การผลิตเอธานอลแบบต่อเนื่องโดยใช้เซลล์ยีสต์ที่ถูกตรึงในตัวพยุงแอลจีเนทเสริมใยบวบ

นาย ภูวิศ บางรักษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรมหาบัณฑิต
สาขาวิชาวิศวกรรมเคมี ภาควิชาวิศวกรรมเคมี
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ปีการศึกษา 2550

CONTINUOUS ETHANOL PRODUCTION USING IMMOBILIZED YEAST CELLS ENTRAPPED IN LOOFA REINFORCED ALGINATE CARRIERS

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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 2007

Thesis Title	CONTINUOUS ETHANOL PRODUCTION USING IMMOBILIZED YEAST CELLS ENTRAPPED IN LOOFA REINFORCED ALGINATE CARRIERS
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Field of Study	Chemical Engineering
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ภูวิศ บางรักษ์: การผลิตเอธานอลแบบต่อเนื่อง โดยใช้เซลล์ยีสต์ที่ถูกตรึงในตัวพยุงแอลจีเนทเสริมใยบวบ (CONTINUOUS ETHANOL PRODUCTION USING IMMOBILIZED YEAST CELLS ENTRAPPED IN LOOFA REINFORCED ALGINATE CARRIERS) อ. ที่ปรึกษา : รศ. ดร. เหมือนเดือน พิศาลพงศ์, 106 หน้า.

เพื่อช่วยแก้ปัญหาวิกฤตการณ์ด้านพลังงาน เอธานอลเป็นทางเลือกใหม่เพื่อเป็นเชื้อเพลิงทดแทนน้ำมันปิโตรเลียม โดยเมื่อพิจารณากระบวนการหมักเอธานอลแบบต่อเนื่องโดยใช้เซลล์ยีสต์ที่ถูกตรึงบนตัวพยุงจะให้ผลดีในหลายๆด้าน เช่น ให้ผลได้ผลิตภัณฑ์ที่เพิ่มขึ้น, ง่ายต่อกระบวนการแยกสารผลิตภัณฑ์, สามารถนำตัวเร่งปฏิกิริยา (เซลล์ยีสต์) กลับมาใช้ใหม่ และให้อัตราการผลิตเอธานอลที่สูงขึ้น ดังนั้นในงานวิจัยนี้จึงได้ศึกษาการหมักเอธานอลแบบต่อเนื่องโดยใช้เซลล์ยีสต์ที่ถูกตรึงในแอลจีเนทเสริมใยบวบ การทดลองได้ทำการเปรียบเทียบขนาดของตัวพยุงแอลจีเนทเสริมใยบวบ 9 x 9 x 3 ลูกบาศก์มิลลิเมตร และ 20 x 20 x 3 ลูกบาศก์มิลลิเมตร ต่อผลของอัตราการผลิตเอธานอล โดยทำการหมักเอธานอลแบบกะในระบบขวดเขย่าขนาด 500 มิลลิลิตรที่อัตราการหมุนรอบ 150 รอบต่อนาที (rpm) และควบคุมอุณหภูมิหมักที่ 33 องศาเซลเซียส โดยใช้ความเข้มข้นของน้ำตาลเริ่มต้นที่ 220 กรัมต่อลิตร จากการทดลองพบว่าขนาดของตัวพยุงแอลจีเนทเสริมใยบวบทั้งสองขนาดไม่ส่งผลให้กิจกรรมของเซลล์แตกต่างกัน สำหรับการหมักเอธานอลแบบต่อเนื่องได้ใช้ถังปฏิกรณ์แบบมีการบรรจุวัสดุตรึงไว้ภายใน และควบคุมอุณหภูมิไว้ที่ 32 ± 1 องศาเซลเซียส โดยศึกษาผลของความเข้มข้นน้ำตาลเริ่มต้นที่ 202, 222 และ 248 กรัมต่อลิตร และอัตราการเจือจางในถังปฏิกรณ์ที่ 0.11, 0.16, 0.20 และ 0.30 ต่อชั่วโมง โดยพบว่าได้อัตราการผลิตเอธานอลสูงสุดที่ 10.57 กรัมต่อลิตรต่อชั่วโมง เมื่อดำเนินการที่ความเข้มข้นของน้ำตาลเริ่มต้น 222 กรัมต่อลิตร และที่อัตราการเจือจางในถังปฏิกรณ์ 0.16 ต่อชั่วโมงโดยได้ความเข้มข้นของเอธานอล 66.06 กรัมต่อลิตร ได้ค่าความเข้มข้นของเอธานอลสูงสุดที่ 81.29 กรัมต่อลิตรเมื่อดำเนินการที่ความเข้มข้นของน้ำตาลเริ่มต้น 222 กรัมต่อลิตรที่อัตราการเจือจางในถังปฏิกรณ์ 0.11 ต่อชั่วโมง จากผลการทดลองแสดงให้เห็นว่าแอลจีเนทเสริมใยบวบเป็นวัสดุตรึงที่มีประสิทธิภาพสูงสำหรับการนำมาใช้ตรึงเซลล์ยีสต์ในถังปฏิกรณ์แบบแพคเบดสำหรับการหมักเอธานอลแบบต่อเนื่อง เนื่องด้วยวัสดุตรึงเซลล์ที่ได้พัฒนาขึ้นมีคุณสมบัติเชิงกลที่ดีและมีโครงสร้างที่มีรูพรุนสูง ทำให้สามารถนำมาใช้กับกระบวนการผลิตเอธานอลแบบต่อเนื่องอย่างน้อย 30 วันที่ให้อัตราการผลิตเอธานอลสูงโดยไม่ทำให้เสถียรภาพของระบบเปลี่ยนแปลง สืบเนื่องจากผลการทดลองจึงควรมีการทดสอบการผลิตในขนาดที่ใหญ่ขึ้นเพื่อใช้เป็นข้อมูลในการพัฒนาสู่การผลิตในระดับอุตสาหกรรมต่อไป

ภาควิชา	วิศวกรรมเคมี	ลายมือชื่อนิสิต _____
สาขาวิชา	วิศวกรรมเคมี	ลายมือชื่ออาจารย์ที่ปรึกษา _____
ปีการศึกษา	2550	

4970507321: MAJOR CHEMICAL ENGINEERING
KEY WORD : IMMOBILIZED CELL/ LOOFA SPONGE / ALGINATE / PACKED-BED REACTOR / ETHANOL / FERMENTATION / *SACCHAROMYCES CEREVISIAE*
PHOOWIT BANGRAK: CONTINUOUS ETHANOL PRODUCTION USING IMMOBILIZED YEAST CELLS ENTRAPPED IN LOOFA REINFORCED ALGINATE CARRIERS. THESIS ADVISOR: ASSOC. PROF. MUENDUEN PHISALAPHONG, Ph.D., 106 pages.

In response the energy crisis, ethanol has re-emerged as an alternative to, or extender for petroleum based liquid fuels. Continuous fermentation using immobilized cell carriers offers many advantages such as higher conversion, relative ease of product separation, reuse of biocatalyst and high productivity. Therefore, in this study continuous ethanol fermentation using immobilized yeast cells (*Saccharomyces cerevisiae* M30) entrapped in loofa reinforced alginate was investigated. To compare productivity of alginate-loofa at sizes of 9 x 9 x 3 mm^3 and 20 x 20 x 3 mm^3, the batch fermentation was carried out in 500 ml Erlenmeyer flask at shaking frequency of 150 rpm and temperature of 33 °C using initial sugar concentration of 220 g/l. It was found that there were no significant differences in cell activity regarding the change of the carrier size. The continuous ethanol fermentation was studied in packed-bed reactor with various initial sugar concentrations (202, 222 and 248 g/l) and dilution rates (0.11, 0.16, 0.20 and 0.30 h^{-1}) of 32 ± 1 °C. At 222 g/l of initial sugar concentration and 0.16 h^{-1} of the dilution rate, the optimum of productivity was obtained (10.57 g/l h) with the ethanol concentration of 66.06 g/l. The maximum of ethanol concentration (81.29 g/l) was obtained at 222 g/l of initial sugar concentration and 0.11 h^{-1} of the dilution rate. The experimental result revealed that the alginate-loofa matrix was successfully used as a cell carrier in packed bed column for continuous ethanol fermentation. With favorable mechanical properties and high porous structure of the developed carrier, a fairly stable operation and high ethanol production over the course of 30 days were achieved. Based on the results of this work, subsequent studies especially in larger scale is recommended to ameliorate for industrial production.

Department	Chemical Engineering	Student's Signature
Field of Study	Chemical Engineering	Advisor's Signature
Academic Year	2007	

ACKNOWLEDGEMENTS

I would like to express my earnest gratitude to my advisor, Assoc. Prof. Muenduen Phisalaphong 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 (Department of Microbiology, Kasetsart University, Bangkok) for her kind and most gratified support to this thesis work by providing the *Saccharomyces cerevisiae* M30 strain.

Thanks to all of my thesis committee, Assoc. Prof. Chirakarn Muangnapoh, Dr. Jirdsak Tscheikuna, and Assoc. Prof. Suwimol Asavapisit 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).

Financial supports from the Department of Chemical Engineering, Chulalongkorn University for providing the partial scholarship for my master study is highly appreciated.

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

Ethanol is a natural component of alcoholic beverages and its use has been continued growth since the late 1970s when it has been applied as a product extender for petroleum fuel due to gasoline shortages. Production of ethanol from renewable carbohydrate materials has been attracting worldwide interest and many researches were directed to the production of ethanol by immobilized microorganisms using continuous culture.

Continuous fermentation offers important advantages, such as higher conversion rates, faster fermentation rates, improved product consistency, reduced product losses and environmental advantages. An important aspect of continuous fermentation is the high volumetric efficiency, which is usually obtained by increased yeast cell concentrations in the reactor compared to traditional batch systems. Immobilizing yeast cells on several support types can provide high cell densities in the bioreactor, which, in combination with high flow rates, leads to short residence times. These economic benefits are the driving force for a global research effort aimed at studying and implementing continuous fermentors with immobilized cells [1].

The immobilized cell system offers many advantages over free cells, such as relative ease of product separation, reuse of biocatalysts, high volumetric productivity, improved process control and reduced susceptibility of cells to contamination. However, the application of the immobilized culture in production scale is still limited on account of inadequate mechanical strength and high cost of the carrier [2].

In our previous study, loofa reinforced gel carriers for yeast (*Saccharomyces cerevisiae* M30) entrapment alginate-loofa cube (EALC) were developed and tested for batch ethanol and repeated batch fermentations. The carriers were fabricated by gelation of peripheral loofa sponge which was previously dipped in alginate-cell mixture. From the study, the final ethanol concentrations of the system with this carrier (EALC) was comparable to the cell immobilized in small calcium alginate beads and the ethanol productivities was relatively stable than the free cell culture.

The immobilized cells were able to remain viable and functioned normally within the alginate-loofa matrix as well as the carrier had good mechanical strength and stability that should be able to apply for a long term use [3].

In order to determine the application of EALC in continuous modes, a packed-bed reactor (PBR) for ethanol production using the alginate-loofa matrix as the cell carrier is developed in this study and the performance of the PBR system is compared to that from the batch system.

1.1Objectives

1. To develop immobilized cell system using loofa reinforced alginate carriers in packed-bed reactor.
2. To gain useful information regarding cell immobilization phenomena in packed-bed reactor.

1.2 Expected benefits

1. Invention of high performance immobilization carrier for large scale commercial ethanol fermentation in packed-bed reactor.
2. Useful information for a better understanding of immobilized cell technology and performance in packed-bed reactor.

1.3 Working scopes

In this study, the immobilized cell fermentation system for ethanol production is carried out in packed-bed reactor with the working volume around 0.64 liters. The optimum condition for ethanol production from our previous study is applied for this study. The working scopes are as follows:

1. Flocculating yeast strain, *Saccharomyces cereviceae* M30 is used as ethanol producer.
2. The fermentation is carried out in packed-bed reactor with the working volume 0.64 liters (6 cm diameter and 42 cm height).
3. Loofa sponge and alginate are applied as materials for constructing immobilized cell carriers and the immobilization method is entrapment cell in the cell carriers.
4. Palm sugar and molasses are utilized as carbon and energy source.

5. The operating condition is as follows:
   - Temperature: 32 ± 1 °C.
   - The dilution rates: 0.11, 0.16, 0.20 and 0.30 h^{-1}.
   - The initial sugar concentrations: varied for 200, 220 and 240 g/l.
   - The initial pH: 5.

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

In traditional fermentation systems, suspended yeast cell is used freely in a batch bioreactor. The bioreactor is filled with unfermented medium and the whole reactor volume (batch) is gradually fermented and subsequently removed from the reactor. Meanwhile, continuous fermentation systems possess a continuous flow of unfermented medium into the fermentor and a corresponding continuous flow of fermented product out of the system. In its simplest one-reactor incarnation, a content that is equal to the finished product that flows out of the system is operated in the continuous fermentor at steady state. A relatively slow inflow and little internal heterogeneity between the points of inflow and outflow in the reactor are needed to evade direct mixing of the unfermented inflow and the finished product. The continuous fermentor can be operated in packed-bed reactor, fluidized bed reactor, gas lift reactor, bubble column reactor (or stirred reactor if stirred); and membrane cell-recycle exhibited in **Figure 2.1** [2].

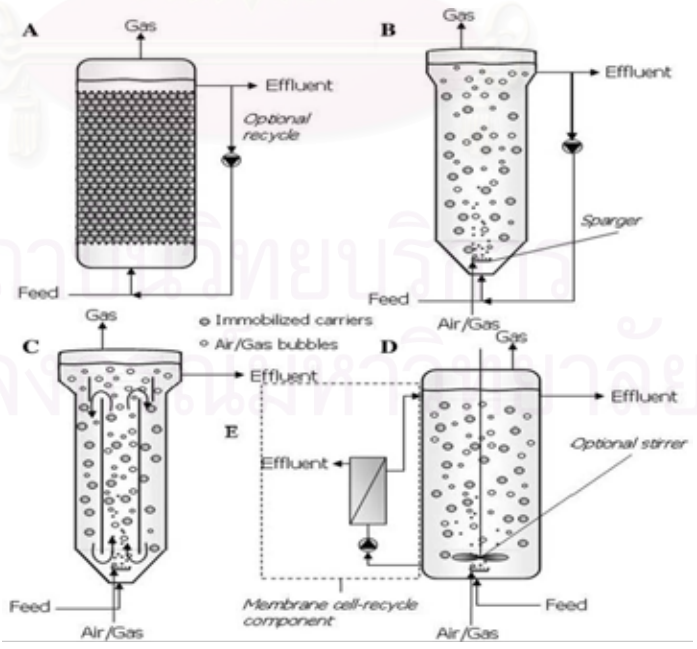


Fig 2.1 Five common types of immobilized cell bioreactor: (A) packed bed reactor, (B) fluidized bed reactor, (C) gas lift reactor, (D) bubble column reactor (or stirred reactor if stirred) and (E) membrane cell-recycle reactor [2].

2.1 *Saccharomyces cereviceae* for ethanol production

The ethanol fermentation by *S. cerevisiae* have been studding 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 as illustrated in Fig. 2.2. 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 [4].

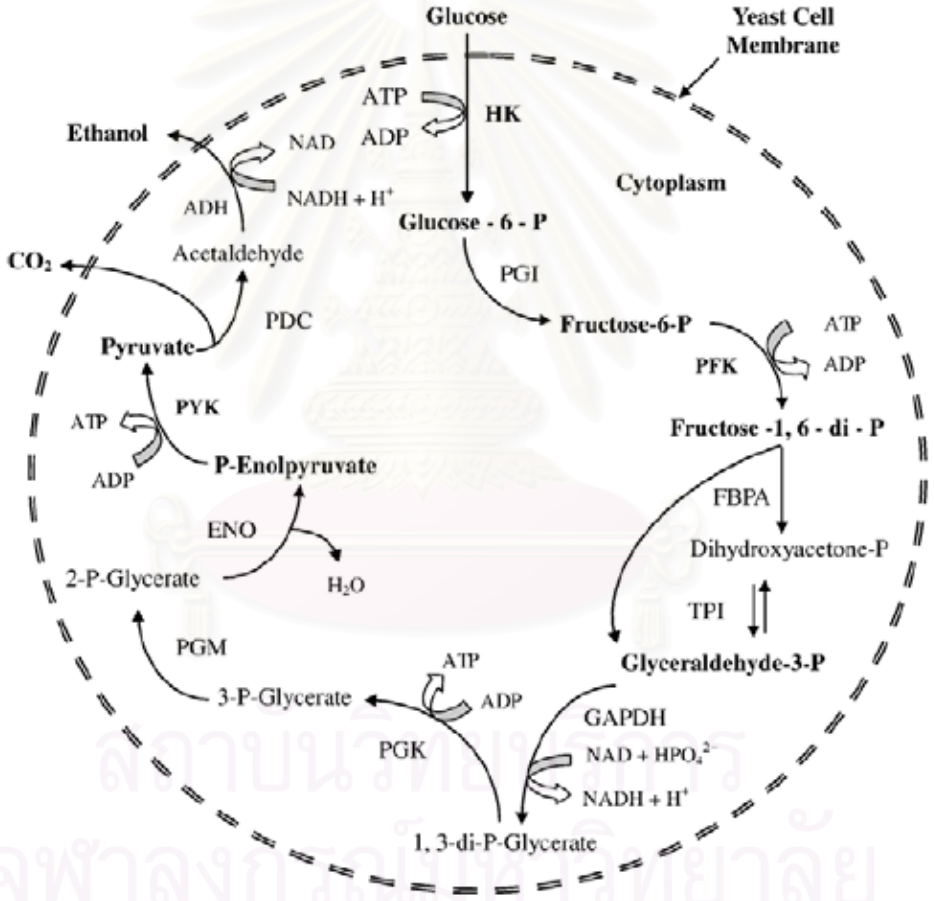


Figure 2.2 Metabolic pathway of ethanol fermentation in S. cerevisiae. (Madigan et al., 200)

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

From metabolic pathway, 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 [4].

2.2 Immobilized cell system

Immobilized cell technology has gained many interests since 1980s. Thousands of documents in various journals and papers are currently available via scientific search websites [5]. Immobilization of cells as biocatalysts is almost as common as enzyme immobilization. It can be defined as the restriction of cell mobility within a finite space [6]. Main application fields of immobilized cell consists of 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 cultures are as following [5-7].

1. Higher cell concentration.
2. Higher possibility for biocatalyst regeneration in hostile condition.
3. Elimination washout problem which in turn enables the fermentation to be carried out at higher dilution rate.
4. Easier downstream processing of the product.
5. Possibility of avoiding costly cells recovery and recycle by reuse of cells.
6. Improving cells genetic stability in some cases.
7. Protection of cells from shear force (especially for shear sensitive cells).
8. More favorable microenvironment conditions.

9. Maintenance of cell activity by protection from toxins and inhibitors.
10. Higher production rates and yields.
11. Smaller fermenter requirements.
12. Capital and energy cost saving.

In a continuous fermentation, productivity in general can be improved by increasing the flow rate of the system which is usually represented as dilution rate. Dilution rate is the ratio between flow rate and volume of reactor. The relationship between productivity and dilution rate is shown in **Figure 2.2**

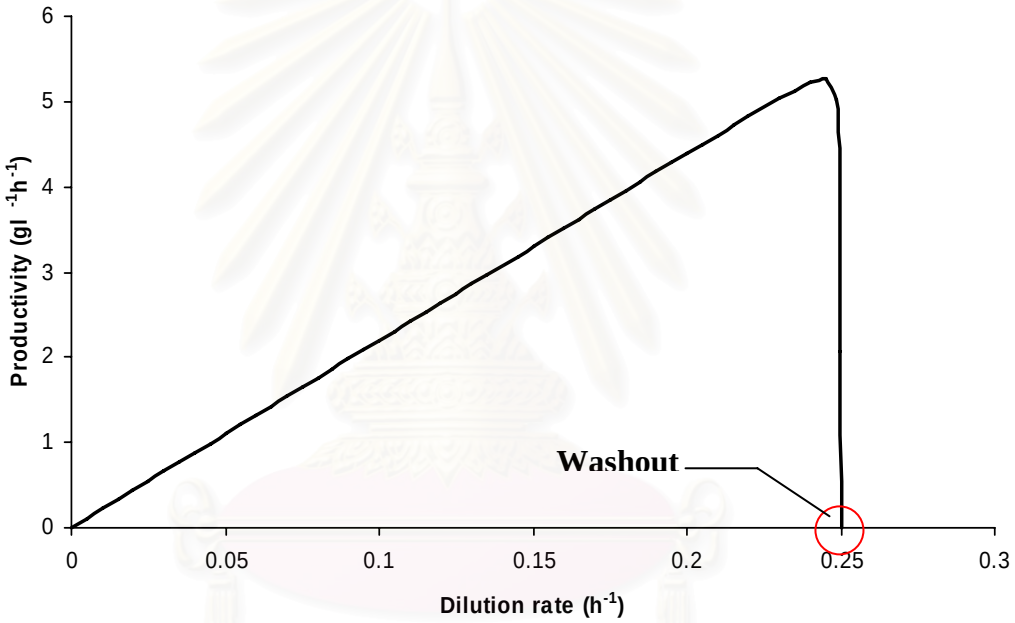


Figure 2.3 Productivity versus dilution rate curve

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 2.3** shows a relationship between substrate, product, and biomass concentration with critical dilution rate.

By immobilizing the cells inside the bioreactor, high cells concentration can be achieved even though the dilution rate has exceeded its critical value. With the combination between high dilution rate and high cell densities inside the reactor,

immobilized cells fermentor can maintain higher productivity as compared to conventional suspended cells culture.

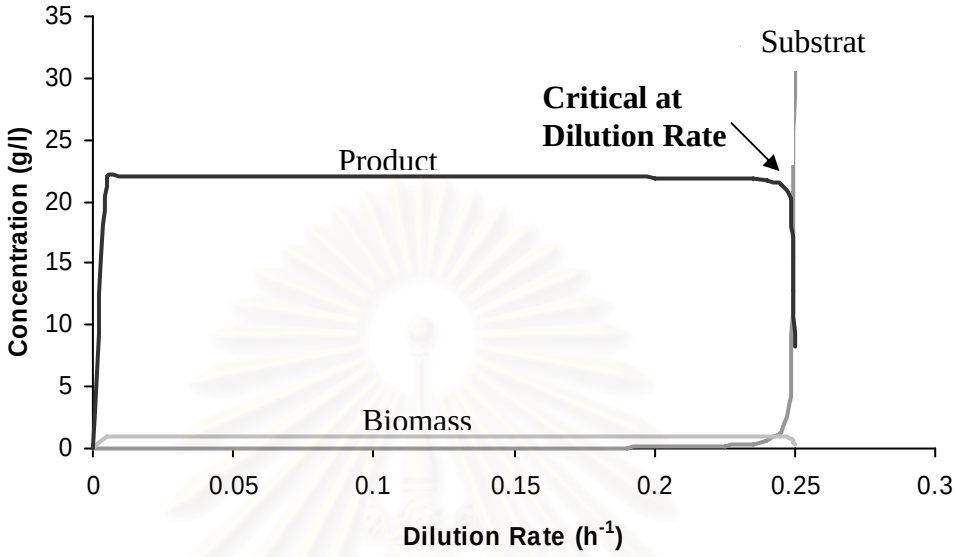


Figure 2.4 Concentration profile with variable dilution rate

Unfortunately, immobilization of cells also has some limitations and drawbacks. It should only be applied when the desired product is excreted by the cells. Furthermore, it often leads to systems for which diffusional restriction are important. In many cases, the control of microenviroment in immobilized biosystems is difficult due to the high degree of heterogeneity within such systems [6].

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 [5]. In artificial immobilized cell system, cells are immobilized by using carriers/supports. 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.

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. 2.4**) [2].

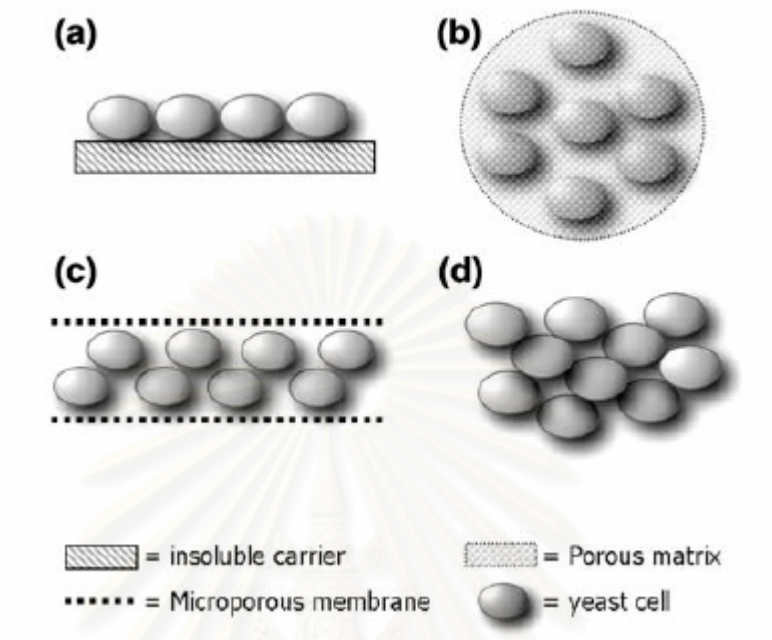


Fig 2.5 Basic methods of yeast immobilization: (a) attachment to a surface, (b) entrapment within a porous matrix, (c) containment behind a barrier and (d) self-aggregation [2].

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 [2].

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 [2]. 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 [8].

2.3.3 Containment behind a barrier

Using of microporous membrane filters or entrapment of cells in microcapsules can attained the containment of yeast cells behind a barrier. This type of immobilization is most suited when high molecular weight products need to be separated from the effluent, or when a cell free product is required. Inherent problems of this technique are possible membrane fouling caused by cell growth and mass

transfer limitations. 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 ethanol production [2].

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 nitrogencompounds could be influent the success of immobilization [2]. 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 [8].

2.4 Packed-Bed Reactor

Columns packed with immobilized biocatalyst particles currently used in numerous applications and additional uses are expected. Such reactors are called packed bed reactors or fixed-bed reactor. Several immobilized cell systems have also been examined in packed-bed configurations.

The simplest and often quite useful description of packed-bed reactor performance uses a plug-flow reactor model modified to account for the influence of the packed catalyst (yeast cells) on flow and kinetics features. The superficial flow velocity through the reactor (fermentor) is equal to the volumetric flow of the feed divided by the void cross-sectional area which is the total cross-sectional area times the void fraction (ε). The appropriate rate expression for use in the tubular reactor material balance is based upon use of effectiveness factor (η). For example, considering a single reaction S $\longrightarrow$ P with intrinsic rate ν = ν(s, p) the rate of product formation per unit volume of immobilized biocatalyst (yeast cells) pellet at a piont in the reactor is:

$$\upsilon = \eta(s_s, p_s)\upsilon(s_s, p_s)$$

Where p_s and s_s are the product and substrate concentrations respectively at the exterior pellet surface at which position inside the reactor. In general, the effectiveness factor (η) which accounts for intraparticle diffusion, and the rate expression (ν) depend upon both and s_s, p_s [9].

2.5 Review of ethanol fermentation by immobilized carriers

Jamuna et al. (1992) studied ethanol fermentation by immobilized cells in a trickle bed reactor with CO_2 ventilation port by consisting of yeast cells entrapped in alginate matrix and varying the substrate concentration, bed volume and inlet flow rate. The trickle bed reactor was provided with gas ventilation ports to eliminate the restrictions imposed by gas holdup and excessive pressure drop. The number of stages

required in a batch operation can easily be computed with the rate equation. The long term performance of the reactor was found to be satisfactory and offered a better alternative to packed-bed reactors.

Tanaka et al. (1997) reported efficient production of ethanol by flocculating (*Saccharomyces cerevisiae* IR2) and non-flocculating (*Candida brassicae*) cells immobilized in loofa (luffa cylindrica) sponge in column-type bioreactors. Immobilization of non-flocculating cells was achieved by addition of chitosan to the reactor. However, the amount of cells per unit sponge was lower and the volumetric ethanol productivity was about 20% less than that of the flocculating cells. In comparison with a fixed bed made of a single cylindrical loofa sponge, the amount of sponge per unit reactor value was 2 time higher cylindrical loofa when sliced loofa sponge was used to construct the fixed bed. The concentration of immobilized non-flocculating cells in the sliced sponge was lower than that of the flocculating cells but it was 4 times higher than the value obtained with a cylindrical sponge.

Nigam. (2000) reported on continuous ethanol product from pineapple cannery waste using immobilized yeast cells in k-carrageenan packed in tapered glasscolumn reactor. From the experimental result, this process was promising in view of high ethanol productivities obtained at relatively high conversions and excellent reactor stability.

Zorlu and Goksungur. (2001) reported on production of ethanol from beet molasses by Ca-alginate immobilized yeast cells in a packed-bed reactor. At the dilution rate of 0.22 h^{-1}. Maximum ethanol concentration of 4.62 %(w/v), 82.9% of theoretical yield and volumetric productivity of 10.16 g/l h were obtained from beet molasses medium containing 10.90% total sugar with 2.0-2.4 mm diameter beads. The reactor was operated for 25 days without loss of original fermentation capacity.

Augusto et al. (2001) reported on continuous fermentation of sugar cane syrup using immobilized yeast cells by adhesion chysotile in packed-bed reactor. The experimental result showed that packed-bed reactors could be assembled using cells immobilized onto chrysotile that had a reasonable operational stability during two months and a higher productivity than the batch and semi-continuous systems.

However, this type of reactor was not suitable for scaling up to current industrial volumes. For scaling up, there is a need to test this support in suspended bed reactors.

Amutha and Gunasekaran. (2001) reported on production of ethanol from liquefied cassava starch using co-immobilized cells of *Zymomonas mobilis* and *Saccharomyces diastaticus in* batch and packed-bed reactor. The co-immobilized cells produced 46.7 g/1 ethanol from 150 g/l liquefied cassava starch, while immobilized cells of yeast *S. diastaticus* produced 37.5 g/l ethanol. 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-batch 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 a packed bed column reactor operated at a flow rate of 15 ml/h (residence time, 4 hour) exhibited a maximum ethanol productivity of 8.9 g/l h.

Ogbonna et al. (2001) studied the scale up of fuel ethanol production from sugar beet juice using loofa sponge immobilized (*Saccharomyces cerevisiae* IR2). Mixing was found not sufficient in an 8 l bioreactor containing a bed of sliced loofa sponges and consequently, the immobilized cells were not uniformly distributed within the bed. The system was scaled up to 50 l and when compared with the 2 l bubble column bioreactor, there were no significant differences in ethanol productivity and yield.

Najafpour et al. (2003) studied that ethanol fermentation using immobilized cell of *Saccharomyces cervisiae* in plug flow tubular column. The results indicated that the immobilization of *Saccharomyces 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. The investigation showed potential application for utilizing concentrated feed with higher rate of ethanol production as the cell was loaded into the gel matrices of sodium alginate.

Alegre et al. (2003) reported ethanol fermentation of a dilute molasses medium by *Saccharomyces cervisiae* immobilized on chrysotile. The fermentation

medium employed consisted only of diluted sugar-cane molasses. In the batch fermentations process with immobilized yeasts, the initial rate of CO_2 production increased roughly 27 % during the first 30 minutes, compared to systems containing no chrysotile. A study of continuous alcoholic fermentation with chrysotile in the reactor bed showed a higher ethanol production rate at the different dilution rates investigated compared to similar fermentations without chrysotile. The effect of chrysotile on bacteria or other fungi is unknown, but its application in other fermentation processes may be advantageous, if a similar effect as on the yeast alcoholic fermentation occurs, especially in those processes in which, unlike alcoholic fermentation, the products do not cause intense inhibition of the microbial activities.

Valach et al. (2005) studied a fixed-bed and a gas-lift three-column reactor for continuous production of ethanol by pectate and alginate immobilized *Saccharomyces cervisiae* cells. For this experiments revealed that the calcium pectate was a material favorable mechanical properties resulting in a fairly stable operation and ethanol production over the course of 630 h of continuous performance. Due to the natural origin of pectins, calcium pectate gel was a promising immobilization material for food applications for its food safety, which represented one of the main concerns in food industry. Out of the two multistage reactor cascade systems, the gas-lift system was proved to be more effective, becoming the overall productivity 7.57 g/l h of ethanol for over 600 hours.

Nishio et al. (2005) studied hydrogen and ethanol production from glycerol containing wastes discharged after biodiesel manufacturing process using *Enterobecter aerogenes* HU-101 in packed-bed reactor. The biodiesel wastes should be diluted with a synthetic medium to increase the rate of glycerol utilization and the addition of yeast extract and tryptone to the synthetic medium accelerated the production of H_2 and ethanol. The yields of H_2 and ethanol decreased with an increase in the concentrations of biodiesel wastes and commercially available glycerol (pure glycerol).

Feng-Wu et al. (2006) reported on continuous ethanol fermentation coupled with recycling of yeast flocs. Composed of three-stage tanks in series coupled with two sedimentation tanks was established. A self-flocculating yeast strain developed

by protoplast fusion from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* was applied. The ethanol fermentation substrate was fed into the first fermentor at the dilution rate of 0.057 h^{-1}. The continuous ethanol fermentation coupled with recycling of yeast flocs in three stages in series-stirred fermentator-tank system evidently enhanced the ethanol productivity at the premises of other fermentation performances.

Phisalaphong et al. (2006) studied mathematical modeling to investigate temperature effect on kinetic parameters of ethanol fermentation by the flocculating yeast, *Saccharomyces cerevisiae* M30, using cane molasses as the substrate. The biomass and ethanol production rates were enhanced slightly by the increase of the isothermal control from 30 to 33 °C but the rates slightly decreased at 35 °C. A significant effect of the initial substrate on the reduction of yield coefficients and the increase of maintenance constant was observed only at high concentration (220 g/l).

Phisalaphong et al. (2007) studied the immobilization *Saccharomyces cerevisiae* M30 in alginate-loofa as carrier matrix for ethanol production using molasses as the substrate. The cell immobilized was been effective for this matrix and had good mechanical 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. The yeast cells remained firmly immobilized and active after a storage period of 4 months.

Yu et al. (2007) have reported the novel immobilization method of *Saccharomyces cerevisiae* to sorghum bagasse for ethanol production. The ethanol productivity of the immobilized cells was 2.24 times higher than the free cells. In repeated batch fermentation with an initial sugar concentration of 200 g/l, nearly 100% total sugar was consumed after 16 hours. The ethanol yield and productivity were 4.9 g/g consumed sugar on average and 5.72 g/l h, respectively. The immobilized cell reactor was operated over a period of 20 days without breakage of the carriers, while the free cell concentration in the effluent remained less than 5 g/l throughout the fermentation. The maximum ethanol productivity of 16.68 g/l h appeared at the dilution rate of 0.3 h^{-1}.

Table 2.1 Studies on ethanol production by immobilized cells

Author	Dilution rate (h^{-1})	Carrier		Diameter/length of Reactor(cm/cm)	Paper Title
		Method	Material		
Jamuna et al. 1992	0.66-5.76 $P_{F,m}$ = 119 $Q_{P,m}$ = 38.25	Entrapment	Alginate	8.0/70	Ethanol fermentation by immobilized cells in a trickle bed reactor
Tanaka et al. 1997	$P_{F,m}$ = 70.8 $Q_{P,m}$ = 6.48	Adsorption	Loofa	1.5 liters (Recirculating column)	Efficient production of ethanol by cells immobilized in loofa (luffa cylindrica) sponge
Nigam 2000	0.2-2.5 $P_{F,m}$ = 42.8 $Q_{P,m}$ = 37	Entrapment	K-carrageenan	-top,5/30 -bottom,3/30	Continuous ethanol production from pineapple waste using immobilized yeast cells
Goksuncur et al. 2001	0.22 $P_{F,m}$ = 46.2 $Q_{P,m}$ = 10.16	Entrapment	Alginate	1.57/49	Production of ethanol from beet molasses by Ca-Alginate immobilized yeast cells in a packed-bed bioreactor
Ogbonna et al. 2001	$P_{F,m}$ = 80 $Q_{P,m}$ = 5	Adsorption	Loofa	9.0/12 for 2 liters 12.0/71 for 8 liters 30/71 for 50 liters	Scale up of fuel ethanol production from sugar beet juice using loofa sponge immobilized bioreactor

* $P_{F,m}$ = maximum final ethanol concentration (g/l) and $Q_{P,m}$ = maximum ethanol productivity (g/l h)

Author	Dilution rate (h^{-1})	Carrier		Diameter/length of Reactor(cm/cm)	Paper Title
		Method	Material		
Augusto et al. 2001	0.25-0.55 $P_{F,m}$ = 75 $Q_{P,m}$ = 27	Adhesion	Chrysotile	4.7/40	Continuous fermentation of sugar cane syrup using immobilized yeast cells
Amutha et al. 2001	0.08-0.42 $P_{F,m}$ = 49.5 $Q_{P,m}$ = 8.9	Entrapment	Alginate	4.6/85	Production of ethanol from liquefied cassava starch using co-immobilized cells of *mobilis* and *Saccharomyces diastaticus*
Najafpour et al. 2004	0.14 and 0.17 $P_{F,m}$ = 47 $Q_{P,m}$ = 6.71	Entrapment	Alginate	3.5/15.7	Ethanol fermentation in an immobilized cell reactor using *Saccharomyces cerevisiae*
Valach et al. 2005	0.1 $P_{F,m}$ = 75.7 $Q_{P,m}$ = 7.57	Entrapment	1.Alginate 2.Pectate	4.5/35	Efficiency of a fixed-bed and a gas-lift three-column reactor for continuous production of ethanol by pectate and alginate immobilized *Saccharomyces cerevisiae* cells

* $P_{F,m}$ = maximum final ethanol concentration (g/l) and $Q_{P,m}$ = maximum ethanol productivity (g/l h)

Author	Dilution rate (h^{-1})	Carrier		Diameter/length of Reactor(cm/cm)	Paper Title
		Method	Material		
Nishio 2005	0.1-1.75 $P_{F,m}$ = 4.37 $Q_{P,m}$ = 5.17	Adsorption	Nagao porcell	2.7/17	Hydrogen and Ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process
Feng-Wu et al. 2006	0.057 $P_{F,m}$ = 101 $Q_{P,m}$ = 5.77	Self-Flocculate		1 liter (CSTR)	Continuous ethanol fermentation coupled with recycling of yeast flocs
Phisalaphong et al. 2006	$P_{F,m}$ = 85 $Q_{P,m}$ = 2.83	Self-Flocculate		500 ml Erlenmeyer flask	Mathematical modeling to investigate temperature effect on kinetic parameters of ethanol fermentation
Phisalaphong et al. 2007	$P_{F,m}$ = 97.4 $Q_{P,m}$ = 2.31	1. Entrapment 2. Adsorption	Alginate Loofa	500 ml Erlenmeyer Flask	Alginate-loofa carrier matrix for ethanol Production
Yu et al. 2007	0.1-0.4 $P_{F,m}$ = 94.7 $Q_{P,m}$ = 16.68	Adsorption	Sorghum	3/30	An novel immobilization method of *Sacharomyces cerevisiae* to sorghum bagasse for ethanol production

* $P_{F,m}$ = maximum final ethanol concentration (g/l) and $Q_{P,m}$ = maximum ethanol productivity (g/l h)

CHPTER III

MATERIALS AND METHODS

Ethanol production in this study was carried out in a packed bed reactor. Palm sugar and molasses were used as carbon and energy source for the producing yeast. A flocculating yeast strain, *Saccharomyces cereviceae* M30 was immobilized in alginate-loofa and its ethanol productivity was investigated.

Most of materials and methods used in this work were of common practice in cell cultivation, cell immobilization, and fermentation technologies. Methods for reinforced carrier's preparation were constructed based on simple sensible aseptic produces that can be readily applied on bench scale fermentation experiments. Except molasses other chemicals are of analytical grade.

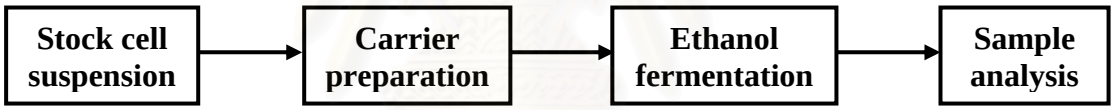


Figure 3.1 General flow diagram of experimental work

3.1 Microorganism and stock cell suspension

S. cereviceae M30 strain was kindly provide by Dr. Savitree Linthong from Deparment of Microbiology, Kasetsart University, Bangkok. Stock cultures were stored in PDA agar slant. Each starter culture was obtained by transfering cells from an agar slant into 500 ml Erlenmeyer flask containing 100 ml sterilized cultivation medium. The cultivation medium was composed of 50 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 20 minutes at 121^oC. Cell cultivation was carried in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33^oC for 24 hours.

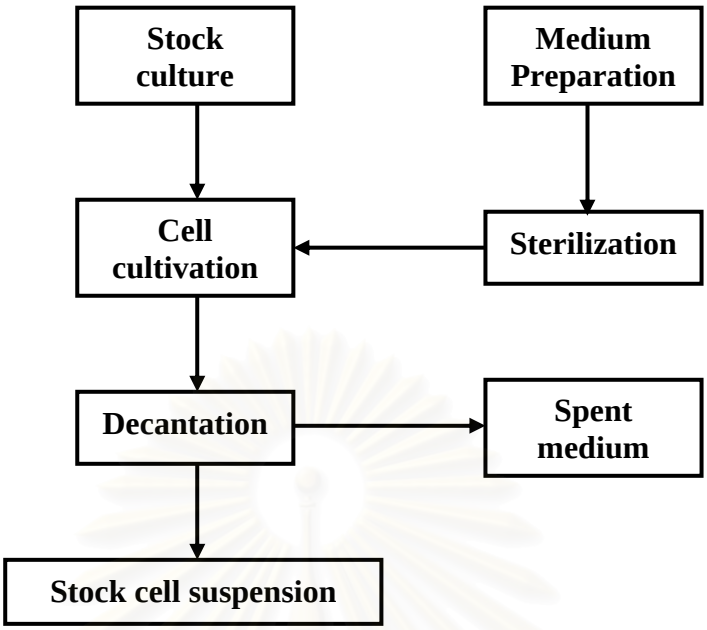


Figure 3.2 Methods of stock cell suspension preparation

3.2 Preparation of alginate-loofa

Sodium alginate of 30 g/l was made by dissolving Na-alginate powder in NaCl 9 g/l solution. It was autoclaved for 15 minutes at 121°C and kept overnight at 4°C to facilitate deaeration. Stock cell suspension was added to the alginate solution to form an alginate-cell mixture with volumetric ratio of 1:10. The mixture was used to construct for entrapment alginate-loofa cube (EALC).

Cubic loofa sponge (19 x 19 x 2 mm^3 and 8 x 8 x 2 mm^3) 2 g for batch fermentation and 18 g for continuous fermentation was dipped into alginate-cell mixture before transferred to $CaCl_2$ 14.7 g/l to form EALC. EALC carriers were left to harden in $CaCl_2$ solution under mild stirring for 15 minutes. The carrier was then rinsed 3 times with NaCl 9 g/l.

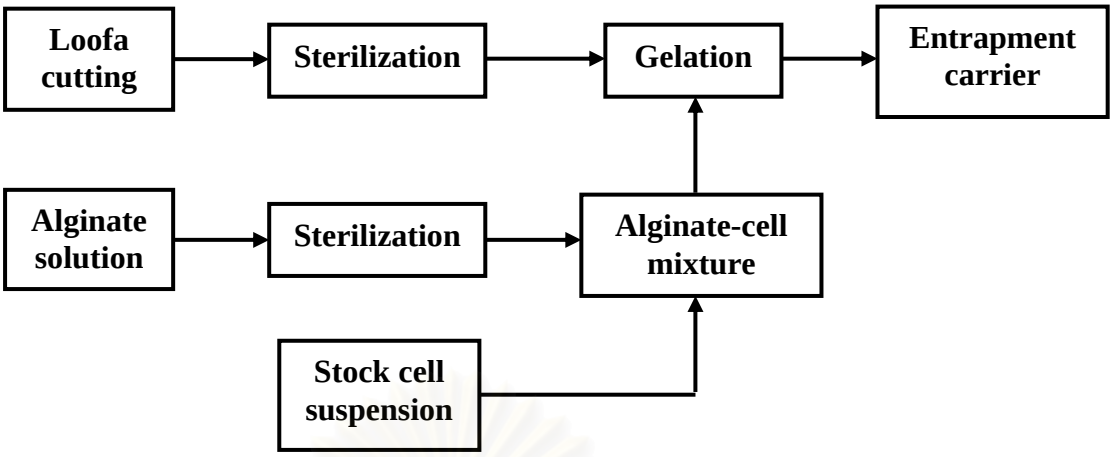


Figure 3.3 Preparation of adsorption based carrier

3.3 Ethanol fermentation

In experiments, molasses was used for substrate in fermentation as it represented the most widely used raw material for fermentative ethanol production.

3.3.1 Bath fermentation

In molasses based medium the composition of fermentation medium was similar with cell cultivation except that the sugar concentration was increased to optimum level of 220 g/l., 0.5 g/l ammonium sulfate was added 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.

3.3.2 Continuous ethanol fermentation

The reactor column with working volume around 0.86 lit containing immobilized cell beds was used for the study. Temperature of the system was controlled at 31 ± 0.5 °C by the passing of 28 °C cooling water inside the reactor jacket. The initial sugar concentration varied of 200, 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 0.11, 0.16, 0.20 and 0.30 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.

3.4 Sample analysis

Sugar concentration was determined by a modified 3, 5-dinitrosalicylic acid (DNS) reagent method through a corresponding standard curve. Briefly, sample was hydrolyzed with HCl 370 g/l in boiling water bath for 10 minutes. After hydrolysis, the sample was neutralized using NaOH 300 g/l. The suspension sample was centrifuged and the supernatant was reacted with DNS reagent before the color intensity was measured by 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 of 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 1μL and injection temperature of 240°C.

The sediment cell was washed with HCl 0.1 N and resuspended in water. The cell concentration was measured by spectrophotometer at 660 nm. A known mass of the carriers was dissolved in sodium citrate 0.5 M. After the sponge was removed, 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°C for 2 hours.

At the beginning and the end of fermentation, samples of carrier were collected for scanning electron microscopy (SEM). The carriers were sputter-coated by gold and examined using JSM 5410LV (JEOL, Japan) scanning electron microscope.

3.5 Calculation of fermentation parameters

Immobilization yield (Y_I, %) was defined as the ratio of immobilized cell concentration (X_I, g/l) to total cell concentration (X_T, g/l). X_T was calculated as summation of free cell concentration (X_E, g/l) and X_I. The yield of sugar consumption (Y_S, %) was considered as the ratio of sugar consumption ($S_0 - S_F$, g/l) to starting sugar level (S_0, g/L). S_F is the value of final sugar concentration of each determinate period. The ethanol yield factor ($Y_{P/S}$, g ethanol/g sugar) is the ratio of ethanol production ($P_F - P_0$, g/l) to sugar consumption ($S_0 - S_F$). P_F and P_0 are ethanol concentration at the end and beginning of each determinate period, respectively. The productivity (Q_P, g/l h) was calculated by multiple of ethanol production and dilution rate (D, h^{-1}).

CHAPTER IV

RESULTS AND DISCUSSION

The purpose of this work was to expand upon the previous research in an attempt to develop rapid production of high concentrations of ethanol from sugar cane molasses by using continuous ethanol production with immobilized *Saccharomyces cerevisiae* M.30 culture. In the construction of the carrier, alginate-loofa was chosen based on its high potential as a cell carrier [3]. This material is biodegradable obtained from natural origin. The carrier was fabricated simply by entrapment of a peripheral loofa sponge that was previously dipped in an alginate cell mixture. The porous structure conferred the new carrier with better mass transfer characteristics. In the previous work [3], an alginate-loofa size of 9 x 9 x 3 mm^3 was effective for cell immobilization. The ethanol production using this carrier was proven to be more stable than that of the suspension cell culture. The alginate-loofa carrier has many advantages including regeneration ability, reusability, altered mechanical strength and high ethanol productivity. The optimal ethanol production rate was obtained by isothermal control at 30- 33 ^oC [10]. The investigation of the effect of the alginate-loofa sizes on the activity of cells was performed in batch fermentation, whereas, the investigation of the effects of the initial sugar concentration and the dilution rate was performed in continuous fermentation.

4.1 Storage effects/Repeated batch Fermentation

First of all, to investigate the stability of the immobilized yeast in alginate-loofa matrix, cell cultures from the previous study [3] were stored for 4 months at 4^oC and reused in this study. For the comparison, three stored cultures were used for ethanol fermentation: suspended cells (SC), Ca-alginate-immobilized cells (AB) with 2 mm diameter, and entrapment alginate-loofa cube (EALC) with carrier size of 9 x 9 x 3 mm^3. The cell cultures were reused by 4-cycle repeated batch fermentation. The suspension cells and immobilized cells were carried out in duplicate using a medium contained 202 g/l sugar and 0.5 g/l ammonium sulfate at pH 5. The experiments were initiated by transferring prepared cell suspension or immobilized

cells into 250 ml of the medium in 500 ml Erlenmeyer flasks. Fermentations were performed in the shaking incubator at 150 rpm, 33^{o}C for 48 hours. The samples were harvested every 24 hours for cell, sugar and ethanol analyses. Table 4.1 and Figure 4.1 to Figure 4.3 show the results of repeated batch fermentation from sugar cane molasses. In the first repeated batch, the ethanol concentration obtained at 48 hours for SC, AB and EALC cultures were 65.70, 76.00, and 76.00 g/l, respectively and residual sugar concentrations were 21.22, 25.10, and 29.58 g/l, respectively. The final ethanol concentration in the suspended cell and the immobilized cell cultures were comparable in the 2nd – the 3rd batch. However, the instability of SC cultures was observed in the 1st and the 4th batch. Especially in the 4th batch, the ethanol concentration obtained at 48 hours for SC, AB and EALC cultures were 3.70, 70.40, and 70.00 g/l, respectively and residual sugar concentrations were 196.24, 30.00, and 33.66, respectively.

Table 4.1 Ethanol and Sugar concentration in repeated batch fermentation using 4-month-stored cultures of SC, AB, and EALC

Batch	Time	Ethanol concentration (g/l)			Sugar concentration (g/l)		
	(hour)	SC	AB	EALC	SC	AB	EALC
I	0	0.00	0.00	0.00	202.00	202.00	202.00
	24	4.40	75.50	76.60	81.60	26.52	24.48
	48	65.70	76.00	76.00	21.22	25.10	29.58
II	0	0.30	0.70	0.70	202.00	202.00	202.00
	24	63.30	66.30	60.30	36.72	42.84	61.20
	48	72.00	80.70	86.10	32.64	23.84	23.26
III	0	0.30	0.70	0.70	202.00	202.00	202.00
	24	47.60	69.00	69.50	88.74	74.46	89.76
	48	86.10	71.30	76.50	20.38	30.60	21.22
IV	0	0.30	0.70	0.70	202.00	202.00	202.00
	24	2.10	65.10	61.80	171.36	45.00	51.00
	48	3.70	70.40	70.00	186.24	30.00	33.66

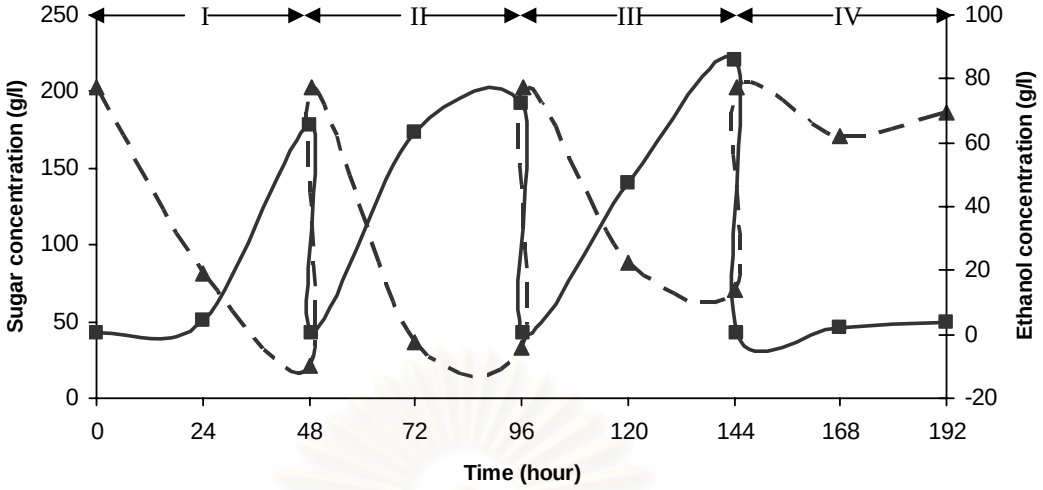


Figure 4.1 Ethanol and Sugar concentration profile in repeated batch fermentation using 4-month-stored cultures of SC; --▲-- = sugar and -■- = ethanol

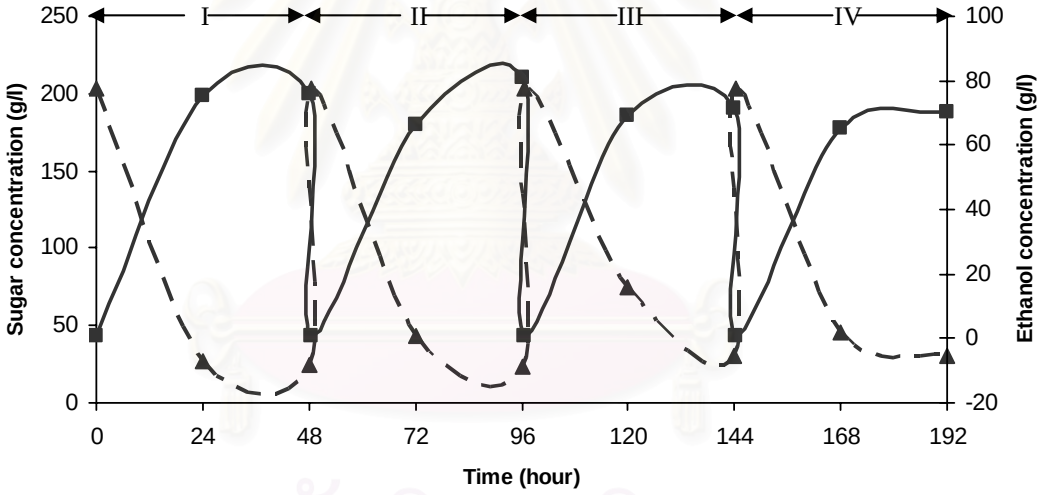


Figure 4.2 Ethanol and Sugar concentration profile in repeated batch fermentation using 4-month-stored cultures of AB; --▲-- = sugar and -■- = ethanol

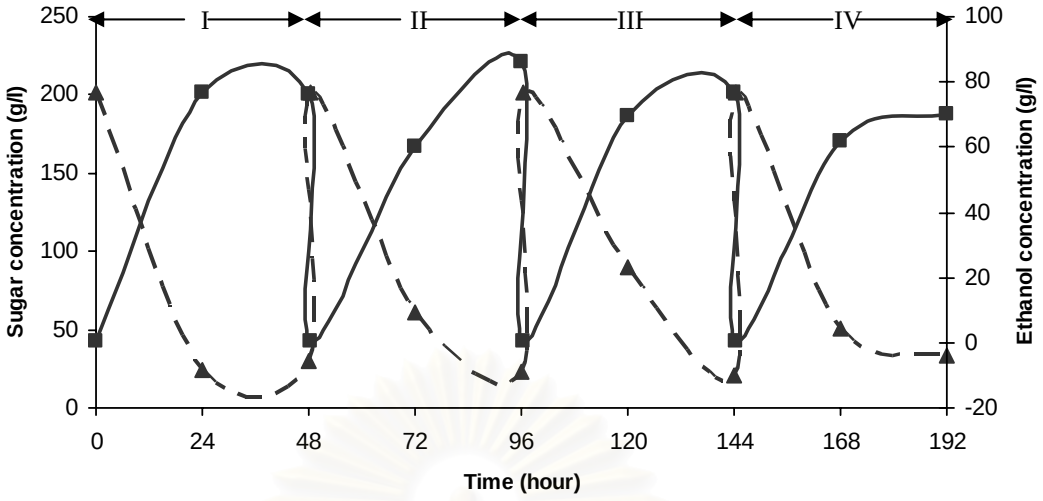
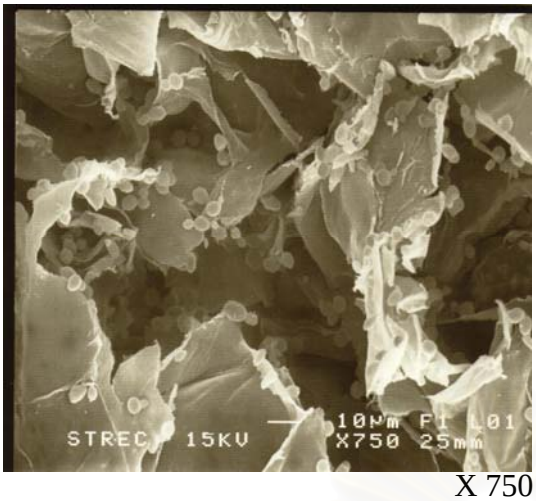


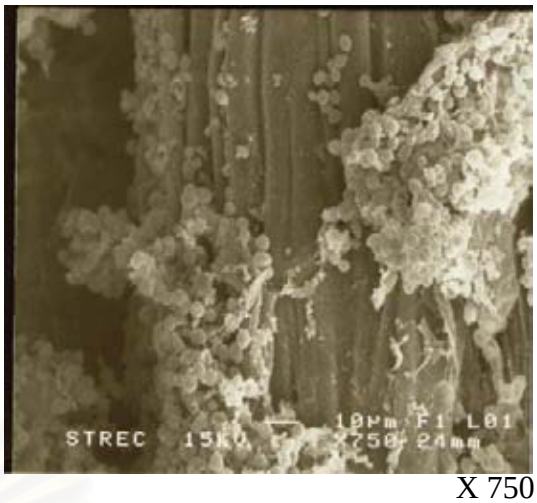
Figure 4.3 Ethanol and Sugar concentration profile in repeated batch fermentation using 4-month-stored cultures of EALC; --▲-- = sugar and -■- = ethanol

In comparison of SC culture with the results from AB and EALC cultures, the stability and average ethanol productivity were significantly improved. The deactivation of suspended yeast cell activity in the medium with high sugar and ethanol concentrations was occurred when they were used for a long time. On the other hand, the yeast cells immobilized or contained in the carriers (AB and EALC) could be protected from the high sugar and ethanol concentration. As a result, the immobilized yeast cells could be used for a long period of time. After the 4-cycle repeated batch, a higher degree of gel degradation could be observed on the surface of the carriers (Figure 4.4 and Figure 4.5). The major cause of gel degradation was from cell growth, CO_2 occurred from the fermentation and shear stress occurred from rotating or shaking in the incubator. However, the majority of cells were still attached to each other within the matrix in EALC. Yeast cells could bind with loofa surface very well in long-term fermentation. With the strong and chemical stable nature of loofa sponge, EALC has good mechanical strength, durability, and stability for long-term use.



X 750

Figure 4.4 AB surface after storage 4 month



X 750

Figure 4.5 EALC surface after storage 4 month

EALC was successfully developed and applied in repeated batch ethanol fermentation. The carriers were fabricated simply by entrapment of a peripheral loofa sponge that was previously dipped in an alginate-cell mixture. The porous structure conferred the new carriers with better mass transfer characteristics. An EALC with a size of 9 x 9 x 3 mm^3 was effective for cell immobilization, which is comparable to a 2 mm diameter alginate bead. Ethanol production using these carriers was proven to be more stable than that using SC cultures. After storage for 4 months, the EALC immobilized cell culture was still active, and the stability of immobilized cell cultures being higher than that of SC culture was confirmed. The result exhibits the potential of EALC carrier in an ethanol fermentation system for a long term use. To simplify the preparation of EALC carrier for a packed bed reactor, ethanol fermentation using a bigger size of EALC carrier (20 x 20 x 3 mm^3) in comparison to a smaller size (9 x 9 x 3 mm^3) was further studied.

4.2 Effect of carrier size in batch fermentation

In the batch fermentation, ethanol production by *S. cerevisiae* M30 immobilized in loofa reinforced alginate with carrier size of 9 x 9 x 3 mm^3 was compared to that with the carrier size of 20 x 20 x 3 mm^3. The initial sugar concentration was set to 220 g/l at pH 5. The fermentation was performed in Innova 4330 Refrigerated Incubator Shaker at 150 rpm, 33 ^oC using 500 ml Erlrnmeyer flask

containing 250 sterilized medium for fermentation. The samples were harvested every 8 hours. Table 4.1 and Figure 4.1 shows the results of batch ethanol production from sugar cane molasses in which an 89 to 90 g/l ethanol was produced within 48-72 hours of the fermentation. The concentration of the residual sugar was about 20 g/l. During the first 16 hours of fermentation, yeast cells in the two systems were acclimatizing with the new microenvironment and increased amount of cells population thus its ethanol yield factor was consequently low (less than 10%). The consumption of sugar for the two systems reached steady state after 48 hours; there were no significant differences in data obtained from the two different sizes of alginate-loofa carrier. The ethanol concentrations were stabilized which corresponded to sugar concentration of about 20-24 g/l. It was proposed that residual sugars available at this level might be the unfermented sugars for the yeast cell [3]. Therefore, it could be assumed that it took between 48-56 hours for most of fermented sugar to convert to ethanol for completing the reaction. The conversion yields of the two systems were not significantly different at any times. The overall conversion yields were 0.44 and 0.45 for 9 x 9 x 3 mm^3 and 20 x 20 x 3 mm^3 alginate-loofa carriers, respectively. From the result, we can concluded that there were no significant difference in cell activity regarding the change of the carrier size from 9 x 9 x 3 mm^3 to 20 x 20 x 3 mm^3. At the end of the fermentation, the ratio of immobilized cell concentration (g/l): suspended cell concentration (g/l) was 3.39: 1.00 for the 9 x 9 x 3 mm^3carrier and 4.33: 0.87 for the 20 x 20 x 3 mm^3 carrier, respectively. The overall ethanol productivities was 1.88 g/l h with the immobilized cell concentration of 1.5 g-cell/g-sponge. From the previous study, ethanol productivities of 6.48 g/l h with the immobilized cell concentration of 0.38 g-cells/g-sponge were obtained when sliced loofa sponge was used for bed construction [11]. The difference results from this study might arise from many factors including the variation in yeast strain, reactor conditions and immobilized techniques.

The suspended (free) cell concentration in the system with alginate-loofa carrier of 9 x 9 x 3 mm^3 has exhibited more value than that of 20 x 20 x 3 mm^3 (Figure 4.6). The final cell concentrations and immobilized yields for the batch fermentations are shown in Table 4.4. It was observed that at the end of fermentation, in the system with 20 x 20 x 3 mm^3 alginate-loofa carrier, the free cell concentration was lower and the immobilized cell concentration was higher in comparison to those with the smaller size carrier.

Table 4.2 Batch fermentation of ethanol production using cell carrier of 9 x 9 x 3 mm^3 alginate-loofa

Time (hour)	Residue sugar concentration (g/l)	Ethanol concentration (g/l)	Free Cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	226.74	0	0		
8	162.13	0.56	0.03	0.01	0.07
16	87.00	11.37	0.28	0.08	0.71
24	62.52	52.57	0.68	0.32	2.19
32	38.04	72.20	0.69	0.38	2.26
40	26.82	80.03	1.02	0.40	2.00
48	22.74	89.03	0.87	0.44	1.85
56	26.82	90.59	1.13	0.45	1.62
64	20.70	87.80	1.06	0.43	1.37
72	24.78	88.82	0.92	0.44	1.23

Table 4.3 Batch fermentation of ethanol production using cell carrier of 20 x 20 x 3 mm^3 alginate-loofa

Time (hour)	Residue sugar concentration (g/l)	Ethanol concentration (g/l)	Free Cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l h)
0	223.68	0	0		
8	158.84	0.48	0.21	0.01	0.06
16	62.52	10.71	0.25	0.07	0.67
24	37.02	52.31	0.50	0.28	2.18
32	28.86	71.25	0.55	0.37	2.23
40	26.82	81.35	0.56	0.41	2.03
48	19.68	89.10	0.73	0.44	1.86
56	30.90	89.32	0.83	0.46	1.60
64	21.72	89.54	0.83	0.44	1.40
72	22.74	89.75	0.87	0.45	1.25

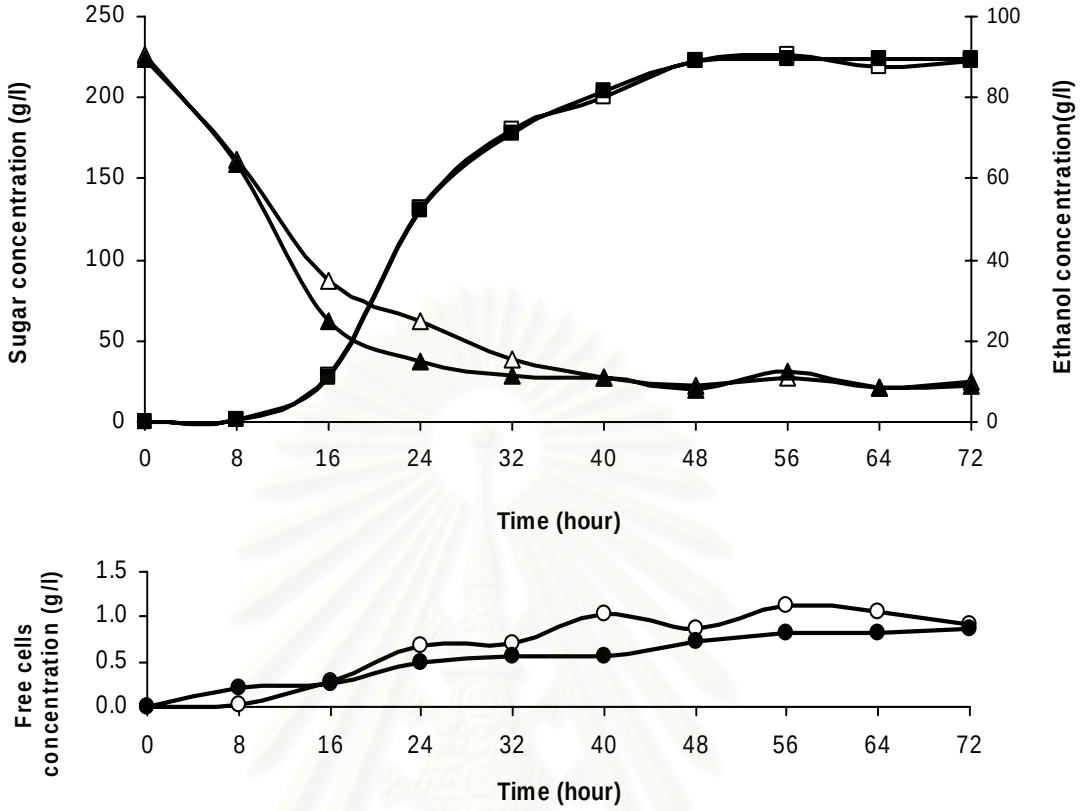


Figure 4.6 Batch fermentations of ethanol production using cell carriers of alginate-loofa with the dimensions of 9 x 9 x 3 mm^3 (opened symbol) and 20 x 20 x 3 mm^3 (crossed symbol); -▲- = sugar, -■- = ethanol and -●- = free cell.

Table 4.4 The final cell concentrations and immobilization yield of batch fermentations using cell carriers of alginate-loofa with the dimensions of 9 x 9 x 3 mm^3 and 20 x 20 x 3 mm^3.

Cell concentration	**Alginate-Loofa sizes**	
(g/l)	**9 X 9 X 3 mm^3**	**20 x 20 x 3 mm^3**
Immobilized cell	3.39	4.33
Free cell	1.00	0.87
Immobilized yield (%)	77.22	82.27

The immobilization yields in the system with the alginate-loofa carrier of 20 x 20 x 3 mm^3 and 9 x 9 x 3 mm^3were 82.27 and 77.22 %, respectively. With the same porous structure, the smaller size carrier had more surface area than the larger one. According to the higher open surface area, more cells leakage was obtained from the smaller carrier.

Ogbonna et al. (2001) [1] reported that the use of loofa sponge with the different sizes for cell immobilization did not show difference of ethanol productivity (5 g/l h). However, their work was performed in bubble column with external loop for recirculation of fermentation broth, while shake flask was used in this work. Higher shear environment in shaking system could cause more excessive cell detachment than that of the bubble column.

4.3 Continuous ethanol fermentation # 1

Continuous ethanol fermentation using a packed-bed reactor was performed under the following condition: working volume 0.64 liters, temperature 32 ± 1^oC, initial sugar concentration about 202 g/l, initial pH 5 and dilution rates 0.11, 0.16, 0.20 and 0.30 h^{-1} (corresponding to a hydraulic retention time, HRT of 9.1, 6.2, 5 and 3.3 h, respectively). From the result of the batch fermentation, the cell carrier of 20 x 20 x 3 mm^3 alginate-loofa was chosen based on its high immobilization yield and its more convenience for the use in packed bed column. Prior to inoculation and start up of the fermentation, the column was sterilized by hot water (100ºC) and circulation of 70% v/v ethanol for 12 hour. The immobilized cell in alginate-loofa matrix was cultivated in Innova 4330 refrigerated incubator shaker at 110 rpm, 33°C for 24 hour to increase the cells concentration in alginate-loofa cube before the carries were aseptically transferred to the sterilized column. The carrier volume was about 35 % (v/v) of the pack bed reactor volume of 960 ml. A start-up procedure was required in order to establish a steady state phase. Initially, the fermentation was started by feeding of the prepared medium of sugarcane molasses, containing about 202 g/l, through the inlet at the bottom of the column at the dilution rate of 0.11 h^{-1}. The dilution rate was changed every 3 days for 0.11 and 0.16 h^{-1} dilution rates and every 2 days for 0.20 and 0.30 h^{-1} dilution rates. The samples were harvested every 8 hours from the 5th port on both sides of the column. After the 2 day of the operation with the

dilution rate of 0.30 h^{-1}, the dilution rate was rolled back to the start point (0.11 h^{-1}) for stability checking of the cell activities.

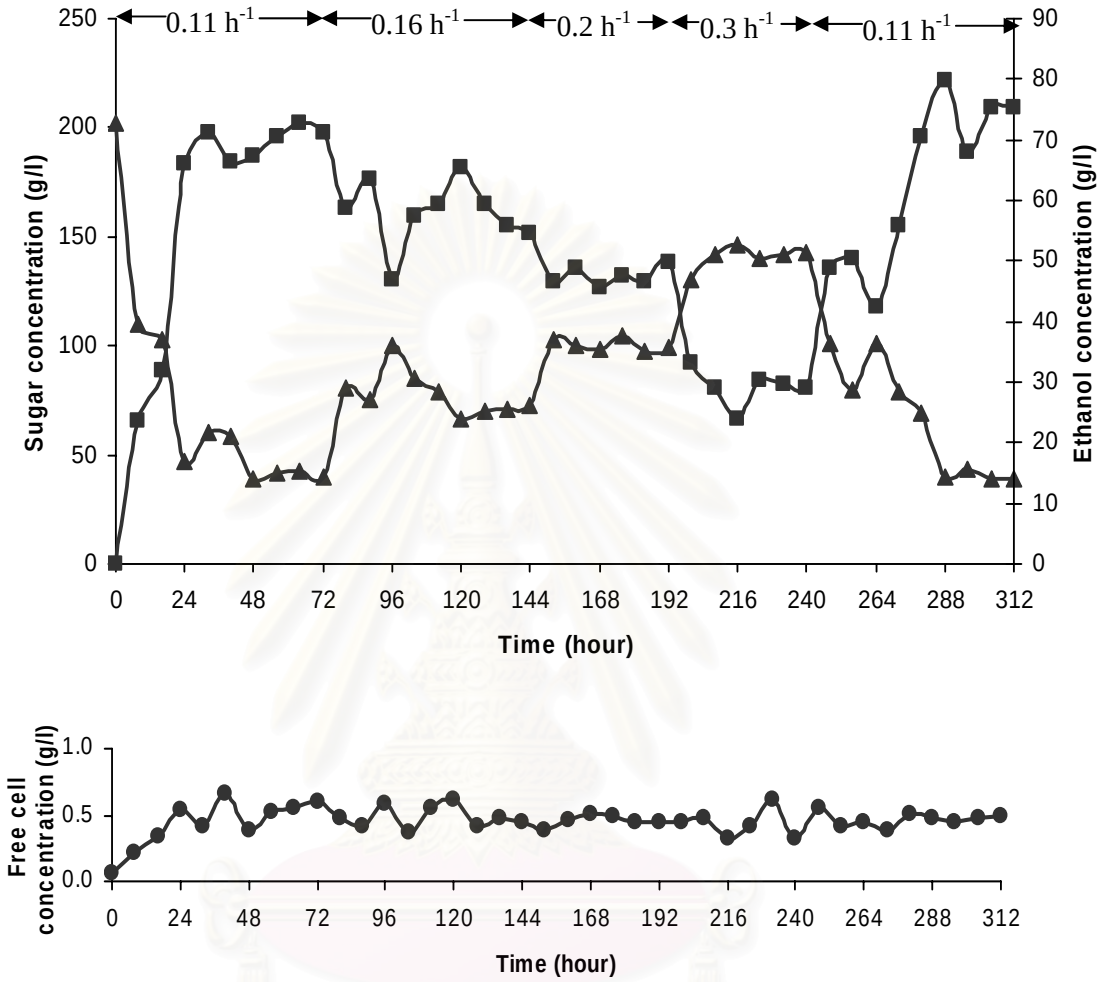


Figure 4.7 Continuous ethanol production in an immobilized cell reactor with initial sugar concentration 202 g/l (-▲- = sugar, -■- = ethanol, and -●- = free cell)

After the steady state of continuous fermentation under the dilution rate of 0.11, 0.16, 0.20 and 0.30 h^{-1}, the average ethanol concentration in the effluent decreased from 71.56 g/l to 56.71 g/l, 48.01 g/l and 29.81 g/l, respectively, whereas, the residual sugar concentration increased from 29.08 g/l, to 72.16 g/l, 113.71 g/l and 142.48 g/l, respectively. Yu et al. (2007) reported on a novel immobilization method of *S. cerevisiae* using sorghum bagasse in packed-bed reactor with the initial sugar concentration of 200 g/l. The maximum ethanol concentration 94.7 g/l was obtained at 0.10 h^{-1} dilution rate with the productivity of 9.5 g/l h (about 1.6 folds compared to

that from the batch process) [12]. The ethanol productivity of 7.87 g/l h was obtained from this work. The major cause of the difference in those results could be due to the variation in the yeast strains. In comparison to the result from the batch cultures, the operation in the pack bed column, using the cells immobilized in alginate-loofa matrix could increase the productivity by 4.2 folds, which was considerable higher relatively to the previous report [12-14]. Moreover, the steady state ethanol concentration in the effluent of the packed bed column obtained from this work was relatively higher than that from many other reports [1, 11, 15-18].

The ethanol fermentation was performed for 13 days and the yeast cells in the reactor were found to be effective and stable within the alginate-loofa matrix through the entire cultivation. This could be confirmed from the results of the ethanol concentration and the sugar consumption rate when the dilution was turned back to 0.11 h^{-1}. There was no difference in ethanol concentration at steady state of the operation at 0.11 h^{-1} dilution rate either at the initial period (48-72 h of cultivation times) or at the end period (288-312 h of cultivation times).

At the end of each dilution rate, the samples were harvested at 5 posts of the bed (5 HRT) (Figure 4.8 and Figure 4.9). The experimental results of each dilution rates showed the increase of ethanol concentration and the decrease of residual sugar concentration with HRT. However, during the operation with the dilution rate in the range of 0.16 -0.30 h^{-1}, at an identical retention time, the concentrations of ethanol and residual sugar remained constant not depending on the variation of the dilution rates. The ethanol concentration profile slightly decreased and the residual sugar concentration profile slightly increased only when the dilution rate was decreased to 0.11 h^{-1}. It could be explained that at a constant working volume, the feed flow rate increased with the dilution rate; the external mass transfer resistance or the resistance of the film layer between the immobilized cell matrix and the solution phase, therefore, increased at a lower dilution rate operation. With the higher mass transfer resistance, the limitation of substrates diffusion could occur and caused the reduction in the overall rates of ethanol production and sugar consumption. According to the result, it could be conclude that in this system, the minor effect of external mass transfer resistant took place at the dilution rate was less than 0.16 h^{-1}. Overall, under steady state condition, there was at a substantial expense of a residual sugar concentration in the effluent. Nonetheless, the nearly complete utilization of the

fermented sugar was obtained at 9.1 h HRT of the 0.11 h^{-1} dilution rate with the average ethanol concentration of 71.56 g/l.

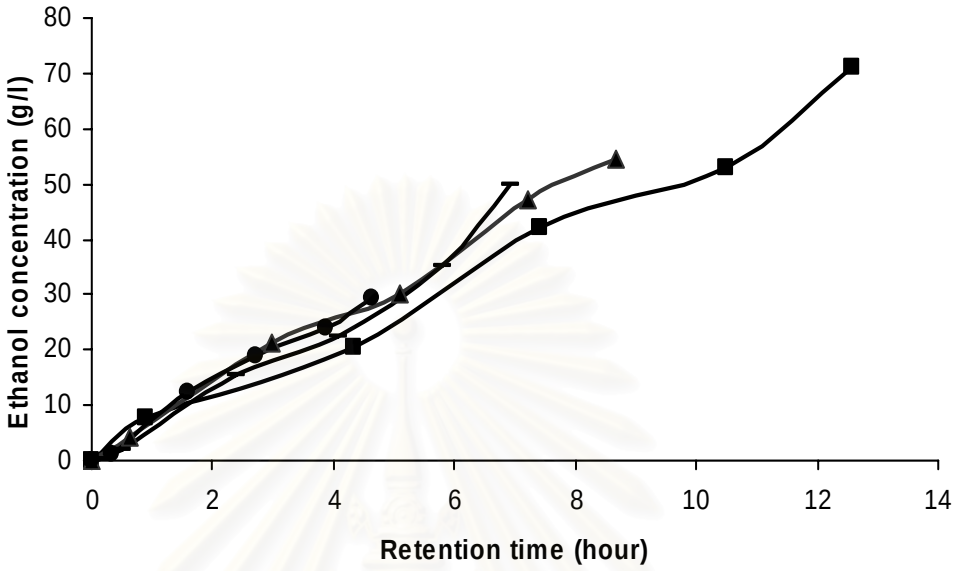


Figure 4.8 The steady state ethanol concentration of 4 dilution rates with initial sugar concentration of 202 g/l (-■- = 0.11h^{-1}, -▲- = 0.16 h^{-1}, ─ = 0.20 h^{-1} and -●- = 0.30 h^{-1})

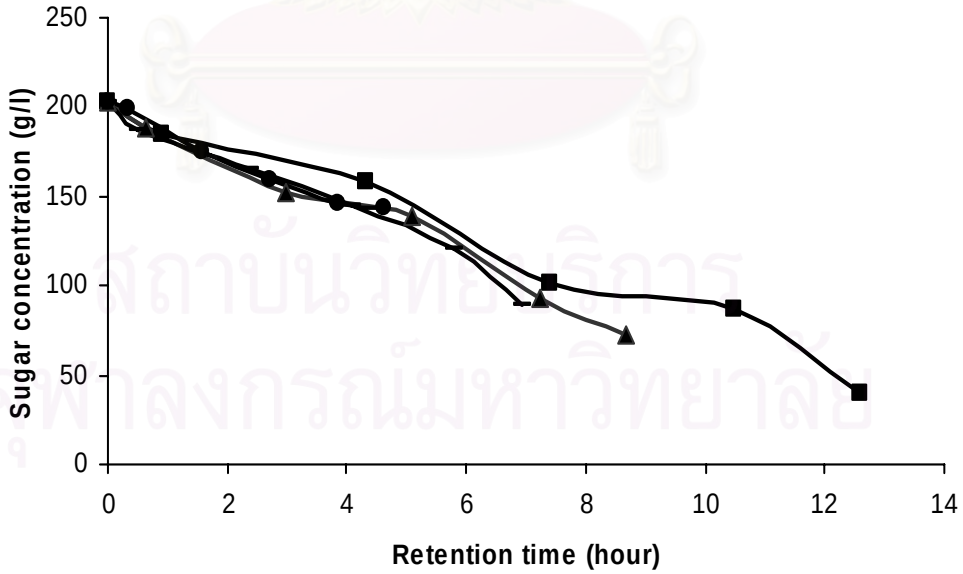


Figure 4.9 The steady state residue sugar concentration of 4 dilution rates with initial sugar concentration of 202 g/l (-■- = 0.11h^{-1}, -▲- = 0.16 h^{-1}, ─ =0.20 h^{-1} and -●- = 0.30 h^{-1})

Table 4.5 The steady state ethanol fermentation of 4 dilution rates with the initial sugar concentration of 202 g/l

Dilution rate (h^{-1})	Retention time (hour)	Ethanol concentration (g/l)	Sugar concentration (g/l)	Productivity (g/l h)
0.11	0.00	0.00	202.30	
0.11	0.90	7.70	185.20	8.56
0.11	3.12	20.47	158.50	6.56
0.11	5.35	41.99	101.10	7.85
0.11	7.57	52.86	86.60	6.98
0.11	9.09	71.28	39.85	7.84
0.16	0.00	0.00	202.30	
0.16	0.62	4.20	188.80	6.77
0.16	2.15	21.10	152.70	9.81
0.16	3.68	30.20	138.90	8.21
0.16	5.21	46.96	93.40	9.01
0.16	6.25	54.67	72.40	8.75
0.20	0.00	0.00	202.30	
0.20	0.49	1.80	187.80	3.67
0.20	1.72	15.49	165.24	9.01
0.20	2.94	22.41	144.80	7.62
0.20	4.17	35.15	120.68	8.43
0.20	5.00	49.76	89.62	9.95
0.30	0.00	0.00	202.30	
0.30	0.33	1.30	199.24	3.94
0.30	1.14	12.40	175.57	10.88
0.30	1.96	19.03	160.01	9.71
0.30	2.78	24.11	145.55	8.67
0.30	3.33	29.20	144.28	8.77

The yeast cell concentration in the effluent remained nearly constant (0.4-0.7 g/l) over the applied range of dilution rates and was lower than that in the batch fermentation (0.9 g/l). The result showed that yeast cells were confined by the bed causing only a few of free cells leaving from the reactor. However, it was difficult to determine the actual yeast cell concentration in the reactor at all times. At the end of fermentation, the free cell and immobilized cell concentrations in the reactor were investigated (Table 4.6). Since almost suspended cells were restricted by the bed, the amount of free cell concentration in the packed-bed reactor after continuous fermentation for 312 h was considerably higher in comparison with that in the batch fermentation for 72 h. The ratio of the free cell concentration in the bed compared to that in the effluent was more than 20.

Table 4.6 Yeast cell concentrations at the end of the continuous ethanol fermentation # 1.

Cell concentrations	**(g/l)**
Immobilized cell	16.03
Free cell in reactor	12.34
Free cell in effluent	0.55
Immobilized yield (%)	56.5

This performance exhibited no contamination by another microorganism. This could be due to the high amount of the yeast cells dominating in the system and at some level; ethanol produced by the yeast cells could inhibit the growth of contaminating microorganisms.

4.4 Continuous ethanol fermentation #2

For the continuous ethanol fermentation # 2, the initial sugar concentration 222 g/l was used for feeding into bottom of the packed bed reactor by peristaltic pump. The studied conditions of the system were exactly similar to the continuous ethanol fermentation # 1 (32 ± 1 ^{o}C, initial pH 5, working volume 0.64 liters, dilution

rate 0.11, 0.16, 0.20 and 0.30 h^{-1} and the samples were harvested every 8 hours). Since the initial sugar concentration increased to 222 g/l, in order to reach the steady state of ethanol concentration, the operation time of 0.11 h^{-1} dilution rate was increased from 3 days to 4 days. From the experimental results, the steady state was reached for all 4 dilution rates (Figure 4.10).

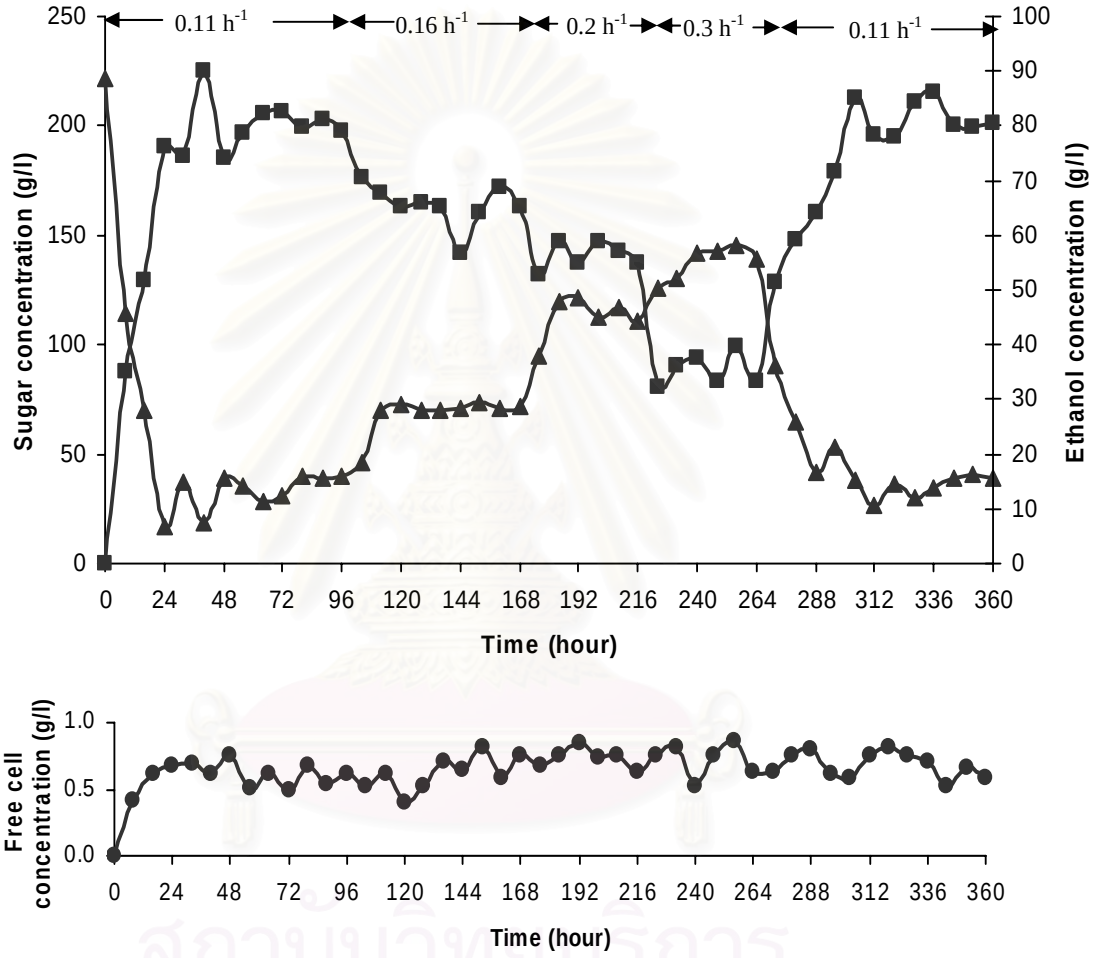


Figure 4.10 Continuous ethanol production in an immobilized cell reactor with initial sugar concentration 222 g/l (-▲- = sugar, -■- = ethanol, and -●- = free cell)

Figure 4.10 shows that the ethanol concentrations in the continuous ethanol fermentation # 2 for 4 dilution rates are higher than those obtained in the continuous ethanol fermentation #1 while the residual sugar concentrations of two systems are evenly. It exhibited the increase of sugar consumption with increasing the initial sugar concentration from 202 to 222 g/l. The steady state ethanol concentrations at dilution

rates of 0.11, 0.16, 0.20 and 0.30 h^{-1} were 81.29, 66.06, 53.69 and 35.38 g/l, respectively with the residual sugar concentration of 41.39, 71.27, 100.60, and 144.73 g/l, respectively. The ethanol and residual sugar concentrations of 5 posts of the reactor were demonstrated in Figure 4.11 and Figure 4.12, respective.

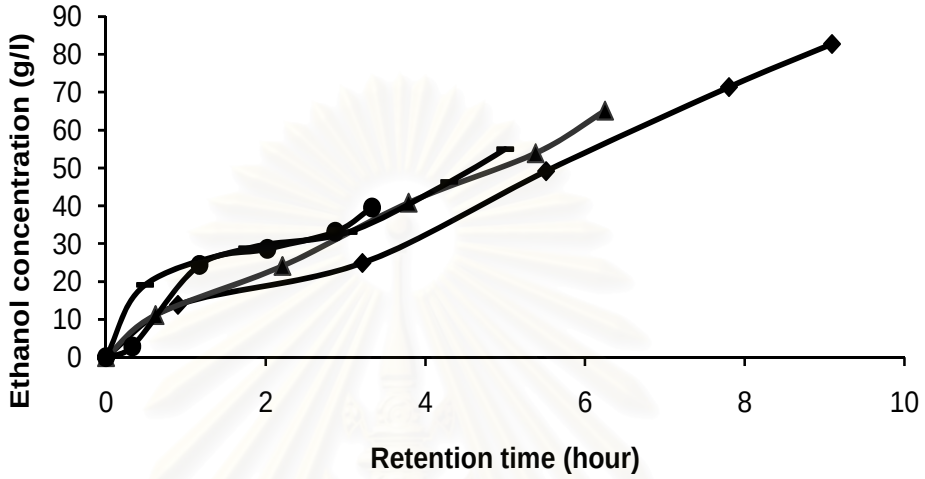


Figure 4.11 The steady state ethanol concentration of 4 dilution rates with initial sugar concentration 222 g/l (-■- = 0.11h^{-1}, -▲- = 0.16 h^{-1}, ━ = 0.20 h^{-1} and -●- = 0.30 h^{-1})

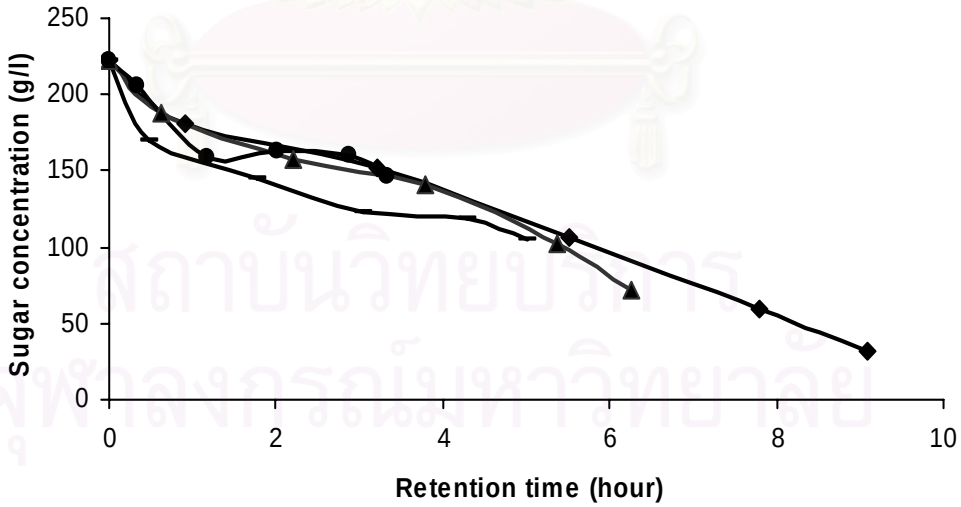


Figure 4.12 The steady state residue sugar concentration of 4 dilution rates with initial sugar concentration of 222 g/l (-■- = 0.11h^{-1}, -▲- = 0.16 h^{-1}, ━ =0.20 h^{-1} and -●- = 0.30 h^{-1})

Table 4.7 The steady state ethanol fermentation of 4 dilution rates with 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)
0.11	0.00	0.00	222.00	
0.11	0.90	13.81	180.55	15.34
0.11	3.12	24.88	152.45	7.97
0.11	5.35	49.13	106.59	9.18
0.11	7.57	71.34	59.21	9.42
0.11	9.09	82.72	31.22	9.10
0.16	0.00	0.00	222.00	
0.16	0.62	11.11	187.77	17.92
0.16	2.15	24.08	157.58	11.20
0.16	3.68	40.79	140.24	11.08
0.16	5.21	53.87	102.55	10.34
0.16	6.25	65.11	72.01	10.42
0.20	0.00	0.00	222.00	
0.20	0.49	19.10	169.91	38.98
0.20	1.72	28.76	145.25	16.72
0.20	2.94	33.07	122.55	11.25
0.20	4.17	46.27	118.55	11.10
0.20	5.00	54.97	105.58	10.99
0.30	0.00	0.00	222.00	
0.30	0.33	2.84	205.47	8.61
0.30	1.14	24.38	158.97	21.39
0.30	1.96	28.61	162.46	14.60
0.30	2.78	33.13	159.86	11.92
0.30	3.33	39.55	145.80	11.88

The system was operated for 15 days. At the end of the fermentation, the free cell concentrations in the reactor and in the effluent were 10.67 and 0.66 g/l, respectively. The immobilized cell concentration was 15.32 and the immobilized yield was 59.0 %. The values of cell concentrations were similar to those of the continuous ethanol fermentation #1 (Table 4.8).

Table 4.8 Yeast cell concentrations at the end of the continuous ethanol fermentation # 2

Cell concentrations	**(g/l)**
Immobilized cell	15.32
Free cell in reactor	10.67
Free cell in effluent	0.66
Immobilized yield (%)	59.0

These advantages suggest that this biosystem is capable of producing high ethanol concentration in continuous operation without contamination. The productivity at high ethanol concentration could be further increased by increasing the initial sugar concentration. Also, more stable continuous ethanol production from molasses may be attained by achieving uniform liquefaction.

In order to increase the ethanol concentration, higher initial sugar concentration (about 250 g/l) using 2 packed bed reactors was employed for continuous ethanol production in the next study.

4.5 Continuous ethanol fermentation # 3

The initial sugar concentration about 248 g/l was used to study the conversion yields of ethanol concentration in the continuous ethanol fermentation # 3. The system was operated under conditions that were identical to the continuous ethanol concentration # 2. The series reactor could be used to enhance the ethanol productivity [15]. In this experimental study, two packed beds connected in series were used to enhance total amount of sugar consumption so as to increase the final ethanol concentration. A combined bioreactor system, composed of a packed-bed

reactor and two-stage bioreactor in series, was established. The combined bioreactor system was shown to be capable of producing ethanol under very high gravity condition using the yeast *S. cerevisiae*; the highest and average ethanol concentrations were achieved over a 2 month continuous operation [19]. The results of ethanol fermentation carried out in the two packed bed column connected in series with the variation of dilution rates from 0.11, 0.16, 0.20 and 0.30 h^{-1} were demonstrated in Figure 4.13 for the 1st packed bed and Figure 4.14 for the 2nd packed bed.

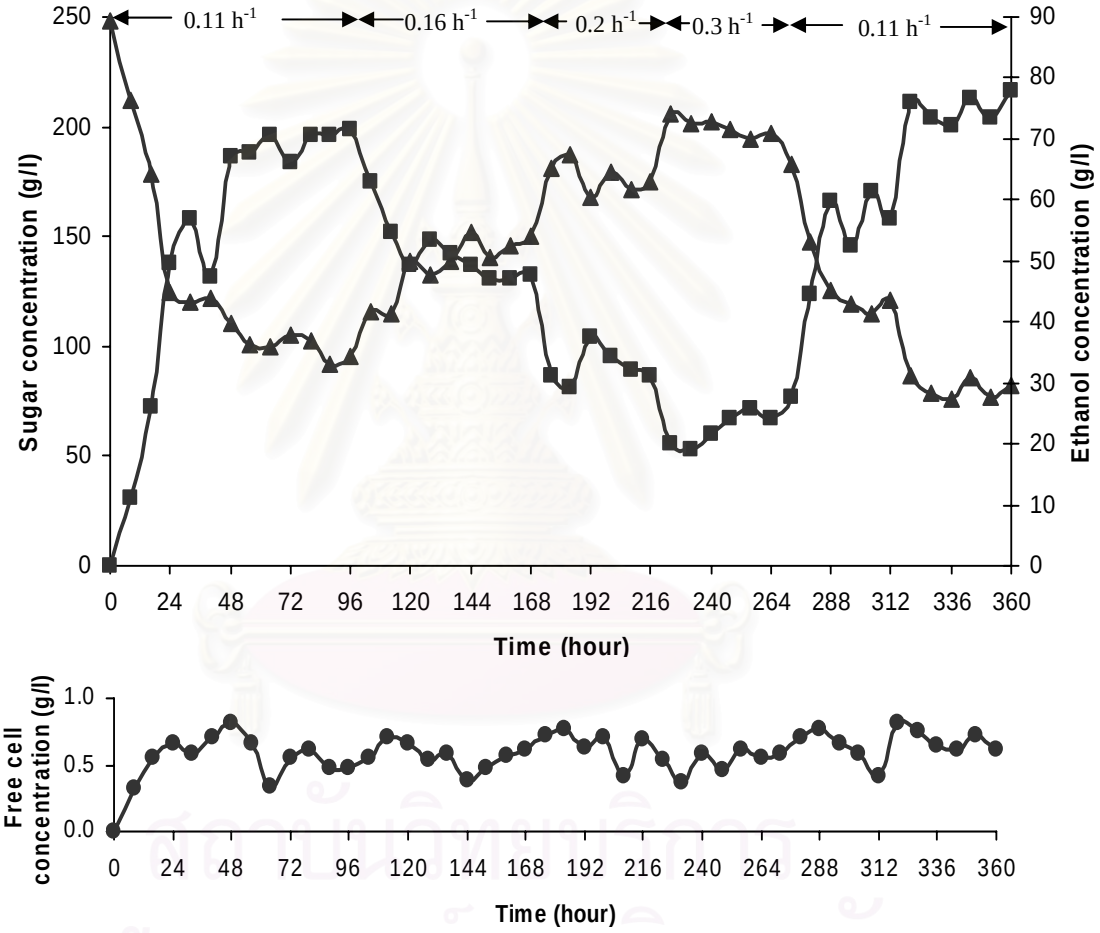


Figure 4.13 Continuous ethanol production of the 1st reactor with initial sugar concentration 248 g/l (-▲- = sugar, -■- = ethanol, and -●- = free cell)

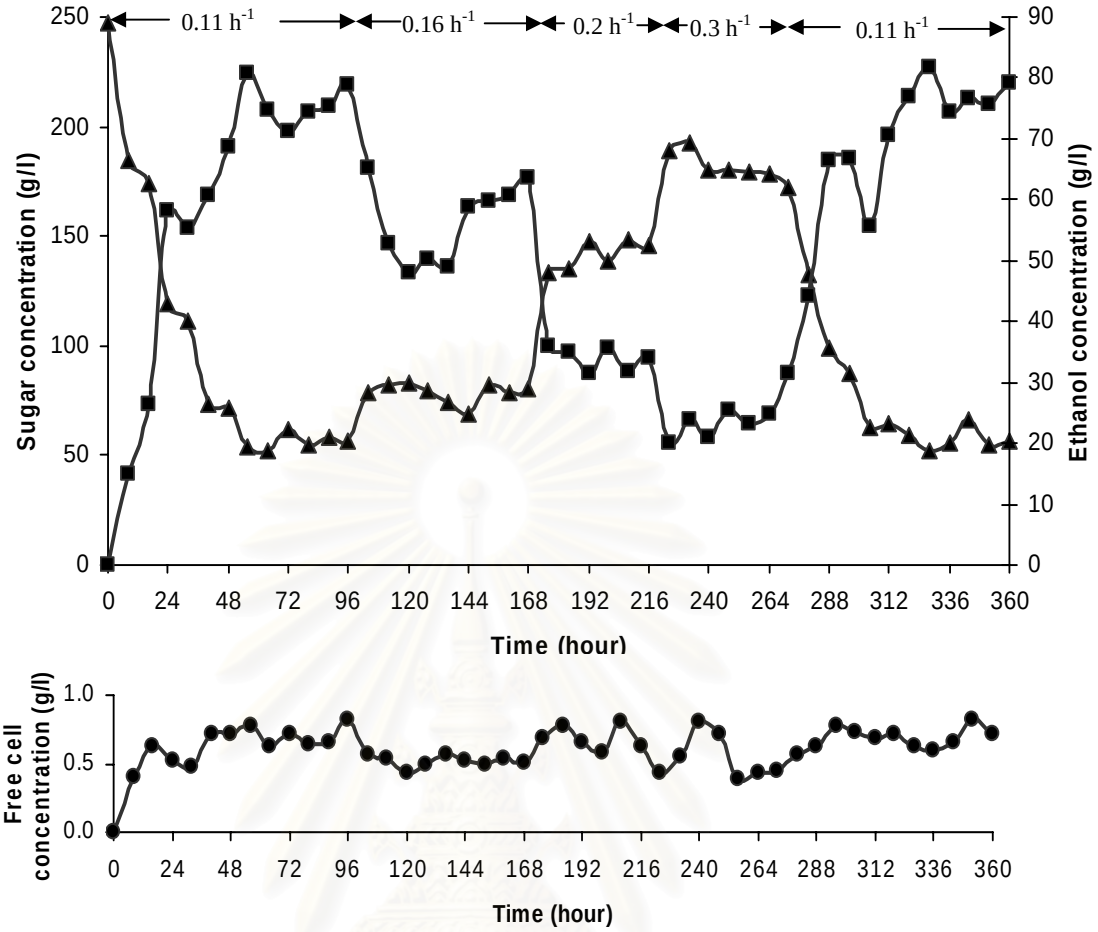


Figure 4.14 Continuous ethanol production of the 2nd reactor with initial sugar concentration 248 g/l (-▲- = sugar, -■- = ethanol, -●- = free cell)

Figure 4.13 shows that the ethanol concentrations at steady state for dilution rates 0.11, 0.16, 0.20 and 0.30 h^{-1} are 69.86, 47.30, 33.91 and 24.25 g/l, respectively with the remained residual sugar concentration in the effluent of 98.50, 145.03, 174.87 and 192.90 g/l, respectively. The sugar conversions in this fermentation exhibited lower value than that obtained in the continuous ethanol fermentation # 1 and # 2. According to the result, it could be explained that the inhibition of cell activities at high initial sugar concentration at 248 g/l could occur. High sugar concentration in the system could cause the occurrence of cell death or inactive conditions including nutritional limitations or high toxic metabolite accumulates. High initial sugar concentration could result in growth inhibition by osmotic stress that occurred when the concentration of a certain solute become so high that a large

osmotic pressure gradient was established across the cell membrane [10]. This might be referred to the reaction rate of this fermentation that was lower than that in the continuous ethanol fermentation # 1 and # 2.

The effluent of the 1st packed bed column was fed to the bottom of the 2nd column. Nonetheless, the ethanol concentrations obtained from the effluent of the 2nd column only slightly increased compared to that of the 1st column (Figure 4.13, and Figure 4.14). The steady state ethanol concentration in the effluent obtained from the 2nd column at the dilution rate of 0.11, 0.16, 0.20 and 0.30 h^{-1} were 78.06, 61.36, 33.91 and 24.85 g/l, or about 11.7, 29.7, 0.0 and 0.0 % increasing from those of the 1st column, respectively. However, the residual sugar in the effluent of the 2nd column at the dilution rat of 0.11, 0.16, 0.20 and 0.30 h^{-1} were 56.95, 80.40, 145.53 and 178.84 g/l, respectively. It was not consistent when the ethanol production was compared with the sugar consumption. The overall conversion yield of ethanol was about 33 percents (maximum 51 percents).

Table 4.9 and Table 4.10 exhibit the cell concentrations for which the distinct result in the 1st and the 2nd reactor was observed. Although the values of the immobilized cell concentration and the free cell concentration of the effluent in the 1st and the 2nd column were similar, the free cell concentration remained in the 2nd reactor was indeed higher than that in the 1st reactor (increasing about 53.47 % or 8 g/l). Therefore, some of the consumed sugar in the 2nd column was used for cell production. In this experimental study, a large amount of foam occurred in the 1st column and the high speed of peristaltic pump was used to draw the effluent from the top of the 1st column and feed into the bottom of the 2nd column. With this operation, the air outside the reactor could flow passing the sterile membrane into the bottom of the 2nd column along with the feed. This operation would supply oxygen for the growth of yeast cell in the 2nd column. Moreover, there might be the limitation of ethanol fermentation in the 2nd column due to the inhibition effect of high ethanol. As a result, a large amount of consumed sugar was used to produce biomass by aerobic respiration in place of anaerobic fermentation of ethanol.

It could be noted that the final concentration of the immobilized cells and free cells in the effluent of the reactor feeding with high initial sugar (248 g/l) were similar to those of the ones with lower initial sugar concentration feeding (202 – 222 g/l). However, the increases of the free cell concentration in the column by 16.7- 40.0 %

and the decrease of the immobilized yield by 8.7-13.5 % in comparison to those of the 1st and the 2nd continuous fermentation were observed.

Table 4.9 Yeast cell concentrations at the end of the continuous ethanol fermentation # 3 in the 1st reactor

Cell concentrations	**(g/l)**
Immobilized cell	16.04
Free cell in reactor	14.48
Free cell in effluent	0.62
Immobilized yield (%)	52.6

Table 4.10 Yeast cell concentrations at the end of the continuous ethanol fermentation # 3 in the 2nd reactor

Cell concentrations	**(g/l)**
Immobilized cell	14.82
Free cell in reactor	22.26
Free cell in effluent	0.52
Immobilized yield (%)	40.0

Figure 4.15 and Figure 4.16 shows the steady state ethanol and residual sugar concentrations in the continuous ethanol fermentation # 3 for 4 dilution rates. At the same HRT, the obtained ethanol concentration was lower than those obtained in the continuous ethanol fermentation # 1 and # 2. In contrast to the results at the feed of initial sugar concentration 200-222 g/l, it exhibited the decrease of sugar consumption and ethanol production at the high dilution rate. Since there was the inhibition effect of high sugar concentration above 222 g/l on the cell activities, the effect of mass transfer resistance turned out to be positive for the ethanol fermentation. Therefore, higher ethanol production and higher sugar consumption were obtained at lower dilution rate in which the flow rate was inferior.

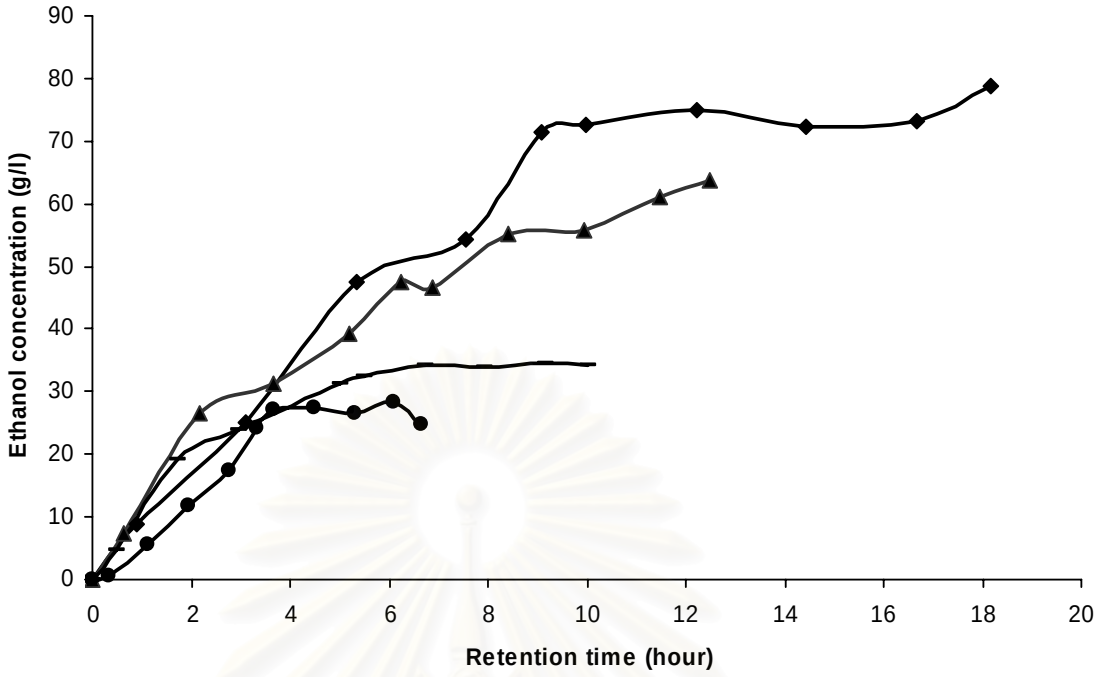


Figure 4.15 The steady state ethanol concentration of 4 dilution rates with initial sugar concentration of 248 g/l (-■- = 0.11 h^{-1}, -▲- = 0.16 h^{-1}, ▬ = 0.20 h^{-1} and -●- = 0.30 h^{-1})

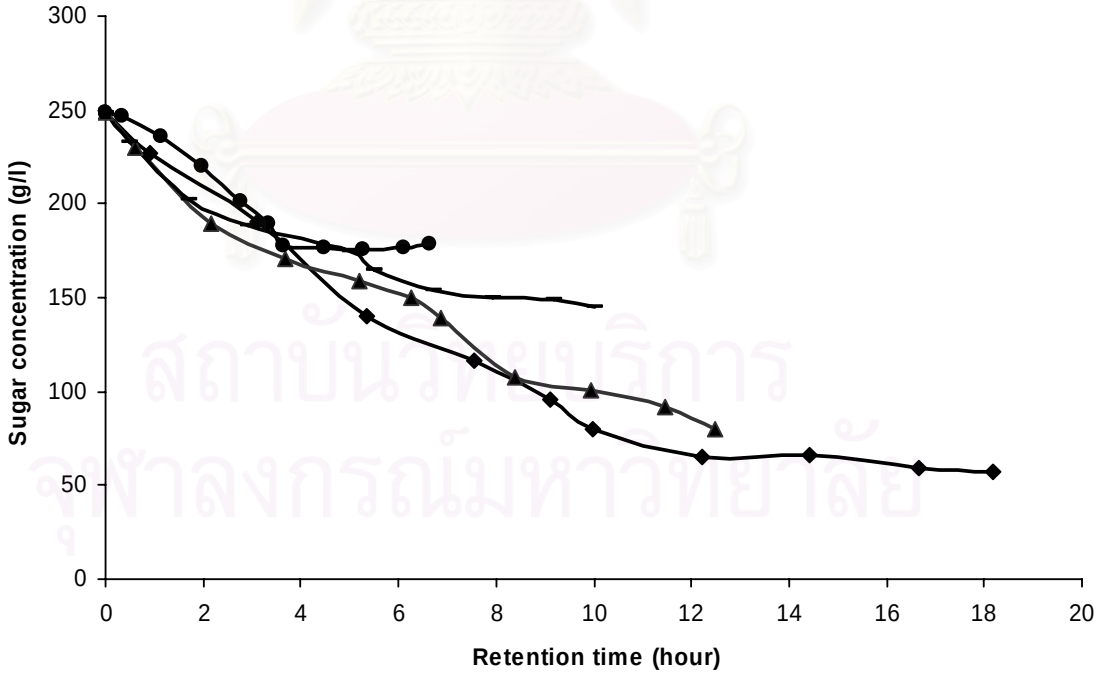


Figure 4.16 The steady state sugar concentration of 4 dilution rates with initial sugar concentration of 248 g/l (-■- = 0.11h^{-1}, -▲- = 0.16 h^{-1}, ▬ =0.20 h^{-1} and -●- = 0.30 h^{-1})

Table 4.11 The steady state ethanol and sugar concentrations of 4 dilution rates with initial sugar concentration 248 g/l

Dilution rate (hr^{-1})	Retention time (hour)	Ethanol concentration (g/l)	Sugar concentration (g/l)	Productivity (g/l h)
0.11	0.00	0.00	248.43	
0.11	0.90	8.78	226.50	9.76
0.11	3.12	25.02	190.10	8.02
0.11	5.35	47.48	140.00	8.87
0.11	7.57	54.31	116.50	7.17
0.11	9.09	71.45	95.50	7.86
0.11	9.99	72.65	80.05	7.27
0.11	12.21	74.90	65.54	6.13
0.11	14.44	72.41	66.48	5.01
0.11	16.66	73.32	59.46	4.40
0.11	18.18	78.86	56.95	4.34
0.16	0.00	0.00	248.43	
0.16	0.62	7.27	230.20	11.73
0.16	2.15	26.56	189.50	12.35
0.16	3.68	31.34	171.10	8.52
0.16	5.21	39.39	158.80	7.56
0.16	6.25	47.59	149.88	7.61
0.16	6.87	46.72	138.83	6.80
0.16	8.40	55.08	107.77	6.56
0.16	9.93	55.68	100.85	5.61
0.16	11.46	60.94	92.25	5.32
0.16	12.50	66.06	80.40	5.09

Dilution rate (hr^{-1})	Retention time (hour)	Ethanol concentration (g/l)	Sugar concentration (g/l)	Productivity (g/l h)
0.20	0.00	0.00	248.43	
0.20	0.49	4.71	233.30	9.61
0.20	1.72	19.08	202.20	11.09
0.20	2.94	23.96	188.80	8.15
0.20	4.17	38.92	155.50	9.33
0.20	5.00	31.18	174.50	6.24
0.20	5.49	32.46	164.43	5.91
0.20	6.72	34.35	154.42	5.11
0.20	7.94	33.85	150.01	4.26
0.20	9.17	34.54	148.89	3.77
0.20	10.00	34.10	145.53	3.41
0.30	0.00	0.00	248.43	
0.30	0.33	0.73	246.62	2.21
0.30	1.14	5.46	235.51	4.79
0.30	1.96	11.93	220.50	6.09
0.30	2.78	17.54	201.10	6.31
0.30	3.33	24.14	189.87	7.25
0.30	3.66	27.19	177.72	7.43
0.30	4.47	27.50	176.64	6.15
0.30	5.29	26.60	175.53	5.03
0.30	6.11	28.21	176.23	4.62
0.30	6.66	24.76	178.84	3.72

4.6 Productivity and Stability of continuous ethanol fermentation

From continuous ethanol fermentation # 1, # 2, and # 3, the productivities were obtained as shown in Figure 4.17. The optimum productivity could be obtained with the suitable dilution rate and initial sugar concentration [12, 15 and 19]. The maximum productivity was obtained in continuous ethanol fermentation # 2 (8.94, 10.57, 10.74, and 10.62 g/l h of dilution rates 0.11, 0.16, 0.20 and 0.30 h^{-1}, respectively). The optimal of initial sugar concentration was 222 g/l.

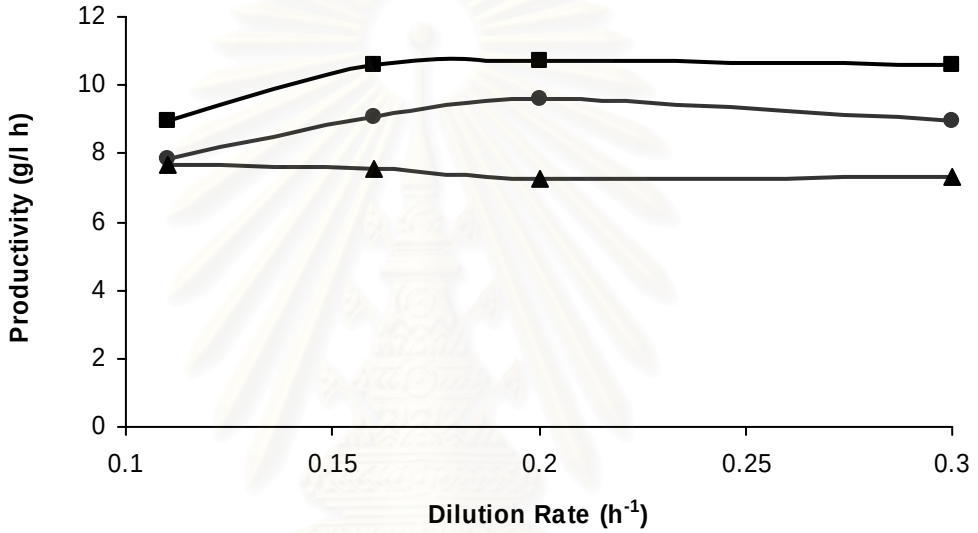


Figure 4.17 The productivities of ethanol fermentation at steady state for the initial sugar concentration of 202 g/l (-●-), 222 g/l (-■-) and 248 g/l (-▲-)

To confirm the consistency of performance and stability of yeast cells, the initial sugar concentration about 228 g/l was used for 30 days continuous fermentation at dilution rate of 0.11 h^{-1} (Figure 4.18). The performance of cell immobilization in alginate-loofa cube for ethanol fermentation was confirmed by satisfactory operational stability during the long term continuous fermentation.

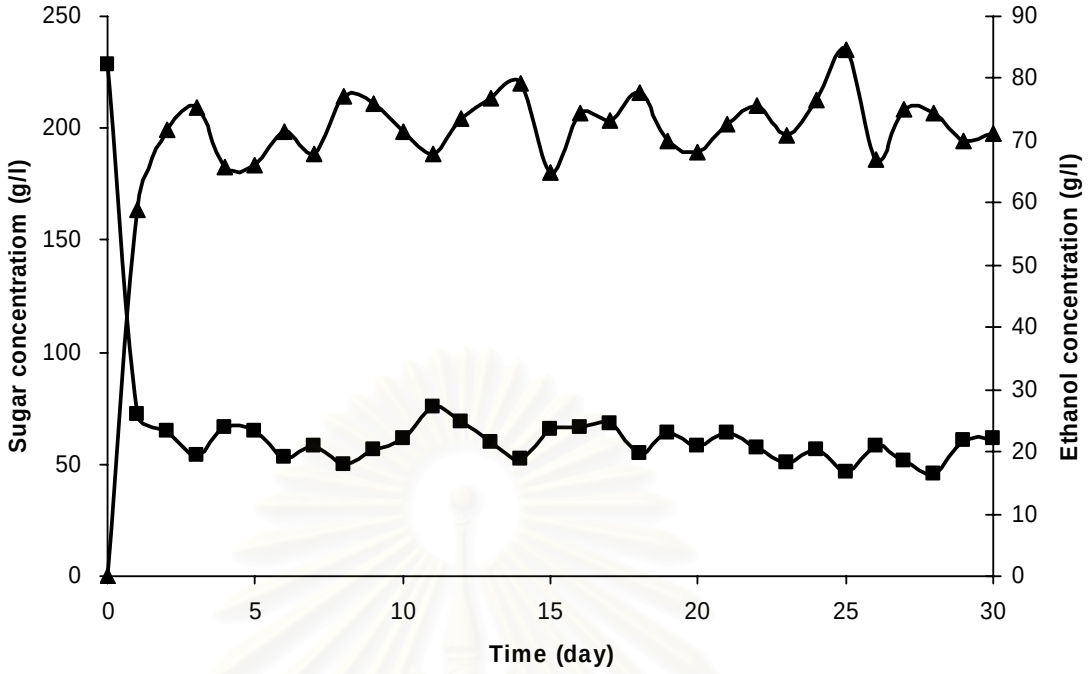


Figure 4.18 The activity of yeast cells in ethanol fermentation at steady state for initial sugar concentration about 228 g/l and dilution rate of 0.11 h^{-1} (-■- = Sugar and -▲- = Ethanol)

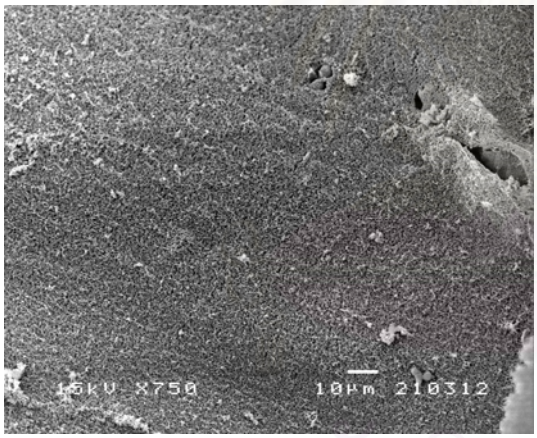
Figure 4.18 exhibits the activity of yeast cells. There was no apparent decline in productivity over 30 days of continuous operation and the average productivity was 8.36 g/l h. The long term performance of the reactor was found to offer a better alternative to packed-bed reactor [11, 12, 13, 15, 16, 19, 20 and 21]. Furthermore, the average conversion yield was about 0.43 throughout the entire operation. It should be noted that in the present study, the operation stoppage after 30 days was due to the experimental plan but not the occurrence of decaying sign in the system.

The cell concentrations in the reactor were determined after 30 days of the fermentation. Although the cell concentration in the effluent in this study was comparable to the other runs, the cell concentrations in the reactor (immobilized cell and free cell) were higher than those in the reactor of continuous ethanol fermentation # 1, # 2 and # 3. The result supported that there was cell regeneration in the packed column throughout the long term fermentation. With the increase of fermentation time, therefore, higher cell concentrations in the reactor and in the effluent were obtained.

Table 4.12 Yeast cell concentrations at the end of 30 days performance

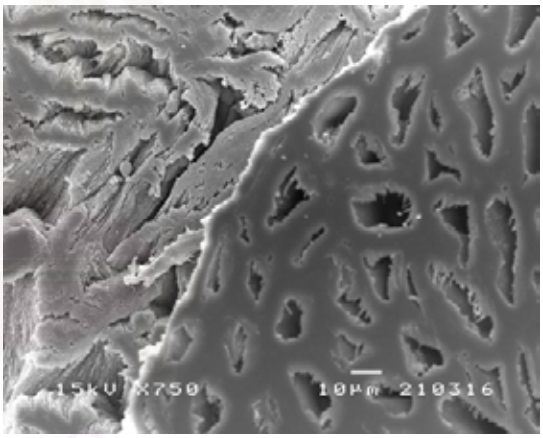
Cell concentrations	**(g/l)**
Immobilized cell	17.71
Free cell in reactor (alive cell)	19.64 (80%)
Free cell in effluent (alive cell)	0.78 (30%)
Immobilized yield (%)	42.2

In this study, alginate and loofa sponge was mutually used for cell immobilization. Loofa sponge alone was not adequate for yeast immobilization. In the same way, alginate alone can not restrict cells for long term performance because of cell growth and CO_2 occurred from the reaction [3]. A scanning electro microscope (SEM) was used to compare the images of the carriers at the completed gelation, the beginning of ethanol fermentation and the end of fermentation.



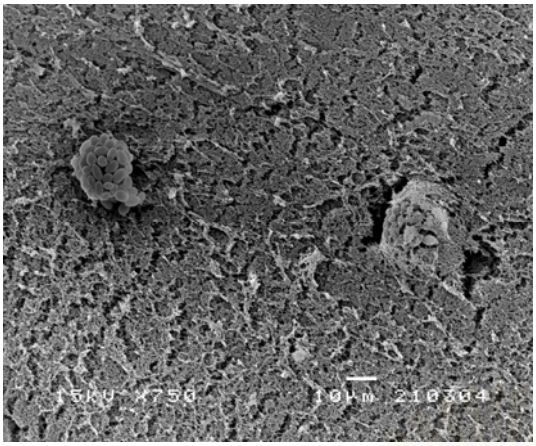
X 750

Figure 4.19 EALC surface at the completed gelation



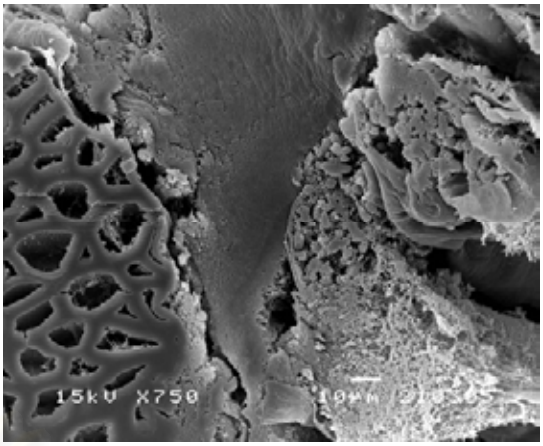
X 750

Figure 4.20 EALC cross section at the completed gelation



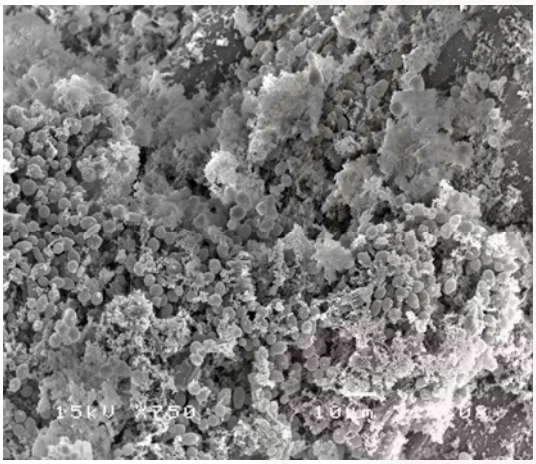
X 750

Figure 4.21 EALC surface at the beginning of ethanol fermentation



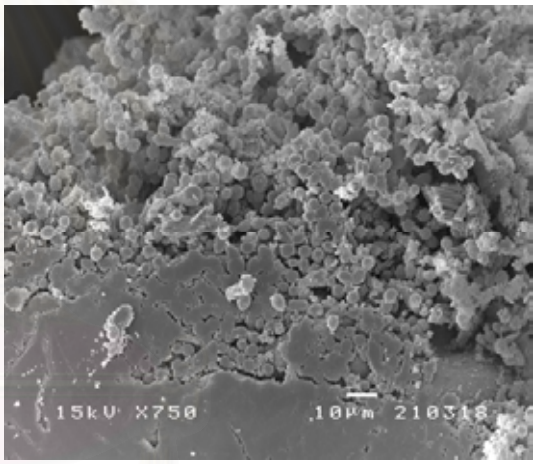
X 750

Figure 4.22 EALC cross section at the beginning of ethanol fermentation



X 750

Figure 4.23 EALC surface at the end of fermentation

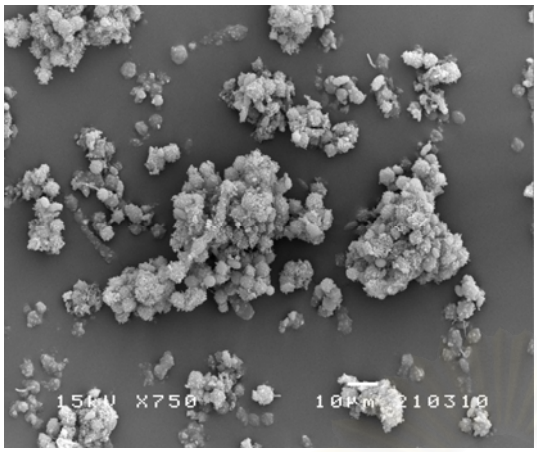


X 750

Figure 4.24 EALC cross section at the end of fermentation

Figure 4.19 to Figure 4.24 indicate the incidence of cell growth and gel degradation during the course of fermentation. The images of carrier from the beginning of performance to the end of performance showed that the amount of cell inside the carrier from time to time was increasing. Yeast cells in carrier were entrapped by film of alginate. For long term performance, severe degradation of alginate films as a result of cell growth and production of CO_2 followed by cell leakage occurred. Such leak can be observed from free cells in the reactor and free cells in the effluent (Figure 4.25 and Figure 4.26).

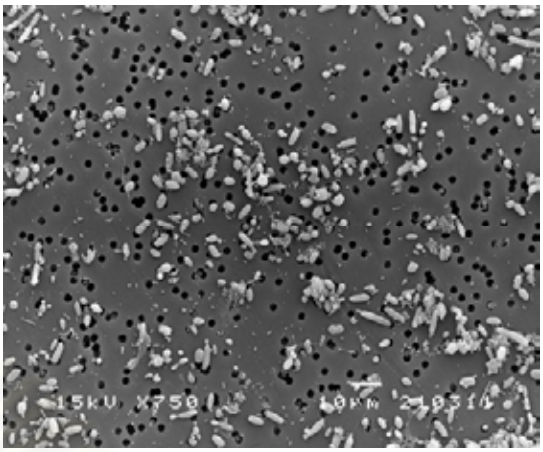
80 % alive cell



X 750

Figure 4.25 Free cell in the reactor

30 % alive cell

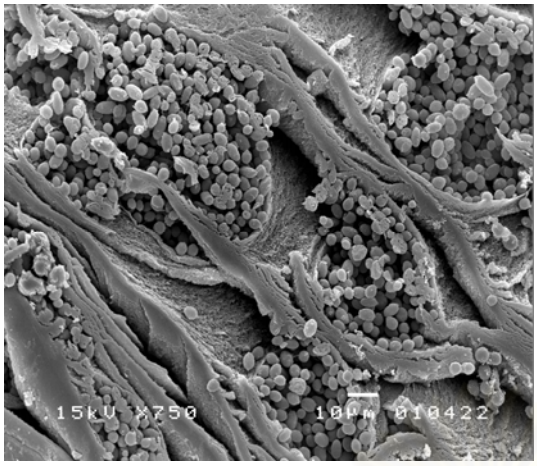


X 750

Figure 4.26 Free cell in the effluent

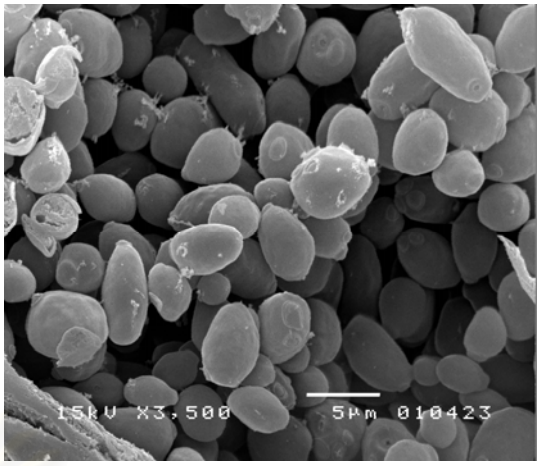
Figure 4.25 and Figure 4.26 show the image of suspended cell culture in the reactor and in the effluent. Figure 4.25 revealed that the cells in the reactor appeared healthy and retained their normal oral shape. In addition, the aggregation of yeast cells with filament connecting was observed. On the other hand, the cells in the effluent (Figure 4.26) were much smaller, not healthy and no aggregation.

Inspection of alginate-loofa carrier cross section in Figure 4.27 demonstrates that yeast can grow well inside the alginate layer. This confirmed that the yeast cells could gain access to substrate needed for growth even though they were located deep inside the alginate-loofa carrier. In addition, the cell was healthy as confirmed by close look image in Figure 4.28. Moreover, there were filaments connecting the cell and the support (Figure 4.29 to Figure 4.32). It is proposed that this filamentous structure promoted firm attachment of cell to support and cell to cell.



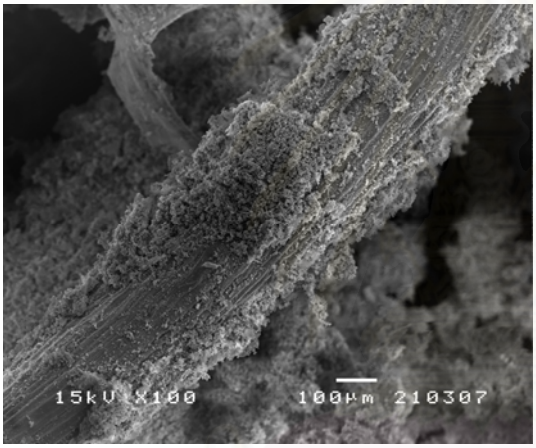
X 750

Figure 4.27 Yeast cell inside the alginate layer 1



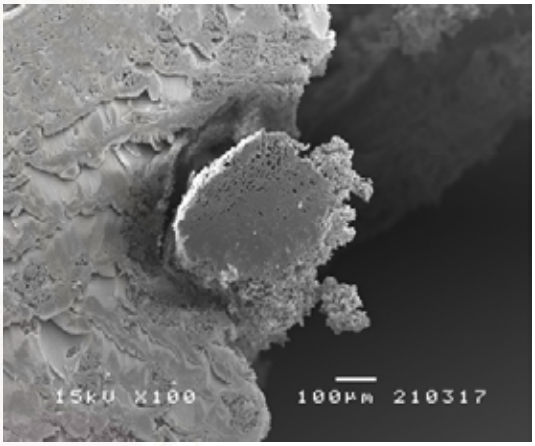
X 3,500

Figure 4.28 Yeast cell inside the alginate layer 2



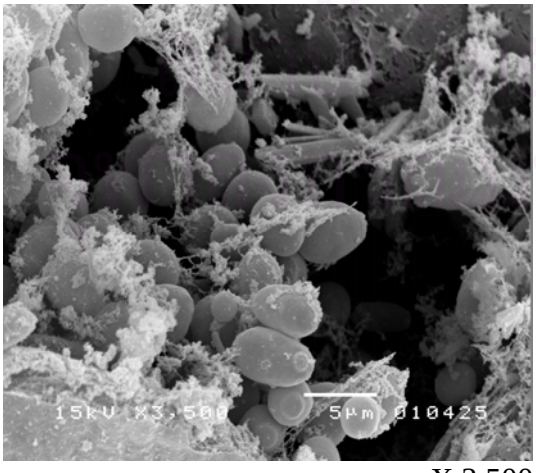
X 100

Figure 4.29 EALC surface 1



X 100

Figure 4.30 EALC cross section



X 3,500

Figure 4.31 EALC surface 2

X 10,000

Figure 4.32 EALC surface 3

The experimental result revealed that the alginate-loofa matrix was a cell carrier with favorable mechanical properties and porous structure resulting in a fairly stable operation and high ethanol production over the course of 30 days of continuous fermentation. Therefore, this carrier was successfully applied for yeast immobilization in packed bed column using molasses as the carbon source in continuous fermentation.

CHAPTER V

CONCLUSION

The data presented in this thesis shows that continuous production of ethanol from molasses using immobilized cell of *Saccharomyces cerevisiae* M30 entrapped in loofa reinforced alginate is promising in view of high conversions and high stability. Compared to the batch fermentation, higher ethanol productivity (5.58 folds) was obtained with packed-bed reactor. By using alginate-loofa cube for cell immobilization, efficient large size of 20 x 20 x 3 mm^3 could be used for cell carrier construction. Continuous ethanol production in a packed-bed reactor was successfully carried out with 202 - 222 g/l of initial sugar concentrations. On the other hand, at 248 g/l of initial sugar concentrations, the inhibition of yeast cells was occurred. The optimum of productivity (10.57 g/l h) and ethanol concentration (66.06 g/l) were obtained with 222 g/l of initial sugar concentration at 0.16 h^{-1} dilution rate and maximum of ethanol concentration 81.29 g/l was obtained with 222 g/l of initial sugar concentration at 0.11 h^{-1} of dilution rate. The packed-bed reactor worked efficiently and was stable for at least 30 days.

With a strong and porous structure of the developed carrier, potential advantages including reusability, altered mechanical strength, cell regeneration and high capacity to trap alive cells in the reactor were achieved. In this study, it was shown that ethanol could be produced from molasses by *S. cerevisiae* M30 in alginate-loofa carrier with a high yield and a high production rate. Indeed, there are some more works could be done to improve this technology for practical applications. For example, development of yeast cell which could be tolerate to high ethanol and high sugar concentration. Further explorations of ethanol producing microorganism which could be able to use low cost agricultural waste such as cellulose as carbon source or could be operated at higher temperature are also required for the technology development.

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APPENDICES

APPENDIX A
EXPERIMENTAL METHODS

A-1 Agar slants preparation

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

1. Mix 7.8 g PDA powder with 200 ml de-ionized (DI) water in 500 ml glass beaker.
2. Stir the solution with magnetic stirrer and heat it up until it is boiling.
3. Boil the solution for 1 minute or until all powder is dissolved as indicated by the formation of clear yellowish agar solution.
4. Transfer ±5 ml agar solution into 16 x 150 mm screw cap culture tube by using 10 ml pipette.
5. Sterilize all agar containing tubes at 121ºC for 20 minutes in autoclave. (Set the tube's cap to be rather loose before autoclaving to facilitate gas expansion inside the tube during sterilization.)
6. After sterilization, tighten the tube's cap and let the tubes to cool down before positioning them in slanted position to obtain agar slant inside the tubes.
7. Precautions:
   a) PDA agar powder is hygroscopic. Minimize exposure time of the powder to the ambient air to avoid excess water absorption.
   b) Sterilization is carried out at high temperature. Wear heat resistant gloves as protection when handling hot materials.
   c) When slanting the agar, provide enough space between tube neck and agar to minimize the risk of contamination from outside the tube.

A-2 Stock cultures preparation

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

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

A-3 Medium preparation

There were 2 varieties of medium used in this study. One was designated for cell cultivation and the other was for ethanol production. 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 50 and 200 g/l for cell cultivation and ethanol production respectively. The resulting sugar solution had a brown color originated from the palm sugar. The color intensity increases with increasing sugar concentration. The amount of palm sugar required to achieve the target level of sugar was estimated from previous trial with 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 $(NH4)_2SO_4$ were added. The compositions were referred to the one which were used by ethanol producing industries. The pH value of the medium was adjusted to 5 with 0.1 M NaOH and HCl solution. The detailed procedures for medium preparation from palm sugar are listed in the following paragraph.

1. Mix palm sugar and nutrients. Add palm sugar until the desired sugar concentration (50 g/l for cell cultivation and 200 g/l for ethanol production) is achieved.
2. Adjust the pH of the solution to 5 by adding NaOH or HCl solution.
3. Pour appropriate volume of medium (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 $(NH4)_2SO_4$ was added as the sole supplement. Before sterilization, centrifugation of diluted molasses mash was necessary to prevent excess mud formation. The mud was created from suspended materials contained in molasses. Palm sugar was still used in inoculums development stage prior to ethanol fermentation. The quantity of molasses needed to reach the intended sugar level was also estimated by DNS trial. The procedures for preparing molasses based fermentation medium are follows:

1. Dilute the molasses mash to intended sugar level with DI water.

2. Centrifuge the solution with Kubota 7820 centrifuge at 2000 rpm for 15 minutes.

3. Mix the diluted sugar solution with appropriate amount of $(NH4)_2SO_4$.
4. Adjust the pH of to 5 with NaOH or HCl solution.
5. Fill 500 ml Erlenmeyer flask with 250 ml medium.
6. Close each flask with cotton plug before sterilization.
7. Autoclave the medium for 15 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 1 day at 150 rpm. After some time, the growing yeast cells could be noticed as brown colored suspended solids inside the sugar solution. The cells were then harvested and concentrated by medium draining. The complete steps are as follows:

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

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

A-5 Cell immobilization

Alginate solution was made by dissolving Na-alginate powder in NaCl 9 g/l solution to obtain clear viscous solution with concentration of 30 g/l. Loofa sponge and alginate solution used for entrapment of cells were sterilized with autoclave for 5 minutes at 121°C prior to usage. Alginate solution was mixed first with concentrated cell suspension before gelation. Formation procedures of alginate-loofa were listed in the following paragraph.

1. Mix concentrated cell suspension with alginate solution with volumetric ratio of 1:10.
2. Add the mixture using a syringe drop wisely into $CaCl_2$ 14.7 g/l solution to form alginate beads.
3. For reinforced gel formation, dip the loofa sponge into alginate solution and then drop it into the $CaCl_2$ solution.
4. Leave the gel to harden with mild stirring for 15 minutes.
5. Rinse the gel 3 times with NaCl 9 g/l solution.
6. When storing is needed, keep the gels in NaCl 9 g/l solution at 4°C.
7. Precautions and notes:
    a) All procedures are conducted aseptically in laminar flow hood.
    b) All equipments including the stirrer and syringe are cleaned and sterilized before use.

A-6 Ethanol fermentation

A-6.1 Bath fermentation

In palm sugar based medium, the composition of fermentation medium was similar with cell cultivation except that the sugar concentration was increased to optimum level of 220 g/l. In case of molasses based medium, 0.5 g/l ammonium sulfate was added 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 ethanol fermentation

The reactor column with working volume around 0.64 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 200, 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 0.11, 0.16, 0.20 and 0.30 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 300 g/l was prepared by dissolving 30 g of NaOH pellets in 100 ml of water. The reaction is highly exothermic so that the preparation should be done in water bath in order to avoid excess heat generation. Weighing time of NaOH pellets should be minimized because of the hygroscopic nature of NaOH. Solution of 370 g/l 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 370 g/l can be also 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, 62.5, 125, 187.5, and 250 g/l) was obtained. The detailed procedures are as follows:

1. Dry ± 2.6 g sucrose at 100-105°C in hot air oven for 2 hours.
2. Put the dried sucrose in desiccators for cooling.
3. Dissolve 2.5 g of the sucrose in 10 ml of water to obtain the source solution.
4. Prepare each 2 ml standard solution in small labeled bottle by serial dilution of suitable amount of source solution and diluting it with water as shown in detail in Table A-7. Use auto pipette for the transfer purpose.

Table A-7 Standard sucrose solution preparation

Source solution (ml)	Water (ml)	Sucrose concentration (g/l)
0	2	0
0.5	1.5	62.5
1	1	125
1.5	0.5	187.5
2	0	250

A-7.4 Sample treatment I

In the first treatment, sample was hydrolyzed using HCl 370 g/l in boiled water bath. After the hydrolysis reaction was stopped, NaOH was added into the solution. The sample was then centrifuged for removing suspended solids. Procedures of the first treatment are:

1. Mix 0.1 ml of sample with 0.9 ml DI water in 16 x 100 mm screw cap tube.
2. Blend the sample with 0.25 ml HCl 370 g/l.
3. Put the tubes in boiling water bath for 10 minutes.
4. Stop the reaction by placing the tubes in ice bath.
5. Add 0.4 ml NaOH 300 g/l and then mix with vortex mixer.
6. Centrifuge the sample at 2500 rpm for 15 minutes.
7. Precautions and notes:
   a) Use vortex mixer for mixing the fluid in the tubes.
   b) Be cautious when handling the hot apparatus.
   c) The level of boiled water and ice bath must be sufficiently higher than the liquid level in the tubes to ensure good heating and cooling of the sample.

A-7.5 Sample treatment II

In treatment II, supernatant obtained from treatment I was reacted with DNS reagent in boiled water bath. In normal circumstances, the mixture of DNS reagent and the sample was readily homogenous. In some cases, yellow precipitates of DNS were formed as an indication of the insufficient basic condition. This can be solved by adding NaOH solution until homogenous solution is obtained.

The solution's color transformed from yellow to reddish brown in the course of reaction. The color intensity represents the corresponding sugar concentration. 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 g/l 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 0.1 ml of supernatant obtained from treatment I with 0.5 ml DNS reagent in 16 x 100 mm screw cap tube.
2. Boil the solution for 10 minutes using water bath.
3. Put the tubes in ice bath to stop the reaction.
4. Dilute with water and transfer to other tube until the desired color intensity is gained.
5. Measure the absorbance at 520 nm. Use sample with 0 g/l sugar as blank.
6. Obtain the standard curve by plotting absorbance versus sucrose concentration of standard sucrose solution.
7. Use the standard curve to gain sugar concentration of the samples.

A-8 Determination of cell concentration

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

concentration (with dry weight basis). The medium in this study could be classified in 2 forms: liquid and gel. The concentration of cells in a liquid medium was referred as free cell concentration. In the case of cells confined in or attached to gels, the gel was first dissolved before separated from cells by centrifugation.

A-8.1 Dry weight of cell

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

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

A-8.2 Free cell concentration

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

1. Dilute sample with DI water in 16 x 100 mm rimless tube.
2. Centrifuge the cell suspension at 2000 rpm for 15 minutes.
3. Remove the supernatant.

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

A-8.3 Immobilized cell concentration

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

1. Dissolve appropriate amount of weighted gel carrier with 6 ml sodium citrate 0.5 M in 16 x 100 mm rimless tube.
2. In case of loofa reinforced carrier, remove the loofa sponge from the suspension after the gel is totally dissolved.
3. Continue with same procedures as step 2-9 of Section A-8.2.
4. Precautions and notes:
    a) Refresh the sodium citrate solution if the gel hasn't been dissolved in a single cycle. Repeat this procedure until all gel has been dissolved.
    b) Intermittent mixing with vortex mixer is recommended to promote faster gel dissolution.
    c) To minimize measurement error caused by sample contamination, the dissolution process can be carried out in at temperature about 4°C.

APPENDIX B

EXPERIMENTAL DATA

B-1 Data of bath fermentation

B-1.1 Data of ethanol and sugar concentration in repeated batch fermentation using 4-month-stored cultures of SC, AB, and EALC

Batch	Time (hour)	Ethanol concentration (g/l)			Sugar concentration (g/l)		
		SC	AB	EALC	SC	AB	EALC
I	0	0.00	0.00	0.00	202.00	202.00	202.00
	24	4.40	75.50	76.60	81.60	26.52	24.48
	48	65.70	76.00	76.00	21.22	25.10	29.58
II	0	0.30	0.70	0.70	202.00	202.00	202.00
	24	63.30	66.30	60.30	36.72	42.84	61.20
	48	72.00	80.70	86.10	32.64	23.84	23.26
III	0	0.30	0.70	0.70	202.00	202.00	202.00
	24	47.60	69.00	69.50	88.74	74.46	89.76
	48	86.10	71.30	76.50	20.38	30.60	21.22
IV	0	0.30	0.70	0.70	202.00	202.00	202.00
	24	2.10	65.10	61.80	171.36	45.00	51.00
	48	3.70	70.40	70.00	186.24	30.00	33.66

B-1.2 Data of batch fermentation of ethanol for 9 x 9 x 3 mm^3 alginate-loofa size

Time (hour)	Sugar concentration (g/l)	Ethanol concentration (g/l)	Free Cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l/h)
0	226.74	0	0		
8	162.13	0.56	0.03	0.01	0.07
16	87.00	11.37	0.28	0.08	0.71
24	62.52	52.57	0.68	0.32	2.19
32	38.04	72.20	0.69	0.38	2.26
40	26.82	80.03	1.02	0.4	2.00
48	22.74	89.03	0.87	0.44	1.85
56	26.82	90.59	1.13	0.45	1.62
64	20.70	87.80	1.06	0.43	1.37
72	24.78	88.82	0.92	0.44	1.23

B-1.3 Data of batch fermentation of ethanol for 20 x 20 x 3 mm^3 alginate-loofa size

Time (hour)	Sugar concentration (g/l)	Ethanol concentration (g/l)	Free Cell concentration (g/l)	$Y_{p/s}$	Productivity (g/l/h)
0	223.68	0	0		
8	158.84	0.48	0.21	0.01	0.06
16	62.52	10.71	0.25	0.07	0.67
24	37.02	52.31	0.50	0.28	2.18
32	28.86	71.25	0.55	0.37	2.23
40	26.82	81.35	0.56	0.41	2.03
48	19.68	89.10	0.73	0.44	1.86
56	30.90	89.32	0.83	0.46	1.60
64	21.72	89.54	0.83	0.44	1.40
72	22.74	89.75	0.87	0.45	1.25

B-2 Data of continuous ethanol fermentation # 1

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

Dilution Rate (h^{-1})	Time (hour)	Sugar Concentration (g/l)	Ethanol Concentration (g/l)	$Y_{p/s}$
0.11	0	202.36	0.00	
0.11	8	109.82	23.57	0.25
0.11	16	103.21	31.86	0.32
0.11	24	47.31	66.13	0.43
0.11	32	60.45	71.06	0.50
0.11	40	58.47	66.43	0.46
0.11	48	38.85	67.26	0.41
0.11	56	41.90	70.49	0.44
0.11	64	42.42	72.91	0.46
0.11	72	39.85	71.28	0.44
0.16	80	80.30	58.79	0.48
0.16	88	75.60	63.55	0.50
0.16	96	99.80	46.82	0.46
0.16	104	85.40	57.45	0.49
0.16	112	78.80	59.49	0.48
0.16	120	66.20	65.45	0.48
0.16	128	70.30	59.46	0.45
0.16	136	71.10	56.00	0.43
0.16	144	72.40	54.67	0.42
0.20	152	102.60	46.49	0.47
0.20	160	100.10	48.78	0.48
0.20	168	98.80	45.68	0.44
0.20	176	105.00	47.71	0.49
0.20	184	97.20	46.55	0.44
0.20	192	99.60	49.76	0.48
0.30	200	130.00	33.22	0.46
0.30	208	142.20	29.16	0.48
0.30	216	145.90	23.88	0.42
0.30	224	140.40	30.40	0.49
0.30	232	142.20	29.82	0.50
0.30	240	142.70	29.19	0.49

Dilution Rate (h^{-1})	**Time** (hour)	**Sugar Concentration** (g/l)	**Ethanol Concentration** (g/l)	$Y_{p/s}$
0.11	248	101.10	48.93	0.48
0.11	256	80.20	50.46	0.41
0.11	264	101.20	42.49	0.42
0.11	272	78.90	55.89	0.45
0.11	280	68.80	70.64	0.53
0.11	288	39.50	79.75	0.49
0.11	296	43.30	68.04	0.43
0.11	304	38.90	75.40	0.46
0.11	312	39.10	75.19	0.46

B-2.2 Data of free cells concentration leaving the reactor

Dilution rate (h^{-1})	**Time (hour)**	**Free cell concentration (g/l)**
0.11	0	0.05
0.11	8	0.22
0.11	16	0.34
0.11	24	0.54
0.11	32	0.42
0.11	40	0.66
0.11	48	0.39
0.11	56	0.52
0.11	64	0.55
0.11	72	0.60
0.16	80	0.47
0.16	88	0.41
0.16	96	0.58
0.16	104	0.37
0.16	112	0.56
0.16	120	0.62
0.16	128	0.42
0.16	136	0.48
0.16	144	0.44
0.20	152	0.38
0.20	160	0.46
0.20	168	0.51

Dilution rate (h^{-1})	Time (hour)	Free cell concentration (g/l)
0.20	176	0.50
0.20	184	0.45
0.20	192	0.44
0.30	200	0.44
0.30	208	0.48
0.30	216	0.33
0.30	224	0.42
0.30	232	0.62
0.30	240	0.33
0.11	248	0.55
0.11	256	0.41
0.11	264	0.44
0.11	272	0.39
0.11	280	0.51
0.11	288	0.47
0.11	296	0.44
0.11	304	0.48
0.11	312	0.50

B-2.3 Data of the stead state ethanol concentration of 4 dilution rates

Dilution rate (h^{-1})	Retention time (hour)	Ethanol concentration (g/l)	Sugar concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$
0.11	0.00	0.00	202.30	0.44	
0.11	0.90	7.70	185.20	0.63	0.45
0.11	3.12	20.47	158.50	0.75	0.47
0.11	5.35	41.99	101.10	0.56	0.43
0.11	7.57	52.86	86.60	0.63	0.46
0.11	9.09	71.28	39.85	0.60	0.44
0.16	0.00	0.00	202.30	0.58	0.00
0.16	0.62	4.20	188.80	0.64	0.31
0.16	2.15	21.10	152.70	0.88	0.43
0.16	3.68	30.20	138.90	0.91	0.48
0.16	5.21	46.96	93.40	0.77	0.41
0.16	6.25	54.67	72.40	0.44	0.42

Dilution rate (h^{-1})	Retention time (hour)	Ethanol concentration (g/l)	Sugar concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$
0.20	0.00	0.00	202.30	0.82	
0.20	0.49	1.80	187.80	0.77	0.12
0.20	1.72	15.49	165.24	0.63	0.42
0.20	2.94	22.41	144.80	0.45	0.39
0.20	4.17	35.15	120.68	0.61	0.43
0.20	5.00	49.76	89.62	0.33	0.44
0.30	0.00	0.00	202.30	0.72	0.00
0.30	0.33	1.30	199.24	0.68	0.42
0.30	1.14	12.40	175.57	0.85	0.46
0.30	1.96	19.03	160.01	0.61	0.45
0.30	2.78	24.11	145.55	0.52	0.42
0.30	3.33	29.20	144.28	0.33	0.50

Immobilized cell

	g	g/l
After immobilized	0.44	0.46
After cells growth in 50 g/l sugar	6.27	6.53
After finished performance	15.39	16.03

Free cell in the reactor

g	g/l
11.85	12.34

B-3 Data of continuous ethanol fermentation # 2

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

Dilution rate (h^{-1})	Time (hour)	Sugar concentration (g/l)	Ethanol concentration (g/l)	$Y_{p/s}$
0.11	0	222.00	0.00	
0.11	8	114.60	35.20	0.33
0.11	16	69.62	51.80	0.34
0.11	24	16.50	76.26	0.37
0.11	32	37.20	74.57	0.40
0.11	40	18.60	90.16	0.44
0.11	48	38.57	74.25	0.40
0.11	56	35.82	78.70	0.42
0.11	64	28.20	82.43	0.43
0.11	72	31.22	82.72	0.43
0.11	80	40.02	79.82	0.44
0.11	88	38.83	81.04	0.44
0.11	96	39.92	78.94	0.43
0.16	104	46.54	70.74	0.40
0.16	112	70.26	67.61	0.45
0.16	120	72.55	65.29	0.44
0.16	128	69.87	66.03	0.43
0.16	136	70.04	65.33	0.43
0.16	144	71.12	56.73	0.38
0.16	152	73.49	64.25	0.43
0.16	160	70.99	68.81	0.46
0.16	168	72.01	65.11	0.43
0.20	176	94.59	52.67	0.41
0.20	184	119.60	59.01	0.58
0.20	192	121.43	54.97	0.55
0.20	200	112.25	59.02	0.54
0.20	208	116.84	57.12	0.54
0.20	216	110.41	54.93	0.49

Dilution rate (h^{-1})	Time (hour)	Sugar concentration (g/l)	Ethanol concentration (g/l)	$Y_{p/s}$
0.30	224	125.58	32.13	0.33
0.30	232	129.90	36.12	0.39
0.30	240	141.43	37.57	0.47
0.30	248	142.65	33.34	0.42
0.30	256	145.80	39.55	0.52
0.30	264	138.99	33.26	0.40
0.11	272	90.83	51.43	0.39
0.11	280	64.62	59.08	0.38
0.11	288	41.45	64.14	0.36
0.11	296	53.04	71.71	0.42
0.11	304	38.41	85.00	0.46
0.11	312	26.82	78.23	0.40
0.11	320	35.97	78.10	0.42
0.11	328	29.86	84.35	0.44
0.11	336	34.26	86.13	0.46
0.11	344	38.88	80.11	0.44
0.11	352	40.56	79.83	0.44
0.11	360	39.00	80.52	0.44

B-3.2 Data of free cells concentration leaving the reactor

Dilution rate (h^{-1})	Time (hour)	Free cell concentration (g/l)
0.11	0	0.00
0.11	8	0.41
0.11	16	0.61
0.11	24	0.68
0.11	32	0.70
0.11	40	0.61
0.11	48	0.75
0.11	56	0.51
0.11	64	0.61
0.11	72	0.50

Dilution rate (h^{-1})	Time (hour)	Free cell concentration (g/l)
0.11	80	0.67
0.11	88	0.54
0.11	96	0.62
0.16	104	0.52
0.16	112	0.62
0.16	120	0.40
0.16	128	0.52
0.16	136	0.71
0.16	144	0.65
0.16	152	0.82
0.16	160	0.59
0.16	168	0.75
0.20	176	0.68
0.20	184	0.75
0.20	192	0.85
0.20	200	0.74
0.20	208	0.75
0.20	216	0.63
0.30	224	0.76
0.30	232	0.82
0.30	240	0.53
0.30	248	0.75
0.30	256	0.86
0.30	264	0.63
0.11	272	0.63
0.11	280	0.75
0.11	288	0.80
0.11	296	0.62
0.11	304	0.59
0.11	312	0.75
0.11	320	0.82
0.11	328	0.75
0.11	336	0.71
0.11	344	0.52
0.11	352	0.66
0.11	360	0.58

B-3.3 Data of the stead state ethanol concentration of 4 dilution rates

Dilution rate (h^{-1})	Retention time (hour)	Ethanol concentration (g/l)	Sugar concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$
0.11	0.00	0.00	222.00	0.35	
0.11	0.90	13.81	180.55	0.46	0.33
0.11	3.12	24.88	152.45	0.62	0.36
0.11	5.35	49.13	106.59	0.70	0.43
0.11	7.57	71.34	59.21	0.85	0.44
0.11	9.09	82.72	31.22	0.66	0.43
0.16	0.00	0.00	222.00	0.66	0.00
0.16	0.62	11.11	187.77	0.62	0.32
0.16	2.15	24.08	157.58	0.50	0.37
0.16	3.68	40.79	140.24	0.72	0.50
0.16	5.21	53.87	102.55	0.79	0.45
0.16	6.25	65.11	72.01	0.63	0.43
0.20	0.00	0.00	222.00	0.80	0.00
0.20	0.49	19.10	169.91	0.63	0.37
0.20	1.72	28.76	145.25	0.70	0.37
0.20	2.94	33.07	122.55	0.59	0.36
0.20	4.17	46.27	118.55	0.82	0.45
0.20	5.00	54.97	105.58	0.66	0.47
0.30	0.00	0.00	222.00	0.60	0.00
0.30	0.33	2.84	205.47	0.66	0.17
0.30	1.14	24.38	158.97	0.75	0.39
0.30	1.96	28.61	162.46	0.71	0.48
0.30	2.78	33.13	159.86	0.66	0.53
0.30	3.33	39.55	145.80	0.85	0.52

Immobilized cell

	g	g/l
After immobilized	0.33	0.34
After cells growth in 50 g/l sugar	5.67	5.90
After finished performance	14.71	15.32

Free cell in the reactor

g	g/l
10.24	12.34

B-4 Data of continuous ethanol fermentation # 3

B-4.1 Data of continuous performance in 1st reactor by every 8 hours harvested the samples

Dilution rate (h^{-1})	Time (hour)	Sugar concentration (g/l)	Ethanol concentration (g/l)	$Y_{p/s}$
0.11	0	248.43	0.00	
0.11	8	212.22	11.09	0.31
0.11	16	178.88	26.06	0.37
0.11	24	124.44	49.74	0.40
0.11	32	120.04	56.98	0.44
0.11	40	122.20	47.24	0.37
0.11	48	110.04	67.24	0.49
0.11	56	100.28	67.68	0.46
0.11	64	99.80	70.46	0.47
0.11	72	105.55	66.24	0.46
0.11	80	102.22	70.55	0.48
0.11	88	92.22	70.46	0.45
0.11	96	95.50	71.45	0.47
0.16	104	115.45	62.85	0.47
0.16	112	114.47	54.72	0.41
0.16	120	138.97	49.44	0.45
0.16	128	132.26	53.52	0.46
0.16	136	138.85	51.27	0.47
0.16	144	152.24	49.27	0.51
0.16	152	140.02	47.10	0.43
0.16	160	145.33	47.21	0.46
0.16	168	149.88	47.59	0.48
0.20	176	181.14	31.19	0.46
0.20	184	187.65	29.32	0.48
0.20	192	167.77	37.37	0.46
0.20	200	178.93	34.33	0.49
0.20	208	171.28	32.20	0.42
0.20	216	174.50	37.18	0.42

Dilution rate (h^{-1})	Time (hour)	Sugar concentration (g/l)	Ethanol concentration (g/l)	$Y_{p/s}$
0.30	224	205.55	20.07	0.47
0.30	232	201.11	19.20	0.41
0.30	240	202.24	21.67	0.47
0.30	248	198.75	24.02	0.48
0.30	256	194.48	25.90	0.48
0.30	264	196.63	24.14	0.47
0.11	272	182.80	27.66	0.42
0.11	280	147.86	44.60	0.44
0.11	288	125.53	59.73	0.49
0.11	296	118.82	52.41	0.40
0.11	304	114.58	61.26	0.46
0.11	312	121.24	56.98	0.45
0.11	320	86.63	75.99	0.47
0.11	328	78.70	73.49	0.43
0.11	336	75.54	72.21	0.42
0.11	344	85.55	76.62	0.47
0.11	352	76.62	73.32	0.43
0.11	360	82.21	78.05	0.47

B-4.2 Data of continuous performance in 2nd reactor by every 8 hours harvested the samples

Dilution rate (h^{-1})	Time (hour)	Sugar concentration (g/l)	Ethanol concentration (g/l)	$Y_{p/s}$
0.11	0	247.20	0.00	
0.11	8	184.45	15.09	0.24
0.11	16	174.43	26.33	0.36
0.11	24	118.88	58.24	0.45
0.11	32	111.20	55.44	0.41
0.11	40	73.33	60.86	0.35
0.11	48	71.19	68.81	0.39

Dilution rate (h^{-1})	**Time (hour)**	**Sugar concentration (g/l)**	**Ethanol concentration (g/l)**	**$Y_{p/s}$**
0.11	56	53.66	80.69	0.42
0.11	64	52.27	74.64	0.38
0.11	72	62.21	71.28	0.39
0.11	80	54.47	74.45	0.39
0.11	88	58.21	75.52	0.40
0.11	96	56.95	78.86	0.41
0.16	104	78.84	65.09	0.39
0.16	112	82.48	52.74	0.32
0.16	120	83.33	47.90	0.29
0.16	128	79.90	50.29	0.30
0.16	136	74.43	48.96	0.28
0.16	144	68.82	58.74	0.33
0.16	152	82.25	59.68	0.36
0.16	160	78.90	60.80	0.36
0.16	168	80.40	63.60	0.38
0.20	176	133.58	35.79	0.31
0.20	184	135.55	34.92	0.31
0.20	192	147.83	31.38	0.32
0.20	200	138.98	35.71	0.33
0.20	208	148.83	31.93	0.32
0.20	216	145.53	34.10	0.34
0.30	224	188.80	19.89	0.34
0.30	232	192.16	23.82	0.43
0.30	240	179.87	20.98	0.31
0.30	248	180.22	25.44	0.38
0.30	256	179.65	23.15	0.34
0.30	264	178.84	24.76	0.36
0.11	272	172.28	31.38	0.42

Dilution rate (h^{-1})	Time (hour)	Sugar concentration (g/l)	Ethanol concentration (g/l)	$Y_{p/s}$
0.11	280	132.53	44.28	0.39
0.11	288	98.70	66.37	0.45
0.11	296	87.55	66.79	0.42
0.11	304	62.57	55.53	0.30
0.11	312	64.42	70.47	0.39
0.11	320	58.80	76.83	0.41
0.11	328	52.12	81.60	0.42
0.11	336	55.22	74.45	0.39
0.11	344	66.22	76.66	0.42
0.11	352	54.41	75.72	0.39
0.11	360	56.64	79.12	0.42

B-3.2 Data of free cells concentration leaving the reactor

Dilution rate (h^{-1})	Time (hour)	Free cell concentration in 1st reactor (g/l)	Free cell concentration in 2nd reactor (g/l)
0.11	0	0.00	0.00
0.11	8	0.33	0.41
0.11	16	0.55	0.63
0.11	24	0.66	0.52
0.11	32	0.58	0.48
0.11	40	0.71	0.72
0.11	48	0.82	0.71
0.11	56	0.66	0.77
0.11	64	0.34	0.63
0.11	72	0.55	0.71
0.11	80	0.62	0.64
0.11	88	0.48	0.65
0.11	96	0.48	0.82
0.16	104	0.55	0.56
0.16	112	0.71	0.54

Dilution rate (h^{-1})	Time (hour)	Free cell concentration in 1st reactor (g/l)	Free cell concentration in 2nd reactor (g/l)
0.16	120	0.66	0.43
0.16	128	0.54	0.49
0.16	136	0.58	0.56
0.16	144	0.39	0.52
0.16	152	0.48	0.49
0.16	160	0.57	0.53
0.16	168	0.62	0.51
0.20	176	0.73	0.68
0.20	184	0.77	0.77
0.20	192	0.63	0.65
0.20	200	0.71	0.58
0.20	208	0.42	0.80
0.20	216	0.69	0.63
0.30	224	0.54	0.44
0.30	232	0.37	0.55
0.30	240	0.58	0.80
0.30	248	0.46	0.71
0.30	256	0.62	0.39
0.30	264	0.55	0.44
0.16	272	0.58	0.45
0.16	280	0.71	0.56
0.16	288	0.77	0.63
0.16	296	0.66	0.77
0.16	304	0.58	0.73
0.16	312	0.42	0.68
0.16	320	0.81	0.71
0.16	328	0.75	0.62
0.16	336	0.64	0.59
0.16	344	0.61	0.66
0.16	352	0.72	0.82
0.16	360	0.62	0.71

B-3.3 Data of the stead state ethanol concentration of 4 dilution rates

Dilution rate (hr^{-1})	Retention time (hour)	Ethanol concentration (g/l)	Sugar concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$
0.11	0.00	0.00	248.43	0.00	
0.11	0.90	8.78	226.50	0.66	0.40
0.11	3.12	25.02	190.10	0.73	0.43
0.11	5.35	47.48	140.00	0.82	0.44
0.11	7.57	54.31	116.50	0.67	0.41
0.11	9.09	71.45	95.50	0.75	0.47
0.11	9.99	72.65	80.05	0.88	0.43
0.11	12.21	74.90	65.54	0.71	0.41
0.11	14.44	72.41	66.48	0.83	0.40
0.11	16.66	73.32	59.46	0.66	0.39
0.11	18.18	78.86	56.95	0.68	0.41
0.16	0.00	0.00	248.43	0.77	0.00
0.16	0.62	7.27	230.20	0.85	0.40
0.16	2.15	26.56	189.50	0.63	0.45
0.16	3.68	31.34	171.10	0.71	0.41
0.16	5.21	39.39	158.80	0.54	0.44
0.16	6.25	47.59	149.88	0.55	0.48
0.16	6.87	46.72	138.83	0.66	0.43
0.16	8.40	55.08	107.77	0.78	0.39
0.16	9.93	55.68	100.85	0.82	0.38
0.16	11.46	60.94	92.25	0.67	0.39
0.16	12.5	63.60	80.4	0.700	0.38

Dilution rate (hr^{-1})	Retention time (hour)	Ethanol concentration (g/l)	Sugar concentration (g/l)	Free cell concentration (g/l)	$Y_{p/s}$
0.20	0.00	0.00	248.43	0.82	
0.20	0.49	4.71	233.30	0.67	0.31
0.20	1.72	19.08	202.20	0.71	0.41
0.20	2.94	23.96	188.80	0.66	0.40
0.20	4.17	38.92	155.50	0.73	0.42
0.20	5.00	31.18	174.50	0.76	0.42
0.20	5.49	32.46	164.43	0.69	0.39
0.20	6.72	34.35	154.42	0.80	0.37
0.20	7.94	33.85	150.01	0.66	0.34
0.20	9.17	34.54	148.89	0.72	0.35
0.20	10.00	34.10	145.53	0.67	0.33
0.30	0.00	0.00	248.43	0.71	0.00
0.30	0.33	0.73	246.62	0.63	0.40
0.30	1.14	5.46	235.51	0.75	0.42
0.30	1.96	11.93	220.50	0.66	0.43
0.30	2.78	17.54	201.10	0.60	0.37
0.30	3.33	24.14	189.87	0.82	0.41
0.30	3.66	27.19	177.72	0.74	0.38
0.30	4.47	27.50	176.64	0.77	0.38
0.30	5.29	26.60	175.53	0.68	0.36
0.30	6.11	28.21	176.23	0.63	0.39
0.30	6.66	24.76	178.84	0.67	0.36

Immobilized cell

	1st reactor (g/l)	2nd reactor (g/l)
After immobilized	0.50	0.50
After cells growth in 50 g/l sugar	6.70	6.70
After finished performance	16.04	14.82

Free cell in the reactor

1st reactor (g/l)	2nd reactor (g/l)
14.48	22.26

B-4.1 Data of the productivity

Dilution rate (h^{-1})	**0.11**	**0.16**	**0.20**	**0.30**
Ethanol concentration for continuous ethanol fermentation # 1 (g/l)	71.56	56.71	48.01	29.81
Sugar concentration for continuous ethanol fermentation # 1 (g/l)	29.08	72.16	113.71	142.48
Ethanol concentration for continuous ethanol fermentation # 2 (g/l)	81.29	66.06	53.69	35.38
Sugar concentration for continuous ethanol fermentation # 2 (g/l)	41.39	71.27	100.60	144.73
Ethanol concentration for continuous ethanol fermentation # 3 (g/l)	69.86	47.30	36.30	24.45
Sugar concentration for continuous ethanol fermentation # 3 (g/l)	98.50	145.03	174.87	192.90
Productivity for continuous ethanol fermentation # 1 (g/l/h)	7.87	9.07	9.60	8.94
Productivity for continuous ethanol fermentation # 2 (g/l/h)	8.94	10.57	10.74	10.62
Productivity for continuous ethanol fermentation # 3 (g/l/h)	7.68	7.57	7.26	7.33

B-4.1 Data of the stability performance

Time (day)	Ethanol concentration (g/l)	Sugar concentration (g/l)	$Y_{p/s}$
0	0.00	228.00	
1	58.82	71.88	0.38
2	71.87	65.14	0.44
3	75.44	54.33	0.43
4	65.93	66.14	0.41
5	66.01	64.58	0.40
6	71.53	53.22	0.41
7	67.73	57.99	0.40
8	77.13	50.01	0.43
9	76.03	56.78	0.44
10	71.36	61.13	0.43
11	67.98	75.55	0.45
12	73.63	68.88	0.46
13	76.85	59.93	0.46
14	79.25	52.24	0.45
15	64.96	65.55	0.40
16	74.49	66.62	0.46
17	73.40	67.77	0.46
18	77.62	54.42	0.45
19	69.99	63.76	0.43
20	68.07	58.29	0.40
21	72.57	63.76	0.44
22	75.72	57.51	0.44
23	70.78	50.86	0.40
24	76.55	56.72	0.45
25	84.68	46.25	0.47
26	67.02	58.12	0.39
27	75.11	51.64	0.43
28	74.55	45.77	0.41
29	69.88	61.03	0.42
30	71.11	61.42	0.43

	Immobilized cell	
	g	g/l
After immobilized	0.50	0.52
After cells growth in 50 g/l sugar	4.84	5.04
After finished performance	17.00	17.71

Free cell in the reactor	
g	g/l
18.85	19.64

APPENDIX C
LISTS OF PUBLICATION

C-1 Thailand conference.

Phoowit Bangrak, Muenduen Phisalaphong, Jirawam Mongkolkajit, “Development of Entrapment-Loofa Matrix Carrier for Ethanol Production ”, Extended Abstract for 17th Thailand Chemical Engineering and Applied Chemistry Conference – Advances in Thailand Organization of Chemical Engineering and Applied Chemistry Cooperated with Faculty of Science and Major Industry Chemical ,Chiangmai University, 29-30 October 2007, Paper ID EFP8.

* He has received the good presentation prizes.

C-2 International paper

Muenduen Phisalaphong, Rusdianto Budirehojo, Phoowit bangrak, Jirawam Mongkolkajit, Savitree Limtong “Alginate-Loofa as carrier matrix for ethanol production”, Journal of Bioscience and Bioengineering. Vol 104, No 3, 214-217: 2007.

การประชุมวิชาการวิศวกรรมเคมีและเคมีประยุกต์แห่งประเทศไทย ครั้งที่ 17

TIChE17

29 – 30 ตุลาคม 2550

ณ โรงแรมดิเอ็มเพรส

จ.เชียงใหม่

จัดโดย

สมาคมวิศวกรรมเคมีและเคมีประยุกต์แห่งประเทศไทย

ร่วมกับ

ภาควิชาเคมีอุตสาหกรรม คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่

การใช้ประโยชน์เศษวัสดุเหลือใช้ทางการเกษตรตามชุมชนเป็นเชื้อเพลิง สำหรับเครื่องยนต์ก๊าซเทอร์ไบน์ขนาดเล็ก วรเทพ สีสัตย์ซื่อ, อรัญ วสันตกรณ์, ฉัตรชัย จันทร์เด่นดวง
การแยกน้ำออกจากเอทานอลด้วยตัวดูดซับจากแป้งมันสำปะหลัง ชลธิรา บุญฟุ้ง, ดวงฤทัย สินเจริญ, พนารัตน์ โทมณี
Production Methyl Esters from Palm Fatty Acids in Supercritical Methanol Akaraphol Petchmala1, Duangkamol Yujaroen, Artiwan Shotipruk, Motonobu Goto, Mitsuru Sasaki
Development of Fuel Briquettes from Biomass-Lignite Blends Suparin Chaiklangmuang, Suwit Supa, and Prattana Kaewpet
Feasibility Survey of Fuel Briquette Demands in Roasting Food Restaurants in Chiang Mai Province, Thailand Suparin Chaiklangmuang, Yaowaluck Chotchaithanakorn and Sanguansak Sri-phalang
การพัฒนาและผลิตไฮโดรเจนจากน้ำมันพืชโดยกระบวนการสตรีมรีฟอร์มมิ่งแบบใช้ตัวเร่งปฏิกิริยา ศุภรินทร์ ไชยกลางเมือง, เอกรินทร์ ปีนธุ, สุนันทา วังกานนท์,, วิษณุ มีอยู่ และ นงคราญ ไชยวงศ์
การศึกษาสมบัติของสารดูดซับที่เตรียมจากกากตะกอนโดยการกระตุ้นด้วยไอน้ำ ศุภรินทร์ ไชยกลางเมือง, ปัทมาภรณ์ ด้วงบุญมา, สุรินทร์ จันทร์มณี และ อรทัย ศรีวรรณ
Development of Entrapment-Loofa Matrix Carrier for Ethanol Production Bangrak P, Mongkolkajit J, Budiraharjo R, Phisalaphong M.

Development of Entrapment-Loofa Matrix Carrier for Ethanol Production

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1. Introduction

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

For improved ethanol production, a new carrier of entrapment-loofa matrix was developed and evaluated in the present study.

2. Materials and mathods

S. cereviceae M30, a flocculating yeast strain was kindly provided by Dr. Savithree Limthong from Department of Microbiology, Kasetsart University, Bangkok. Stock cell suspension is added to the alginate or chitosan solutions with volumetric ratio of 1:10. The mixture is used to construct for entrapment of loofa matrix with dimension of 8 x 8 x 3 mm.

Batch fermentation was performed in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 150 rpm, 33°C for 48 hours for a batch. Samples of fermentation broth were analyzed for sugar, ethanol, and cell concentration by 3,5-dinitrosalicylic acid (DNS) reagent, gas chromatography, and dry weight respectively. At the end of fermentation, samples of carriers were taken for immobilized cell concentration measurement and scanning electron microscopy (SEM).

3. Results and discussion

3.1 Yeasts immobilization by loofa sponge

The preliminary test with loofa sponge demonstrated that it was not effective for immobilizing flocculating yeast cells. Excessive cells detachment from the sponges was observed and there were no significant differences among different shapes of sponges used for cells immobilization. High shear environment was thought to be the possible cause for excessive cells' detachment from loofa sponge carrier in this study.

Fig. 1. Loofa sponge(http://www.myristica.it[2006,January,5]**).**

3.2 Comparison of alginate and chitosan

As loofa sponge alone was not adequate for yeast immobilization, new hybrid carriers were formed by combining loofa sponge with natural biopolymers: alginate and high viscosity chitosan. Preliminary evaluation and comparison between the two polymers was conducted by 3 days batch fermentation. At the end of the fermentation, the broth was analyzed for sugar content by DNS method and the pH value was measured by pH meter. It was demonstrated that chitosan based carriers had low performance in term of sugar consumption. On the other hand, sugar consumption and ethanol production were slightly higher for entrapment alginate-loofa matrix(ALM) system than suspended cells culture (SC).

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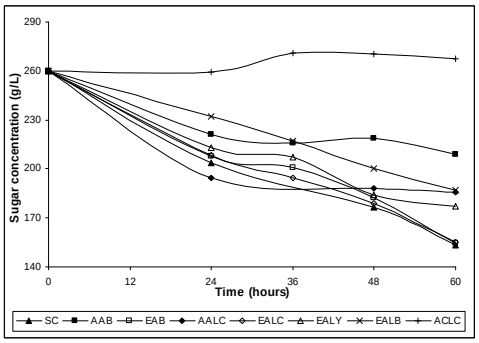


Fig.2. Sugar concentration profile of fermentation.

3.3. Reusability and Storage Effects

To investigate the reusability and storage effects, cell cultures in forms of suspension cells and immobilized cells (IC) were stored for 4 months at 4°C and reused. The experimental study was performed in a 4-cycle repeated batch fermentation. Overall, the IC cultures remained stable; a maximum ethanol concentration of 70-80 g/l(90 g/l for fresh catalysts) was achieved with an ethanol yield ($Y_{P/S}$) of 0.44- 0.49. The instability of SC cultures was observed in the first and the fourth batches as shown in Fig.3. Compared with the SC cultures, the stability and average ethanol productivity of the ALM-immobilized cell were significantly improved. After the 4th batch (194 h), a higher degree of gel degradation on the surface of the carriers was observed; however, the majority of cells were still attached to each other within the matrix(Fig.5.). The immobilization yield of ALM carrier was 81%.

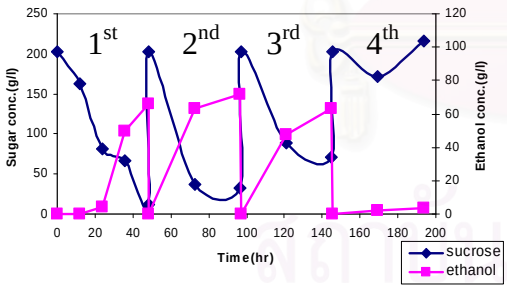


Fig.3. Sugar and ethanol concentration profile using 4-month-stored cultures of SC.

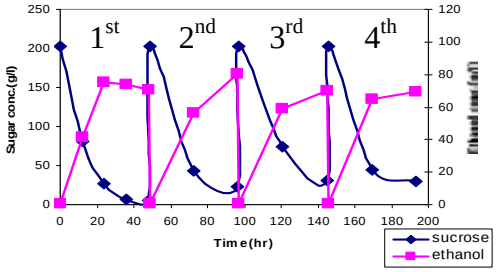
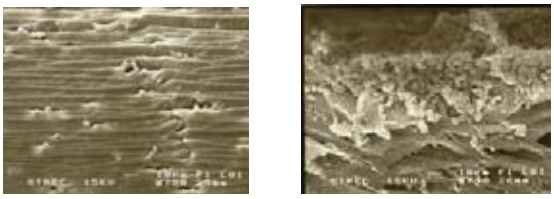


Fig.4. Sugar and ethanol concentration profile using 4-month-stored cultures of ALM.



(A)x750 (B)x750

Fig.5. Cross section of ALM (A) fresh carrier and (B) carrier after the 4th batch.

4. Conclusion

A novel immobilization method of *S. cerevisiae* M30 in alginate-loofa matrix was investigated. In repeated batch fermentation at an initial sugar concentration of 200-220 g/L, the immobilized cells could be stored for at least 4 month retaining about 80% of its original activity. Thus, the process could be a promising way for efficient production of ethanol. The matrix was effective for yeast immobilization and had good mechanical strength and stability for long-term use.

5.Acknowledgment

The authors acknowledged the support by the Thailand Research fund (TRF)-Chulalonkorn University(RSA 5080011).

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JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 104, No. 3, 214–217, 2007
DOI: 10.1263/jbb.104.214

Alginate-Loofa as Carrier Matrix for Ethanol Production

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Received 7 May 2007/Accepted 22 June 2007

An alginate-loofa matrix was developed as a cell carrier for ethanol fermentation owing to its porous structure and strong fibrous nature. The matrix was effective for cell immobilization and had good mechanical strength and stability for long-term use. After a storage period of 4 months, yeast cells remained firmly immobilized and active.

[**Key words:** loofa sponge, alginate, yeast, ethanol production]

In response to energy crisis, ethanol has re-emerged as an alternative to, or extender for, petroleum-based liquid fuels. Ethanol production using an immobilized cell system offers many advantages such as higher productivity and protection of cells from inhibitions. Cell entrapment within alginate is one of the most widely studied because cell viability and activity are kept very high (1). However, the practical application of polymeric gel carriers including alginate beads has been limited by the problems of gel degradation, low physical strength, and severe mass transfer limitation (1–8). Furthermore, large-scale production of these carriers often requires complex and sophisticated equipment leading to high cost of production (2). On the other hand, loofa sponges, lignocellulosic matrices from *Luffa cylindrica*, were found to be promising cell carriers for ethanol production by flocculating cells (2–5). The sponges are light, strong, chemically stable, and composed of intereonnecting voids within an open network of fibers. Because of the random lattices of small cross sections of the sponges coupled with high porosity, the sponges are suitable for cell adhesion. Continuous fuel ethanol production had been realized using yeast cells immobilized in loofa sponges in a bubble column configuration (5). However, a low-shear environment and large aggregates of cells were required in order to prevent excessive cell sloughing from the carriers (3, 9).

On the basis of the above, in the present work, we focused on developing a new cell carrier by combining alginate gel and loofa sponge namely, the alginate-loofa matrix (ALM). Ethanol production by repeated batch fermentation using yeast cells immobilized within the ALM was then examined and compared with that using suspended cells and cells immobilized in conventional calcium alginate beads.

MATERIALS AND METHODS

Yeast strains *Saccharomyces cerevisiae* M30 selected on the basis of its high efficiency in ethanol production from molasses at high temperature was used in this study.

Culture media and cell preparation Starter cultures were prepared by transferring cells from stock PDA slants to 150 ml of sterilized medium followed by incubation at 33°C, 150 rpm for 20 h. The medium for the starter culture contained 0.05% ammonium sulfate and 5% inverse sugar from palm sugar at pH 5.0. After that, the obtained cell suspension was concentrated by decantation and then transferred to the main culture.

Cells immobilized on alginate-loofa matrix Sodium alginate (3% w/v) solution was formulated by dissolving Na-alginate powder in 0.9% (w/v) NaCl solution. It was autoclaved for 5 min at 121°C and kept overnight at 4°C to facilitate deaeration. Cell suspension of 5 ml was then added to 50 ml of 3% (w/v) alginate solution to form an alginate-cell mixture. To form ALM, 2 g of sterilized cubic sponges of loofa (8×8×2 mm) was dipped into the alginate-cell mixture. The gel carriers were transferred to 1.47% (w/v) $CaCl_2$ solution and left to harden in this solution with mild stirring for 15 min. The carriers were then rinsed 3 times with 0.9% (w/v) NaCl solution. Carriers were prepared under aseptic conditions and the average size of ALM was 9×9×3 mm^3.

Fermentations Repeated batch fermentations were carried out in duplicate using a medium contained 0.05% ammonium sulfate and 21% (w/v) inverse sugar from cane molasses at pH 5.0. The prepared medium was sterilized at 121°C for 20 min. Experiments were initiated by transferring prepared cell suspension or immobilized cells into 250 ml of the medium in 500 ml Erlenmeyer flasks. Fermentation flasks were then shaken in the incubator at 150 rpm, 33°C for 48 h. The experiments were monitored by removing 2 ml samples every 6 h for cell, sugar and ethanol analyses.

Analytical methods Free cell dry weight was determined from the absorbance at 660 nm with a UV-2450 UV-visible spectrophotometer and converted to dry cell concentration on the basis of a corresponding standard curve. For immobilized cells, a known mass of cell carriers was dissolved in 0.05 M sodium citrate. After the sponge was removed, immobilized cell concentration was determined similarly for the free cells. The concentration of ethanol was determined by gas chromatography using a Shimadzu model GC $7A_{II}$ (Shimadzu, Kyoto) equipped with a flame ionization detector. To measure reducing sugar concentration, the sample solution was hydrolyzed in 33% HCl at 100°C for 10 min and neutralized with NaOH solution. Reducing sugar content was then determined by the dinitrosalicylic acid method (10).

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TABLE 1. Yields and end products of repeated batch ethanol fermentation for 48 h for each batch using the cultures of suspended cells (SC), Ca-alginate-immobilized cells (AB), and alginate-loofa-matrix-immobilized cells (ALM)

Batch	P (g/l)	X (g/l)		Y_i (%)	Y_s (%)	Y_{PS} (g/g)
		X_v	X_i			
I						
SC	91.7	3.5	–	–	86	0.47
AB	70.4	0.6	6.0	91	87	0.40
ALM	77.8	0.7	4.6	87	84	0.44
II						
SC	47.7	5.1	–	–	57	0.33
AB	72.7	0.7	8.5	93	86	0.40
ALM	75.9	0.9	6.1	87	86	0.40
III						
SC	97.4	6.5	–	–	85	0.46
AB	89.5	0.8	8.5	92	87	0.44
ALM	90.6	1.1	7.1	87	88	0.46

Immobilization yield (Y_i) is the ratio of immobilized cell concentration (X_i) to total cell concentration (X), and X_v is the free cell concentration. Sugar consumption yield (Y_s) is the ratio of sugar consumption (S_0-S) to the initial sugar concentration (S_0). Ethanol yield (Y_{PS}) is the ratio of ethanol accumulation ($P-P_0$) to the sugar consumption.

Scanning electron microscopy (SEM) Samples of immobilized cells in alginate beads (AB) and ALM were frozen in liquid nitrogen, immediately snapped, vacuum-dried, and then sputtered with gold and photographed. Images were taken on a JEOL JSM-5410LV (JEOL, Tokyo) scanning electron microscope.

RESULTS AND DISCUSSION

Ethanol production using ALM as a carrier for *S. cerevisiae* M30 was examined by a 3-cycle repeated batch fermentation using cane molasses as the C source. The duration of each batch was 48 h. There were three cultures in this study: suspended cells (SC), Ca-alginate-immobilized (AB) cells and ALM-immobilized cells. The results of the fermentations are summarized in Table 1. For the first batch, after 48 h the ethanol concentration of the SC system was 91.7 g/l, whereas the final ethanol concentrations of immobilized cells (IC) in AB and ALM carriers were 70.4 and 77.8 g/l, respectively. At the end of the first batch, the total cell concentrations of IC cultures were higher than that of the SC culture. The increase in cell concentration in AB and ALM carriers owing to cell growth inside the carriers during the course of fermentation was observed previously (11). The final total cell concentration of the system with ALM carriers was 5.3 g/l with an immobilization yield (Y_i) of 87%, which was slightly lower than that of the system with AB carriers (X 6.6 g/l with Y_i 91%); however, the ethanol production was 10% higher.

Instability of SC culture was observed in the second batch. It was found that there was no lag phase in IC systems, whereas an approximately 30 h lag phase was observed in SC systems. Corresponding to its sugar consumption, the ethanol concentration of SC cultures in the second batch was only half of that of the first batch, whereas the ethanol productions by IC cultures were similar to those of the first batch. Free-cell concentrations of IC systems slightly increased from the first to the second batch, which can be attributed to cell leakage and growth in the medium.

In the third batch, all the systems exhibited high ethanol productions without any occurrence of the lag phase. In most cases, the majority of sugar was consumed within 36 h with the final ethanol concentrations being 97.4, 89.5 and 90.6 g/l for SC, AB-immobilized cell and ALM-immobilized cell cultures, respectively. The ethanol concentration profile followed the trend of a normal microbial growth curve. At the end of the third batch, partial gel degradation on the surfaces of AB and ALM carriers was observed. However, the immobilization yields (Y_i) remained constant.

A marked instability of the SC culture in the repeated batch fermentation was observed from the comparison of its final ethanol concentration from batch to batch, which may be attributable to the negative effect of high ethanol concentration on cell activity and viability. In contrast, the ethanol production of IC cultures in AB and ALM carriers were relatively stable. It was suggested from previous studies (2, 7) that the matrix of carriers can protect yeast by functioning as a fortification against toxins and inhibitors. In terms of immobilization yield (Y_i), ALM carriers exhibited a slightly lower immobilization capacity (average Y_i=87%) than AB carriers (average Y_i=92%). However, the final ethanol concentration and the ethanol yield factor (Y_{PS}) were comparable. Changes in physical or chemical parameters such as temperature and the concentrations of sugar and ethanol affected cell growth and product formation during fermentation (12). To improve yield, substrates, metabolite products, and conditions should be maintained or controlled at optimal levels under steady-state condition by continuous bioreactor fermentation.

A series of SEM images were taken to provide visual description and information of fermentation systems. The SEM images of the initial AB and ALM are shown in Fig. 1. From the external surface, most of the yeast cells were covered by alginate film; only a few cells were absorbed on the surface of the carriers. The AB and ALM carriers displayed different gel morphologies. ALM carriers were composed of stacked layers of thin alginate films whereas the structure of AB carriers were dense and less porous (Figs. 1 and 2). Consequently, fewer cells were observed in the central part

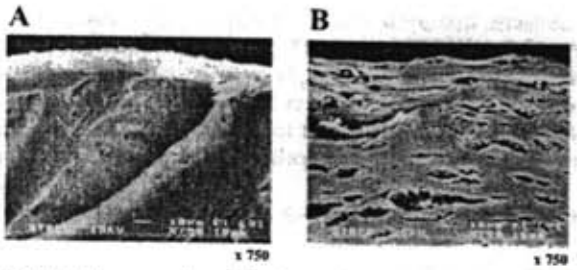

FIG. 1. Cross section of fresh carriers after the entrapment of yeasts (0 h): (A) alginate bead, AB; (B) alginate-loofa matrix, ALM. Bars: 10 μm.

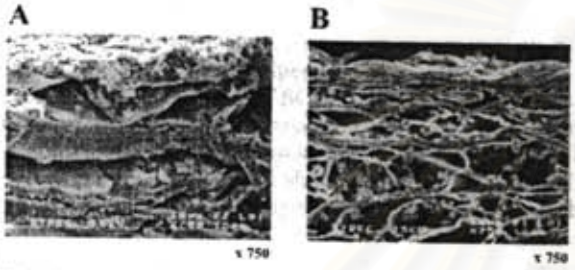

FIG. 2. Cross section of carriers after the third batch (144 h): (A) alginate bead, AB; (B) alginate-loofa matrix, ALM. Bars: 10 μm.

of AB carriers. The preference of cells to grow near a surface other than the middle of a gel bead was previously reported (1, 11). The high concentration of substrates near the surface as a consequence of mass transfer limitation was believed to be the main driving force for this phenomenon.

High porosity and better cell distribution were observed in ALM carriers. Because of their highly porous structure, mass transfer limitation in ALM carriers was less severe than that in AB carriers despite their larger size. An examination of the center of ALM carriers revealed that yeast cells were located in the middle of ALM carriers although ALM carriers were relatively large (about 70 times the size of AB carriers). Despite the partial degradation of alginate films after several fermentation cycles, cells were still firmly immobilized due to the aggregation of yeasts and their adhesion to the ALM matrix. Many cells in the space between a loofa fiber and an alginate film were observed (Fig. 3). In a previous study of cellulose carriers (13), the structure consisting of small pores distributed on the outer surface and large pores distributed in the interior was found to be effective for yeast immobilization.

The beneficial properties of IC systems, such as the protection of cells from solvent inhibitions and promotion of cell productivity as demonstrated in this study, were also reported elsewhere (14). The ability of cells to grow in an immobilized state made it possible for cell regeneration under hostile conditions such as a high ethanol concentration. In this study, the regeneration and protection of entrapped cells by ALM were proposed as the main factors that work synergistically to preserve cell activity. Thus, a stability of ALM-immobilized cell culture higher than that of SC culture was achieved.

Ogbonna *et al.* (4, 5) reported that loofa sponge alone can

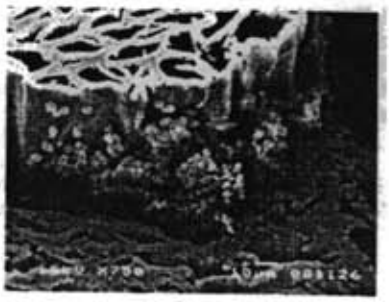

FIG. 3. Yeast cells in a space between loofa fiber and alginate gel of alginate-loofa matrix (ALM) carrier. Bar: 10 μm.

be used to achieve 99% immobilization of a flocculating yeast strain for ethanol production in column-type bioreactors. On the other hand, the results from our preliminary study in conventional shake flask cultures at 150 rpm showed that loofa sponge alone was not effective for the immobilization of yeast cells owing to the large pores of loofa sponges (500–1000 μm) with respect to the size of yeast cells (3–7 μm). Therefore, in a high-shear environment arising from agitation or high fluid velocity, excessive cell detachment from the sponges was observed regardless of the shape or size of sponges used. The difference in the obtained results might arise from differences in the yeast strain used and system characteristics.

To investigate storage effects, cell cultures from the third batch (a total time span of 144 h) were stored for 4 months at 4°C and reused. Ethanol productions by a 4-cycle repeated batch fermentation (a total time span of 192 h) using the stored cultures of AB-immobilized cells, ALM-immobilized cells and SCs, were examined. Overall, the IC cultures remained stable; a maximum ethanol concentration of 70–80 g/l was achieved with an ethanol yield ($Y_{P/S}$) of 0.44–0.49. The instability of SC cultures was again observed in the first and the fourth batches as shown in Table 2. In comparison with the results shown in Table 1, the average productivities of the stored AB- and ALM-immobilized cell cultures (Table 2) slightly decreased. However, compared with the SC cultures, the stability and average ethanol productivity of the ALM-immobilized cell culture were significantly improved. After the 4-cycle repeated batch, a higher degree of gel degradation occurred on the surface of the car-

TABLE 2. Ethanol concentration in repeated batch fermentation using 4-month-stored cultures of suspended cells (SCs), Ca-alginate-immobilized cells (AB), and alginate-loofa-matrix-immobilized cells (ALM)

Batch	Time (h)	Ethanol concentration (P, g/l)		
		SC	AB	ALM
I	24	4.4	76.6	75.5
	48	65.7	76.0	76.0
II	24	63.3	60.3	66.3
	48	72.0	86.1	80.7
III	24	47.6	69.5	69.0
	48	86.1	76.5	71.3
IV	24	2.1	61.8	65.1
	48	3.7	70.0	70.4

riers; however, the majority of cells were still attached to each other within the matrix. The immobilization yields (Y_I) of AB and ALM carriers slightly decreased to 86% and 81%, respectively. From the results together with the strong and chemical stable nature of loofa sponge, ALM has good mechanical strength, durability, and stability for long-term use.

In conclusion, ALM was successfully developed and applied in repeated batch ethanol fermentation. The carriers were fabricated simply by entrapment of a peripheral loofa sponge that was previously dipped in an alginate-cell mixture. The porous structure conferred the new carriers with better mass transfer characteristics. An ALM with a size of $9\times9\times3$ mm^3 was effective for cell immobilization, which is comparable to a 2-mm-diameter alginate bead. The immobilization yield of the new carriers was approximately 87%. Ethanol production using these carriers was proven to be more stable than that using SC cultures. After storage for 4 months, the ALM-immobilized cell culture was still active, and the stability of IC cultures being higher than that of SC culture was confirmed. As shown in this study, the ALM carriers have many advantages including regeneration abiity, reusability, stability, altered mechanical strength, and high ethanol productivity. The results demonstrated the potential use of ALM carriers in an ethanol fermentation system for a long period of time. To improve productivity and yield, the evaluation of ALM as carrier matrix in a packed column for continuous fermentation is on going.

ACKNOWLEDGMENTS

We thank the Thailand Research Fund (TRF) and Chulalongkorn University (RSA5080011) for financial support, the Thailand-Japan Technology Transfer Project (TJTTP-OECF) for the support of analytical equipments.

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APPENDIX D

MATH MODEL FOR PACKED-BED REACTOR

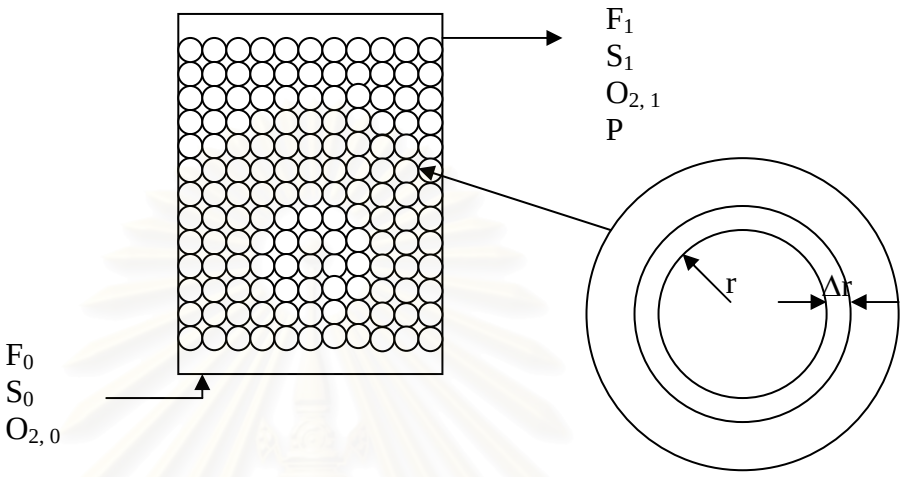
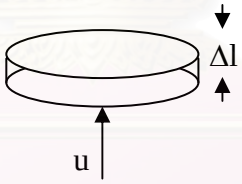


Fig.D Packed-bed reactor and immobilized cell sperical

Particular calculation



$\Delta V = A\Delta l$

$V_{particle} = 4\pi r_s^2 \Delta r$

Reaction for ethanol fermentation

- Glucose for cell growth at yield coefficient = 0.6

$C_6H_{12}O_6 + 1.95O_2 + 0.9NH_3 + cell \longrightarrow 1.5CO_2 + 4.65H_2O + 4.5cell(CH_{1.2}O_{0.5}N_{0.2})$

- Glucose for ethanol fermentation

$C_6H_{12}O_6 + cell \longrightarrow 2C_2H_5OH + 2CO_2 + cell$

Assumption

1. Cell density constant.
2. Concentration of substrate not depend radius of reactor.
3. No reaction in liquid phase

Liquid phase:

$$\frac{\partial}{\partial t}(1-\varepsilon)A\Delta l S(l,t) = uA(1-\varepsilon)\big(S(l-\Delta l,t)-S(l,t)\big) - k_S aA\Delta l\big(S_S(R,l)-S(l,t)\big)$$

$$\frac{\partial}{\partial t}S(l,t) = \frac{u\big(S(l-\Delta l,t)-S(l,t)\big)}{\Delta l} - \frac{k_S a\big(S_S(R,t)-S(l,t)\big)}{1-\varepsilon}$$

$$\frac{\partial}{\partial t}S(l,t) = -u\frac{\partial}{\partial l}S(l,t) - \frac{k_S a\big(S_S(R,t)-S(l,t)\big)}{1-\varepsilon}$$

$$\frac{\partial}{\partial t}S(l,t) = -u\frac{\partial}{\partial l}S(l,t) - \frac{k_S a\big(S_S(surface,t)-S(l,t)\big)}{1-\varepsilon}$$

At steady state

$$\frac{\partial}{\partial t}S(l,t) = 0$$

Boundary condition

$$S(0,t) = S_0$$

Solid phase:

- **Sphere bed**

$$\frac{\partial}{\partial t}\left(4\pi r^2 \Delta r S_S(r,t)\right)=\left(-D_{eff,s}\,4\pi r^2\frac{\partial}{\partial r}S_S(r,t)\right)-\left(-D_{eff,s}\,4\pi\left(r+\Delta r^2\right)\frac{\partial}{\partial r}S_S(r+\Delta r,t)\right)$$

$$-\upsilon_S\,4\pi r^2\Delta r$$

$$\frac{\partial}{\partial t}\left(S_s(r,t)r^2\Delta r\right)=\left(-D_{eff,s}r^2\frac{\partial}{\partial r}S_s(r,t)\right)-\left(-D_{eff,s}\left(r+\Delta r\right)^2\frac{\partial}{\partial r}S_s(r+\Delta r,t)\right)-\upsilon_s r^2\Delta r$$

$$\frac{d}{dt}S_s(r,t)=\frac{D_{eff,s}}{r^2\Delta r}\left[\left(\left(r+\Delta r\right)^2\frac{\partial}{\partial r}S_s(r+\Delta r,t)\right)-\left(r^2\frac{\partial}{\partial r}S_s(r,t)\right)\right]-\upsilon_s$$

$$\frac{\partial}{\partial t}S_s(r,t)=\frac{D_{eff,s}}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial}{\partial r}S_s(r,t)\right)-\upsilon_s$$

$$\frac{\partial}{\partial t}S_s(r,t)=\frac{D_{eff,s}}{r^2}\left(r^2\frac{\partial^2}{\partial r^2}S_s(r,t)+2r\frac{\partial}{\partial r}S_s(r,t)\right)-\upsilon_s$$

$$\frac{\partial}{\partial t}S_s(r,t)=D_{eff,s}\left(\frac{\partial^2}{\partial r^2}S_s(r,t)+\frac{2}{r}\frac{\partial}{\partial r}S_s(r,t)\right)-\upsilon_s$$

$$0=D_{eff,s}\left(\frac{\partial^2}{\partial r^2}S_s(r)+\frac{2}{r}\frac{\partial}{\partial r}S_s(r)\right)-\upsilon_s \qquad \text{At steady state}$$

At steady state

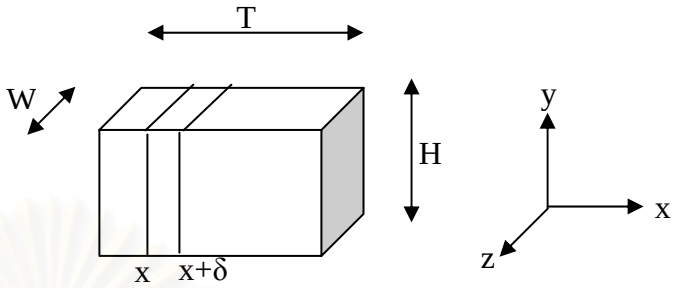
$$\frac{\partial}{\partial t}S_s(0,t)=0$$

Boundary condition

$$\frac{\partial}{\partial r}S_S(0,t)=0$$

$$-D_{eff,s}\,4\pi R^2\frac{\partial}{\partial r}S_s(R,t)=k_s aA\Delta l\left(S_s(R,t)-S(l,t)\right)$$

- **Cubic bed**



$$(WH\delta)\frac{\partial}{\partial t}S_s(x,t)=-D_{eff,s}(WHX)\frac{\partial}{\partial x}S_{s,}(x,t)-\left(-(WH(X+\delta))D_{eff,s}\frac{\partial}{\partial x}S_{s,}(x+\delta,t)\right)$$

$$-(WH\delta)\upsilon_s(x,t)$$

$$\frac{\partial}{\partial t}S_s(x,t)=\lim_{x\to 0}D_{eff,s}\frac{\left((X+\delta)\frac{\partial}{\partial x}S_s(x+\delta,t)-X\frac{\partial}{\partial x}S_s(x,t)\right)}{(WH\delta)}-\upsilon_s(x,t)$$

$$\frac{\partial}{\partial t}S_s(x,t)=D_{eff,s}\frac{\partial^2}{\partial x^2}S_s(x,t)-\upsilon_s(x,t)$$

$$\frac{\partial}{\partial t}S_s(y,t)=D_{eff,s}\frac{\partial^2}{\partial x^2}S_s(y,t)-\upsilon_s(y,t)$$

$$\frac{\partial}{\partial t}S_s(z,t)=D_{eff,s}\frac{\partial^2}{\partial x^2}S_s(z,t)-\upsilon_s(z,t)$$

At steady state

$$\frac{\partial}{\partial t}S_s(i,t)=0$$

Boundary condition

$$\frac{\partial}{\partial i} S_s(0,t) = 0,\ \frac{\partial}{\partial i} S_s(j,t) = 0$$

$$-D_{eff,s}\left(Surface.area\right)\frac{\partial}{\partial i} S_s(Surface,t) = k_s aA\Delta l\left(S_s(surface,t) - S(l,t)\right)$$

* i = x, y, z and j = H ,W, T

Where

F = volumetric flow rate

D_{eff} = effectiveness diffusivity

t = time

k_s = masstransfer coefficient

a = area of solid per volume of liquid

u = velocity of liquid

υ_s = rate of reaction for substrate

r = radius in bed that attention

ε = volume of solid phase per volume of liquid phase

S = substrate concentration in liquid phase

S_s = substrate concentration in solid phase

P = product concentration

l = height of reactor that attention

A = cross-section area of reactor

R = radius of particle

T, W and H = length of dimensions x, y and z of cubic bed

VITA

Phoowit Bangrak was born on 1 June 1983, in Suratthani, Thailand. He earned a Bachelor of Engineering Degree from the Department of Chemical Engineering, Prince of Songkhla University., Thailand, in 2005. He then subsequently fulfilled the requirements for a Master of Engineering Degree at the Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Thailand, in 2007