effect of the product and by-products from this fermentation such as butyric acid and acetic acid, which could be directly toxic to the cell. The stability of the AC culture was slightly higher than that of the SC culture; however 1, 3-propanediol production of the AC culture was much lower than that of the SC culture. The stability of the AEC culture was significantly decreased due to the negative effect of γ-Al_2O_3 on the cell activity together with the reduced diffusivity of product and by products due to the alginate entrapment.

CONCLUSIONS

This study indicated that the ethanol fermentation by immobilized *S. cerevisiae* M30 in the AC and AEC carriers was promising. The high ethanol production from the immobilized cultures was achieved owing to the high density of cells and high stability of the cell activities for long term operation. In 4-cycles of repeated batch ethanol fermentation, the AC and AEC cultures exhibited a good potential of reusability. However, the AC and AEC carriers were found unfavorable for *C. butyricum* DSM 5431 immobilization. High positive charge density on the surface of the carrier (γ-Al_2O_3) was considered to be the possible negative impact on *C. butyricum* activities. It was found that with the use of the immobilized carriers incorporated with γ-Al_2O_3, the production of 1, 3-propanediol was much lower than that of the SC system. The 4-cycle repeated batch of 1, 3-propanediol fermentation revealed that the instability of *C. butyricum* DSM 5431 was very high in all three systems.

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