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APPENDICES

APPENDIX A

EXPERIMENTAL METHODS

A-1 Agar slants preparation

In this study, Potato Dextrose Agar (PDA) was used as medium for stock cultures. For sterilization, TOMY SS-325 autoclave was used. The preparation steps of PDA agar slants in details are:

1. Mix 7.8 g PDA powder with 200 ml de-ionized (DI) water in 500 ml glass beaker.
2. Stir the solution with magnetic stirrer and heat it up until it is boiling.
3. Boil the solution for 1 minute or until all powder is dissolved as indicated by the formation of clear yellowish agar solution.
4. Transfer 4 ml agar solution into 16 x 150 mm screw cap culture tube by using 10 ml pipette.
5. Sterilize all agar containing tubes at 121°C for 15 minutes in autoclave. (Set the tube's cap to be rather loose before autoclaving to facilitate gas expansion inside the tube during sterilization.)
6. After sterilization, tighten the tube's cap and let the tubes to cool down before positioning them in slanted position to obtain agar slant inside the tubes.
7. Precautions:
    a) PDA agar powder is hygroscopic. Minimize exposure time of the powder to the ambient air to avoid excess water absorption.
    b) Sterilization is carried out at high temperature. Wear heat resistant gloves as protection when handling hot materials.
    c) When slanting the agar, provide enough space between tube neck and agar to minimize the risk of contamination from outside the tube.

A-2 Stock cultures preparation

Stock cultures were prepared by aseptic inoculation of the flocculating yeast *S. cerevisiae* M30 on the PDA agar slants. The procedures are as follows:

1. Sterilize all equipments and agar slants with ultraviolet (UV) light with air flow for about 1 hour in the ISSCO VS-124 laminar flow hood.
2. After the UV lamp is turned off, clean all apparatus and the hood's compartment with alcohol 70% v/v solution to ensure asepticity.
3. Open the caps of source culture and fresh agar tubes then heat up the tubes' neck with an alcohol burner.
4. Heat up the inoculation loop thoroughly until it reds up.
5. Cool down the loop by contacting with fresh medium.
6. Transfer the yeast cells from source culture to fresh agar slant. Inoculate the cells on fresh agar by zigzag movement.
7. Heat the tube neck again before securing the cap.
8. Repeat step 4-8 again for other fresh medium until sufficient amounts of stock cultures is obtained.
9. Leave the stock cultures to grow at room temperature for 20-24 hours before use.
10. Precautions:
    a) Be cautious with the UV light as it is harmful for human eyes and skin.
    b) Wear protective gloves during inoculation for safety and aseptic reasons.

A-3 Medium preparation

There were 2 varieties of medium used in this study. One was designated for cell cultivation and the other was for ethanol production.

A-3.1 Preculture medium preparation

The main component of the medium in earlier experiments was palm sugar which was used as carbon and energy source for the yeast. For 1 L of cell cultivation consisted of palm sugar 50 g, 0.1 g KH_2PO_4, 0.035 g $MgSO_4.7H_2O$, and 0.5 g $(NH_4)_2SO_4$. The pH

value of the medium was adjusted to 5 with 0.1 M NaOH and HCl solution. To the adsorption of cells onto the surface of Al_2O_3 powder, Al_2O_3 is add into the palm sugar medium to intended the concentration of Al_2O_3 (Table A-3.1). The detailed procedures for medium preparation from palm sugar are listed in the following paragraph.

Table A-3.1 The quantity of Al_2O_3 was added into the palm sugar medium.

Al_2O_3 concentration (% w/v of alginate)	**Al_2O_3 g/ 100 palm sugar medium**
3.3	1.0
5.0	1.5
6.7	2.0
8.3	2.5

1. Palm sugar (50 g/l) and nutrients are mixed with DI water.
2. Adjust the pH of the solution to 5 by adding NaOH or HCl solution.
3. Pour appropriate volume of medium (100 ml and 250 ml for inoculums development and ethanol fermentation respectively into 500 ml Erlenmeyer flask.
4. Mix the palm sugar medium with appropriate amount of Al_2O_3 that required for experiment.
5. Close each flask with cotton plug and wrap with aluminum foil before sterilization.
6. Sterilize the mediums with autoclave for 15 min at 121°C.
7. Precautions and notes:
   a) Avoid wetting the flasks' neck when pouring the solution as the heated solution may act as adhesive so that the plug is difficult to be removed after sterilization.
   b) The pH of the solution may be quite altered after sterilization.
   c) Some precipitates may be formed after sterilization from the sugar solution.

A-3.2 Fermentation medium preparation

For 1 L of fermentation medium consisted 0.5 g $(NH_4)_2SO_4$ and 220 g sugar from sugar cane molasses. The quantity of molasses needed to reach the intended sugar level was also estimated by DNS trial. Sugar cane molasses was diluted to obtain sugar concentration of about 220 g/l for ethanol fermentation. The procedures for preparing molasses based fermentation medium are follows:

1. Dilute the molasses with DI water until the desired sugar concentration (220 g/l) is achieved.
2. Mix the diluted sugar solution with appropriate amount of $(NH_4)_2SO_4$.
1. Adjust the pH of the solution to 5 with NaOH or HCl solution.
2. Fill 500 ml Erlenmeyer flask with 250 ml medium.
3. Close each flask with cotton plug before sterilization.
4. Autoclave the medium for 15 min at 121°C.
5. Precautions and notes are same with palm sugar based medium preparation.

A-4 Cell cultivation and harvesting

Cell cultivation was initiated with the transfer of cells from stock culture tube aseptically to Erlenmeyer flask containing fresh medium by using Gilson Pipetman auto pipette. Thus, sterile pipette tips should be prepared in advance by autoclaving or dry heat in hot air oven. Active yeast cells with generation time (age) 20-24 hours were used for cultivation purpose. After inoculation, cell cultivation was carried out in Innova 4330 Refrigerated Incubator Shaker for 20-24 hours at 150 rpm. After some time, the growing yeast cells could be noticed as brown colored suspended solids inside the sugar solution. The cells were then harvested and concentrated by medium draining. The complete steps are as follows:

1. Sterilize equipments and the laminar flow hood with UV and by wiping with alcohol 70% v/v solution.
2. Heat up the neck of stock culture tube and medium flask after removing the tube cap and cotton plug.

3. Heat up the inoculation loop evenly and then slightly deep it into the fresh medium in the Erlenmeyer flask to cool it down before touching the yeast cells.
4. Scratch the yeast culture on the tube to detach the cells from the surface of the agar using the loop.
5. Transfer the cell at the loop into the Erlenmeyer flask and then close the flask using cotton plug.
6. Repeat steps 3-5 for the other flasks.
7. Put all flasks in the incubator shaker and then operate the shaker at 150 rpm 33°C for a day before harvesting the cells.
8. Let the cells to settle for a while after incubation and then carefully take out 130 ml of the medium from each flask by using 10 ml of auto pipette.
9. Combine the concentrated cells suspension from several flasks by pouring it into one flask.
10. Further draining can be done to concentrate cells by the same method until the desired volume of concentrated cells suspension is obtained.
11. Precautions and notes:
    a) Except the stock culture and the fresh medium, all equipments should be cleaned and sterilized using UV light and alcohol to ensure asepticity.
    b) Clean the outer surface of the tubes and flasks using alcohol before use.
    c) Keep the tube neck and flask opening hot by regular heating after removal of the cap or plug to prevent contamination originated from ambient air.

A-5 Cell immobilization

Immobilization of cells was investigated using two general methods. The first was the adsorption of cells onto the surface of Al_2O_3 powder. Sterilized Al_2O_3 powder was immersed in palm sugar medium and incubated with cell for 20-24 hours to induce natural cells adhesion. The second was entrapment of Al_2O_3-cells in Ca-alginate matrix. Thus, Al_2O_3 and Na-alginate was used for cell adsorption and entrapment purposes. To the entrapment of Al_2O_3-cells in Ca-alginate matrix, 30 ml of Alginate solution was

prepared for each flask with appropriate concentration that required for experiment (Table A-5.1). Preparation of Na-alginate steps are as follows:

1. Mix Na-alginate, palm sugar medium and water at appropriate concentration that required for experiment, into 50 ml Erlenmeyer flask.
2. Close each flask with cotton plug before sterilization.
3. Autoclave the medium for 15 min at 121°C.
4. Keep the solution at 4°C before use.

Table A-5.1 The concentration of Na-alginate was prepared for each experiment.

Na-alginate concentration (% w/v of alginate)	**Na-alginate (g)**	**Palm sugar medium (ml)**	**Water (ml)**	**Al_2O_3-cells solution (ml)**
1.5	0.45	2.5	12.5	15
2.0	0.60	2.5	12.5	15
2.5	0.75	2.5	12.5	15
3.0	0.90	2.5	12.5	15

Preparation of gelation of alginate-Al_2O_3-cells (AEC) was listed in the following:

1. Na-alginate mixture was mixed with Al_2O_3-cells solution that was provided from cell cultivation with volumetric ratio of 1:1.
2. Drop alginate solution by using auto pipette into 0.12M $CaCl_2$ solution to form alginate beads.
3. Keep the beads in the solution for 30 minute to ensure that precipitation reaction reaches completion.
4. Rinse the gel 3 times with NaCl 0.9% w/v solution.
5. Precautions and notes:
   a) All procedures are conducted aseptically in laminar flow hood.
   b) All equipments are cleaned and sterilized before use.

A-6 Ethanol fermentation

A-6.1 Batch fermentation

Sugar cane molasses was used as carbon source for the fermentation medium. The medium contained initial sugar concentration of 220 g/L, 0.05% w/v $(NH_4)_2SO_4$ and the initial pH was adjusted at 5.0. Experiments were initiated by transferring prepared cell suspension or immobilized cells into 500 ml Erlenmeyer flasks containing 250 ml of the fermentation medium in order to promote anaerobic condition which was favorable ethanol fermentation. Batch fermentation in shake flasks was performed in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C for 72 hours.

A-6.2 Continuous fermentation

Prior to inoculation and start up of the continuous fermentation, the column was sterilized by circulation of 70% v/v ethanol for 1 hour and then was kept under UV light overnight. The immobilized cells in AEC carrier was prepared by suitable condition from batch system was cultivated with initial sugar concentration about 223 g/l in Innova 4330 refrigerated incubator shaker at 150 rpm, 33°C for 24 hour that to increase the cells concentration in AEC carrier before the carries were aseptically transferred to the sterilized column. The carrier volume was about 40 % (v/v) of the pack bed reactor volume of 1 L.

The bioreactor was sterilized by circulation of 70% v/v ethanol for 1 hour and then was kept under UV light overnight. The column was packed by immobilized cell bed (AEC carrier) with working volume around 0.6 L. Temperature of the system is controlled at 32 ± 1 °C by the passing of 28 °C cooling water inside the reactor jacket. The sterile molasses solution with the initial sugar concentration of 220 g/l and the initial pH at 5.0 was fed to the bottom of the fermentor continuously by means of a peristaltic pump through sterile silicon tubing. Effluent liquid overflowed from an outlet port at the top of the bioreactor, maintaining a constant level of fermentation broth in the column.

The carriers were trapped inside the bioreactor with a metal mesh filter covered. The samples were harvested with volume of 5 ml every 8 hours from the 5th port of the column.

Before and during fermentation, regular sampling of the fermentation broth and carrier was done in aseptic condition. The liquid samples were taken by auto pipette. They were then frozen in freezer. Before analysis, the frozen samples were melted in room temperature. Centrifugation of the samples with Kubota 5100 centrifuge was carried out at 2000 rpm for 15 minutes. The supernatant was divided into 2 portions; one was used for sugar analysis while the other was sent to be analyzed for ethanol content by gas chromatography. The yeast pellet was further used for free cells concentration determination. Samples of carrier were taken by sterilized stainless spoon. They were then stored in refrigerator at 4°C before immobilized cell determination. Carriers from the start and end of each fermentation cycle were sent for scanning electron microscopy (SEM)

A-7 Sugar analysis

Sugar (sucrose) concentration was determined using a modified DNS reagent method. All disaccharides in the samples and standard sucrose solutions were first hydrolyzed to their monomers by using acid solution at elevated temperature. The acid residue was then neutralized using a basic solution and the resulting precipitates were settled by centrifugation. After centrifugation, the supernatant was reacted with DNS reagent at high temperature resulting in the formation of brown colored solution. The solution was then diluted before being analyzed by using spectrophotometer. The absorbance of the sample was compared with standard sucrose solutions to obtain the corresponding sucrose concentration. Complete step by step procedures are provided in the following sections.

A-7.1 NaOH and HCl solution preparation

NaOH 20% w/v was prepared by dissolving 200 g of NaOH pellets in 100 mL of water. The reaction is highly exothermic so that the preparation should be done in water bath in order to avoid excess heat generation. Weighing time of NaOH pellets should be minimized because of the hygroscopic nature of NaOH. Solution of 37% w/v HCl was obtained by diluting concentrated HCl solution with DI water. Beware of the acid vapor and wear protective gloves when preparing the solutions. Commercially available HCl 37% can be also be used directly.

A-7.2 DNS reagent preparation

DNS powder is toxic and easy to airborne so that it should be handled with caution. This powder should be added slowly in the mixing process because it is not easy to dissolve. After preparation, the resulting yellow colored reagent is best used in fresh condition so that it is not suggested to keep unused for long time (more than 1 month). The reagent is usually kept in brown bottle to protect it from degradation originated from light for example sun light. The complete preparation steps are:

1. Dissolve 1.633 g NaOH 98% w/w in 20 ml of water. Mix the solution with magnetic stirrer.
2. Under stirring, slowly add 1 g of 3,5-dinitrosalicylic acid powder into the solution.
3. Dilute by adding 50 ml of water. Stir until it is homogeneous.
4. Add 30 g Na-K tartrate & mix it thoroughly.
5. Adjust the volume to 100 ml.
6. Keep the reagent for 3 days before use.

A-7.3 Standard sucrose solution preparation

Standard sucrose solutions were prepared first by making the source solution which was the solution with the highest sucrose concentration as the upper limit. The source solution was then diluted with water so that a set of standard solution with

increasing sucrose concentration (for instance 0, 6.25, 12.5, 18.75, and 25% w/v) was obtained. The detailed procedures are as follows:

1. Dry 3.0 g sucrose at 100-105°C in hot air oven for 2 hours.
2. Put the dried sucrose in desiccator for cooling.
3. Dissolve 2.5 g of the sucrose in 10 ml of water to obtain the source solution.
4. Prepare each 2 ml standard solution in small labeled bottle by serial dilution of suitable amount of source solution and diluting it with water as shown in detail in Table A-7. Use auto pipette for the transfer purpose.

Table A-7.3 Standard sucrose solution preparation

Sucrose concentration (% w/v)	**Source solution (ml)**	**Water (ml)**
0	0	2.0
6.25	0.5	1.5
12.50	1.0	1.0
18.75	1.5	0.5
25.00	2.0	0

A-7.4 Sample treatment I

In the first treatment, sample was hydrolyzed using HCl 37% in boiled water bath. After the hydrolysis reaction was stopped, NaOH was added into the solution. The sample was then centrifuged for removing suspended solids. Procedures of the first treatment are:

1. Mix 0.2 ml of sample with 0.8 ml DI water in screw cap tube.
2. Blend the sample with 0.5 ml HCl 37%.
3. Put the tubes in boiling water bath for 10 minutes.
4. Stop the reaction by placing the tubes in ice bath.
5. Add 0.5 ml NaOH 20% w/v and then mix with vortex mixer.
6. Add 10 ml DI water and then mix with vortex mixer.

7. Centrifuge the sample at 2000 rpm for 20 minutes.
8. Precautions and notes:
   a) Use vortex mixer for mixing the fluid in the tubes.
   b) Be cautious when handling the hot apparatus.
   c) The level of boiled water and ice bath must be sufficiently higher than the liquid level in the tubes to ensure good heating and cooling of the sample.

A-7.5 Sample treatment II

In treatment II, supernatant obtained from treatment I was reacted with DNS reagent in boiled water bath. The solution's color transformed from yellow to reddish brown in the course of reaction. The color intensity represents the corresponding sugar concentration. Solution with higher sugar content will have darker color. After the reaction was ended, the solution was diluted with sufficient amount of water until its absorbance spectrum obtained by spectrophotometer was well distributed along the range of concentration being considered (the absorbance measured was not more 0.7). Shimadzu UV-2450 UV-Visible spectrophotometer was used for absorbance measurement. Sample containing only water (0% sugar) which had been treated in the same manner as the other samples was used as blank. At every absorbance measurement, fresh standard solution should be used. Complete procedures are described in the following paragraph.

1. Mix 0.2 ml of supernatant obtained from treatment I with 1.0 ml DNS reagent in screw cap tube.
2. Boil the solution for 10 minutes using water bath.
3. Put the tubes in ice bath to stop the reaction.
4. Add 10 ml DI water and then mix with vortex mixer.
5. Measure the absorbance at 520 nm. Use sample with 0% sugar as blank.
6. Obtain the standard curve by plotting absorbance versus sucrose concentration of standard sucrose solution.
7. Use the standard curve to gain sugar concentration of the samples.

A-8 Determination of cell concentration

Cell concentration was determined by separation of cell from its carrier or medium followed by measurement by spectrophotometer. The cell concentration was obtained by comparing the absorbance of sample with its corresponding standard curve. The standard curve was made by measuring a set of samples of known cell concentration (with dry weight basis).

A-8.1 Dry weight of cell

Dry weight of cell was determined by separating the cells from their suspending liquid medium by centrifugation. The cells were then dried and their weight was measured as the representative of their concentration in the initial suspension. The procedures are:

1. Centrifuge the cell containing medium at 2000 rpm for 15 minutes.
2. Remove the supernatant (discarded or to be used for other analysis).
3. Add HCl 0.1 N to the cell pellet and mix with vortex mixer.
4. Centrifuge the suspension at 2000 rpm for 15 minutes.
5. Discard the supernatant.
6. Disperse the cell pellet with DI water.
7. Repeat step 4-6.
8. Transfer the cell suspension to a pre-weighted aluminum dish.
9. Dry the cell in hot air oven at 100°C for 2 hours.
10. Measure the weight of the cells.
11. Precautions and notes:
    a) The cells cake is fragile. Pour out all of the supernatant in one cycle instead of several cycles.
    b) Dry and measure the weight of aluminum dishes before use.
    c) The dry weight of the cells is obtained as the difference between the weight of the aluminum dish which contains cells and the weight of empty dish.

A-8.2 Free cell concentration

A set of cell suspension with known cell concentration was used as standard. This solution was analyzed at the same time with samples of fermentation and used to generate standard curve of cell concentration. The complete procedures are:

1. Dilute sample with DI water in 16 x 100 mm rimless tube.
2. Centrifuge the cell suspension at 2000 rpm for 15 minutes.
3. Remove the supernatant.
4. Add HCl 0.1 N and mix with vortex mixer.
5. Centrifuge the suspension at 2000 rpm for 15 minutes.
6. Discard the supernatant.
7. Disperse the cell pellet with DI water.
8. Repeat step 5-8.
9. Measure the absorbance of sample at 660 nm.
10. Precautions and notes:
    a) Dilute the sample with DI water before optical density measurement if the cell concentration is too high (its absorbance value is too high).
    b) Mix every sample with vortex mixer before spectrophotometry to ensure homogeneity of the sample.

A-8.3 Immobilized cell concentration

Before the cell concentration could be measured, a measured amount of carrier should be dissolved to obtain cell suspension. The dissolution of the gel can be done by immersing the gel in several chemicals such as EDTA, sodium citrate, potassium citrate, and phosphate buffer. In this study, the dissolution of gel was carried out using sodium citrate 0.5 M solution. In the case of AEC (alginate-Al_2O_3-cell) carriers, the Al_2O_3 particle was removed from the suspension after the gel was dissolved. The cells suspension was then treated with the same procedures as for free cells suspension in order to obtain its corresponding immobilized cell concentration. The complete procedures are as follows:

1. Dissolve appropriate amount of weighted AEC carrier with 6 ml 0.5 M sodium citrate in 16 x 100 mm rimless tube.
2. Heat the solution at 80°C for 15 minutes using water bath.
3. When the gel is totally dissolved, Continue with same procedures as step 2-9 of Section A-8.2.
4. Precautions and notes:
   a) Intermittent mixing with vortex mixer is recommended to promote faster gel dissolution.
   b) Al_2O_3 particle was subtracted from the immobilized cell value by the measurement of sample containing only Al_2O_3 particle at 660 nm.
   c) To minimize measurement error caused by sample contamination, the dissolution process can be carried out in at temperature about 4°C.

APPENDIX B

EXPERIMENTAL DATA

B-1 Experimental data of batch fermentation

Table B-1.1 Experimental data of ethanol and residual sugar concentration in batch fermentation of SC, EC and ACE with 4mm bead diameter.

Time	Ethanol concentration (g/l)			Residual sugar concentration (g/l)		
(hours)	SC	EC	AEC	SC	EC	AEC
0	0	0	0	225.13	225.13	225.13
8	2.13	3.01	3.64	188.70	208.54	193.61
16	28.03	16.55	22.39	137.90	177.44	170.06
24	58.29	54.02	50.07	81.70	123.86	117.82
32	64.36	67.12	77.98	60.60	96.06	73.17
40	71.70	82.07	86.95	36.30	45.39	38.09
48	77.44	85.11	91.07	34.60	36.11	30.57
56	79.11	85.59	91.21	34.78	27.41	25.43
64	82.14	87.41	90.80	33.86	23.58	22.46
72	85.07	89.52	91.67	33.62	21.45	20.29

Table B-1.2 Experimental data of ethanol and residual sugar concentration in batch fermentation of SC, EC and ACE with 6mm bead diameter.

Time	Ethanol concentration (g/l)			Residual sugar concentration (g/l)		
(hours)	SC	EC	AEC	SC	EC	AEC
0	0	0	0	225.13	225.13	225.13
8	2.13	0.17	0.34	188.70	209.61	209.84
16	28.03	7.64	8.29	137.90	189.13	171.40
24	58.29	30.34	34.21	81.70	152.01	137.30
32	64.36	53.67	58.29	60.60	91.86	82.00
40	71.70	68.93	74.60	36.30	49.27	50.06
48	77.44	78.26	81.42	34.60	43.59	38.10
56	79.11	78.84	84.03	34.78	42.11	36.14
64	82.14	79.05	86.17	33.86	40.83	30.78
72	85.07	81.93	86.76	33.62	35.84	28.38

Table B-1.3 Experimental data of ethanol and residual sugar concentration in batch fermentation of ACE carrier with 2 mm, 4 mm and 6 mm bead diameter.

Time	Ethanol concentration (g/l)			Residual sugar concentration (g/l)		
(hours)	Ø 2 mm	Ø 4 mm	Ø 6 mm	Ø 2 mm	Ø 4 mm	Ø 6 mm
0	0	0	0	225.07	225.07	225.07
8	0	0.32	0.41	188.45	201.07	214.79
16	3.64	3.01	1.28	150.32	173.75	183.62
24	22.39	13.72	18.10	121.77	127.98	146.08
32	59.78	36.55	33.02	67.45	84.62	96.47
40	77.98	72.13	71.70	39.16	46.63	58.05
48	86.95	83.89	83.54	36.29	39.89	45.79
56	90.76	87.21	84.94	32.08	37.16	41.52
64	91.81	87.98	87.34	29.34	34.61	39.58
72	92.03	89.30	88.00	27.58	29.33	30.46

Table B-1.4 Experimental data of immobilized cell and free cell concentration in batch fermentation of ACE carrier with 2 mm, 4 mm and 6 mm bead diameter.

Time	Immobilized cell concentration (g/l)			Free concentration (g/l)		
(hours)	Ø 2 mm	Ø 4 mm	Ø 6 mm	Ø 2 mm	Ø 4 mm	Ø 6 mm
0	0.18	0.17	0.17	0	0	0
8	0.57	0.51	0.44	0.20	0.16	0.19
16	1.23	1.09	1.14	0.33	0.29	0.23
24	2.84	2.45	2.19	0.53	0.47	0.44
32	3.44	3.04	2.83	0.62	0.54	0.57
40	3.88	3.49	2.94	0.74	0.61	0.67
48	4.07	3.65	2.98	0.89	0.69	0.75
56	4.02	3.72	3.22	0.84	0.73	0.72
64	4.05	3.75	3.42	0.85	0.76	0.74
72	4.19	3.68	3.50	0.86	0.79	0.73

Table B-1.5 Experimental data of ethanol and residual sugar concentration in batch fermentation of ACE carrier with 1.5%, 2%, 2.5% and 3% (w/v) of Na-alginate.

Time	Ethanol concentration (g/l)				Residual sugar concentration (g/l)			
(hours)	1.5%	2%	2.5%	3%	1.5%	2%	2.5%	3%
0	0.37	0.13	0.12	0.39	217.57	217.57	217.57	217.57
8	1.35	0.63	0.27	0.26	200.10	193.15	183.41	205.63
16	10.93	13.25	11.86	10.45	146.04	148.87	135.80	164.86
24	31.81	30.55	29.68	31.37	112.82	106.03	96.51	129.91
32	53.03	58.59	57.21	51.81	73.55	72.46	67.05	78.67
40	63.56	65.82	65.00	61.98	50.07	52.38	48.27	56.10
48	65.78	68.82	72.42	65.66	48.92	46.66	45.79	53.20
56	69.89	72.04	74.25	68.10	46.78	45.95	43.75	52.43
64	70.95	74.28	75.72	71.09	45.57	43.81	42.53	49.80
72	73.29	75.65	77.76	71.56	44.88	41.95	41.70	49.58

Table B-1.6 Experimental data of immobilized cell and free cell concentration in batch fermentation of ACE carrier with 1.5%, 2%, 2.5% and 3% (w/v) of Na-alginate.

Time	Immobilized cell concentration (g/l)				Free concentration (g/l)			
(hours)	1.5%	2%	2.5%	3%	1.5%	2%	2.5%	3%
0	0.21	0.22	0.24	0.22	0.00	0.00	0.00	0.00
8	0.51	0.49	0.39	0.31	0.20	0.17	0.19	0.11
16	1.39	1.23	1.09	0.87	0.29	0.29	0.23	0.19
24	2.23	2.44	2.38	1.72	0.58	0.47	0.44	0.32
32	2.88	2.79	3.14	2.50	0.67	0.54	0.53	0.45
40	3.13	3.37	3.50	2.91	0.74	0.61	0.60	0.51
48	3.17	3.47	3.66	3.10	0.87	0.69	0.70	0.63
56	3.38	3.54	3.72	3.17	0.88	0.73	0.72	0.67
64	3.47	3.67	3.70	3.30	0.89	0.76	0.73	0.66
72	3.55	3.69	3.72	3.42	0.90	0.78	0.73	0.67

Table B-1.7 Experimental data of ethanol and residual sugar concentration in batch fermentation of ACE carrier with 3.3%, 5.0%, 6.7% and 8.3% (w/v of alginate) of Al_2O_3.

Time	Ethanol concentration (g/l)				Residual sugar concentration (g/l)			
(hours)	3.3%	5.0%	6.7%	8.3%	3.3%	5.0%	6.7%	8.3%
0	0.00	0.00	0.00	0.00	208.55	208.55	208.55	208.55
8	2.46	1.91	1.41	1.54	186.14	197.16	198.47	201.73
16	11.33	9.30	11.02	8.88	149.61	158.01	167.46	173.63
24	31.74	36.21	35.20	33.29	102.55	109.11	111.27	125.55
32	51.37	48.86	48.17	46.72	60.66	63.58	66.88	73.18
40	59.93	59.45	56.25	58.89	44.25	42.50	44.72	52.09
48	67.95	68.73	66.82	66.85	41.29	39.81	40.54	48.36
56	70.01	71.44	71.47	66.45	38.84	38.73	39.16	45.34
64	70.48	72.38	71.07	67.16	35.95	37.95	37.42	42.44
72	72.31	75.04	74.73	67.69	33.91	36.61	35.51	40.07

Table B-1.8 Experimental data of immobilized cell and free cell concentration in batch fermentation of ACE carrier with 3.3%, 5.0%, 6.7% and 8.3% (w/v of alginate) of Al_2O_3.

Time	Immobilized cell concentration (g/l)				Free concentration (g/l)			
(hours)	3.3%	5.0%	6.7%	8.3%	3.3%	5.0%	6.7%	8.3%
0	0.22	0.21	0.21	0.19	0.00	0.00	0.00	0.00
8	0.51	0.42	0.49	0.31	0.16	0.16	0.15	0.16
16	1.63	1.29	1.19	0.88	0.20	0.23	0.29	0.29
24	2.50	2.71	2.63	2.03	0.32	0.47	0.44	0.58
32	2.80	3.04	2.89	2.52	0.52	0.61	0.59	0.64
40	2.88	3.12	3.01	2.66	0.61	0.65	0.67	0.71
48	3.02	3.26	3.10	2.86	0.66	0.67	0.71	0.76
56	3.18	3.30	3.25	2.94	0.70	0.71	0.72	0.81
64	3.24	3.41	3.34	3.10	0.72	0.73	0.73	0.87
72	3.30	3.48	3.41	3.10	0.72	0.75	0.76	0.89

Table B-1.9 Experimental data of ethanol and residual sugar concentration in batch fermentation of ACE carrier with square and sphere shape.

Time	Ethanol concentration (g/l)		Residual sugar concentration (g/l)	
(hours)	Square	Sphere	Square	Sphere
0	0.00	0.00	226.90	226.90
8	2.30	6.32	222.66	222.32
16	13.67	17.97	207.26	194.54
24	51.81	56.56	106.17	85.08
32	71.29	76.27	69.69	51.95
40	81.19	81.99	49.27	43.24
48	82.00	89.07	37.22	32.20
56	87.80	90.68	35.54	30.52
64	88.99	90.82	29.85	28.51
72	89.83	90.92	26.84	25.84

Table B-1.10 Experimental data of immobilized cell and free cell concentration in batch fermentation of ACE carrier with square and sphere shape.

Time (hours)	Immobilized cell concentration (g/l)		Free concentration (g/l)	
	Square	Sphere	Square	Sphere
0	0.2522	0.2689	0.0420	0.0708
8	-	-	0.2283	0.1233
16	-	-	0.2892	0.2029
24	-	-	0.4363	0.3279
32	2.5713	2.8039	0.5771	0.4566
40	-	-	0.6106	0.5869
48	-	-	0.6696	0.6462
56	-	-	0.7258	0.6970
64	3.4522	3.5285	0.8019	0.7478
72	3.5615	3.7215	0.8646	0.7782

Table B-1.11 Experimental data of tensile strength and elongation at break of the square shape with EC and ACE carrier

Sample	Tensile strength (MPa)		Elongation at break (%)	
	EC	AEC	EC	AEC
1	0.17	0.21	55.50	72.70
2	0.18	0.23	58.20	80.30
3	0.17	0.26	61.80	85.50
4	0.19	0.21	56.40	78.20
5	0.16	0.28	53.60	87.30
Mean	**0.17**	**0.24**	**57.10**	**80.80**
SD	0.0111	0.0292	3.1064	5.8515

Table B-1.12 Experimental data of BJH desorption pore distribution of EC carrier ACE carrier and Al_2O_3 powder

System	Average diameter (nm)	Pore volume (cc/g)
EC	52.89	0.022177
	36.37	0.033918
	25.03	0.081275
	14.40	0.081246
	8.85	0.023629
	4.91	0.007896
	3.01	0.003305
	1.79	0.004586
AEC	55.67	0.058464
	36.99	0.080095
	25.38	0.140590
	14.24	0.150299
	8.79	0.039371
	4.95	0.013025
	3.07	0.003601
	1.86	0.008610
Al_2O_3 powder	102.76	0.000628
	65.12	0.000645
	40.77	0.000607
	22.48	0.000686
	14.11	0.000399
	8.71	0.000287
	4.86	0.000160
	2.97	0.000082
	1.88	0.000075

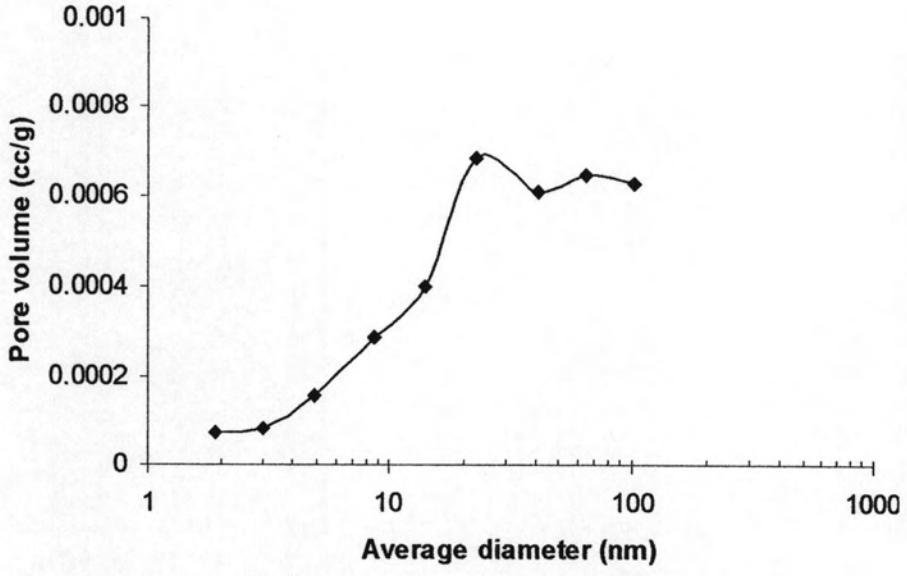


Figure B-1 Pore volume versus average diameter curve of Al_2O_3 powder

B-2 Experimental data of continuous fermentation

Table B-2.1 Experimental data of ethanol and residual sugar concentration in packed bed reactor of ACE carrier with dilution rate of 0.09, 0.16, 0.22 and 0.28 h^{-1}, stability test.

Dilution rate (h^{-1})	Time (hours)	Ethanol concentration (g/l)	Residual sugar concentration (g/l)	$Y_{P/S}$ (%)
0.09	0	0	223.00	-
	8	33.17	108.99	29.09
	16	48.07	85.95	35.07
	24	69.38	54.50	41.17
	32	78.12	44.53	43.77
	48	85.82	35.14	45.68
	56	82.85	35.70	44.23
	64	86.15	22.45	42.96
	72	89.19	18.40	43.59
	80	86.11	21.78	42.79
	88	87.88	20.24	43.34
	96	88.05	19.14	43.19
0.16	104	75.30	30.89	39.19
	112	61.86	70.83	40.65
	120	65.87	67.31	42.31
	128	68.77	66.73	44.00
	136	69.74	65.26	44.21
	144	69.67	65.17	44.14
	152	67.61	61.46	41.85
	160	69.26	63.47	43.42
	168	68.54	62.63	42.74
0.22	176	49.79	64.27	31.37
	184	59.10	75.73	40.13

Dilution rate (h^{-1})	Time (hours)	Ethanol concentration (g/l)	Residual sugar concentration (g/l)	$Y_{P/S}$ (%)
	192	56.17	100.05	45.68
	200	53.07	104.91	44.94
	208	51.31	105.60	43.71
	216	53.62	102.82	44.61
	224	50.36	106.99	43.41
	232	54.86	99.70	44.49
	240	54.06	97.61	43.11
0.28	248	49.38	97.61	39.39
	256	46.36	116.02	43.34
	264	41.92	129.17	44.68
	272	43.23	127.49	45.27
	280	44.84	126.92	46.67
	288	41.31	128.53	43.73
	296	44.49	124.01	44.95
	304	45.47	122.63	45.30
	312	45.05	124.36	45.67
0.09	336	75.87	50.45	43.97
	360	93.09	17.13	45.22
	384	91.74	18.03	44.76
	408	82.08	19.62	40.36
	432	92.22	17.44	44.86
	456	82.30	27.23	42.04
	480	82.02	32.30	43.01
	504	84.09	28.96	43.34
	528	85.40	25.16	43.17
	552	91.18	22.42	45.46
	576	82.07	35.50	43.77

Dilution rate (h^{-1})	Time (hours)	Ethanol concentration (g/l)	Residual sugar concentration (g/l)	$Y_{P/S}$ (%)
	600	79.46	41.11	43.68
	624	81.55	33.49	43.03
	648	81.96	28.96	42.24
	672	78.99	39.43	43.03
	696	84.14	33.72	44.45
	720	84.86	34.59	45.04

Table B-2.2 Experimental data of free cell (effluent) concentration in packed bed reactor of ACE carrier with dilution rate of 0.09, 0.16, 0.22 and 0.28 h^{-1}, stability test.

Dilution rate (h^{-1})	Time (hours)	Free cell concentration (g/l)
0.09	0	0.03
	8	0.11
	16	0.17
	24	0.21
	32	0.31
	48	0.42
	56	0.46
	64	0.41
	72	0.47
	80	0.49
	88	0.46
	96	0.50
0.16	104	0.56
	112	0.54
	120	0.51
	128	0.55
	136	0.50

Dilution rate (h^{-1})	Time (hours)	Free cell concentration (g/l)
	144	0.53
	152	0.54
	160	0.52
	168	0.53
0.22	176	0.60
	184	0.59
	192	0.61
	200	0.57
	208	0.57
	216	0.58
	224	0.56
	232	0.59
	240	0.58
0.28	248	0.67
	256	0.61
	264	0.63
	272	0.65
	280	0.60
	288	0.63
	296	0.64
	304	0.67
	312	0.66
0.09	336	0.71
	360	0.66
	384	0.70
	408	0.67
	432	0.74
	456	0.70

Dilution rate (h^{-1})	Time (hours)	Free cell concentration (g/l)
	480	0.72
	504	0.66
	528	0.70
	552	0.73
	576	0.72
	600	0.68
	624	0.71
	648	0.66
	672	0.69
	696	0.71
	720	0.70
	Mean	0.61
	SD	0.0827

Table B-2.3 Experimental data of ethanol productivity in packed bed reactor of ACE carrier with dilution rate of 0.09, 0.16, 0.22 and 0.28 h^{-1}.

Dilution rate (h^{-1})	Ethanol concentration (g/l)	Productivity (g/l)
0.09	86.58 (± 1.33)	7.79 (± 0.19)
0.16	68.49 (± 1.37)	10.96 (± 0.22)
0.22	53.35 (± 2.00)	11.74 (± 0.44)
0.28	44.09 (± 1.77)	12.34 (± 0.50)

; Ethanol concentration was calculated from the average at steady state.

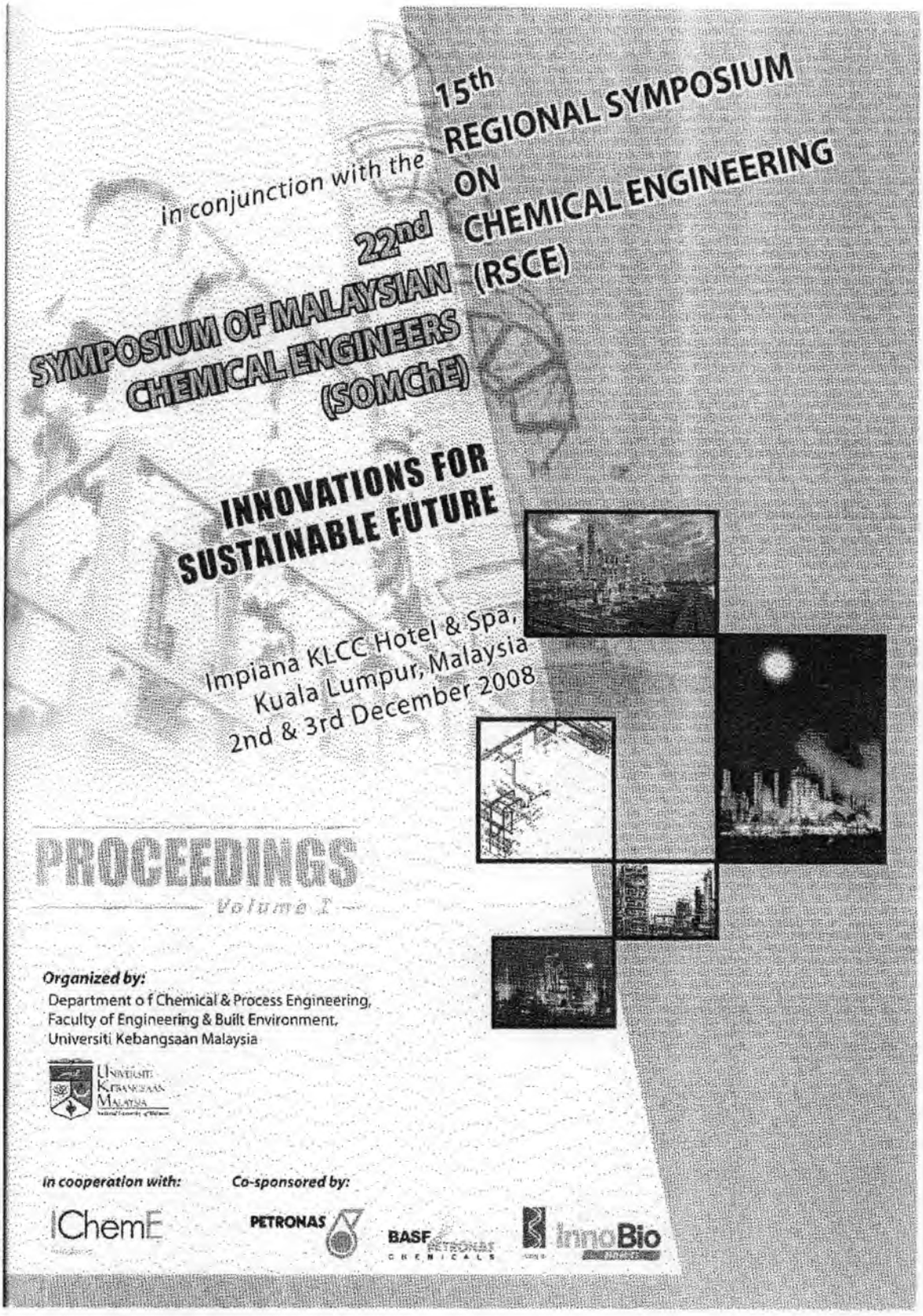
APPENDIX C

LIST OF PUBLICATION

International conferences

1. Jirawan Mongkolkajit, Jiranan Pullsirisombat, Seeroong Prichanont, Veerapat Tantayakom, Phatthanon Prasitchoke, and Muenduen Phisalaphong, "γ – ALUMINA DOPED ALGINATE GEL FOR CELL IMMOBILIZATION IN FERMENTATION PROCESSES" , Full text proceeding for 15th Regional Symposium on Chemical Engineering In Conjunction With 22nd Symposium of Malaysian Chemical Engineering, Malaysia, 2 - 3 December 2008, Paper ID BIO010-O.

2. Jirawan Mongkolkajit and Muenduen Phisalaphong, "Development of Alumina Doped Alginate Gel as a Cell Carrier for Ethanol Fermentation" , Full text proceeding for The 2nd Thammasat University International Conference on Chemical, Environmental and Energy Engineering, Thailand, 3 - 4 March 2009, Paper ID ChE-074.


15th REGIONAL SYMPOSIUM ON CHEMICAL ENGINEERING (RSCE)
in conjunction with the
22nd SYMPOSIUM OF MALAYSIAN CHEMICAL ENGINEERS (SOMChE)
INNOVATIONS FOR SUSTAINABLE FUTURE
Impiana KLCC Hotel & Spa,
Kuala Lumpur, Malaysia
2nd & 3rd December 2008
PROCEEDINGS
Volume 1
Organized by:
Department o f Chemical & Process Engineering,
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RSCE-SOMCHE 2008
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γ – ALUMINA DOPED ALGINATE GEL FOR CELL IMMOBILIZATION IN FERMENTATION PROCESSES

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

ABSTRACT

γ-Alumina (γ-Al_2O_3) doped alginate gel (AEC) was developed as a cell carrier in fermentation process 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 repeated batch modes, 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 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, the instability of AEC cultures for reuse in 1,3-propanediol fermentation was revealed in the repeated batch mode. Interfering of positive charge of γ-Al_2O_3 on the cell membrane was thought as the cause of inactivity of *C. butyricum* DSM 5431 on 1,3-propanediol production.

INTRODUCTION

Due to the world energy crisis, which increased industrial focus on renewable energy resource and by-product valuable. The production of ethanol from renewable carbohydrate materials has become interesting worldwide (Bai et al., 2008, Yu et al., 2007). Sugar cane molasses is a low cost and abundant 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 because glycerol is a main by-product of biodiesel production. The conversion 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 that helps to convert glycerol into products of high value. Under anaerobic condition, glycerol can be converted to 1,3-propanediol by bacterial cells. 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 production. The immobilization of cells leads to 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 also 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 simplicity and non-toxic (Verbelen et al., 2006, Arasaratnam, 1994).

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

MATERIALS AND METHODS

Microorganism

S.cereviceae M30 strain was kindly provided by Assoc. 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.cereviceae M30 was grown in 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 5, and sterilized at 121 °C for 15 min. Cell cultivation was carried in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C for 20 hours in order to obtain high cell density. The late exponential phase cells were harvested by decantation to obtain stock cell suspension.

C.butyricum DSM 5431 was grown in 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. The late exponential phase cells were harvested by decantation to obtain stock cell suspension.

Immobilization

Immobilization of cells was investigated using two general methods. The first was the adsorption of cells onto the surface of 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 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 mixture was used to construct γ-alumina doped alginate gel (AEC) carriers. The formation 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 (⌀) 3 mm were left to harden in $CaCl_2$ solution for 30 minutes and then rinsed 3 times with 0.9% w/v NaCl.

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 5. Immobilized cells were culture in 500 ml Erlenmeyer flask containing 250 ml fermentation medium. Batch fermentation was performed in shaker at 150 rpm, 33°C.

For 1,3-propanediol fermentation, The composition of the fermentation 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 $CaCl_2.2H_2O$; 1 g yeast extract; 80 g glycerol; 1 ml trace element solution; 2 ml Fe solution at pH 7. Immobilized cells were culture 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). Residual sugars were measured using the 3,5-dinitrosalicylic acid (DNS) method through a corresponding standard curve. 1,3-Propanediol assay was measured by HPLC (LC-3A, Shimadzu, Japan). Biomass concentrations were measured as optical density (UV-2450, Shimadzu, Japan) at 660 nm for *S.cereviceae* M30 and 650 nm for *C.butyricum* DSM 5431, and correlated directly with cell dry weight. Free cells were measured by centrifuge liquid samples from fermentation. For immobilized cell determination, immobilized cell were separated from the support by dissolved in 0.5 M sodium citrate and then, the suspension was treated similarly as fermentation broth to obtain its corresponding immobilized cell concentration.

RESULTS AND DISCUSSION

Ethanol fermentation

Ethanol fermentation was carried out with 220 g/l of initial sugar concentration from molasses as a carbon source for *S.cereviceae* M30. The fermentation in this study were performed using suspended cell (SC) culture, immobilized cells on γ-Al_2O_3 (AC) culture and adsorption a γ-Al_2O_3-entrapment alginate (AEC) culture in a single batch for 60 h and a 4-cycle repeated batch with the duration of each batch of 48 h.

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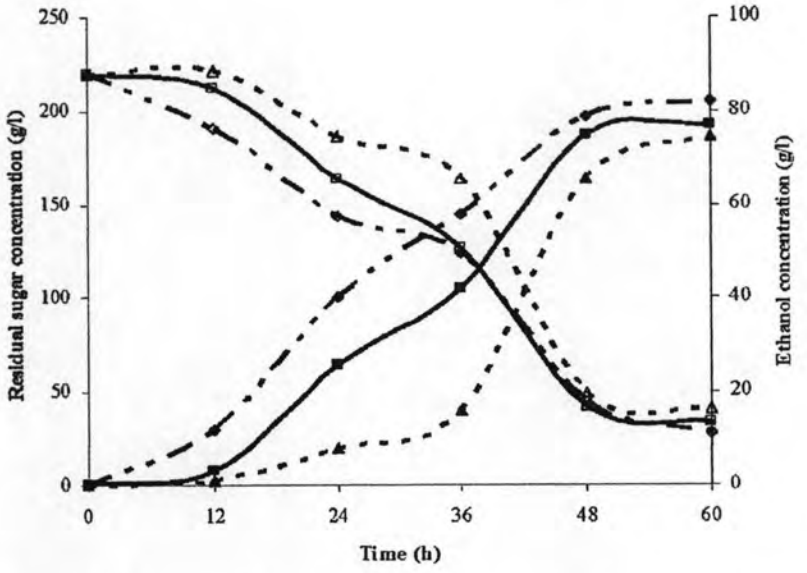

FIGURE 1: Time-course of single batch fermentation by *S.cerevisiae* M30 at 220 g/l of initial sugar concentration:
—◆··, SC-ethanol; —◇··, SC-sugar; —■—, AC-ethanol;
—□—, AC-sugar; --▲--·, AEC-ethanol and --△--·, AEC-sugar

In a single batch mode, the total cell concentration of AC (4.3 g/l) and AEC (4.12 g/l) were obtained, which were slightly higher than that of SC (3.9 g/l). The immobilization yields of the AC and AEC were 89.3% and 85.6% respectively. It was demonstrated that γ-Al_2O_3 has a positive effect on the growth of *S.cereviceae* M30. This is similar to the finding of M. 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 3-6.5 owing to the opposite electric charges (K. Kana et al., 1989). As shown in Fig. 1, the concentration of sugar was gradually decreased while the ethanol concentration was increased for a duration of 60 h. The final ethanol concentration of the SC, AC and AEC system were 82.4, 77.1 and 74.6 g/l, respectively ($Y_{P/S}$ 43%, 41% and 42%, respectively). In a range of fermentation time from 12 to 36 h, it was shown that the ethanol concentration of AEC system was lower than that of AC system. This could be indicated that mass transfer resistance did affect cell growth and product formation. However, the final ethanol concentration of AEC increased until it reached to similar level with AC at the end of fermentation.

TABLE 1: Yields and end products of repeated batch ethanol fermentations for 48 h, using the cultures of suspended cell (SC) culture, immobilized cells on γ-Al_2O_3 (AC) culture and adsorption γ-Al_2O_3-entrapment alginate (AEC) culture

Batch	P (g/l)	X (g/l)		Y_I (g/g)	Y_S (g/g)	$Y_{P/S}$ (g/g)
		X_E	X_I			
I						
SC	77.06	-	-	-	0.79	0.43
AC	67.25	-	-	-	0.78	0.39
AEC	69.73	-	-	-	0.78	0.40
II						
SC	72.88	-	-	-	0.80	0.40
AC	70.27	-	-	-	0.78	0.40
AEC	67.17	-	-	-	0.76	0.39
III						
SC	72.75	-	-	-	0.79	0.40
AC	69.46	-	-	-	0.77	0.40
AEC	71.80	-	-	-	0.79	0.40
IV						
SC	70.87	4.50	-	-	0.78	0.39
AC	71.80	0.56	5.25	0.90	0.78	0.41
AEC	70.74	0.78	4.96	0.86	0.78	0.40

For a repeated batch mode, the results of the fermentation are summarized in Table 1. In the first batch, after 48 h ethanol concentration of the SC system was 77.06 g/l, whereas the final ethanol concentrations of immobilized cells (IC) in AC and AEC systems were 67.25 g/l and 69.73 g/l, respectively. In the second and the third batch, all system exhibited the ethanol productions without any occurrence of the lag phase. In the fourth batch, the majority of sugar was consumed with the final ethanol concentrations being 70.87, 71.80 and 70.74 g/l for SC, AC and AEC carriers, respectively. The final total cell concentration of AC (5.81g/l, Y_I 90%) and AEC (5.74 g/l, Y_I 86%) were higher than that of SC (4.50 g/l). Increases in cell concentrations in AC and AEC carriers were due to 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 SC system dropped from 77.06 g/l in the first batch to 71-73 g/l in the second to the fourth batch, which maybe attributable 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 IC carriers in AC and AEC carriers were relatively stable because the ethanol yield factors ($Y_{P/S}$) were quite stable in all batches. The

stability of IC cultures were higher than SC since the matrix of the IC carriers can protect yeast by fortification from toxins and inhibitor (Verbelen et al., 2006, Kourkoutasa et al., 2004, Phisalaphong et al., 2007).

1,3-propanediol fermentation

The fermentation of *C.butyricum* DSM 5431 was carried out in the 1 L glass fermenter with 80 g/l initial glycerol concentration. The fermentation in this study were SC, AC and AEC carriers. 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 h.

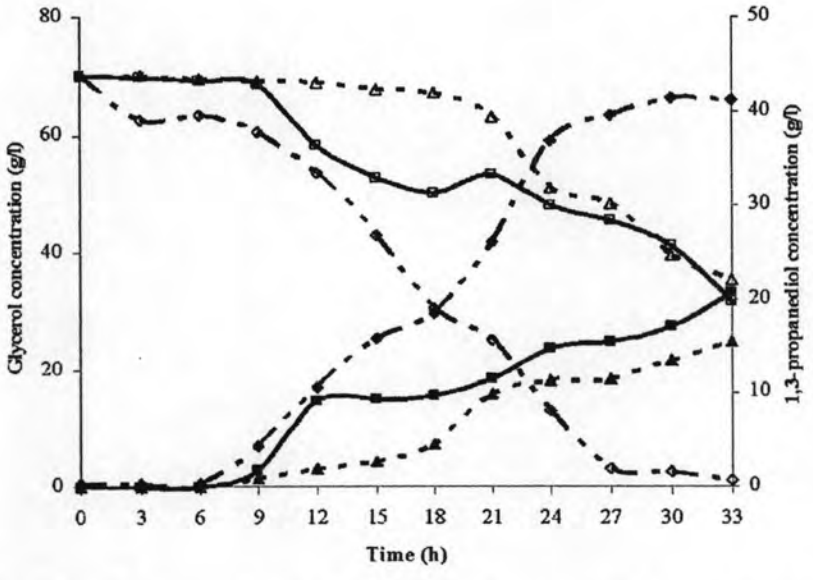

FIGURE 2: Time-course of single batch fermentation by *C.butyricum* DSM 5431 at 80 g/l of initial glycerol concentration:
—◆··, SC-1,3-propanediol; —◇··, SC-glycerol;
—■—, AC-1,3-propanediol; —□—, AC-glycerol ;
--▲--, AEC-1,3-propanediol and --△--, AEC-sugar

At the end of single batch fermentation, the total cell concentration of AC (4.37 g/l) and AEC (3.57 g/l) were higher than that of SC (3.11 g/l), the immobilization yield of the AC and AEC were 79.6% and 83.2% respectively. It was confirmed that IC carriers can be preserved and promoted the growth of *C.butyricum* DSM 5431.This result agrees with the ethanol fermentation experiment. As shown in Fig. 2, the final 1,3-propanediol concentration of the SC, AC and AEC system were 41.37, 20.61 and 15.47 g/l, respectively. The 1,3-propanediol yield ($Y_{P/S}$) of AC (25.76%) and AEC (19.34%) carriers were lower than that of SC (51.71%)

culture. It was found that in the system with IC carriers, there were occurrence of inhibition effect on 1,3-propanediol production, caused by γ-Al_2O_3 based carrier. High positive charge density on the surface of the carrier (γ-Al_2O_3) was considered negative interfering in the activities of the cells. The adverse effects on cell membrane could affect its enzyme activities.

TABLE 2: Yields and end products of repeated batch 1,3-propanediol fermentation for 24 h each batch using the cultures of suspended cell (SC) culture, immobilized cells on γ-Al_2O_3 (AC) culture and adsorption γ-Al_2O_3-entrapment alginate (AEC) culture

Batch	P (g/l)	X (g/l)		Y_I (g/g)	$Y_{P/S}$ (g/g)
		X_E	X_I		
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	4.38	-	-	0.33
AC	12.5	1.14	4.56	0.80	0.16
AEC	3.3	0.8	3.91	0.83	0.04

In 4-cycle repeated batch fermentations, the experimental results are shown in Table 2. The 1,3-propanediol concentration 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 (46.12% to 33%). It can be attributable to the effect of the product and by-product from this process such as butyric acid and acetic acid which could also be toxic directly to cells. The stability of the AC culture was relatively higher than that of SC culture, however 1,3-propanediol production of the AC culture was much lower than that of SC culture. The stability of AEC culture was significantly decreased due to the negative effect of γ-Al_2O_3 on the cell activity together with the decrease in diffusivity from alginate entrapment.

CONCLUSION

This study indicated that the production of ethanol by immobilized *S. cerevisiae* M30 in the AC and AEC carriers was promising to apply for high ethanol production with high density of biomass and high stable cell activity. In 4-cycle repeated batch ethanol fermentation, AC and AEC carriers illustrated a good potential of reusability. The ethanol production of AC and AEC carriers were more stable than SC culture. However, the AC and AEC based carriers were found unfavorable for *C.butyricum* DSM 5431 immobilization because of the inhibition effect of γ-Al_2O_3 on the cell activity. In the 4-cycle repeated batch of 1,3-propanediol fermentation, AC and AEC carrier revealed the instability of the cell cultures. The overall 1,3-propanediol productions using the immobilized cell systems were much lower than that in the suspended cell system.

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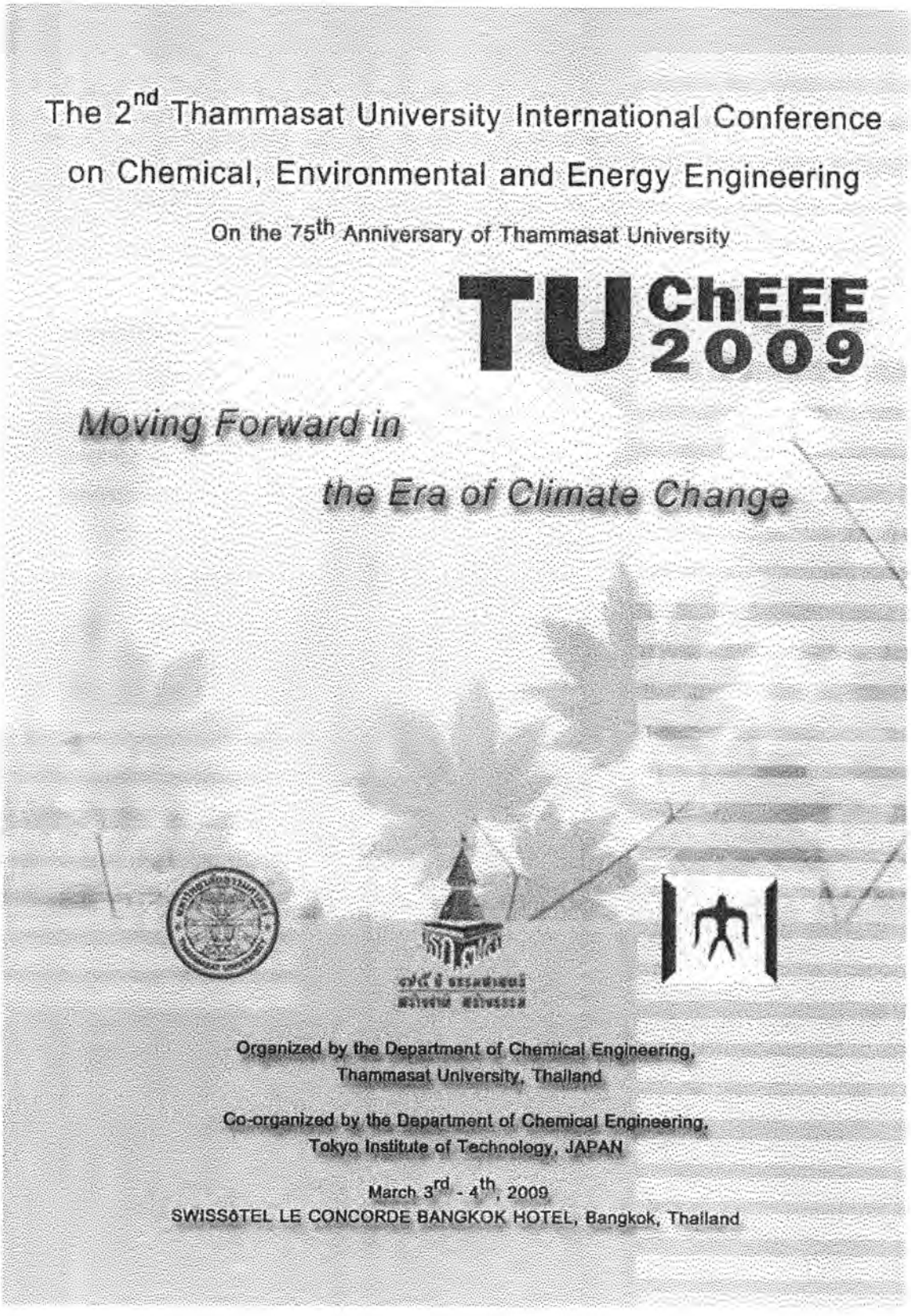
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Development of Alumina Doped Alginate Gel as a Cell Carrier for Ethanol Fermentation

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Abstract

Alumina (Al_2O_3) doped alginate gel (AEC) was developed as a cell carrier in fermentation process of *Saccharomyces cerevisiae* M30 for ethanol production. The immobilized cell carrier was performed in two bead diameter sizes (4 mm and 6 mm). The AEC carrier was found to be more effective for yeast immobilization than conventional calcium alginate beads. In terms of ethanol fermentation performance, the addition of Al_2O_3 could promote cell activities resulting in higher ethanol production in AEC culture (86.8 - 91.7 g/l) than that in Ca-alginate bead (81.9 - 89.5 g/l) or suspended cell (85.1 g/l) cultures. The AEC carriers have many advantages for long-term use including good mechanical strength, chemical stability and high immobilization yield.

Keywords: Alumina, alginate, immobilization, strength, *Saccharomyces cerevisiae*

1. Introduction

Due to the world energy crisis, the production of ethanol from renewable carbohydrate materials has become interesting worldwide. Sugar cane molasses is a low cost and abundant material in Thailand. It is a by-product from sugar industries that can be fermented by yeast to produce ethanol under anaerobic condition.

Ethanol fermentation by conventional batch suffers from various constrains such as low cell density, nutritional limitation and rather time consuming. Immobilized cell technology has been suggested as an effective mean for improved production. The immobilization of cells leads to 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 [1, 2]. For improving the performance of immobilized cell carrier, alumina (Al_2O_3) particle and calcium alginate were applied as materials for constructing immobilized cell carriers. Al_2O_3 has also been reported as a good support for cells because of the electrostatic attraction between Al_2O_3 and cells [3]. Calcium alginate is the most wildly used material for entrapment because of simplicity and non-toxic [1].

In this study, Adsorption and entrapment techniques are used together for improving the drawback of immobilization for fermentation process. Al_2O_3 doped alginate gel (AEC) is developed as a new type of cell carrier for fermentation processes. To test this immobilization technique, the immobilization system of *Saccharomyces cerevisiae* M30 for ethanol production from sugar cane molasses is used to evaluate the performance of the new carrier. This system is examined in batch fermentation. The activities of the immobilized cells within the AEC are then compared to the systems of suspended cells (SC) and immobilized cells by entrapped in calcium

alginate (EC). The immobilized cell carrier (AEC and EC) is examined in two bead diameter sizes that are 4 mm and 6 mm.

2. Experimental

2.1 Microorganism

S. cerevisiae M30 strain was kindly provided by Assoc. Prof. Dr Savitree Limtong, from Department of Microbiology, Kasetsart University, Bangkok. The culture was stored in Potato Dextrose Agar (PDA) slant at 4 °C.

2.2 Pre-culture and immobilization

S. cerevisiae M30 was grown in 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 and 0.035 g $MgSO_4.7H_2O$. The medium was adjusted to pH 5, and sterilized at 121 °C for 15 min. Cell cultivation was carried in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C for 20 hours. In order to obtain high cell density, the late exponential phase cells were harvested by decantation to obtain stock cell suspension.

2.3 Immobilization

Immobilization of cells was investigated using two general methods. The first was the adsorption of cells onto the surface of Al_2O_3 powder. The second was entrapment of Al_2O_3 - cells in calcium alginate matrix. To prepare AEC carrier, sterilized Al_2O_3 powder was immersed in pre-culture medium and incubated with cell for 20 hours to induce natural cells adhesion. After that, Al_2O_3-cell mixture and cells were added to 3% w/v sodium alginate solution with volumetric ratio of 1:1 to form an alginate-Al_2O_3-cell mixture and alginate-cell mixture, respectively. The mixture was used to construct AEC and EC carriers. The formation was initiated by adding the mixture drop wisely into 500 mL sterile 0.12M $CaCl_2$ solution by using Gilson Pipetman auto pipette. The carriers were left to harden in $CaCl_2$ solution for 30 minutes and then rinsed three times with sterile 0.9% w/v NaCl solution to remove excess Ca^{2+} and untrapped cells.

2.4 Fermentation

Sugar cane molasses was used as carbon source for the fermentation medium. The composition of the fermentation medium per liter was: 220 g reducing sugar from molasses; 0.5 g $(NH_4)_2SO_4$ at pH 5. The prepared medium was sterilized at 121°C for 15 min. Experiments were initiated by transferring prepared cell suspension or immobilized cells into 500 ml Erlenmeyer flasks containing 250 ml of the fermentation medium in order to promote anaerobic condition which was favourable ethanol fermentation. Batch fermentation in shake flasks was performed in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C for 72 hours.

2.5 Analytical methods

Ethanol concentration was determined using gas chromatography (GC-7AG, Shimadzu, Japan). Residual sugars were measured using the 3,5-dinitrosalicylic acid (DNS) method through a corresponding standard curve. Cell concentration was determined by cell dry weight method. The sample of fermentation broth was centrifuged and washed with HCL 0.1 N and water respectively. The cell concentrations were measured as optical density (UV-2450, Shimadzu, Japan) at 660 nm and converted to dry cell concentration on the basis of a corresponding standard curve. For immobilized cell determination, immobilized cell were separated from the support by dissolved in 0.5 M sodium citrate and then, the suspension was treated similarly as fermentation broth to obtain its corresponding immobilized cell concentration. The mechanical properties of EC and AEC carrier were evaluated in term of tensile strength and percent elongation at brake by Universal Testing Machine-H 10 KM (Hounsfield). The test condition follows ASTM D882. The sample was cut into strip-shaper specimens 10 mm in width and 10 cm in length. At least five specimens were used for each carrier tested.

3. Results and Discussions

Ethanol fermentation was carried out with 220 g/l of initial sugar concentration from molasses as a carbon source for *S.cerevisiae* M30. The fermentation were performed using

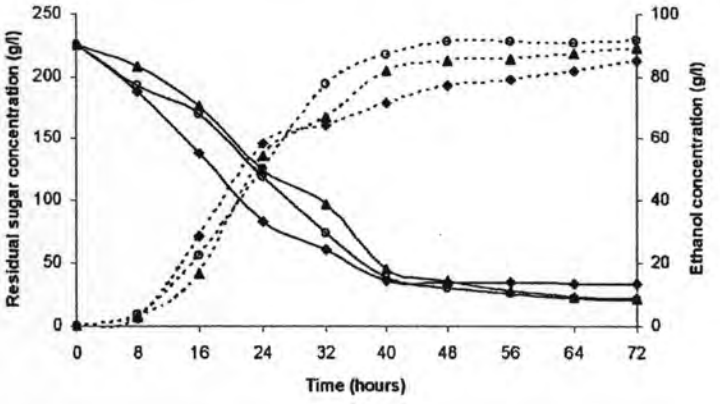

Fig. 1 Residual sugar concentration and ethanol concentration profile in fermentation of 4 mm bead diameter size; Residual sugar concentration (solid lines) and Ethanol concentration (dash lines); ◆, SC; ▲, EC and O, AEC

suspended cell (SC) culture, immobilized cells by entrapment in Ca-alginate (EC) culture and adsorption a Al_2O_3-entrapment alginate (AEC) culture in batch fermentation for 72 hours. The samples were harvested every eight hours for cell, sugar and ethanol analyses.

As shown in Figure 1, the concentration of sugar was gradually decreased while the ethanol concentration was increased for duration of 72 hours. In comparison between the result of SC system and that from the immobilized systems, the sugar consumption and ethanol production in the immobilized systems were relatively lower than those of the SC system during the first period of 24 hours, but became higher at the end of the fermentation. The higher cell activities of AEC and EC system than that of SC system should be owing to the maintenance of cell activity by protection from inhibitors such as ethanol of the immobilized cell system. The ability of cells to grow inside the carriers made it possible for cell regeneration and product formation under hostile conditions such as high ethanol concentration. It has been previously suggested that the matrix of the immobilized cells carriers protected yeast by fortification from toxins and inhibitor [1, 4]. Overall, the final ethanol concentrations of AEC carrier were relatively higher than that of EC carrier. Therefore, the addition of Al_2O_3 (AEC) could promote cell activities resulting in higher ethanol production.

The structure and size of cell carrier are important factors affected cell activities. The yields and the end product concentrations from the system of SC, and AEC and EC with bead diameter of 4 mm and 6 mm are summarized in Table 1. At the end of the fermentation, it was found that the total cell concentrations of the immobilized cell systems were slightly higher than that of the SC system. The increase of cell concentrations inside immobilized cell carriers during the fermentation indicated that yeast cells could regenerate inside the immobilized cell carriers. It was confirmed that the AEC and EC carriers preserved the cells and promoted the cell growth.

The total cell concentrations and immobilization yields of AEC carrier of both sizes were relatively higher than that of EC carrier. The result supported that Al_2O_3 has a positive effect on the growth of *S.cerevisiae* M30. This was similar to the finding of Kana et al. (1989) who reported that Al_2O_3 was a good supporter of ethanol fermentation because of the electrostatic attraction between positive charge of Al_2O_3 particles and negative charged on the yeast cell wall [5].

Table 1 Yield and end products of batch ethanol fermentation for 72 hours using the cultures of SC, EC and AEC

System	Ethanol (g/l)	Residual sugar (g/l)	Free cell (g/l)	Immobilized cell (g/l)	Y_I (%)	$Y_{P/S}$ (%)
SC	85.07	33.62	3.74	-	-	44.42
Ø 4 mm						
EC	89.52	21.44	0.76	3.26	81.09	43.95
AEC	91.67	20.29	0.81	3.54	81.38	44.75
Ø 6 mm						
EC	81.93	35.84	0.91	2.96	76.49	43.28
AEC	86.76	28.38	0.76	3.36	81.55	44.10

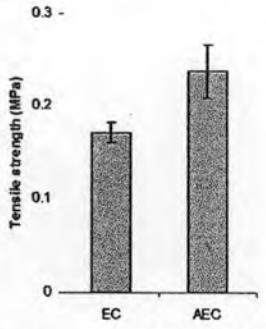

Fig. 2 Tensile strength of EC and AEC carriers

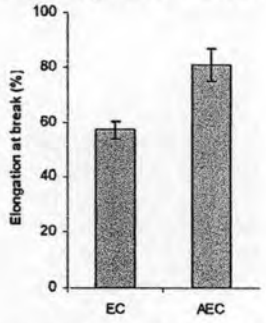

Fig. 3 Elongation at break of EC and AEC carriers

From the comparison of diameter sizes, the increasing of bead diameter from 4 mm to 6 mm resulted in the decrease of ethanol concentration at 5.4 % in AEC carrier system and 8.5 % in the EC carrier system. While the immobilization yield (Y_I) of the AEC remained constant at around 81 %, the Y_I of the EC dropped from 81% to 76 % with the increase of the diameter from 4 to 6 mm. The decrease of ethanol production and immobilization yield (Y_I) of the EC carrier at Ø 6 mm could be due to the drop of cell growth and metabolite inside the carrier. At large size, accessibility of the nutrient into inner portion of the carrier might become the limiting factor for cell activities, especially for the carrier with the dense structure. This result revealed that the mass transfer limitation through the porous structure of the AEC carrier was less severe than that of the EC carrier.

Moreover, with the addition of Al_2O_3, the mechanical property of the carrier was also improved. The mechanical properties of AEC and EC carrier were compared in term of tensile strength and percentage elongation at break. Tensile strength measures material strength, whereas elongation at break is an indicator of toughness and stretch-ability prior to breakage. These parameters dictate the end-use handling properties and mechanical performance of the films [6]. The mechanical properties of AEC and EC carriers were summarized in Figure 2 and 3. Tensile strength and % elongation at break of the AEC carrier (0.24 MPa and 80.8%, respectively) were significantly higher than those of the EC carrier (0.17 MPa and 57.1%, respectively). The greater strength and flexibility of the AEC carrier might be caused by the presence of Al_2O_3 particle that constructed the network inside the gel and help to support, link or hold the molecule together. It exhibited that the AEC carrier had better mechanical property than the EC carrier.

4. Conclusion

Alumina (Al_2O_3) doped alginate gel (AEC) has been developed as a cell carrier in fermentation process of *Saccharomyces cerevisiae* M30 for ethanol production. The AEC carrier was proven to be more effective for cell immobilization than the conventional Ca-alginate bead (EC). Higher ethanol production and higher cell density of the AEC cultures indicated that the carrier could protect the cells from unfavourable environment and promoted the cell growth. The mass transfer limitation through the porous structure of the AEC carrier was less severe than that of the EC carrier. Moreover, with the integration of Al_2O_3 inside the AEC carrier, it exhibited better mechanical property than that of the EC carrier.

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