การตรึงยีสต์โดยใช้เปลือกรังไหมบางสำหรับการผลิตเอทานอลย่างต่อเนื่อง

นาย อนุชิต รัตนพันธุ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรมหาบัณฑิต
สาขาวิชาวิศวกรรมเคมี ภาควิชาวิศวกรรมเคมี
คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551

YEAST IMMOBILIZATION USING THIN SHELL SILK COCOON FOR CONTINUOUS ETHANOL PRODUCTION

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Engineering Program in Chemical Engineering
Department of Chemical Engineering
Faculty of Engineering
Chulalongkorn University
Academic Year 2008

Thesis Title	YEAST IMMOBILIZATION USING THIN SHELL SILK COCOON FOR CONTINUOUS ETHANOL PRODUCTION
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อนุชิต รัตนพันธุ์: การตรึงยีสต์โดยใช้เปลือกรังไหมบางสำหรับการผลิตเอทานอลอย่างต่อเนื่อง. (YEAST IMMOBILIZATION USING THIN SHELL SILK COCOON FOR CONTINUOUS ETHANOL PRODUCTION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. เหมือนเดือน พิศาลพงศ์, 89 หน้า.

พลังงานหมุนเวียนเอทานอลได้รับความสนใจเพิ่มขึ้น เนื่องจากมีคุณสมบัติที่เป็นประโยชน์หลายประการ เช่น เป็นพลังงานที่สะอาด และผลิตได้จากกระบวนการหมักสารชีวมวล โดยเซลล์ยีสต์ที่ถูกตรึงทำหน้าที่ตัวเร่งปฏิกิริยาของกระบวนการผลิตเอทานอลมีข้อดีเด่นหลายประการรวมถึง การให้อัตราการผลิตเอทานอลที่สูง และทำให้สามารถนำเซลล์กลับมาใช้ใหม่ได้ สืบเนื่องจากการตรึงเซลล์ยีสต์ที่ใช้วิธีการหุ้มไว้ภายในโครงข่ายที่มีรูพรุนส่วนใหญ่จะมีปัญหาเรื่องการจำกัดของการถ่ายโอนมวลผ่านตัวพยุง ดังนั้นในการศึกษานี้จึงได้นำเสนอวิธีการตรึงเซลล์ยีสต์โดยใช้วิธีการดูดซับหรือยึดติดกับพื้นผิวเปลือกรังไหมบาง การศึกษาทดลองทำโดยใช้ขวดเขย่าขนาด 500 มิลลิลิตร ภายใต้สภาวะการหมักแบบกะ(batch) ทำการหมักเอทานอลโดยใช้เซลล์ยีสต์ *Saccharomyces cerevisiae* M30 และใช้กากน้ำตาล เป็นตัวผลิตเอทานอลและเป็นแหล่งคาร์บอน ตามลำดับ โดยที่ใช้ความเข้มข้นน้ำตาลเริ่มต้น 220-280 กรัมต่อลิตร เขย่าที่ 150 รอบ/นาที และที่อุณหภูมิ 33 องศาเซลเซียส การทดลองพบว่ากระบวนการหมักเอทานอลโดยใช้เซลล์ตรึงบนวัสดุเปลือกรังไหมบาง (TSI) มีประสิทธิภาพสูงกว่าเซลล์ที่ถูกตรึงบนเปลือกรังไหมบางหุ้มด้วยอัลจิเนท (ETSI) และเซลล์แขวนลอย (SC) ซึ่งส่งผลให้ได้อัตราการผลิตเอทานอลที่สูงกว่า ยิ่งกว่านั้นพบว่าโดยการใช้ความเข้มข้นน้ำตาลเริ่มต้น 240 กรัมต่อลิตร จะทำให้ได้ความเข้มข้นเอทานอลสูงสุดถึง 98.6 กรัมต่อลิตร หลังจากการหมักเป็นเวลา 64 ชั่วโมง ผลการประเมินโดยการหมักแบบกะที่ทำซ้ำจำนวน 5 รอบ (repeated batch) แสดงให้เห็นว่า เซลล์แบบ TSI มีศักยภาพในการนำเซลล์กลับมาใช้ (reusability) ที่ดีกว่าเซลล์แขวนลอย จากนั้นผลจากกระบวนการหมักเอทานอลแบบต่อเนื่องในถังปฏิกรณ์แพคเบด (packed-bed) ขนาด 1 ลิตร แสดงให้เห็นว่า เมื่อป้อนสารตั้งต้นน้ำตาลที่ 220 กรัมต่อลิตร ที่ อัตราเจือจาง 0.36 ต่อชั่วโมง สามารถทำให้ได้อัตราผลผลิตสูงสุดถึง 19.02 กรัมต่อลิตรต่อชั่วโมง ที่ความเข้มข้นเอทานอล 52.83 กรัมต่อลิตร ในขณะที่จะได้ความเข้มข้นเอทานอลสูงสุดที่ 80.72 กรัมต่อลิตร ที่อัตราการเจือจาง 0.034 ต่อชั่วโมง โดยรวมแล้ว วัสดุ TSI ที่พัฒนาขึ้นนี้ สามารถใช้เป็นตัวพยุงเซลล์ได้เป็นอย่างดีทั้งในกระบวนการผลิตแบบกะ แบบกะแบบทำซ้ำ และ แบบต่อเนื่อง โดยคุณสมบัติที่เป็นที่พึงพอใจคือการที่เข้าได้เป็นอย่างดีกับเซลล์สิ่งมีชีวิตและคุณสมบัติที่ดีเชิงกล ส่งผลทำให้ได้ อัตราการผลิตเอทานอลที่สูง อัตราการตรึงเซลล์ที่สูง ความหนาแน่นของเซลล์ที่สูง และระบบมีความเสถียรสำหรับการใช้งานเป็นเวลานาน

ภาควิชา.....วิศวกรรมเคมี..... ลายมือชื่อนิสิต…….....

สาขาวิชา.....วิศวกรรมเคมี..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2551……...

5070505521 : MAJOR CHEMICAL ENGINEERING
KEYWORDS : IMMOBILIZED CELL/ THIN SHELL SILK COCOON/ PACKED-BED REACTOR/ ETHANOL/ *SACCHAROMYCES CEREVISIAE*

ANUCHIT RATTANAPAN: YEAST IMMOBILIZATION USING THIN SHELL SILK COCOON FOR CONTINUOUS ETHANOL PRODUCTION. ADVISOR: ASSOC. PROF. MUENDUEN PHISALAPHONG, Ph.D., 89 pp.

A renewable energy, ethanol gains more interest because of its benefits such as clean energy and production from biomass fermentation. As a biocatalyst of ethanol fermentation, immobilized yeast offers many advantages including high ethanol productivity and reuse ability of cells. Since, yeast immobilization using method of entrapment within porous matrix always encounters with a mass transfer limitation problem, therefore, in this study, yeast immobilization using the method of adsorption or attachment to the surface of thin shell silk cocoon was proposed. Under batch fermentations in 500 ml Erlenmeyer flask, the experimental studies were carried out using *Saccharomyces cerevisiae M30* and molasses as the ethanol producer and carbon source, respectively with the initial sugar concentration of 220- 280 g/l at shaking frequency of 150 rpm and temperature of 33 ^{o}C. The ethanol fermentation using thin shell silk cocoon immobilized cell (TSI) culture was found to be more effective than that using thin shell silk cocoon immobilized cell entrapment within alginate (ETSI) and suspension cell (SC) cultures, resulting in higher ethanol production. Moreover, by using TSI culture with the initial sugar concentration of 240 g/l, the maximum ethanol concentration of 98.6 g/l was obtained after 64 hours of the fermentation. From the evaluation in the 5-cycle repeated batch, the TSI culture demonstrated a good potential of reusability than that of the SC culture. The further continuous ethanol fermentation in a 1-litre packed-bed reactor revealed that the maximum ethanol productivity of 19.02 g/l h with ethanol concentration of 52.83 g/l could be obtained with the feed of 220 g/l sugar concentration at 0.36 h^{-1} dilution rate, while the highest ethanol concentration of 80.72 g/l was obtained at the dilution rate of 0.034 h^{-1}. Overall, the developed TSI was successfully used as the cell carrier for the ethanol fermentations in batch, repeated batch and continuous processes. Its favorable biocompatible and mechanical properties resulted in high ethanol production, high cell immobilized yield, high density of biomass and high stability for long-term use.

Department :…...Chemical Engineering.. Student's Signature : .....

Field of Study :...Chemical Engineering.. Advisor's Signature : .....

Academic Year :……2008……..

ACKNOWLEDGEMENTS

I would like to express my earnest gratitude to my advisor, Assoc. Prof. Muenduen Phisalaphong, Ph.D. for her encouragement, support, guidance, and unfailing faith all the way through my thesis work and study.

Special appreciation is addressed to Assoc. Prof. Savitree Limtong, Dr.Eng. (Department of Microbiology, Kasetsart University, Bangkok) for her kind and most gratified support to this thesis work by providing the *Saccharomyces cereviceae* M30 strain.

Thanks to all of my thesis committee, Prof. Piyasan Praserthdam, Dr.Ing., Assoc. Prof. Suwimol Asavapisit, Ph.D. and Asst. Prof. Artiwan Shotipruk, Ph.D. for their kind advices and recommendations which are invaluable for improving my work.

Many thanks are also addressed to Mrs. Sunee Pakprapan and Mrs. Rujiporn Prateepasin (Scientific and Technological Research Equipment Centre, Chulalongkorn University) for their kind assistance in commencing Gas Chromatography (GC) and Scanning Electron Microscopy (SEM).

Sincere thanks are given to all members of the Biochemical Engineering Research Laboratory and all my friends and staffs in the Department of Chemical Engineering, Chulalongkorn University for their assistance, support, and warm collaborations.

Finally, I would like to express my highest gratitude to my parents for their affectionate support, blessings, inspiration, and love which guide me all the way throughout my life and study.

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CHAPTER I

INTRODUCTION

According to the diminishing of crude oil and the increasing of fuel cost in recent years, ethanol has re-emerged as an alternative to or extender for petrochemical based liquid fuels. Ethanol is renewable and clean fuel produced by biomass fermentation process. Its high octane and high heat of vaporization make alcohol more efficient as a pure fuel than gasoline. The Thai government has announced that it plans to double the amount of ethanol used in petroleum in order to curb Thailand imports of oil. In addition, the plan to promote ethanol-based fuel is of great importance in helping to stabilize agricultural product prices, raise farmers' income and reducing petrol- induced air pollution. Nowadays, gasohol E 10, a mixture of 10% ethanol and 90% gasoline has been widely used in vehicles and there is an attempt to promote the use of E20 or E85 in the vehicles in the near future. Beside that, ethanol is used as solvent and chemical feedstock in various industries. Therefore the demand of ethanol has increased rapidly.

In the past, almost of fuel ethanol was produced from petrochemical process, whereas, currently this process is considered as a very high investment process and it is replaced by biomass fermentation process. The biomass fermentation process can used many type of agricultural feed stock such as molasses, tapioca, sugar cane, sugar beet, corn, sorghum etc. Traditional ethanol industries produce ethanol by batch or fed batch process. However, for large quantities of ethanol, high production rate is achieved in a continuous process. The use of immobilization cells has been suggested as an effective means for improved continuous ethanol fermentation [1]. The immobilization of cells leads to protection of cells from inhibitions, maintaining of high cell densities with consequent increase in reaction rates and enzyme productivities. As a result, shorter residence time and smaller reactor size can be employed [1].

In this study, thin shell silk cocoon (TSSC), a residual agricultural material, is selected to be an immobilized material due to many advantages such as bio-

degradability, light but strongly structure, low cost, chemically stability, high porosity and high surface area. The possibility of using TSSC as cell carrier for yeast immobilization in ethanol fermentation is investigated. The fermentations are carried out in repeated batch mode in a shaking flask and continuous mode in a packed bed reactor using *Saccharomyces cerevisiae M30* as a cell culture. To optimize the process, the effect of controlled condition such as, initial sugar concentration, dilution rate and retention time on the cell activities are examined. It is expected that the information gained from the study will be useful for the development of high performance cell carrier for ethanol production.

1.1 Objectives

1.1.1 To develop a novel immobilized cell carrier for continuous ethanol production using thin shell silk cocoon.

1.1.2 To study optimal condition for ethanol fermentation by immobilized *Saccharomyces cerevisiae* M30 in thin shell silk cocoon.

1.2 Expected benefits

1.2.1 To develop a novel immobilized cells carrier for ethanol fermentation.

1.2.2 To add value of thin shell silk cocoon, a residual agriculture product.

1.2.3 To gain useful information for a better understanding of immobilization technology.

1.3 Working scopes

1.3.1 Flocculating yeast strain, *Saccharomyces cereviceae* M30 is used as ethanol producer.

1.3.2 For batch and fed batch fermentation, the experiment is carried out in 500 mL shaking flasks.

1.3.2 For continuous fermentation, the experiment is carried out in a packed-bed reactor with the working volume 0.67 liters (6 cm diameter and 34 cm height).

1.3.3 Thin shell silk cocoon is applied as materials for constructing immobilized cell carriers and the immobilization method is attachment cell to a surface of carriers.

1.3.4 Palm sugar and cane molasses are utilized as carbon and energy source.

1.3.5 The operating condition is as follows:

- Temperature: 32 ± 1 °C.
- The dilution rates: 0.034, 0.15, 0.24 and 0.36 h^{-1}.
- The initial sugar concentrations: varied for 222 and 241 g/l.
- The initial pH: 5.

CHAPTER II
BACKGROUND AND LITERATURE REVIEW

In recent years, a new round of enthusiasm in biomass and bioenergy has been initiated with the recognition that the global crude oil reserve is finite, and its depletion is occurring much faster than previously predicted. In addition, the environmental deterioration resulting from the over-consumption of petroleum-derived products, especially the transportation fuels is one of the most important environmental concerning issues today. Ethanol, both renewable and environmentally friendly, is believed to be one of the best alternatives, leading to a dramatic increase in its production capacity [2].

The continuous fermentation systems offer important economical advantages in comparison with traditional systems. Fermentation rates are significantly improved, especially when continuous fermentation is combined with cell immobilization techniques to increase the yeast concentration in the fermentor [3].

2.1 Microorganisms for ethanol production.

In recent years, many microorganisms were used to produced ethanol. For example, *Saccharomyces cerevisiae, Zymomonas mobilis* and *Saccharomyces diastaticus* etc. Especially, *Saccharomyces cerevisiae* that has many advantages than other microorganisms. It is a flocculent yeast, that easy to concentrate for immobilization method. It is tolerant to high sugar and ethanol concentration. Although many researchers studied the ethanol fermentation with *Saccharomyces cerevisiae*, in some cases a lack of recognition of its metabolic pathway led to approaches that are unlikely to yield significant improvements. The main metabolic pathway involved in the ethanol fermentation is glycolysis (Embden–Meyerhof Parnas or EMP pathway), through which one molecule of glucose metabolized can be produced to two molecules of pyruvate [4], as illustrated in **Fig. 2.1.**

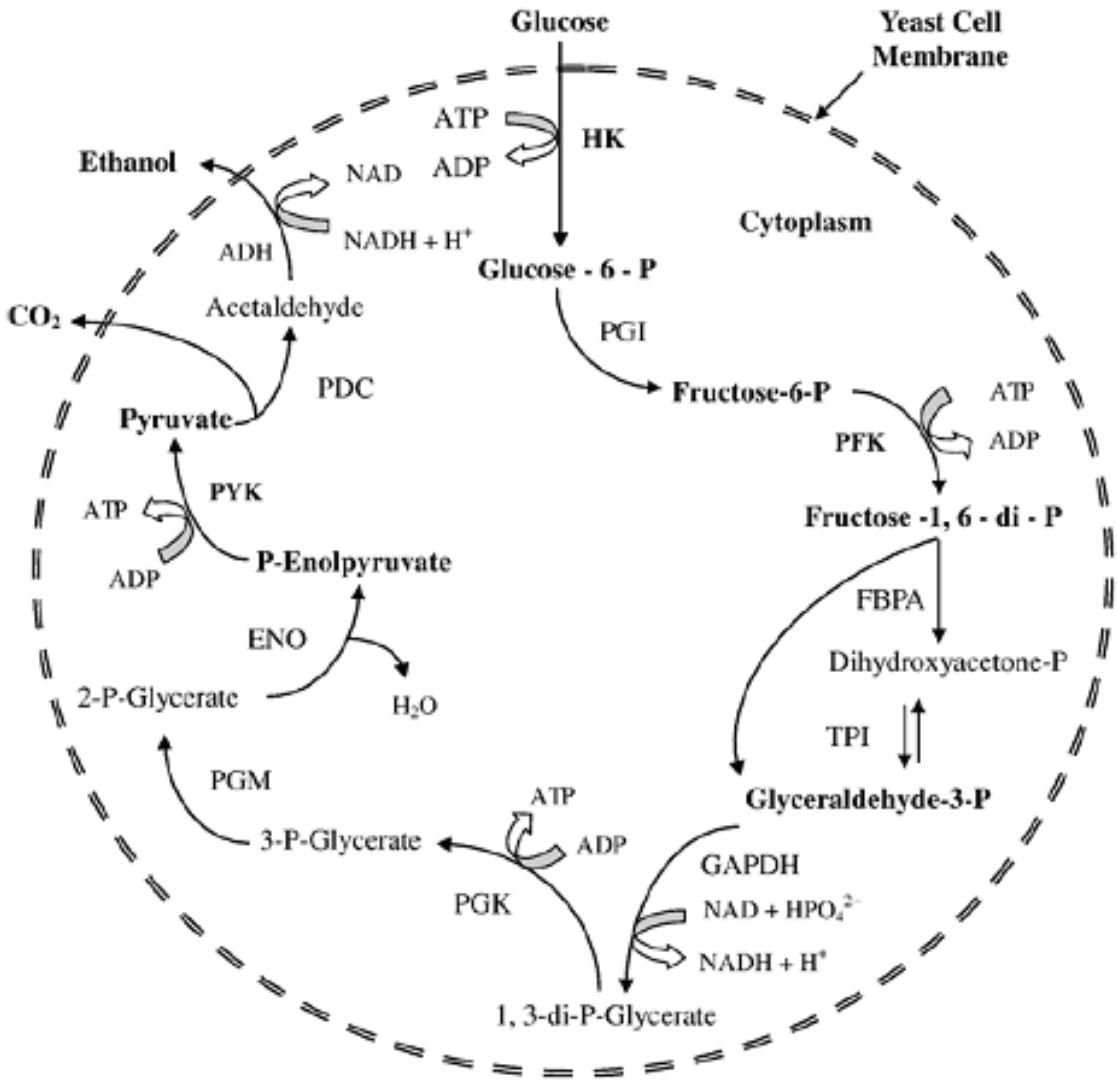


Figure 2.1 Metabolic pathway of ethanol fermentation in S. cerevisiae. Abbreviations: HK: hexokinase, PGI: phosphoglucoisomerase, PFK: phosphofructokinase, FBPA: fructose bisphosphate aldolase, TPI: triose phosphate isomerase, GAPDH: glyceraldehydes-3-phosphate dehydrogenase, PGK: phosphoglycerate kinase, PGM: phosphoglyceromutase, ENO: enolase, PYK: pyruvate kinase, PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase [4].

Under anaerobic conditions, the pyruvate is reduced to ethanol with the release of CO_2. Theoretically, the ethanol yield is 0.511 and CO_2 yield is 0.489 on a mass basis of glucose metabolized.

From path way, two ATPs produced in the glycolysis are used to drive the biosynthesis of yeast cells which involves a variety of energy-requiring bioreactions. Consequently, ethanol production is tightly coupled with yeast cell growth, which means yeast must be produced as a co-product. Without the continuous consumption of ATPs by the growth of yeast cells, the glycolytic metabolism of glucose will be interrupted immediately, because of the intracellular accumulation of ATP, which inhibits phosphofructokinase (PFK), one of the most important regulation enzymes in the glycolysis. This very basic principle contradicts the ethanol fermentation with the yeast cells immobilized by supporting materials, particularly by gel entrapments, which physically restrict the yeast cells and significantly retard their growth [2].

Although the researcher has more interest in *Saccharomyces cerevisiae* than *Zymomonas mobilis* . In the other hand, *Zymomonas mobilis* exhibits higher ethanol yield and productivity. *Zymomonas mobilis* is an anaerobic, gram-negative bacterium which produces ethanol from glucose via the Entner– Doudoroff (ED) pathway in conjunction with the enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) [5], as illustrated in **Fig 2.2.**

However, *Zymomonas mobilis* is not suitable for the industrial ethanol production. Firstly, thisspecies has a very specific substrate spectrum including only three sugars: D-glucose, D-fructose, and sucrose. The ethanol fermentation industry cannot use pure glucose as its raw material like many researchers did in their laboratory studies. Secondly, although *Zymomonas mobilis* is generally regarded as safe (GRAS) [6], its biomass is not commonly acceptable to be used as animal feed, which nevitably generates the problem for its biomass disposal if it replaces *Saccharomyces cerevisiae* in the industrial ethanol production. And finally, the continuous ethanol fermentation with *Zymomonas mobilis* tends to be oscillatory.

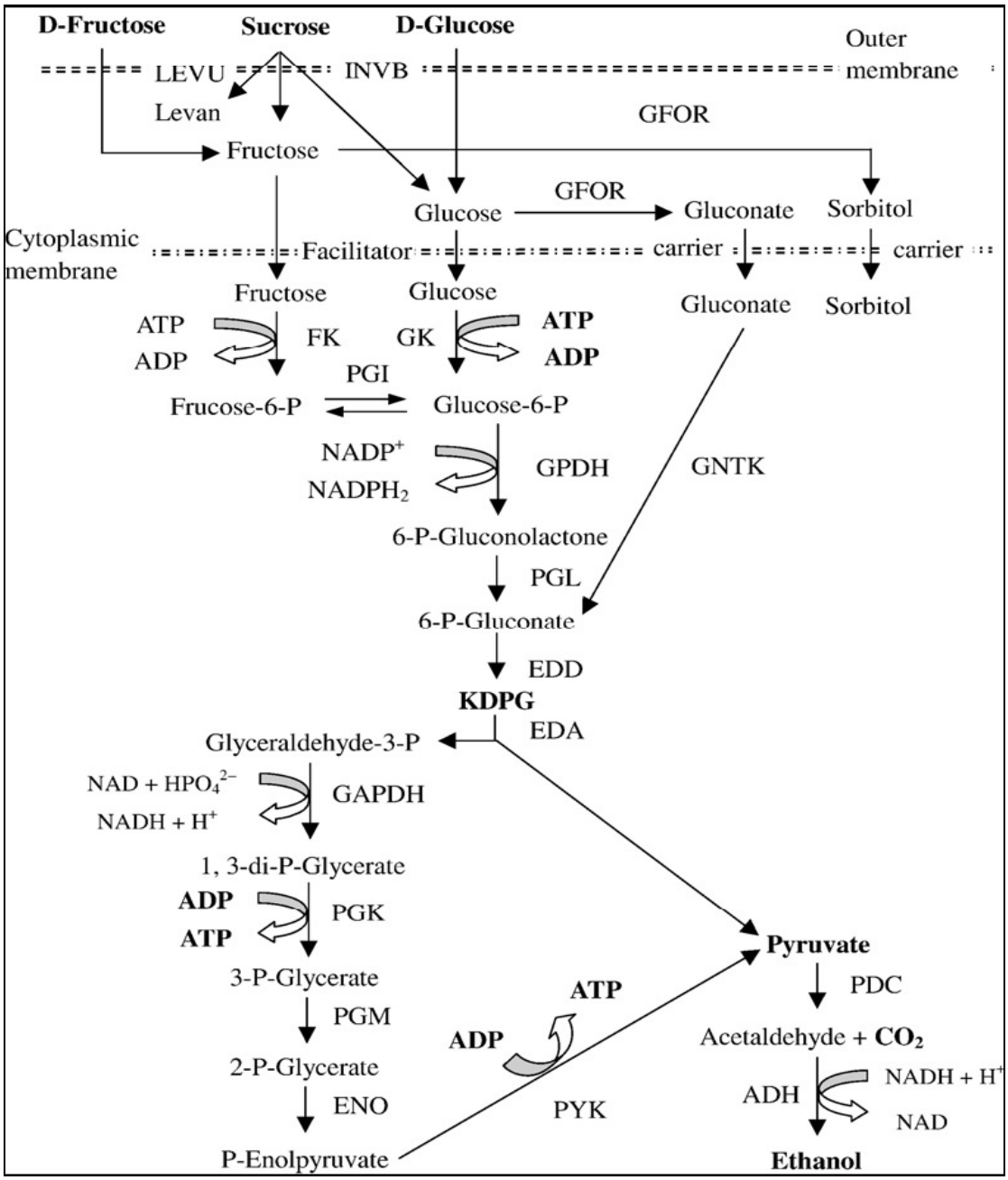


Fig. 2.2. Carbohydrate metabolic pathways in Z. mobilis Abbreviations: LEVU: levansucrase, INVB: invertase, GFOR: glucose–fructose oxidoreductase, FK: fructokinase, GK: glucokinase, GPDH: glucose-6-phosphate dehydrogenase, PGL: phosphogluconolactonase, EDD:6-phosphogluconate dehydratase, KDPG: 2-keto-3-deoxy-6-phosphogluconate, EDA: 2-keto-3-deoxy-gluconate aldolase, GNTK: gluconatekinase. See Fig. 2 for PGI, GAPDH, PGK, PGM, ENO, PYK, PDC and ADH [7].

2.2 Immobilized cell system

Nowadays, development of immobilized cell technology has more increasing. The researchers attempt to increase performance of immobilization method for using on large scale of production. The immobilized cell are used various application fields. For example, biosyntheses, bioconversions, environment, food processing, biosensors, and optical. Motivation for development of immobilized cell systems emerged from their potential advantages. Some potential advantageous characteristics of immobilized cell over suspension fermentations including:

1. Higher cell concentration.
2. Higher production rates and yields.
3. Smaller bioreactor requirements.
4. Capital and energy cost saving.
5. Prolonged activity and stability of the biocatalyst.
6. Increased tolerance to high substrate and product concentration.
7. Elimination washout problem which in turn enables the fermentation to be carried out at higher dilution rate.
8. Easier downstream processing of the product.
9. Protection of cells from toxins and inhibitors.
10. Feasibility of continuous processing.

The productivity can be improved by increasing the flow rate of the system which is usually represented as dilution rate. Dilution rate can calculated from the ratio between flow rate and volume of reactor. The relationship between productivity and dilution rate is shown in **Figure 2.3**

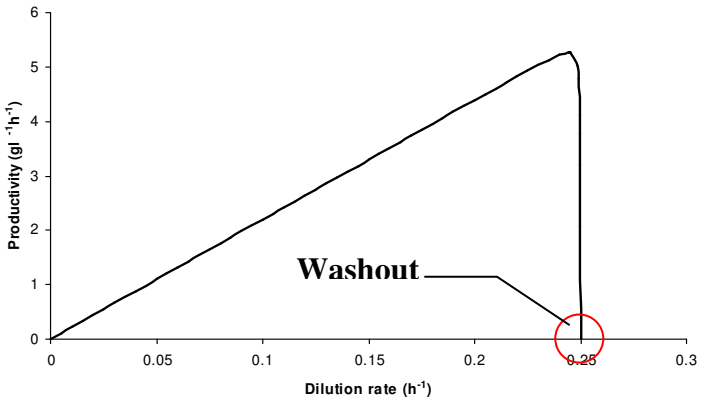


Figure 2.3 Productivity versus dilution rate curve.

After reaching an optimum value, the productivity will decrease drastically until it reaches nearly zero. In some cases, the optimum bioreactor volume is set by the critical dilution rate which corresponds to the dilution rate at which washout occurs. **Figure 2.4** shows a relationship between substrate, product, and biomass concentration with critical dilution rate.

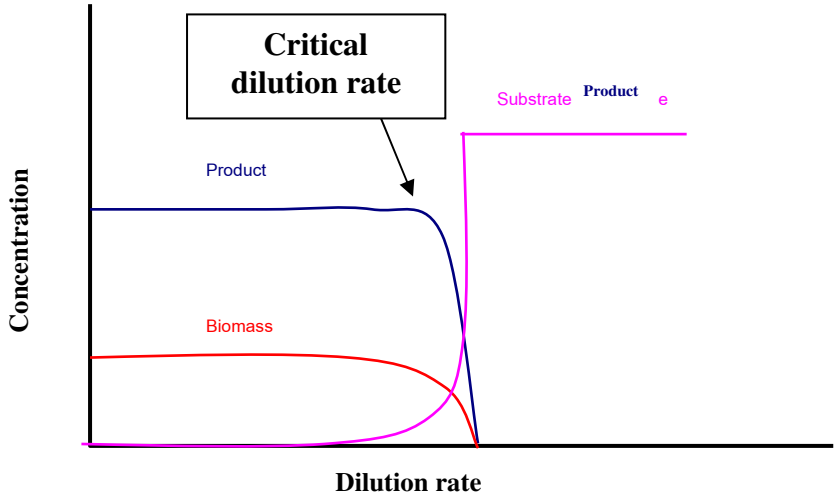


Figure 2.4 Concentration profile with variable dilution rate.

Besides these advantages, the use of immobilized microorganism has some disadvantages. One of the major problems is susceptibility to diffusion limitation on reaction rate and possible loss in the yield of the desired product. In such case, the control of micro-environmental conditions is difficult because of the resulting heterogeneity in the system. With viable cells, growth and gas evolution can lead to significant mechanical disruption of the immobilizing matrix [8].

Immobilized systems can be classified into natural and artificial occurring ones. In nature, some microorganisms can form biofilm by attaching to one another or even to surfaces. This attachment is facilitated by secretion of adhesive substance called glycocalyx by the cells. In artificial immobilized cell system, cells are immobilized by using carriers/supports. Proper selection of carrier is extremely important for immobilized cell application because it will affect greatly on the performance of the system. As every organism exhibits different interaction with different carriers, evaluation of carrier performance for an individual organism should be done in case by case basis [9].

2.3 Immobilization materials and methods

Generally, four categories of immobilization techniques can be divided, based on the physical mechanism of cell localization and the nature of support mechanisms: attachment to a surface, entrapment within a porous matrix, containment behind a barrier and self-aggregation (**Fig. 2.5**) [3].

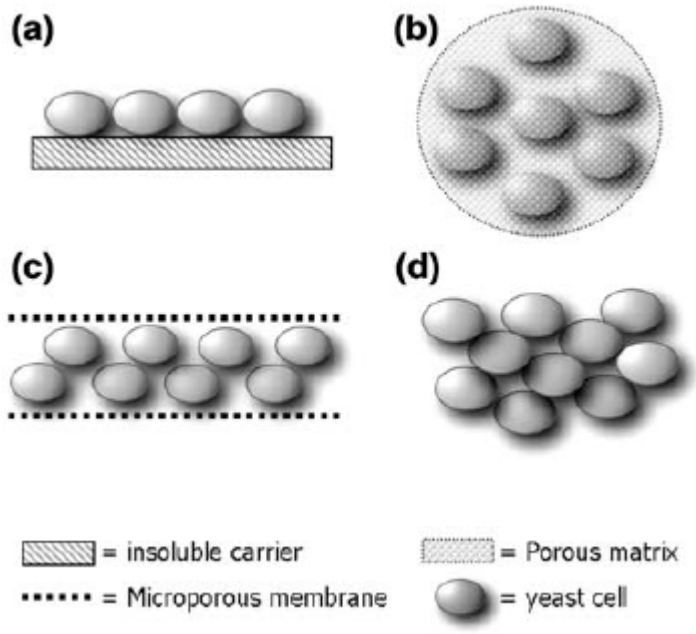


Fig 2.5 Basic methods of yeast immobilization: (a) attachment to a surface, (b) entrapment within a porous matrix, (c) containment behind a barrier and (d) self-aggregation

2.3.1 Surface attachment of yeast cells

In this type of immobilization, yeast cells are permitted to attach to a solid support. Many different carrier materials have been using. Using linking agents (such as metal oxides, glutaraldehyde or aminosilanes) can induce cellular attachment to the carrier. However, for the production of ethanol and beverages, natural adhesion is often preferred over the use of inducers which are considered potentially harmful or unstable. Natural immobilization is very simple and the conditions are mild, but cell

loadings are usually not as high as those obtained in systems in which the cells are entrapped. Moreover, as there are no barriers between the cells and the solution, cell relocation and detachment is possible.

While the natural adhesion of yeast cells to substrates remains uncertainly established, several mechanisms have been submitted. The adhesion phenomenon could, for example, be conferred by electrostatic, ionic (Lewis acid/base) and hydrophobic (Lifshitz–van der Waals) interactions, but retention within carrier cavities and yeast flocculation can also play an important role in the process of immobilization on preformed, roughly shaped carriers. Hence, when designing new immobilization carriers the physicochemical properties of the yeast cell wall and the carrier, such as hydrophobicity, charge, electron-donor and electronacceptor properties, should be considered [3].

2.3.2 Entrapment within porous matrices

Entrapment within porous matrices is the second major category of yeast immobilization. Two methods of entrapment exist. In the first, yeast cells are allowed to diffuse into a preformed porous matrix. After the yeast cells begin to grow, their mobility is hindered by the presence of other cells and the matrix and they are thus effectively entrapped. Attachment on this material surface is also possible. Sponge, silicon carbide, sintered glass, ceramics, chitosan, polyurethane foam and stainless steel fibres are commonly used materials.

In the second method, the porous matrix is synthesized in situ around the yeast cells. Most often, natural and synthetic polymeric hydrogels such as Ca-alginate, polyurethane, j-carrageenan, agar, polyvinylalcohol and polystyrene are being used. The polymeric beads are usually spherical with diameters ranging from 0.3 to 3 mm. Although high biomass loadings can be obtained, gel entrapment receive less attention in the fermentation industry because of several drawbacks, such as diffusion limitations of nutrients, metabolites and oxygen due to the gel matrix and the high cell densities in the gel beads, the chemical and physical instability of the gel and the non-regenerability of the beads, making this immobilization type rather expensive [3]. Recently, attempts are made to solve most of these drawbacks by the introduction of

new techniques that are able to adjust the size (microbeads) and shape (lenticular shape) of the hydrogels [10].

2.3.3 Containment behind a barrier

Containment of yeast cells behind a barrier can be attained either by the use of microporous membrane filters or by entrapment of cells in microcapsules. This type of immobilization is most suited when a cell free product is required, or when high molecular weight products need to be separated from the effluent. Inherent problems of this technique are mass transfer limitations and possible membrane fouling caused by cell growth. This type of immobilization is attractive in terms of productivity, but it seems that the cost/benefit ratio for low-added-value fermentations like beer will remain unfavorable as long as high-performance membranes remain expensive. Several research groups have nevertheless investigated their use for the production of ethanol [3].

2.3.4 Yeast flocculation

The common brewer's yeast, *Saccharomyces cerevisiae*, has the natural ability to adhere to inert surfaces as well as other yeast cells; the latter process called flocculation. Yeast flocculation is a reversible, asexual and calcium depend process in which cells adhere to form flocs consisting of thousands of cells. It involves lectin-like proteins, which stick out of the yeast cell wall and selectively bind mannose residues present on the cell walls of adjacent yeast cells. Yeast flocculation is a complex process that depends on the expression of several specific genes such as Lg-FLO1, FLO8, FLO5 and FLO1. Other genes, such as FLO11, confer adhesion to inert substrates and the formation of biofilms on nutrient sources. Because of their macroscopic size and their mass, the flock of yeast is rapidly sediment from the fermenting medium, thus providing a natural immobilization of the cells.

The use of flocculating yeast is very attractive, due to its simplicity and low cost. However, things are more complex than they may seem. Flocculation is affected by several parameters, such as nutrient conditions, agitation, Ca^{2+}-concentration, pH, fermentation temperature, yeast handling and storage conditions. Hence, the

fermentation medium itself, and more specifically the content of glucose, sucrose and nitrogen compounds could be influent the success of immobilization [3]. However these parameters have not yet been systematically studied and it is hard to predict the impact of the medium on cell adhesion. Above all, flocculation is a strain-specific phenomenon. The ability of yeast cells to flocculate is of considerable importance for the brewing industry, as it affects fermentation productivity and beer quality in addition to yeast removal and recovery. The growing interest in flocculation bioreactors, because of the prospect of high cell densities in continuous processes, further intensifies the need for controlling yeast flocculation. In this case, constitutive flocculent yeast strains (by genetic engineering) are desired, because normal strains only flocculate in the stationary phase and thereby the exponentially growing cells would be washed out [11].

2.4 Packed-Bed Reactor

Generally, in continuous immobilized yeast fermentation systems, 5 types of bioreactors are being used, which are depicted schematically in Fig. 2. The bioreactors contain three phases: solid (the carrier or aggregate), liquid (the medium) and gas (air, oxygen or other gas feeds). The choice of bioreactor is related to the type of immobilization, to the metabolism of cells, and to the mass and heat transfer requirements [3].

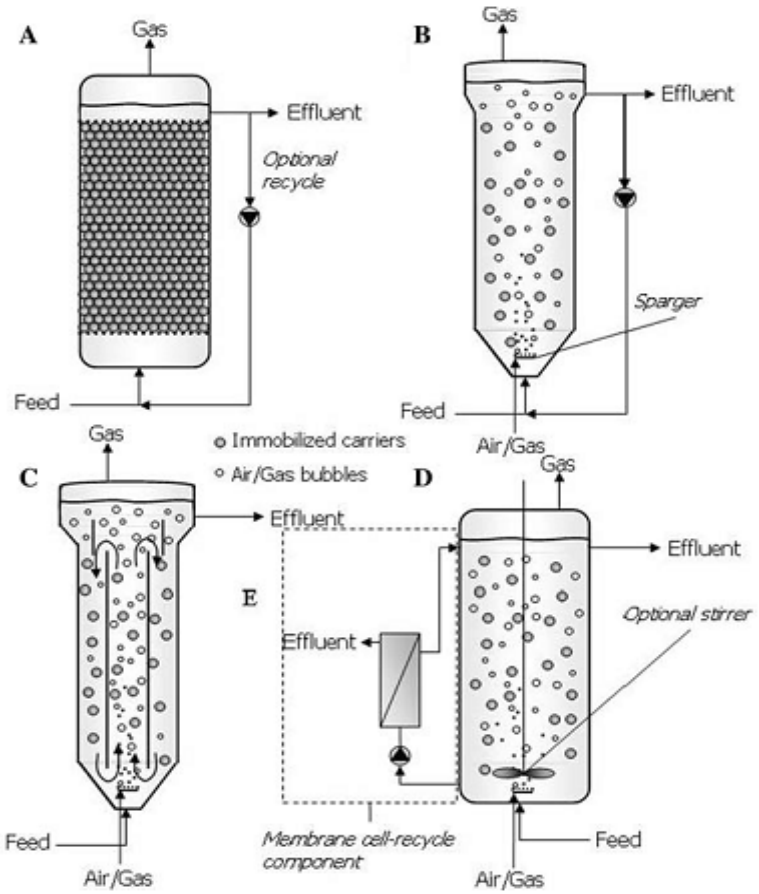


Fig 2.6 Five common types of immobilized cell bioreactors [3].

(A) : Packed-bed reactor

(B) : Fluidized bed reactor

(C) : Gas lift reactor

(D) : Bubble column reactor

(E) : Membrane cell-recycle reactor

In a packed-bed reactor (Fig. 2A), the fermenting meduim is passed either upward or downward through the reactor which is packed with immobilized yeast. This type of reactor has the advantages of simplicity and the ability of realizing a plug flow. Thepretically, maintenance of ideal plug flow conditions would allow the various stages of a batch fermentation to be mimicked. This is especially useful when a balanced flavor profile must be formed during the fermentation. In practice, these ideal conditions are difficult to achieve and, in addition, fixed bed reactors are prone

to channeling, mass transfer limitations, difficulties in CO_2-evacuation, compresssion of some carrier materials and fouling [3].

2.5 Review of ethanol fermentation by immobilized carriers

Ogbonna et al. (2000) studied scale up of fuel ethanol production from sugar beet juice using loofa sponge immobilized bioreactor. The effects of the initial pH, nitrogen source, cultivation time, repeated batch, broth circulation and size of reactor were studied on ethanol production. It was found that addition of nitrogen source to the juice and adjusting the pH of the juice from the original 4.5 to 6.5 had no significant effect on ethanol production. Furthermore, from a comparison of ethanol production from sucrose medium and sugar beet juice, there were no significant differences in both the rate of fermentation and ethanol yield from the consumed sucrose. From the repeated batch fermentation indicated the stability of cell activity without any sigh of decrease from three repeated batches. In the large scale of ethanol production, the broth circulation must be considered to achieve uniform cell distribution within the bed. Therefore external loop bioreactor can be constructed in large scale production systems.

Amutha and Gunasekaran. (2001) reported on production of ethanol from liquefied cassava starch using co-immobilized cells of *Zymomonas mobilis* and *Sacharomyces diastaticus* in bath and packed-bed reactor. From the experimental result, the co-immobilized cells can produced ethanol concentration higher than immobilized cells (*S. diastaticus*). The concentration of ethanol produced by immobilized cells was higher than that by free cells of *S. diastaticus* and *Z. mobilis* in mixed-culture fermentation. In repeated-bath fermentation using co-immobilized cells, the ethanol concentration increased to 53.5 g/l. The co-immobilized gel beads were stable up to seven successive batches. Continuous fermentation using co-immobilized cells in packed-bed reactor operated at a flow rate of 15 ml/h (residence time, 4 h) exhibited a maximum ethanol productivity of 8.9 g/l h.

Shindo et al. (2001) reported on development of novel carrier using natural Zeolite for continuous ethanol fermentation with immobilized *Saccharomyces cerevisiae* in a bioreactor. The experimental result showed that the maximum

concentration of yeast cells immobilized on a natural zeolite carrier was 3.6 x 10^8 cells ml^{-1} carrier at 1300 ^oC calcination temperature. Moreover, the capacity for immobilization and ethanol fermentation activity of natural zeolite carrier higher than glass beads 2'-fold and 1.2'-fold respectively. Continuous ethanol fermentation was stable for over 21 days without breakage of the carrier.

Najafpour et al. (2003) reported ethanol fermentation in an immobilized cell reactor using *Saccharomyces cerevisiae*. Calcium alginate were chosen as an immobilized cell material. The residual glucose concentration (g/l), cell dry weight (g/l) and ethanol production (%v/v) were investigated as a function of time in batch fermentation and retention time in immobilized cell reactor (ICR). In addition they were studied the effected of initial glucose concentration to the ethanol production. From above, it was found that the continuous ethanol production in an ICR was successfully with high sugar concentration. In suspended culture, the substantial substrate inhibition strongly occurred when the concentration of glucose was increased in batch fermentation. While in an ICR, the substrate inhibition of substrate and product were not apparent even with high glucose concentration in the fresh feed. The ICR system exhibited a higher yield of ethanol production (38%) compared to the batch system and experimental runs resulted in glucose consumption of 82-85%.The results indicated that the immobilization of *S. cerevisiae* possesses the capacity not only to utilize high concentration of sugar but also to yield higher ethanol productivities during the course of continuous fermentation.

Baptista et al. (2005) studied a natural immobilization of microorganism for continuous ethanol production. Three kinds of support (glassy coke, expanded clay, polyurethane foam cubes) were tested to immobilized yeast cells. The fluidized-bed reactor and two strains of *Saccharomyces cerevisiae* (adhesive and non-adhesive strain) were used to produced ethanol. The result indicated that the polyurethane foam cubes was a good support material than glassy coke and expanded clay. Moreover, the productivity of the adhesive strain was higher than that of the non-adhesive one.

Valach et al. (2005) reported on efficiency of Fixed-bed and a gas-lift three-column reactor for continuous production of ethanol by pectate and alginate immobilized *Saccharomyces cerevisiae* cells. Calcium pectate and calcium alginate were used to immobilized *Saccharomyces cerevisiae* C11-3 cells for ethanol

fermentation in a three reactor system. The result revealed that the gas-lift system was more efficiency than fixed-bed system due to a better mass transport between the phases. Beside that, the calcium pectate gel more suitable as an immobilization material in comparison with calcium alginate due to its mechanical resistance and favourable diffusion parameters, providing an ethanol production of more than 7.57 g/l h over a period of 630 h.

Cazetta et al. (2006) reported on fermentation of molasses by *Zymomonas mobilis*. Effects of total reducing sugars (TRS) concentrations in the molasses, temperature, agitation and culture time on ethanol production were studied. It was found that, when the sugar concentration in the molasses increased, the ethanol production decreased, because of an increase in the osmotic pressure that is one of the essential factors for by-products synthesis such as sorbitol and levan. In addition, the temperatures above 37 ^{o}C are detrimental for ethanol production. The results showed that the condition of 200 g L^{-1} of TRS and temperature 30 ^{o}C was the most favorable, achieving 54.83 g L^{-1} of ethanol production after a 48-hour-culture time.

Phisalaphong et al. (2007) studied the immobilization cells of *Saccharomyces cerevisiae M30* in alginate-loofa as carrier matrix for ethanol production. Molasses was used as a substrate. The cell immobilized was been effective and good strength and stability for long term use. The carrier was fabricated simply by entrapment of a peripheral loofa sponge that was previously dipped in an alginate cell mixture. After a storage period of 4 month, yeast cells remained firmly immobilized and active.

Yu et al. (2007) studied an novel immobilization method of *Saccharomyces cerevisiae* to sorghum bagasse for ethanol production. Natural Sorghum bagasse was used to immobilize *Saccharomyces cerevisiae* for continuous ethanol production. The effects of cultivation time, reuse number of immobilized cell and dilution rate were studied to improved performance of ethanol production. From the experimental result, nearly 100% total sugar was consumed after 16 hours with the ethanol yield 0.49 g/g consumed sugar on average, with ethanol productivity of 5.72 g/(L h) at an initial sugar concentration of 200 g/L in repeated batch fermentation. The immobilized cells could be reused for at least 30 days retaining about 95% of its original activity. In continuous ethanol fermentation using a filled-bed reactor, complete conversion of total sugar to ethanol was obtained at a dilution rate of 0.1 h^{-1} and the maximum

ethanol productivity 16.68 g/(L h) appeared at the dilution rate of 0.3 h^{-1}. Continuous ethanol production was maintained for up to 20 days. The results showed that the sorghum bagasse has a potential as a carrier for the whole yeast cell immobilization using this innovative method. There are several advantages such as low carrier cost, simplicity of immobilization procedure, high carrier strength and durability.

Liu et al. (2008) reported ethanol fermentation in a magnetically fluidized bed reactor with immobilized *Saccharomyces cerevisiae* in magnetic particles. Sodium alginate and Mn-Zn ferrite powder were used in magnetically stabilized fluidized bed bioreactor (MSFBR) as a carrier. The effects of concentration of $CaCl_2$, particle loading rate and dilution rate were studied. The experimental result revealed that at a $CaCl_2$ concentration of 2%, these particles were flexible and enough strong to hold their weight of packing in the MSFBR. The ethanol fermentation efficiency was highest at 41% (v/v) of particle loading rate and 0.4 h^{-1} of dilution rate respectively.

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Microbial Strains

The *Saccharomyces cerevisiae M30* was kindly provided by Dr. Savitree Linthong from Department of Microbiology, Kasetsart University, Bangkok. Stock cultures were stored in PDA agar slant.

3.1.2 Chemicals

The details of chemicals used in this experiment are shown in Table 3.1

Table 3.1 The chemicals used in this experiment

Chemical	Supplier
- Sucrose	Ajax Finechem
- Sodium hydroxides (NaOH)	Merck
- Hydrochloric acid (HCl)	J.T. Baker
- Ammonium sulfate $(NH_4)_2SO_4$	Ajax Finechem
- Magnesium sulfate (MgSO4.7H2O)	APS
- 3,5-dinitrosalicylic acid (DNS)	Fluka
- Potassium dihydrogen ortho-phosphate (KH2PO4)	Ajax Finechem
- Potato dextrose agar (PDA)	Himedia
- Absolute ethanol	Merck
- Na-K tartrate	Carlo Erba

3.1.3 Equipments

-Scanning electron microscopy, SEM (JOEL JSM-5410LV, Japan).

-UV-visible spectrophotometer, UV-Vis (UV 2450, Shimadzu, Japan)

-Gas chromatography (Shimadzu Model GC $7A_G$, Japan)
-Autoclave (Model Tomy Autoclave SS-325, Ner ima-ku, Tokyo, Japan).

-Refrigerated incubator shaker,(Innova 4330, New Brunswick Scientific, USA)

-Peristaltic pump (WATSON MARLOW 505U, England)

3.2 Methods for fermentation.

3.2.1 Methods for stock cell suspension preparation

The stock cultures from an agar slant tube was aseptically transferred into a 500 ml Erlenmeyer flask containing 150 ml sterilized cultivation medium. The cultivation medium was composed of 100 g/l sugar from palm sugar, 0.5 g/l $(NH_4)_2SO_4$, 0.1 g/l KH_2PO_4, and 0.035 g/l $MgSO_4.7H_2O$ at pH 5. The medium was sterilized in autoclave for 15 minutes at 121^oC. Cell cultivation was carried out in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33 ^oC for 24 hours.

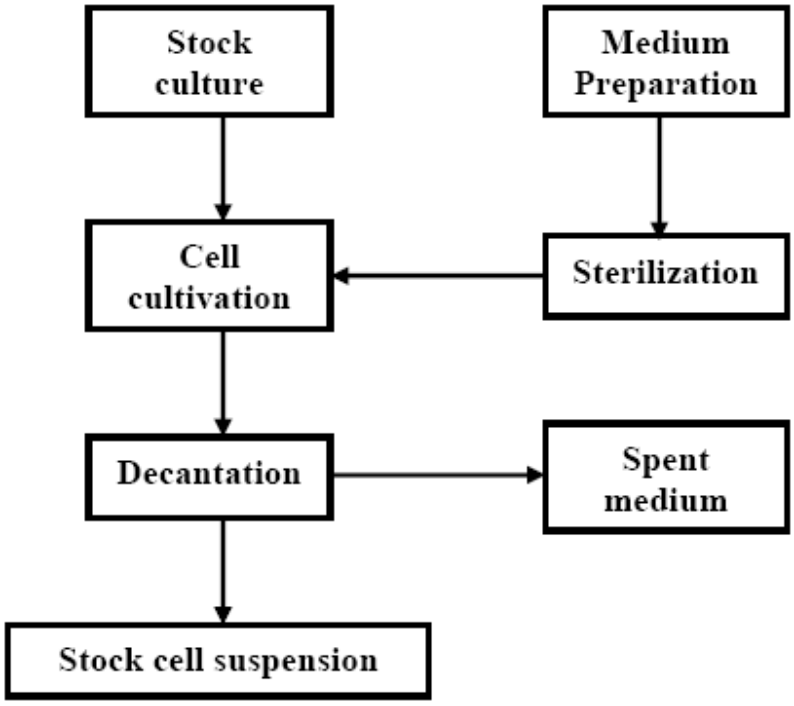


Figure 3.1 Diagram for methods of stock cell suspension preparation

3.2.2 Methods for cells immobilization.

Thin shell silk cocoons of 2.5 g and 250 ml of culture medium in a 500 ml flask were separately autoclaved for 15 minutes at 121°C. Stock cell suspension 10 ml was added to the culture medium. After that the steriled thin shell silk cocoons were added into the mixture. The thin shell silk immobilized cells were obtained after the incubation of the suspension mixture for 24 hr. The method are shown in **Figure 3.2**

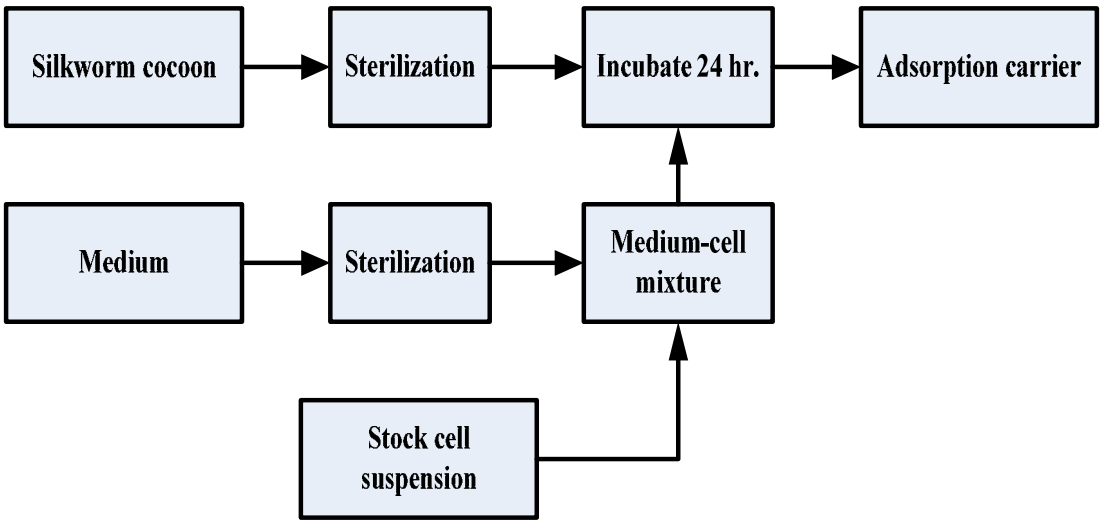


Figure 3.2 Diagram for methods of cells immobilization.

3.2.3 Methods for ethanol fermentation.

Cane molasses was used as a substrate for the ethanol fermentation. An amount of ammonium sulfate for 0.5 g/l was added in the molasses solution as the nitrogen source. For batch fermentation, the volume of molasses solution was 250 ml in 500 ml Erlenmeyer flask. The flask was shaken in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33 ^{o}C. The substrate for continuous fermentation was similar to that for the batch fermentation. However, the continuous fermentation was performed in a packed-bed reactor with the liquid working volume of 0.67 liters. Temperature of the system was controlled at 31 ^{o}C by the passing of 28 ^{o}C cooling water through the reactor jacket. Sampling was aseptically taken with the volume of 2 ml for every 8 hours. The sample were frozen before analysis of sugar, ethanol and cell concentration in order to enable all samples to be analyzed at the same time.

3.2.4 Methods for sample analysis.

Modified 3,5-dinitrosalicylic acid (DNS) reagent method was used to determine sugar concentration. Briefly, the sample was hydrolyzed with HCl 370 g/l in boiling water bath for 10 minutes. After that, the sample was neutralized with NaOH 300 g/l. The sample was centrifuged and the supernatant was reacted with DNS reagent before the color intensity was measured by UV-visible spectrophotometer at 520 nm.

Ethanol assay was conducted by gas chromatography using a Shimadzu Model GC $7A_G$ equipped with Flame Ionization Detector (FID). A column with length 2 m, outer diameter of 3.3 mm, and packed with Porapak Q 80-100 mesh was used in collaboration with N_2 as carrier gas. Flow rate of N_2 was 50 ml/min. The oven and detector temperature were 190 ^oC and 240 ^oC respectively. The sample were injected with volume of 1 μL and injection temperature of 240 ^oC.

Cell concentration was determined by cell dry weight method. The sample was washed with HCL 0.1 N and water respectively. The cell concentration was measured by UV-visible spectrophotometer at 660 nm for determined free cell leakage concentration. The carrier was cut into small pieces and stirred in DI water for 1 hour. After that, the carrier was removed and the suspension was treated similarly as fermentation broth to obtain its corresponding immobilized cell concentration. Drying of all cells was performed in oven at 100 ^oC for 2 hours. At the beginning and the end of fermentation, samples of carrier were collected for SEM.

The examination of the surface properties was performed by scanning electron microscopy (SEM). Scanning electron micrographs were taken with JOEL JSM-5410LV microscope at Scientific and technological research equipment centre, Chulalongkorn University. The TSI were frozen in liquid nitrogen, immediately snapped, and vacuum-dried. Then, the TSI were sputtered with gold and photographed. The coated specimens were kept in dry place before experiment. SEM was obtained at 15 kV which is considered to be a suitable condition since too high energy can be burn the samples.

3.3 Methods for calculation of fermentation parameters.

Fermentation efficiency for bioreactor system were expressed as follows:

- Immobilization yield (Y_I, %)

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

- Yield of sugar consumption (Y_S, %)

$$Y_S = \frac{S_0 - S_F}{S_0}$$

- Yield of ethanol production ($Y_{P/S}$, g ethanol/g sugar)

$$Y_{P/S} = \frac{P_F - P_0}{S_0 - S_F}$$

- Ethanol productivity (Q_P, g/L h)

$$Q_P = \frac{P_F}{fermentation\ \ time} = P_F \times D$$

X_I ; immobilized cell concentration (g/l)

X_E ; free cell concentration (g/l)

X_T ; total cell concentration (g/l)

S_0 ; initial sugar concentration (g/l)

S_F ; final sugar concentration (g/l)

P_0 ; initial ethanol concentration (g/l)

P_F ; final ethanol concentration (g/l)

D ; Dilution rate (h^{-1})

CHAPTER IV

RESULTS AND DISCUSSION

Almost all of cell immobilization methods are based on adsorption or entrapment techniques. However, the entrapment method always encounters with the problems of nutrients and metabolites mass transfer limitations, degradation of gel and cell detachment. Therefore, this work attempts to develop an effective ethanol production process by using the method of immobilized cell by adsorption or attachment to the surface of a low-cost agricultural material in order to reduce the effect of mass transfer diffusion. The yeast strain, *Saccharomyces cerevisiae M30* and cane molasses were selected as an ethanol producer and carbon source, respectively. Thin shell silk cocoon, a byproduct from silk industry, was chosen to be an immobilized cell material because of its many advantages. It was cheap, simple to use, non-toxic, high biocompatibility, chemical and mechanical stable.

4.1 Comparison of yeast immobilization method

To compare the performance of yeast immobilization method, the ethanol production using immobilized *S. cerevisiae M30* attachment to thin shell silk cocoon (TSI) was compared to that using cell attachment to thin shell silk cocoon and entrapment with alginate (ETSI) and suspended cell cultures (SC). The initial sugar concentration from sugar cane molasses was set to 200 g/l at pH 5. The fermentation was performed in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33 ^{o}C for 72 hours using 500 ml Erlenmeyer flask containing 250 ml of medium for fermentation. The samples were harvested every 8 hours for cell, sugar and ethanol analyses. Table 4.1 to 4.2 and Figure 4.1 to 4.2 show the results of batch fermentation of ethanol production using the cultures of TSI, ETSI and SC. The ethanol production and sugar consumption of the TSI culture was higher than those of the ETSI culture after 24 hours of fermentation time. Moreover, the free cell concentration in the ETSI system was about 2 times higher than that of the TSI

system (Figure 4.2). The overall conversion yields were 0.32 and 0.29 for the TSI and ETSI cultures, respectively.

Table 4.1 Batch fermentation of ethanol production using TSI carrier.

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	Yp/s	Productivity (g/l h)
0	200.00	0.00	0.00		
8	184.60	7.20	1.16	0.45	0.90
16	97.30	31.40	1.17	0.30	1.96
24	30.10	44.30	1.42	0.26	1.85
32	26.10	49.40	0.83	0.28	1.54
40	19.10	49.80	0.68	0.27	1.25
48	20.10	51.40	0.64	0.28	1.07
56	18.10	51.90	0.61	0.28	0.93
64	20.10	53.80	0.62	0.30	0.84
72	21.10	58.20	0.62	0.32	0.81

Table 4.2 Batch fermentation of ethanol production using ETSI carrier.

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	Yp/s	Productivity (g/l h)
0	200.00	0.00	0.00		
8	159.50	8.70	1.23	0.21	1.09
16	82.30	31.90	1.6	0.27	1.99
24	35.10	42.10	1.63	0.25	1.75
32	12.00	46.00	1.64	0.24	1.44
40	25.10	47.60	1.74	0.27	1.19
48	22.10	48.90	1.68	0.27	1.02
56	21.10	48.90	1.53	0.27	0.87
64	20.10	50.50	1.38	0.28	0.79
72	22.10	52.00	1.39	0.29	0.72

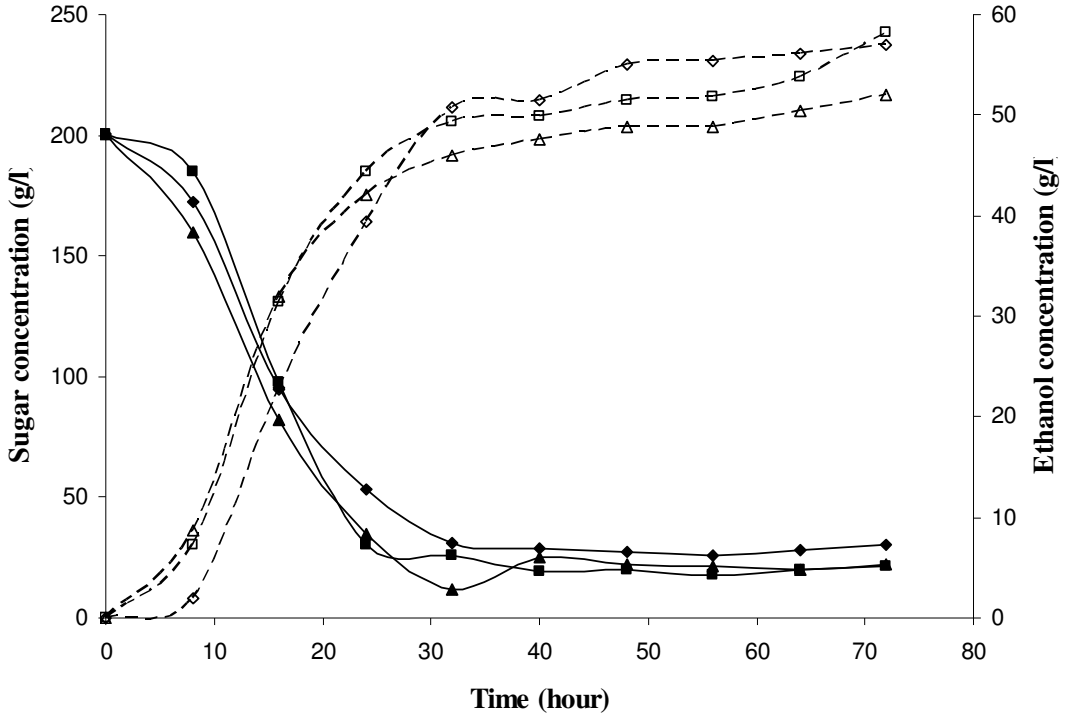


Figure 4.1 Ethanol (dash line, -----) and sugar (solid line, ─) concentration profiles with initial sugar concentrations of 200 g/l; (♦, ◊) = SC; (■, □) = TSI ;(▲, Δ) = ETSI.

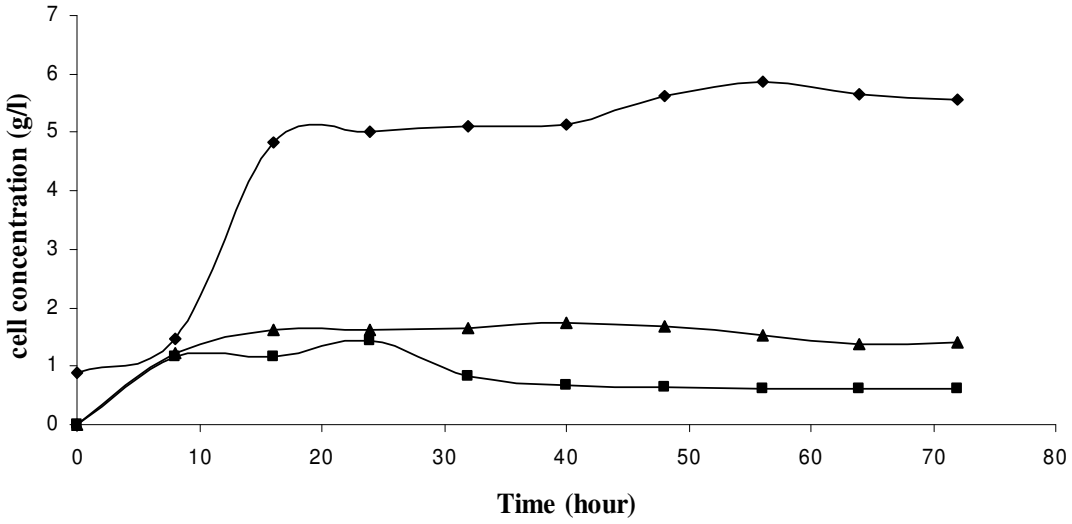


Figure 4.2 Cell concentration profile with initial sugar concentration 200 g/l; ─♦─ = suspended cell (SC), ─■─ = free cell concentration of TSI, ─▲─ = free cell concentration of ETSI.

The results indicated that the TSI culture was more effective than the ETSI culture, resulting in higher ethanol concentration, lower residual sugar concentration, and lower free cell leakage concentration. Moreover, the process for preparation of the TSI carrier was very simple and much easier than that of the ETSI. Cell immobilization using TSI method has less effect of mass transfer limitation than that of the ETSI method as this method, cells were absorbed or attached on surface of thin shell silk cocoon; not entrapment within the gel matrix, which was different from that of the ETSI. During the fermentation, some breakages and degradation of alginate thin film was inevitable and the cells entrapment in those parts leaked out of the carrier, resulting in higher free cell concentration in comparison to that of the TSI method. Due to the lower performance of the ETSI, it was no longer evaluated in the further studies.

4.2 Effect of initial sugar concentration on batch fermentation

To investigate the effect of initial sugar concentration, batch fermentations in 500 ml shaking flasks using two cultures for ethanol production: suspended cells (SC) and thin shell silk immobilized cells (TSI) at initial sugar concentration varied from 220 to 240, 260 and 280 g/l were carried out and the results were shown in the Table 4.3 -4.6 and Figure 4.3 -4.4.

Table 4.3 Batch fermentation of ethanol production using TSI carrier at initial sugar concentration 220 g/l

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	229.80	0.00	0.98		
8	202.60	6.90	1.23	0.25	0.86
16	133.80	33.40	1.67	0.35	2.09
24	75.20	61.80	1.22	0.40	2.58
32	58.20	69.30	1.08	0.40	2.17
40	49.40	74.60	0.76	0.41	1.87
48	48.50	84.40	0.71	0.47	1.76
56	49.70	88.00	0.74	0.49	1.57
64	48.10	83.80	0.70	0.46	1.31
72	49.90	84.50	0.75	0.47	1.17

Table 4.4 Batch fermentation of ethanol production using TSI carrier at initial sugar concentration 240 g/l

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	248.70	0.00	0.86		
8	207.00	13.20	0.84	0.32	1.65
16	135.40	40.90	0.82	0.36	2.56
24	90.40	71.00	1.49	0.45	2.96
32	50.50	85.60	1.22	0.43	2.68
40	54.80	86.30	1.14	0.45	2.16
48	50.20	89.80	1.59	0.45	1.87
56	53.30	93.20	1.51	0.48	1.66
64	50.20	100.80	1.82	0.51	1.58
72	51.50	96.30	1.56	0.49	1.34

Table 4.5 Batch fermentation of ethanol production using TSI carrier at initial sugar concentration 260 g/l

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	269.30	0.00	0.83		
8	237.90	6.00	0.77	0.19	0.75
16	151.40	41.20	1.04	0.35	2.58
24	103.50	63.10	1.26	0.38	2.63
32	84.40	78.10	0.99	0.42	2.44
40	64.10	88.50	0.84	0.43	2.21
48	63.60	87.10	1.61	0.42	1.81
56	62.30	94.40	1.67	0.46	1.69
64	56.40	93.20	1.76	0.44	1.46
72	62.00	92.60	1.51	0.45	1.29

Table 4.6 Batch fermentation of ethanol production using TSI carrier at initial sugar concentration 280 g/l

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	292.00	0.00	0.74		
8	268.30	7.30	0.77	0.31	0.91
16	228.10	28.50	1.27	0.45	1.78
24	165.50	60.40	1.35	0.48	2.52
32	118.20	73.60	1.49	0.42	2.30
40	71.80	79.40	1.50	0.36	1.99
48	80.80	92.30	1.86	0.44	1.92
56	74.70	96.80	1.94	0.45	1.73
64	71.80	91.10	2.00	0.41	1.42
72	71.60	89.10	1.86	0.40	1.24

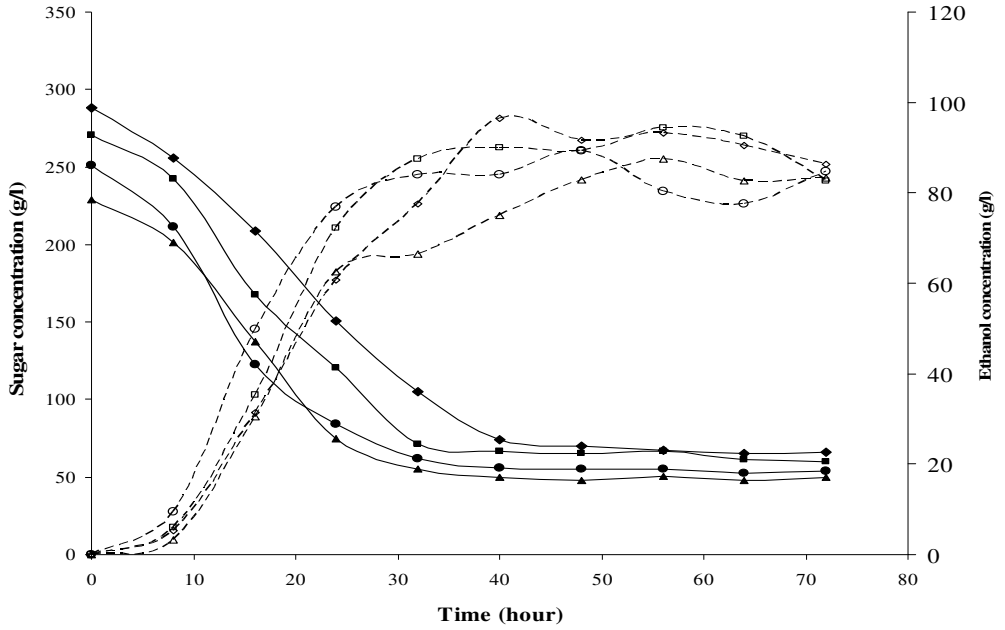


Figure 4.3 Ethanol (dash line, ----) and sugar (solid line, ─) concentration profiles at various initial sugar concentration of SC cultures; (▲,Δ) = 220 g/l , (●,○) = 240 g/l, (■, □) = 260 g/l, (♦,◊) = 280 g/l.

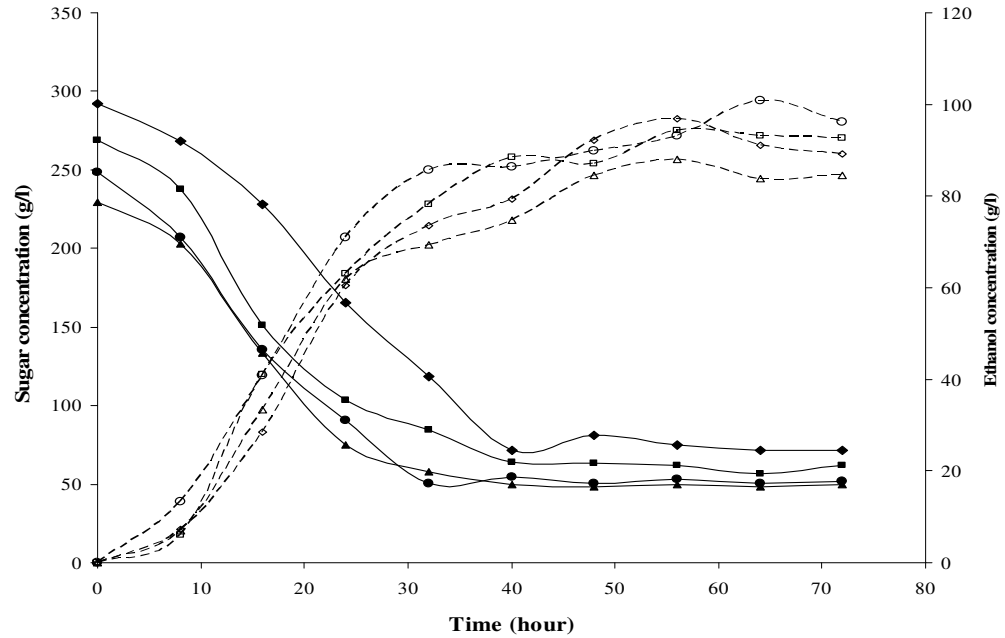


Figure 4.4 Ethanol and Sugar concentration profile at various initial sugar concentration of TSI carriers; (▲,Δ) = 220 g/l , (●,○) = 240 g/l, (■, □) = 260 g/l, (♦,◊) = 280 g/l.

The maximum ethanol concentration of 98.6 g/l with the productivity of 1.5 g/l h was obtained from the medium with the 240 g/l initial reducing sugar concentration. The initial sugar concentration increased from 240 g/l to 260 g/l and 280 g/l resulted in a decrease of the final ethanol concentration to 92.9 g/l and 90.1 g/l, respectively. The overall conversion yields were 0.47, 0.49, 0.45 and 0.40 for the initial sugar of 220 g/l, 240 g/l, 260 g/l and 280 g/l, respectively. The decrease of ethanol production with the increase initial sugar concentrations could occur from the substrate or product inhibition which has been previously reported [10]. It is worth to note that under the immobilization with TSI method, the final ethanol concentration was higher than those of our previously reports on the suspension culture and the immobilized cell cultures in Ca-alginate (1), Loofa reinforced gel carrier [12] and alumina doped alginate gel [13].

4.3 Repeated batch fermentation

Ethanol production using TSI as a carrier for *Saccharomyces cerevisiae M30* was evaluated by a 5-cycle repeated batch fermentation using 240 g/l of cane molasses as the carbon source. The duration of each batch was 48 h. For the comparison, two cell cultures were used for ethanol fermentation: suspended cells (SC) and thin shell silk cocoon-immobilized cells (TSI). Figure 4.5, Figure 4.6, Table 4.7 and Table 4.8 show the results of the repeated batch fermentation. In the first repeated batch, the ethanol concentration obtained at 48 hours for SC and TSI cultures were 88.8 g/l ($Y_{P/S}$ 47%)and 88.1 g/l ($Y_{P/S}$ 49%), respectively and the residual sugar concentration were at 53.1 and 59.2 g/l, respectively. The final ethanol concentration in the suspended cell and the immobilized cell cultures were comparable in the 2^{nd}, 3^{rd} and the 4^{th} batch.

Table 4.7 Ethanol and Sugar concentration in repeated batch fermentation using cultures of SC and TSI.

Batch	Time (hour)	Ethanol concentration (g/l)		Sugar concentration (g/l)	
		SC	TSI	SC	TSI
I	0	0.00	0.00	247.60	250.30
	12	18.90	14.00	179.80	208.10
	24	66.90	50.30	80.40	107.70
	36	81.10	55.80	55.10	67.50
	48	88.70	88.10	53.10	59.20
II	0	11.90	13.10	242.10	251.20
	12	14.40	13.20	238.90	180.90
	24	15.50	35.00	138.10	134.60
	36	61.80	65.30	103.70	104.60
	48	65.90	67.90	88.40	81.00
III	0	4.70	12.30	239.90	251.20
	12	20.00	26.80	198.20	172.30
	24	56.90	55.00	127.90	77.50
	36	76.80	84.80	65.10	54.20
	48	79.80	88.30	63.30	53.50
IV	0	6.80	8.50	243.60	249.70
	12	23.20	21.40	206.00	169.80
	24	51.80	48.70	138.00	83.20
	36	67.40	82.80	102.90	58.10
	48	72.30	87.60	81.40	59.70
V	0	14.60	9.00	242.90	249.10
	12	9.90	24.40	231.20	155.40
	24	12.00	65.30	223.70	90.10
	36	9.90	75.00	223.50	66.90
	48	9.40	76.10	215.40	57.80

Table 4.8 Yields and end products of repeated batch ethanol fermentation for 48 h for each batch using the cultures of suspended cell (SC) culture, thin shell silk cocoon immobilized cells (TSI) culture.

Batch	P (g/l)	X (g/l)		Y_I (g/g)	Y_S (g/g)	$Y_{P/S}$ (g/g)
		X_F	X_I			
I						
SC	88.75	n	n	n	0.78	0.47
TSI	88.10	n	n	n	0.75	0.49
II						
SC	65.87	n	n	n	0.63	0.43
TSI	67.92	n	n	n	0.66	0.43
III						
SC	79.77	n	n	n	0.73	0.45
TSI	88.27	n	n	n	0.78	0.47
IV						
SC	72.30	n	n	n	0.66	0.46
TSI	87.59	n	n	n	0.75	0.49
V						
SC	9.40	7.35	n	n	0.10	0.38
TSI	76.12	1.37	13.36	0.91	0.76	0.42

Ethanol concentration (P); Free cell concentration (X_F); Immobilized cell concentration (X_I); Immobilization yield (Y_I, g immobilized cell/g total cell); Sugar consumption yield (Y_S, g consumed sugar /g total sugar); Ethanol yield ($Y_{P/S}$, g ethanol/g consumed sugar); not detected (n)

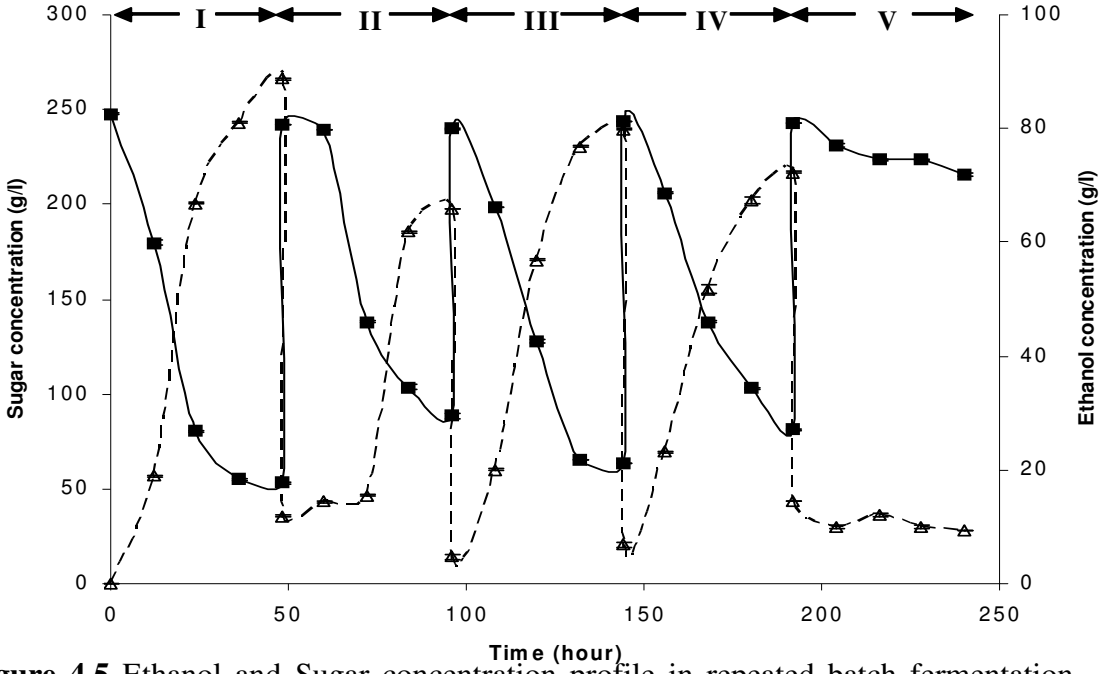


Figure 4.5 Ethanol and Sugar concentration profile in repeated batch fermentation using cultures of SC; —■— = sugar and --Δ-- = ethanol

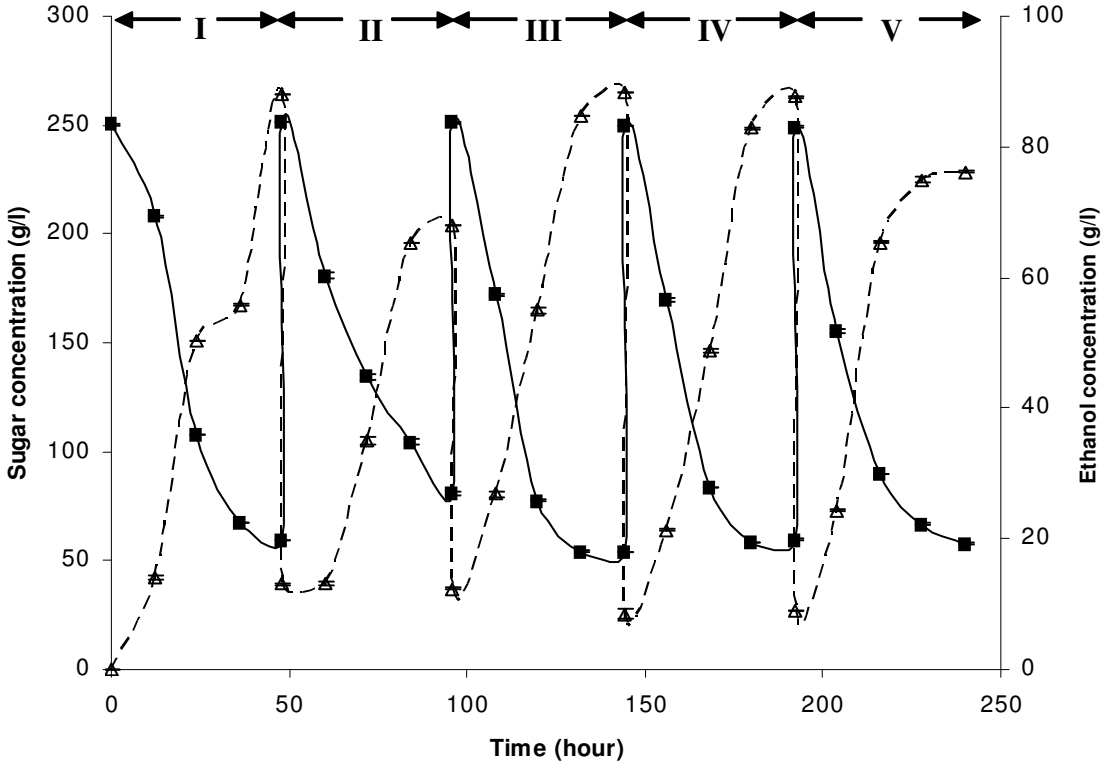


Figure 4.6 Ethanol and Sugar concentration profile in repeated batch fermentation using cultures of TSI; —■— = sugar and --Δ-- = ethanol

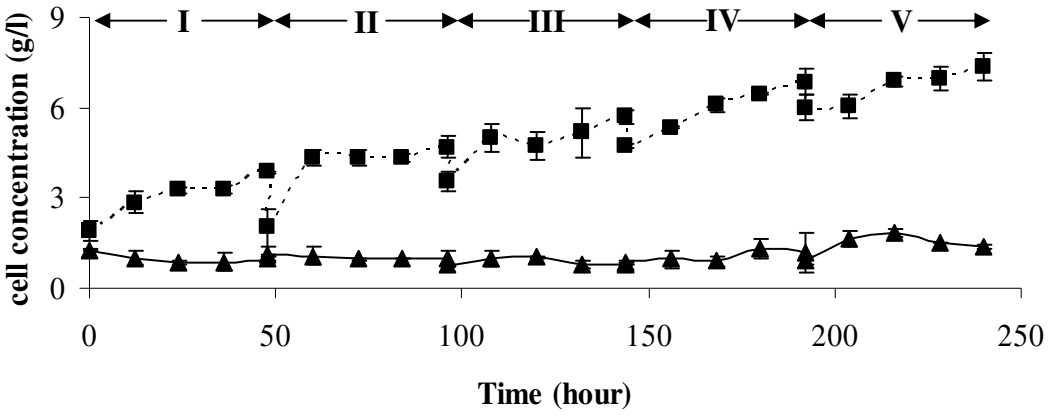
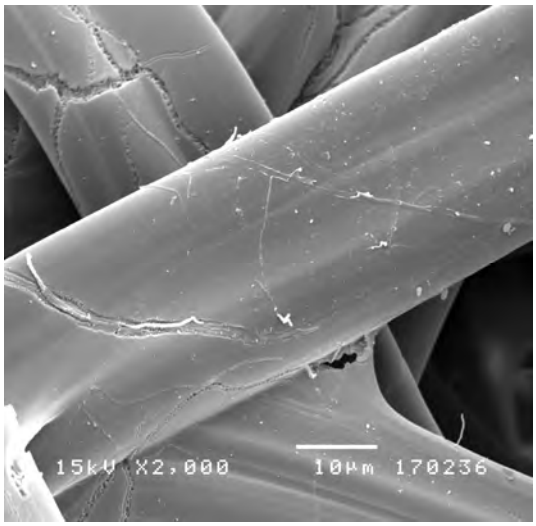


Figure 4.7 Cell concentration profile in repeated batch fermentation using SC and TSI; --■-- = SC and —▲— = free cell in TSI.

Table 4.9 Yeast cell concentrations at the end of repeated batch ethanol fermentation.

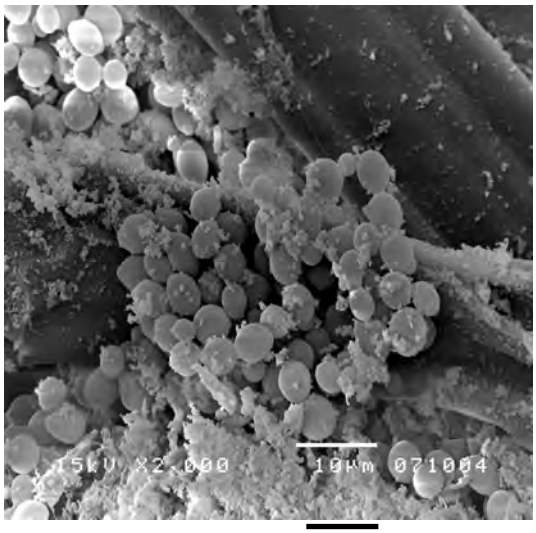
Cell concentartions	**(g/l)**
Immobilized cell	13.36
Free cell	1.37
Immobilized yield (%)	90.70

The thin shell silk cocoon was compatible for yeast immobilization. After the 5th batch, the final total cell concentration of system using TSI culture (14.7 g/l) was considerably higher than that of SC (7.4 g/l). The increase of the cell concentration in TSI carrier indicated the growth of immobilized cells in the carriers during the fermentation. Moreover, the cell carrier exhibited the high immobilization yield (Y_I) of 91% and the higher stability of the TSI cultures over the SC cultures was observed, especially in the 5th batch. The ethanol concentration obtained at 48 hours of the 5th batch using SC and TSI cultures were at 9.4 g/l ($Y_{P/S}$ 38%) and 76.1 g/l ($Y_{P/S}$ 42%), respectively and the final residual sugar concentrations were at 215.4 and 57.8 respectively. The negative effect of high ethanol and sugar concentration on SC activities has been previously reported [10]. The higher stability of TSI cultures implied that the TSI carriers could protect yeast cells from toxins and/or inhibitor during the fermentation process.



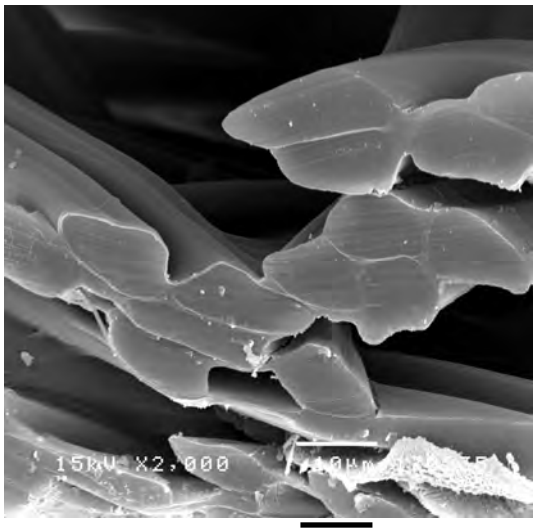
15kv X2,000 10µm

Figure 4.8 TSI surface at the initial stage.



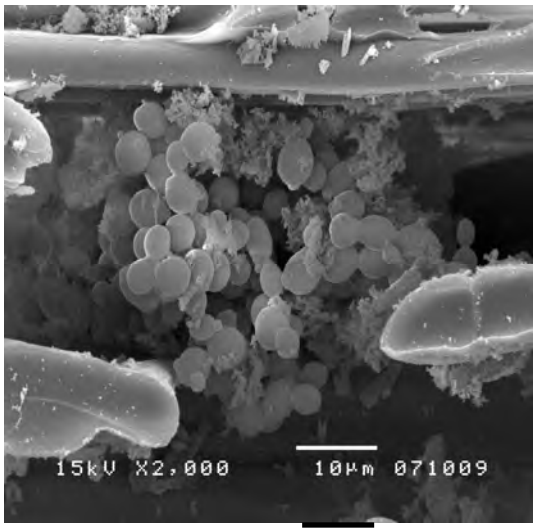
15kv X2,000 10µm

Figure 4.9 TSI surface after 5th batch.



15kv X2,000 10µm

Figure 4.10 TSI cross section at the Initial stage.



15kv X2,000 10µm

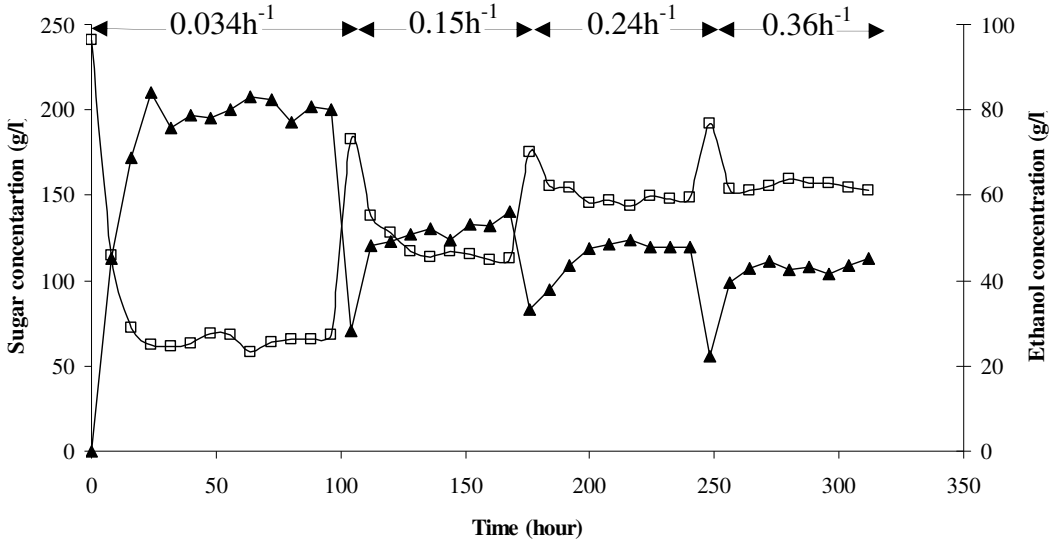
Figure 4.11 TSI cross section after 5th batch.

The comparison of SEM images of TSI carriers at the initial stage and after the 5th batch demonstrates that the immobilized yeast cells were attached to outer surface of shell silk cocoon (Figure 4.9) and also accessed and grew well in the space

of its matrix (Figure 4.11). Therefore, the porous structure of the carrier provided suitable living environment for yeast cells without severe mass transfer problem.

4.4 Continuous ethanol fermentation #1

The optimum condition from the batch fermentation was further used in continuous ethanol fermentations. For the continuous ethanol fermentation # 1, a packed-bed reactor with the total working volume of 0.96 liters was used for study with the operated condition of: 32 ± 1°C, initial feeding sugar concentration of 241 g/l, initial pH of 5.0 at the varied dilution rates from 0.034, 0.15, 0.24 to 0.36 h^{-1}. Prior to inoculation and start up of the fermentation, the column was sterilized by 70% v/v ethanol. The immobilized cell in thin shell silk cocoon was prepared by the cultivation in Innova 4330 refrigerated incubator shaker at 150 rpm, 33°C for 24 hours in order to increase the cells concentration before the cell carries were aseptically transferred into the sterilized column. The carrier volume was about 30 % (v/v) of the pack bed reactor volume of 960 ml.



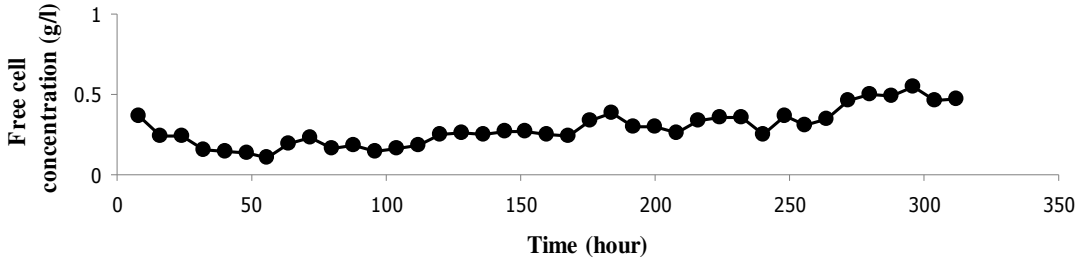


Figure 4.12 Continuous ethanol production in an immobilized cell reactor with initial sugar concentration 241 g/l (—□— = sugar, —▲— = ethanol and —●— = free cell)

The fermentation was started by feeding of the prepared medium of sugarcane molasses, containing about 241 g/l reducing sugar, through the inlet at the bottom of the column at the dilution rate of 0.034 h^{-1}. For the first dilution rate (0.034 h^{-1}), the fermentation was maintained for 96 hours before the change to the next dilution rate. For other dilution rates of 0.15, 0.24 and 0.36 h^{-1}, the fermentation was maintained for 72 hours for each dilution rate. The recycle process was performed at the end of each dilution rate. To recycle, the fermented broth was circulated by a peristaltic pump from the outlet tube at the top of the packed column to the inlet tube at the bottom of the column and flowed upward through the packed column back into the outlet tube. The circulation was performed for 10 cycles. The samples were harvested every 8 hours from the outlet port on both sides of the column. After the 3 day of the operation with the dilution rate of 0.36 h^{-1}, the dilution rate was rolled back to the start point (0.034 h^{-1}) for stability checking of the cell activities.

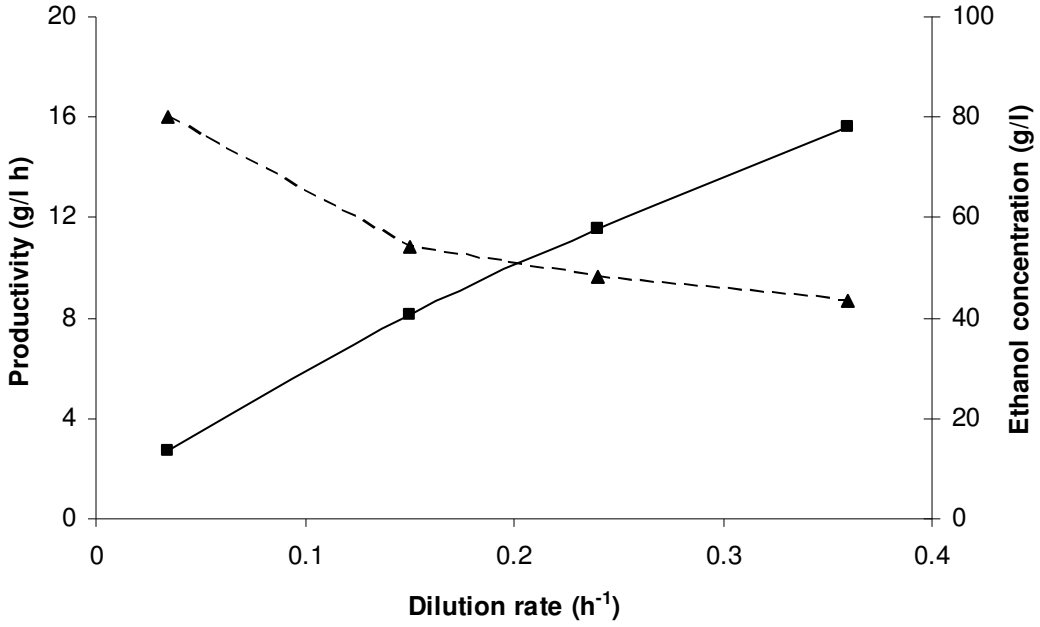


Figure 4.13 The ethanol productivities of ethanol fermentation at steady state for the initial sugar concentration of 241 g/l (—■— = ethanol productivity, --▲-- = ethanol concentration)

The pseudo-steady steady was reached at about 64, 32, 24 and 24 hours for the dilution rate of 0.034, 0.15, 0.24 and 0.36, respectively. The experimental results showed that the average ethanol concentration after the pseudo-steady state of continuous fermentation decreased from 80.03 g/l to 54.12 g/l, 48.24 g/l and 43.76 g/l under dilution rate of 0.034, 0.15, 0.24 and 0.36 h^{-1} respectively, while the residual sugar concentration increased from 67.26 g/l to 114.38 g/l, 147.07 g/l and 156.08 g/l, respectively. The experimental trend lines for concentration of ethanol and residual sugar all in agreement with the previous published reports (Yu et al., 2007; Liu et al., 2008). At the steady state, the maximum productivity of 15.60 g/l h was obtained from the dilution rate of 0.36 h^{-1}. The ethanol productivity linearly increased with the dilution rate. The maximum ethanol concentration (80.03 g/l) was obtained from dilution rate of 0.034 h^{-1}.

Table 4.10 Yeast cell concentrations at the end of continuous fermentation # 1.

Cell concentartions	**(g/l)**
Immobilized cell	40.02
Free cell in reactor	5.48
Free cell in effluent	0.46
Immobilized yield (%)	87.08

The free cell leakage concentration slightly increased with the fermentation time from 0.2 – 0.5 g/l (for 312 hours of the operation). To compare with the previous batch fermentation, the free cell leakage concentration in the continuous fermentation was much lower than that in the batch fermentation (1.37 g/l). The result indicated that yeast cells were restricted by the bed, resulting in only a few of free cells leaving from the reactor. At the end of the fermentation, the free cell in effluent, in reactor and the total immobilized cell in the reactor were investigated (Table 4.10). Since almost suspended cells were confined by the bed, after continuous fermentation for 312 hours, the ratio of the free cell concentration in the bed compared to that in the effluent was more than 80. In addition, the immobilized yield was very high at 87.1 %.

4.5 Continuous ethanol fermentation #2

In the continuous ethanol fermentation # 2, the lower reducing sugar concentration of 222 g/l was used for feeding into the bottom of the packed bed reactor by peristaltic pump.. The studied conditions of the system were exactly similar to the continuous ethanol fermentation # 1 (temperature 32 ± 1 ^{o}C, initial pH 5, total working volume of 0.96 liters, dilution rate varied from 0.034, 0.15, 0.24 to 0.36 h^{-1} and the samples were harvested every 8 hours). From the experimental results, the pseudo-steady state was reached for all 4 dilution rates (Figure 4.14).

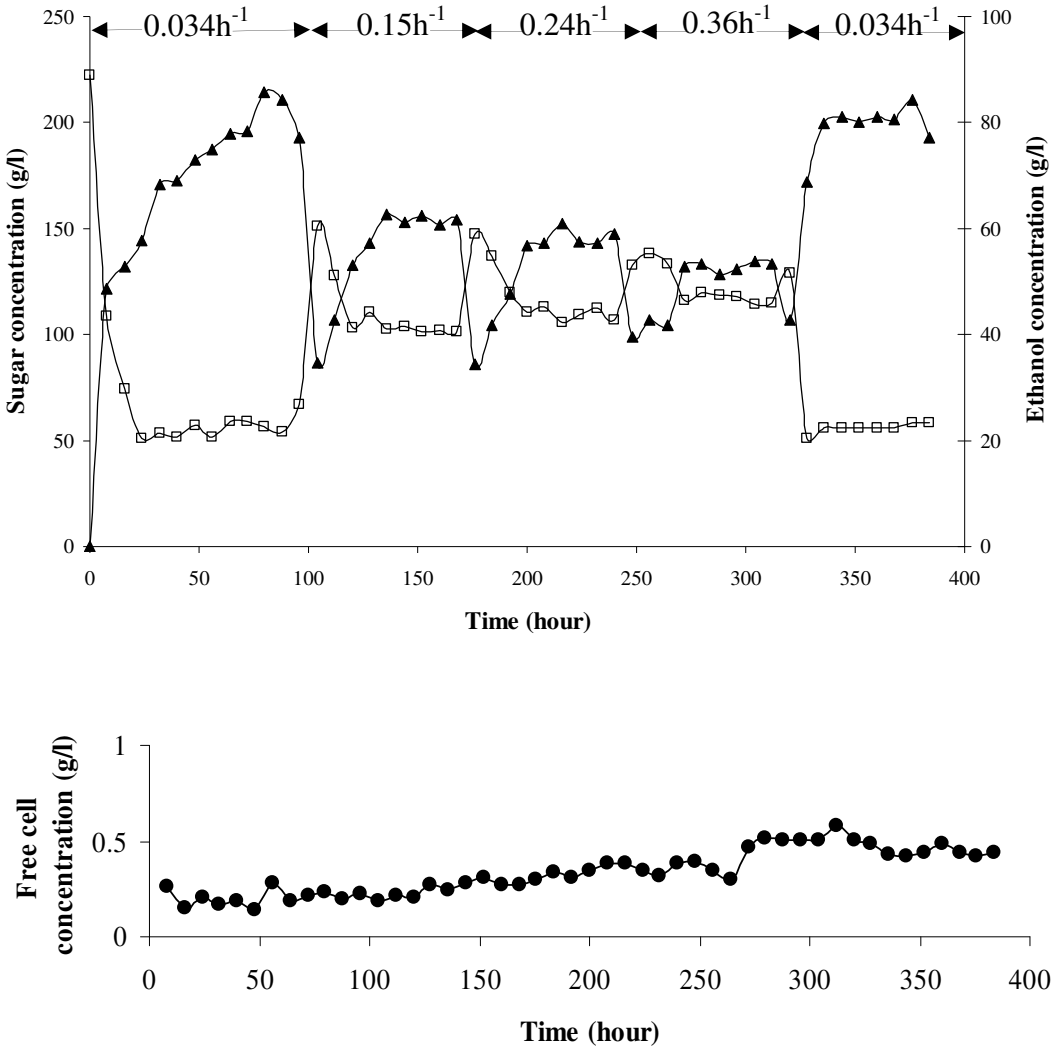


Figure 4.14 Continuous ethanol production in an immobilized cell reactor with initial sugar concentration 222 g/l (—□— = sugar, —▲— = ethanol and —●— = free cell)

Figure 4.14 shows that the ethanol concentrations in the continuous ethanol fermentation # 2 for all four dilution rates were higher than those obtained in the continuous ethanol fermentation #1 while the residual sugar concentrations are lower. The steady state ethanol concentrations at dilution rates of 0.034, 0.15, 0.24 and 0.36 h^{-1} were 80.72, 61.72, 58.12 and 52.83 g/l respectively with the residual sugar concentration of 59.13, 102.32, 109.64 and 116.96 g/l, respectively.

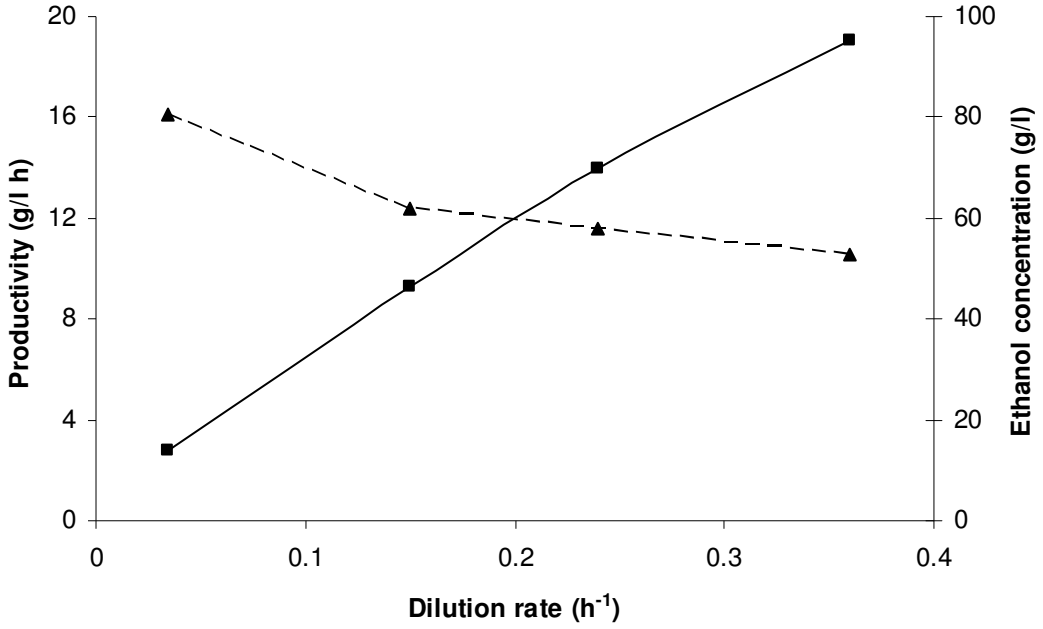


Figure 4.15 The ethanol productivities of ethanol fermentation at steady state for the initial sugar concentration of 222 g/l (—■— = ethanol productivity, --▲-- = ethanol concentration)

At the steady state, the maximum productivity (19.02 g/l h) and the maximum ethanol concentration (80.72 g/l) were obtained from dilution rate of 0.36 h^{-1} and 0.034 h^{-1}, respectively which were higher than those of the continuous fermentation # 1. The system was continuously operated for 16 days. It was found that the free cell leakage concentration slightly increased with the fermentation time from 0.2 – 0.5 g/l in the similar way as observed in the continuous fermentation # 1. At the end of fermentation, the free cell in effluent, in reactor and immobilized cell in the reactor were investigated (Table 4.11). The amount of the free cell in reactor (3.39 g/l) and the free cell in effluent (0.44 g/l) and immobilized yield (88.3 %) were in the similar levels of those from the continuous fermentation # 1. However, the amount of total immobilized cell was slightly lower than that in the system with the higher sugar concentration. Although the ethanol productivities at the low dilution of 0.034 h^{-1} in both continuous fermentation # 1 and # 2 were almost equivalent, the ethanol productivities from feeding with 220 g/l sugar concentration at the high dilution rate of 0.36 h^{-1} was 21.9 % higher than that from feeding with 240 g/l sugar concentration.

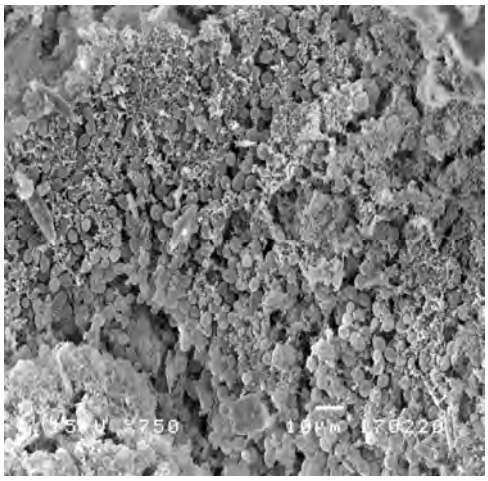
Therefore, at higher dilution rate, the cell activity was more influenced by the inhibition effect of the high sugar concentration.

Table 4.11 Yeast cell concentrations at the end of continuous fermentation # 2.

Cell concentartions	**(g/l)**
Immobilized cell	28.93
Free cell in reactor	3.39
Free cell in effluent	0.44
Immobilized yield (%)	88.31

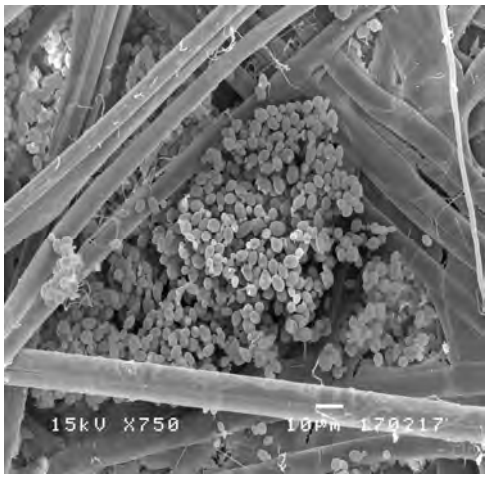
15kv X750 10µm

Figure 4.16 TSI outer surface before fermentation



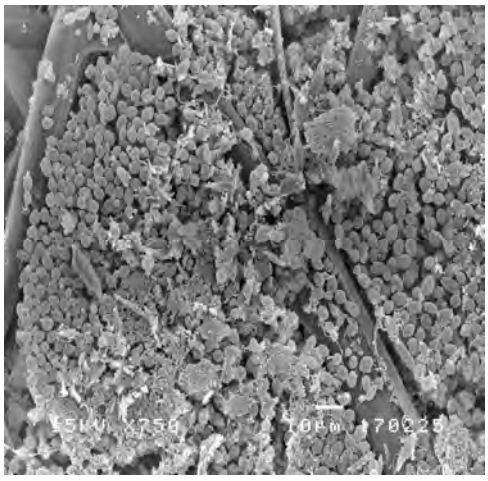
15kv X750 10µm

Figure 4.17 TSI outer surface at the end of continuous fermentation



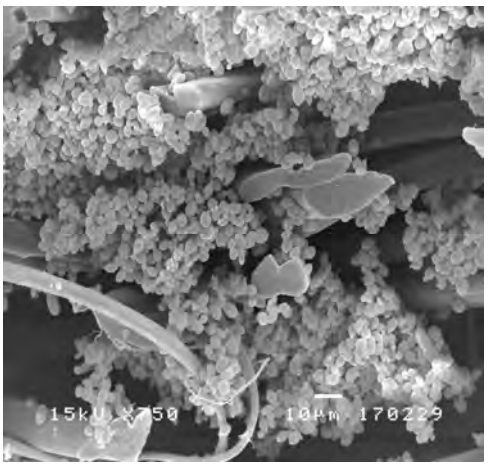
15kv X750 10µm

Figure 4.18 TSI inner surface before thefermentation.



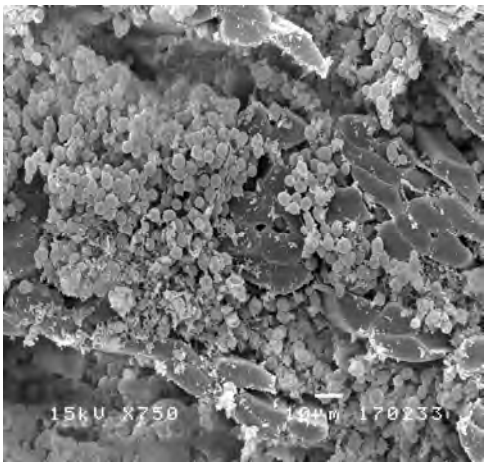
15kv X750 10µm

Figure 4.19 TSI inner surface at end of continuous fermentation



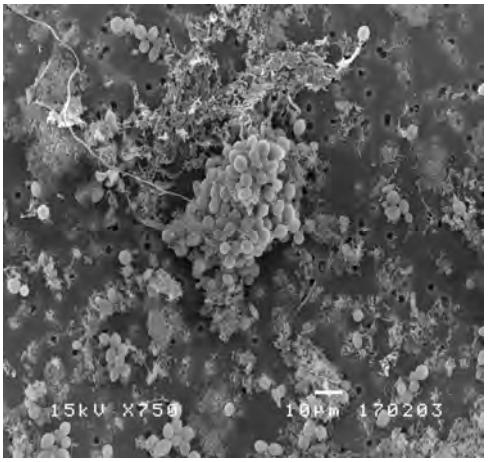
15kv X750 10µm

Figure 4.20 TSI cross section before fermentation.



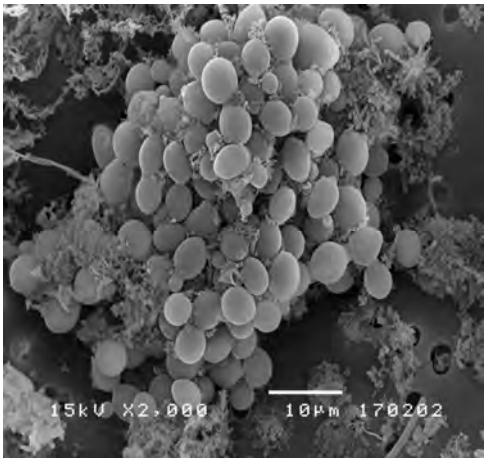
15kv X750 10µm

Figure 4.21 TSI cross section at the end of continuous fermentation.



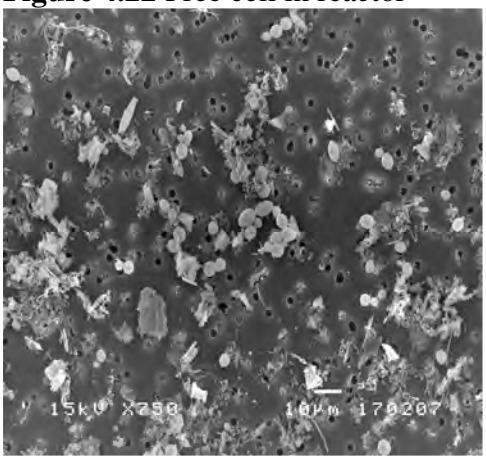
15kv X750 10µm

Figure 4.22 Free cell in reactor



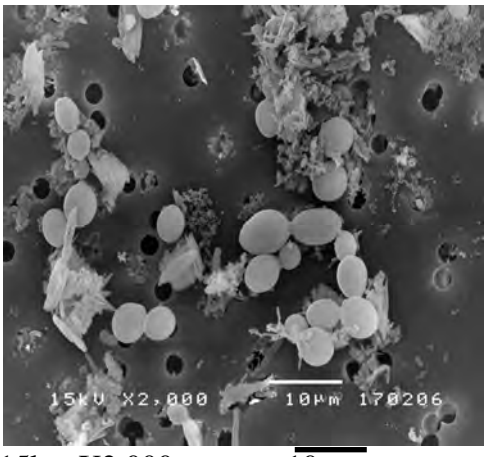
15kv X2,000 10µm

Figure 4.5.23 Free cell in reactor



15kv X750 10µm

Figure 4.24 Free cell in the effluent



15kv X2,000 10µm

Figure 4.25 Free cell in the effluent

A scanning electron microscope (SEM) was used to compare the images of the TSI carriers before ethanol fermentation and at the end of fermentation. Figure 4.16 to Figure 4.21 represent the images of carrier from the beginning of the fermentation to the end of fermentation, the amount of cell inside and outside the carriers from time to time were increasing. For long term performance, the free cell leakage occurred. Such leak can be observed from free cells in the reactor and free cells in the effluent. Figure 4.22 to Figure 4.25 show the image of suspension cell culture in the reactor and in the effluent. Figure 4.22 and Figure 4.23 showed that the cells in the reactor appeared healthy, retained their normal oral shape and flocculated inform of big groups of 100 - 1000 cells while the cells in the effluent (Figure 4.24 and Figure 4.25) were mostly separated as a single cell or small groups of 2-5 cells.

Overall, by continuous fermentation using TSI culture, the ethanol productivity could be improved up to 19.0 g/l h or about 12.6 times of that from the batch fermentation. The experimental result showed that TSI culture could be used as a cell carrier with favorable mechanical and biocompatibility properties and porous structure, resulting in a stable operation, high ethanol production and high density of biomass. The TSI carrier has a good potential of reusability to produce continuous ethanol production. Therefore, this carrier was successfully applied for yeast immobilization in ethanol fermentation using cane molasses as the carbon source in continuous fermentation. It is also worth to note that at the optimal condition, the ethanol productivity, immobilized yield and cell density of the continuous fermentation in the packed bed column using TSI culture were higher than those of our previously reports using the immobilized cell cultures in Loofa reinforced gel carrier [12] and alumina doped alginate gel [13].

CHAPTER V
CONCLUSIONS AND RECOMMENDATIONS

Conclusions

In an effort to develop a high performance ethanol fermentation process, in this study, a new immobilized method using thin shell silk cocoon was developed and evaluated. Under batch fermentations in 500 ml Erlenmeyer flask, the ethanol fermentation using thin shell silk cocoon immobilized cell (TSI) culture was found to be more effective than that using thin shell silk cocoon immobilized cell entrapment within alginate (ETSI) and suspension cell (SC) cultures, resulting in higher ethanol production. Moreover, by using TSI culture with the initial sugar concentration of 240 g/l, the maximum ethanol concentration of 98.6 g/l was obtained after 64 hours of the fermentation. From the evaluation in the 5-cycle repeated batch, the TSI culture demonstrated a good potential of reusability than that of the SC culture. Continuous ethanol production was successfully carried out in a 1-litre packed-bed reactor and revealed that the maximum ethanol productivity of 19.02 g/l h with ethanol concentration of 52.83 g/l could be obtained with the feed of 220 g/l sugar concentration at 0.36 h^{-1} dilution rate, while the highest ethanol concentration of 80.72 g/l was obtained at the dilution rate of 0.034 h^{-1}.

With a strong and porous structure together with high biocompatibility of TSI carrier, many advantages including reusability, altered mechanical strength, cell regeneration and high capacity to attach alive cells were achieved. In this research, it was found that ethanol could be produced from cane molasses by *Saccharomyces cerevisiae M30* in TSI carrier with a high yield and a very high production rate.

Recommendations

To improve this technology, further works could be done such as the improvement of yeast strain which could be tolerate to high ethanol and high sugar concentration or could be operated at higher temperature. The development of yeast or another microorganism which could be able to use agriculture waste to be a carbon source such as cellulose or other low cost of substrate is suggested. Moreover, the potential use of TSI carrier in other similar biosystems is recommended.

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APPENDICES

APPENDIX A
EXPERIMENTAL METHODS

A-1 Agar slants preparation

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

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

A-2 Stock cultures preparation

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

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

A-3 Medium preparation

Palm sugar was designated for cell cultivation. 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 100 g/l for cell cultivation . 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 3,5-dinitrosalicylic acid (DNS) method (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 $(NH_4)_2SO_4$ 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 (100 g/l for cell cultivation) is achieved.
2. Adjust the pH of the solution to 5 by adding NaOH or HCl solution.
3. Pour appropriate volume of medium (100 ml and 250 ml for inoculums development and ethanol fermentation respectively) 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 the fermentation, molasses was used for fermentation medium. For 1 liter of the medium 0.5 g $(NH_4)_2SO_4$ 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 7820 centrifuge at 2000 rpm for 15 minutes.

3. Mix the diluted sugar solution with appropriate amount of $(NH_4)_2SO_4$

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 minutes 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 20-24 hours at 150 rpm. After some time, the growing yeast cells could be noticed as brown colored suspended solids inside the sugar solution. The cells were then harvested and concentrated by medium draining. The complete steps are as follows:

1. Sterilize equipments and the laminar flow hood with UV and by wiping with alcohol 70% v/v solution.
2. Heat up the neck of stock culture tube and medium flask after removing the tube cap and cotton plug.
3. Heat up the inoculation loop evenly and then slightly deep it into the fresh medium in the Erlenmeyer flask to cool it down before touching the yeast cells.
4. Scratch the yeast culture on the tube to detach the cells from the surface of the agar using the loop.
5. Transfer the cell at the loop into the Erlenmeyer flask and then close the flask using cotton plug.
6. Repeat steps 3-5 for the other flasks.
7. Put all flasks in the incubator shaker and then operate the shaker at 150 rpm 33°C for a day before harvesting the cells.

8. Let the cells to settle for a while after incubation and then carefully take out 130 ml of the medium from each flask by using 10 ml of auto pipette.
9. Combine the concentrated cells suspension from several flasks by pouring it into one flask.
10. Further draining can be done to concentrate cells by the same method until the desired volume of concentrated cells suspension is obtained.
11. Precautions and notes:
    a) Except the stock culture and the fresh medium, all equipments should be cleaned and sterilized using UV light and alcohol to ensure asepticity.
    b) Clean the outer surface of the tubes and flasks using alcohol before use.
    c) Keep the tube neck and flask opening hot by regular heating after removal of the cap or plug to prevent contamination originated from ambient air.

A-5 Cell immobilization

Thin shell silk cocoon and palm sugar medium were sterilized with autoclave for 15 minutes at 121 ^{o}C prior to usage. Preparation of TSI carrier were listed in the following paragraph.

1. Mix 10 ml of concentrated cell suspension with 250 ml of palm sugar medium.
2. Add the thin shell silk cocoon in the mixture.
3. incubated suspension mixture for 20-24 hours.
4. Precautions and notes:
    a) All procedures are conducted aseptically in laminar flow hood.
    b) All equipments are cleaned and sterilized before use.

A-6 Ethanol fermentation

A-6.1 Batch fermentation

The molasses which has 240 g/l of initial sugar concentration was added by 0.5 g/l of ammonium sulfate as the nutrient. The volume of medium was adjusted to 250 ml in 500 ml Erlenmeyer flask in order to promote anaerobic condition which was favorable ethanol fermentation by yeast. Batch fermentation in shake flasks was

performed in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C.

A-6.2 Continuous fermentation

The reactor column with working volume around 0.67 liters containing immobilized cell bed is used for the study. Temperature of the system was controlled at 32 ± 1 °C by the passing of 28 °C cooling water inside the reactor jacket. The initial sugar concentration varied of 220 and 240 g/l at room temperature continuously fed into the bottom of reactor for each dilution rate. The dilution rate was varied from 0.034, 0.15, 0.24 and 0.36 h^{-1}. Sampling was done regularly with volume of 5 ml for every 8 hours. The samples were frozen before analysis of sugar, ethanol, and cell concentration in order to enable all samples to be analyzed at the same time.

A-7 Sugar analysis

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

A-7.1 NaOH and HCl solution preparation

NaOH 20% w/v was prepared by dissolving 200 g of NaOH pellets in 100 mL of water. The reaction is highly exothermic so that the preparation should be done in water bath in order to avoid excess heat generation. Weighing time of NaOH pellets should be minimized because of the hygroscopic nature of NaOH. Solution of 37% w/v HCl was obtained by diluting concentrated HCl solution with DI water. Beware

of the acid vapor and wear protective gloves when preparing the solutions. Commercially available HCl 37% can be also be used directly.

A-7.2 DNS reagent preparation

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

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

A-7.3 Standard sucrose solution preparation

Standard sucrose solutions were prepared first by making the source solution which was the solution with the highest sucrose concentration as the upper limit. The source solution was then diluted with water so that a set of standard solution with increasing sucrose concentration (for instance 0, 6.25, 12.5, 18.75, and 25% w/v) was obtained. The detailed procedures are as follows:

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

Table A-7.3 Standard sucrose solution preparation

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

A-7.4 Sample treatment I

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

1. Mix 0.2 ml of sample with 0.8 ml DI water in screw cap tube.
2. Blend the sample with 0.5 ml HCl 37%.
3. Put the tubes in boiling water bath for 10 minutes.
4. Stop the reaction by placing the tubes in ice bath.
5. Add 0.5 ml NaOH 20% w/v and then mix with vortex mixer.
6. Add 10 ml DI water and then mix with vortex mixer.
7. Centrifuge the sample at 2000 rpm for 20 minutes.
8. Precautions and notes:
    a) Use vortex mixer for mixing the fluid in the tubes.
    b) Be cautious when handling the hot apparatus.
    c) The level of boiled water and ice bath must be sufficiently higher than the liquid level in the tubes to ensure good heating and cooling of the sample.

A-7.5 Sample treatment II

In treatment II, supernatant obtained from treatment I was reacted with DNS reagent in boiled water bath. The solution's color transformed from yellow to reddish brown in the course of reaction. The color intensity represents the corresponding sugar concentration. Solution with higher sugar content will have darker color. After

the reaction was ended, the solution was diluted with sufficient amount of water until its absorbance spectrum obtained by spectrophotometer was well distributed along the range of concentration being considered (the absorbance measured was not more 0.7). Shimadzu UV-2450 UV-Visible spectrophotometer was used for absorbance measurement. Sample containing only water (0% sugar) which had been treated in the same manner as the other samples was used as blank. At every absorbance measurement, fresh standard solution should be used. Complete procedures are described in the following paragraph.

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

A-8 Determination of cell concentration

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

A-8.1 Dry weight of cell

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

1. Centrifuge the cell containing medium at 2000 rpm for 15 minutes.
2. Remove the supernatant (discarded or to be used for other analysis).

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

A-8.2 Free cell concentration

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

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

b) Mix every sample with vortex mixer before spectrophotometry to ensure homogeneity of the sample.

A-8.3 Immobilized cell concentration

Before the cell concentration could be measured, a measured amount of carrier should be dissolved to obtain cell suspension. The dissolution of TSI was carried out using water. The thin shell silk cocoon 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. Cut the TSI carrier in to the small size.
2. Dissolve appropriate amount of TSI carrier with 10 ml water in 25 ml beaker.
3. Stir TSI carrier in the beaker with magnetic stirrer for 30 minutes.
4. Remove the TSI carrier from the suspension and continue with same procedures as step 2-9 of Section A-8.2.

APPENDIX B

EXPERIMENTAL DATA

B-1 Experimental data of batch fermentation

Table B-1.1 Data of batch fermentation of ethanol production using TSI carrier.

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	Yp/s	Productivity (g/l h)
0	200.00	0.00	0.00		
8	184.60	7.20	1.16	0.45	0.90
16	97.30	31.40	1.17	0.30	1.96
24	30.10	44.30	1.42	0.26	1.85
32	26.10	49.40	0.83	0.28	1.54
40	19.10	49.80	0.68	0.27	1.25
48	20.10	51.40	0.64	0.28	1.07
56	18.10	51.90	0.61	0.28	0.93
64	20.10	53.80	0.62	0.30	0.84
72	21.10	58.20	0.62	0.32	0.81

Note : Ethanol yield ($Y_{P/S}$, g ethanol/g consumed sugar)

Table B-1.2 Data of batch fermentation of ethanol production using ETSI carrier.

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	Yp/s	Productivity (g/l h)
0	200.00	0.00	0.00		
8	159.50	8.70	1.23	0.21	1.09
16	82.30	31.90	1.6	0.27	1.99
24	35.10	42.10	1.63	0.25	1.75
32	12.00	46.00	1.64	0.24	1.44
40	25.10	47.60	1.74	0.27	1.19
48	22.10	48.90	1.68	0.27	1.02
56	21.10	48.90	1.53	0.27	0.87
64	20.10	50.50	1.38	0.28	0.79
72	22.10	52.00	1.39	0.29	0.72

Note : Ethanol yield ($Y_{P/S}$, g ethanol/g consumed sugar)

Table B-1.3 Data of batch fermentation of ethanol production using TSI carrier at initial sugar concentration 220 g/l

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	229.80	0.00	0.98		
8	202.60	6.90	1.23	0.25	0.86
16	133.80	33.40	1.67	0.35	2.09
24	75.20	61.80	1.22	0.40	2.58
32	58.20	69.30	1.08	0.40	2.17
40	49.40	74.60	0.76	0.41	1.87
48	48.50	84.40	0.71	0.47	1.76
56	49.70	88.00	0.74	0.49	1.57
64	48.10	83.80	0.70	0.46	1.31
72	49.90	84.50	0.75	0.47	1.17

Note : Ethanol yield ($Y_{P/S}$, g ethanol/g consumed sugar)

Table B-1.4 Data of batch fermentation of ethanol production using TSI carrier at initial sugar concentration 240 g/l

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	248.70	0.00	0.86		
8	207.00	13.20	0.84	0.32	1.65
16	135.40	40.90	0.82	0.36	2.56
24	90.40	71.00	1.49	0.45	2.96
32	50.50	85.60	1.22	0.43	2.68
40	54.80	86.30	1.14	0.45	2.16
48	50.20	89.80	1.59	0.45	1.87
56	53.30	93.20	1.51	0.48	1.66
64	50.20	100.80	1.82	0.51	1.58
72	51.50	96.30	1.56	0.49	1.34

Note : Ethanol yield ($Y_{P/S}$, g ethanol/g consumed sugar)

Table B-1.5 Data of batch fermentation of ethanol production using TSI carrier at initial sugar concentration 260 g/l

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	269.30	0.00	0.83		
8	237.90	6.00	0.77	0.19	0.75
16	151.40	41.20	1.04	0.35	2.58
24	103.50	63.10	1.26	0.38	2.63
32	84.40	78.10	0.99	0.42	2.44
40	64.10	88.50	0.84	0.43	2.21
48	63.60	87.10	1.61	0.42	1.81
56	62.30	94.40	1.67	0.46	1.69
64	56.40	93.20	1.76	0.44	1.46
72	62.00	92.60	1.51	0.45	1.29

Note : Ethanol yield ($Y_{P/S}$, g ethanol/g consumed sugar)

Table B-1.6 Data of batch fermentation of ethanol production using TSI carrier at initial sugar concentration 280 g/l

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	292.00	0.00	0.74		
8	268.30	7.30	0.77	0.31	0.91
16	228.10	28.50	1.27	0.45	1.78
24	165.50	60.40	1.35	0.48	2.52
32	118.20	73.60	1.49	0.42	2.30
40	71.80	79.40	1.50	0.36	1.99
48	80.80	92.30	1.86	0.44	1.92
56	74.70	96.80	1.94	0.45	1.73
64	71.80	91.10	2.00	0.41	1.42
72	71.60	89.10	1.86	0.40	1.24

Note : Ethanol yield ($Y_{P/S}$, g ethanol/g consumed sugar)

Table B-1.7 Data of batch fermentation of ethanol production using SC culture at initial sugar concentration 220 g/l

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	228.90	0.00	0.98		
8	201.30	3.19	1.23	0.12	0.40
16	137.30	30.51	1.67	0.33	1.91
24	74.70	62.54	1.22	0.41	2.61
32	55.20	66.53	1.08	0.38	2.08
40	49.50	75.07	0.76	0.42	1.88
48	48.10	82.89	0.71	0.46	1.73
56	50.60	87.56	0.74	0.49	1.56
64	48.10	82.66	0.70	0.46	1.29
72	49.50	83.30	0.75	0.46	1.16

Note : Ethanol yield ($Y_{P/S}$, g ethanol/g consumed sugar)

Table B-1.8 Data of batch fermentation of ethanol production using SC culture at initial sugar concentration 240 g/l

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	250.80	0.00	0.98		
8	211.10	9.40	1.23	0.24	1.18
16	122.60	49.80	1.67	0.39	3.11
24	83.90	76.80	1.22	0.46	3.20
32	62.00	84.10	1.08	0.45	2.63
40	55.90	84.00	0.76	0.43	2.10
48	55.40	89.40	0.71	0.46	1.86
56	55.40	80.20	0.74	0.41	1.43
64	52.30	77.60	0.70	0.39	1.21
72	53.60	84.60	0.75	0.43	1.18

Note : Ethanol yield ($Y_{P/S}$, g ethanol/g consumed sugar)

Table B-1.9 Data of batch fermentation of ethanol production using SC culture at initial sugar concentration 260 g/l

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	270.80	0.00	0.98		
8	242.50	5.90	1.23	0.21	0.74
16	167.30	35.30	1.67	0.34	2.21
24	120.70	72.20	1.22	0.48	3.01
32	71.10	87.40	1.08	0.44	2.73
40	66.40	90.10	0.76	0.44	2.25
48	65.40	89.40	0.71	0.44	1.86
56	66.90	94.50	0.74	0.46	1.69
64	61.00	92.50	0.70	0.44	1.45
72	60.20	82.70	0.75	0.39	1.15

Note : Ethanol yield ($Y_{P/S}$, g ethanol/g consumed sugar)

Table B-1.10 Data of batch fermentation of ethanol production using SC culture at initial sugar concentration 280 g/l

Time (hour)	Residual sugar concentration (g/l)	Ethanol concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	288.40	0.00	0.98		
8	255.90	5.40	1.23	0.17	0.68
16	208.50	31.50	1.67	0.39	1.97
24	150.90	60.70	1.22	0.44	2.53
32	104.80	77.60	1.08	0.42	2.43
40	74.10	96.50	0.76	0.45	2.41
48	69.80	91.60	0.71	0.42	1.91
56	67.50	93.30	0.74	0.42	1.67
64	65.10	90.50	0.70	0.41	1.41
72	66.20	86.20	0.75	0.39	1.20

Note : Ethanol yield ($Y_{P/S}$, g ethanol/g consumed sugar)

Table B-1.11 Data of ethanol and sugar concentration in repeated batch fermentation using cultures of SC and TSI.

Batch	Time (hour)	Ethanol concentration (g/l)		Sugar concentration (g/l)	
		SC	TSI	SC	TSI
I	0	0.00	0.00	247.60	250.30
	12	18.90	14.00	179.80	208.10
	24	66.90	50.30	80.40	107.70
	36	81.10	55.80	55.10	67.50
	48	88.70	88.10	53.10	59.20
II	0	11.90	13.10	242.10	251.20
	12	14.40	13.20	238.90	180.90
	24	15.50	35.00	138.10	134.60
	36	61.80	65.30	103.70	104.60
	48	65.90	67.90	88.40	81.00
III	0	4.70	12.30	239.90	251.20
	12	20.00	26.80	198.20	172.30
	24	56.90	55.00	127.90	77.50
	36	76.80	84.80	65.10	54.20
	48	79.80	88.30	63.30	53.50
IV	0	6.80	8.50	243.60	249.70
	12	23.20	21.40	206.00	169.80
	24	51.80	48.70	138.00	83.20
	36	67.40	82.80	102.90	58.10
	48	72.30	87.60	81.40	59.70
V	0	14.60	9.00	242.90	249.10
	12	9.90	24.40	231.20	155.40
	24	12.00	65.30	223.70	90.10
	36	9.90	75.00	223.50	66.90
	48	9.40	76.10	215.40	57.80

B-2 Experimental data of continuous fermentation #1

Table B-2.1 Data of continuous performance by every 8 hours harvested the sample

Dilution rate (h^{-1})	Time (hour)	Sugar concentration (g/l)	Ethanol concentration (g/l)	$Y_{p/s}$
0.034	0	241.24	0.00	
0.034	8	114.27	45.18	0.36
0.034	16	72.25	68.73	0.41
0.034	24	62.58	84.17	0.47
0.034	32	61.67	75.78	0.42
0.034	40	62.88	78.78	0.44
0.034	48	68.62	77.91	0.45
0.034	56	68.26	80.08	0.46
0.034	64	58.42	83.19	0.46
0.034	72	63.79	82.26	0.46
0.034	80	65.87	77.21	0.44
0.034	88	65.58	80.85	0.46
0.034	96	67.96	79.93	0.46
0.15	104	182.38	28.24	0.48
0.15	112	138.24	48.12	0.47
0.15	120	127.72	49.21	0.43
0.15	128	117.20	50.69	0.41
0.15	136	113.69	52.00	0.41
0.15	144	117.49	49.55	0.40
0.15	152	115.84	53.23	0.42
0.15	160	111.89	52.90	0.41
0.15	168	113.02	56.22	0.44
0.24	176	175.46	33.21	0.50
0.24	184	155.68	38.03	0.44
0.24	192	154.55	43.68	0.50
0.24	200	145.51	47.61	0.50
0.24	208	146.92	48.37	0.51
0.24	216	144.03	49.44	0.51
0.24	224	149.48	47.90	0.52
0.24	232	147.84	47.82	0.51
0.24	240	148.66	47.68	0.51

Dilution rate (h^{-1})	Time (hour)	Sugar concentration (g/l)	Ethanol concentration (g/l)	$Y_{p/s}$
0.36	248	191.68	22.11	0.45
0.36	256	153.56	39.48	0.45
0.36	264	152.47	42.74	0.48
0.36	272	155.19	44.59	0.52
0.36	280	159.24	42.53	0.52
0.36	288	157.33	43.17	0.51
0.36	296	157.05	41.58	0.49
0.36	304	154.52	43.63	0.50
0.36	312	153.12	45.10	0.51

Table B-2.2 Data of free cells concentration leaving the reactor.

Dilution rate (h^{-1})	Time (hour)	Free cell concentration (g/l)
0.034	8	0.36
0.034	16	0.24
0.034	24	0.24
0.034	32	0.15
0.034	40	0.14
0.034	48	0.13
0.034	56	0.11
0.034	64	0.19
0.034	72	0.23
0.034	80	0.17
0.034	88	0.18
0.034	96	0.14
0.15	104	0.17
0.15	112	0.18
0.15	120	0.25
0.15	128	0.26
0.15	136	0.25
0.15	144	0.26
0.15	152	0.27
0.15	160	0.25
0.15	168	0.24

Dilution rate (h^{-1})	Time (hour)	Free cell concentration (g/l)
0.24	176	0.34
0.24	184	0.38
0.24	192	0.30
0.24	200	0.29
0.24	208	0.26
0.24	216	0.34
0.24	224	0.36
0.24	232	0.36
0.24	240	0.25
0.36	248	0.37
0.36	256	0.31
0.36	264	0.34
0.36	272	0.46
0.36	280	0.50
0.36	288	0.49
0.36	296	0.55
0.36	304	0.46
0.36	312	0.47

Table B-2.3 Experimental data of ethanol productivity in packed bed reactor of TSI carrier with dilution rate of 0.034, 0.15, 0.24 and 0.36 h^{-1}.

Dilution rate (h^{-1})	Ethanol concentration (g/l)	Productivity (g/l)
0.034	80.03	2.72
0.15	54.12	8.12
0.24	48.24	11.58
0.36	43.76	15.60

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

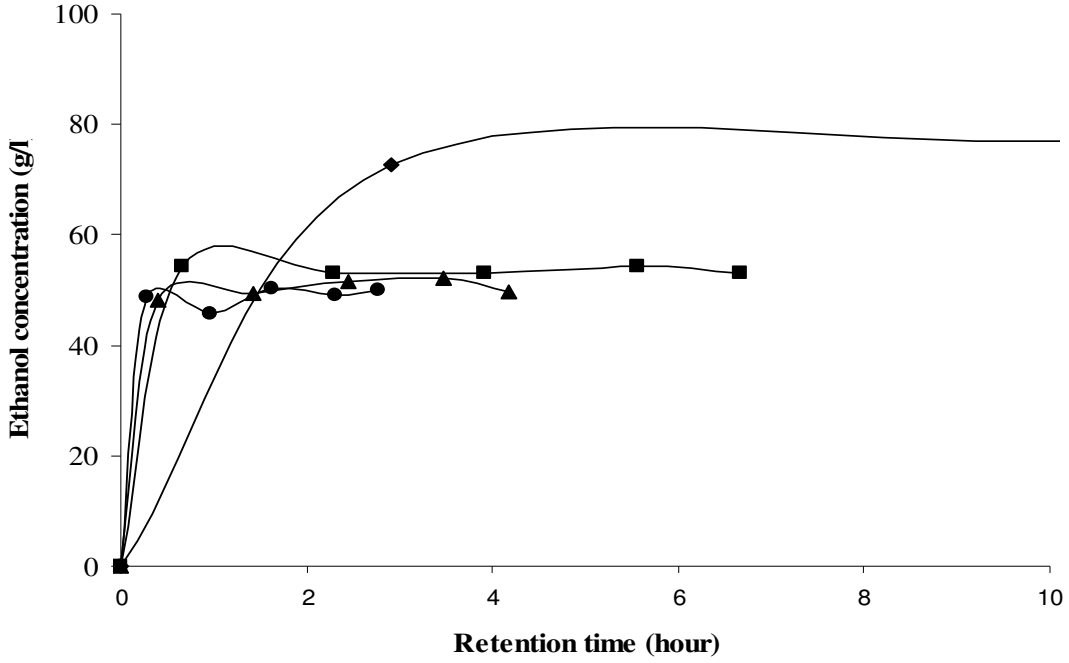


Figure B-2.1 The steady state ethanol concentration of 4 dilution rates with initial sugar concentration of 241 g/l (—♦— = 0.034h^{-1}, —■— = 0.15 h^{-1}, —▲— = 0.24 h^{-1} and —●— = 0.36 h^{-1})

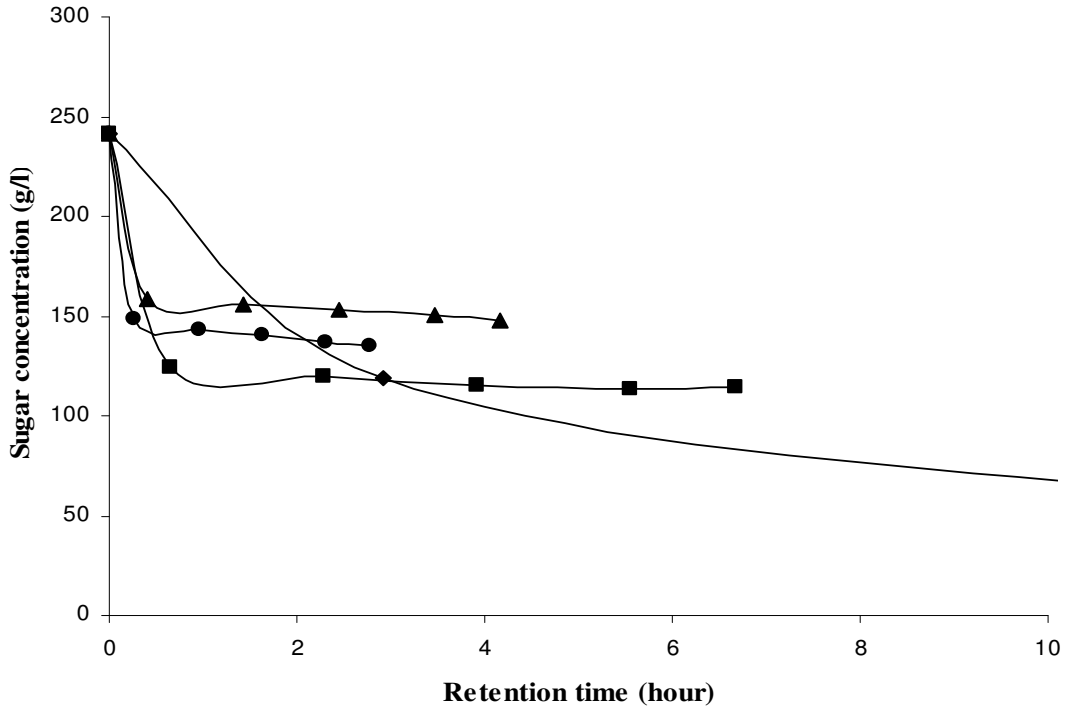


Figure B-2.2 The steady state residue sugar concentration of 4 dilution rates with initial sugar concentration of 241 g/l (—♦— = 0.034h^{-1}, —■— = 0.15 h^{-1}, —▲— =0.24 h^{-1} and —●— = 0.36 h^{-1})

Table B-2.4 The steady state ethanol fermentation of 4 dilution rates with the initial sugar concentration 241 g/l.

Dilution rate (h^{-1})	Retention time (hour)	Ethanol concentration (g/l)	Sugar concentration (g/l)	Productivity (g/l h)
0.034	0.00	0.00	241.24	
0.034	2.91	72.64	119.33	24.96
0.034	10.12	77.04	67.76	7.61
0.034	17.29	78.68	65.38	4.55
0.034	24.49	80.29	63.49	3.28
0.034	29.41	82.05	65.67	2.79
0.15	0.00	0.00	241.24	
0.15	0.66	54.28	124.41	82.24
0.15	2.29	52.97	119.80	23.13
0.15	3.92	53.03	115.56	13.53
0.15	5.56	54.33	113.77	9.77
0.15	6.67	53.00	114.15	7.95
0.24	0.00	0.00	241.24	
0.24	0.41	48.13	158.98	117.39
0.24	1.43	49.52	155.59	34.63
0.24	2.45	51.43	153.04	20.99
0.24	3.47	52.28	150.12	15.07
0.24	4.17	49.81	147.30	11.95
0.36	0.00	0.00	241.24	
0.36	0.28	48.62	148.28	176.80
0.36	0.96	45.79	143.23	47.92
0.36	1.63	50.38	140.51	30.84
0.36	2.31	49.06	136.96	21.20
0.36	2.78	49.93	134.90	17.97

B-3 Experimental data of continuous fermentation #2

Table B-3.1 Data of continuous performance by every 8 hours harvested the sample

Dilution rate (h^{-1})	Time (hour)	Sugar concentration (g/l)	Ethanol concentration (g/l)	$Y_{p/s}$
0.034	0	222.48	0.00	
0.034	8	108.54	48.60	0.43
0.034	16	74.07	52.82	0.36
0.034	24	50.71	57.82	0.34
0.034	32	53.27	68.34	0.40
0.034	40	51.56	69.12	0.40
0.034	48	56.97	72.88	0.44
0.034	56	51.56	74.94	0.44
0.034	64	58.96	77.78	0.48
0.034	72	58.67	78.35	0.48
0.034	80	56.65	85.85	0.52
0.034	88	54.33	84.34	0.50
0.034	96	67.05	77.26	0.50
0.15	104	151.15	34.76	0.49
0.15	112	127.74	42.68	0.45
0.15	120	103.47	53.00	0.45
0.15	128	110.86	57.23	0.51
0.15	136	102.54	62.56	0.52
0.15	144	103.80	61.10	0.51
0.15	152	101.65	62.50	0.52
0.15	160	101.96	60.67	0.50
0.15	168	101.65	61.78	0.51
0.24	176	147.72	34.45	0.46
0.24	184	136.97	41.73	0.49
0.24	192	119.77	47.64	0.46
0.24	200	110.70	56.82	0.51
0.24	208	113.07	57.13	0.52
0.24	216	105.74	60.86	0.52
0.24	224	109.52	57.59	0.51
0.24	232	112.18	57.27	0.52
0.24	240	106.62	59.03	0.51

Dilution rate (h^{-1})	Time (hour)	Sugar concentration (g/l)	Ethanol concentration (g/l)	$Y_{p/s}$
0.36	248	132.90	39.65	0.44
0.36	256	137.93	42.82	0.51
0.36	264	133.49	41.86	0.47
0.36	272	116.32	52.77	0.50
0.36	280	119.69	53.40	0.52
0.36	288	118.48	51.32	0.49
0.36	296	118.17	52.35	0.50
0.36	304	114.23	53.84	0.50
0.36	312	114.84	53.32	0.50
0.034	320	128.78	42.83	0.46
0.034	328	51.21	68.84	0.40
0.034	336	56.06	79.97	0.48
0.034	344	56.06	81.14	0.49
0.034	352	55.75	80.09	0.48
0.034	360	56.06	81.11	0.49
0.034	368	56.06	80.70	0.48
0.034	376	58.48	84.22	0.51
0.034	384	58.18	77.23	0.47

Table B-3.2 Data of free cells concentration leaving the reactor.

Dilution rate (h^{-1})	Time (hour)	Free cell concentration (g/l)
0.034	0	0.35
0.034	8	0.27
0.034	16	0.15
0.034	24	0.21
0.034	32	0.17
0.034	40	0.18
0.034	48	0.14
0.034	56	0.28
0.034	64	0.19
0.034	72	0.22
0.034	80	0.23
0.034	88	0.19
0.034	96	0.22

Dilution rate (h^{-1})	Time (hour)	Free cell concentration (g/l)
0.15	104	0.18
0.15	112	0.22
0.15	120	0.20
0.15	128	0.27
0.15	136	0.24
0.15	144	0.28
0.15	152	0.31
0.15	160	0.27
0.15	168	0.27
0.24	176	0.29
0.24	184	0.34
0.24	192	0.31
0.24	200	0.34
0.24	208	0.38
0.24	216	0.39
0.24	224	0.35
0.24	232	0.32
0.24	240	0.38
0.36	248	0.39
0.36	256	0.35
0.36	264	0.30
0.36	272	0.47
0.36	280	0.52
0.36	288	0.50
0.36	296	0.51
0.36	304	0.50
0.36	312	0.58
0.034	320	0.50
0.034	328	0.49
0.034	336	0.43
0.034	344	0.42
0.034	352	0.44

Dilution rate (h^{-1})	Time (hour)	Free cell concentration (g/l)
0.034	360	0.49
0.034	368	0.44
0.034	376	0.42
0.034	384	0.44

Table B-3.3 Experimental data of ethanol productivity in packed bed reactor of TSI carrier with dilution rate of 0.034, 0.15, 0.24 and 0.36 h^{-1}

Dilution rate (h^{-1})	Ethanol concentration (g/l)	Productivity (g/l)
0.034	80.72	2.74
0.15	61.72	9.26
0.24	58.12	13.95
0.36	52.83	19.02

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

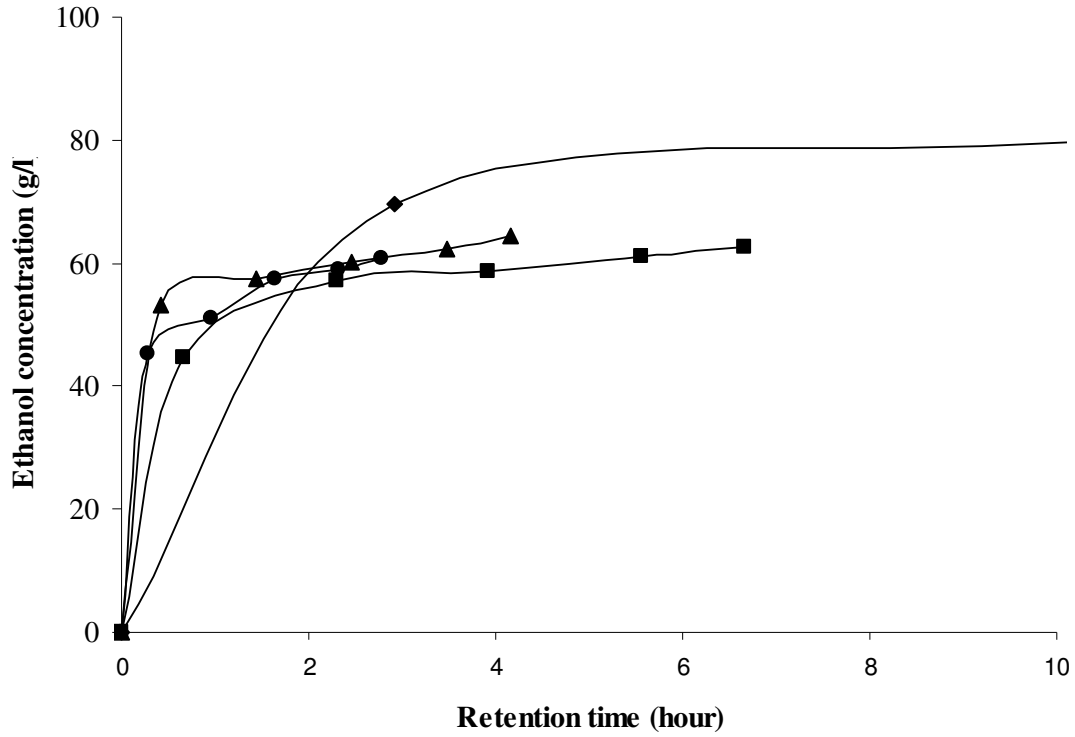


Figure B-3.1 The steady state ethanol concentration of 4 dilution rates with initial sugar concentration of 222 g/l (–♦– = 0.034h^{-1}, –■– = 0.15 h^{-1}, –▲– =0.24 h^{-1} and –●– = 0.36 h^{-1})

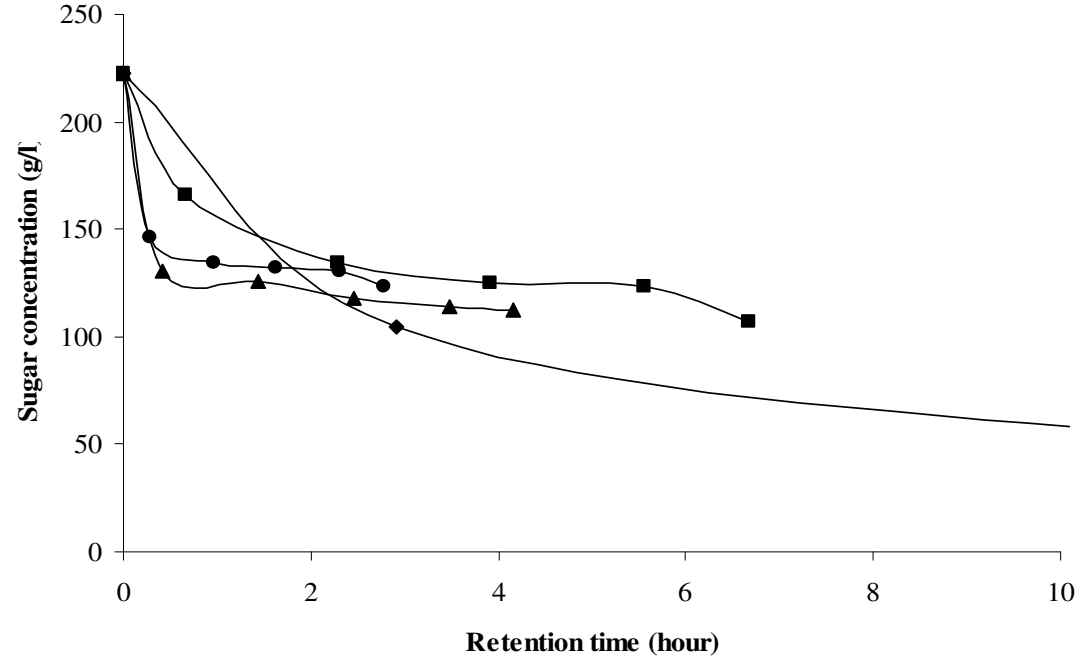


Figure B-3.2 The steady state residue sugar concentration of 4 dilution rates with initial sugar concentration of 222 g/l (–♦– = 0.034h^{-1}, –■– = 0.15 h^{-1}, –▲– =0.24 h^{-1} and –●– = 0.36 h^{-1})

Table B-3.4 The steady state ethanol fermentation of 4 dilution rates with the initial sugar concentration 222 g/l.

Dilution rate (h^{-1})	Retention time (hour)	Ethanol concentration (g/l)	Sugar concentration (g/l)	Productivity (g/l h)
0.034	0.00	0.00	222.48	
0.034	2.91	69.62	104.17	23.92
0.034	10.12	79.68	58.30	7.87
0.034	17.29	85.51	57.45	4.95
0.034	24.49	87.12	56.69	3.56
0.034	29.41	90.05	53.65	3.06
0.15	0.00	0.00	222.48	
0.15	0.66	44.55	166.25	67.50
0.15	2.29	57.07	134.21	24.92
0.15	3.92	58.68	124.79	14.97
0.15	5.56	61.13	123.25	10.99
0.15	6.67	62.61	106.77	9.39
0.24	0.00	0.00	222.48	
0.24	0.41	53.09	130.53	129.49
0.24	1.43	57.40	125.99	40.14
0.24	2.45	60.18	117.90	24.56
0.24	3.47	62.20	113.86	17.93
0.24	4.17	64.56	112.77	15.49
0.36	0.00	0.00	222.48	
0.36	0.28	45.29	145.95	164.69
0.36	0.96	51.02	134.64	53.39
0.36	1.63	57.38	132.21	35.13
0.36	2.31	58.88	130.70	25.45
0.36	2.78	60.92	123.73	21.93

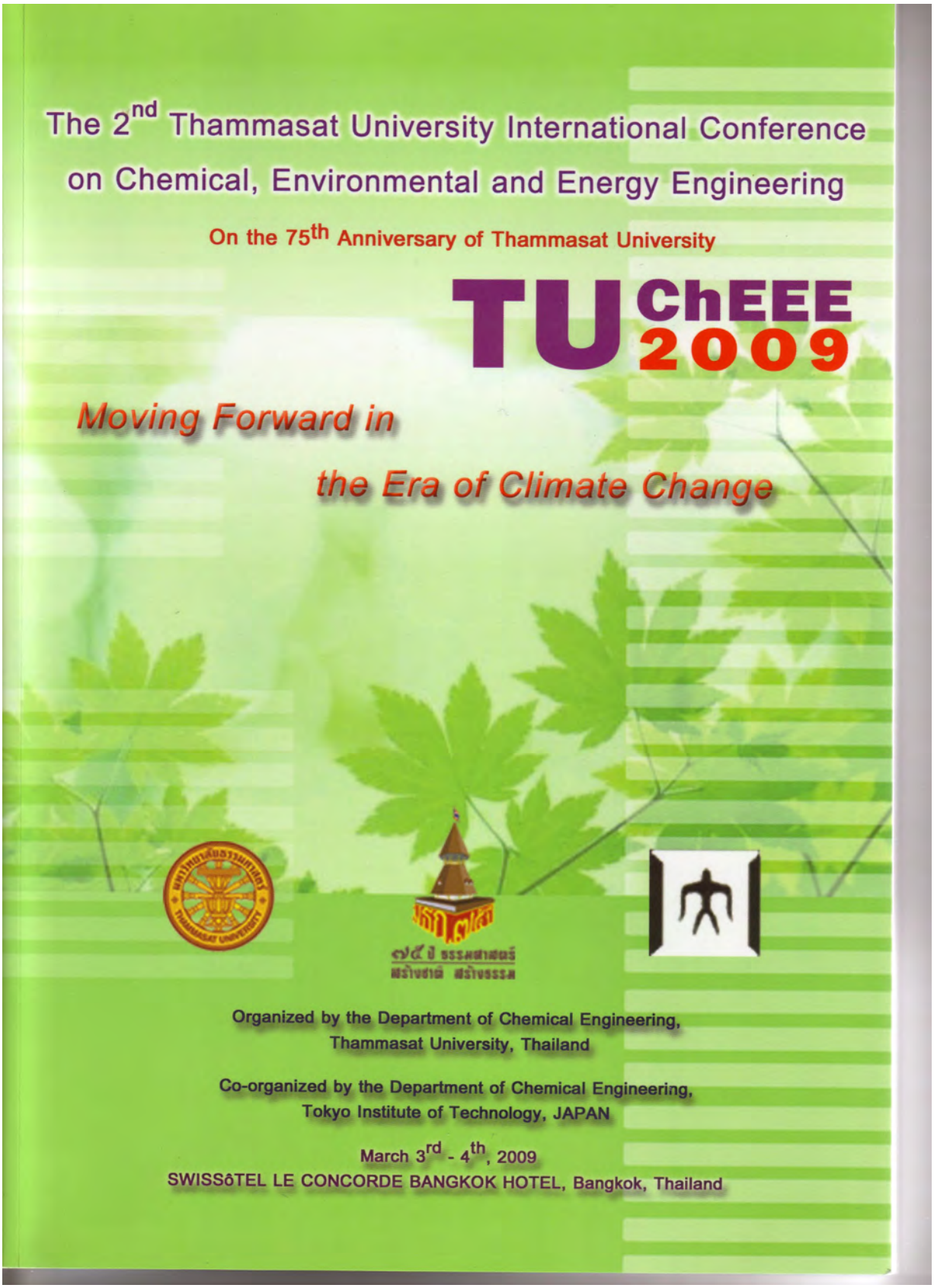
APPENDIX C
LIST OF PUBLICATION

International conference

1. Anuchit Rattanapan and Muenduen Phisalaphong, "Yeast Immobilization Using Thin Shell Silk Cocoon" , Proceeding for The 2nd Thammasat University International Conference on Chemical, Environmental and Energy Engineering, Thailand, 3 - 4 March 2009, Paper ID ChE-060.

Thai Patent

1. Muenduen Phisalaphong and Anuchit Rattanapan, "A process for ethanol fermentation with immobilized yeasts using thin shell silk cocoon", (Submitted by Chulalongkorn University Intellectual Property Institute)


The 2nd Thammasat University International Conference
on Chemical, Environmental and Energy Engineering
On the 75th Anniversary of Thammasat University
TU ChEEE 2009
Moving Forward in
the Era of Climate Change
Organized by the Department of Chemical Engineering,
Thammasat University, Thailand
Co-organized by the Department of Chemical Engineering,
Tokyo Institute of Technology, JAPAN
March 3rd - 4th, 2009
SWISSôTEL LE CONCORDE BANGKOK HOTEL, Bangkok, Thailand

Time	Paper No.	March 3, 2009 - Energy Technology and Management (Biodeisel and Ethanol) – SAKTHONG			
		Article Title	Authors	Affiliation	Country
14:15-14:30	ChE-090	Utilization of Glycerol as a Fuel of Extender of Etherified with FCC Gasoline	S. Suwanmanee, W. Kiatkittipong and S. Assabumrungrat	Center of Excellence in catalysis and Catalytic Reaction Engineering, Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok	Thailand
14:40 -15:00		Coffee Break			
15:00-15:15	ChE-064	Glycerin Reforming using Atmospheric Pressure Microwave Plasma	Sang Ryun Kim, Hidetoshi Sekiguchi	Department of Chemical Engineering, Faculty of Engineering, Tokyo Institute of Technology	Japan
15:15-15:30	ChE-094	The Possibility of the Useful Chemicals Production from Sewage Sludge Derived Oil	Ratanaporn Yuangsawad, Walairat Suksamai and Duangkamol Na-Ranong	Department of Chemical Engineering, Faculty of Engineering, King Mongkut's Institute of Technology Ladkrabang, Bangkok	Thailand
15:30-15:45	ChE-074	Development of Alumina Doped Alginate Gel as a Cell Carrier for Ethanol Fermentation	J.Mongkolkajit and M.Phisalaphong	Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok	Thailand
15:45-16:00	ChE-060	Yeast Immobilization Using Thin Shell Silk Cocoon	A. Rattanapan and M.Phisalaphong	Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok	Thailand
16:00-16:15	ENM-086	The Effect of Processing Conditions of Bagasse Biomass on Its Characterization	Patcharin Worathanakul, Yananthorn Thongchai, Khemakorn Niruttimaytee, Wisaroot Payubnop and Akhapon Muangpet	Department of Chemical Engineering, Faculty of Engineering, King Mongkut's University of Technology North Bangkok, Bangsue, Bangkok	Thailand

The 2nd Thammasat University International Conference
on Chemical, Environmental and Energy Engineering
TU ChEEE 2009

Yeast Immobilization Using Thin Shell Silk Cocoon

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Abstract

A natural organic material, thin shell silk cocoon (TSSC) was tested as a cell carrier in fermentation process for ethanol production. TSSC exhibited many advantageous characteristics. It was cheap, simple to use, non-toxic, high porosity, chemically stable and had good mechanical strength for long-term use. From the evaluation in a 5-cycle repeated batch system, thin shell silk cocoon immobilized cell (TSI) culture demonstrated a good potential for reusability. Ethanol fermentation by the TSI cultures was proven to be stable than that using the suspended cell cultures.

Keywords: silk cocoon, yeast, immobilization, ethanol production

1. Introduction

According to the diminishing of crude oil and the increasing of fuel cost in recent years, ethanol has re-emerged as an alternative to or extender for petrochemical based liquid fuels. Ethanol is renewable and clean fuel produced by biomass fermentation process. Its high octane and high heat of vaporization make alcohol more efficient as a pure liquid fuel. The Thai government has announced that it plans to double the amount of ethanol used in petroleum in order to curb Thailand imports of oil. In addition, the plan to promote ethanol-based fuel is of great importance in helping to stabilize agricultural product prices, raise farmers' income and reducing petrol- induced air pollution. Nowadays, gasohol E 10, a mixture of 10% ethanol and 90% gasoline has been widely used in vehicles and there is an attempt to promote the use of E20 or E85 in the vehicles in the near future. Beside that, ethanol is used as solvent and chemical feedstock in various industries. Therefore, the demand of ethanol has increased rapidly.

In the past, most of fuel ethanol was produced from petrochemical process, whereas, currently this process is considered as a very high invested process and it is replaced by biomass fermentation process. The biomass fermentation process can be used many types of agricultural feedstock such as molasses, tapioca, sugar cane, sugar beet, corn, sorghum etc. Traditional ethanol industries produce ethanol by batch or fed-batch process. However, for large quantities of ethanol, high production rate is achieved in a continuous process. The use of immobilization cells has been suggested as an effective means for improved continuous ethanol fermentation [6]. The immobilization of cells leads to protection of cells from inhibitions, maintaining of high cell densities with consequent increase in reaction rates and enzyme productivities. As a result, shorter residence time and smaller reactor size could be employed [6].

In this study, thin shell silk cocoon (TSSC), a residual agricultural material, was selected to be an immobilized material due to many advantages such as bio-degradability, light, but strong structure, low cost, chemical stability, high porosity and high surface area. The possibility of using TSSC as cell carries for yeast immobilization in ethanol fermentation was investigated. The fermentation experiments were carried out in repeated batch mode in a shake flask using *Saccharomyces cerevisiae M30* as a cell culture. Ethanol production using yeast cells immobilized with TSSC was compared with

that using suspended cells. The information gained from the study will be useful for the development of high performance cell carrier for ethanol production.

2. Experimental

2.1 Microorganism and culture media

The flocculate yeast, strain *Saccharomyces cerevisiae M30* provided by the laboratory of Dr. Savithree Limthong (Department of Microbiology, Kasetsart University, Bangkok) was used in this study. The cultivation medium was composed of 100 g/l sugar from palm sugar, 0.5 g/l $(NH_4)_2SO_4$, 0.1 g/l KH_2PO_4, and 0.035 g/l $MgSO_4.7H_2O$ at pH 5. The medium was sterilized in autoclave for 15 minutes at 121°C. The stock cell suspension was prepared by transferring cells from a PDA agar slant into 500 ml Erlenmeyer flask containing 150 ml sterilized cultivation medium. Cell cultivation was carried in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33 °C for 24 hours.

2.2 Cells immobilized on thin shell silk cocoon

The thin shell silk cocoons of 2.5 g and the 250 ml of culture medium in 500 ml flasks were separately autoclaved for 15 minutes at 121°C. Stock cell suspension of 10 ml was then added into the prepared culture medium. After that the thin shell silk cocoons were added into the culture broth. The immobilized cells were obtained after the cultivation was carried at 150 rpm, 33 °C for 24 hours.

2.3 Fermentations

A 5-cycle repeated batch fermentation experiment was carried out in duplicate using cane molasses as the carbon source. The experiment was initiated by transferring prepared cell suspension or immobilized cells into 250 ml of culture medium containing 24% (w/v) of initial reducing sugar and 0.5 g/l $(NH_4)_2SO_4$ in 500 ml Erlenmeyer flask. The flasks were shaken in the incubator at 150 rpm, 33 °C. The duration of each batch was 48 hr. The experiment was monitored by removing 2 ml samples every eight hours. The samples were frozen before the analysis of sugar, ethanol and cell concentration in order to enable all samples to be analyzed at the same time.

2.4 Analytical methods

Modified 3,5-dinitrosalicylic acid (DNS) reagent method was used to determined sugar concentration. Briefly, the sample was hydrolyzed with HCl 370 g/l in boiling water bath for 10 minutes. Then, the sample was neutralized with NaOH 300 g/l. The sample was centrifuged and the supernatant was reacted with DNS reagent before the color intensity was measured by UV-visible spectrophotometer at 520 nm.

Ethanol assay was conducted by gas chromatography using a Shimadzu Model GC 7AG equipped with Flame Ionization Detector (FID). A column with length 2 m, outer diameter of 3.3 mm, and packed with Porapak Q 80-100 mesh was used in collaboration with N_2 as carrier gas. Flow rate of N_2 was 50 ml/min. The oven and detector temperature were 190 °C and 240 °C, respectively. The samples were injected with volume of 1 µL with the injection temperature of 240 °C.

Cell concentration was determined by cell dry weight method. The sample was washed with HCL 0.1 N and water respectively. The cell concentration was measured by UV-visible spectrophotometer at 660 nm for determined free cell leakage concentration. To determine the immobilized cell concentration, the carrier was cut into small pieces and stirred in DI water for 1 hour. Then, the carrier was removed and the suspension was treated similarly as fermentation broth to obtain its corresponding immobilized cell concentration. Drying of all cells was performed in oven at 90 °C for 2 hours. At the beginning and the end of fermentation, samples of carrier were collected for SEM.

3. Results and Discussions

Ethanol production using TSSC as a carrier for *Saccharomyces cerevisiae M30* was evaluated by a 5-cycle repeated batch fermentation using 240 g/l of cane molasses as the carbon source. The duration of each batch was 48 h. For the comparison, two cell cultures were used for ethanol fermentation: suspended cells (SC) and thin shell silk cocoon-immobilized cells (TSI). Figure 3.1 and Table 3.1 show the results of the repeated batch

fermentation. In the first repeated batch, the ethanol concentration obtained at 48 hours for SC and TSI cultures were 88.8 g/l ($Y_{P/S}$ 47%)and 88.1 g/l ($Y_{P/S}$ 49%), respectively and the residual sugar concentration were at 53.1 and 59.2 g/l, respectively. The final ethanol concentration in the suspended cell and the immobilized cell cultures were comparable in the 2nd, 3rd and the 4th batch.

A thin shell silk cocoon was compatible for yeast immobilization. After the 5th batch, the final total cell concentration of system using TSI culture (14.7 g/l) was considerably higher than that of SC (7.4 g/l). The increase of the cell concentration in TSI carrier indicated the growth of immobilized cells in the carriers during the fermentation. Moreover, the cell carrier exhibited the high immobilization yield (Y_T) of 91% and the higher stability of the TSI cultures over the SC cultures was observed, especially in the 5th batch. The ethanol concentration obtained at 48 hours of the 5th batch using SC and TSI cultures were at 9.4 g/l ($Y_{P/S}$ 38%) and 76.1 g/l ($Y_{P/S}$ 42%), respectively and the final residual sugar concentrations were at 215.4 and 57.8 respectively. The negative effect of high ethanol and sugar concentration on SC activities has been previously reported [3]. The higher stability of TSI cultures implied that the TSI carriers could protect yeast cells from toxins and/or inhibitor during the fermentation process.

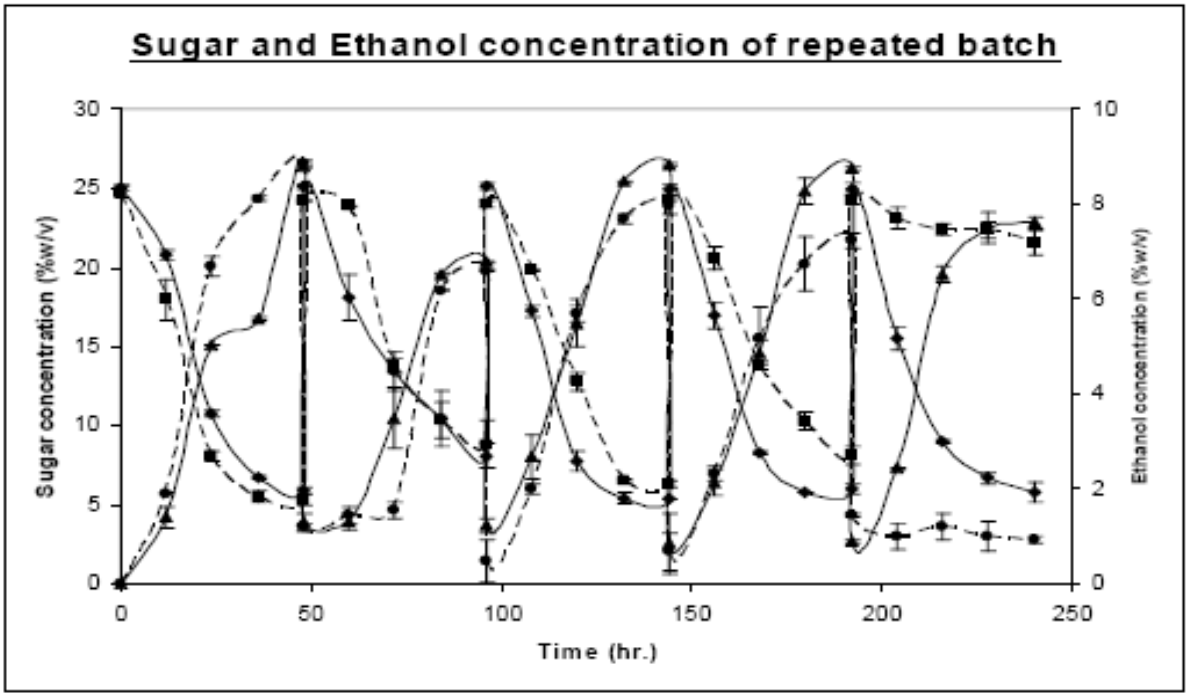
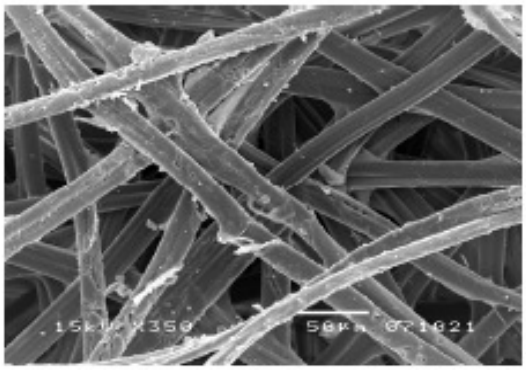


Figure 3.1 Ethanol and sugar concentration profiles in the repeated batch fermentation by *Saccharomyces cerevisiae M30* at 240 g/l of initial sugar concentration:
-- ■ -- = SC-sugar; -- ● -- = SC-ethanol; • ◆ • = TSI-sugar; • • • • = TSI-ethanol

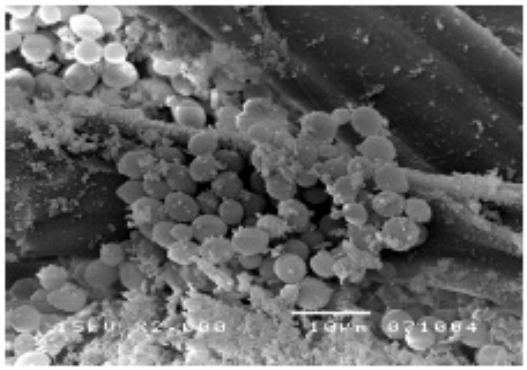
Table 3.1: Yields and end products of repeated batch ethanol fermentation for 48 h for each batch using the cultures of suspended cell (SC) culture, thin shell silk cocoon immobilized cells (TSI) culture.

Batch	P (g/l)	X (g/l)		Y_I (g/g)	Y_S (g/g)	$Y_{P/S}$ (g/g)
		X_F	X_I			
I						
SC	88.75	n	n	n	0.78	0.47
TSI	88.10	n	n	n	0.75	0.49
II						
SC	65.87	n	n	n	0.63	0.43
TSI	67.92	n	n	n	0.66	0.43
III						
SC	79.77	n	n	n	0.73	0.45
TSI	88.27	n	n	n	0.78	0.47
IV						
SC	72.30	n	n	n	0.66	0.46
TSI	87.59	n	n	n	0.75	0.49
V						
SC	9.40	7.35	n	n	0.10	0.38
TSI	76.12	1.37	13.36	0.91	0.76	0.42

Ethanol concentration (P); Free cell concentration (X_F); Immobilized cell concentration (X_I); Immobilization yield (Y_I, g immobilized cell/g total cell); Sugar consumption yield (Y_S, g consumed sugar /g total sugar); Ethanol yield ($Y_{P/S}$, g ethanol/g consumed sugar); not detected (n)

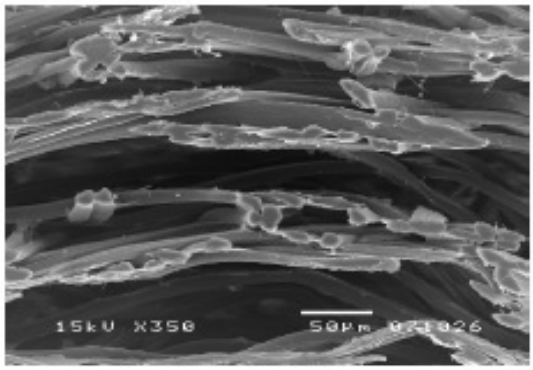


(A)

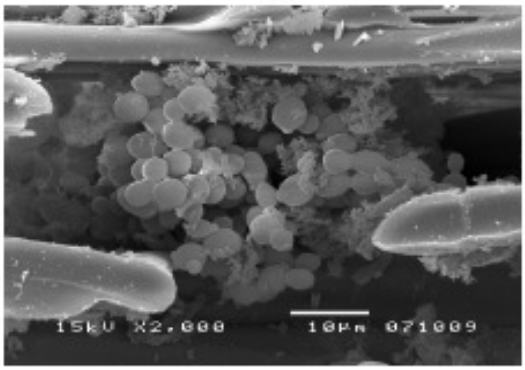


(B)

Figure 3.2 Outer surfaces of TSI carriers at (A) the initial stage and (B) after the 5th batch.



(C)



(D)

Figure 3.3 Cross section of TSI carriers at (C) the initial stage and (D) after the 5th batch.

The comparison of SEM images of TSI carriers at the initial stage and after the 5th batch demonstrates that the immobilized yeast cells were attached to outer surface of shell silk cocoon (Figure 3.2) and also accessed and grew well in the space of its matrix (Figure 3.3). Therefore, the porous structure of the carrier provided suitable living environment for yeast cells without severe mass transfer problem.

4. Conclusion

The thin shell silk cocoon (TSSC) was successfully applied for yeast immobilization in ethanol fermentation using cane molasses as the carbon source. The experimental result showed that TSSC could be used as a cell carrier with favorable mechanical properties and porous structure resulting in a stable operation, high ethanol production and high density of biomass. From the evaluation in the 5-cycle repeated batch ethanol fermentation, the TSI carrier has a good potential of reusability. The ethanol production from the TSI cultures was more stable than that of the SC cultures.

5. Acknowledgement

The authors are grateful for the financial support of the Thailand Research Fund/Chulalongkorn University; contract grant number: RSA5080011 and the support of analytical equipments from the Thailand-Japan Technology Transfer Project (TJTTP-OECF) and S. Limthong for provision of cell cultures.

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