Chapter IV
Results and Discussion

In the construction of the new carriers, alginate and chitosan were chosen based on their high potential as cell carriers. All materials chosen for the construction of these new carriers are biodegradable because of their natural origin. By doing so, environmental concern related to the use of these materials can be eliminated. The methods for cell immobilization were diversified into adsorption/adhesion and entrapment method. By doing so, comparison between the two methods could be done simultaneously.

Instability of polymeric gels including alginate and chitosan is often a problem for their application in immobilized biosystems. Reinforcement of alginate and chitosan with strong fibers of loofa sponge was done to alter their mechanical strength. When compared to previous attempts to enhance the strength of polymeric carriers such as the use of a hard core and a soft gelatin shell [6], the use of loofa sponge as reinforcement material is simpler and inexpensive. Furthermore, the occurrence of inactive zone inside the carrier as reported in the use of a hard plastic core can be avoided in the new carriers because of the macroporous nature of loofa sponge [6]. The organic nature of loofa sponge is expected to have positive effects on cells attachment to the supports as organic materials are suggested to possess rich functional groups suitable for cells adsorption [8]. Much saving in term of work and cost are obtainable if the immobilized cell carriers can be used repeatedly. To evaluate this possibility, reusability of the carriers was evaluated by using repeated batch mode.

Flocculating yeast strain was chosen in this work as it was reported as favorable for cell immobilization by Ogbonna et al. (1996). The use flocculating cell might in turn be beneficial considering degradation of gel part of the carriers was expected after a certain period during the fermentation. At the time that gel degradation occurred, cells near the surface of the carriers were exposed. Because of their flocculating property, the cells might have formed binding to each other and to the carrier. This binding would help to prevent excessive cell sloughing from the carrier.

Ethanol productivity and yield of the system are considered as the key values to be evaluated before any other characteristics are taken into consideration. If any of the new

carriers can fulfill the productivity criteria, it can be further evaluated in subsequent studies for other characteristics including mechanical strength and scale up.

4.1 Yeasts immobilization by loofa sponge

Ogbonna et al. (1997) reported that by using loofa sponge as a cell carrier, 99% immobilization of flocculating yeast was achieved. On contrary, preliminary test with loofa sponge in this study demonstrated that it was not effective for immobilizing flocculating yeast. Excessive cells detachment from the sponges was observed and there were no significant differences among different shapes of sponges used for cells immobilization. The contrast might arise from different setting of experiment. In their work, Ogbonna et al. (1997) used bubble column configuration for ethanol fermentation while shake flask culture was used in this work. Thus, shear forces acting on the immobilized cells were not identical in both sets. High shear environment may be the possible cause for excessive cells' detachment from loofa sponge carrier in this study. Nevertheless, it cannot be denied that by direct comparison, the pore size of loofa sponge is much larger than the size of yeasts' flocs. In this view, a special treatment is required to create strong bond between yeast cells and loofa sponge.

4.2 Comparison of alginate and chitosan

As loofa sponge alone was not adequate for yeast immobilization, new hybrid carriers were formed by combining loofa sponge with natural biopolymers: alginate and high viscosity chitosan (viscosity = 1,044 cps). Percent de-acetylation (%DAC) and molecular weight (MW) of the chitosan used in this experiment was 88% and 814,000 respectively. Preliminary evaluation and comparison between the two polymers was conducted by 3 days batch fermentation. At the end of the fermentation, the broth was analyzed for sugar content by DNS method and the pH value was measured by pH meter.

It was demonstrated that chitosan based carriers had low performance in term of sugar consumption which corresponded to low ethanol production. The absorbance gained from their fermentation broth was slightly differed from the initial fresh medium. On the other hand, sugar consumption was higher for entrapment alginate-loofa cube (EALC) system than suspended cells culture (SC). This fact demonstrated for the first time that EALC was a promising yeast cell carrier.

There can be many factors that caused low performance in chitosan based systems. The first suspect was the pH value of the fermentation broth, considering that strong base

solution (sodium hydroxide) was used in the gelation of chitosan. Basic condition was known as unfavorable for yeast growth. The normal pH range for yeast is 4 to 7. However, the suspect over highly basic condition in chitosan based systems was ruled out as the measurement by pH meter showed that the pH values were still ideal for yeast growth.

As it was confirmed that high pH value was not the cause for low yeast activity in chitosan based systems, there must be other mechanisms of inhibition in these systems. As a matter of fact, there were reports on the issue of chitosan inhibitory effect towards microorganisms [9, 20]. Chitosan is known to restrain the growth of some pathogenic bacteria. On the other hand, it promotes the growth of plant cells and has been used as material for wound healing scaffold for human. Although the exact mechanism of chitosan inhibition on yeast growth has not yet been ameliorated, some preposition can be made based on the nature of chitosan itself. Chitosan is well known as a cationic polymer. Cationic charge of chitosan may cause instability for yeast cell's membrane which has a net negative charge. This may lead to cell disruption which in the end causes inactivity of the yeast cells.

Another possible cause for low cell activity in chitosan based systems is the occurrence of endotoxin as contaminant in the chitosan itself. Although such endotoxin is not explicitly reported in the certificate of analysis of the chitosan used in this work (Appendix C), there is still a possibility that such compound does exist considering that the chitosan was not of pharmaceutical grade. Therefore, it is suggested that high purity chitosan should be used in direct use of chitosan (without modification) as cells carrier. However, this may imply that the cost of the material is increased which in turn can make chitosan less preferable than other cheap biopolymers such as alginate if their performance in the fermentation process is comparable.

4.3 Fermentation 1

In fermentation 1, both adsorption and entrapment methods were compared. Based on the unfavorable results of chitosan based carriers gained from previous experiment (comparison of alginate and chitosan), the focus of fermentation 1 was on the use of alginate in combination with loofa sponge. Only one culture of low viscosity chitosan carriers was tested in parallel with alginate bead and alginate-loofa carriers. The low viscosity chitosan had viscosity of 218 cps, %DAC of 90%, and MW 550,000. Complete specification of the chitosan used in this experiment is provided in Appendix C. The use of

chitosan carrier in fermentation 1 was aimed to confirm the result of previous experiment and to investigate the effect of low viscosity chitosan. Suspended cells culture was conducted as control. Eight cultures were evaluated in fermentation run 1. Labels of cultures investigated in fermentation 1 are given in Table 4.1.

Table 4.1 List of samples and labels for fermentation 1

Sample's Name	Label
Suspended cells culture	SC
Adsorption alginate bead	AAB
Entrapment alginate bead	EAB
Adsorption alginate-loofa cube	AALC
Entrapment alginate-loofa cube	EALC
Entrapment alginate-loofa cylinder	EALY
Entrapment alginate-loofa bead	EALB
Adsorption chitosan-loofa cube	ACLC

The sugar concentration profile of fermentation 1 is given in Figure 4.1. From the graph, it is obvious that chitosan based carrier (ACLC) was not suitable for immobilizing yeast. The flat profile of ACLC indicated that the yeast in this system was inactive since it couldn't consume sugar from fermentation medium. This result was in agreement with the previous experiment and confirmed that unmodified chitosan was not suitable for yeast immobilization.

Modification of chitosan based carrier could be done to alter its properties to better suit immobilization purpose. Such modification has been demonstrated successfully for instance by Shinonaga et al. (1992). However, the modification procedure introduces additional cost for producing the carrier. Moreover, toxic chemicals are often used in the process. These chemicals can be detrimental to the activity of the yeast.

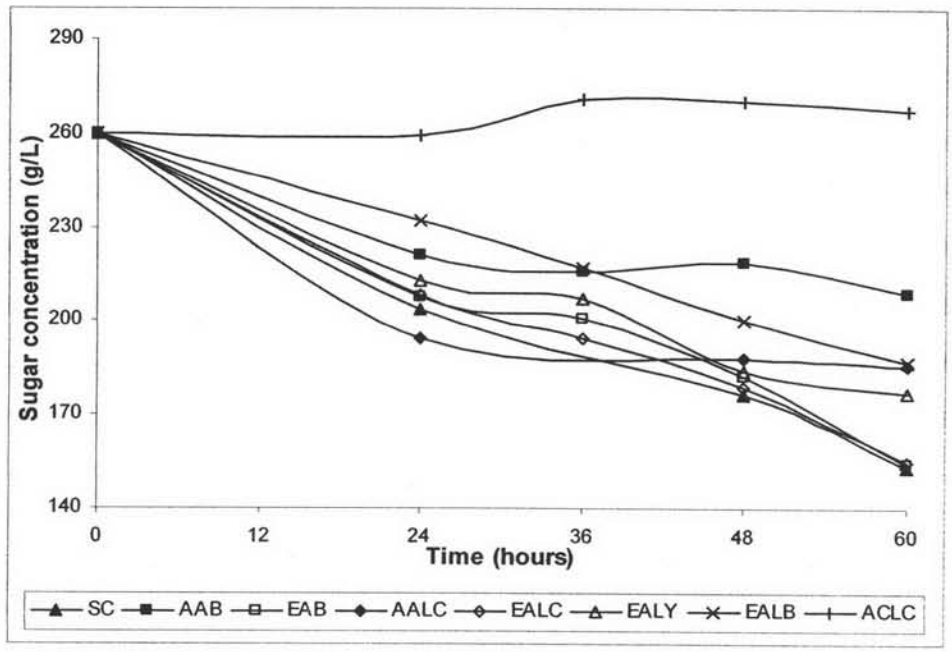

Figure 4.1 Sugar concentration profile of fermentation 1

In general, performances of adsorption based systems (AAB, AALC, and ACLC) were lower than entrapment ones (EAB, EALC, EALY, and EALB). One possible cause of this event was low adsorption yield of yeast cells on the supports. SC, EAB, and EALC showed the most promising results. It was anticipated that suspended cell culture (SC) produced a good result as it was characterized by high initial cells loading and good mass transfer characteristics when compared to other systems [6]. Even though the loading of EAB was small, it sugar consumption profile was good. This result indicated that the EAB system may possess some beneficial characteristics for yeast immobilization such as high cell concentration inside the carrier. Similar result for EAB system was reported by Najafpour et al. (2004).

The sugar concentration profile of EALC was less anticipated. On contrary to initial worry concerning mass transfer limitation in large size carriers, EALC system was considered impressive in term of sugar consumption. High yeasts activity is thought as the main driving force for the good performance of EALC. As good performance can be obtained by EALC, it is less significant to use a carrier with more complicated geometry such as EALY for performance improvement. In practical point of view, it is more difficult to form cylindrical sponge from loofa as it has a natural tendency to expand. On the other hand, EALB was maintained for the next experiment as an anticipation of its possible benefit which originated from its geometrical similarity with EAB.

4.4 Fermentation 2

EALY and chitosan based carriers were no longer evaluated in fermentation 2 due to their low performances in fermentation 1. Sugar concentrations were determined and the results are summarized in Figure 4.2. In addition, cell and ethanol concentration data were evaluated to provide a better insight on fermentation characteristic of the various systems. Free cell and ethanol concentration profile are shown in Figure 4.3 and Figure 4.4 respectively. Except adsorption alginate-loofa beads (AALB), labels used in this experiment are same as listed in Table 4.1.

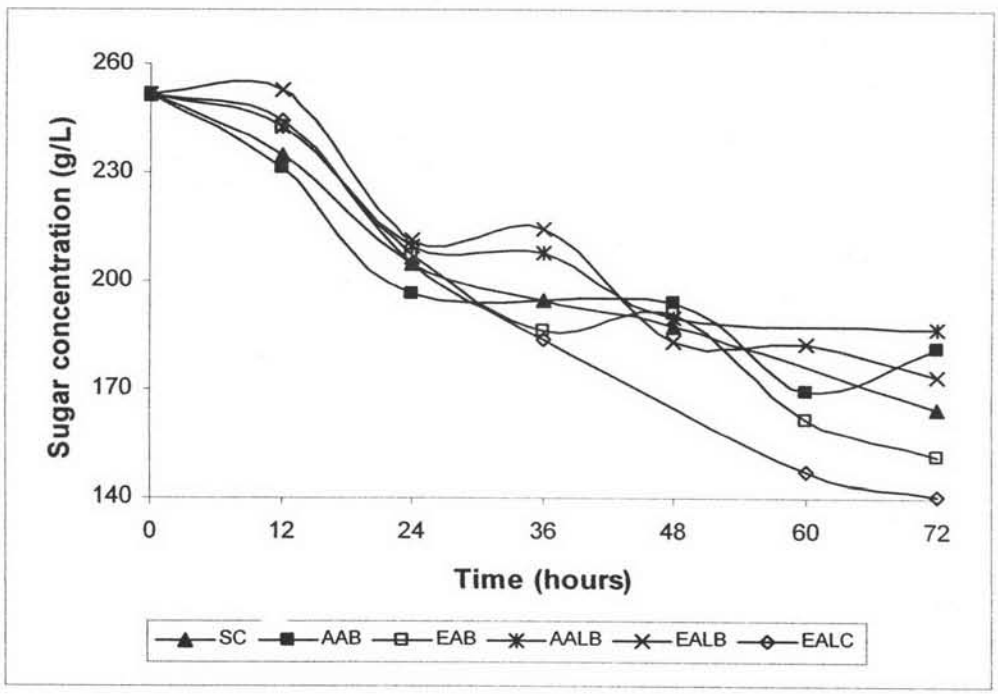

Figure 4.2 Sugar concentration profile of fermentation 2

The sugar concentration profile of fermentation 2 exhibits similar pattern as the previous experiment (fermentation 1). Adsorption carriers were proven once more to be inferior for ethanol production purpose. As proposed by Shuler and Kargi (2001), limited cell loading capacity and rather weak binding forces were the main cause for low performance of adsorption based systems. The results of adsorption systems might also suggest that hydrodynamic shear stress around adsorbed cells in this work should not be mild enough to prevent cells detachment from surface of cell carrier [4].

EALC and EAB represented the most promising systems. This result resembles the one obtained in the fermentation 1. At the beginning of the fermentation process, sugar

consumption of EALC was lower than SC. It was then increasing with higher rate so that by the end, sugar consumption of EALC exceeded SC.

Free cell concentration in entrapment systems in general was lower than suspended cell culture. As shown in Figure 4.3, EAB system had the lowest free cell concentration, suggesting good entrapment of yeast cells inside alginate beads. Higher free cell concentration was observed for adsorption based carriers which indicated yeast cells detachment from the supports. On contrary to EAB, high free cell concentration was observed for EALB and EALC cultures. Cells leakage from these carriers was thought as the most possible cause.

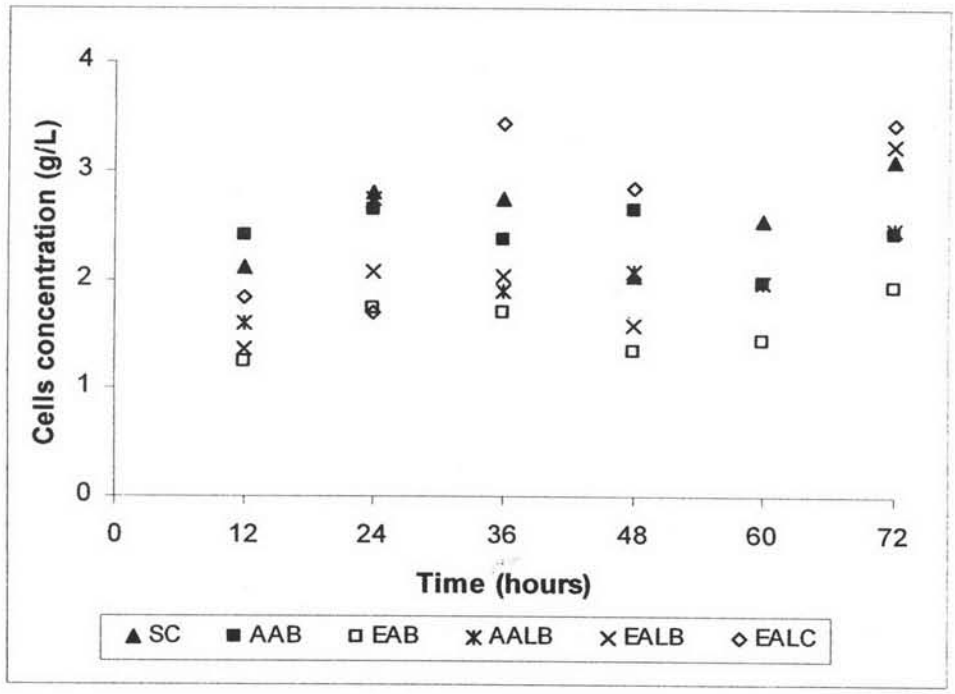

Figure 4.3 Free cell concentration profile of fermentation 2

Highest ethanol concentration was obtained with EALC and EAB, greater than that of SC (Figure 4.4). Corresponding to sugar consumption trend, ethanol concentration of EALC system at 12 hours was lower as compared to that of SC but then became higher by the end. Therefore, high cell concentration and activity was expected from the system with EALC. EALB performance was not satisfactory in term of ethanol yield. However, this result may arise from low cell loading in EALB.

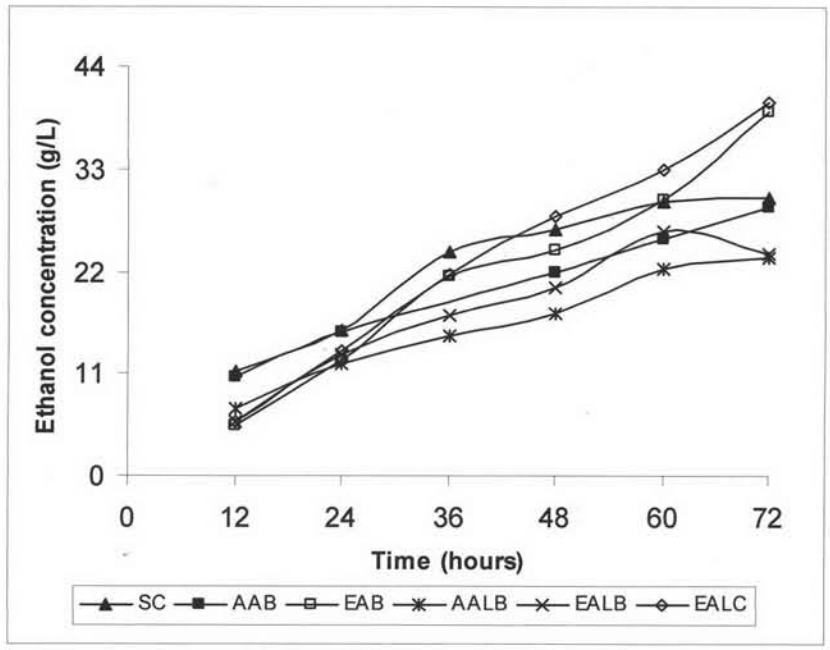

Figure 4.4 Ethanol concentration profile of fermentation 2

Immobilized cell concentrations at the end of fermentation 2 are provided in Table 4.2. Stock cell suspension obtained from cell harvesting process had a very high cell concentration (40.1 g/L). This level of cell concentration was considered adequate for inducing adsorption of yeast cells onto the supports. However, the amount of cells which were attached to the surface of adsorption based carriers was small. Thus, low immobilization yield for adsorption based carriers was not caused by inadequate concentration of initial stock solution but by the low immobilization capacity of the carriers themselves.

Table 4.2 Final immobilized cell concentrations of fermentation 2

Carrier	Cell concentration (mg cell/g carrier)
AAB	2
EAB	81
AALB	10
EALB	41
EALC	31

For alginate-cell mixture, low cell concentration (1 mg cell/g solution) was obtained due to dilution effect which occurred when stock cell suspension was combined

with alginate solution. Final cell concentration inside entrapment carriers (EAB, EALB, and EALC) on the other hand was much higher than the initial alginate-cell mixture. This was a clear indication of yeast growth inside the entrapment carriers. Moreover, it was obvious from direct observations that by the end of fermentation, the entrapment carriers were heavier, darker, and bigger than the initial carriers. This fact also demonstrates that there was an intensive yeast growth inside the carriers. Occurrence of cell growth inside immobilized cell carriers was previously reported by Junter and Jouenne (2004) and Najafpour et al. (2004)

Concentration of cell inside EAB and EALB was higher than the one inside EALC. This result indicated that carriers with small size such as EAB and EALB might be more beneficial in promoting cell growth. High rate of cell growth was desirable until an optimum or saturation point was achieved. Afterward, additional growth inside the carrier is considered as overgrowth. Najafpour et al. (2004) reported overgrowth as an unwanted phenomenon as it might lead to cell leakage from the supports.

A series of electronic micrographs were taken for immobilized cell carriers, showing the appearances of outer surface and cross section of the various carriers at the end of fermentation 2. Figure 4.5 shows an overall view of AAB. EAB as shown in Figure 4.6 was bigger in size than AAB. The surface of EAB was not as smooth as AAB because of yeast growth inside the carrier. Yeast cells in EAB were protected by film of alginate (Figure 4.8). However, some cells may escape from the carrier if there was a leak on the surface. Such leak can be observed at the bottom right of Figure 4.8. Fewer amounts of yeast were attached to the surface of AAB. As shown in Figure 4.7, the cells were fully exposed to the surroundings.

Figure 4.5 Overall view of AAB

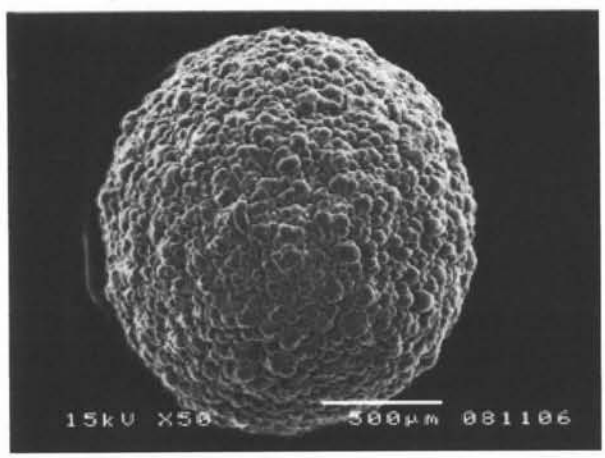

Figure 4.6 Overall view of EAB

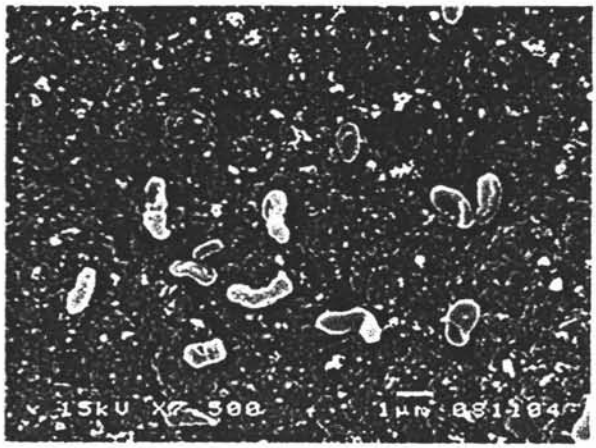

Figure 4.7 Surface of AAB

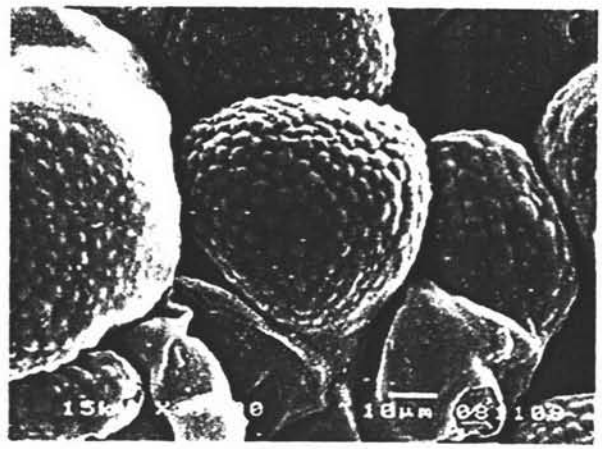

Figure 4.8 Surface of EAB

Some cells on the surface of AAB didn't maintain their natural oval shape which can be an indicator that the cells were not in good condition. The condition may be caused by strong shear force originated from the shaking of medium during fermentation. This opinion is supported by Figure 4.9 which provides a closer look at the form of yeast living in a cavity inside AAB. The cavity was actually undesirable and probably formed from air bubble trapped inside the gel during the gelation process. As the cells in the void were protected from destructive shear force, it is well expected that the morphology was better when compared with cells in Figure 4.7.

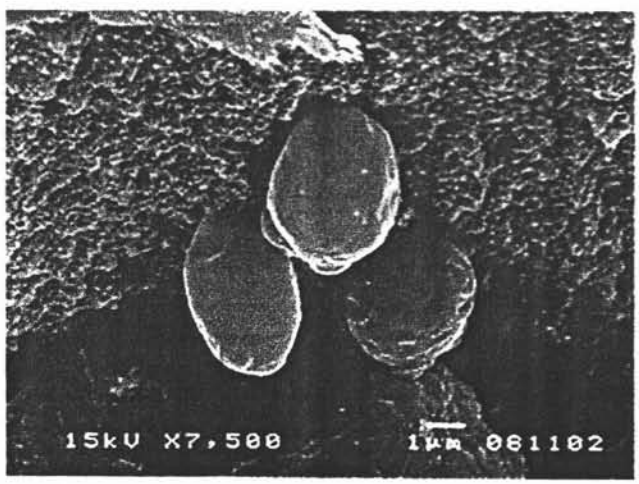

Figure 4.9 Yeast cells inside cavity of AAB

The absence of growth inside the thick layer of gel in AAB was verified by Figure 4.10. In contrast, inspection of EAB cross section in Figure 4.11 demonstrates that yeast can grow well inside the gel structure of EAB. This confirms that the yeast could gain access to substrate needed for growth even though they were located deep inside the carrier. Moreover, the yeast was healthy as confirmed by close look image in Figure 4.12.

In addition, there were filaments connecting the yeast and the support. It is proposed that they were advantageous to promote firm attachment of cell to support.

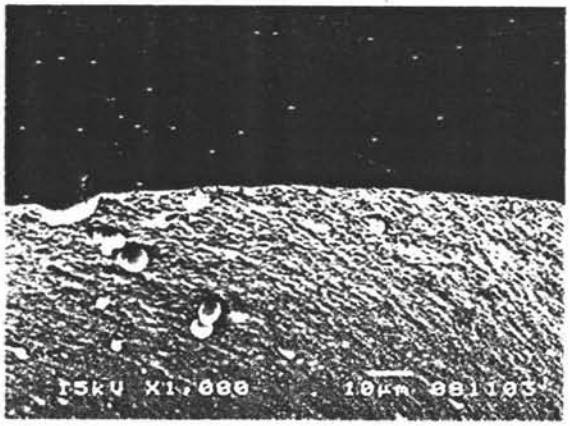

Figure 4.10 Cross section of AAB

Figure 4.11 Cross section of EAB

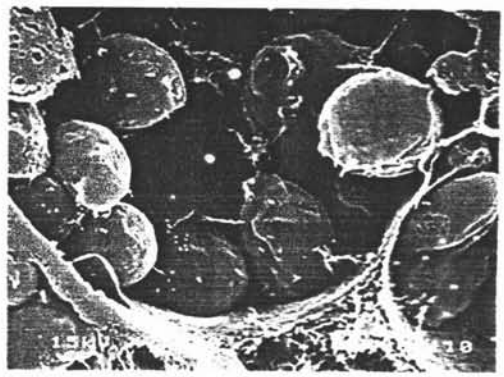

Figure 4.12 Close look on EAB cross section

Figure 4.13 is an image showing a bare fiber part of AALB. As can be seen from the picture, there were not many cells attached on the surface. This result demonstrates the lack of adsorption capacity for AALB. Figure 4.14 proves that yeast could not penetrate into the core of loofa fiber so that no cell was observed there.

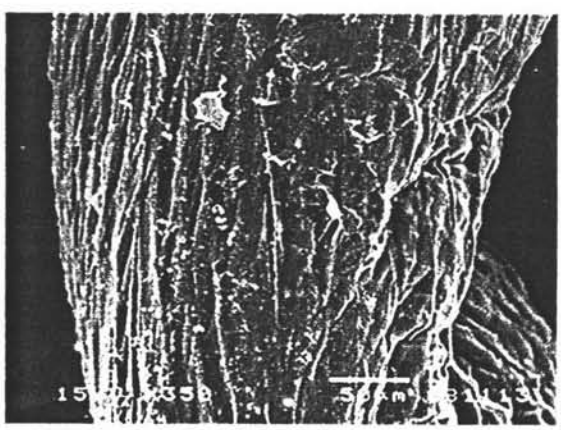

Figure 4.13 Fiber part of AALB

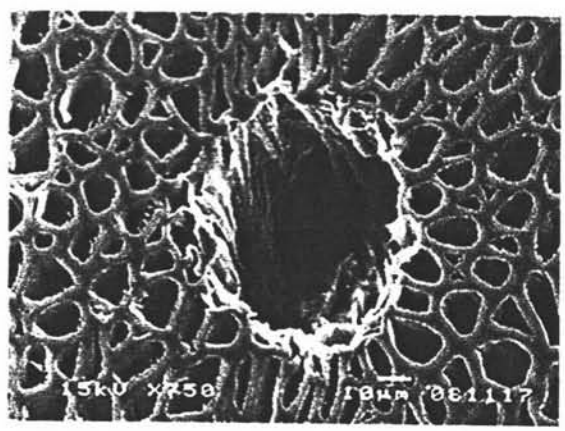

Figure 4.14 Core of AALB fiber

Figure 4.15 shows a picture of EALB surface. Cell leakage is obvious as holes were found on the exterior of the carrier. Such hole is depicted on top right corner of Figure 4.15. When compared to the surface of EAB (Figure 4.8), the surface of EALB was flatter.

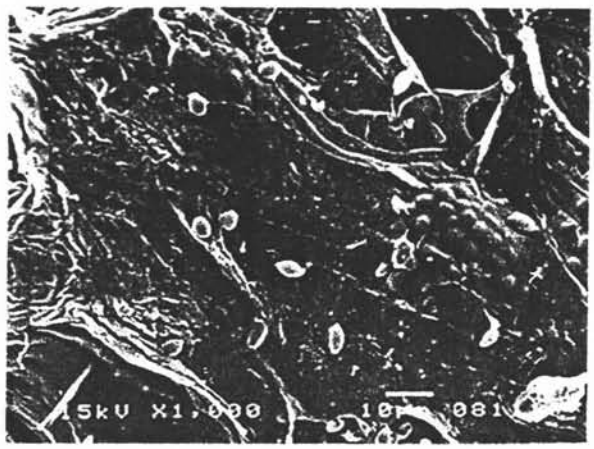

Figure 4.15 Surface of EALB

EALB had an irregular shape (Figure 4.16). The inner structure of EALB was not as dense as EAB. There were big cavities inside the carrier. Most of the cavities were found at the loofa fiber and alginate gel interface. Thus, it is proposed that loofa fiber had double function inside entrapment based carriers: as reinforcement and providing additional space for cell habitation. Closer observation as depicted in Figure 4.17 proves that the cavity inside EALB was indeed functional for yeast habitation as many cells were found living inside it.

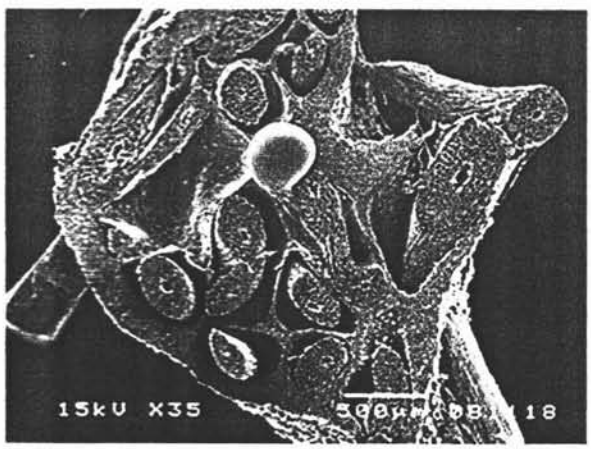

Figure 4.16 EALB cross section

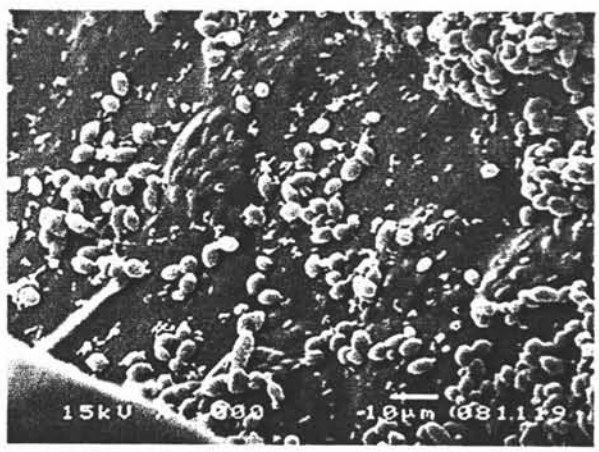

Figure 4.17 Cells in EALB cavity

The central part of a loofa fiber was hollow. This part when it is accessible by yeast, for example in the case of exposed fiber tip, can be used by the yeast as a living

space (Figure 4.18). Filaments connecting the cells to support are shown in Figure 4.19. These filamentous structures are also previously observed in EAB (Figure 4.12).

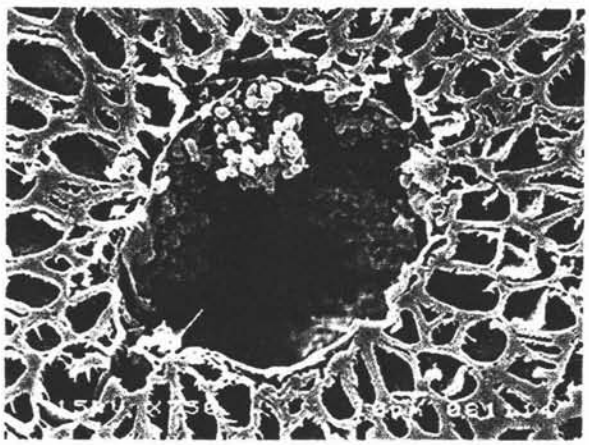

Figure 4.18 Center of fiber of EALB

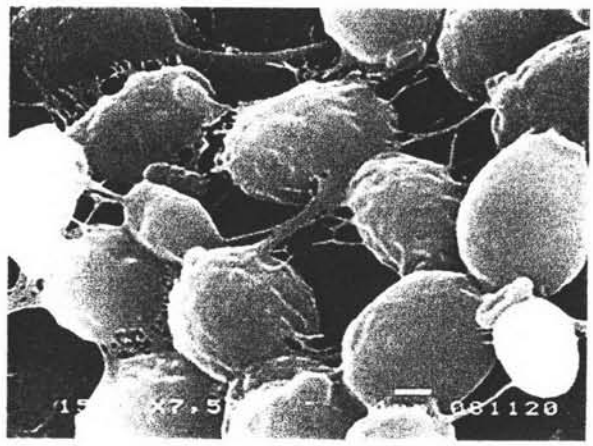

Figure 4.19 Yeast inside EALB

The exterior of EALC (Figure 4.20) was similar with EAB (Figure 4.8). In the center of Figure 4.20, sign of cell leakage is obvious. Figure 4.21 shows an image of EALC cross section which resembles the view of EALB (Figure 4.16) at a bigger scale.

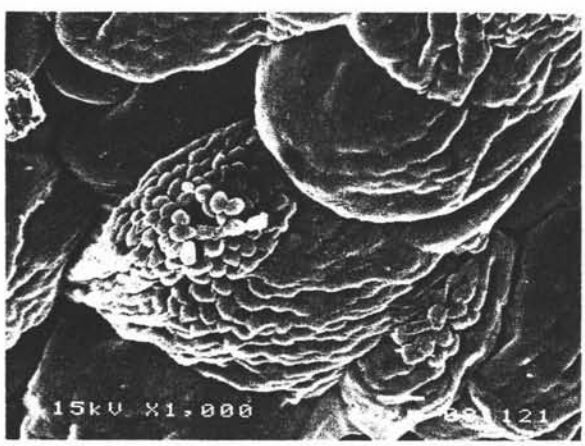

Figure 4.20 Exterior of EALC

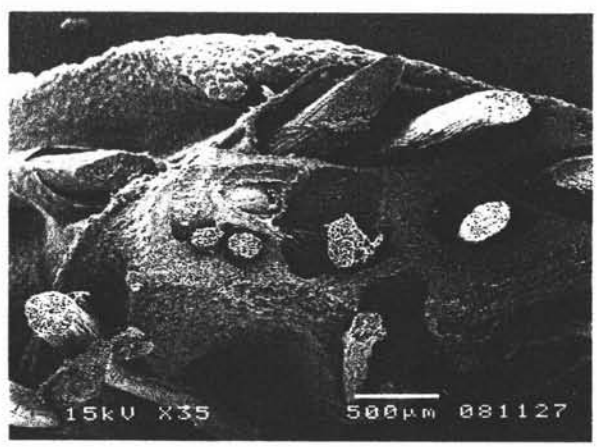

Figure 4.21 Cross section of EALC

Similar to Figure 4.13, Figure 4.22 gives another impression on loofa fiber capacity for yeast immobilization. As seen in the picture, there were only few cells attached to the fiber. Moreover, it is obvious from the picture that the ratio between the fiber and yeast size is huge. This fact supports previous proposal in section 4.1 that loofa sponge matrix alone in general is insufficient for yeast entrapment. The space between fibers is too big for yeast retention.

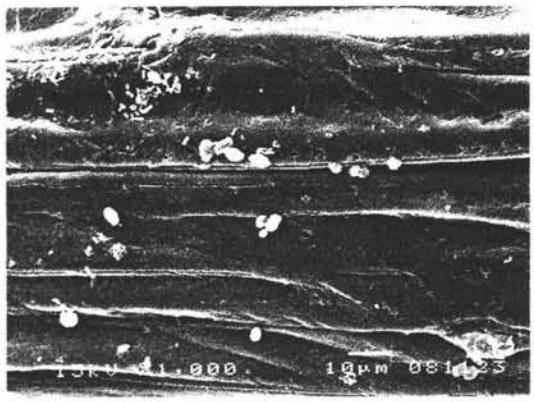

Figure 4.22 Exterior of EALC fiber

As found in EALB (Figure 4.16), there were also many cavities in EALC (Figure 4.23 and 4.24). The cavities also served as ideal spaces for cell growth. Gel layer in EALC was less dense than EAB. This can be seen by comparing Figure 4.24 and Figure 4.11. The loose gel structure of EALC enabled the yeast to have more spacious living space inside the carrier (lower part of Figure 4.24). On the other hand, the core part of loofa fiber in EALC (Figure 4.25) was in general not accessible by yeast.

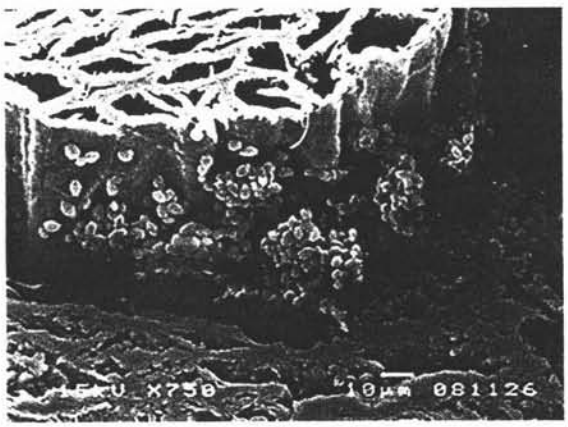

Figure 4.23 Cavity in EALC

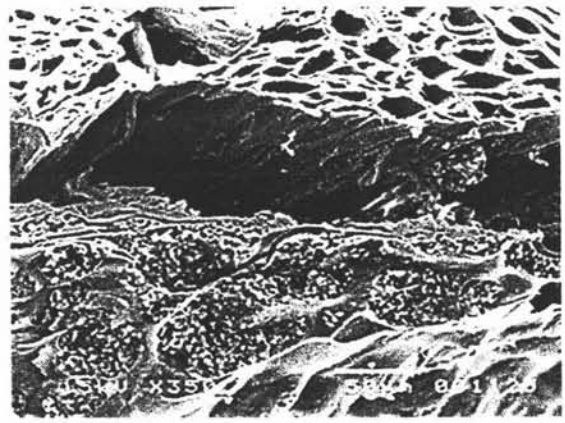

Figure 4.24 Cross section of EALC

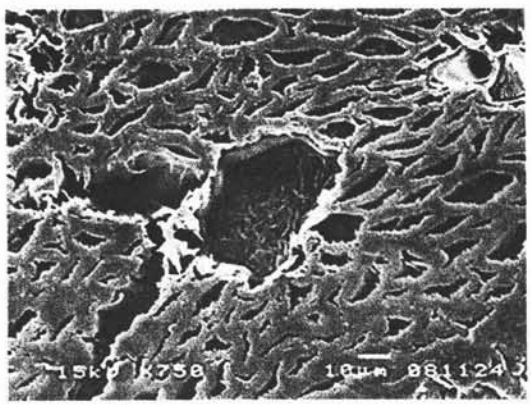

Figure 4.25 Cross section of EALC fiber

4.5 Fermentation 3

In fermentation 2, adsorption based carriers exhibited low performance and high degree of cell detachment. Therefore, they were considered ineffective for ethanol production by immobilized yeast and no longer evaluated in fermentation 3. Ethanol fermentation in this experiment was carried out for 120 hours in order to gain information on carriers' performance in extended period. Four cultures were conducted: SC, EAB, EALB, and EALC. Palm sugar was used as the carbon source.

Parameters of fermentation 3 are given in Table 4.3. Complete definitions of the parameters were previously described in section 3.4. After decantation, cells in stock cell suspension were highly concentrated as the cell concentration reached 16.9 g/L. Cell concentration in alginate-cell mixture was 2 g/L. Final cell concentrations inside the carriers (X_I) were significantly higher than alginate-cell mixture. This fact is a positive confirmation on cell growth inside the carrier and is in agreement with the result of fermentation 2.

Free cell concentration (X_E) of EAB system was the lowest (Table 4.3 and Figure 4.26), an early indication of efficient yeast immobilization. On the other hand, free cell concentration of EALB was significantly higher than EALC and EAB. At this stage, cell leakage as a consequence of fragile gel structure of EALB was suspected as the primary cause.

Table 4.3 Parameters of fermentation 3

System	P_F (g/L)	X_T (g/L)		Y_I (%)	Y_S (%)	$Y_{P/S}$ (g/g)
		X_E	X_I			
SC	68.3	4.03	-	-	89.5	0.382
EAB	79.3	0.69	3.62	83.92	97.8	0.405
EALB	81.3	2.15	1.08	33.37	89.6	0.453
EALC	83.5	1.38	2.53	64.72	97.9	0.427

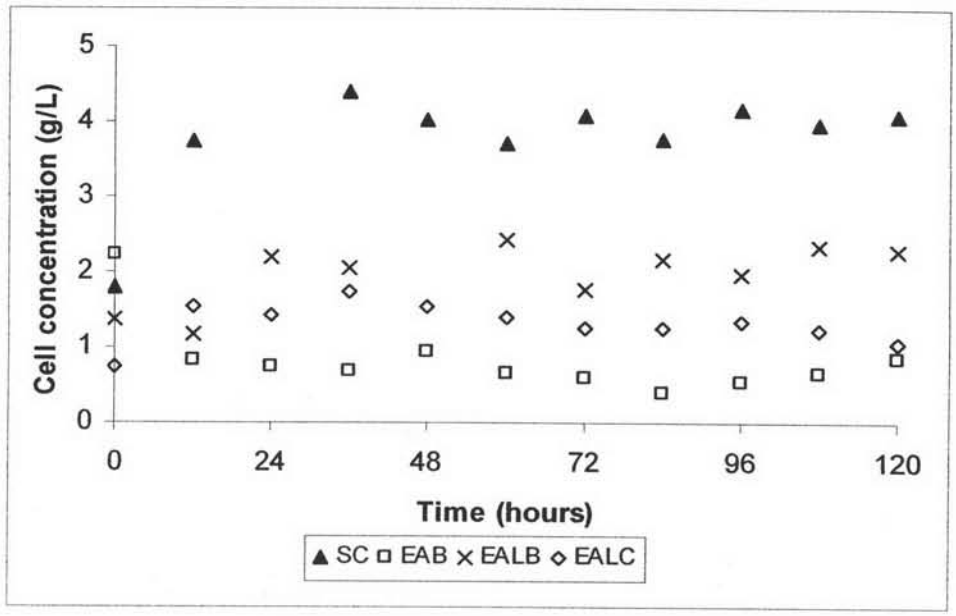

Figure 4.26 Free cell concentration of fermentation 3

Sugar concentration was set to 200 g/L for fermentation 3. Consumption of sugar for all systems reached steady state after a certain period. SC was the first to achieve steady state (Figure 4.27). Initial concentration of cell was intentionally high for SC so that it was no surprise that this system reached steady state at the highest rate. At 72 hours period, a stable sugar concentration of about 20 g/L was obtained for SC. Both EAB and EALC cultures consumed sugar at slower pace than SC but their final sugar concentrations were lower than SC. This fact demonstrates that these immobilized cell systems can exhibit higher sugar utilization than SC. If product inhibition is considered destructive for yeast activity, better ability of sugar utilization for EAB and EALC may suggest that alginate matrix of EAB and EALC may enhance yeast activity. Yeast immobilized in alginate was found to contain higher amount of fatty acid than its freely suspended counterpart, causing it to be more osmotolerant [44]. Alginate matrix was also reported to act as activity enhancer for metabolic enzymes of yeast such as invertase. In case of EALB, slower rate of sugar consumption was expected as the loading of this carrier at the start of fermentation was significantly lower than the others.

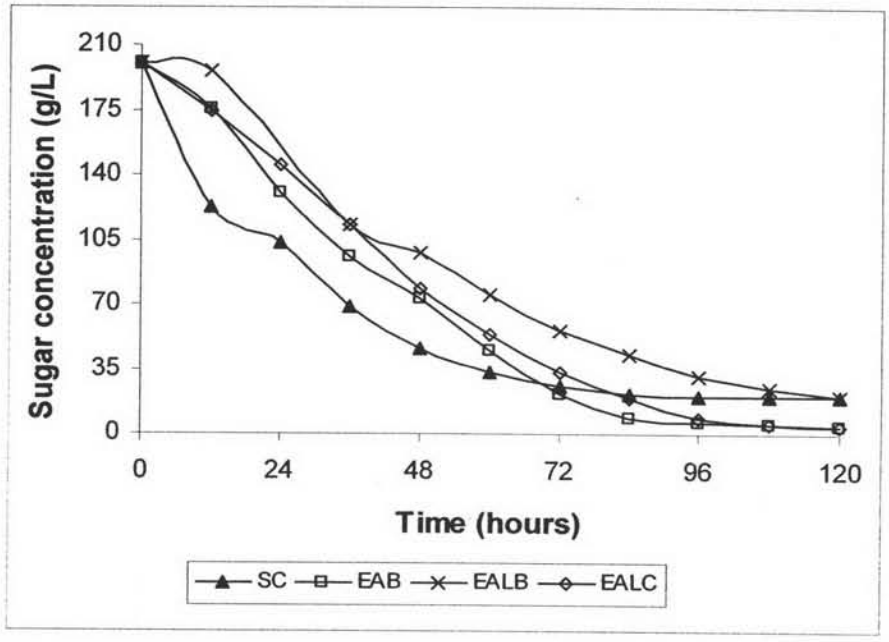

Figure 4.27 Sugar concentration profile of fermentation 3

As in fermentation 2, final ethanol concentrations were higher in immobilized cell systems than SC (Figure 4.28). Although sugar consumption of EAB and EALC was higher than EALB, the final ethanol concentrations (P_F) of the three systems were equivalent (Table 4.3). After 72 hours, the ethanol concentration of EAB and EALC was stabilized which corresponded to sugar concentration of about 20 g/L. It was proposed that the sugars available at this level might be the unfermented sugars for yeast.

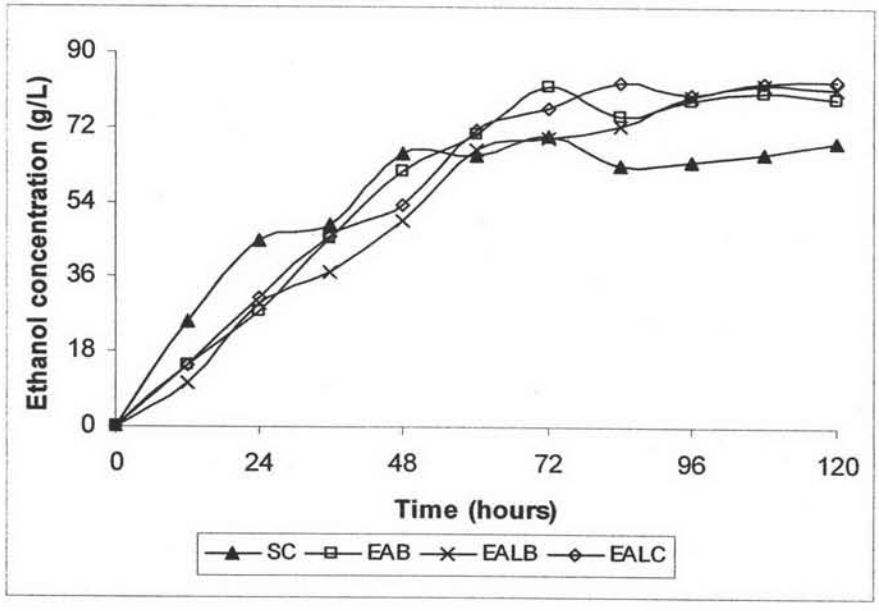

Figure 4.28 Ethanol concentration profile of fermentation 3

From the immobilization yield (Y_I) data (Table 4.3), it can be concluded that the new carriers (EALB and EALC) were less efficient in immobilizing yeast than alginate bead (EAB). Structural differences among the carriers were proposed as the main separating factor and will be further analyzed by comparing the SEM images. Y_I of EALB (33.37 %) was too low thus this carrier was considered ineffective for yeast immobilization purpose.

By analyzing the values of sugar consumption yield (Y_S) and ethanol yield factor ($Y_{P/S}$) simultaneously, it is suggested that there might be physiological differences on immobilized yeast in this study. Low Y_S and $Y_{P/S}$ in SC system indicate the occurrence of inhibition, most probably caused by high ethanol level.

Higher final ethanol concentration (P_F) and Y_S obtained in EAB, EALB, and EALC suggests that these systems might have better resistance to inhibition than free cell culture (SC). When compared with EAB, Y_S and $Y_{P/S}$ were higher in alginate-loofa carrier (EALB and EALC) cultures. This result indicates that larger fraction of sugar was utilized for ethanol production in alginate-loofa carrier systems than SC. In this view, the new carriers are proposed to be more advantageous in promoting ethanol production by yeast than conventional alginate bead (EAB). Beneficial properties of immobilized cell systems, such as protection from inhibition and promotion of cell activity had also been reported elsewhere [6,7,44,45]. Junter and Jouenne (2004) proposed that the ability of cell to grow in the immobilized state makes it possible for the regeneration of the culture in hostile condition such as high ethanol concentration.

Figure 4.29 shows an image of yeast living in suspended cell culture (SC). The cells appeared healthy and retained their normal oval shape. The whole view of EAB is provided in Figure 4.30. Considerable amounts of opening were observed on the surface of EAB as shown in detail in Figure 4.31. As shown in Figure 4.32, the yeast cells inside the opening could escape to surroundings, leaving only few cells attached to the carrier.

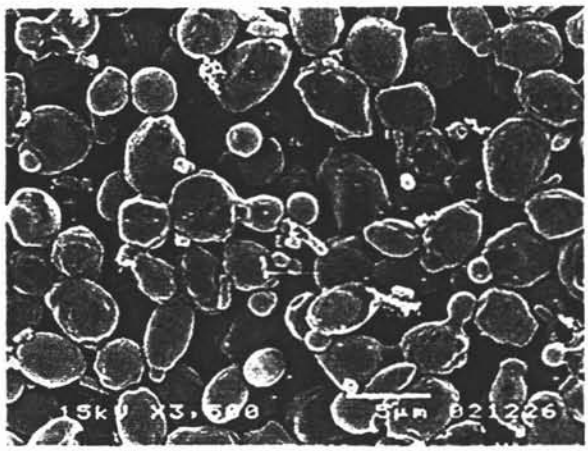

Figure 4.29 Yeast from SC broth

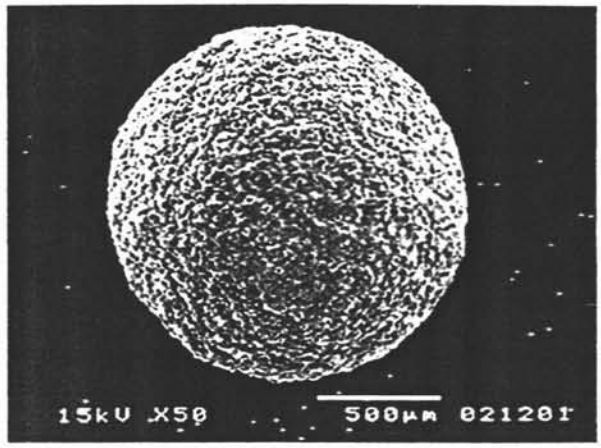

Figure 4.30 Whole view of EAB

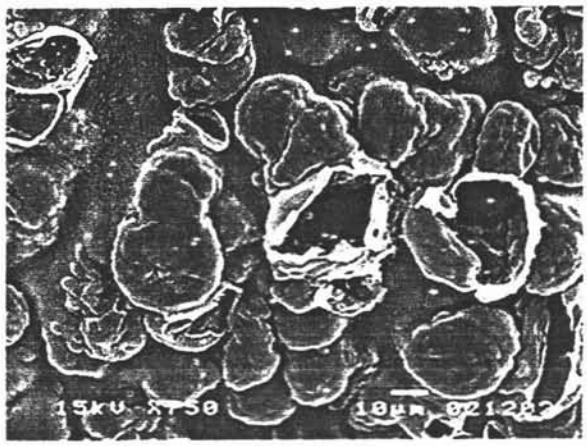

Figure 4.31 Surface of EAB

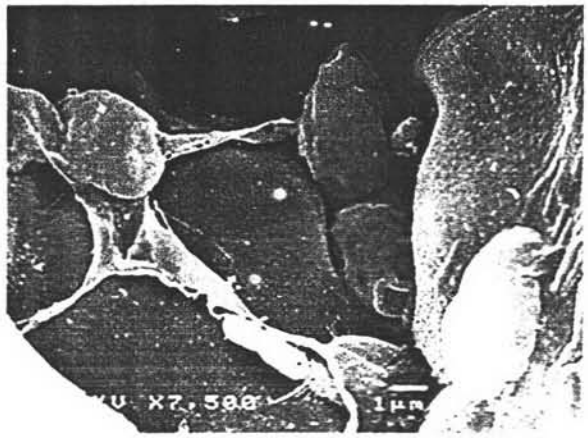

Figure 4.32 Surface cavity of EAB

Figure 4.33 shows near surface appearance of EAB in cross sectional point of view. Close to the surface, the gel was highly porous. Many cells as shown in greater detail in Figure 4.35 were found living near the surface of the bead. On contrary, the gel in the center of the bead was less porous (Figure 4.34); consequently, fewer cells were observed. However, the cells found in the center of the bead were in good condition (Figure 4.36). The preference of the cells to live near the surface rather than deep inside the bead had also been reported by Najafpour et al. (2004). High accessibility to food as a consequence of better mass transfer near the surface is proposed as the main driving force. However, the intense growth might in turn be the cause of cell leakage. As more cells are grown near the surface, the film of gel at the surface expands and becomes thinner. After a maximum yielding point, the tensile strength of the film is no longer sufficient to stand the expansion force caused by cells' growth. At this point, the break of the polymer film is inevitable.

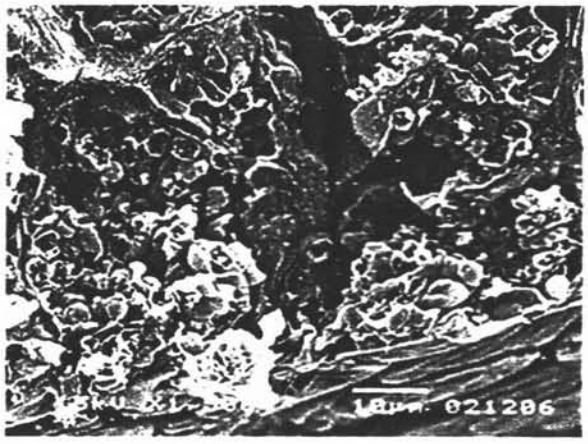

Figure 4.33 EAB surface cross section

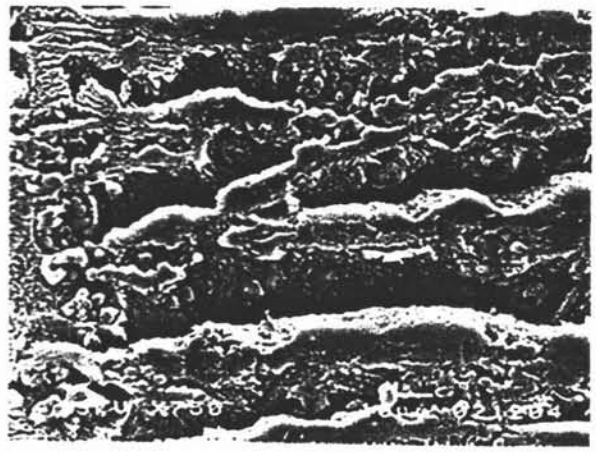

Figure 4.34 EAB central cross section

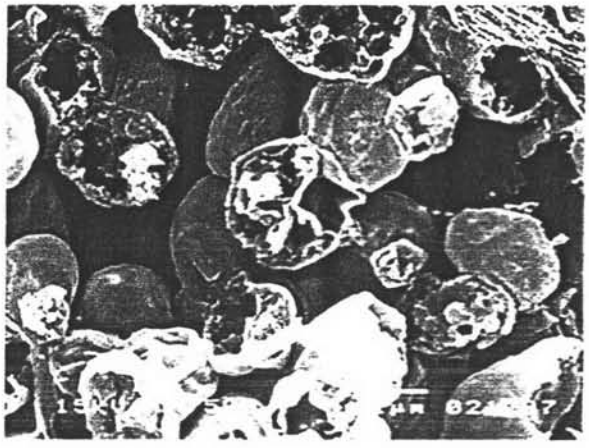

Figure 4.35 Yeasts near EAB surface

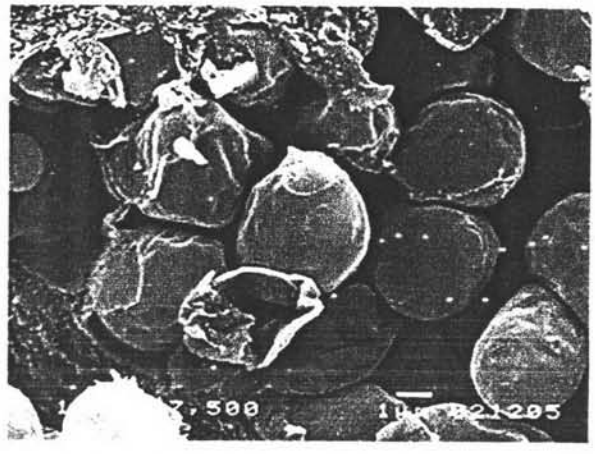

Figure 4.36 Yeasts in EAB center

Figure 4.37 shows an overall view of EALB which had an irregular shape. Figure 4.38 and 4.39 shows that there was huge leak of cells from the surface of EALB. Although some cells were attached to the surface of EALB, they were no longer protected by the film of alginate. Closer look to these cells as provided by Figure 4.40 confirms that the cells were in good condition even without coverage of alginate. Before the leakage occurred, the yeasts might have gathered in aggregate. This aggregate form might somehow grant the cells a better endurance towards external forces so that the cells could maintain their good condition.

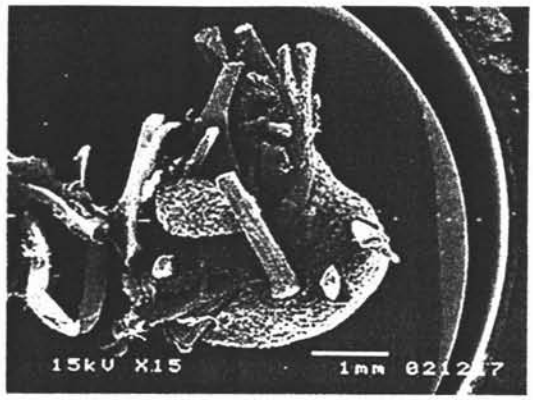

Figure 4.37 Whole view of EALB

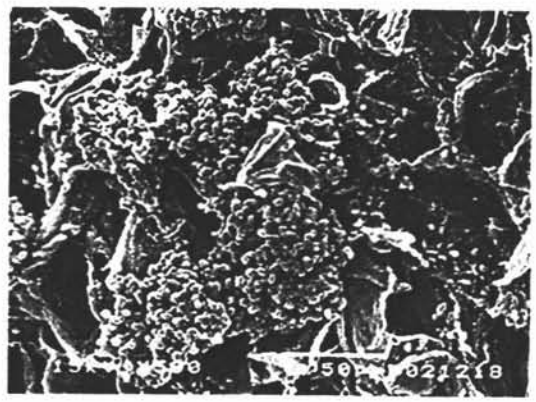

Figure 4.38 Surface of EALB

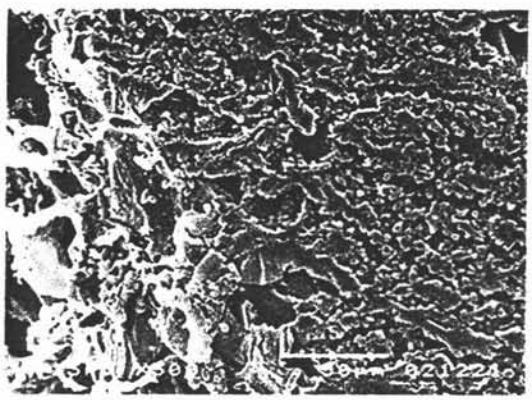

Figure 4.39 EALB surface cross section

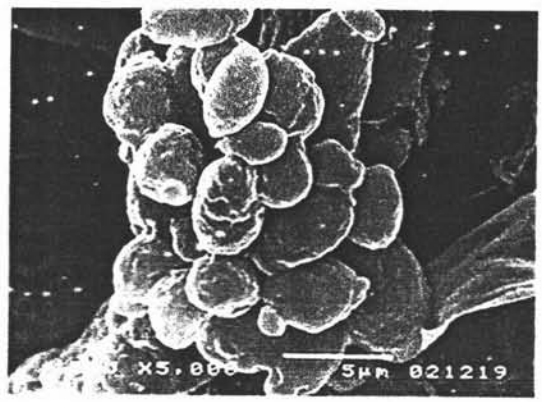

Figure 4.40 Yeasts on EALB surface

The cross sectional view of EALB is provided in Figure 4.41, showing the gel thickness across the carrier was uneven. There was also a big vacant space inside the carrier. The cavity together with uneven thickness of gel and overgrowth of yeast might have played a major role in causing excessive leak of cells from EALB. A better look into the center of the carrier (Figure 4.42) suggests that the cells could access essential food even they were located deep inside the carrier. This result suggests that mass transfer limitation inside the carrier was not severe.

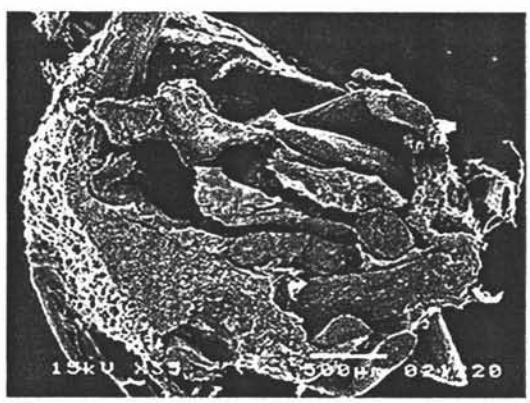

Figure 4.41 Cross section of EALB

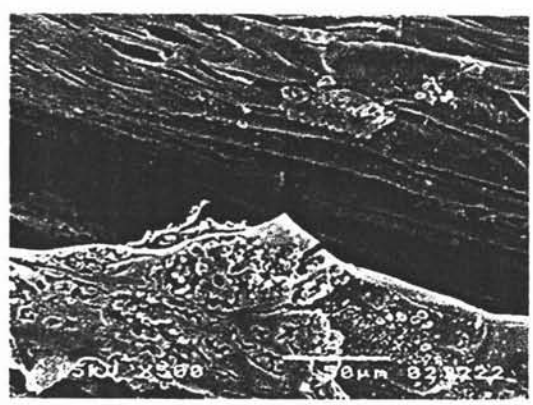

Figure 4.42 EALB central cross section

Figure 4.43 shows an image of EALC surface. Some exposed fibers of loofa can be seen on the top right corner of the image. These fibers were utilizable by yeast as a living space (Figure 4.45 and 4.46). Cracks were also observed on EALB surface (Figure 4.44) although the amount was fewer than EALB (Figure 4.38). This fact suggests that overgrowth of yeast in EALC was less severe than EALB. Figure 4.47 provides a better sight of yeasts' morphology inside the hole.

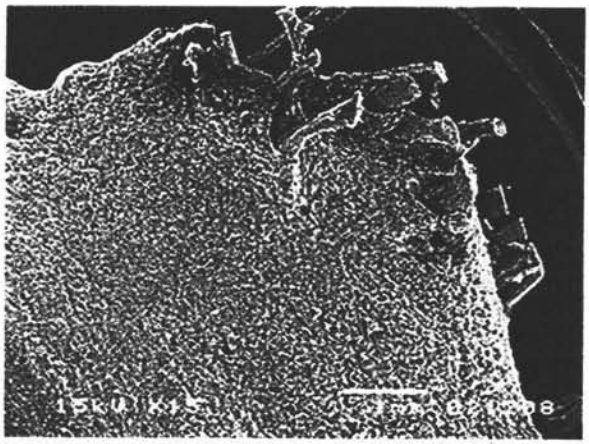

Figure 4.43 EALC view

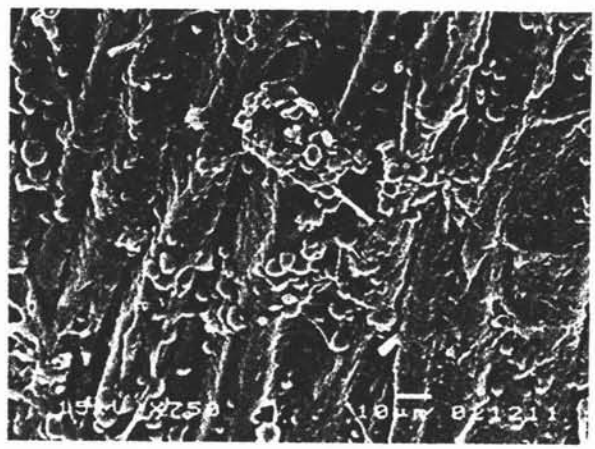

Figure 4.44 Surface of EALC

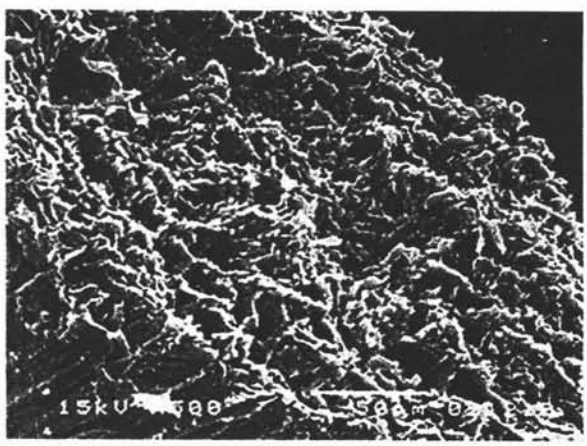

Figure 4.45 Exposed fiber of EALC

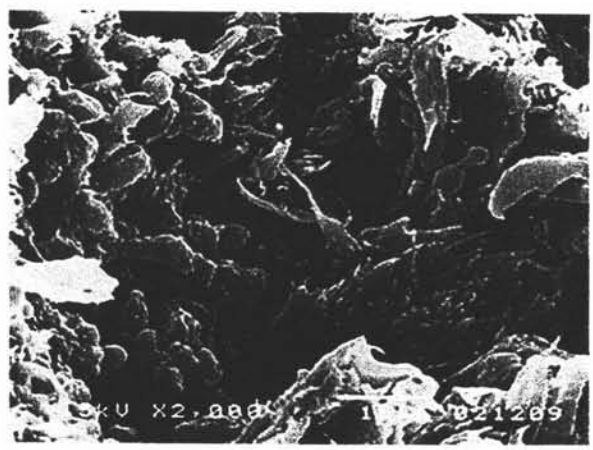

Figure 4.46 Yeasts on exposed fiber

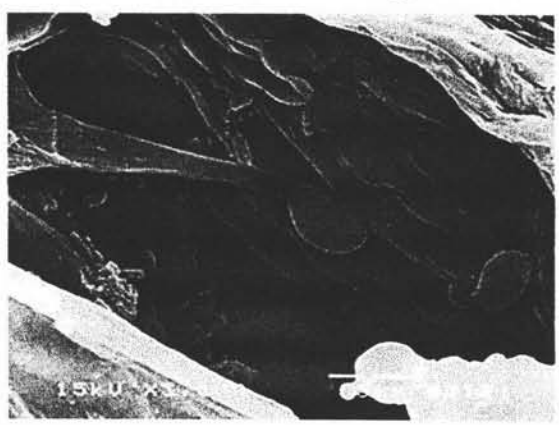

Figure 4.47 Surface cavity of EALC

The overall cross section of EALC is shown in Figure 4.48. When compared to EALB, gel coverage for EALC was better, as indicated by more even thickness of gel across the carrier. The structure inside EALC (Figure 4.49) was more porous than EAB (Figure 4.34). This porous structure could provide convenient living environment for the yeast resulting in many yeasts found in the center of the carrier (Figure 4.50). Figure 4.51 indicates that there was no severe mass transfer problem as the cells in the center of the carrier were in good condition.

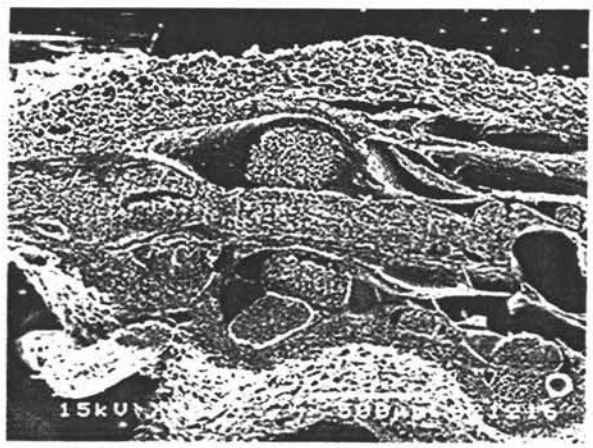

Figure 4.48 Cross section of EALC

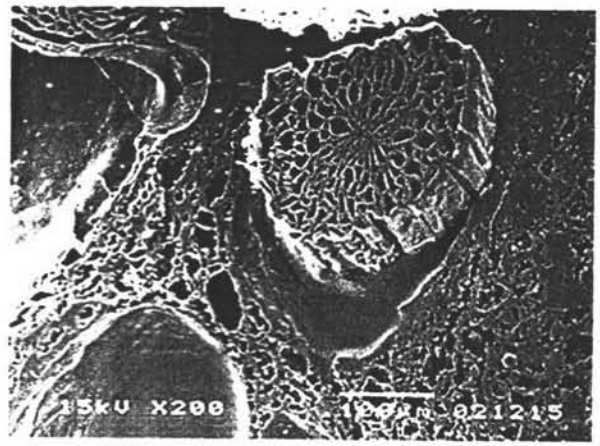

Figure 4.49 Porous structure of EALC

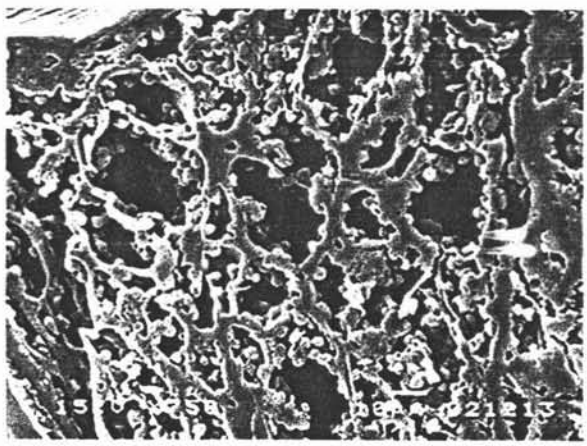

Figure 4.50 EALC central cross section

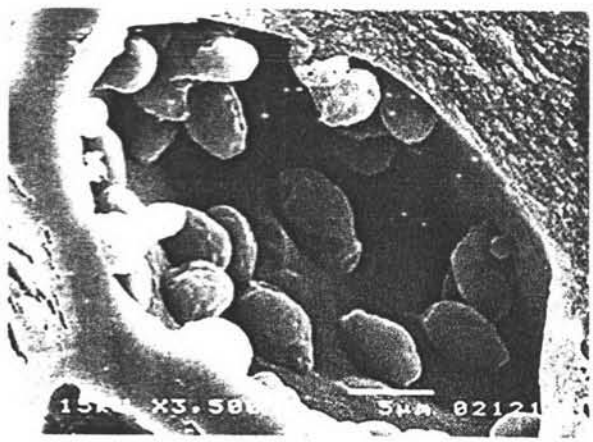

Figure 4.51 Yeasts at EALC center

4.6 Fermentation 4

Fermentation 4 was designated for early examination of carrier reusability in molasses medium. Due to its low immobilization yield in fermentation 3, EALB was not used in fermentation 4. Sugar concentration was about 50 g/L in this experiment, a level which is considered ideal for yeast growth. SC, EAB, and EALC systems were carried out in 2 sets, resulting in total 6 flasks evaluated in parallel at every cycle. For reusability testing, 3 repeated batches were carried out after the main batch.

Reusability of the carriers was successfully demonstrated as steady ethanol production was detected in all batches. Moreover, major carrier deformation (for example change of carrier shape and break of carrier) was not observed during the total time span of 216 hours of repeated batch fermentation. Based on this result, the matrix of alginate-loofa carrier is durable and suitable for reuse.

By comparing the images of the carriers at the beginning of main batch (Figure 4.52 and 4.53) and the end of repeated batch 3 (Figure 4.54 and 4.55), the incidence of cell growth and gel degradation during the course of fermentation is confirmed. Images of carriers from main batch to repeated batch 3 show that the amount of cell inside the carriers from time to time was increasing. Evolution of carrier in fermentation 4 from batch to batch can be observed from a complete set of SEM images provided in Appendix B.

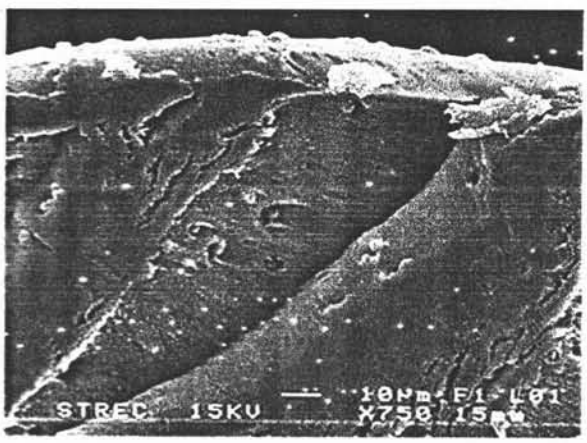

Figure 4.52 Initial EAB cross section

Figure 4.53 Initial EALC cross section

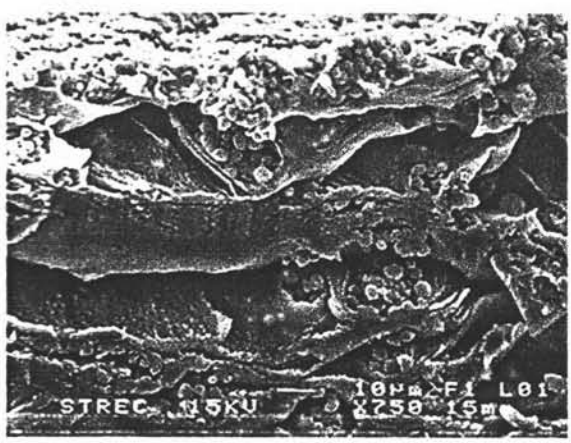

Figure 4.54 EAB cross section after repeated batch 3

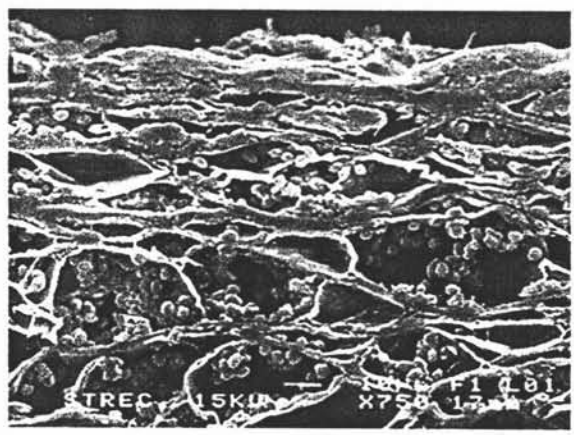

Figure 4.55 EALC cross section after repeated batch 3

4.7 Fermentation 5

Fermentation 4 confirmed that EALC could be reused. Further investigation of EALC performance in repeated batch mode was conducted in fermentation 5. There were 4 cultures in fermentation 5: SC, EAB, EALC 1, and EALC 2. The initial sugar concentration of 220 g/L was used in this experiment. Before sterilization, the medium was centrifuged to remove suspended solid originated from the raw molasses. Same stock cell suspension with cell concentration of 20.7 g/L was used for SC, EAB, and EALC 1. In case of EALC 2, another stock cell suspension with lower cell concentration (1.75 g/L) was used. In all inoculums preparation, 5 ml of stock cell suspension was utilized. With this configuration, it is convenient to compare SC, EAB, and EALC 1 as they were inoculated with the same amount of cells. EALC 2 is used in comparison with EALC 1 to demonstrate the capability of EALC carriers to perform under lower cell loading.

Free cell concentration of SC was considerably higher than immobilized cell systems (EAB, EALC 1, and EALC 2) in all batches (Figure 4.56 to 4.58). For SC, intensive cell growth took place during the first 24 hours in the main batch. In the experiment, the intensive growth period was indicated by massive forming of foam in the fermentation broth, especially for the SC culture. After 24 hours, the cell concentration profile of SC for the main batch became flat. As most of the cells in EAB, EALC 1, and EALC 2 were entrapped in the carriers, minor increase of free cell concentration was observed in these systems as indicated by flat profiles of cell concentration in all batches. In average, the free cell concentration was increased from main batch to repeated batch. This increase can be attributed to cell's growth and cell leakage from the carriers.

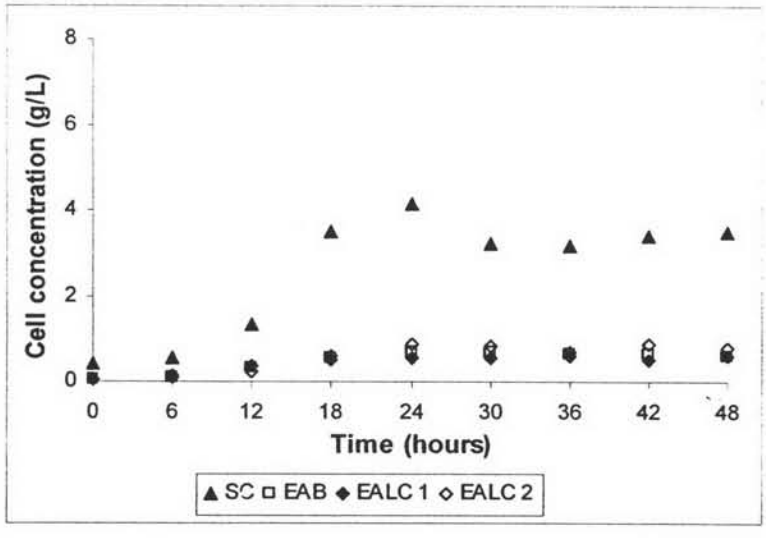

Figure 4.56 Free cell concentration profile for main batch

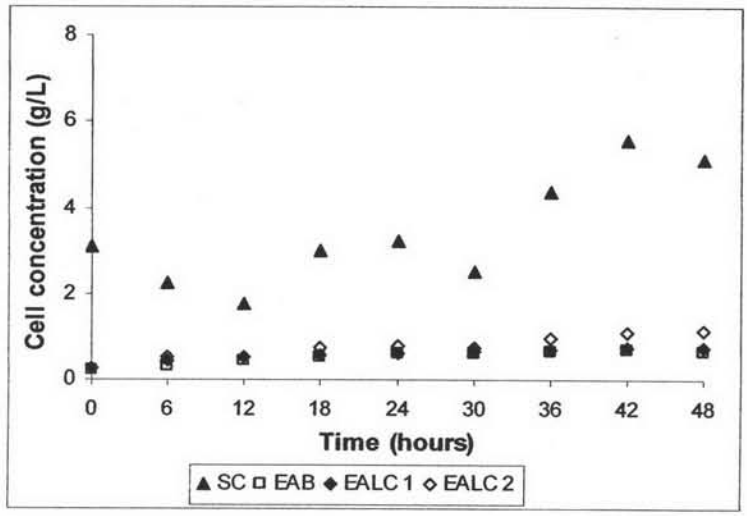

Figure 4.57 Free cell concentration profile for repeated batch 1

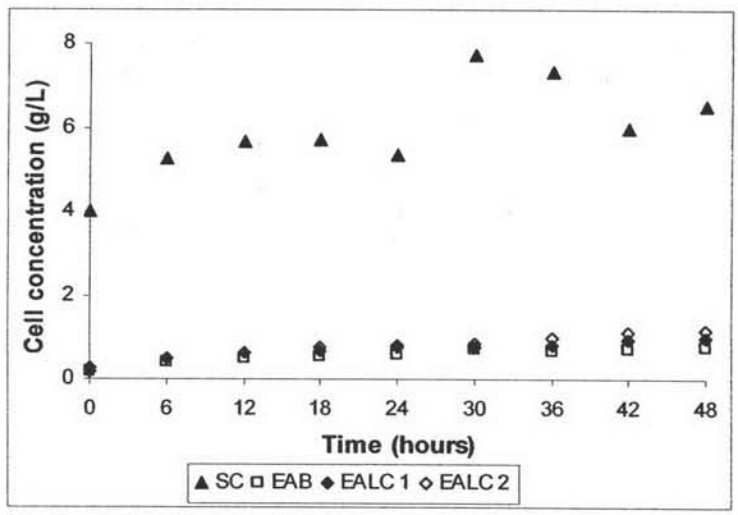

Figure 4.58 Free cell concentration profile for repeated batch 2

In case of immobilized cell concentration, EAB was superior to EALC in all batches. It is proposed that cell growth was better in EAB than EALC. This fact is demonstrated in Figure 4.59 to 4.61. Initially, cell concentration inside EALC 2 was much smaller (approximately 10 times) than EALC 1 because of its less cell loading. Following the course of fermentation, immobilized cell concentration of EALC 2 eventually increase until it reached similar level with EALC 1 within 30 hours. This result demonstrated that in case of lower cell loading, longer time is needed to reach the saturated cell concentration. For all systems, major cell growth happened in the initial period of main batch and repeated batch 1 at which the medium was still in a fresh condition. After 12 hours in repeated batch 1, the immobilized cell concentration for EAB, EALC 1, and EALC 2 was stabilized. Moreover, this level of immobilized cell concentration was practically

unchanged in repeated batch 2. This can be an indicator that the cell concentration in the respective carriers had reached a certain saturation point. It is understandable that the carriers themselves were limited in size and volume so that they had restricted capacity for cell retention.

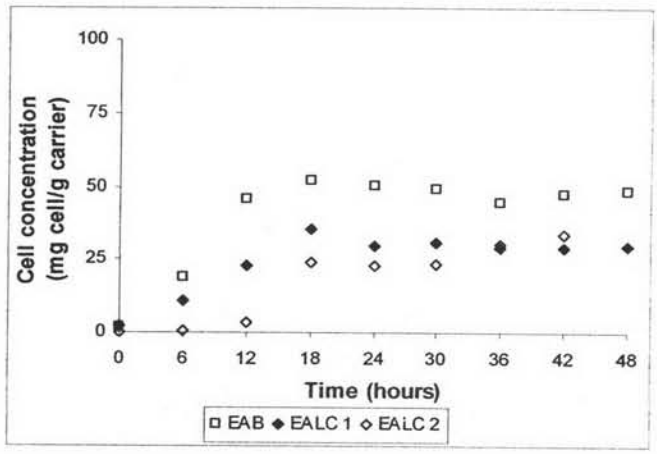

Figure 4.59 Immobilized cell concentration profile for main batch

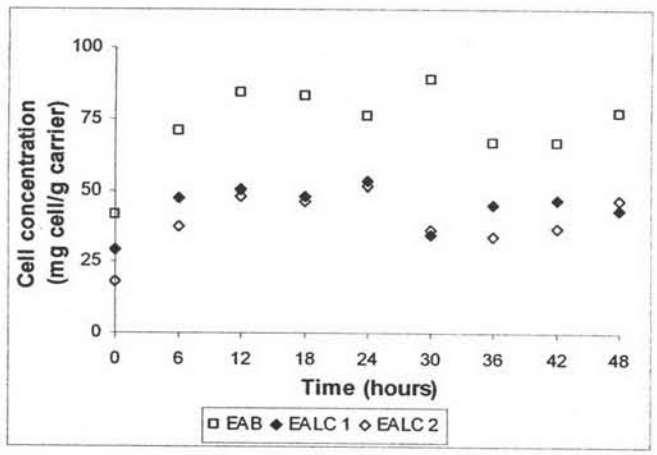

Figure 4.60 Immobilized cell concentration profile for repeated batch 1

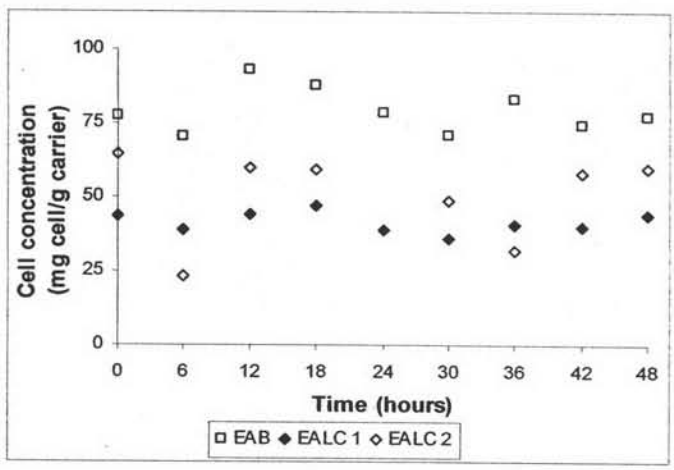

Figure 4.61 Immobilized cell concentration profile for repeated batch 2

Sugar concentration profiles for the main batch resemble the reversed version of a normal microbial growth curve which consists of lag, logarithmic, and stationary phase (Figure 4.62 to 4.64). This is well anticipated as the substrate consumption in general is proportional with cell concentration. The shape of the sugar concentration curve was similar for all systems, indicating that the yeast was behaving similarly in term of sugar consumption. It is suggested that there might be the same limiting factors in all systems so that by the end of the main batch, the sugar concentration reached a similar level.

In repeated batch 1, lag phase in sugar concentration profile diminished for EAB, EALC 1, and EALC 2. On contrary, there was 30 hours lag phase in case of SC. At the end of main batch, free yeast cells were directly exposed to low sugar concentration and high level of ethanol. As this condition was not suitable for the viability of the free cells, a long lag phase was required for the cells to revitalize themselves. In case of the immobilized systems (EAB, EALC 1, and EALC 2), most of the active cells were protected inside the carrier matrix which in turn enabled them to retain their vitality. In repeated batch 2, all systems exhibited high sugar consumption without the occurrence of lag phase. In most cases except SC in repeated batch 2, majority of sugar was consumed during the first 36 hours of each batch. This demonstrated that the yeast used in this study was highly active.

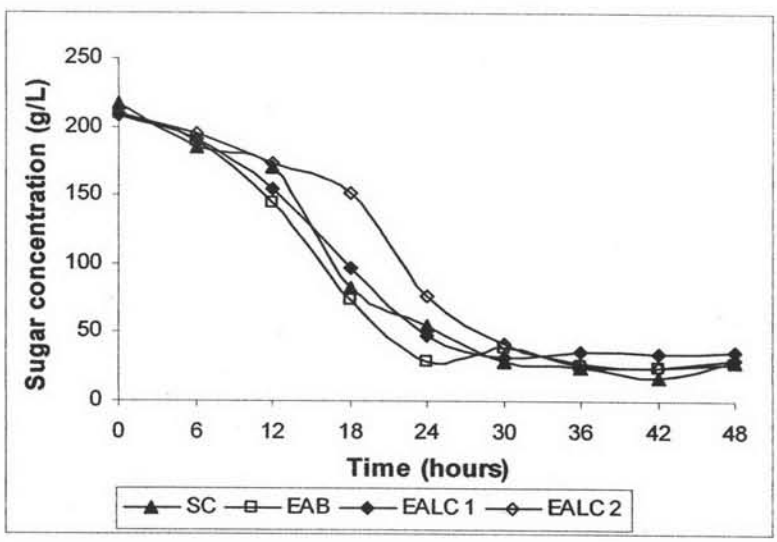

Figure 4.62 Sugar concentration profile for main batch

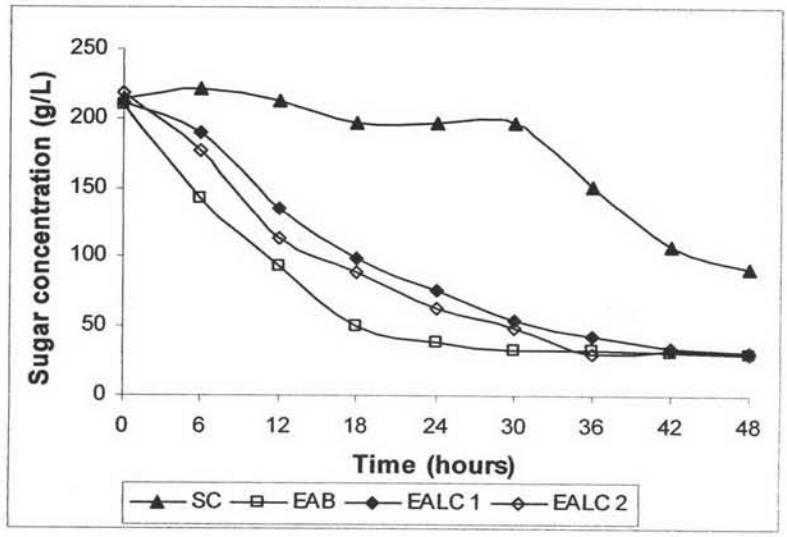

Figure 4.63 Sugar concentration profile for repeated batch 1

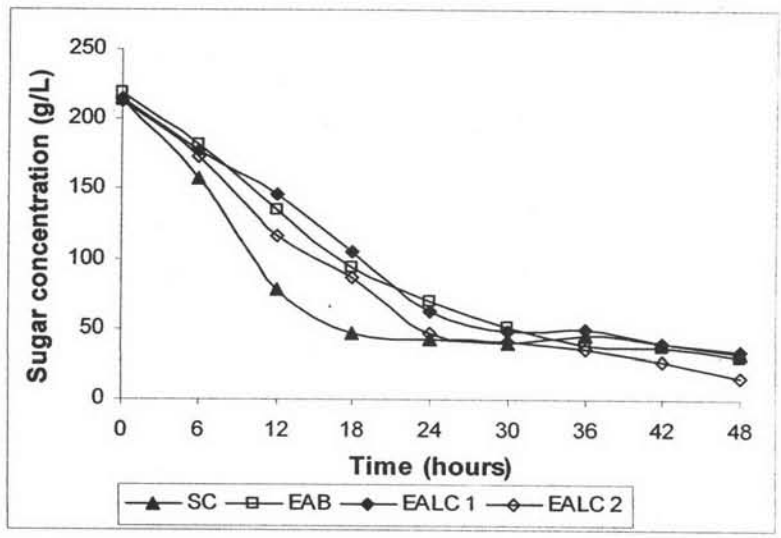

Figure 4.64 Sugar concentration profile for repeated batch 2

Similar with sugar concentration curve, ethanol concentration profile (Figure 4.65 to 4.67) also followed the trend of the normal microbial growth curve. This is understandable for ethanol as it is a growth associated product. Because of its lower cell loading, ethanol production rate of EALC 2 was slower than EALC 1. By the end of the main batch, ethanol concentrations or immobilized cell systems were similar to each other but were lower than SC.

Corresponding to its sugar concentration profile, ethanol production rate of SC was greatly reduced in repeated batch 1. Other cultures behaved accordingly to their respective sugar consumption level and produced ethanol to the similar level as the main batch without passing through a lag phase. In repeated batch 2, all systems exhibited slightly higher final ethanol concentration as compared to the main batch. This was possible

because lag phase was absent in repeated batch 2. The ethanol concentration profiles in this batch were also in good agreement with their respective sugar concentration profiles. At logarithmic phase, SC culture had steeper slope in its ethanol concentration profile than EAB and EALC. Thus, volumetric ethanol productivity of SC in this stage was higher than the others. Unfortunately, this high volumetric productivity of SC was not supported by stable production. This fact was eminently proven in repeated batch 1.

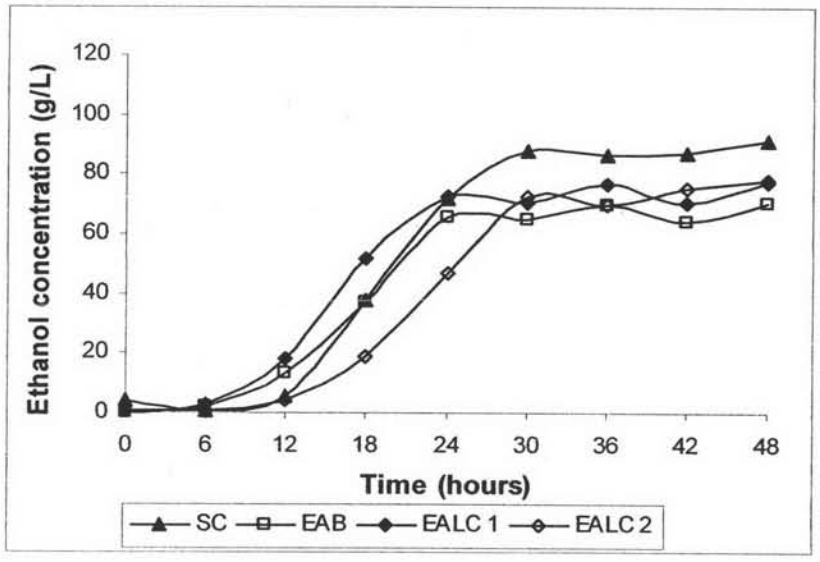

Figure 4.65 Ethanol concentration profile for main batch

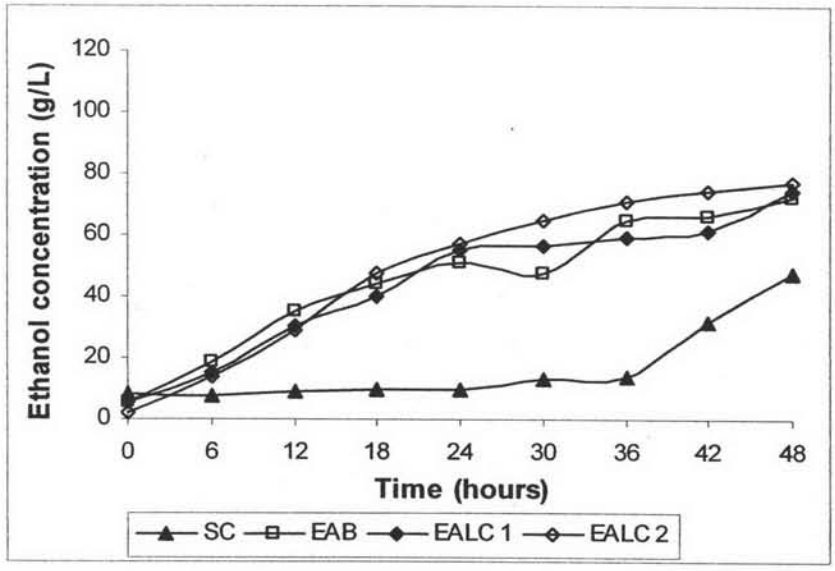

Figure 4.66 Ethanol concentration profile for repeated batch 1

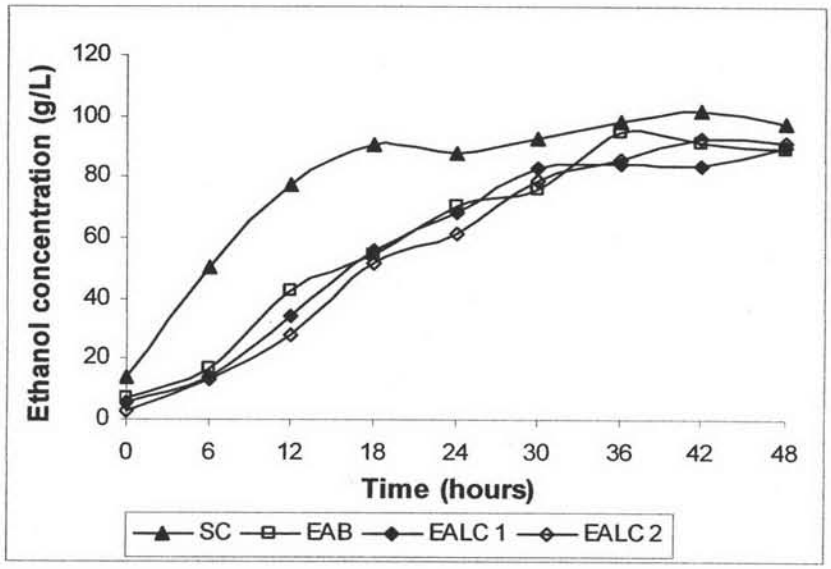

Figure 4.67 Ethanol concentration profile for repeated batch 2

Summary of results of fermentation 5 is provided in Table 4.4 to 4.6. All data were evaluated at the end of every batch. Instability of suspended culture in term of ethanol yield is obvious by comparing its final ethanol concentration (P_F) from batch to batch. P_F of SC fluctuated significantly from 97.4 g/L in repeated batch 2 to 47.7 g/L in repeated batch 1. On contrary, ethanol yield of EAB and EALC was quite stable during the main batch and repeated batch 2. Moreover, the performance of EAB and EALC systems was more comparable to SC in repeated batch 2 than main batch as the difference between their P_F values was reduced.

Table 4.4 Parameters of fermentation 5 in main batch

System	P_F (g/L)	X_T (g/L)		Y_I (%)	Y_S (%)	$Y_{P/S}$ (g/g)
		X_E	X_I			
SC	91.7	3.48	-	-	86.4	0.468
EAB	70.4	0.60	6.00	90.9	86.8	0.388
EALC 1	77.7	0.60	4.56	88.3	82.6	0.448
EALC 2	77.8	0.80	4.56	85.1	85.9	0.428

Table 4.5 Parameters of fermentation 5 in repeated batch 1

System	P_F (g/L)	X_T (g/L)		Y_I (%)	Y_S (%)	$Y_{P/S}$ (g/g)
		X_E	X_I			
SC	47.7	5.13	-	-	57.0	0.319
EAB	72.7	0.66	8.47	92.8	85.9	0.375
EALC 1	74.3	0.74	5.94	88.9	85.2	0.384
EALC 2	77.4	1.14	6.25	84.6	86.4	0.397

Table 4.6 Parameters of fermentation 5 in repeated batch 2

System	P_F (g/L)	X_T (g/L)		Y_I (%)	Y_S (%)	$Y_{P/S}$ (g/g)
		X_E	X_I			
SC	97.4	6.50	-	-	85.0	0.456
EAB	89.5	0.77	8.49	91.7	86.5	0.438
EALC 1	89.7	0.99	6.10	86.1	84.0	0.467
EALC 2	91.5	1.18	7.99	87.1	92.4	0.450

It is suggested that during the main batch and repeated batch 1, the yeast in the carriers was still growing and adapting to its new microenvironment. Thus, full potential of the cell had not been achieved. This preposition was supported by final cell concentration data which were increasing from batch to batch. Although the dry weight of cell method can not provide information on the active fraction of the cells, it is proven that the cell densities of immobilized cell systems was indeed higher than suspended cell culture. Even though Chien and Sofer (1985) reported that specific ethanol productivity of immobilized yeast was two-thirds than free cell, higher ethanol yield as demonstrated in this study was able to be achieved. The repeated batch study also revealed protective ability of the immobilization matrix. In the medium of same composition, more cells were grown in the carriers than in the liquid broth. The matrix of the carriers may protect the yeast by fortification from toxins and inhibitor [3, 5-7].

Total cell concentration (X_T) and final ethanol concentration (P_F) of EALC 2 was equivalent with EALC 1 at the end of main batch although longer time was needed to reach this result because of its low initial cell loading (Figure 4.65). As EALC 1 and EALC 2 culture started with comparable total amount of cell in repeated batch 1 and 2, it is well expected that their ethanol concentration profiles (Figure 4.66 and 4.67) and final ethanol concentrations are similar. It is suggested that in case of low initial cell loading, optimum ethanol fermentation can be achieved after saturated cell concentration is reached. In this experiment, the optimum performance of EALC 2 was achieved after the main batch.

In term of immobilization yield, EALC exhibited comparable immobilization capacity with EAB. Sugar consumption (Y_S) of EALC was also equivalent with EAB and SC, suggesting that the new EALC system was reliable in term of substrate utilization.

Ethanol yield factor ($Y_{P/S}$) of EALC culture was higher than EAB in all batches. This result is in agreement with cell concentration data. As more cells were produced in EAB than EALC, the fraction of substrate used for ethanol production was higher in

EALC. When compared with SC, $Y_{P/S}$ of EALC was at first lower than SC in the main batch. It was then higher and comparable with SC in repeated batch 1 and 2 respectively.

During the first batch, the yeast in immobilized systems was still acclimatizing with the new microenvironment thus its ethanol yield factor was consequently lower than SC. Because of the harsh condition induced by metabolic products accumulation at the end of the main batch, the yeast could spent more energy from the substrate to maintain its viability through repeated batch 1. As the cells in the carrier were better protected from their environment than free cell, they could utilize more fraction of sugar for ethanol production as indicated by higher $Y_{P/S}$ for EAB and EALC than SC. In repeated batch 2, the yeast in all systems regained its vitality thus high value of $Y_{P/S}$ was accomplished. It is interesting to note that the level of $Y_{P/S}$ of immobilized systems especially EALC was more comparable to SC in repeated batch 2 than main batch. This result is proposed as an indication that the immobilized yeast had been well acclimatized to environmental condition in repeated batch 2.

SEM micrographs of the initial EAB and EALC (before fermentation was started) show that the carriers were sparsely populated by yeast (Figure 4.68 to 4.73). Moreover, the cells were randomly distributed inside the carriers, across the surface until the core. From the surface view provided by Figure 4.68 and 4.69, most of the cells were covered by alginate film. In addition, free cells might also adsorb on the surface of the carrier (Figure 4.68). This is in good agreement with previous experiment which showed that the alginate carriers had some extent of adsorbing capacity. Some cells were aggregated, demonstrating flocculating properties of the yeast strain. From the cross sectional view (Figure 4.70 to 4.73), it is obvious that EAB and EALC had different gel morphology. Cutting mark of EAB proved that this carrier was denser. On the other hand, it was shown that gel of EALC was lamellar. The gel was constructed by stacked layers of thin alginate film.

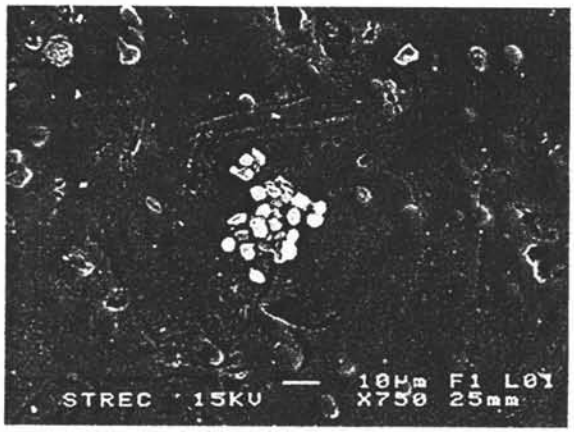

Figure 4.68 Initial EAB surface

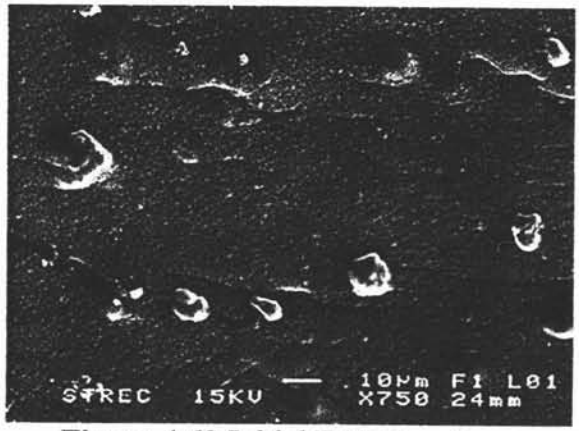

Figure 4.69 Initial EALC surface

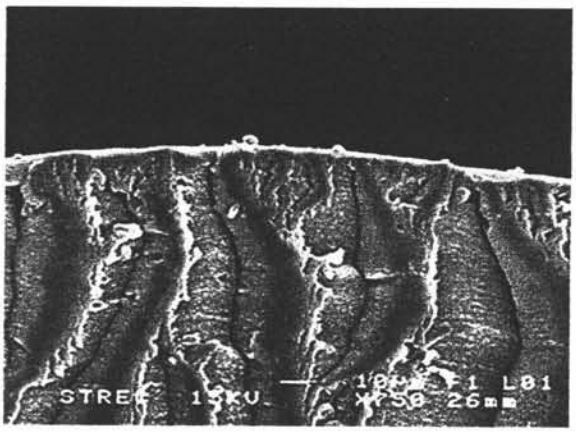

Figure 4.70 Initial EAB surface cross section

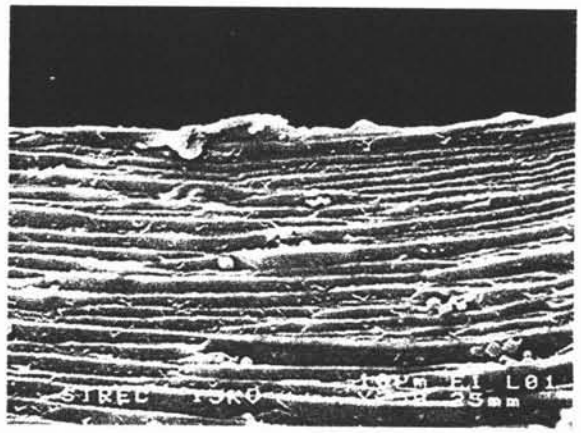

Figure 4.71 Initial EALC surface cross section

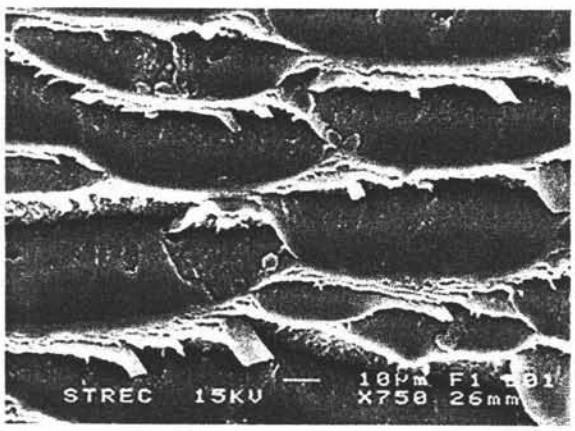

Figure 4.72 Initial EAB central cross section

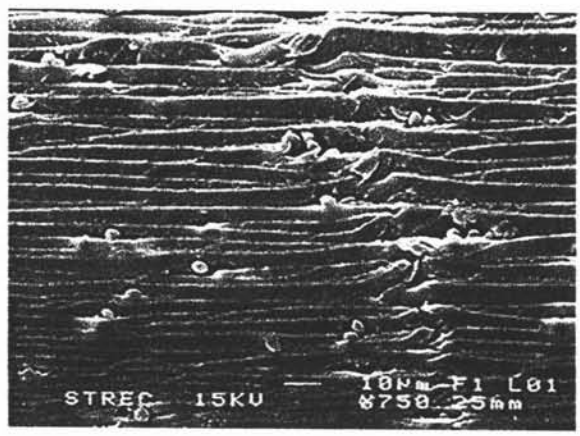

Figure 4.73 Initial EALC central cross section

After the main batch, there was a massive population of cells inside EAB and EALC (Figure 4.74 to 4.79). This is in contradiction with initial carrier's condition (Figure 4.68 to 4.73). Cell leakage from the surface of the carriers is again confirmed by the SEM images (Figure 4.74 and 4.75). It is shown that cell leakage was more severe on EALC than EAB as Figure 4.74 and 4.75 were compared. It is suggested that this fact

corresponded with the lamellar structure of EALC gel. As the cells growth, they caused expansion in the gel by producing gas and by their own volume. This could be observed in the form of bumps on the carrier surface (Figure 4.74 and 4.75) and pores inside the carrier (Figure 4.76 to 4.77). Until a certain maximum point, the elasticity of the alginate film was no longer sufficient to withstand the stress caused by the gel expansion. Thus, gel breakage or degradation was inevitable and the cells inside was able to leak out of the carrier. By comparing Figure 4.76 and 4.78, there was a tendency of cells to grow more on the surface than the inner core of the carrier. The same event was previously reported by Najafpour et al. (2004). On contrary, large cell population and high porosity was observed both on surface and core of EALC (Figure 4.77 and 4.79). This demonstrates that because of its unique structure, mass transfer in EALC might be better than EAB despite of its larger size. The preposition was supported by Sakurai et al. (2000) who stated that physical structure is significant in cell immobilization.

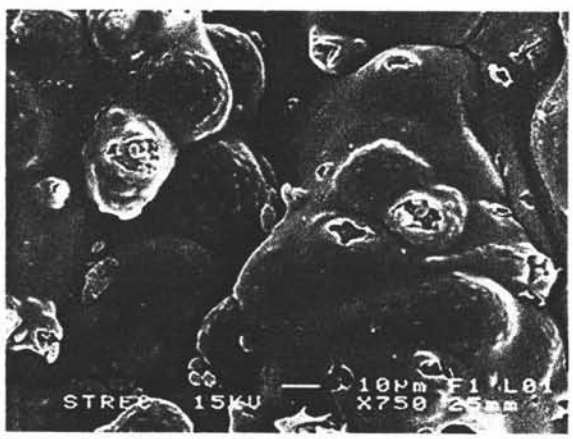

Figure 4.74 EAB surface after main batch

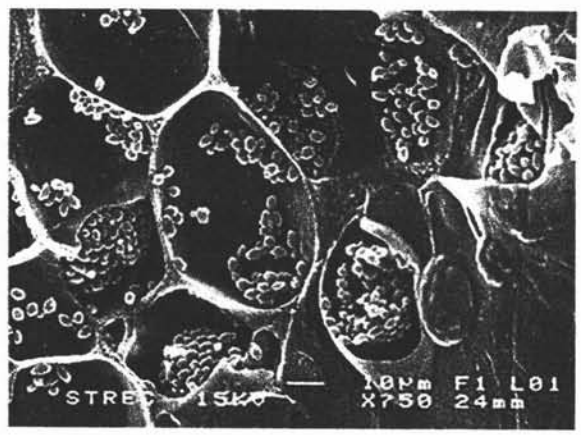

Figure 4.75 EALC surface after main batch

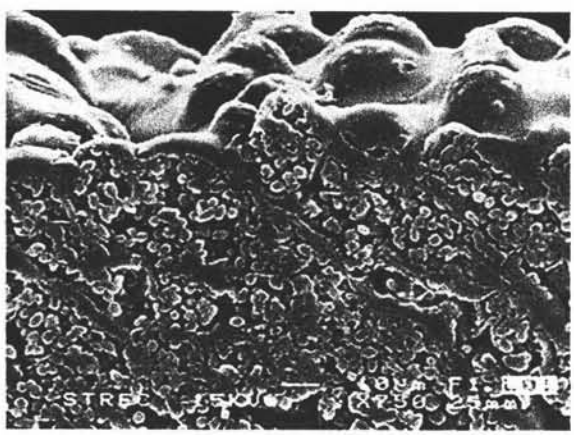

Figure 4.76 EAB surface cross section after main batch

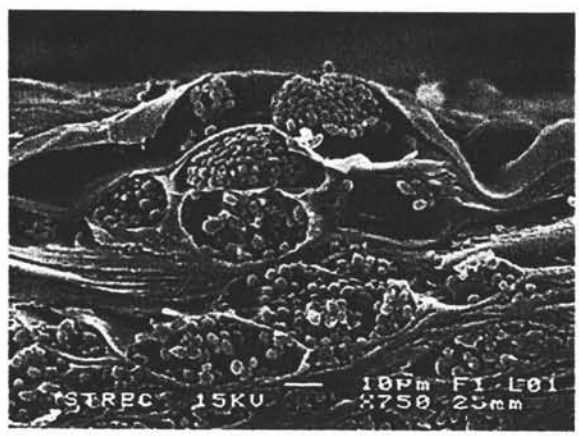

Figure 4.77 EALC surface cross section after main batch

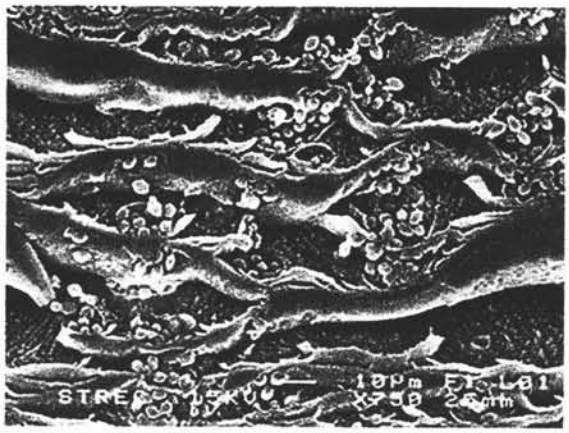

Figure 4.78 EAB central section after main batch

Figure 4.79 EALC central cross section after main batch

As minor growth took place during the repeated batch 1, the appearance of the carriers' structure after this batch (Figure 4.80 to 4.85) was in general similar with the main batch (Figure 4.74 to 4.79). Figure 4.81 depicts a massive exposure of cells on the surface of EALC. It is shown in this picture that even though the cells were no longer contained by the alginate film, they were firmly attached to the carrier surface because of their flocculating nature. In flocculating yeast, the cells are connected to each other by binding structure. This structure played a major contribution in forming a network between the cells as well as forming a bond between the cells and the carrier. On contrary, it was also shown in Figure 4.83 that in case of excess sloughing, the cells were wiped out of the surface. It is interesting also to note that the big space between the fiber of loofa and alginate in EALC, as shown in left part of Figure 4.85 was also usable for cell habitation.

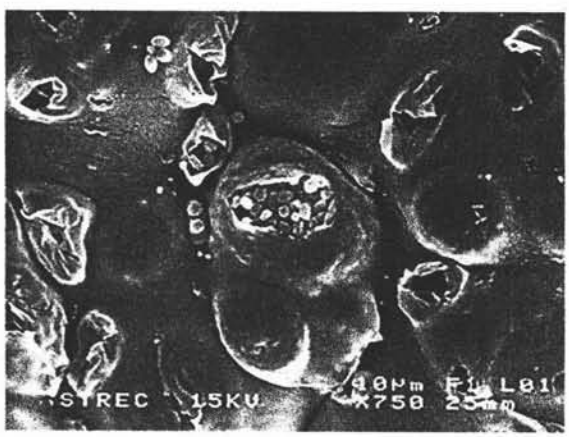

Figure 4.80 EAB surface after repeated batch 1

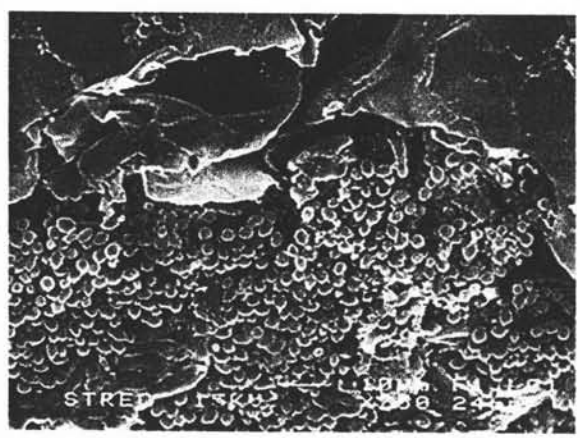

Figure 4.81 EALC surface after repeated batch 1

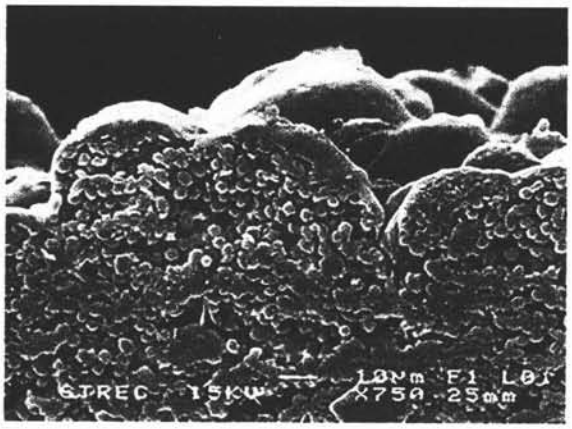

Figure 4.82 EAB surface cross section after repeated batch 1

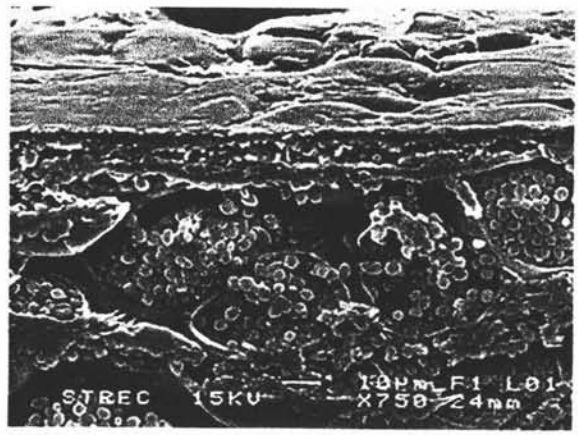

Figure 4.83 EALC surface cross section after repeated batch 1

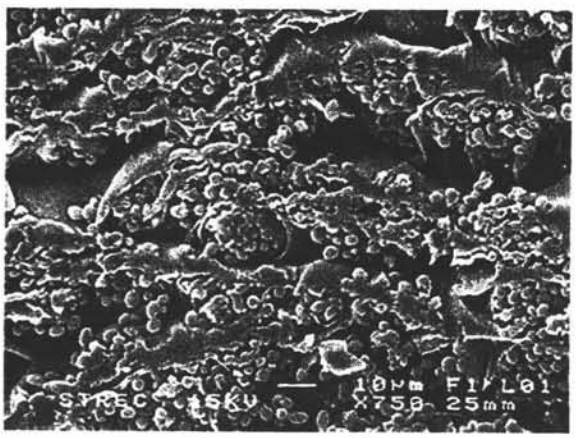

Figure 4.84 EAB surface cross section after repeated batch 1

Figure 4.85 EALC surface cross section after repeated batch 1

SEM micrographs of the EAB and EALC after the repeated batch 2 were shown in Figure 4.86 to 4.91. Higher degree of gel degradation occurred on the surface of the carriers as the cells were reused in the repeated batch 2. For EALC, severe gel degradation followed by cell leakage was observed (Figure 4.87 and 4.89). Figure 4.91 demonstrates that dense gel structure could also be found in EALC, even though the possibility was lower than in EAB.

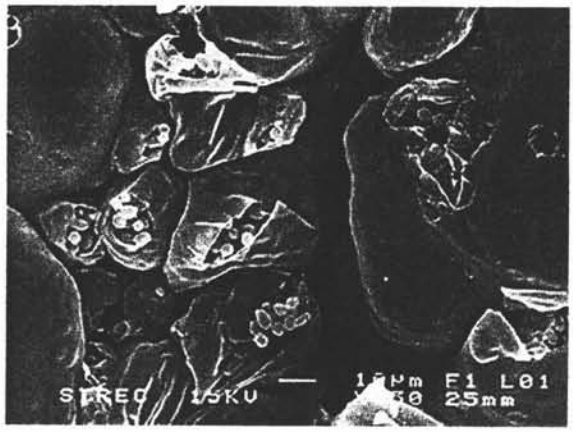

Figure 4.86 EAB surface after repeated batch 2

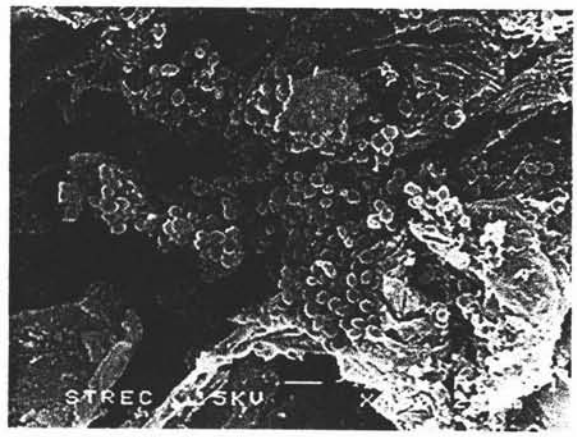

Figure 4.87 EALC surface after repeated batch 2

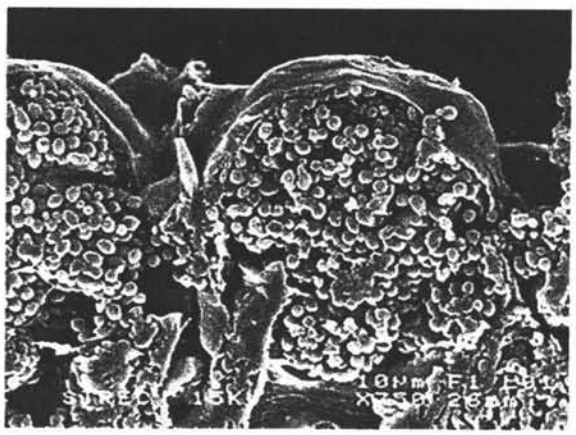

Figure 4.88 EAB surface cross section after repeated batch 2

Figure 4.89 EALC surface cross section after repeated batch 2

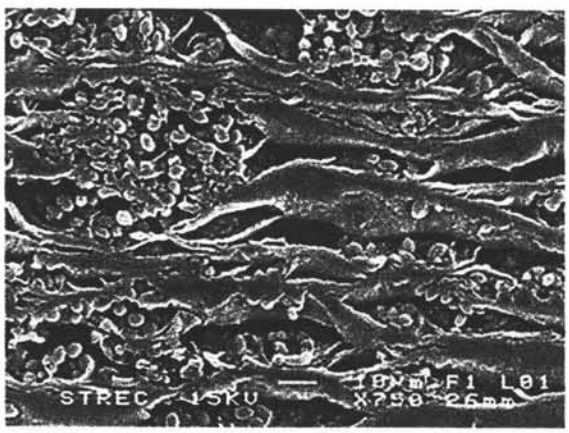

Figure 4.90 EAB central cross section after repeated batch 2

Figure 4.91 EALC central cross section after repeated batch 2

4.8 Working scheme

Outline of remarkable experimental configurations and results of every experiment carried out in this study is provided in the form of a working scheme (Figure 4.92).

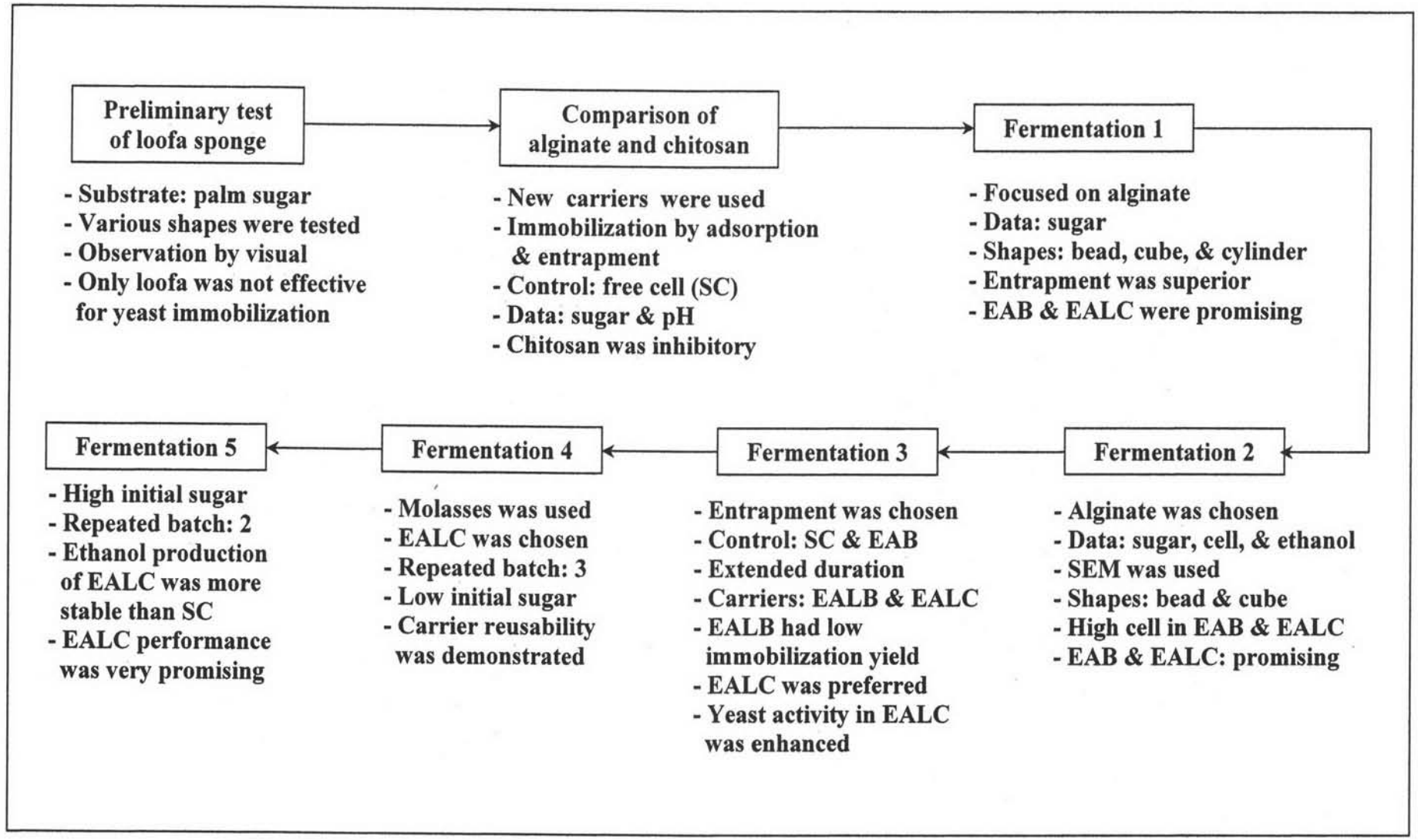

Figure 4.92 Working scheme of experimental works