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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 ±5 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 20 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 flocculating yeast M30 on the PDA agar slants. The procedures are as follows.

1. Sterilize all equipments and agar slants with UV (ultraviolet) 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. The main component of the medium in earlier experiments (until fermentation 3) was palm sugar which was used as carbon and energy source for the yeast. Palm sugar was dissolved to obtain sugar concentration of about 5% and 20% w/v for cell cultivation and ethanol production respectively. The resulting sugar solution had a brown color originated from the palm sugar. The color intensity increases with increasing sugar concentration. The amount of palm sugar required to achieve the target level of sugar was estimated from previous trial with DNS method (see Section A-7)

For 1 liter of sugar solution, nutrients consisted of 0.1 g KH_2PO_4, 0.035 g $MgSO_4.7H_2O$, and 0.5 g $(NH4)_2SO_4$ were added. The compositions were referred to the one which were used by ethanol producing industries. The pH value of the medium was adjusted to 5 with 0.1 M NaOH and HCl solution. The detailed procedures for medium preparation from palm sugar are listed in the following paragraph.

1. Mix palm sugar and nutrients. Add palm sugar until the desired sugar concentration (5% for cell cultivation and 20% w/v for ethanol production) is achieved.
2. Adjust the pH of the solution to 5 by adding NaOH or HCl solution.
3. Pour appropriate volume of medium (150 mL and 250 mL for inoculums development and ethanol fermentation respectively) through a sieve or screen into 500 mL Erlenmeyer flask.
4. Close each flask with cotton plug and wrap with aluminum foil before sterilization.
5. Sterilize the mediums with autoclave for 20 min at 121°C.
6. 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.

In fermentation 4 and 5, molasses was used instead of palm sugar in the fermentation medium. For 1 liter of the medium 0.5 g $(NH4)_2SO_4$ was added as the sole supplement. Before sterilization, centrifugation of diluted molasses mash was necessary to prevent excess mud formation. The mud was created from suspended materials contained in molasses. Palm sugar was still used in inoculums development stage prior to ethanol fermentation. The quantity of molasses needed to reach the intended sugar level was also estimated by DNS trial. The procedures for preparing molasses based fermentation medium are follows:

1. Dilute the molasses mash to intended sugar level with DI water.
2. Centrifuge the solution with Kubota 6700 centrifuge at 2000 rpm for 15 minutes.
3. Mix the diluted sugar solution with appropriate amount of $(NH4)_2SO_4$.
4. Adjust the pH of to 5 with NaOH or HCl solution.
5. Fill 500 mL Erlenmeyer flask with 250 mL medium.
6. Close each flask with cotton plug before sterilization.
7. Autoclave the medium for 15 min at 121°C.
8. 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 1 day 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. Transfer ±4 mL of medium from the Erlenmeyer flask to the tube with auto pipette.
4. 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.
5. Scratch the yeast culture on the tube to detach the cells from the surface of the agar using the loop.
6. Pour the cell suspension into the Erlenmeyer flask and then close the flask using cotton plug.
7. Repeat steps 2-6 for the other flasks.
8. Put all flasks in the incubator shaker and then operate the shaker at 150 rpm 33°C for a day before harvesting the cells.
9. Let the cells to settle for a while after incubation and then carefully pour out 130 mL of the medium from each flask.
10. Combine the concentrated cells suspension from several flasks by pouring it into one flask.
11. Further draining can be done to concentrate cells by the same method until the desired volume of concentrated cells suspension is obtained.
12. 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

Alginate gel was used for both cell adsorption and entrapment purposes. Cell immobilization for chitosan was done only by adsorption because of the harsh condition encountered in chitosan gelation which might be harmful to cell activity. Alginate solution was made by dissolving Na-alginate powder in NaCl 0.9% w/v solution to obtain clear viscous solution with concentration of 30 g/L. Low and high viscosity chitosan flakes was slowly added to 1 M acetic acid solution to form 2% w/v yellowish chitosan solution. Alginate solution used for entrapment of cells was sterilized with autoclave for 5 minutes at 121°C prior to usage. Before gelation, chitosan and alginate solutions were kept overnight in refrigerator at 4°C to facilitate deaeration. For adsorption experiment, the polymeric gel of alginate and chitosan was formed first and then sterilized before proceeding with cell immobilization procedure. In entrapment process, alginate solution was mixed first with concentrated cell suspension before gelation. Formation procedures of chitosan gel were listed in the following paragraph.

1. To form chitosan beads, gradually drop chitosan solution into NaOH 1 M solution using syringe.
2. For reinforced gel formation, dip the preformed loofa sponge into chitosan solution and then drop it into the chelating NaOH 1 M solution. The shape and size of the gel depends on the shape and size of the dipped loofa sponge.
3. Leave the gel to harden for 24 hours.
4. Rinse the gel with deionized water. Immerse the washed gel in deionized water.
5. Sterilize the gel in autoclave for 5 min at 121°C.
6. Add the gel into concentrated yeast cells suspension.
7. Leave the mixture for 1 hour to let the adsorption to proceed naturally.
8. Precautions and notes:
   a) Chitosan solution is highly viscous so that chitosan flakes should be added slowly into acetic acid solution when preparing the solution.

b) The gel should be thoroughly rinsed with water to ensure proper removal of NaOH as basic condition is not suitable for yeast growth.

Gelation steps of alginate for cell adsorption and entrapment purpose were similar. The main difference between the two methods laid on the occurrence of initial mixing step between the concentrated cell suspensions with alginate solution in entrapment method. Preparation of adsorbing alginate gels are as follows:

1. Add alginate solution using a syringe drop wisely into $CaCl_2$ 14.7 g/L solution to form alginate beads.
2. For reinforced gel formation, dip the loofa sponge into alginate solution and then drop it into the $CaCl_2$ solution.
3. Leave the gel to harden under mild stirring for 15 minutes.
4. Rinse the gel 3 times with NaCl 0.9% w/v solution.
5. Sterilize the gel in autoclave for 5 min at 121°C.
6. Add the gel into concentrated yeast cells suspension.
7. Leave the mixture for 1 hour to let the natural adsorption to occur.
8. When storing is needed, keep the gels in NaCl 0.9% w/v solution at 4°C.

Entrapment of yeast cells in alginate is carried out similarly:

1. Mix concentrated cell suspension with alginate solution with volumetric ratio of 1:10.
2. Add the mixture using a syringe drop wisely into $CaCl_2$ 14.7 g/L solution to form alginate beads.
3. For reinforced gel formation, dip the loofa sponge into alginate solution and then drop it into the $CaCl_2$ solution.
4. Leave the gel to harden with mild stirring for 15 minutes.
5. Rinse the gel 3 times with NaCl 0.9% w/v solution.
6. When storing is needed, keep the gels in NaCl 0.9% w/v solution at 4°C.
7. Precautions and notes:
   a) All procedures are conducted aseptically in laminar flow hood.
   b) All equipments including the stirrer and syringe are cleaned and sterilized before use.

A-6 Ethanol fermentation

As in cell cultivation step, the procedure was initiated by aseptic inoculation of cells into the fermentation medium with sugar concentration about 20% w/v (200 g/L). Likewise, in the immobilized cells system, the cells were added along with their supporting materials into each flask containing the fermentation medium. The amount of immobilized cells carriers added was carefully measured as their wet weight before the start of every fermentation cycles. Ethanol fermentation was carried out in Innova 4330 Refrigerated Incubator Shaker at 150 rpm, 33°C.

In repeated batch mode, carriers and suspended cells from preceding batch were reused in subsequent cycles. For suspended cell culture, major portion (more than 80%) of the preceding fermentation broth was drained after decantation. The concentrated cell suspension was then transferred into 500 mL Erlenmeyer flask containing 250 mL fresh medium. Used carriers from previous run were screened and then transferred to flask containing fresh medium. The amount of carrier loaded into each flask was carefully controlled to maintain the same proportion (in wet weight basis) as in the main batch. This was done to incite similarity required for comparison between different fermentation cycles. All procedures were done carefully in aseptic condition.

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 3, 5-dinitrosalicylic acid (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 30% w/v was prepared by dissolving 30 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 ±2.6 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 Standard sucrose solution preparation

Source solution (mL)	Water (mL)	Sucrose concentration (% w/v)
0	2	0
0.5	1.5	6.25
1	1	12.5
1.5	0.5	18.75
2	0	25

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 0.1 mL of sample with 0.9 mL DI water in 16 x 100 mm screw cap tube.
2. Blend the sample with 0.25 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 0.4 mL NaOH 30% w/v and then mix with vortex mixer.
6. Centrifuge the sample at 3000 rpm for 10 minutes.
7. 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. In normal circumstances, the mixture of DNS reagent and the sample was readily homogenous. In some cases, yellow precipitates of DNS were formed as an indication of the insufficient basic condition. This can be solved by adding NaOH solution until homogenous solution is obtained.

The solution's color transformed from yellow to reddish brown in the course of reaction. The color intensity represents the corresponding sugar concentration which means that solution with higher sugar content can be recognized visually as the one with 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). Sample containing only water (0% sugar) which had been treated in the same manner as the other samples was used as blank for absorbance measurement. Complete procedures are described in the following paragraph.

1. Mix 0.1 mL of supernatant obtained from treatment I with 0.5 mL DNS reagent in 16 X 100 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. Dilute with water and transfer to other tube until the desired color intensity is gained.
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). The medium in this study could be classified in 2 forms: liquid and gel. The concentration of cells in a liquid medium was referred as free cell concentration. In the case of cells confined in or attached to gels, the gel was first dissolved before separated from cells by centrifugation.

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 0.05 M solution. In the case of loofa reinforced gel carriers, the loofa sponge 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 gel carrier with 6 mL sodium citrate 0.05 M in 16 x 100 mm rimless tube.
2. In case of loofa reinforced carrier, remove the loofa sponge from the suspension after the gel is totally dissolved.
3. Continue with same procedures as step 2-9 of section A-8.2.
4. Precautions and notes:
    a) Refresh the sodium citrate solution if the gel hasn't been dissolved in a single cycle. Repeat this procedure until all gel has been dissolved.
    b) Intermittent mixing with vortex mixer is recommended to promote faster gel dissolution.
    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 SEM images of Fermentation 4

B-1.1 Initial carriers

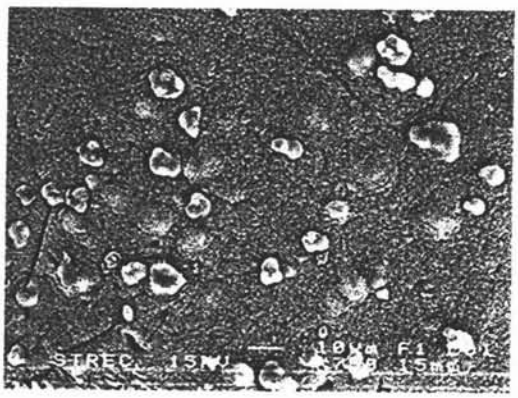

Figure B-1 EAB surface 1

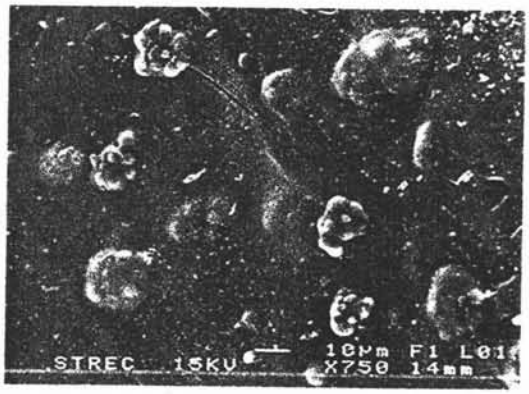

Figure B-2 EALC surface 1

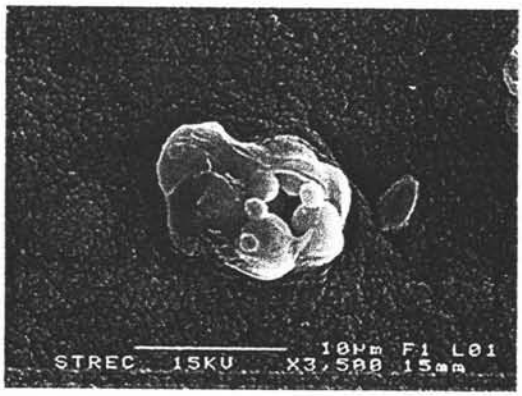

Figure B-3 EAB surface 2

Figure B-4 EALC surface 2

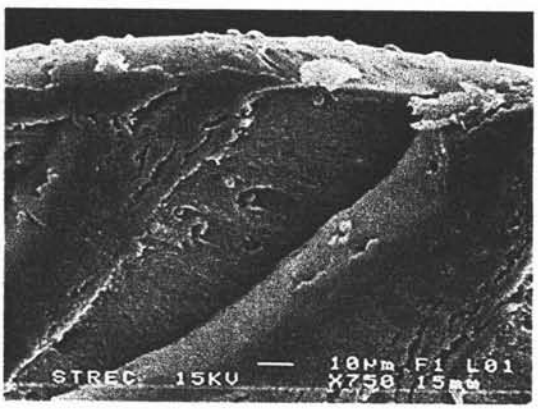

Figure B-5 EAB surface cross section 1

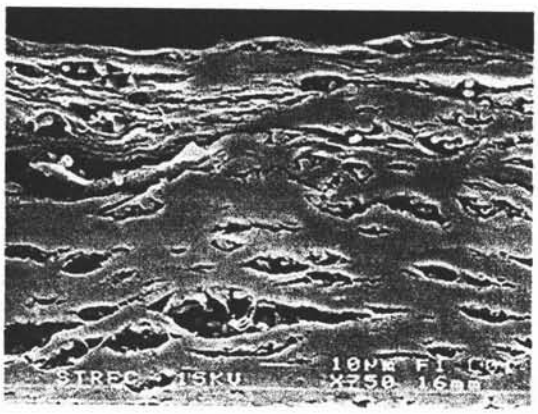

Figure B-6 EALC surface cross section

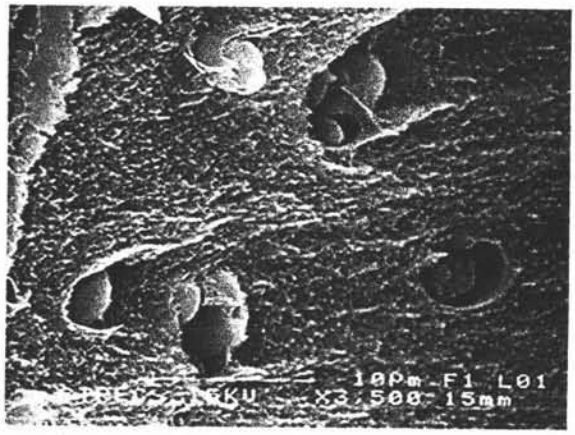

Figure B-7 EAB surface cross section 2

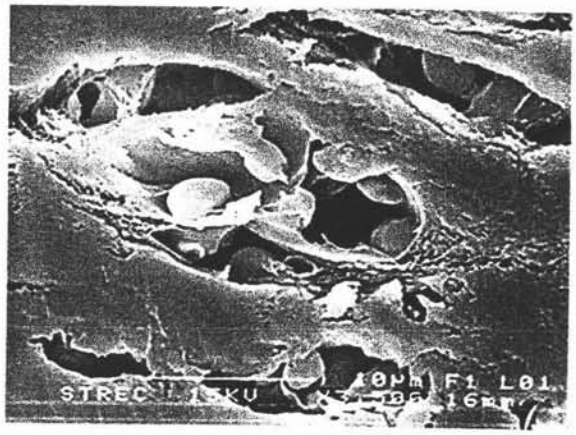

Figure B-8 EALC surface cross section 2

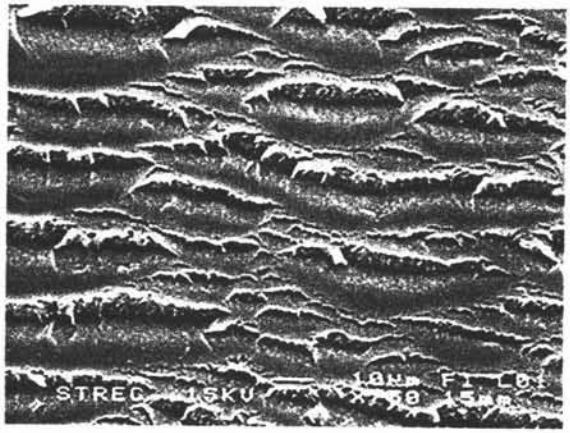

Figure B-9 EAB central cross section

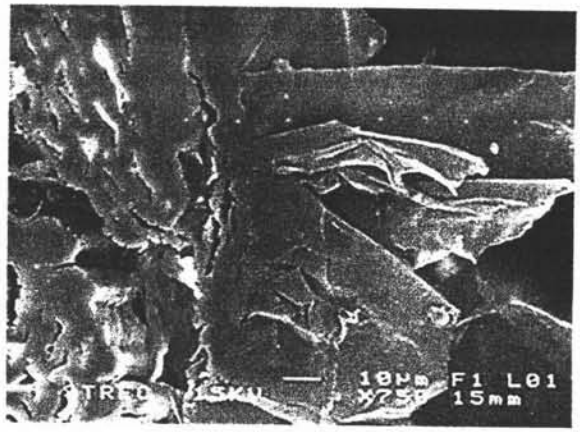

Figure B-10 EALC central cross section

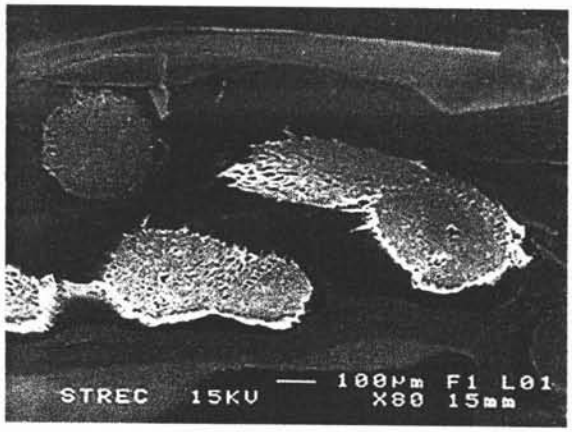

Figure B-11 EALC cross section

B-1.2 Carriers after main batch

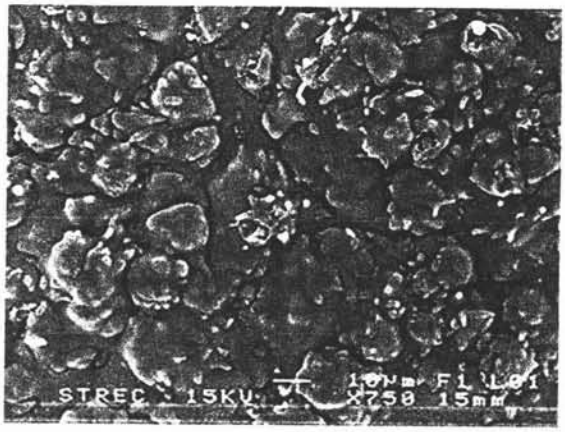

Figure B-12 EAB surface 1

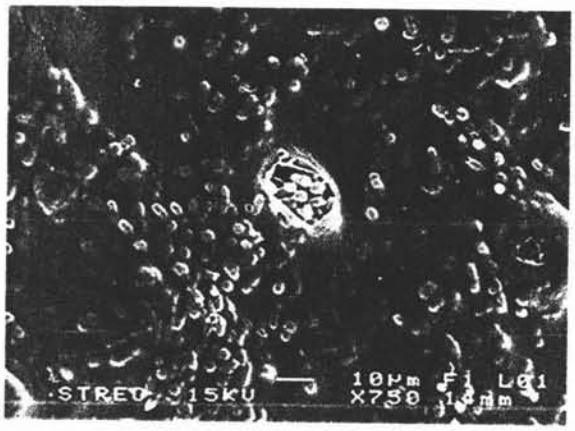

Figure B-13 EALC surface 1

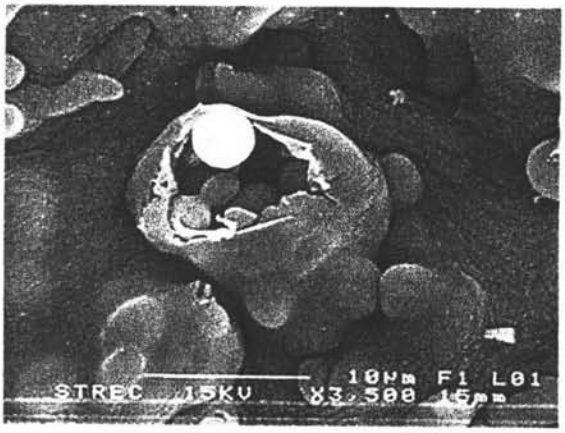

Figure B-14 EALC surface 2

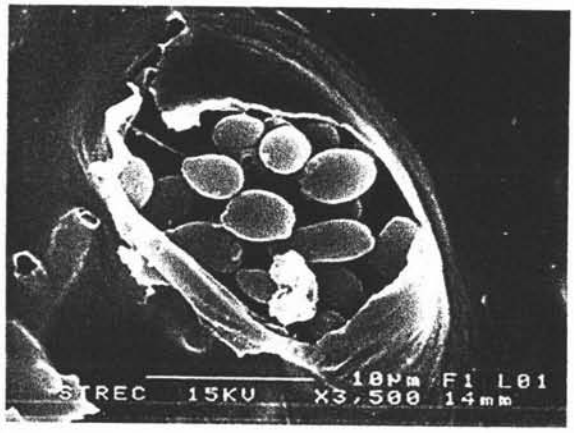

Figure B-15 EALC surface 2

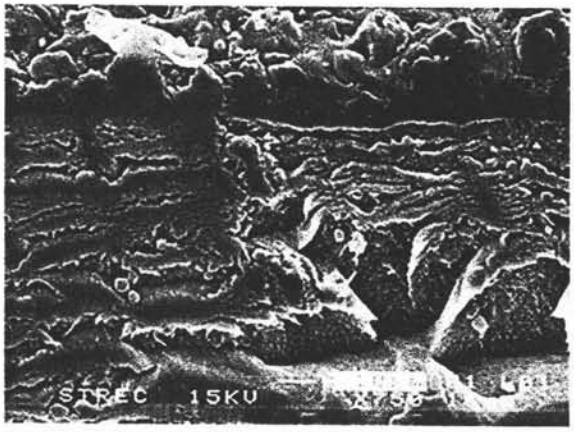

Figure B-16 EAB surface cross section 1

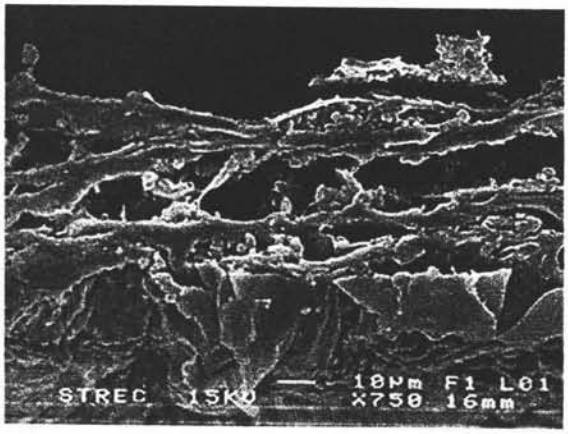

Figure B-17 EALC surface cross section 1

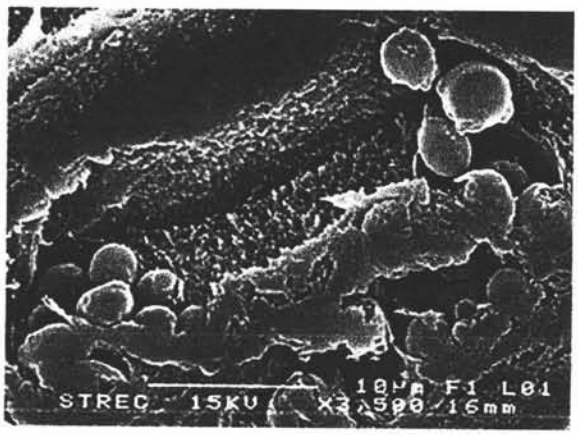

Figure B-18 EAB surface cross section

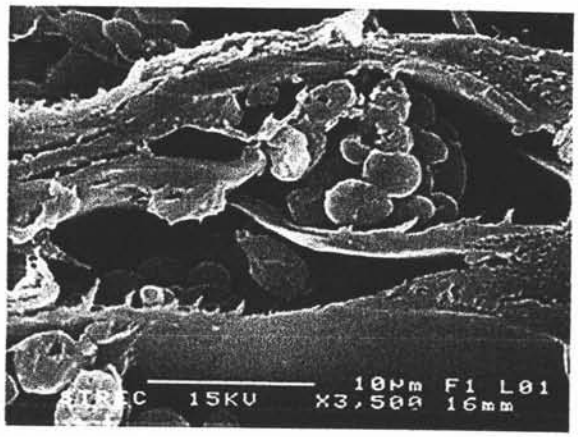

Figure B-19 EALC surface cross section 2

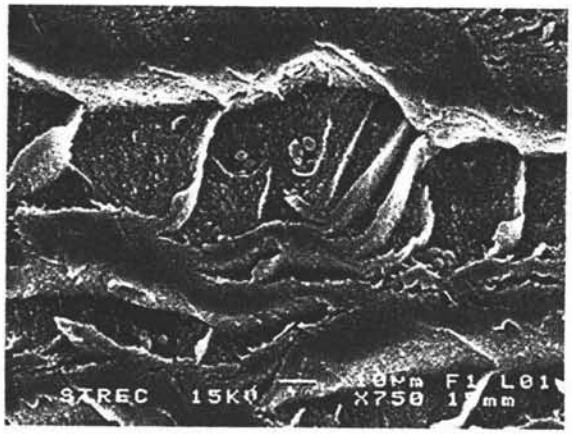

Figure B-20 EAB central cross section

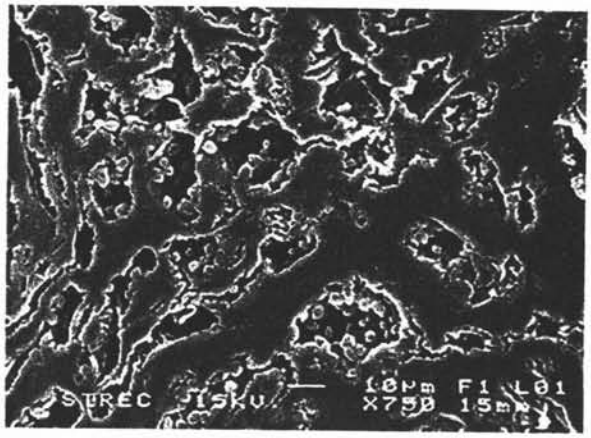

Figure B-21 EALC central cross section

B-1.3 Carriers after repeated batch 1

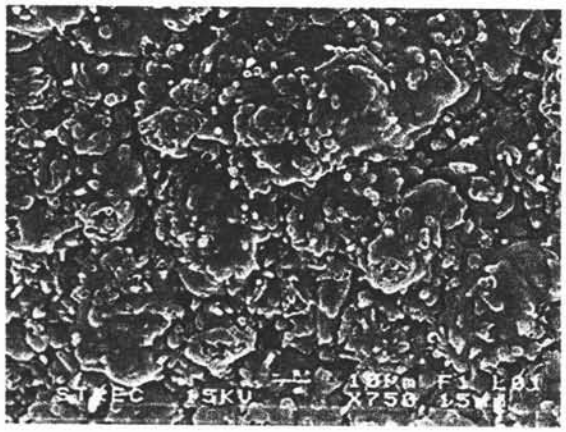

Figure B-22 EAB surface 1

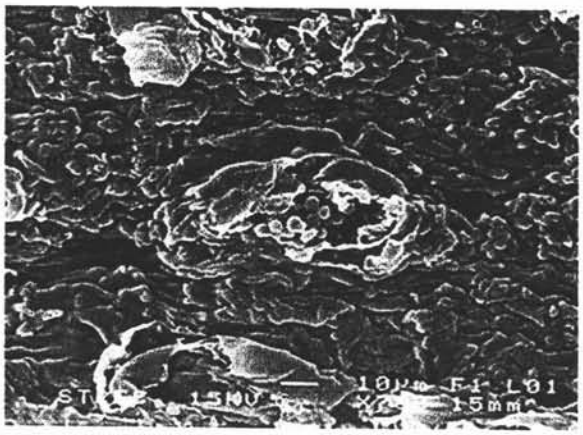

Figure B-23 EALC surface 1

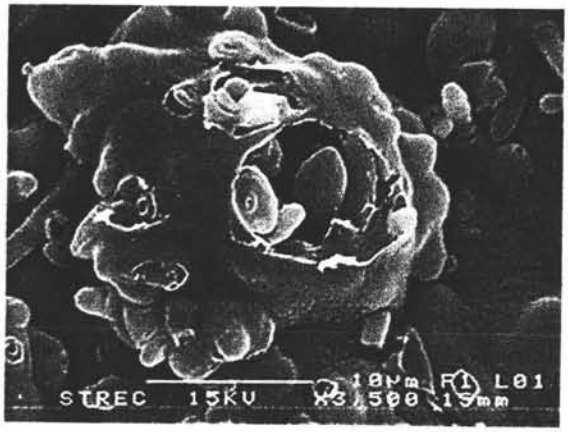

Figure B-24 EAB surface 2

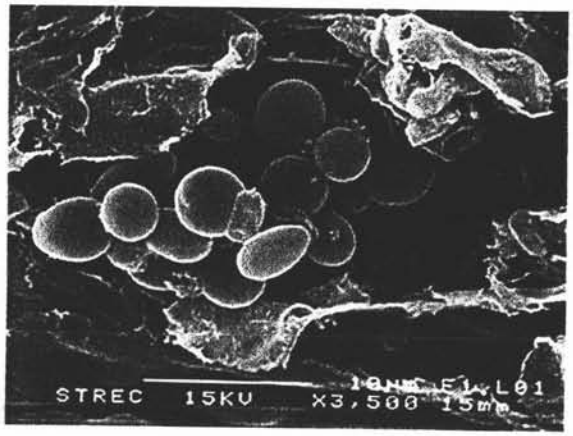

Figure B-25 EALC surface 2

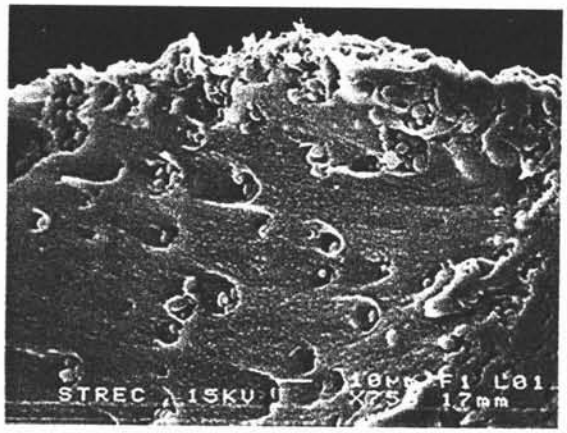

Figure B-26 EAB surface cross section 1

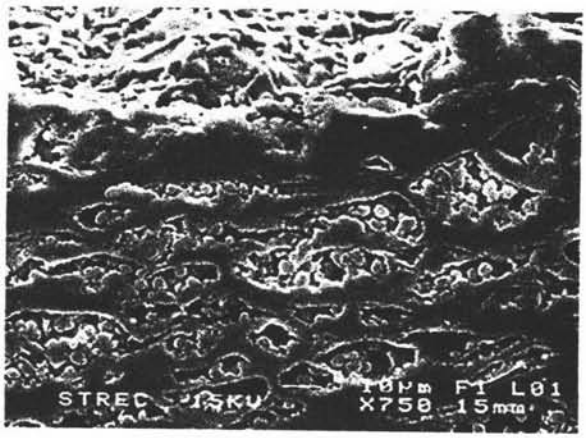

Figure B-27 EALC surface cross section 1

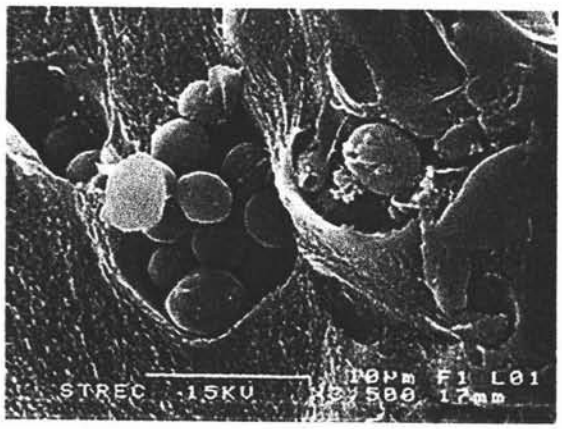

Figure B-28 EAB surface cross section 2

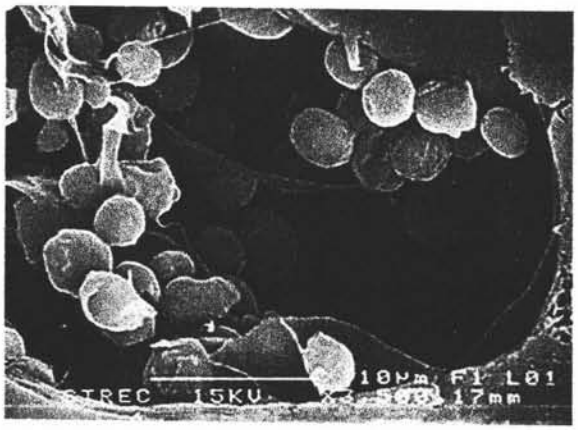

Figure B-29 EALC surface cross section 2

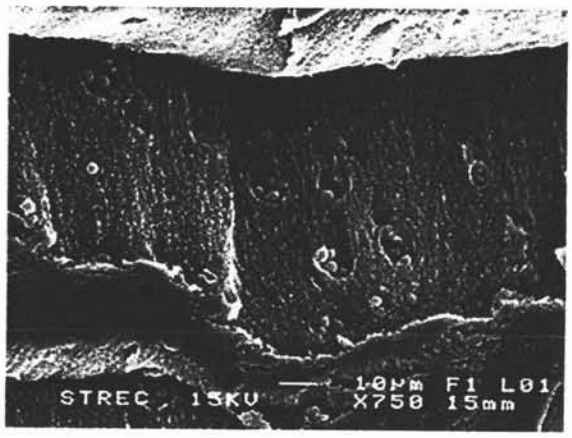

Figure B-30 EAB central cross section

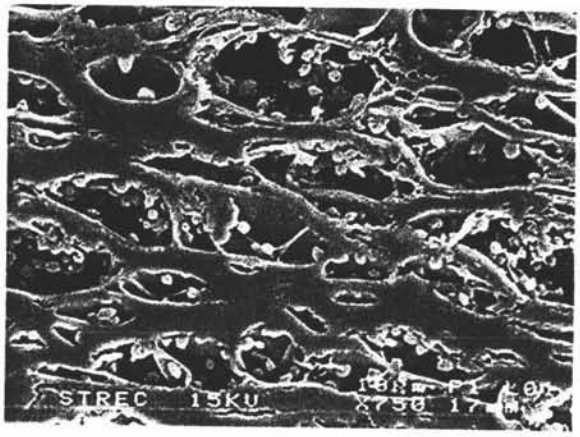

Figure B-31 EALC central cross section

B-1.4 Carriers after repeated batch 2

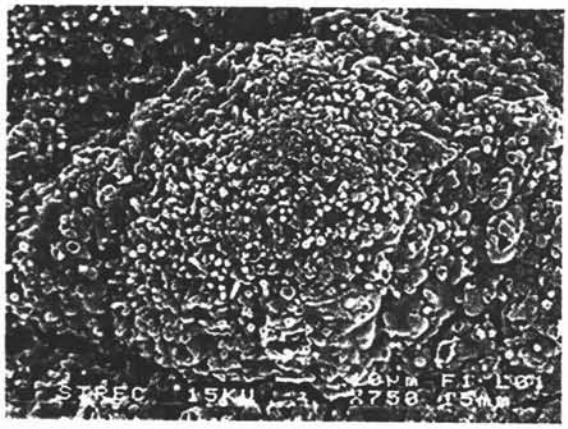

Figure B-32 EAB surface 1

Figure B-33 EALC surface 1

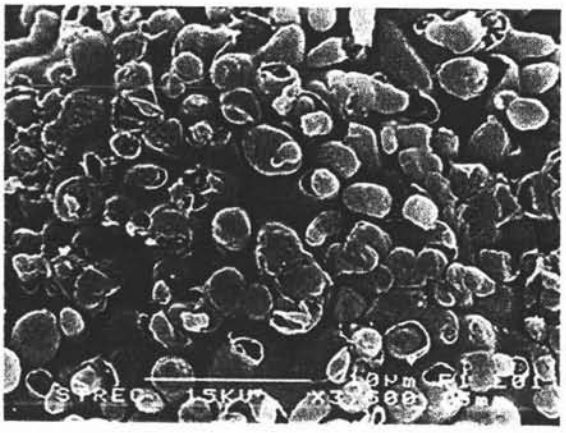

Figure B-34 EAB surface 2

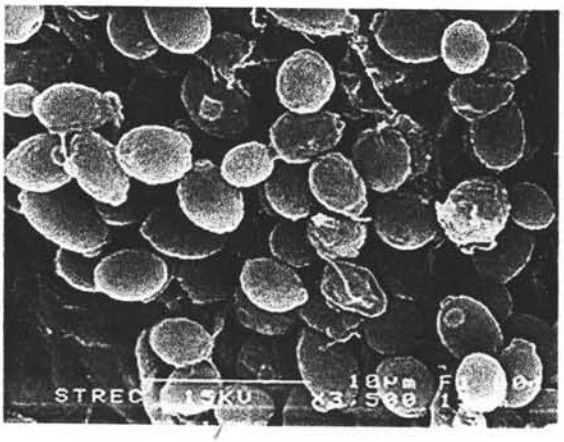

Figure B-35 EALC surface 2

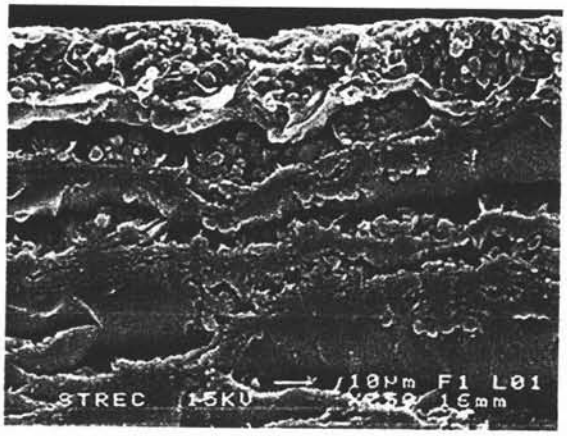

Figure B-36 EAB surface cross section 1

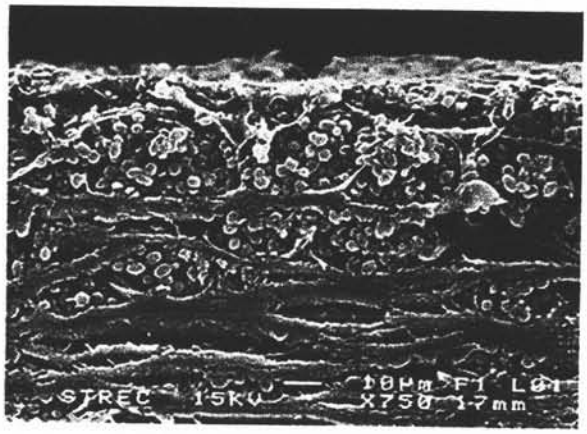

Figure B-37 EALC surface cross section 1

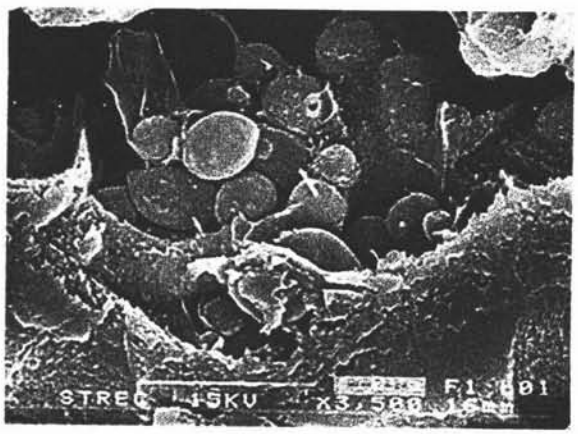

Figure B-38 EAB surface cross section 2

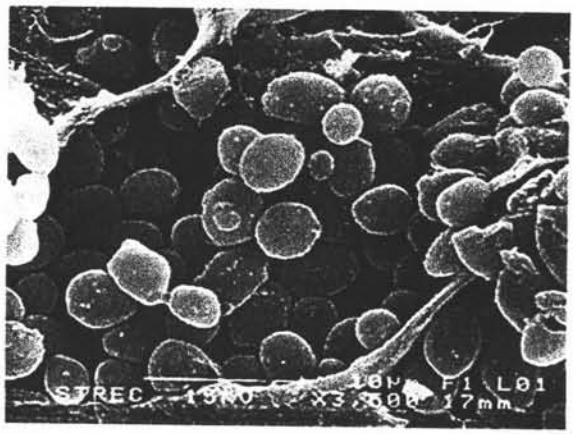

Figure B-39 EALC surface cross section 2

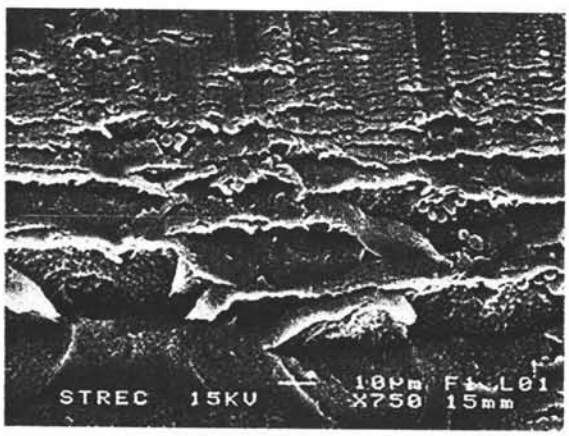

Figure B-40 EAB central cross section

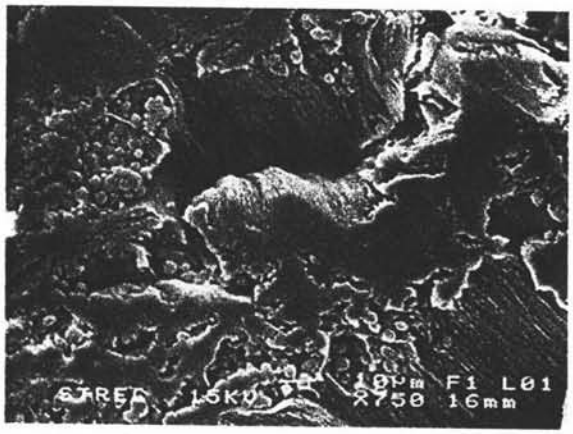

Figure B-41 EALC central cross section

B-1.5 Carriers after repeated batch 3

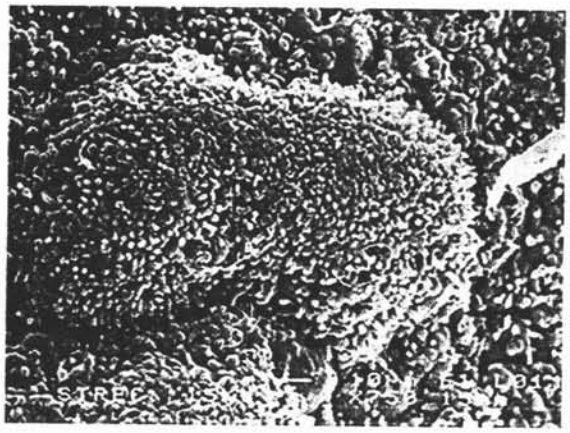

Figure B-42 EAB surface 1

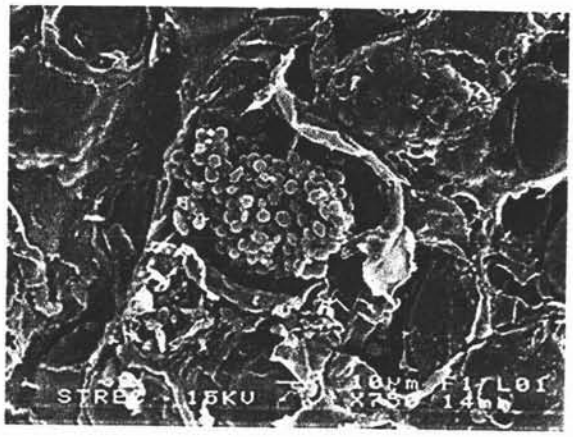

Figure B-43 EALC surface 1

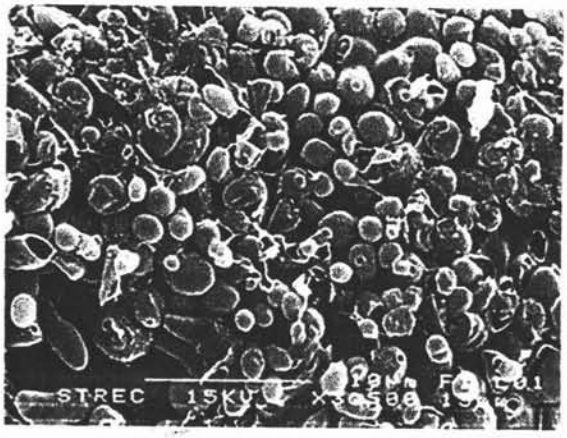

Figure B-44 EAB surface 2

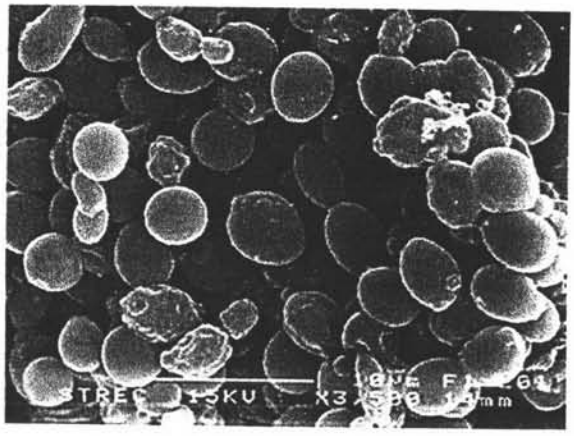

Figure B-45 EALC surface 2

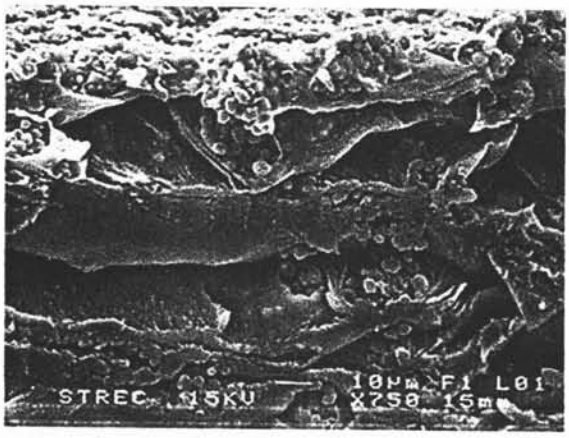

Figure B-46 EAB surface cross section 1

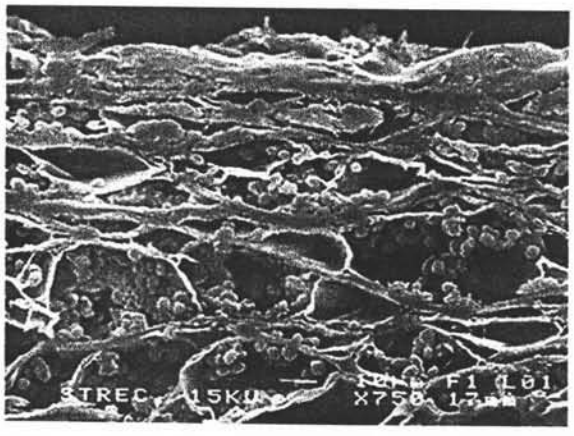

Figure B-47 EALC surface cross section 1

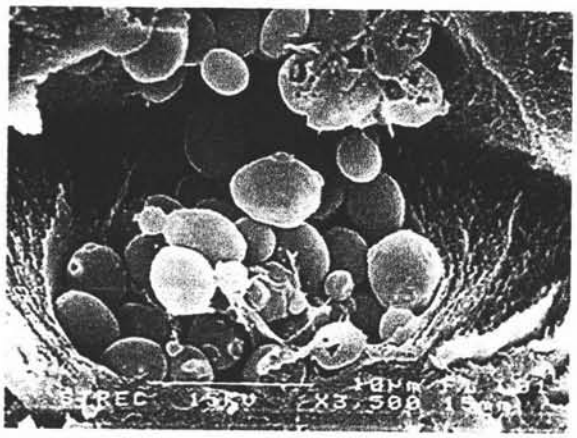

Figure B-48 EAB surface cross section 2

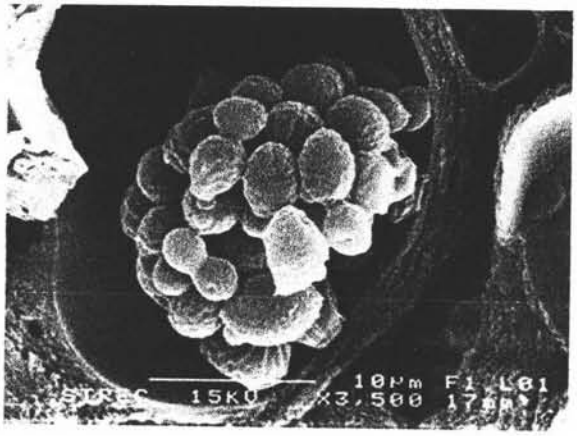

Figure B-49 EALC surface cross section 2

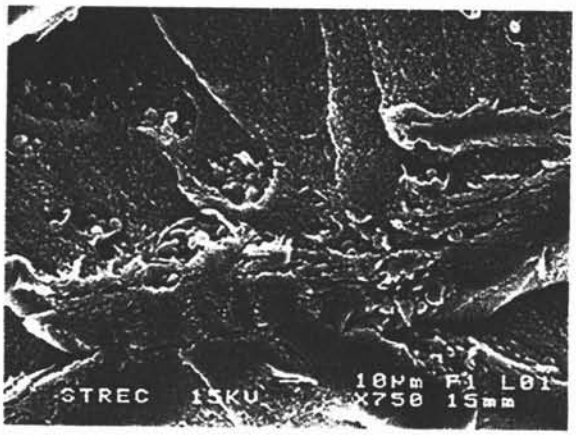

Figure B-50 EAB central cross section

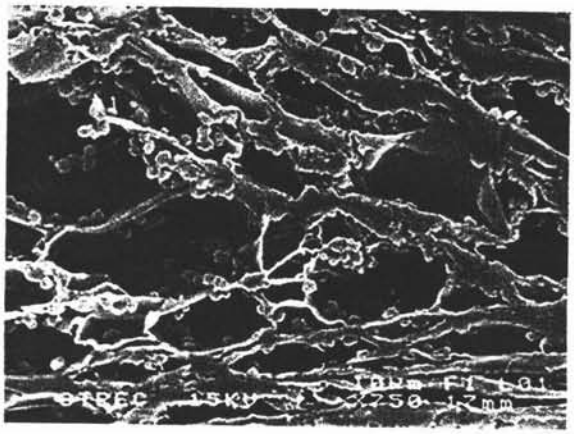

Figure B-51 EALC central cross section

APPENDIX C

CERTIFICATE OF ANALYSIS OF CHITOSAN

C-1 High viscosity chitosan

Date 25/04/2005

Item	Specification	Chitosan
1. Appearance	Yellowish	Yellowish
2. Particle size	Mesh No. 18	Mesh No. 18
3. Ash content	Less than 1.0%	0.2%
4. Moisture content	Less than 10%	9%
5. Deacetylation degree (%DAC)	85% Minimum	88%
6. Solution (1% in 1% acetic acid)		
Insoluble (%)	Less than 1.0%	0.52%
Viscosity	Higher than 200 cps	1,044 cps
7. Molecular weight	800,000 – 900,000	814,000
8. Heavy metal	Less than 20 ppm	0 ppm
9. Microbial content		
Total Plate Count	Less than 1,000 cfu/g	300
Yeast and Mold	Less than 100 cfu/g	10
E. coli	Nil	Nil
Salmonella	Nil	Nil

Manufacturer:
SEAFRESH CHITOSAN (LAB) CO., LTD.
31st floor Chartered Square Building, 152/25 North Sathon Road
Bangkok 10500, THAILAND
Tel : (662) 637-8888 (12 Lines) Fax : (662) 6378801-2
E-mail : seafresh@thai.com Homepage : http://www.seafresh.com

C-2 Low viscosity chitosan

Date 25/04/2005

Item	Specification	Chitosan
1. Appearance	Yellowish	Yellowish
2. Particle size	Mesh No. 18	Mesh No. 18
3. Ash content	Less than 1.0%	0.75%
4. Moisture content	Less than 10%	9%
5. Deacetylation degree (%DAC)	90% Minimum	90%
6. Solution (1% in 1% acetic acid)		
Insoluble (%)	Less than 1.0%	0.47%
Viscosity	Less than 200 cps	218 cps
7. Molecular weight	500,000 – 600,000	550,000
8. Heavy metal	Less than 20 ppm	0 ppm
9. Microbial content		
Total Plate Count	Less than 1,000 cfu/g	10
Yeast and Mold	Less than 100 cfu/g	20
E. coli	Nil	Nil
Salmonella	Nil	Nil

Manufacturer:
SEAFRESH CHITOSAN (LAB) CO., LTD.
31st floor Chartered Square Building, 152/25 North Sathon Road
Bangkok 10500, THAILAND
Tel : (662) 637-8888 (12 Lines) Fax : (662) 6378801-2
E-mail : seafresh@thai.com Homepage : http://www.seafresh.com

APPENDIX D
LIST OF PUBLICATION

Rusdianto Budiraharjo, Muenduen Phisalaphong, "Loofa Reinforced Alginate Carriers for Ethanol Fermentation by Flocculating Yeast", Extended Abstract for 13th Regional Symposium on Chemical Engineering 2006 – Advances in Chemical and Biomolecular Engineering, Singapore, 3-5 December 2006, Paper ID H-8-OR4.

NANYANG TECHNOLOGICAL UNIVERSITY

School of Chemical and Biomedical Engineering

Reg. No. 200604393R

Dear Mr. Rusdianto Budiraharjo

Paper ID : **H-8-OR4**
Paper Title : **Loofa reinforced alginate carriers for ethanol fermentation by flocculating yeast**

The review process for the 13th Regional Symposium on Chemical Engineering 2006 - Advances in Chemical and Biomolecular Engineering has been completed. Based on the recommendations of the reviewers, we are pleased to inform you that your paper identified above has been accepted for presentation in RSCE 2006. You are cordially invited to present the paper at RSCE 2006 to be held on **3 – 5 December 2006*** in Singapore.

This notification email serves as our formal acceptance of your paper as well as an invitation to present your work at RSCE 2006.

Please kindly refer to RSCE 2006 website (http://www.ntu.edu.sg/scbe/cbe/rsce2006/) for further information on the registration, accommodation reservation, venue, program and preparation of extended abstract.

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Xu Rong (Assistant Professor)

For RSCE Secretariat

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Loofa reinforced alginate carriers for ethanol fermentation by flocculating yeast

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ABSTRACT

Two new immobilized cell carriers namely entrapment alginate-loofa bead (EALB, ∅=3 mm) and entrapment alginate-loofa cube (EALC, 8 x 8 x 1 mm) were developed and tested for batch ethanol fermentation by flocculating yeast. The carriers were fabricated by gelation of preformed peripheral loofa sponge which was previously dipped in alginate-cell mixture. The 72 hours fermentation performances of the new carriers were compared to suspended cell (SC) and conventional entrapment alginate bead (EAB, ∅=2 mm) cultures. At steady state, final cell and ethanol concentration of the new carriers were comparable to SC and EAB culture. Because of their distinctive porous structure, the large new carriers can exhibit fermentation properties similar to compact EAB.

INTRODUCTION

Ethanol production by *Saccharomyces cereviceae* immobilized within alginate beads in packed bed was higher than batch system (Najafpour et al., 2004). However, some limitations such as gel degradation, low physical strength, and severe mass transfer limitation were often found in the use of alginate based carriers (Shuler and Kargi, 2001). On the other hand, loofa sponge was demonstrated as an excellent cell carrier for ethanol fermentation by flocculating cells (Ogbonna et al., 2001). Its strength, abundance, low price, biodegradability, and natural origin have become the main sources of interest. However, low shear environment and large aggregate of cells were required in the application loofa sponge in order to prevent excessive cell sloughing from the carrier (Ogbonna et al., 1996).

In this study, development of a new type of cell carrier namely loofa reinforced alginate carriers was attempted and their feasibility in term of ethanol productivity was evaluated by comparing with suspended cell and conventional alginate bead culture.

MATERIALS AND METHODS

S. cereviceae M30, a flocculating yeast strain was kindly provided by Dr. Savitree Limtong from Department of Microbiology, Kasetsart University, Bangkok. Alginate-cell mixture was made by mixing sterilized alginate 3%w/v solution with concentrated yeast suspension. Entrapment alginate beads (EAB, ∅=2 mm) was formed by adding alginate-cell mixture drop wisely into $CaCl_2$ 1.47% w/v using a syringe with internal needle diameter of 1.2 mm. Small pieces of peripheral loofa sponge (4 x 4 mm) were rolled into spherical shape and dipped into alginate-cell mixture before transferred to $CaCl_2$ 1.47% w/v to form entrapment alginate-loofa beads (EALB, ∅=3 mm). Entrapment alginate-loofa cube (EALC) was made in the same manner as EALB by using cubic sponge (8 x 8 x 1 mm) instead of spherical sponge. All carriers were left to harden in $CaCl_2$ solution under mild stirring for 15 minutes and then rinsed 3 times with NaCl 0.9% w/v.

Four fermentation cultures were evaluated in this study: suspended cell (SC), EAB, EALB, and EALC. Medium composition was 20% w/v palm sugar, 0.05% w/v $(NH_4)_2SO_4$, 0.01% w/v KH_2PO_4, and 0.0035% w/v $MgSO_4.7H_2O$ at pH 5. Batch fermentation was performed in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C for 72 hours. Samples of fermentation broth were analyzed for sugar, ethanol, and cell concentration by 3,5-dinitrosalicylic acid (DNS) reagent, gas chromatography, and dry weight respectively. At the end of fermentation, samples of carriers were taken for immobilized cell concentration measurement and scanning electron microscopy (SEM).

RESULTS AND DISCUSSION

Table 1 Fermentation parameters of all evaluated systems

System	P_F (g/L)	X_F (g/L)		Y_S (%)	$Y_{P/S}$ (g/g)
		Free	Immobilized		
SC	68.3	4.03	-	89.5	0.382
EAB	79.3	0.69	3.62	97.8	0.405
EALB	81.3	2.15	1.08	89.6	0.453
EALC	83.5	1.38	2.53	97.9	0.427

Fermentation results are summarized in Table 1. Final ethanol concentrations (P_F) of the new carrier based cultures were slightly higher than SC and EAB. At the end of fermentation, total cell concentrations (free plus immobilized, X_F) were similar for all four systems. Sugar consumption (Y_S) and ethanol yield ($Y_{P/S}$) in EALB and EALC systems were higher than SC culture.

From SEM images, it was shown that the new loofa reinforced alginate carriers had a more porous structure than EAB (Figure 1 and 2). It is proposed that this distinction had made it possible for the new carriers to possess fermentation properties comparable to compact sized alginate bead. With a strong structure of cellulose matrix inside, potential advantages including reusability, altered mechanical strength, and high ethanol productivity are expected in the application of this new carriers

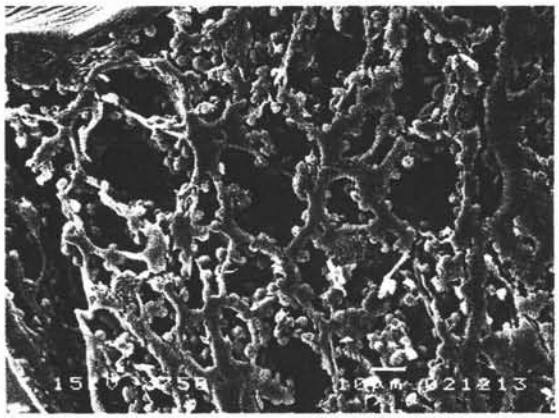

Figure 1 Cross section of EALC (magnification: 750 times)

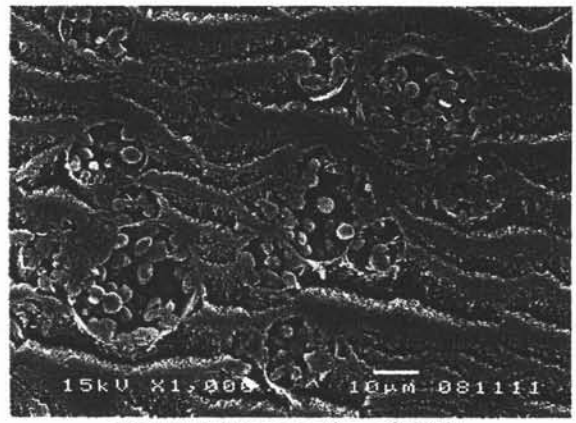

Figure 2 Cross section of EAB (magnification 1000 times)

Nomenclature

P_F	final ethanol concentration (g/L)
X_F	final cell concentration (g/L)
Y_S	sugar consumption yield (%)
$Y_{P/S}$	ethanol yield (g ethanol/g substrate)
$Y_{X/S}$	cell yield (g cell/g substrate)

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VITA

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