การศึกษาการผลิตเอทานอลในระดับกึ่งอุตสาหกรรมของเชื้อผสม *Saccharomyces cerevisiae* M30

และ *Kluyveromyces marxianus* DMKU 3-1042 ในถังปฏิกรณ์แบบแพคเบด

นายบุญฤทธิ์ เกียรติไพบูลย์

A PILOT-SCALE STUDY OF ETHANOL PRODUCTION BY MIXED CULTURE
OF *SACCHAROMYCES CEREVISIAE* M30 AND *KLUYVEROMYCES MARXIANUS*
DMKU 3-1042 IN A PACKED-BED REACTOR

Mr. Boonyarit Kiatpaiboon

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 2010
Copyright of Chulalongkorn University

Thesis Title   A PILOT-SCALE STUDY OF ETHANOL PRODUCTION BY MIXED CULTURE OF *SACCHAROMYCES CEREVISIAE* M30 AND *KLUYVEROMYCES MARXIANUS* DMKU 3-1042 IN A PACKED-BED REACTOR

By   Mr. Boonyarit Kiatpaiboon

Field of Study   Chemical Engineering

Thesis Advisor   Associate Professor Muenduen Phisalaphong, Ph.D.

Accepted by the Faculty of Engineering, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

………………………………… Dean of the Faculty of Engineering

(Associate Professor Boonsom Lerdhirunwong, Dr.Ing.)

THESIS COMMITTEE

………………………………… Chairman

(Associate Professor Tharathon MongKhonsi, Ph.D.)

………………………………… Thesis Advisor

(Associate Professor Muenduen Phisalaphong, Ph.D.)

………………………………… Examiner

(Associate Professor Bunjerd Jongsomjit, Ph.D.)

………………………………… External Examiner

(Professor Savitree Limtong, D.Eng.)

บุญฤทธิ์ เกียรติไพบูลย์ : การศึกษาการผลิตเอทานอลในระดับกึ่งอุตสาหกรรมของเชื้อผสม *SACCHAROMYCES CEREVISIAE* M30 และ *KLUYVEROMYCES MARXIANUS* DMKU 3-1042 ในถังปฏิกรณ์แบบแพคเบด (A PILOT-SCALE STUDY OF ETHANOL PRODUCTION BY MIXED CULTURE OF *SACCHAROMYCES CEREVISIAE* M30 AND *KLUYVEROMYCES MARXIANUS* DMKU 3-1042 IN A PACKED-BED REACTOR.) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. เหมือนเดือน พิศาลพงศ์, 84 หน้า.

พลังงานหมุนเวียนเอทานอลได้รับความสนใจเพิ่มขึ้น เนื่องจากมีคุณสมบัติที่เป็นประโยชน์หลาย ประการ เช่น เป็นพลังงานสะอาดและผลิตได้จากการหมักชีวมวล ขณะที่ประเทศไทยตั้งอยู่ในเขตร้อน, มี ศักยภาพในการผลิตอ้อยซึ่งเป็นแหล่งคาร์บอนที่สำคัญ นอกจากนี้การหมักเอทานอลที่อุณหภูมิสูงได้รับ ความสนใจมากสำหรับการผลิตเอทานอลมีผลตั้งแต่วันเวลาอุณหภูมิเฉลี่ยมักจะสูงตลอดทั้งปี ข้อดีของ การหมักเอทานอลที่อุณหภูมิสูงจะลดค่าใช้จ่ายในการทำความเย็นและลดความเสี่ยงจากการ ปนเปื้อน ดังนั้นในงานนี้จึงทำการศึกษาการหมักเอทานอลโดยใช้ยีสต์ทนร้อน, *Kluyveromyces marxianus* DMKU 3-1042 และ *Saccharomyces cerevisiae* M30 ในระบบเซลล์เดี่ยวและระบบเซลล์ผสม ทำการ ตรวจสอบโดยใช้ระบบเซลล์แขวนลอยและระบบเซลล์ตรึงบนเส้นใยของรังไหมบาง โดยใช้น้ำตาลทราย แดงเป็นแหล่งพลังงานคาร์บอนที่ความเข้มข้นน้ำตาลเริ่มต้น   220 กรัมต่อลิตร พบว่าการหมักเอทานอล โดยระบบเซลล์ผสมที่ถูกตรึงโดยใช้รังไหมบางเป็นระบบที่มีประสิทธิภาพมากที่สุด ภายใต้สภาวะการ หมักแบบกะโดยใช้ขวดเขย่าขนาด 500 มิลลิลิตร พบว่าการตรึงเซลล์ของระบบเซลล์ผสม สามารถผลิตเอ ทานอลได้อย่างมีประสิทธิภาพสูงที่หลากหลายอุณหภูมิ (33 - 40 องศาเซลเซียส) สำหรับการใช้การตรึง เซลล์ระบบเซลล์ผสมบนรังไหมบางในถังปฏิกรณ์แบบแพคเบดขนาด 1 ลิตรสำหรับการหมัก แบบต่อเนื่องภายใต้สภาวะไม่ควบคุมอุณหภูมิ พบว่าได้ผลผลิตสูงสุดที่ 16.55 กรัมต่อลิตรต่อชั่วโมงที่ อัตราการเจือจาง 0.40 ต่อชั่วโมง โดยได้ความเข้มข้นของเอทานอล 41.86   กรัมต่อลิตร และได้ความ เข้มข้นของเอทานอลสูงสุด 70.95 กรัมต่อลิตร เมื่อดำเนินการที่อัตราการเจือจาง 0.10 ต่อชั่วโมง การตรึง เซลล์ระบบเซลล์ผสมบนรังไหมสำหรับกระบวนการหมักอย่างต่อเนื่องภายใต้การทำงานที่ไม่มีการ ควบคุมอุณหภูมิ นอกจากนั้นการผลิตเอทานอลแบบต่อเนื่องดำเนินการอย่างดีในถังปฏิกรณ์แบบแพคเบด ขนาด 100 ลิตร สามารถผลิตความเข้มข้นเอทานอล 85.34±3.00 กรัมต่อลิตรและผลผลิต 8.53 กรัมต่อ ลิตรต่อชั่วโมง

ภาควิชา........วิศวกรรมเคมี........   ลายมือชื่อนิสิต………………………………........
สาขาวิชา........วิศวกรรมเคมี........   ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก...........................
ปีการศึกษา..........2553..........

5270367421 : MAJOR CHEMICAL ENGINEERING

KEYWORDS : IMMOBILIZED CELL/ DARK BROWN SUGAR/ MIXED CULTURE/ ETHANOL/ *SACCHAROMYCES CEREVISIAE/ KLUYVEROMYCES MARXIANUS/*

BOONYARIT KIATPAIBOON: A PILOT-SCALE STUDY OF ETHANOL PRODUCTION BY MIXED CULTURE OF *SACCHAROMYCES CEREVISIAE* M30 AND *KLUYVEROMYCES MARXIANUS* DMKU 3-1042 IN A PACKED-BED REACTOR. ADVISOR: ASSOC. PROF. MUENDUEN PHISALAPHONG, Ph.D., 84 pp.

A renewable energy, ethanol earns more interest because of its benefits such as clean energy and production from biomass fermentation. As Thailand is located in the tropics, sugarcane has the potential to serve as an important carbon source. Moreover, ethanol fermentation at high temperature has received much attention for effective ethanol production since average day-time temperatures are usually high throughout the year. The advantages of ethanol fermentation at high temperature are reduced in cooling costs and decreased risk of contamination. Hence, in the present work, ethanol fermentations using thermotolerant yeast, *Kluyveromyces marxianus* DMKU 3-1042 and *Saccharomyces cerevisiae* M30 in monoculture and mixed cultures were studied. The cultures in forms of suspended cells and immobilized cells adsorption on the fibrous of thin shell silk cocoon (TSSC) were used for the investigation with dark brown sugar as a carbon source at the initial sugar concentration of 220 g/l. The ethanol fermentation by the mixed culture system using TSSC as a carrier was found to be the most effective system. Under batch fermentation in 500 ml Erlenmeyer shaking-flasks, the immobilized mixed culture system was capable of highly efficient ethanol production at various temperatures (33-40°C).Under uncontrolled temperature condition in 1-L continuous packed-bed reactor using the mixed culture immobilized on TSSC, the highest productivity (16.55 g/L h) was obtained at 0.40 h^{-1} of dilution rate with ethanol concentration of 41.86 g/L, whereas, the maximum of ethanol concentration (70.95 g/L) was obtained at 0.10 h^{-1} of dilution rate The immobilized mixed culture on TSSC was promising for continuous fermentation process under uncontrolled operating temperature. Moreover the continuous ethanol production was excellently performed carried out in a 100-L packed bed, the ethanol concentration and productivity were 85.34±3.00 g/L and 8.53 g/L h, respectively.

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

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

Academic Year :   2010

ACKNOWLEDGEMENTS

CONTENTS

CHAPTER

LIST OF TABLES

LIST OF FIGURES

PAGE

CHAPTER I

INTRODUCTION

Global energy crisis is a major problem worldwide due to the decline in fossil fuel supplies. In addition, the massive utilization of fossil fuels has led to the problem of gas pollution. The reduction of greenhouse gas emissions has become a primary focus of environmental concern. Therefore, alternative energy sources need to be renewable, sustainable, efficient and cost-effective with less greenhouse gas emissions than fossil fuels. In the past decades, bioethanol produced from biomass has been interested as an alternative fuel for the future [1].

As Thailand is located in the tropics, sugarcane has the potential to serve as an important carbon source. Fresh sugar cane juice, dark brown sugar and cane blackstrap molasses can be used as a raw material for ethanol fermentation. Moreover, ethanol fermentation at high temperature has received much attention for effective ethanol production since average day-time temperatures are usually high throughout the year. The advantages of ethanol fermentation at high temperature are reduced in cooling costs and decreased risk of contamination [2]. Several microorganisms, including the well known yeast ethanol producer, *Saccharomyces cerevisiae*, *Leuconostoc oenos* and *Zymomonas mobilis* are candidates for ethanol production [3]. *Saccharomyces cerevisiae* M30 has been reported previously as a strain capable of producing ethanol at temperature ranging from 30 to 35°C when cultured on media containing cane molasses [4]. In addition *Kluyveromyces marxianus* DMKU 3-1042 was an effective strain that could be employed for ethanol production at elevated temperature up to 45 ºC when sugar cane juice was used as a raw material [2]. Among various techniques for enhanced ethanol production, cell immobilization is one of the effective means. Immobilized cell systems present many advantages over free cell systems, such as relative simplicity of product separation, high volumetric productivity, improved process control and cellular stability [3]. In our previous work, thin shell silk cocoons (TSSC) were used as cell carrier for immobilization of *S.*

cerevisiae M30 and *K. marxianus* DMKU 3-1042. TSSC is shown to be a very economical and excellent cell carrier for ethanol fermentation [5].

The aim of this work is to study the ethanol production by mixed culture system of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 using dark brown sugar medium. Preliminary studies are performed using the suspended mixed cell culture in batch shaking-flasks. Furthermore, continuous ethanol fermentation using the immobilized mixed culture on TSSC are performed in packed-bed reactors for both laboratory and pilot-scale testing.

1.1 Objectives

    1.1.1   To evaluate ethanol production from dark brown sugar medium by the mixed cultures of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042.

    1.1.2   To develop continuous ethanol fermentation process using the immobilized mixed culture of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 on TSSC in a pilot-scale packed bed reactor.

1.2 Expected benefits

    1.2.1   Invention of high performance ethanol fermentation process for large scale commercial ethanol fermentation in a packed bed reactor.

    1.2.2   Useful information for a better understanding of the mixed culture system of ethanol fermentation.

1.3 Working scopes

In this study, the immobilized mixed cell culture for ethanol production is carried out in a packed bed reactor with the working volumes of 1-L and 100-L. The optimum condition for ethanol production from the previous work [5] is applied for this study. The working scopes are as follows:

    1.3.1   Flocculating yeasts strains, *S. cereviceae* M30 and *K. marxianus* DMKU 3-1042 are used as ethanol producers.

    1.3.2   The study of suspended cell culture is carried out in batch mode in a temperature range of 33 - 40°C.

    1.3.3   Dark brown sugar are utilized as carbon and energy source.

13.4 Batch fermentation is carried out in shake flask culture system at shaking frequency of 200 rpm and at initial sugar concentration of 220g/l with the initial pH of 5.0.

1.3.5 Continuous fermentation is carried out in a pack-bed reactor with the working volume of 1-L (6 cm diameter and 34 cm height). The dilution rate is varied from 0.1, 0.2, 0.3 to 0.4 h^{-1}. TSSC is used for the cell immobilization.

1.3.6 The configuration of the 1-L packed-bed reactor is used for the scale up of the 100-L packed-bed reactor. The suitable condition from the experimental study in the 1-L reactor is used for the study in the 100-L reactor.

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

2.1. Ethanol fermentation

Due to fast depleting of petroleum reserves and the environmental pollution resulting from the over-utilization of petroleum-derived products [6], in recent years, ethanol has been interested as one of the most important alternative resources. Renewable resources such as agricultural, industrial and urban wastes could be used for ethanol fermentation in an ecofriendly and benefit manner. Moreover, the tropical countries have the potential to serve for agricultural resources such as sugar cane, corn and cassava which are used as raw materials for the ethanol fermentation [7].

2.1.1 Raw materials

Raw materials used in the manufacture of ethanol via fermentation are conveniently classified under three types of agricultural raw materials: sugar, starches, and cellulose materials.

1. Sugars (from sugar cane, sugar beets, molasses and fruits) can be converted to ethanol directly.

2. Starches (from grains, potatoes and root crops) must first be hydrolyzed to fermentable sugars by the action of enzymes from malt or molds.

3. Cellulose (from wood, agricultural residues, waste sulfite liquor from pulp and paper mills) must likewise be converted to sugars, generally by the action of mineral acids. Once simple sugars are formed, enzymes from yeast can readily ferment them to ethanol [8].

2.1.2 Microorganisms

Many microorganisms can be used for the production of ethanol. However, yeasts especially *S. cerevisia*e, still remain as the major species [6].

2.1.2.1 *Saccharomyces cerevisiae*

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. 2.1. 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 [6].

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 [6].

2.1.2.2 *Kluyveromyces marxianus*

K. marxianus, described as a homothallic and hemiascomycetous yeast, is phylogenetically related to *S. cerevisiae*. *K. marxianus* has been widely adopted by industry, mainly because it possesses traits that are desirable for biotechnology applications. These include the capacity to assimilate key sugars, namely lactose and inulin; an extremely rapid growth rate, with typical generation times of ~70min; thermotolerance, with the ability to growth up to 52 ^{o}C; and a high secretory capacity [9]. As a consequence, several different biotechnological applications have been investigated with this yeast, such as for production of enzymes (β-galactosidase, β-glucosidase, inulinase, and polygalact-uronases), single-cell protein, aroma compounds, and ethanol (including high-temperature and simultaneous saccharification-fermentation processes); reduction of lactose content in food

products; production of bioingredients from cheese-whey; bioremediation; or as an anticholesterolemic agent; and as a host for heterologous protein production [10].
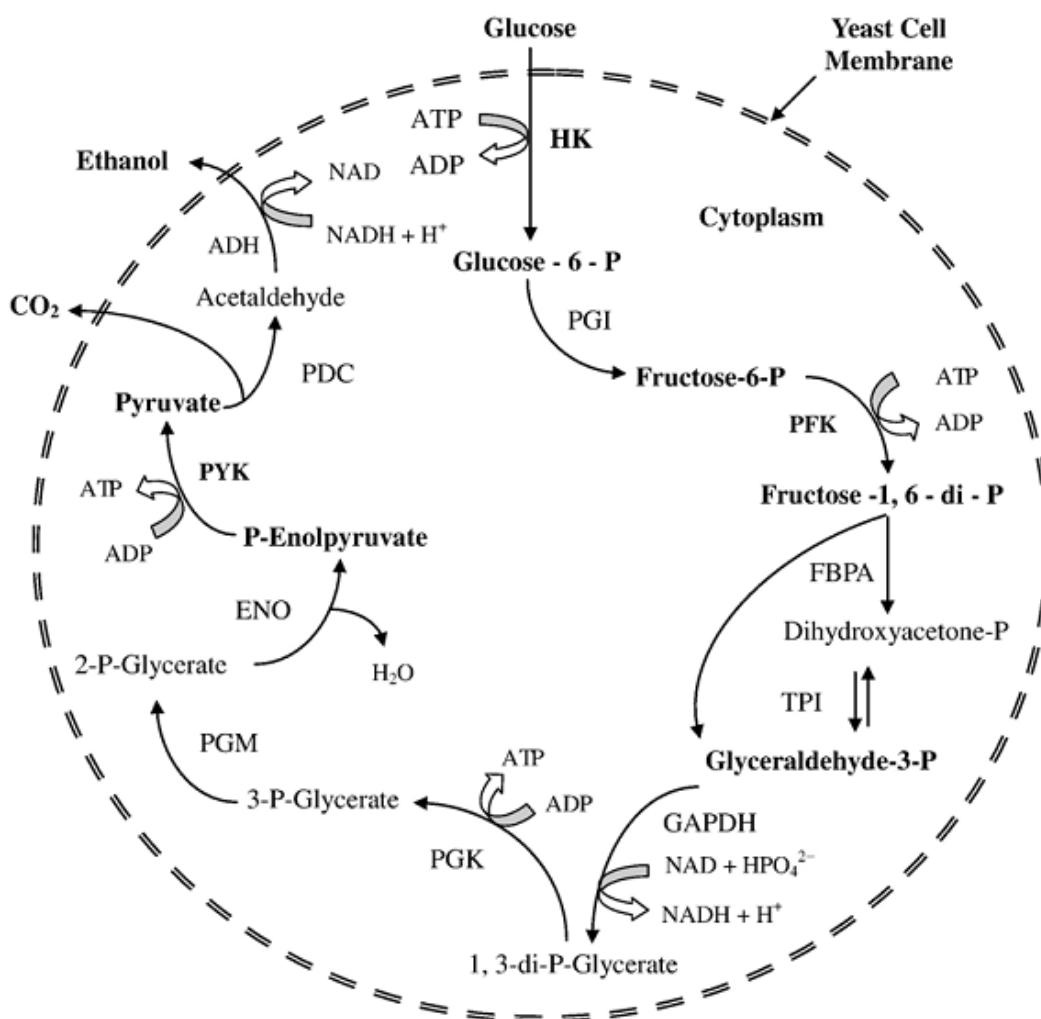
Fig. 2.1. Metabolic pathway of ethanol fermentation in *S. cerevisiae.* [9].

Abbreviations: HK: hexokinase, PGI: phosphoglucoisomerase,
PFK: phosphofructokinase, FBPA: fructose bisphosphate aldolase,
TPI: triose phosphate isomerase, GAPDH: glyceraldehydes-3-phosphate dehy-
drogenase, PGK: phosphoglycerate kinase, PGM: phosphoglyceromutase,
ENO: enolase, PYK: pyruvate kinase,
PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase.

2.2 Cell immobilization

Cell immobilization was defined as ''the physical confinement or localization of intact cells to a certain region of space with preservation of some desired catalytic activity''. Immobilization often mimics what occurs naturally when cells grow on surfaces or within natural structures. Many microorganisms own the ability to adhere to different kinds of surfaces in nature. Immobilization techniques can be divided into four major categories based on the physical mechanism employed (Fig. 2): (a) attachment or adsorption on solid carrier surfaces, (b) entrapment within a porous matrix, (c) self-aggregation by flocculation (natural) or with cross-linking agents (artificially induced), and (d) cell containment behind barriers [11].
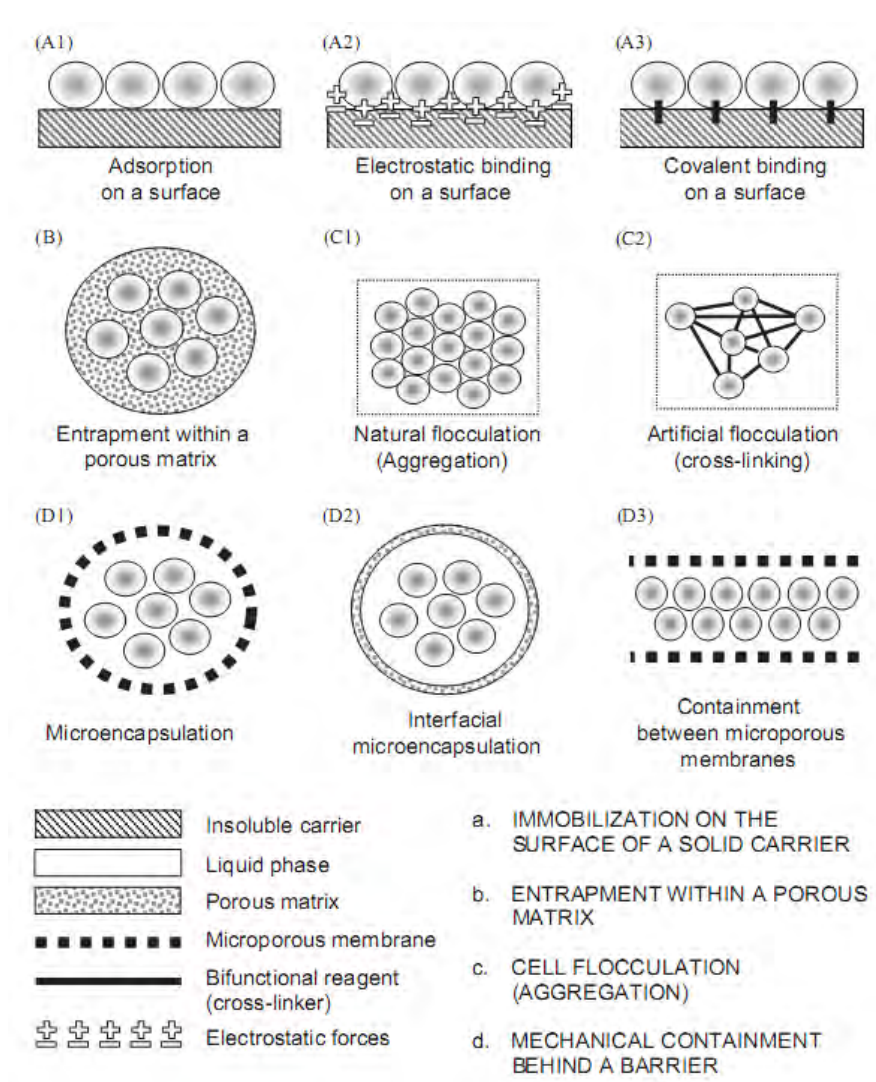
Fig. 2.2. Basic methods of cell immobilization [11].

2.2.1. Immobilization on solid carrier surfaces

Cell immobilization on a solid carrier is carried out by physical adsorption due to electrostatic forces or by covalent binding between the cell membrane and the carrier. Systems using immobilized cells on a surface are popular due to the relative ease of carrying out this type of immobilization. The strength with which the cells are bonded to the carrier as well as depth of the biofilm often varies and is not readily determined. As there are no barriers between the cells and the solution, cell detachment and relocation is possible with potential establishment of equilibrium between adsorbed and freely suspended cells Examples of solid carriers used in this type of immobilization are cellulosic materials (DEAE-cellulose, wood, sawdust, delignified sawdust), inorganic materials (polygorskite, montmorilonite, hydromica, porous porcelain, porous glass), etc. Solid materials like glass or cellulose can also be treated with polycations, chitosan or other chemicals (pre-formed carriers) to enhance their adsorption ability [11].

2.2.2 Entrapment within a porous matrix

In this type of immobilization, the cells are either allowed to penetrate into the porous matrix until their mobility is obstructed by the presence of other cells, or the porous material is formed in situ into a culture of cells. Both entrapment methods are based on the inclusion of cells within a rigid network to prevent the cells from diffusing into the surrounding medium, while still allowing mass transfer of nutrients and metabolites. Cell growth in the porous matrix depends on diffusion limitations imposed by the porosity of the material and later by the impact of accumulating biomass. Examples of this type of immobilization are the entrapment into polysaccharide gels like alginates, k-carrageenan, agar, chitosan and polygalac-turonic acid or other polymeric matrixes like gelatin, collagen and polyvinyl alcohol [11].

2.2.3 Cell flocculation (Aggregation)

Flocculation can be considered as an immobilization technique as the large size of the aggregates makes their potential use in reactors possible. Yeast flocculation is a property of major importance for the ethanol industry as it affects fermentation

productivity and yeast removal and recovery. Because of their macroscopic size and their mass, the yeast flocs 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, flocculation can be affected by numerous parameters, such as nutrient conditions, agitation, Ca^{2+}-concentration, pH, fermentation temperature, yeast handling and storage conditions. Artificial flocculating agents or cross-linkers however can be used to enhance aggregation in cell cultures that do not naturally flocculate [11].

2.2.4 Mechanical containment behind a barrier

Containment of cells behind a barrier can be attained either by use of microporous membrane filters or by entrapment of cells in a microcapsule or by cell immobilization on to an interaction surface of two immiscible liquids. The major disadvantages of cells immobilization between microporous membranes are mass transfer limitations and possible membrane biofouling caused by cell growth [11].

2.2.5 Prerequisites for cell immobilization

Many materials are suitable for cell immobilization for use in the production of ethanol, when the following prerequisites are satisfied [11]:

1. The carrier should have high surface area for cell attachment.

2. The carrier must be easy to handle and regenerate.

3. Cell viability and operational stability of the immobilized cell must be high and retained for longer times.

4. The biological activity of the immobilized cells should not be negatively affected by the immobilization process.

5. The carrier should maintain good mechanical, chemical, thermal and biological stability and not be easily degraded by enzymes, solvents, pressure changes or shearing forces.

6. The carrier and immobilization technique should be easy, cost effective and amenable to scale-up.

2.2.6 Advantages of immobilized cells over free cell systems

The use of immobilized systems for ethanol production presents many advantages over conventional free cells fermentations including:

1. Prolonged activity and stability of the biocatalyst. The immobilization support may act as a protective agent against physicochemical effects of pH, temperature, solvents or even heavy metals.

2. Superior cell densities per unit bioreactor volume, which leads to high volumetric productivity, increased substrate uptake, yield improvement, shorter fermentation times and elimination of non-productive cell growth phases.

3. Reduction of risk of microbial contamination due to high cell densities and fermentation activity.

4. Feasibility of continuous processing.

5. Easier product recovery through reduction of separation and filtration requirements, thus reducing cost for equipment and energy demands.

6. Regeneration and reuse of the biocatalyst for extended periods in batch operations, without removing it from the bioreactor.

2.3 Bioreactor

The overall productivity of an immobilized bioparticle process, and hence the feasibility of its industrial use, depend to a large extent on the choice of the reactor system. In the case of the production of ethanol by fermentation, which is only attractive if the costs involved are no more than those of the usual petrochemical process, a ago study has concluded that replacement of batch processes by continuous immobilized cell systems may result in higher production and investment costs [12].

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 2.3**.
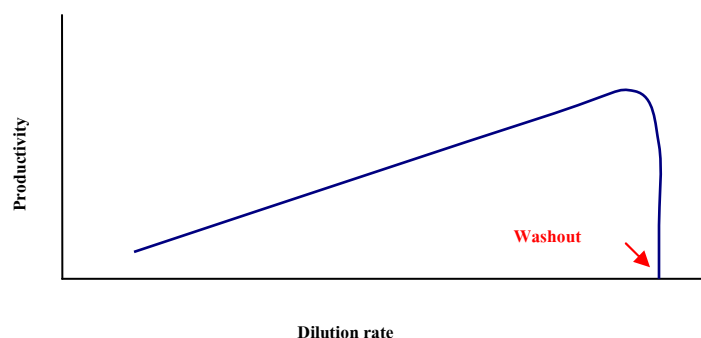
Figure 2.3. Productivity versus dilution rate curve

After reaching an optimum value, the productivity will decrease drastically until it reaches nearly zero. In some cases, the optimum bioreactor volume is set by the critical dilution rate which corresponds to the dilution rate at which washout occurs. Figure 2.4 shows a relationship between substrate, product, and biomass concentration with critical dilution rate [13].

The design of fermentors must therefore take into account the need to maintain the activity of the fermenting microorganisms. Most of the continuous bioreactor systems currently being evaluated in laboratory experiments or pilot plants work with entrapped cells. The chief problem they face is that of ensuring adequate diffusion of substrate and oxygen through the matrix toward the immobilized cells, and adequate diffusion of carbon dioxide, ethanol and other metabolites out of the matrix. Poor diffusion may severely limit the productivity of a bioreactor [12].
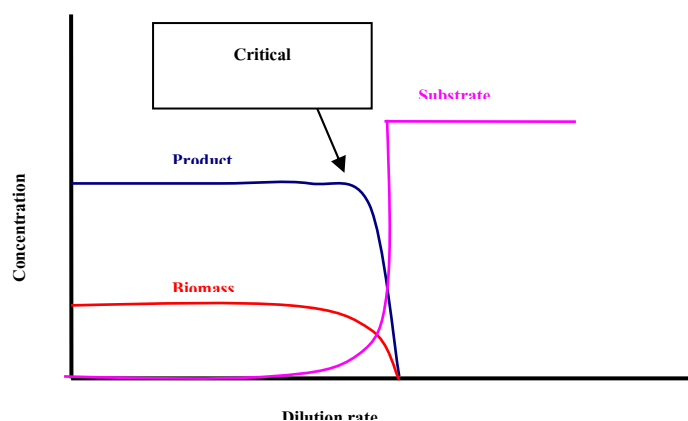
Figure 2.4 Concentration profile with variable dilution rate

As is well known, the production of alcohol by fermentation is inhibited by the products. Together with economic considerations, this circumstance is crucial in deciding on the type of reactor system in which to perform fermentation. Continuous fermentation by free cells in stirred tank reactors (CSTRs) has the drawback of concentrating the product to a maximum. 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 can maintain higher productivity as compared to conventional suspended cells culture [12, 14].
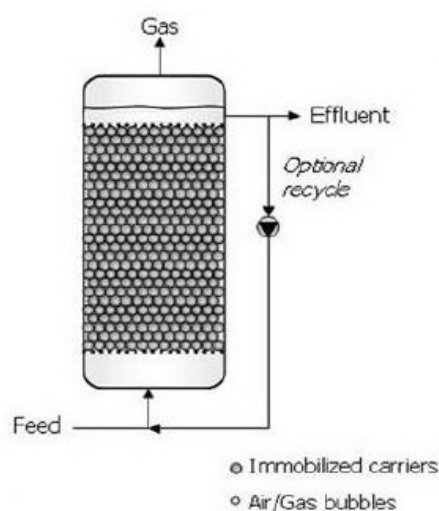
Figure 2.5. Packed bed reactor [15].

Packed bed reactors (PBR) are one of the most frequently employed types of bioreactor for immobilization systems. The reactor consists of a tube, usually vertical, packed with catalyst particles. Medium can be fed either at the top or bottom of the column and forms a continuous liquid phase between the particles. This type of bioreactor has the advantages of simplicity of operation and high reaction rates. Cells are immobilized in appropriate carriers, which are packed in the fixed reactors, resulting in high solid-liquid specific interfacial contact areas, and the velocity of liquid creeping over the static solid particles substantially alleviates the film resistance to mass transfer. The major disadvantages of the PBR are mass transfer

limitations, difficulties in CO_2 evacuation, compression of some carrier materials and fouling [14].

The most important characteristic of a PBR is that material flows through the reactor as a plug; they are also called plug flow reactors (PFR). Ideally, all of the substrate stream flows at the same velocity, parallel to the reactor axis with no back - mixing. All material present at any given reactor cross-section has had an identical residence time. The longitudinal position within the PBR is, therefore, proportional to the time spent within the reactor; all product emerging with the same residence time and all substrate molecule having an equal opportunity for reaction. The conversion efficiency of a PBR, with respect to its length, behaves in a manner similar to that of a well-stirred batch reactor with respect to its reaction time [14].

2.4   Scale-up strategies of bioreactors [17, 18].

Scale-up means reproducing in large-scale equipment based on the results from a successful fermentation made in laboratory- or pilot-scale equipment. The scale-up strategy directly influences the production capacity and efficiency of bioprocesses. The scale-up procedures are usually developed in three stages or scales:

1. Bench or laboratory scale, where basic screening procedures are carried out.

2. Pilot plant, where the optimal operating conditions are ascertained.

3. Plant scale, where the process is brought to economic fruition.

The process characteristics which have been suggested to be maintained constant during scale-up include: reactor geometry, volumetric oxygen transfer coefficient, maximum shear, power input per unit volume of liquid, volumetric gas flow rate per unit volume of liquid, superficial gas velocity, mixing time, impeller Reynolds number and momentum factor.

2.5 Review of ethanol fermentation

Limtong et al. (2007) reported the newly isolated *K. marxianus* strain DMKU 3-1042 for ethanol production at high temperatures. *K. marxianus* strain DMKU 3-1042 was an effective strain for ethanol production from sugar cane juice medium. It produced 8.7% (w/v) ethanol at 37 °C and 6.78% (w/v) at 40 °C. In addition, under the ethanol fermentation at 37°C in a 5-L jar fermenter with an agitation speed of 300 rpm and an aeration rate of 0.2 vvm, *K. marxianus* DMKU 3-1042 yielded a final ethanol concentration of 6.43% (w/v).

Ogbonna et al. (2001) demonstrated that efficient ethanol production from sugar beet juice was possible even without any pretreatment of the juice by using immobilized cells, *Saccharomyces cerevisiae* IR2 on loofa sponge and an external loop bioreactor to achieve uniform cell distribution within the bed. The efficient large scale production systems can be constructed. There were no significant differences in ethanol productivity and yield from the 50-L external loop bioreactor when compared with those from the 2-L bubble column bioreactor.

Yu et al. (2007) studied a novel immobilization method of *S. cerevisiae* to natural sorghum bagasse for ethanol production. Natural sorghum bagasse without any treatment was used to immobilize *S. cerevisiae* at 0.6±0.2 g dry cell weight (DCW)/g dry sorghum bagasse weight (DSW). The companionship between yeast cells and sorghum bagasse are adsorption and embedding. 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. The maximum ethanol productivity of 16.68 g/(L h) appeared at the dilution rate of 0.3 h^{-1}.

Guo et al. (2008) studied ethanol production by the mixed immobilized cells of *K. marxianus* and *S. cerevisiae*. From the experimental study at 30°C, the use of the mixed culture of immobilized cells improved ethanol fermentation using cheese whey powder (CWP) as medium. This system achieved higher ethanol productivity than that from fermentations by free cells or coimmobilized cells.

Cáceres-Farfán et al. (2008) studied ethanol production from henequen (*Agave fourcroydes Lem*.) juice and molasses by the mixture of two yeasts: *K. marxianus* (isolated from the henequen plant) and *S. cerevisiae* (commercial strain). Ethanol

production of 5.22 $\pm$ 1.087% v/v with residual reducing sugars of 2-4 g/l were obtained. The best results were obtained when the mixture of 25% *K. marxianus* and 75% *S. cerevisiae* or *S. cerevisiae* alone were used with an initial inoculum concentration of 3×10^7 cell mL^{-1}.

Saccharum spontaneum (wild sugarcane) was reported as potential, renewable and low cost biomass for the production of ethanol [Chandel et al., 2009]. Aqueous ammonia pretreated *S. spontaneum* showed the increased enzymatic digestibility of total carbohydrate content of the substrate using the enzyme solution of *A. oryzae* MTCC1846. Enzymatic hydrolysate of *S. spontaneum* was then tested for ethanol production under batch and repeated batch production system using ''in-situ'' entrapped *S. cerevisiae* VS3 cells in *S. spontaneum* stalks (1 cm x 1 cm). Batch fermentation of VS3 free cells and immobilized cells showed ethanol production, 19.45 ± 0.55 g/L and 21.66 ± 0.62 g/L respectively. Immobilized VS3 cells showed maximum ethanol production (22.85 ± 0.44 g/L) up to 8th cycle during repeated batch fermentation followed by a gradual reduction in subsequent cycles of fermentation.

Kandylis et al. (2010) studied the immobilization of *S. cerevisiae* AXAZ-1 yeast cells on whole wheat grains for wine making in a wide range of temperatures in laboratory scale and in a scale-up system of 80-L. When the 30 repeated batch fermentations were completed, the support was used in a scale-up system, which retained its operational stability for a period longer than 5 months. The scale-up process did not affect the fermentative ability of biocatalyst, even at low temperatures.

Behera et al. (2011) reported the ethanol production from mahula (*Madhuca latifolia* L.) flowers with immobilized cells of *S. cerevisiae* in *Luffa cylindrica* L. sponge discs. In this method, no pretreatment of luffa matrix was required. Cells not only survived but also were physiologically active in three more cycles of fermentation without significant reduction (<5%) in ethanol production. After 96 h, there was 91.1% sugar conversion producing 223.2 g ethanol/kg flowers (1st cycle), which was 0.99%, 2.3% and 3.2% more than the 2nd (221 g ethanol/kgflowers), the 3rd (218 g ethanol/kg flowers) and the 4th (216 g ethanol/kg flowers) cycle of fermentation, respectively. Also in comparison with the commercially available synthetic immobilization carriers, the use of luffa sponge does not lead to any additional technical problems during the scale up process.

Sodium-alginate immobilized yeast was employed to produce ethanol continuously using cane molasses as a carbon source in an immobilized cell reactor (ICR) (Ghorbani et al., 2011). The immobilization of *S.cerevisiae* was performed by entrapment of the cell cultured media harvested at exponential growth phase (16 h) with 3% sodium alginate. The ethanol production was affected by the concentration of the cane molasses (50, 100 and 150 g/L), dilution rates (0.064, 0.096, 0.144 and 0.192 h^{-1}) and hydraulic retention time (5.21, 6.94, 10.42 and 15.63 h) of the media. The pH of the feed medium was set at 4.5 and the fermentation was carried out at an ambient temperature. The maximum ethanol production, theoretical yield ($Y_{E/S}$), volumetric ethanol productivity (Q_P) and total sugar consumption was 19.15 g/l, 46.23%, 2.39 g l^{-1} h^{-1} and 96%, respectively.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Microbial Strains

Stock cultures of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 strains were kindly provided by Prof. Savitree Limtong, Dr.Eng. (Department of Microbiology, Kasetsart University, Bangkok). The stock cultures were stored in PDA agar slants at 4 °C.

3.1.2 Chemicals

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

Table 3.1 The chemicals used in this experiment

Chemical	Supplier
- Sucrose	Ajax Finechem
- Sodium hydroxides (NaOH)	Merck
- Hydrochloric acid (HCl)	J.T. Baker
- Ammonium sulfate $(NH_4)_2SO_4$	Ajax Finechem
- Magnesium sulfate $(MgSO_4.7H_2O)$	APS
- Potassium dihydrogen ortho-phosphate (KH_2PO_4)	Ajax Finechem
- Potato dextrose agar (PDA)	Himedia
- Absolute ethanol	Merck
- Na-K tartrate	Carlo Erba

3.1.3   Equipments

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

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

3. Gas chromatography (Shimadzu Model GC 7A$_G$, Japan).

4. Autoclave (Model Tomy Autoclave SS-325, Ner ima-ku, Tokyo, Japan).

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

6. Peristaltic pump (WATSON MARLOW 505U, England).

7. YSI (Model 2700 SELECT Biochemistry Analyzer, Yellow Springs, Ohio 45387 USA).

3.2 Methods for fermentation.

3.2.1 Methods for stock cell suspension preparation

The cell culture was transferred from an agar slant into 500 ml Erlenmeyer flask containing 150 ml sterilized cultivation medium. The cultivation medium was composed of 10% w/v sugar from palm sugar, 0.05%w/v $(NH_4)_2SO_4$, 0.05%w/v KH_2PO_4, and 0.15%w/v $MgSO_4.7H_2O$ at pH of 5.0. The medium was sterilized in autoclave for 15 minutes at 121°C. Cell cultivation was carried out in the Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 200 rpm, with the controlled temperatures of 33, 37, and 40 ^{o}C for 24 hours.
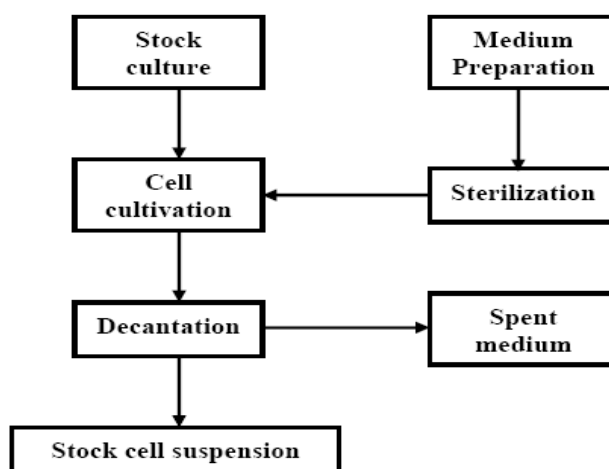
Figure 3.1 Diagram for methods of stock cell suspension preparation

3.2.2 Methods of coimmobilization

Thin shell silk cocoons of 2.5 g and 250 ml of culture medium in 500 ml flask were autoclaved for 15 minutes at 121°C. For coimmobilization, equal volume of 5 ml of each *K. marxianus* and *S. cerevisiae* stock cell suspension were mixed together. The mixed cell suspension was then added to the medium, which was called mixed culture. After that the sterilized thin shell silk cocoons was added into the mixed culture. The immobilized cells were obtained after the incubation of the suspension mixture for 24 hr. The method are shown in **Figure 3.2**
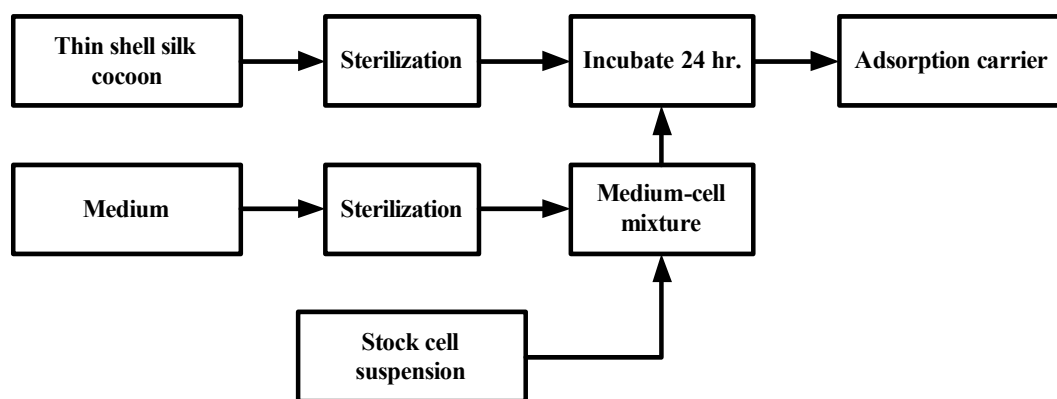
Figure 3.2 Preparation of thin shell silk carrier

3.2.3 Methods for ethanol fermentation.

3.2.3.1 Batch Fermentation

Dark brown sugar was used as carbon source for the fermentation. The medium contained initial sugar concentration of 220 g/L, 0.05%w/v $(NH_4)_2SO_4$, 0.05%w/v KH_2PO_4, and 0.15%w/v $MgSO_4.7H_2O$ and the initial pH was adjusted at 5.0. The prepared medium was sterilized at 121°C for 15 min. Experiments were initiated by transferring the prepared cell suspension into 500 ml Erlenmeyer flasks containing 250 ml of the fermentation medium in order to promote anaerobic condition which was favorable ethanol fermentation. Batch fermentation in shake flasks was performed in Innova 4330 Refrigerated Incubator Shaker (New Brunswick Scientific, USA) at 200 rpm. The fermentation was performed at the temperature varied from 33 to 45°C for 72 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. The considerable variables in batch system were including:

1. The substrate used as carbon source is dark brown sugar.

2. The effect of temperature on fermentation was studied in the temperatures range of 33 - 40°C.

3. The effect of mono and mixed cell cultures was studied by using suspension in monocultures of *K. marxianus* DMKU 3-1042 or mixed cultures of *K. marxianus* DMKU 3-1042 and *S. cerevisiae* M30.

3.2.3.2 Continuous Fermentation

The suitable condition from the batch system was applied for the continuous fermentation by coimmobilized on thin shell silk cocoons in packed bed columns. By using two different size reactors, one working volume has sized 0.7 L and another has sized 100 L. The concentrated sugar solutions with the initial sugar concentration of 220 g/l, 0.05%w/v $(NH_4)_2SO_4$, 0.05%w/v KH_2PO_4, and 0.15%w/v $MgSO_4.7H_2O$ with the adjusted pH at 5.0 was used as a fermentative medium. Temperature of systems is uncontrolled. Sampling was aseptically took 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.

3.2.4 Analytical methods

Ethanol assay is 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 is used in collaboration with N_2 as carrier gas. Flow rate of N_2 is 50 ml/min. The oven and detector temperatures are 190 ^{o}C and 240 ^{o}C respectively. The samples are injected with volume of 1μL and injection temperature of 240 ^{o}C.

The residual glucose concentration was determined by the YSI (Model 2700 SELECT Biochemistry Analyzer, Yellow Springs, Ohio 45387 USA). Firstly, 0.1 ml of sample is hydrolyzed with 0.5 ml of 37% w/v HCl. After the hydrolysis is stopped, the sample is neutralized using 0.5 ml of 30% w/v NaOH and diluted with 10 mL DI

water. After that, the samples were injected with volume of 25 μL in order to determine the total glucose concentration.

Cell concentration was determined by cell dry weight method. Yeast cells were harvested by centrifugation for 15 min at 20,000 rpm. The pellets were washed with HCl 0.1 N; after that it was washed twice with distilled water and weighed after 24 h of drying at 100°C. At the beginning and the end of fermentation, samples of carrier were collected for Scanning Electron Microscopy (SEM).

3.2.5 Calculation of fermentation parameters

Fermentation efficiency for bioreactor system was expressed as follows:

1   Immobilization yield (Y_I, %)

$$Y_I \quad = \quad \frac{X_I}{X_T} \times 100$$

2   Yield of sugar consumption (Y_S, %)

$$Y_S \quad = \quad \frac{S_0 - S_F}{S_0} \times 100$$

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

$$Y_{P/S} \quad = \quad \frac{P_F - P_0}{S_0 - S_F} \times 100$$

4   Ethanol productivity (Q_P, g/l h)

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

X_I	;	immobilized cell concentration (g/l)
X_E	;	free cell concentration (g/l)
X_T	;	total cell concentration (g/l)
S_0	;	initial sugar concentration (g/l)
S_F	;	final sugar concentration (g/l)
P_0	;	initial ethanol concentration (g/l)
P_F	;	final ethanol concentration (g/l)
D	;	dilution rate (h^{-1})

CHAPTER IV

RESULTS AND DISCUSSION

The purpose of this work was to develop continuous ethanol fermentation process from dark brown sugar using the immobilized mixed culture of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 on TSSC in a pilot-scale packed bed reactor. The experiments were divided into 3 parts. Firstly, prestudying the effect of temperature on ethanol fermentation from dark brown sugar medium by the suspension cell cultures of monocultures of *S. cerevisiae* M30, *K. marxianus* 3-1042 and the mixed culture of *S.cerevisiae* M30 and *K. marxianus* DMKU 3-1042 was carried out in batch mode. Secondly, in order to evaluate the performance of continuous ethanol fermentation process using the immobilized mixed culture of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 on TSSC, in a 1-L packed bed reactor and the effect of dilution rate was investigated. Thirdly, the pilot-scale study of the immobilized mixed cultured system was carried out in 100-L packed bed reactor.

4.1 Batch fermentation of suspended cells: monoculture vs. mixed culture

Batch fermentations in the shaking incubator at 200 rpm for ethanol production were carried out in duplicate for 72 h. The effect of temperature on ethanol fermentation in a medium contained 220 g/l sugar from dark brown sugar at temperatures of 33, 37 and 40 °C by the single cultures of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 and the mixed culture of both strains was studied. The samples were harvested every 8 hours for cell, sugar and ethanol analyses. The sugar and ethanol concentration profiles during the fermentation of 72 h are presented in Fig. 4.1 to 4.3. Under steady state, at 33°C, the single culture system of *S.cerevisiae* M30, a mesophilic yeast produced the maximal average concentration of ethanol

(79.56 g/L). In contrast, at 40°C, the maxium ethanol concentration was obtained by the use of *K.marxianus* DMKU 3-1042, a thermotolerant strain. With controlled temperature at 33, 37 and 40°C, it was found that at the end of the fermentations, the ethanol concentrations in the mixed cell culture system were 75.29, 66.87 and 62.66 g/L respectively and residual sugar concentrations were 40.20, 31.49, and 44.17 g/l, respectively, which were relatively equivalent or higher in comparison to those of the single culture systems. The result demonstrated the capability of the mixed cell culture system for producing ethanol over a wide range of temperature. The summary of ethanol fermentations of the mixed culture and the single culture systems is shown in Table 4.2.

Table 4.1 List of samples and labels for this study

Sample's Name	Label
Suspended cells of *S.cerevisiae* M30	SS
Suspended cells of *K.marxianus* DMKU 3-1042	SK
Suspended cells of Mixed culture	SM

Table 4.2 Batch fermentation of ethanol production at constant temperature of 33-40°C

System	Ethanol concentration (g/l)	Residual sugar concentration (g/l)	Free cell Concentration (g/l)	$Y_{P/S}$ (%)	Productivity (g/l h)
T = 33 °C					
SS	79.56	39.33	5.72	44.04	1.11
SK	54.93	60.45	4.53	34.43	0.76
SM	75.29	40.20	5.90	41.87	1.05
T = 37 °C					
SS	55.12	60.58	5.27	31.42	0.77
SK	59.73	51.88	6.27	35.53	0.83
SM	66.87	31.49	6.23	35.47	0.93
T = 40 °C					
SS	43.64	86.01	4.83	32.57	0.61
SK	63.85	51.81	6.10	37.96	0.89
SM	62.66	44.17	6.47	35.63	0.87
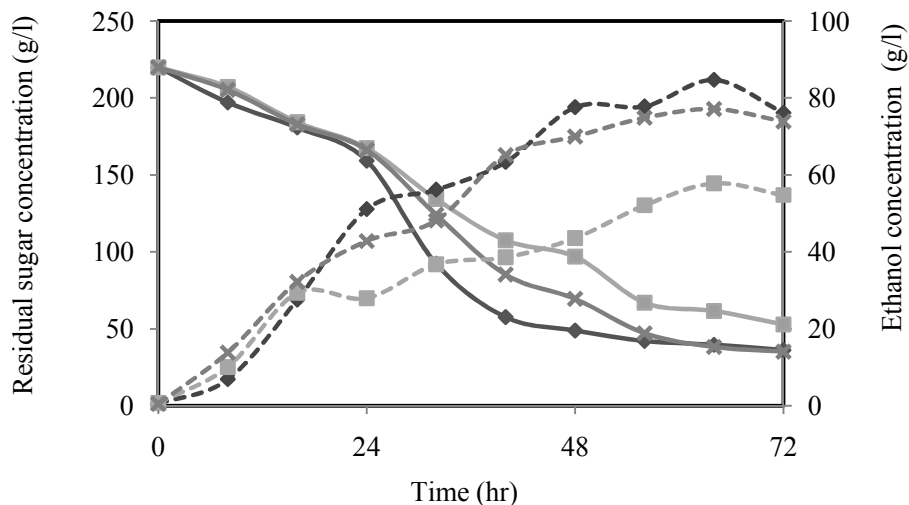
Figure 4.1 Ethanol and residual sugar concentration during the fermentation by *S. cerevisiae* M30 (♦), *K.marxianus* DMKU 3-1042 (■) and the mixed culture of *S.cerevisiae* and *K.marxianus* (×) using dark brown sugar medium by reciprocating flask cultivation at 33 °C ; the residual sugar concentration (—), Ethanol concentration (----). The proportion of *S.cerevisiae* M30 and *K.marxianus* DMKU 3-1042 in mixed culture was 1:1.
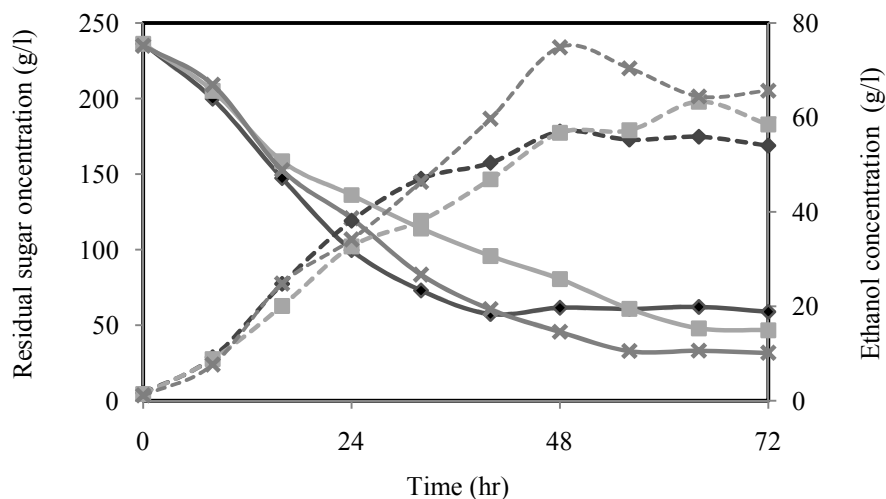
Figure 4.2 Ethanol and residual sugar concentration during the fermentation by *S. cerevisiae* M30 (♦), *K.marxianus* DMKU 3-1042 (■) and the mixed culture of *S.cerevisiae* and *K.marxianus* (×) using dark brown sugar medium by reciprocating flask cultivation at 37 °C ; the residual sugar concentration (—), Ethanol concentration (----). The proportion of *S.cerevisiae* M30 and *K.marxianus* DMKU 3-1042 in mixed culture was 1:1.
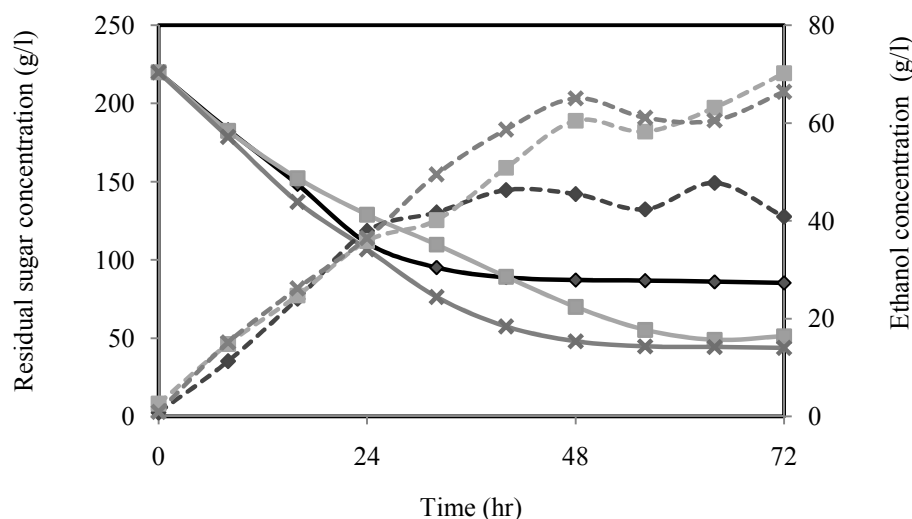
Figure 4.3 Ethanol and residual sugar concentration during the fermentation by *S. cerevisiae* M30 (♦), *K.marxianus* DMKU 3-1042 (■) and the mixed culture of *S.cerevisiae* and *K.marxianus* (×) using dark brown sugar medium by reciprocating flask cultivation at 40°C ; the residual sugar concentration (—), Ethanol concentration (----). The proportion of *S.cerevisiae* M30 and *K.marxianus* DMKU 3-1042 in mixed culture was 1:1.

Successful efforts for ethanol fermentation by mixed cultures have been reported by several researches. For example, fermentation of Jerusalem artichoke tubers at 30 ^{o}C by mixed cultures of *S. cerevisiae* and *Kluyveromyces fragilis* expressed 9.4 % v/v ethanol (Szambelan et al., 2004). Cáceres-Farfán et al. (2008) reported ethanol production from henequen juice and molasses by the mixture of *K. marxianus* and *S. cerevisiae* produced   5.22±1.087% v/v ethanol at 35 ± 2 ^{o}C. Ethanol production from cheese whey powder by the mixed immobilized cells of *K. marxianus* and *S. cerevisiae* at 30°C produced 5.3% v/v ethanol (Guo et al. 2008).

4.2 Continuous ethanol fermentation in packed bed reactor

Continuous fermentation in packed bed reactor presents 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. Immobilized cell reactors have been extensively used due to their shows several advantages for industrial fermentation, such as the relative ease of product separation, improved process control and reduced susceptibility of cells to contamination and developed to eliminate inhibition caused by high concentration of substrate and product, also to enhance productivity and yield of ethanol production. Moreover, the reactor can be operated at dilution rates higher than the washout to achieve increased [3, 15]. From our previous work, TSSC were used as cell carrier for immobilization of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042. TSSC is shown to be excellent cell carrier for ethanol fermentation in a wide range of temperature and very economical for the use in packed bed column [5].

4.2.1 Continuous ethanol fermentation in 1-L packed bed reactor #1

Continuous fermentation in 1-L packed-bed reactor with the mixed culture of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 immobilized on TSSC was investigated. The 25 g TSSC was tightly packed in the reactor. The working volume of reactor after packing was 0.55 L. The experimental study was carried out under the following condition: at the initial temperature about 31±1 °C, the initial sugar concentration about 220 g/l from dark brown sugar and initial pH 5. The effects of dilution rate from 0.10, 0.20, 0.3 and 0.40 h^{-1} was tested. 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 1 hour and then was kept under UV light overnight. The immobilized mixed cell culture on TSSC carrier was cultivated with initial sugar concentration about 250 g/l from palm sugar in incubator shaking at 200 rpm, 33°C for 24 hour and then the immobilized cell carries were aseptically transferred to the

sterilized column. The carrier volume was about 50% (v/v) of the pack bed reactor volume of 1-L. A start-up procedure was required in order to establish a steady state condition. Initially, the fermentation was started by feeding of the sterilized medium of dark brown sugar (220 g/l) through the inlet at the bottom of the column at the dilution rate of 0.10 h^{-1}. The dilution rate was changed after 4 days of 0.10 h^{-1} dilution rate and after 3 days of 0.20, 0.30 and 0.40 h^{-1} dilution rates. After the operation with sterilized medium for the dilution rates of 0.10, 0.20, 0.30 and 0.40 h^{-1}, then the substrate has been changed to unsterile medium. The dilution rate was increased after the system reached the steady state, from 0.10, 0.20, 0.30 and 0.40 h^{-1}, respectively. The samples were harvested every 8 hours.

The continuous fermentation system was performed under uncontrolled temperature condition. The average daytime temperature was 31±1 °C. The temperatures within the packed bed reactor are rather higher than the environmental temperature due to exothermic metabolic reactions of yeasts during active growth. The packed bed temperatures at 5 positions were monitored as shown Figure 4.4. The ethanol production and sugar consumption profiles at various dilution rates using dark brown sugar as feedstock are shown in Figure 4.5
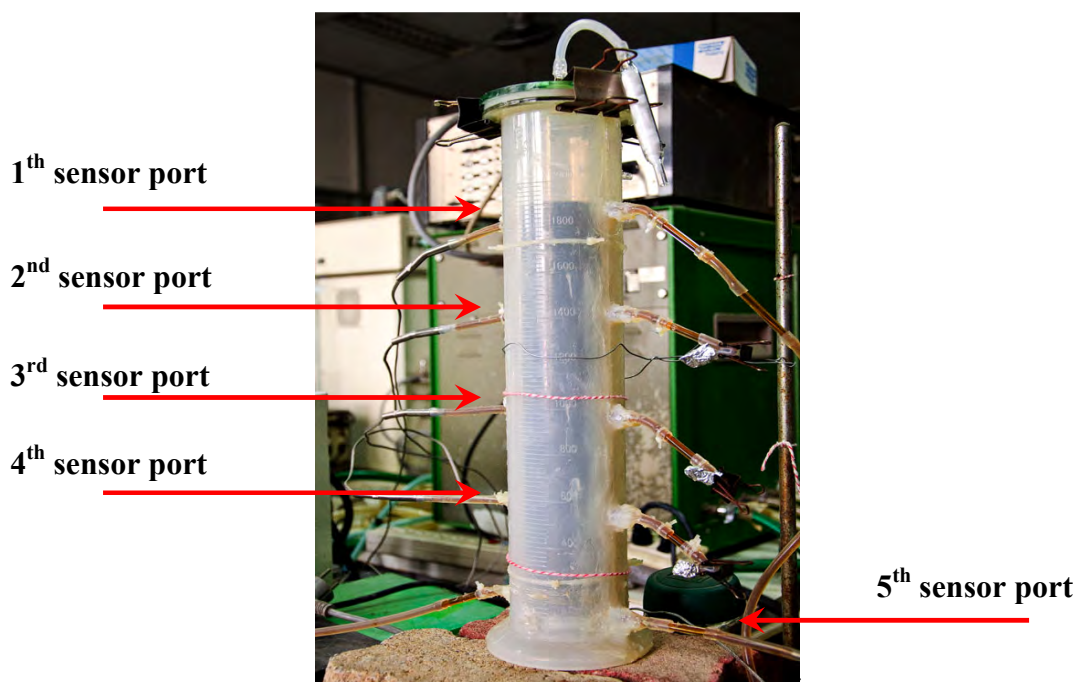
Figure 4.4 Picture of a 1-L packed bed reactor

Figure 4.5 Continuous ethanol fermentation in the tightly packed bed reactor at the dilution rate of 0.10, 0.20, 0.30 and 0.40 h^{-1}; ○, Residual sugar concentration and ■, Ethanol concentration

The average ethanol concentration after the steady state of continuous fermentation decreased from 54.84, 48.90, 45.35 and 37.21 g/L with dilution rate of 0.10, 0.20, 0.30 and 0.40 h^{-1} respectively, whereas the residual sugar concentration increased from 89.29, 92.71, 104.78 and 122.08 g/L respectively for using the sterilized medium as feedstock. By using the unsterilized medium after steady state, the average ethanol concentrations were 53.14, 49.29, 45.28 and 36.98 g/L and the residual sugar concentrations were 85.84, 102.72, 117.11 and 125.27 g/L at dilution rate of 0.10, 0.20, 0.30 and 0.40 h^{-1} respectively. The maximum productivity of the unsterilized medium was 14.79 g/L h, which was relatively equivalent when compared with the sterilized medium (14.89 g/L h). At the end of the fermentation, the free cell in effluent, in the reactor and the total immobilized cell in the reactor were 4.80, 6.47 and 27.85 g/L, respectively. This result indicated that the unsterilized medium could be used as feedstock for the ethanol fermentation.

Table 4.3 Effect of dilution rate on continuous in the tightly packed bed reactor ethanol production and ethanol productivity, average at steady state

Dilution rate (h⁻¹)	Ethanol concnentration (g/L)		Residual sugar concnentration (g/L)		$Y_{P/S}$ (%)	Productivity (g/L h)
Sterilized medium						
0.1	54.84 ±	0.77	89.29 ±	0.42	41.97	5.48
0.2	48.90 ±	1.53	92.71 ±	0.38	38.42	9.78
0.3	45.35 ±	1.20	104.78 ±	0.39	39.46	13.60
0.4	37.21 ±	1.18	122.08 ±	0.38	38.04	14.89
Unsterilized medium						
0.1	53.14 ±	0.45	85.84 ±	0.40	39.62	5.31
0.2	49.29 ±	1.08	102.72 ±	0.42	42.06	9.86
0.3	45.28 ±	0.48	117.11 ±	0.44	44.02	13.58
0.4	36.98 ±	0.77	125.27 ±	0.39	39.04	14.79

.
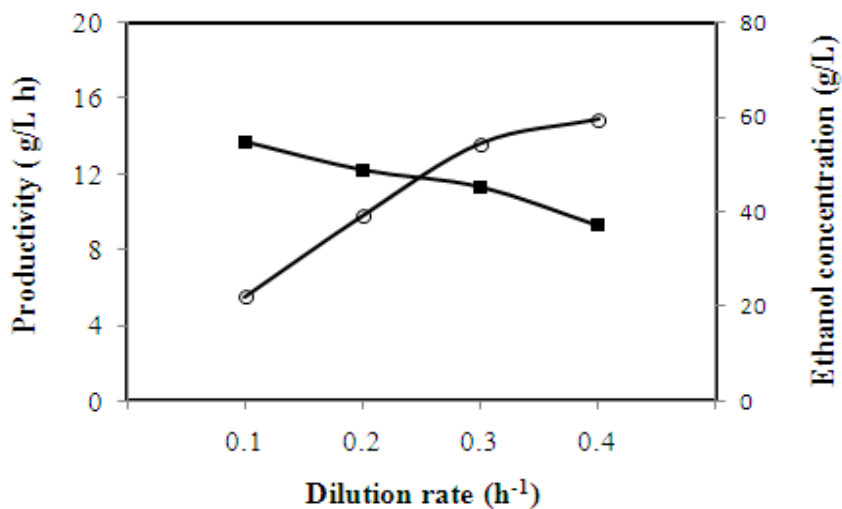
Figure 4.6 The ethanol productivities of ethanol fermentation by the sterilized medium of dark brown sugar at steady state; ○, ethanol productivities and ■, Ethanol concentration
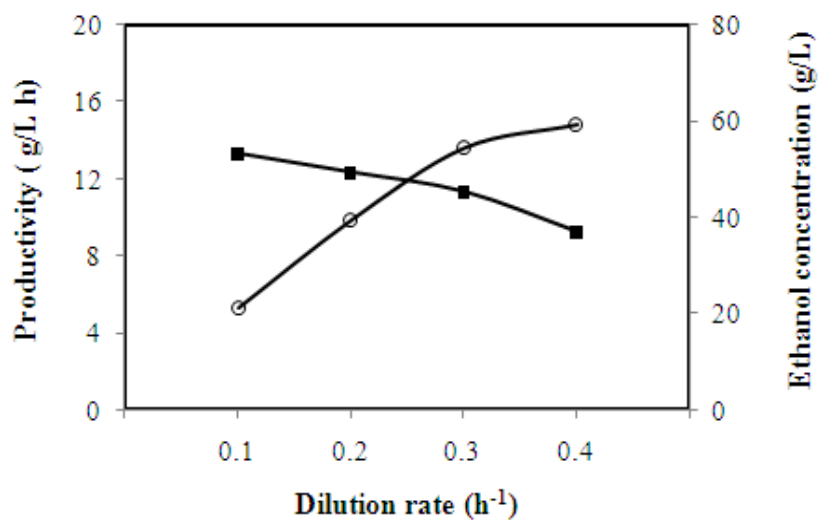
Figure 4.7 The ethanol productivities of ethanol fermentation by the unsterilized medium of dark brown sugar at steady state; ○, ethanol productivities and ■, Ethanol concentration

4.2.2 Continuous ethanol fermentation in 1-L packed bed reactor #2

In the continuous ethanol fermentation in 1-L packed bed reactor #2**,** the amount of TSSC was reduced to half of the continuous ethanol fermentation #1 and fitted to the reactor. About 30% of the 1-L packed bed reactor was packed with TSSC. The immobilized mixed cell culture on TSSC carrier was cultivated with initial sugar concentration about 250 g/l from palm sugar in incubator shaking at 200 rpm, 33°C for 24 hour and then the immobilized cell carries were aseptically transferred to the sterilized column. From the continuous ethanol fermentation in 1-L packed bed reactor #1, this result confirmed that the unsterilized medium could use as feedstock for the ethanol fermentation. Therefore, in this experiment used the unsterilized medium of dark brown sugar throughout the operation. The average daytime temperature was 29±1 °C. The studied conditions of the systems were exactly similar to continuous ethanol fermentation in 1-L packed bed reactor #1 (the initial sugar concentration about 220 g/l, initial pH 5, the dilution rate was investigated with was varied from 0.10, 0.20, 0.30 and 0.40 h^{-1} and the samples were harvested every 8 hours.)
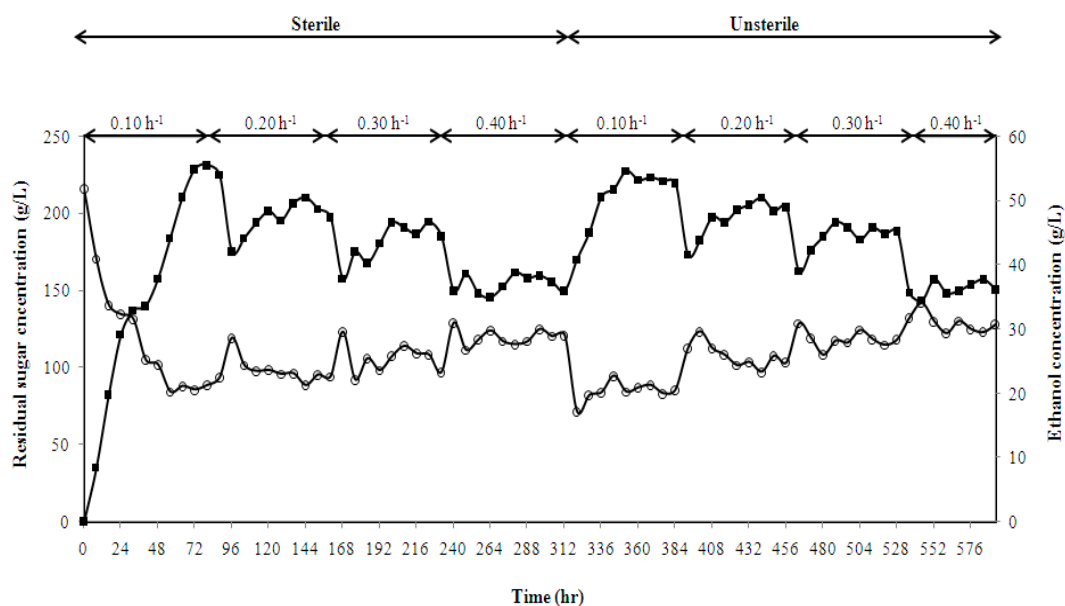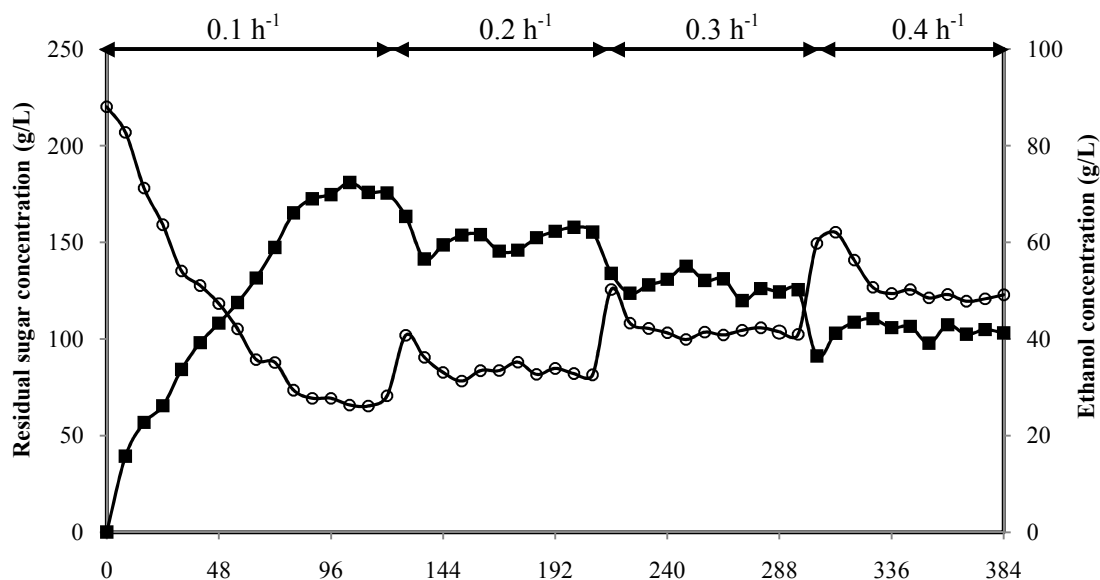

Figure 4.8 Continuous ethanol fermentation in the fit packed bed reactor at the dilution rate of 0.10, 0.20, 0.30 and 0.40 h^{-1}; ○, Residual sugar concentration and ■, Ethanol concentration

Under the steady state of continuous fermentation, the maximal ethanol concentration and the lowest residual sugar concentration at the lowest dilution rate (0.10 h^{-1}) were 70.96 and 67.14 g/L respectively. For further dilution rate of 0.20, 0.30 and 0.40 h^{-1} under steady state, the ethanol concentrations were at 62.49, 50.11 and 41.38 g/L, respectively and the residual sugar concentrations were at 85.33, 103.88 and 120.98 g/L, respectively. This result indicated that the ethanol concentration decreased with the dilution rate increased due to the decrease of the retention time.

Table 4.4 Effect of dilution rate on continuous production and ethanol productivity, average at steady state in the fit packed bed reactor ethanol

Dilution rate (h^{-1})	Ethanol concentration (g/L)			Residual sugar concentration (g/L)			$Y_{P/S}$ (%)	Productivity (g/L h)
0.1	70.96	±	1.24	67.14	±	2.90	44.20	7.10
0.2	62.49	±	0.51	82.67	±	1.75	43.60	12.50
0.3	50.11	±	0.34	103.88	±	1.72	41.04	15.03
0.4	41.38	±	0.50	120.98	±	1.68	39.41	16.55

The effect of dilution rate on ethanol productivity is shown in Figure 4.6. The ethanol productivity increased when the dilution rate was increased while the ethanol concentration was decreased because the reduction of the retention time. The ethanol productivities were increased from 7.10 to 12.50, 15.03 and 16.55 g/L h under dilution rate of 0.10, 0.20, 0.30 and 0.40 h^{-1} respectively.

The temperature profiles in the packed bed reactor during the continuous fermentation were monitored by the temperature sensors installed at 5 different positions of the packed bed column. The space between each was 8 cm. The average surroundings temperature was 29±1 °C. The temperatures within the column were

rather higher than the environmental temperature due to exothermic metabolic reactions of yeasts during active growth. The maximum temperatures during the operation were observed at the highest dilution rate (0.40 h^{-1}) according with the highest productivity. The average temperatures at dilution rate of 0.40 h^{-1} from the positions 1st to 5th were 34.67±0.61, 35.44±0.58, 35.36±0.57, 33.70±0.48, and 31.72±0.31 ^{o}C, respectively.
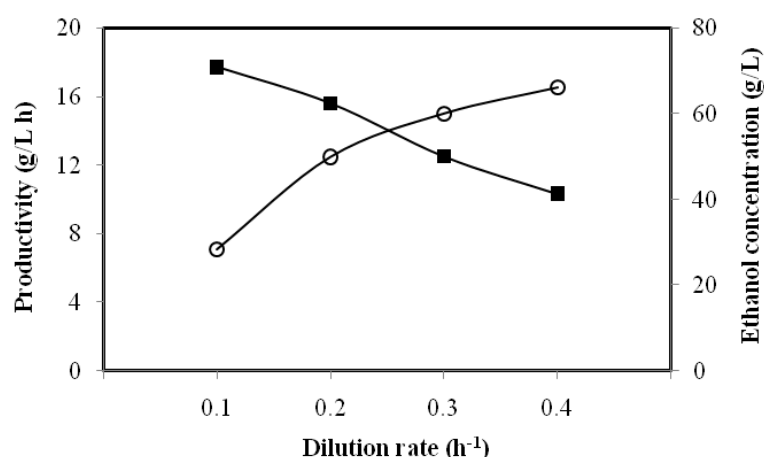
Figure 4.9 The ethanol productivities of the continuous ethanol fermentation at steady state; ○, ethanol productivities and ■, Ethanol concentration

Table 4.5 Average temperature at 1st - 5th position of the 1-L Packed bed reactor as shown in the Figure 4.4

Dilution rate (h^{-1})	1st sensor port (^{o}C)	2nd sensor port (^{o}C)	3rd sensor port (^{o}C)	4th sensor port (^{o}C)	5th sensor port (^{o}C)
0.1	29.81 ± 1.20	30.10 ± 1.33	30.61 ± 1.45	30.63 ± 1.19	28.91 ± 0.62
0.2	32.91 ± 0.88	33.99 ± 1.27	33.97 ± 1.29	33.66 ± 1.06	30.92 ± 0.61
0.3	34.17 ± 0.91	35.30 ± 0.71	35.28 ± 0.62	34.26 ± 0.67	31.66 ± 0.43
0.4	34.67 ± 0.61	35.44 ± 0.58	35.36 ± 0.57	33.70 ± 0.48	31.72 ± 0.31
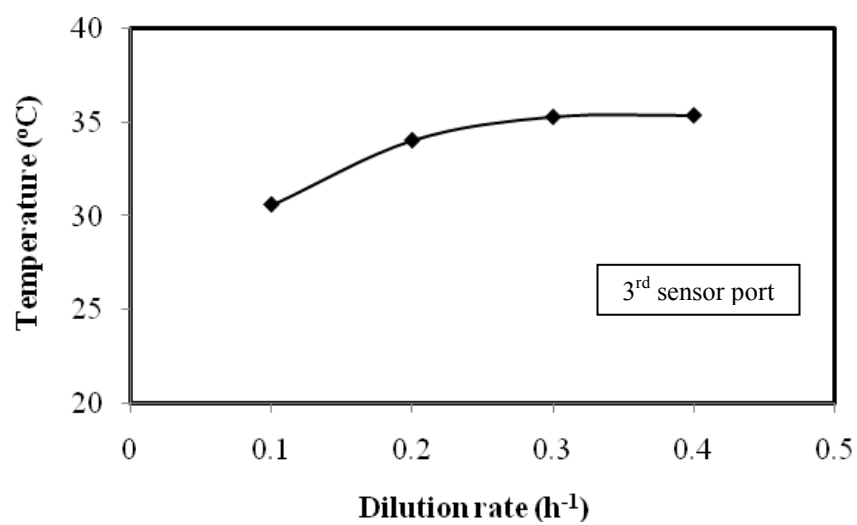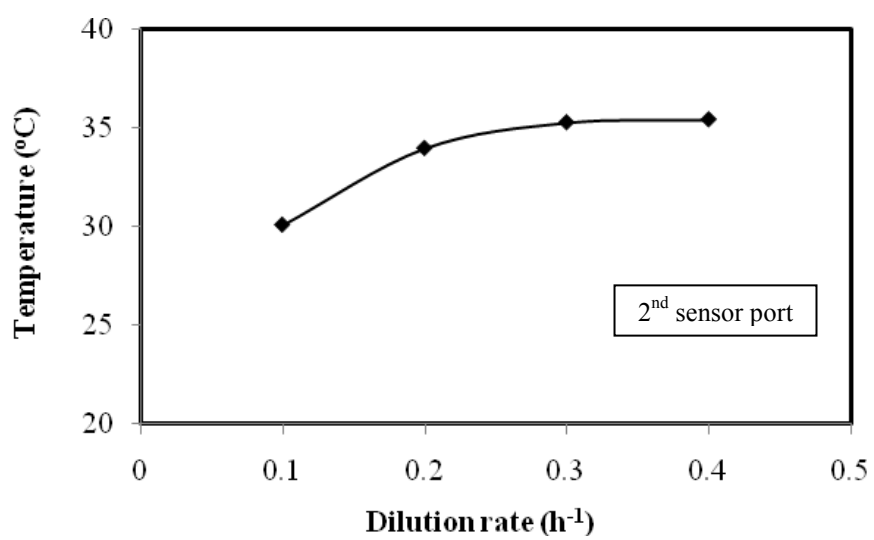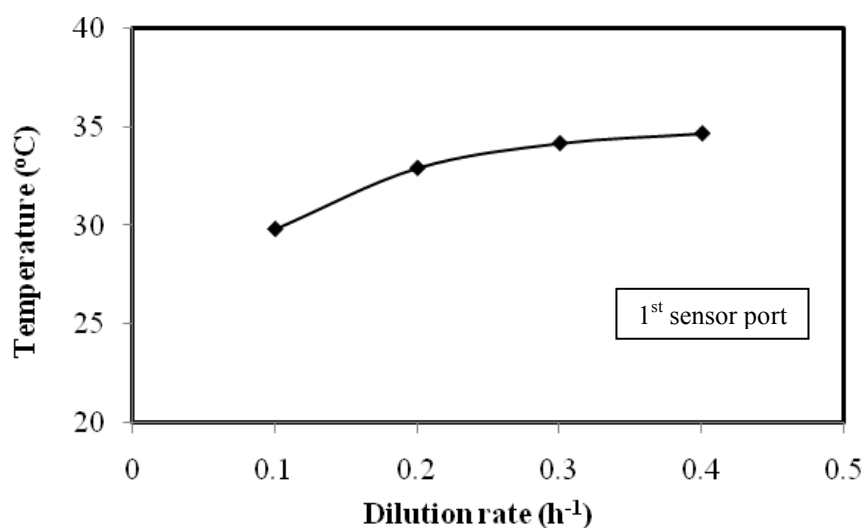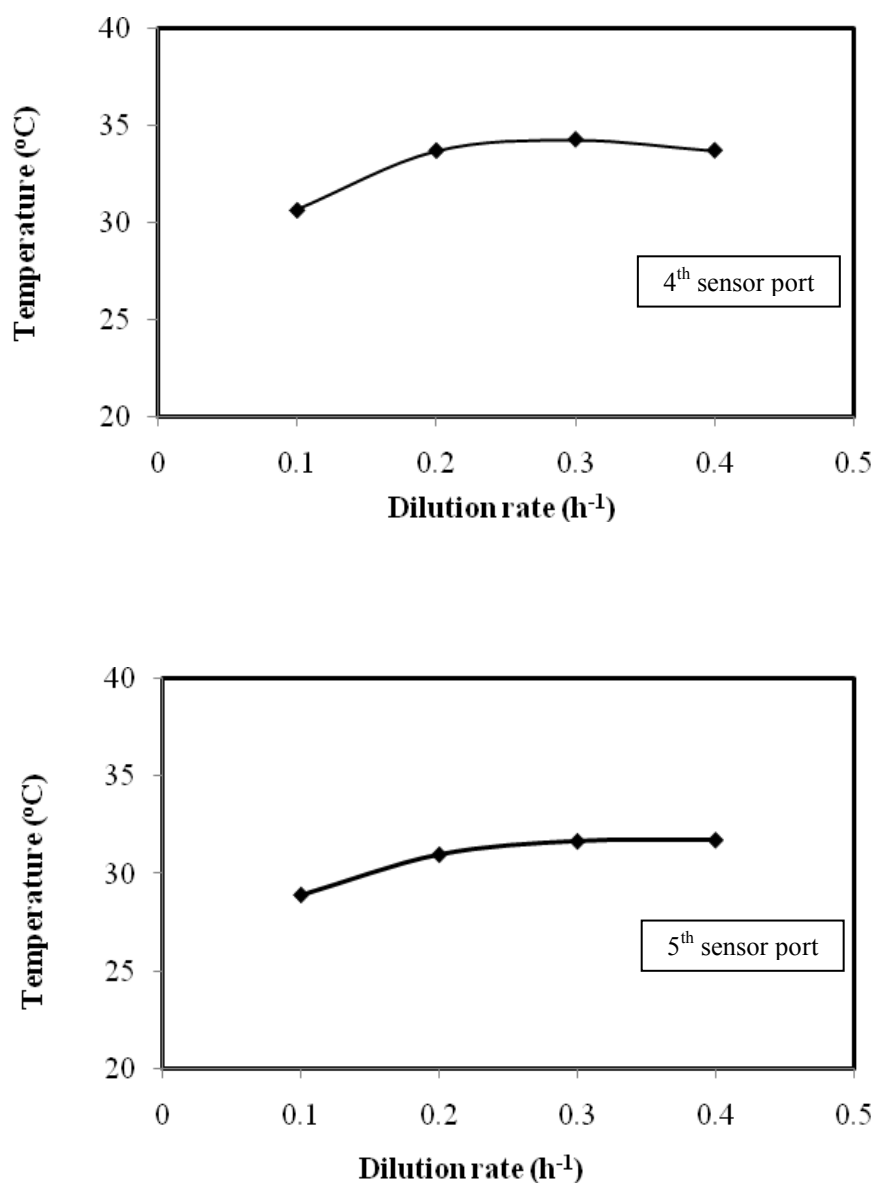
Figure 4.10 Temperature profiles at 1st - 5th position of the 1-L Packed bed reactor as shown in the Figure 4.4

.
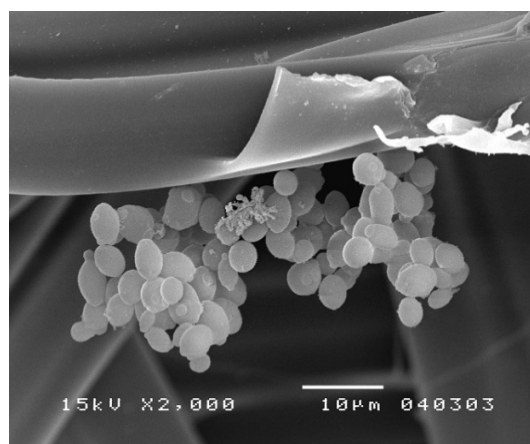
15 kv X750   10 μm   15 kv X2000   10 μm

Figure 4.11 TSSC outer surface before the continous fermentation
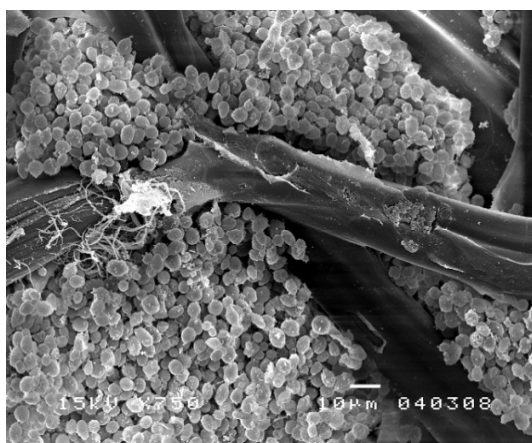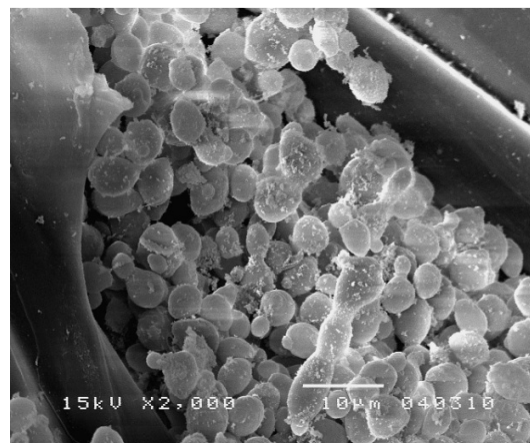
15 kv X750   10 μm   15 kv X2000   10 μm

Figure 4.12 TSSC outer surface at the end of the continuous fermentation (16 days)
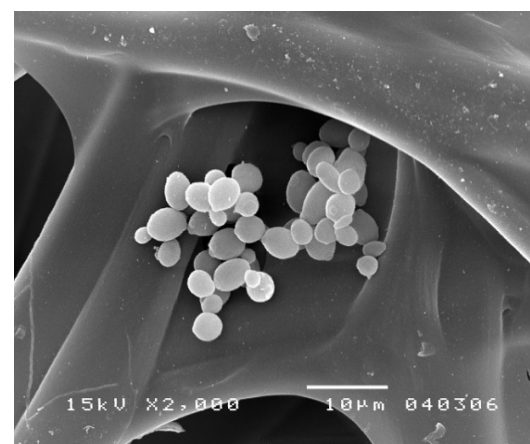
15 kv X750   10 μm   15 kv X2000   10 μm

Figure 4.13 TSSC inner surface before the continuous fermentation
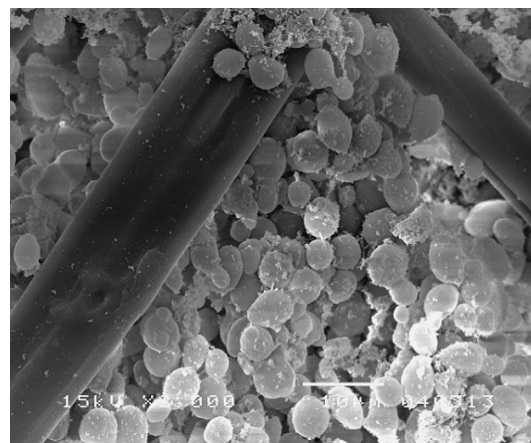
15 kv X750   10 µm   15 kv X2000   10 µm

Figure 4.14 TSSC inner surface at the end of the continuous fermentation (16 days)
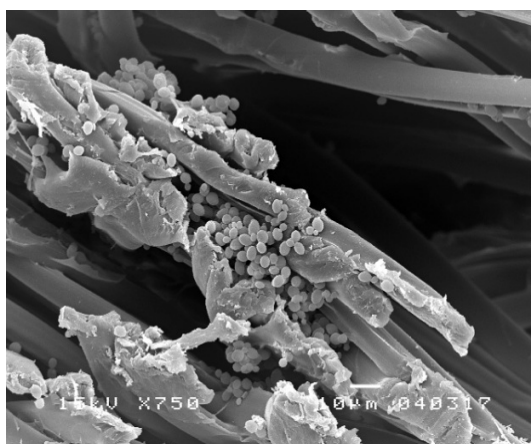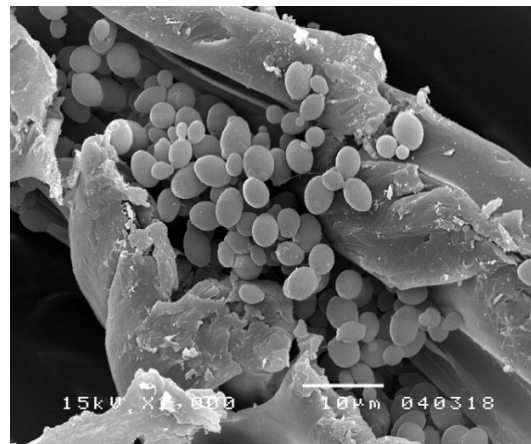
15 kv X750   10 µm   15 kv X2000   10 µm

Figure 4.15 TSSC cross section surface before the continuous fermentation
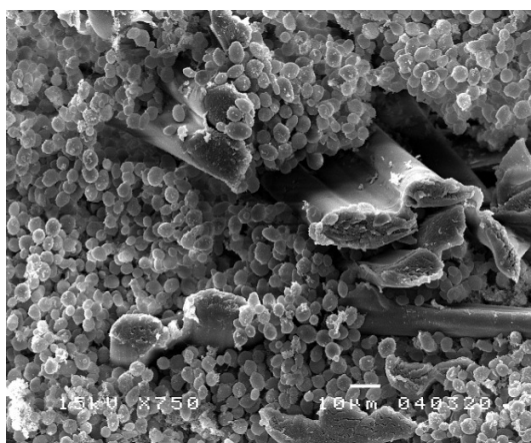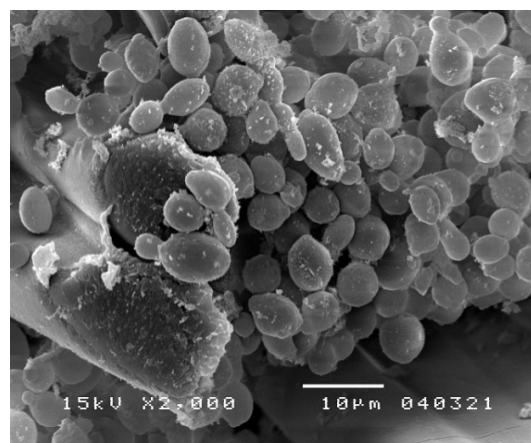
15 kv X750   10 µm   15 kv X2000   10 µm

Figure 4.16 TSSC cross section surface at the end of the continuous fermentation
(16 days)
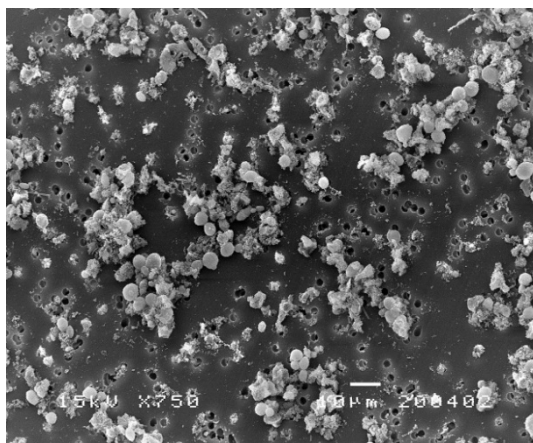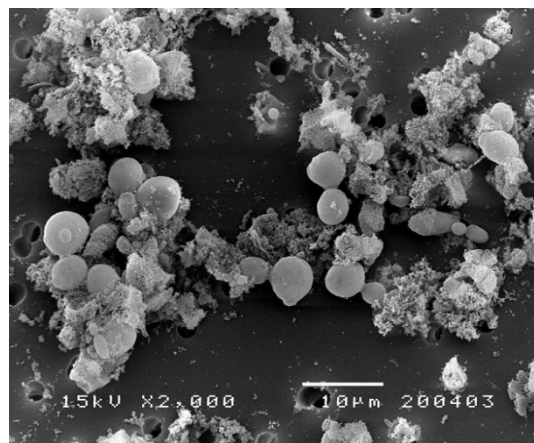
15 kv X750   10 µm   15 kv X2000   10 µm

Figure 4.17 Free cell in th reactor
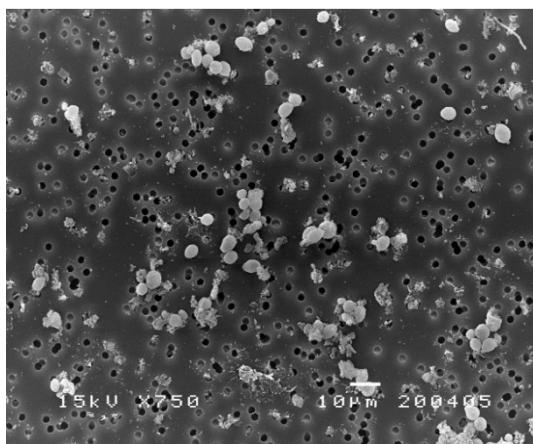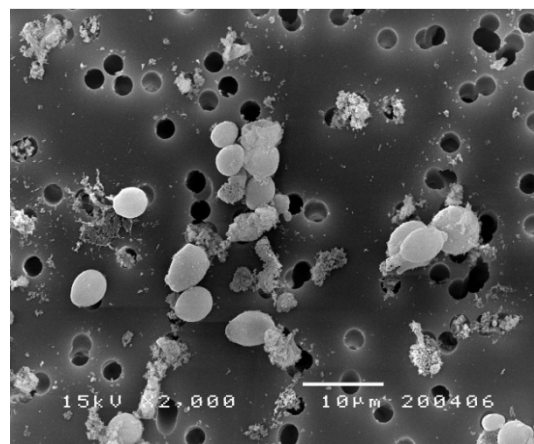
15 kv X750   10 µm   15 kv X2000   10 µm

Figure 4.18 Free cell in the effluent

A scanning electron microscope (SEM) was as a comparative indicator of yeast growth on the TSSC carriers before ethanol fermentation and at the end of the continuous fermentation. Figure 4.8 to Figure 4.13 represent the images of carrier from the beginning of the fermentation to the end of the fermentation. The amount of cells inside and outside of the carriers from time to time was increased. For long term performance, the free cell leakage occurred, which could be observed from the free cells in the reactor and the free cells in the effluent. Figure 4.14 and Figure 4.15 show the image of suspended cell culture in the reactor and in the effluent

At the end of the continuous fermentation (16 days), the concentrations of free cells in the effluent, in the reactor and immobilized cells in the reactor are shown in Table 4.11. The immobilized cell concentration was 31.06 g/l with 7.10 g/l free cell in the reactor and 4.07 g/l free cell in the effluent. The overall immobilized yield was 73.55%. This result revealed that yeast cells could grow and regenerate on the TSSC carrier in the packed bed reactor. The immobilized mixed cell culture system was found effective with high stability throughout the long period of continuous fermentation.

Table 4.6 Yeast cell concentrations at the end of continuous fermentation

Cell concentrations	(g/l)
Immobilized cell	31.06
Free cell in reactor	7.10
Free cell in effluent	4.07
Immobilized yield (%)	73.55

Many studies were performed for continuous ethanol fermentation using immobilized yeast cells. For instance, Ghorbani et al., (2011) reported the case of Sodium-alginate immobilized yeast was employed to produce ethanol continuously from cane molasses in an immobilized cell reactor. The maximum ethanol production and volumetric ethanol productivity were 19.15 g/L and 2.39 g/L.h, respective with the initial sugar concentration and lowest dilution rate of 150 g/l and 0.064 h^{-1}, respectively. In this study result of the continuous fermentation in 1-L packed bed reactor with the mixed culture immobilized on TSSC demonstrated good performance and stability for ethanol production under a wide range of operating temperatures. The next step, the configuration of the 1-L packed bed reactor was used for the scale up of a 100-L packed bed reactor in order to study the TSSC coimmobilized cell for a pilot-scale ethanol production.

4.2.3 Continuous ethanol fermentation in 100-L packed bed reactor

The configuration of the 100-L packed bed reactor was scaled up from the of the 1-L packed bed reactor by geometric similarity**.** The 100-L packed bed reactor was 200 cm high and 30 cm in diameter. To ensure good liquid flow distribution, the bed was divided into ten stages with porous stainless steel sheets. A magnetic stirrer was placed at the bottom of the reactor for feed mixing. The optimal condition fermentation system of a 1-L packed bed reactor fermentation was used in this studied. The unsterilized medium of dark brown sugar, initial sugar concentration about 220 g/l was used as feedstock for the ethanol fermentation. The operation system was performed under uncontrolled temperature condition. The average daytime temperature was 30±1 °C. The optimum of the dilution rate, 0.1 h^{-1} was carried out. The working volume of reactor after packing was 70 L. Prior to inoculation and start up of the fermentation, the column was sterilized by hot water, 10 minute twice. Cell immobilization could be done by transferring the 30 L broth in pre-cultured process and the unsterilezed medium into a reactor and then holding them for 24 hours. The samples were harvested every 8 hours.
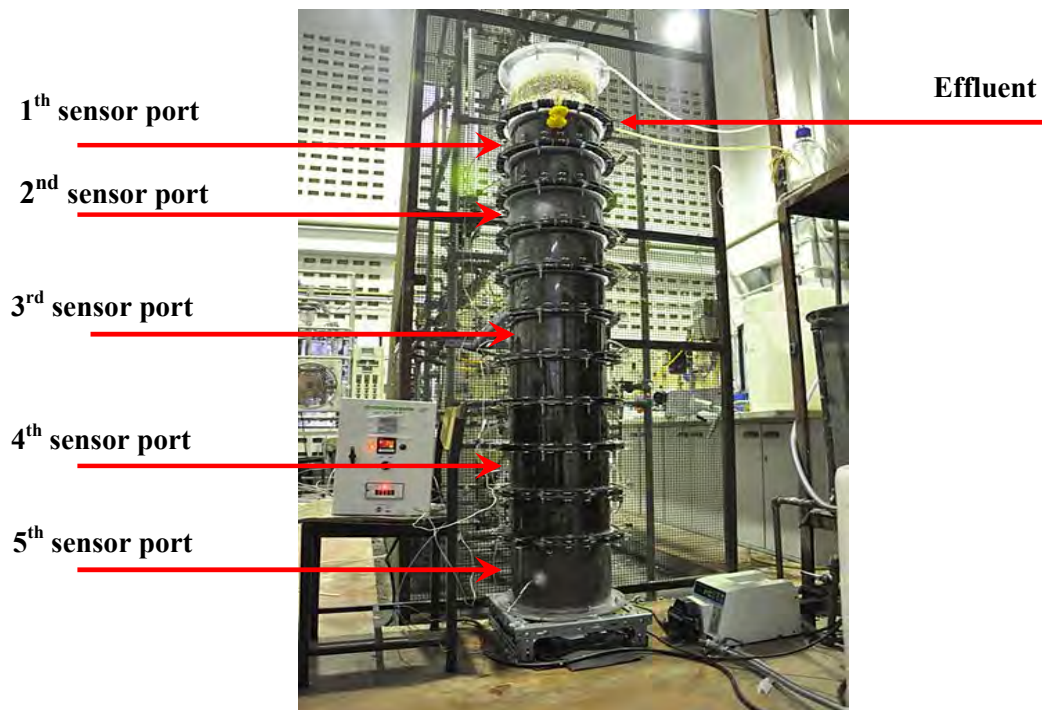
Figure 4.19 Picture of a 100-L packed bed reactor

Table 4.7 Ethanol concentrations at effluent and 1st - 4th position of the 100-L packed-bed reactor

Time (h)	Effluent (g/L)			1st position (g/L)			2nd position (g/L)			3rd position (g/L)			4th position (g/L)		
0	28.26			33.49			36.64			31.43			33.23		
8	31.52			34.31			28.47			36.10			20.92		
16	32.89			34.26			32.96			31.34			28.22		
24	41.55			39.07			35.73			38.81			34.23		
32	43.74			44.94			41.46			39.21			42.41		
40	89.32			94.06			90.88			83.92			81.37		
48	93.97	±	0.57	92.91	±	1.15	90.91	±	2.80	91.05	±	3.33	92.38	±	0.34
56	95.31	±	4.41	89.74	±	1.63	88.63	±	2.70	86.71	±	6.53	93.53	±	7.17
64	92.10	±	0.62	92.76	±	2.28	92.40	±	3.15	92.88	±	0.80	94.63	±	1.13
72	83.64	±	5.76	86.61	±	9.38	83.91	±	7.80	86.11	±	3.02	88.93	±	2.71
80	93.47	±	0.39	87.68	±	4.52	93.60	±	2.46	83.46	±	4.51	89.02	±	11.21
88	83.52	±	4.18	79.43	±	7.67	83.65	±	2.28	85.15	±	1.00	76.21	±	2.39
96	86.58	±	1.18	83.92	±	3.17	85.61	±	0.77	83.90	±	6.31	83.40	±	0.97
104	85.91	±	1.31	90.11	±	4.47	81.99	±	0.85	83.34	±	2.20	84.29	±	5.79
112	88.03	±	5.44	83.09	±	1.53	85.74	±	2.60	84.07	±	1.34	80.93	±	3.91
120	82.10	±	0.88	83.40	±	4.22	82.53	±	0.09	78.35	±	4.25	77.76	±	1.40
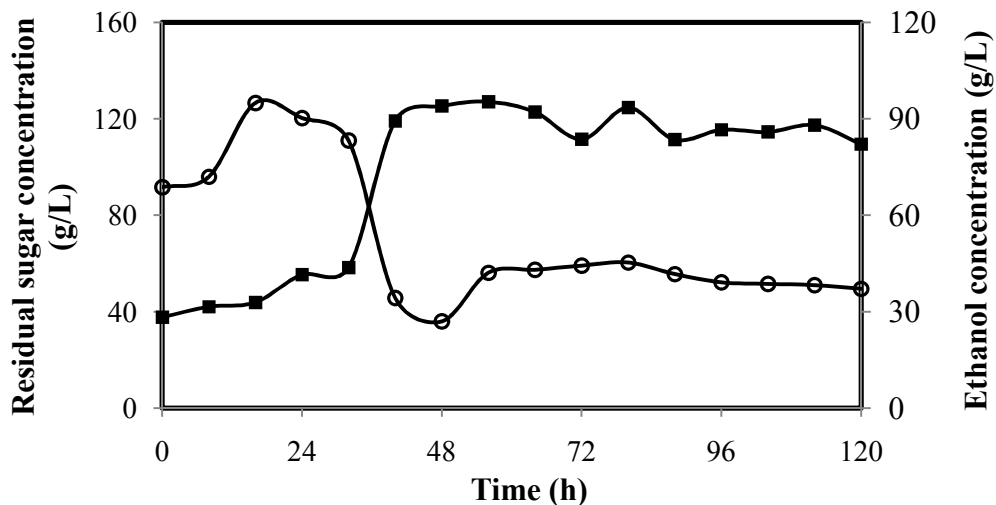
Figure 4.20 Continuous ethanol fermentation in a 100-L packed bed reactor at the dilution rate of 0.10 h^{-1}; ■, Ethanol concentration

Table 4.8 Temperature at 1st - 5th position of the 100-L packed-bed

Time (h)	1st sensor port (^{o}C)	2nd sensor port (^{o}C)	3rd sensor port (^{o}C)	4th sensor port (^{o}C)	5th sensor port (^{o}C)
0	30.8	30.4	30.4	30.0	28.5
8	30.8	30.2	30.5	30.0	28.7
16	31.6	31.3	31.2	30.9	29.6
24	32.1	31.4	31.8	31.4	30.5
32	32.8	32.5	32.4	32.0	31.2
40	34.2	33.5	33.8	33.8	33.8
48	34.2	33.7	34.1	34.2	34.3
56	35.2	34.9	35.1	35.2	35.2
64	35.1	34.6	34.9	34.9	35.0
72	35.0	34.7	34.7	34.6	34.5
80	34.2	33.9	34.3	34.6	34.6
88	34.4	34.1	34.5	34.8	34.8
96	34.7	34.5	34.8	34.8	34.7
104	35.1	34.8	35.1	35.2	35.1
112	35.0	34.8	34.9	35.1	35
120	34.1	33.7	34.0	34.0	34.0

Under the steady state of continuous fermentation, the average ethanol concentration and residual sugar concentration at the effluent were 85.34±3.00 g/L and 50.70±1.08 g/L, respectively. The ethanol productivity was 8.53 g/L h. The average ethanol concentrations from the positions 1st to 4th were 85.20±3.41, 83.42±1.18, 81.92±2.60 and 80.99±3.70 g/L, respectively and the average temperatures from the positions 1st to 5th were 34.73±1.53, 34.37±1.59, 34.70±1.65, 34.77±1.88 and 34.67±2.42 ^{o}C, respectively. The equivalence of ethanol concentration and temperatures in the reactor occurred because of mixed flow in the process.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

In an attempt to improve continuous ethanol fermentation process, in this study, a new technique ethanol fermentation from dark brown sugar by immobilization of the mixed cultures of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 using thin shell silk cocoon carriers was developed and evaluated. Under batch fermentations in 500 ml Erlenmeyer flask, *S. cerevisiae* M30 was more efficient at 33 ºC whereas *K. marxianus* DMKU 3-1042 was found to be the better strain for higher-temperature (37-40 ºC). Whereas the mixed culture system of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 was capable of highly efficient ethanol production from dark brown sugar at various temperatures (33-40°C).

Continuous ethanol production could be carried out in a 1-L packed bed reactor under uncontrolled operating temperature. The maximum ethanol productivity of 16.55 g/L h with ethanol concentration of 41.63 g/L were obtained at 0.40 h^{-1} dilution rate, while the highest ethanol concentration of 70.96 g/l was obtained at 0.10 h^{-1} dilution rate under the range of operating temperature of the packed bed column between 29.91 to 35.44 °C. In this research, the immobilized mixed culture of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 was promising for continuous fermentation process under uncontrolled operating temperature with a high production rate.

Moreover the continuous ethanol production with the mixed culture of *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 immobilized on TSSC was excellently performed carried out in a 100-L packed bed. At the dilution rate of 0.10 h^{-1}, the average ethanol concentration and productivity were 85.34±3.00 g/L and 8.53 g/L h , respectively. Finally, It can conclude that the outcome of 100-L was better than 1-l reactor because of more mixed flow in the process. In addition, this process

used the unsterilized medium of dark brown sugar as feedstock and operated under uncontrolled temperature that advanced method could reduce the costs associated with heating and cooling system during the fermentation process.

5.2 RECOMMENDATIONS

1. Experimental study with lower initial sugar concentration.

2. Experimental study with other raw materials, ex. hydrolysate from cassava strach.

APPENDICES

APPENDIX A
EXPERIMENTAL METHODS

A-1 Agar slants preparation

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

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

A-2 Stock cultures preparation

Stock cultures were prepared by aseptic inoculation of the flocculating yeast *S. cerevisiae* M30 and *K. marxianus* DMKU 3-1042 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 other was for ethanol production.

A-3.1 Preculture medium precaration

Palm sugar was designated for cell cultivation. The main component of the medium in earlier experiments (until fermentation 3) was palm sugar which was used as carbon and energy source for the yeast. Palm sugar was dissolved to obtain sugar concentration of about 100 g/l for cell cultivation. The resulting sugar solution had a

brown color originated from the palm sugar. The color intensity increases with increasing sugar concentration. The amount of palm sugar required to achieve the target level of sugar was estimated from previous trial with 3,5-dinitrosalicylic acid (DNS) method (Section A-7).

For 1 liter of sugar solution, nutrients consisted of 0.5 g KH_2PO_4, 1.5 g $MgSO_4.7H_2O$, and 0.5 g $(NH_4)_2SO_4$ were added Limtong et al. (2007). The compositions were referred to the one which were used by ethanol producing industries. The pH value of the medium was adjusted to 5 with 0.1 M NaOH and HCl solution. The detailed procedures for medium preparation from palm sugar are listed in the following paragraph.

1. Mix palm sugar and nutrients. Add palm sugar until the desired sugar concentration (100 g/l for cell cultivation) is achieved.

2. Adjust the pH of the solution to 5 by adding NaOH or HCl solution.

3. Pour appropriate volume of medium (100 ml and 250 ml for inoculums development and ethanol fermentation respectively) through a sieve or screen into 500 ml Erlenmeyer flask.

4. Close each flask with cotton plug and wrap with aluminum foil before sterilization.

5. Sterilize the mediums with autoclave for 20 min at 121°C.

6. Precautions and notes:

   a) Avoid wetting the flasks' neck when pouring the solution as the heated solution may act as adhesive so that the plug is difficult to be removed after sterilization.

   b) The pH of the solution may be quite altered after sterilization.

   c) Some precipitates may be formed after sterilization from the sugar solution.

A-3.2 Fermentation medium preparation

For 1 liter of fermentation medium consisted 0.5 g KH_2PO_4, 1.5 g $MgSO_4.7H_2O$, and 0.5 g $(NH_4)_2SO_4$ and 220 g sugar from sugar cane molasses. The quantity of molasses needed to reach the intended sugar level was also estimated by DNS trial. Sugar cane molasses was diluted to obtain sugar concentration of about 220 g/l for ethanol fermentation. The procedures for preparing molasses based fermentation medium are follows:

1. Dilute the molasses with DI water until the desired sugar concentration (220 g/l) is achieved.
2. Mix the diluted sugar solution with appropriate amount of KH_2PO_4, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$.
3. Adjust the pH of to 5 by adding NaOH or HCl solution.
4. Fill 500 ml Erlenmeyer flask with 250 ml medium.
5. Close each flask with cotton plug before sterilization.
6. Autoclave the medium for 15 min at 121 ºC.
7. Precautions and notes are same with palm sugar based medium preparation.

A-4 Cell cultivation and harvesting

Cell cultivation was initiated with the transfer of cells from stock culture tube aseptically to Erlenmeyer flask containing fresh medium by using Gilson Pipetman auto pipette. Thus, sterile pipette tips should be prepared in advance by autoclaving or dry heat in hot air oven. Active yeast cells with generation time (age) 20-24 hours were used for cultivation purpose. After inoculation, cell cultivation was carried out in Innova 4330 Refrigerated Incubator Shaker for 20-24 hours at 200 rpm. After some time, the growing yeast cells could be noticed as brown colored suspended solids inside the sugar solution. The cells were then harvested and concentrated by medium draining. The complete steps are as follows:

1. Sterilize equipments and the laminar flow hood with UV and by wiping with alcohol 70% v/v solution.

2. Heat up the neck of stock culture tube and medium flask after removing the tube cap and cotton plug.

3. Heat up the inoculation loop evenly and then slightly deep it into the fresh medium in the Erlenmeyer flask to cool it down before touching the yeast cells.

4. Scratch the yeast culture on the tube to detach the cells from the surface of the agar using the loop.

5. Transfer the cell at the loop into the Erlenmeyer flask and then close the flask using cotton plug.

6. Repeat steps 3-5 for the other flasks.

7. Put all flasks in the incubator shaker and then operate the shaker at 200 rpm 33°C for a day before harvesting the cells.

8. Let the cells to settle for a while after incubation and then carefully take out 130 ml of the medium from each flask by using 10 ml of auto pipette.

9. Combine the concentrated cells suspension from several flasks by pouring it into one flask.

10. Further draining can be done to concentrate cells by the same method until the desired volume of concentrated cells suspension is obtained.

11. For mix culture, the proportion *S. cerevisiae*/*K marxianus* was adjusted volumetric ratio of 1:1.

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

Immobilization of cells was investigated using two general methods. The first was the adsorption of cells onto the surface of thin shell silk cocoon. Thin shell silk cocoon and palm sugar medium were sterilized with autoclave for 15 minutes at 121°C prior to usage. Preparations of TSSC carrier were listed in the following paragraph.

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

The second was entrapment of cells in Alginate loofa matrix. To the entrapment of cells in Alginate loofa matrix, 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.

i. Mix concentrated cell suspension with alginate solution with volumetric ratio of 1:10.
ii. Add the mixture using a syringe drop wisely into CaCl$_2$ 14.7 g/l solution to form alginate beads.
iii. For reinforced gel formation, dip the loofa sponge into alginate solution and then drop it into the CaCl$_2$ solution.
iv. Leave the gel to harden with mild stirring for 15 minutes.
v. Rinse the gel 3 times with NaCl 9 g/l solution.
vi. When storing is needed, keep the gels in NaCl 9 g/l solution at 4°C.
vii. Precautions and notes:
   1. All procedures are conducted aseptically in laminar flow hood.

2. All equipments including the stirrer and syringe are cleaned and sterilized before use.

A-6 Ethanol fermentation

A-6.1 Batch fermentation

Sugar cane molasses was used as carbon source for the fermentation medium. The medium contained initial sugar concentration of 220 g/l, 0.05% w/v KH_2PO_4, 0.15% w/v $MgSO_4.7H_2O$, 0.05% w/v $(NH_4)_2SO_4$ and the initial pH was adjusted at 5.0. 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 200 rpm, 33, 37, 40 and 45°C for 72 hours.

A-6.2 Continuous fermentation

The reactor column was sterilized by circulation of 70% v/v ethanol for 1 hour and then was kept under UV light overnight. The column was packed by immobilized cell (TSSC carrier) with working volume around 0.7 L. Temperature of the system is controlled at 37°C. The sterile molasses solution with the initial sugar concentration of 220 g/l and the initial pH at 5.0 was fed to the bottom of the fermentor continuously by means of a peristaltic pump through sterile silicon tubing. Effluent liquid overflowed from an outlet port at the top of the column, maintaining a constant level of fermentation broth in the column. The temperatures inside diameter of the column 5 position are measured by temperature sensors, which will recorded every 8 hours. The samples were harvested with volume of 5 ml every 8 hours from the 5th port of the column.

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. The residual glucose concentration was also determined by the glucose analyzer YSI.

A-7.1 NaOH and HCl solution preparation

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

A-7.2 DNS reagent preparation

DNS powder is toxic and easy to airborne so that it should be handled with caution. This powder should be added slowly in the mixing process because it is not easy to dissolve. After preparation, the resulting yellow colored reagent is best used in fresh condition so that it is not suggested to keep unused for long time (more than 1

month). The reagent is usually kept in brown bottle to protect it from degradation originated from light for example sun light. The complete preparation steps are:

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

A-7.3 Standard sucrose solution preparation

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

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

Table A-7.3 Standard sucrose solution preparation

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

A-7.4 Sample treatment I

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

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

A-7.5 Sample treatment II

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

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

A-8. Dry weight cell

A-8.1 Free cell concentration

Cell concentration was determined by separation of cell from its medium followed by dry weight cell. Dry weight of cell concentration 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 24 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 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 TSSC was carried out using water. The thin shell silk cocoon was removed from the suspension after the gel was dissolved. The cells suspension was then treated with the same procedures as for free cells suspension in order to obtain its corresponding immobilized cell concentration. The complete procedures are as follows:

1. Cut the TSSC carrier in to the small size.
2. Dissolve appropriate amount of TSSC carrier with 10 ml water in 25 ml beaker.
3. Stir TSSC carrier in the beaker with magnetic stirrer for 30 minutes.
4. Remove the TSSC carrier from the suspension and continue with same procedures as step 2-9 of Section A-8.2.

APPENDIX B
EXPERIMENTAL DATA

B-1 Experimental data of suspension cell in batch fermentation

Table B-1.1 Data of batch fermentation of ethanol production in dark brown sugar medium by SS, SK and SM system at 33 º C.

Time (hours)	Ethanol concentration (g/l)			Residual sugar concentration (g/l)		
	SS	SK	SM	SS	SK	SM
0	0.56	0.74	0.49	220	220	220
8	6.92	10.10	13.83	197.15	207.32	205.36
16	27.57	29.35	32.22	180.89	184.35	182.94
24	51.11	27.98	42.81	159.32	167.55	166.32
32	56.17	36.81	48.13	92.41	134.35	124.24
40	63.36	38.60	65.14	57.64	107.55	85.31
48	77.60	43.61	69.96	48.91	96.92	69.48
56	77.82	52.13	74.83	42.13	66.99	47.18
64	84.70	57.86	77.13	39.67	61.58	38.25
72	76.16	54.79	73.89	36.20	52.79	35.16

Table B-1.2 Data of batch fermentation of ethanol production in dark brown sugar medium by SS, SK and SM system at 37°C.

Time (hours)	Ethanol concentration (g/l)			Residual sugar concentration (g/l)		
	SS	SK	SM	SS	SK	SM
0	1.60	1.45	1.12	236.00	220.00	220.00
8	9.21	8.85	7.65	199.97	205.23	208.52
16	24.76	20.10	24.82	147.40	158.57	152.65
24	38.14	32.71	34.17	99.75	136.22	121.18
32	47.05	38.17	46.36	73.00	114.08	84.37
40	50.42	46.91	59.76	57.43	95.87	60.06
48	57.06	56.77	74.89	61.57	80.56	44.81
56	55.34	57.31	70.44	60.78	60.98	31.73
64	55.95	63.36	64.47	62.09	47.96	31.66
72	54.07	58.52	65.70	58.87	46.71	31.07

Table B-1.3 Data of batch fermentation of ethanol production in dark brown sugar medium by SS, SK and SM system at 40°C.

Time (hours)	Ethanol concentration (g/l)			Residual sugar concentration (g/l)		
	SS	SK	SM	SS	SK	SM
0	0.79	2.58	0.92	220.00	220.00	220.00
8	11.30	14.78	15.11	183.17	182.52	178.56
16	24.05	24.69	26.18	148.25	152.21	137.05
24	37.92	35.84	36.47	110.76	128.95	106.74
32	41.72	40.12	49.47	95.08	109.77	76.30
40	46.28	50.82	58.65	88.82	89.22	57.39
48	45.46	60.46	65.03	87.11	69.98	47.90
56	42.34	58.23	61.08	86.78	55.28	44.61
64	47.73	63.14	60.51	85.99	48.89	44.21
72	40.85	70.19	66.38	85.26	51.26	43.69

B-2 Experimental data of continuous fermentation: Tightly packed

Table B-2.1 Experimental data of ethanol and residual sugar concentration in the tightly packed bed reactor of TSSC carrier with dilution rate of 0.1, 0.2, 0.3 and 0.4 h^{-1}.

Dilution rate (h^{-1})	Time (hours)	Sugar concentration (g/l)	Ethanol concentration (g/l)	$Y_{P/S}$ (%)
Sterilized medium				
0.1	0	215.90	0.00	
0.1	8	170.22	8.42	16.92
0.1	16	140.66	19.73	24.87
0.1	24	134.55	29.16	34.12
0.1	32	131.49	32.94	37.22
0.1	40	104.99	33.60	29.22
0.1	48	101.93	37.85	32.06
0.1	56	84.30	44.19	32.56
0.1	64	87.97	50.63	38.35
0.1	72	85.72	54.94	40.92
0.1	80	88.58	55.55	42.27
0.1	88	93.57	54.02	42.73
0.2	96	119.21	42.03	41.70
0.2	104	101.52	44.19	37.30
0.2	112	97.75	46.60	38.12
0.2	120	98.36	48.35	39.75

Dilution rate (h^{-1})	Time (hours)	Sugar concentration (g/l)	Ethanol concentration (g/l)	Y$_{P/S}$ (%)
0.2	128	95.81	46.95	37.81
0.2	136	96.22	49.62	40.09
0.2	144	88.58	50.51	38.43
0.2	152	95.41	48.74	39.12
0.2	160	94.16	47.46	37.71
0.3	168	123.14	37.87	39.09
0.3	176	92.25	42.03	32.90
0.3	184	106.01	40.32	35.37
0.3	192	98.26	43.36	35.62
0.3	200	107.41	46.60	41.39
0.3	208	113.97	45.78	43.18
0.3	216	109.07	44.83	40.41
0.3	224	108.24	46.71	41.80
0.3	232	97.04	44.49	36.18
0.4	240	129.07	36.02	39.61
0.4	248	111.42	38.61	35.56
0.4	256	118.11	35.60	34.94
0.4	264	123.97	34.99	36.43
0.4	272	117.03	36.65	35.59
0.4	280	115.18	38.83	37.05
0.4	288	117.03	38.01	36.91
0.4	296	124.99	38.33	40.34
0.4	304	120.28	37.34	37.44
0.4	312	120.97	35.98	36.33

Dilution rate (h^{-1})	Time (hours)	Sugar concentration (g/l)	Ethanol concentration (g/l)	Y$_{P/S}$ (%)
Unsterilized medium				
0.1	320	71.48	40.83	27.49
0.1	328	81.99	45.04	32.63
0.1	336	83.94	50.63	37.21
0.1	344	94.67	51.83	41.35
0.1	352	84.28	54.62	40.24
0.1	360	87.07	53.24	40.05
0.1	368	88.53	53.62	40.79
0.1	376	83.27	53.07	38.81
0.1	384	85.71	52.74	39.27
0.2	392	112.42	41.60	38.67
0.2	400	123.41	43.88	45.43
0.2	408	112.42	47.46	44.12
0.2	416	108.20	46.64	41.72
0.2	424	101.44	48.57	40.96
0.2	432	103.51	49.37	42.38
0.2	440	97.21	50.47	41.11
0.2	448	107.67	48.35	43.05
0.2	456	103.28	49.05	42.02

Dilution rate (h⁻¹)	Time (hours)	Sugar concentration (g/l)	Ethanol concentration (g/l)	Y P/S (%)
0.3	464	128.49	39.04	42.66
0.3	472	119.19	42.30	41.96
0.3	480	108.20	44.44	39.75
0.3	488	117.50	46.69	45.55
0.3	496	116.22	45.78	44.12
0.3	504	124.38	43.97	45.98
0.3	512	118.38	45.78	45.05
0.3	520	114.63	44.83	42.55
0.3	528	118.32	45.22	44.47
0.4	536	132.42	35.67	40.73
0.4	544	141.76	34.48	44.07
0.4	552	129.89	37.75	41.89
0.4	560	122.28	35.62	36.45
0.4	568	130.12	36.02	40.07
0.4	576	124.82	37.01	38.88
0.4	584	122.95	37.74	38.89
0.4	592	128.05	36.19	39.36

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

Dilution rate (h^{-1})	Time (hours)	Free cell concentration (g/l)
Sterilized medium		
0.1	0	0.00
0.1	8	0.40
0.1	16	0.40
0.1	24	0.60
0.1	32	0.80
0.1	40	0.80
0.1	48	0.80
0.1	56	1.00
0.1	64	1.20
0.1	72	1.20
0.1	80	1.00
0.1	88	1.20
0.2	96	1.40
0.2	104	1.40
0.2	112	1.20
0.2	120	1.60
0.2	128	1.20
0.2	136	1.80
0.2	144	1.80

Dilution rate (h^{-1})	Time (hours)	Free cell concentration (g/l)
0.2	152	2.00
0.2	160	2.00
0.3	168	1.80
0.3	176	2.00
0.3	184	2.20
0.3	192	2.20
0.3	200	2.10
0.3	208	2.60
0.3	216	2.4
0.3	224	2.6
0.3	232	2.6
0.4	240	2.60
0.4	248	2.60
0.4	256	3.00
0.4	264	3.20
0.4	272	3.40
0.4	280	3.80
0.4	288	3.60
0.4	296	4.40
0.4	304	4.20
0.4	312	4.20

Dilution rate (h^{-1})	Time (hours)	Free cell concentration (g/l)
Unsterilized medium		
0.1	320	4.20
0.1	328	3.60
0.1	336	4.40
0.1	344	4.40
0.1	352	3.40
0.1	360	2.60
0.1	368	2.80
0.1	376	2.80
0.1	384	2.40
0.2	392	2.60
0.2	400	2.20
0.2	408	2.00
0.2	416	3.20
0.2	424	2.60
0.2	432	2.60
0.2	440	2.40
0.2	448	4.00
0.2	456	3.20
0.3	464	5.00
0.3	472	3.20
0.3	480	3.20
0.3	488	4.20

Dilution rate (h⁻¹)	Time (hours)	Free cell concentration (g/l)
0.3	496	3.00
0.3	504	3.60
0.3	512	4.00
0.3	520	5.00
0.3	528	4.60
0.4	536	4.60
0.4	544	3.20
0.4	552	3.40
0.4	560	3.80
0.4	568	4.40
0.4	576	5.00
0.4	584	4.80
0.4	592	4.80

B-3 Experimental data of continuous fermentation: Fit packed

Table B-3.1 Experimental data of ethanol and residual sugar concentration in the fit packed bed reactor of TSSC carrier with dilution rate of 0.10, 0.20, 0.30 and 0.40 h^{-1}

Dilution rate (h^{-1})	Time (hours)	Sugar concentration (g/l)	Ethanol concentration (g/l)	$Y_{P/S}$ (%)
0.10	0	220.05	0.00	
0.10	8	206.81	15.74	0.82
0.10	16	177.96	22.70	0.47
0.10	24	159.04	26.15	0.39
0.10	32	135.15	33.69	0.37
0.10	40	127.55	39.22	0.40
0.10	48	118.20	43.26	0.40
0.10	56	105.16	47.52	0.39
0.10	64	89.20	52.60	0.38
0.10	72	87.78	58.94	0.43
0.10	80	73.41	66.08	0.43
0.10	88	69.21	69.01	0.44
0.10	96	69.21	69.87	0.45
0.10	104	65.72	72.39	0.44
0.10	112	65.24	70.31	0.44
0.10	120	70.48	70.17	0.45

Dilution rate (h^{-1})	Time (hours)	Sugar concentration (g/l)	Ethanol concentration (g/l)	$Y_{P/S}$ (%)
0.20	128	101.80	65.33	0.53
0.20	136	90.36	56.55	0.42
0.20	144	82.58	59.45	0.41
0.20	152	78.15	61.47	0.42
0.20	160	83.53	61.57	0.43
0.20	168	83.58	58.17	0.41
0.20	176	87.89	58.37	0.42
0.20	184	81.59	60.94	0.42
0.20	192	84.66	62.28	0.44
0.20	200	81.99	63.08	0.44
0.20	208	81.35	62.12	0.43
0.30	216	125.53	53.57	0.53
0.30	224	108.10	49.46	0.42
0.30	232	51.17	105.38	0.44
0.30	240	52.32	103.09	0.43
0.30	248	55.06	99.62	0.42
0.30	256	52.09	103.44	0.39
0.30	264	52.43	102.01	0.42
0.30	272	47.94	104.41	0.41
0.30	280	50.41	105.70	0.41
0.30	288	49.73	103.65	0.44
0.30	296	50.18	102.28	0.43

Dilution rate (h^{-1})	Time (hours)	Sugar concentration (g/l)	Ethanol concentration (g/l)	Y$_{P/S}$ (%)
0.40	312	41.17	155.13	0.58
0.40	320	43.46	140.73	0.51
0.40	328	44.17	126.65	0.44
0.40	336	42.32	123.41	0.41
0.40	344	42.59	125.45	0.42
0.40	352	39.09	121.14	0.37
0.40	360	42.93	122.93	0.42
0.40	368	40.97	119.46	0.38
0.40	376	41.94	120.69	0.40
0.40	384	41.23	122.79	0.40

Table B-3.2 Data of free cells concentration leaving the1-L packed bed reactor.

Dilution rate (h^{-1})	Time (hours)	Free cell concentration (g/l)
0.10	0	0.20
0.10	8	0.20
0.10	16	0.20
0.10	24	0.40
0.10	32	0.45
0.10	40	1.00
0.10	48	1.10
0.10	56	1.15
0.10	64	1.00
0.10	72	1.40
0.10	80	1.40
0.10	88	1.40
0.10	96	1.40
0.10	104	1.40
0.10	112	1.40
0.10	120	1.40
0.20	128	1
0.20	136	1.6
0.20	144	1.6
0.20	152	1.4

Dilution rate (h⁻¹)	Time (hours)	Free cell concentration (g/l)
0.20	160	1.4
0.20	168	1.4
0.20	176	1.8
0.20	184	2
0.20	192	1.6
0.20	200	1.4
0.20	208	1.4
0.30	232	1.4
0.30	240	1.4
0.30	248	1.6
0.30	256	1.6
0.30	264	2.2
0.30	272	2.2
0.30	280	1.8
0.30	288	2.2
0.30	296	2.2
0.40	304	2.6
0.40	312	3
0.40	320	2.6
0.40	328	3
0.40	336	3
0.40	344	2.8

Dilution rate (h^{-1})	Time (hours)	Free cell concentration (g/l)
0.40	352	3.6
0.40	360	3.2
0.40	368	3.8
0.40	376	3.8
0.40	384	4.2

Table B-3.3 Experimental data of temperature profiles

Time (hours)	Dilution rate (h^{-1})	1st sensor port (°C)	2nd sensor port (°C)	3rd sensor port (°C)	4th sensor port (°C)	5th sensor port (°C)
0.10	0	28.32	29.38	29.64	29.17	28.60
0.10	8	28.08	28.33	28.53	28.93	28.12
0.10	16	29.25	28.09	28.20	28.52	28.04
0.10	24	28.72	29.39	29.88	30.37	28.63
0.10	32	28.47	28.95	29.37	29.98	28.25
0.10	40	30.15	28.32	28.84	29.75	28.30
0.10	48	29.70	30.55	30.97	31.41	29.12
0.10	56	29.65	30.25	30.58	30.79	28.87
0.10	64	30.40	29.82	30.32	30.38	28.53
0.10	72	30.01	30.91	31.80	31.53	29.51
0.10	80	29.69	30.40	31.17	30.88	29.03
0.10	88	31.56	29.95	30.89	30.22	28.96
0.10	96	31.19	31.97	32.55	32.29	29.91
0.10	104	31.19	31.65	32.29	31.87	29.34
0.10	112	30.88	31.21	31.87	31.33	29.04
0.10	120	32.14	32.47	32.84	32.59	30.23
0.20	128	31.87	32.07	32.40	32.49	30.12
0.20	136	31.49	31.67	31.58	31.87	30.43
0.20	144	33.41	34.23	34.19	34.00	31.49
0.20	152	32.58	33.85	33.63	33.72	30.69

Time (hours)	Dilution rate (h⁻¹)	1st sensor port (°C)	2nd sensor port (°C)	3rd sensor port (°C)	4th sensor port (°C)	5th sensor port (°C)
0.20	168	33.79	35.14	35.46	34.76	31.90
0.20	176	33.25	34.56	34.87	33.96	31.40
0.20	184	32.60	33.87	34.59	33.54	31.27
0.20	192	34.39	35.74	35.44	35.15	31.40
0.20	200	33.65	35.07	35.16	34.80	31.10
0.20	208	32.80	34.60	34.24	33.86	30.98
0.30	216	34.16	35.72	35.93	35.42	31.75
0.30	224	33.61	35.49	35.65	35.20	31.43
0.30	232	32.84	34.54	34.69	33.24	30.95
0.30	240	33.76	34.95	35.14	34.03	31.42
0.30	248	33.48	34.40	34.77	33.80	31.57
0.30	256	33.00	34.23	34.37	33.53	31.24
0.30	264	35.10	36.12	35.98	34.70	32.07
0.30	272	34.92	35.63	35.16	34.38	31.81
0.30	280	34.40	34.97	34.56	33.96	31.51
0.30	288	35.59	36.12	36.00	34.53	32.47
0.30	296	35.02	36.09	35.87	34.11	32.07
0.40	304	34.02	35.31	35.06	33.32	31.70
0.40	312	35.27	35.70	35.81	34.19	32.16
0.40	320	34.77	35.23	35.46	33.96	31.90
0.40	328	33.83	34.68	34.15	33.51	31.48

Time (hours)	Dilution rate (h⁻¹)	1st sensor port (°C)	2nd sensor port (°C)	3rd sensor port (°C)	4th sensor port (°C)	5th sensor port (°C)
0.40	336	35.40	36.03	35.90	34.03	32.07
0.40	344	34.95	35.94	35.64	33.53	31.56
0.40	352	33.98	34.86	34.70	32.97	31.72
0.40	360	35.07	35.96	35.52	33.88	32.02
0.40	368	35.02	35.44	35.92	33.89	31.66
0.40	376	33.87	34.53	34.98	32.98	31.10
0.40	384	35.16	36.19	35.85	34.44	31.54

B-4 Experimental data of continuous fermentation: Pilot-scale

Table B-4.1 Experimental data of residual sugar concentration at effluent and 1st - 4th position of the 100-L packed-bed reactor

Time (h)	Effluent (g/L)			1st position (g/L)			2nd position (g/L)			3rd position (g/L)			4th position (g/L)		
0	91.61			92.65			86.84			98.87			92.23		
8	95.97			114.54			114.64			131.76			157.70		
16	126.58			124.50			127.61			128.65			146.29		
24	120.35			117.24			118.28			133.84			141.10		
32	111.01			112.05			114.13			120.35			127.61		
40	45.75			44.41			44.41			45.44			52.50		
48	36.16	±	0.22	32.58	±	0.15	43.16	±	0.15	37.82	±	3.45	53.54	±	0.59
56	56.08	±	0.07	60.54	±	0.37	55.56	±	0.95	57.17	±	0.88	58.41	±	0.15
64	57.27	±	0.15	57.11	±	0.22	55.40	±	0.15	51.10	±	0.07	55.20	±	1.17
72	58.83	±	0.44	57.89	±	0.15	57.01	±	0.22	59.40	±	0.37	55.09	±	0.15
80	60.28	±	0.15	59.03	±	0.73	61.21	±	0.73	56.03	±	3.67	60.75	±	0.95
88	55.77	±	0.22	54.99	±	0.29	55.40	±	1.91	53.33	±	0.15	61.11	±	0.29
96	52.45	±	0.37	52.71	±	0.15	52.39	±	0.15	52.19	±	0.15	57.29	±	0.46
104	51.77	±	0.29	59.14	±	0.29	52.96	±	0.37	56.18	±	0.22	59.14	±	0.73
112	51.41	±	0.51	51.15	±	0.29	52.45	±	0.81	54.57	±	0.59	60.07	±	0.29
120	49.80	±	0.44	52.50	±	0.15	54.52	±	0.07	54.73	±	0.22	57.11	±	0.37

Table B-4.2 Data of free cells concentration leaving the100-L packed bed reactor

Dilution rate (h^{-1})	Time (hours)	Free cell concentration (g/l)
0.10	0	2.8
0.10	8	3.2
0.10	16	2
0.10	24	2.4
0.10	32	3.4
0.10	40	3.4
0.10	48	3.2
0.10	56	2.4
0.10	64	2.6
0.10	72	3.8
0.10	80	3.2
0.10	88	3.1
0.10	96	4.5
0.10	104	4.8
0.10	112	3.5
0.10	120	3.5

APPENDIX C
LIST OF PUBLICATION

International conference

Pure and Applied Chemistry International Conference 2011 (PACCON 2011)

Suitable Development: from Basic to Applied Chemistry

The Department of Chemistry, Faculty of Science, Srinakharinwirot University and

The Chemical Society of Thailand under the Patronage of her Royal Highness
Princess Chulabhorn Mahidol

5-7 January 2011, Bangkok, Thailand

Ethanol productivity from dark brown sugar by the mixed culture of *Kluyveromyces marxianus* DMKU 3-1042 and *Saccharomyces cerevisiae* M30.

Boonyarit Kiatpaiboon, Kijchai Kanjanaprapakul and Muenduen Phisalaphong*

Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University,

Bangkok, 10330, Thailand

* E-mail: muenduen.p@chula.ac.th

VITA

Boonyarit Kiatpaiboon was born on March 27, 1987, in Songkhla, Thailand. He earned a Bachelor of Engineering Degree from the Department of Chemical Engineering, Prince of Songkhla University., Thailand, in 2008. 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 2009.