PRODUCTION OF ETHANOL BY IMMOBILIZED *SACCHAROMYCES CEREVISIAE*

NOORDIANA BINTI ARIFFIN

BACHELOR OF CHEMICAL ENGINEERING (BIOTECHNOLOGY)
UNIVERSITI MALAYSIA PAHANG

Thesis Access Form

No_____Location_____

Author : NOORDIANA BINTI ARIFFIN

Title : PRODUCTION OF ETHANOL BY IMMOBILIZED *S. CEREVISIAE*

Status of access OPEN / RESTRICTED / CONFIDENTIAL

Moratorium period:_____ years, ending_____/_____200____

Conditions of access proved by (CAPITALS): DR. NUR HIDAYAH BINTI MAT YASIN

Supervisor (Signature)……

Faculty: FACULTY OF CHEMICAL ENGINEERING AND NATURAL RESOURCES

Author's Declaration: *I agree the following conditions:*

OPEN access work shall be made available in the Universiti Malaysia Pahang only and not allowed to reproduce for any purposes.

*The statement itself shall apply to **ALL** copies:*

This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Restricted/confidential work: All access and any photocopying shall be strictly subject to written permission from the University Head of Department and any external sponsor, if any.

Author's signature……..Date: ……

users declaration: for signature during any Moratorium period (Not Open work): ***I undertake to uphold the above conditions:***			
Date	Name (CAPITALS)	Signature	Address

PRODUCTION OF ETHANOL BY IMMOBILIZED *SACCHAROMYCES CEREVISIAE*

NOORDIANA BINTI ARIFFIN

Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

JANUARY 2015

SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

Signature	:
Name of main supervisor	: DR. NUR HIDAYAH BINTI MAT YASIN
Position	: SENIOR LECTURER
Date	: 9 JANUARY 2015

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature :
Name : NOORDIANA BINTI ARIFFIN
ID Number : KE11031
Date : JANUARY 2015

Dedication

Dedicated to my beloved family for their love and encouragement and to my supportive supervisors and friends

ACKNOWLEDGEMENT

I would like to express my sincere appreciation to my main supervisor, Dr. Nur Hidayah Binti Mat Yasin and to my co-supervisor, Dr. Wan Salwanis Binti Wan Md Zain for their supervision, encouragement, advice and patience during my study. Their support and guidance through an effective well-arranged weekly meeting helped me facing the hard times throughout my study. I am also thankful to Mr. Zainal Giman from the Safety and Health unit of FKKSA's Laboratory of University Malaysia Pahang for the useful advices and providing training in the microbiology work on the equipment and apparatus handling.

I am highly indebted to my colleagues, Atiqah, Imla and Faezahtul for the words of encouragement, experience and knowledge sharing. Their help and support have been very valuable and appreciated. Credits are also extended to the academic and support staff at the FKKSA's Laboratory department, especially all the technicians (Mr. Firdaus, Mr. Anuar, Mr. Hafiz and Miss Farah).

My thanks and appreciations also goes to the Centre of Graduate Studies UMP that has always been very responsive in providing necessary information, and without their generous support plan would lack in accurate information. I am also been thankful for the chance and opportunity that has been given to me to finish this study.

ABSTRACT

This research was conducted on the fermentation of ethanol by *Saccharomyces cerevisiae* using immobilization technique. *S. cerevisiae* which is also known as Baker's yeast is a single-celled eukaryote that is frequently used in scientific research. It is an attractive model organism due to the fact that its genome has been sequenced, its genetics are easily manipulated and very easy to maintain in the laboratory. Besides, immobilization has already been approved as useful method in maintaining high cell concentration within the reactor, increases the cell's ability to tolerate high ethanol concentration in the medium, allowing higher conversion yield and increased volumetric productivities. Therefore, the purpose of this study is to immobilize the yeast using various kind of supports (e.g: muslin cloth, membrane and sugarcane stalk) in batch processes to determine the best support for ethanol production. In this method, immobilized fermentation was carried out through shake flask seeding technique while the duration of fermentation process was fixed at 10 hours with sampling made at every 2 hours intervals. The germination of the stock and seed culture preparations were conducted followed by the immobilization technique in order to produce ethanol. Samples taken were analyzed for ethanol and glucose concentration, colony forming unit (CFU), biomass concentration, optical density and viewing of *S. cerevisiae* attachment on immobilized supports by Scanning Electron Microscope (SEM). From the result obtained, it showed that immobilized cloth was capable to increase cell optical density up to 1.602 after 10 hours of fermentation. Moreover, analysis performed by using biomass has revealed that immobilized cloth at 10 hours of fermentation gave the highest biomass concentration which is 39.64 gL^{-1} and colonies of cells forming at 2.57 x 10^8/mL of viable cells compared to other materials. Besides, its ethanol productivity was enhanced to 0.42 $gL^{-1}h^{-1}$, being over 9.6% higher than that observed in the batch culture (0.38 $gL^{-1}h^{-1}$). The consumption of glucose was improved by 6.9% (compared to batch operation), where nearly 67% of glucose conversion at 10 hours period of immobilized fermentation through muslin cloth. The increment might be associated with the altered metabolical functions in the immobilized cells. This alteration is attributed to the reduction of the diffusion path of the growth nutrient that enhanced the availability and promoted the growth of yeast, thus improving the catalytic conversion of glucose to ethanol. Therefore, the supported muslin cloth was proved to increase the number of cells and glucose inhibition is able to reduce with the utilization of immobilized *S. cerevisiae*, thus increasing ethanol production.

ABSTRAK

Kajian ini telah dijalankan ke atas penapaian etanol oleh *Saccharomyces cerevisiae* menggunakan teknik imobilisasi. *S. cerevisiae* yang juga dikenali sebagai yis roti adalah satu eukariot unisel yang sering digunakan dalam penyelidikan saintifik. Ia adalah satu organisma model yang menarik berdasarkan fakta bahawa genomnya yang telah disusun, genetik yang mudah dimanipulasi dan sangat mudah untuk dikekalkan di dalam makmal. Selain itu, kaedah imobilisasi telah diluluskan kerana ia berguna dalam mengekalkan kepekatan sel yang tinggi dalam reaktor, meningkatkan keupayaan sel untuk bertolak ansur dengan kepekatan etanol yang tinggi di dalam media, yang memberikan hasil penukaran yang lebih tinggi dan meningkatkan isipadu produktiviti. Oleh itu, tujuan kajian ini adalah untuk mengimobilisasikan yis dengan menggunakan pelbagai jenis sokongan (cth: kain kasa, membran dan batang tebu) dalam proses berkelompok bagi menentukan sokongan yang terbaik untuk pengeluaran etanol. Dalam kaedah ini, penapaian secara imobilisasi dilakukan melalui teknik pembenihan kelalang manakala tempoh proses penapaian telah ditetapkan pada 10 jam dengan pensampelan dibuat pada setiap selang 2 jam. Persediaan bagi stok dan kultur benih telah dijalankan secara imobilisasi untuk menghasilkan etanol. Sampel yang diambil telah dianalisis untuk kepekatan etanol dan glukosa, pembentukan koloni unit (CFU), kepekatan sel, ketumpatan optik dan paparan *S. cerevisiae* yang berada di atas sokongan imobilisasi dilihat menggunakan SEM. Daripada keputusan yang diperolehi, ia menunjukkan bahawa imobilisasi menggunakan kain kasa mampu untuk meningkatkan kepadatan sel optik sehingga 1.602 selepas 10 jam penapaian. Selain itu, analisis yang dijalankan menggunakan sel jisim telah mendedahkan bahawa kain setelah 10 jam penapaian memberikan kepekatan sel jisim yang tertinggi iaitu 39.64 gL^{-1} dan pembentukan sel koloni pada 2.57 x 108 / mL sel apabila di bandingkan dengan bahan imobilisasi yang lain. Tambahan lagi, produktiviti etanol telah dipertingkatkan kepada 0.42 $gL^{-1}h^{-1}$, menunjukkan 9.6% lebih tinggi daripada yang diperhatikan dalam kultur berkelompok (0.38 $gL^{-1}h^{-1}$). Penggunaan glukosa telah meningkat sebanyak 6.9% (berbanding dengan operasi kelompok), di mana hampir 67% daripada penukaran glukosa pada 10 jam tempoh penapaian secara imobilisasi adalah melalui kain kasa. Peningkatan ini boleh dikaitkan dengan fungsi metabolik yang terubah di dalam sel-sel imobilisasi. Perubahan ini adalah disebabkan oleh pengurangan laluan resapan nutrien pertumbuhan yang meningkatkan tahap kesediaan dan pertumbuhan yis, sekali gus menyebabkan pertambahan terhadap penukaran pemangkin glukosa kepada etanol. Oleh itu, sokongan kain kasa telah terbukti mampu meningkatkan jumlah sel dan perencatan glukosa mampu dikurangkan dengan penggunaan imobilisasi *S. cerevisiae*, oleh itu pengeluaran ethanol dapat ditingkatkan.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

% w/v	percentage of weight per volume
%v/v	percentage of volume per volume
CDW	dry cell weight
CFU	colony forming unit (cells/mL)
C_P	concentration of product (ethanol)
C_S	concentration of substrate (glucose)
C_X	concentration of biomass (cells)
DNS	dinitrosalicylic
G	glucose
GC	gas chromatography
H_3PO_4	phosphoric acid
HCl	hydrochloric acid
O/W	oil in water emulsion
O_2	oxygen
OD	optical density
P	peptone
PBS	phosphate buffered saline
SEM	scanning electron microscopy
SF	shake flask
V	volume (cm^3/mL)
W	weight of biomass (g)
W/O	water in oil emulsion
YE	yeast extract
$Y_{P/S}$	yield of product form over substrate consumed
$Y_{P/X}$	yield of product form over biomass produced
YPG	media containing yeast extract, peptone and glucose.
$Y_{X/S}$	yield of biomass produced over substrate consumed
μ_{max}	specific maximum growth (hr^{-1})

1 INTRODUCTION

1.1 Introduction

Ethylene hydration process is being used in the early days to produce commercial ethanol, derived from fossil fuel by using phosphoric acid as a catalyst. However, nowadays, 90% of the total ethanol is derived from sugar through microbial action (fermentation process). Three major steps that involved in the process are: i) the production of simple sugar from the main feedstock via enzyme or chemical hydrolysis, ii) conversion of sugar to ethanol by microorganisms, and iii) a treatment process to separate ethanol from the fermentation broth (Balat *et al*., 2008). Conventional batch or fed-batch modes are usually used to carry out the fermentation operation by utilising various feedstocks such as sugarcane, corn, wheat, beet root and tapioca. But currently, ethanol is frequently produced by fermentation when certain species of yeast (*Saccharomyces cerevisiae*) metabolize sugar in the absence of oxygen they will produce ethanol and carbon dioxide (Anxo *et al*., 2008).

Since ethanol has been chosen as an important industrial chemical with emerging potential as a biofuel to replace fossil fuels, the full attention has been paid especially to the economics and energy consumption factor (Demirbas, 2006). Due to its characteristic of being friendly to environment, ethanol has been labelled as the one of the most advanced liquid fuels in the world. Ethanol (C_2H_5OH) is a monohydric primary alcohol which also known as ethyl alcohol. It is the second member of the aliphatic alcohol series consist of clear, colourless liquid with characteristic odour and taste of pleasant smell which commonly called grain alcohol or simply alcohol. In dilute aqueous solution, it has a sweet flavour, but in more concentrated solutions it has a burning taste (Patil 1991). It is categorized in a group of chemical compounds whose molecules contain an OH group, bonded to a carbon atom (Kaur & Kocher, 2002).

Ethanol is particularly useful in industrial applications because of its relatively high affinity for both water and organic compounds (Anxo *et al*., 2008). Beyond its use as an automotive fuel, ethanol can be produced for use in a various applications in industries including chemistry, pharmaceutical and food industries as a form of raw materials, solvents and fuel. One of the largest users of industrial ethanol is including

personal care products industry especially in many hand sanitizers, soaps, and shampoos. Besides, it also gave major contribution in processing antibiotics, vaccines, tablets, pills, and vitamins due to its properties that act as a solvent for the pharmaceutical industry.

Various processes have been developed for ethanol production but worldwide demand of ethanol is generally satisfied by biotechnological fermentation process (Walker *et al*., 1990). Recently, cell immobilization techniques have become increasingly important and are being successfully applied in production of ethanol through fermentation process (Reddy *et al.*, 2008). These procedures frequently improve catalyst stability and the immobilized cells can be concentrated to higher densities within the immobilization support that is impossible in normal suspension cultures, resulting in potentially high reactor productivities. However, this technique has also shown a few disadvantages in terms of the stability and fermentation performance. Several problems have been identified, which are mainly due to the longer operating time and cell losses. The poor mechanical strength of the support enhances cell leakage during prolonged operation. Due to the problems occurred, applying the laboratory works into the real industrial process is almost impossible if the problems have not been resolved.

Therefore, the aim of this study is to investigate the effect of immobilized yeast (*Saccharomyces cerevisiae*) on the production of ethanol using various kind of supports (e.g: muslin cloth, sugarcane stalk and membrane) in batch processes thus, determine the best support for ethanol production. The ethanol produced is proportional to the growth of yeast, so preparing suitable housing with unlimited spaces that permit the proliferation of yeast is crucial for a long term continuous operation. Immobilization technique and the types of supports will be thoroughly studied.

1.2 Motivation and statement of problem

Presently, fossil fuel is the main source of world energy production. But, the depletion of fossil fuels due to the environmental problems has become a serious energy crisis to the world. Therefore, biomass has been utilized for the production of friendly biofuels such as ethanol. Since the ethanol market has developed fantastically, the diversification of processes in terms of operating mode, feedstock, and searching for an effective and efficient microorganism has been extensively studied. The major research and development challenges for ethanol production from biomass basically involve several factors such as the improvement of quality and feedstock for its production and techno-economic along with environmental assessment by minimising the negative impact on the environment relating to agricultural issues on land and wastes (Gnansounou, 2010). Unfortunately, ethanol fermentation nowadays encountering more disadvantages rather than advantages such as the cost of production is too high, more energy is required, production of the process is too slow and the product obtained is impure so additional pretreatment are needed. This problem occurs will lead to the time consuming factor and decrease the product yield. Although continuous process helps to reduce the cost and operation time by terminating some of the production steps in the batch operation, the washout effect and contamination resulting from the long operation time reduces its effectiveness. This kind of problem can be improved by applying the immobilization technique. It is a technique where the microorganisms were grown in insoluble matrix (support) which permits the flow of liquid in and out but retains the cells within its capsules. Due to this technique, the cell's ability can be increased to tolerate high ethanol concentration in the fermentation medium, allowing higher conversion yield and increased volumetric productivities (Qi *et al.*, 2006). Besides, the immobilization technique allows the reuse of cells, so the operating cost can be reduced. Therefore, ethanol production by immobilized cells has been intensively investigated during last few years because this technique showed certain technical and economic advantages over free cells system that can be more profitable rather than past years of operation used.

1.3 Objectives

The objectives of this study are to investigate the effect of immobilized yeast (*Saccharomyces cerevisiae*) on the production of ethanol using various kind of supports (e.g: muslin cloth, sugarcane stalk and membrane) in batch processes and to determine the best support for the production thus reducing the effect of glucose inhibition and increasing the ethanol production.

1.4 Scope of this research

In this research study, the scopes function as a guideline to achieve the objectives. The study has been divided into several scopes which are:

1.4.1 To perform growth profile of ethanol production through submerged fermentation using free cell *Saccharomyces cerevisiae* in batch processes.

1.4.2 To carry out fermentation process using immobilized cells on various kind of supports (e.g: muslin cloth, membrane and sugarcane stalk).

1.4.3 To study the effects of glucose concentration on the ethanol production using the best support in (1.4.2).

1.5 Main contribution of this work

This research was capable to investigate the effect of various kinds of supports (eg: muslin cloth, membrane and sugarcane stalks) on the productivity of ethanol production. According to the previous researchers, the production of ethanol by immobilizing organism had been demonstrated to have greater advantages over a suspended culture of free cells of *S. cerevisiae*. Although it is consider as a time consuming technique that needed additional equipment and materials, it is one of the best method since it enable to contain the cells within its capsulation, thus avoiding the cells to be present in products, therefore an additional step of purification can be discarded. Hence, ethanol production was effectively enhanced to yield the maximum amount of product through immobilized supports if compared to batch operation. Nevertheless, it was able to help people effectively improve their knowledge and understanding on the immobilized fermentation of ethanol.

1.6 Organization of the thesis

The structure of the thesis was outlined as follow:

Chapter 2 provided a description on the ethanol characteristic and its application. Moreover, the benefits of using *S. cerevisiae* as the free immobilized cells and also the advantages rather than disadvantages of immobilization technique were explained. This chapter also provided a brief discussion on ethanol producers, characteristic of yeast (*Saccharomyces cerevisiae*) and its growth profile along with the future demand of ethanol production. A summary of the previous reported experimental work on ethanol fermentation process through immobilization technique was also presented.

Chapter 3 gave a review on the chemicals, materials and all the experimental works employed for fermentation stage.

Chapter 4 was devoted on the result and observation obtained from the analyzes performed with samples taken from the fermentation process to study the effect of immobilized yeast (*Saccharomyces cerevisiae*) on the production of ethanol using various kind of supports (e.g: muslin cloth, sugarcane stalk and membrane). Therefore, the analysis data that was conducted were through direct and indirect methods including determination of glucose concentration by DNS method and ethanol concentration using Gas Chromatography, colony forming unit (CFU), biomass concentration, optical density and viewing of *S. cerevisiae* attachment on immobilized supports by SEM was demonstrated in this chapter. Besides, the kinetic growth profile of the immobilized cells has also been illustrated to examine the trend of growth curve of the yeast cells.

Chapter 5 was concluded the overall study result and observation related to the immobilized fermentation in terms of glucose and ethanol concentration, its yield and productivity. Besides, the best support to immobilize the *S. cerevisiae* was determined by performing analysis on the cell absorbance, biomass concentration and the cells forming unit. The recommendation regarding the best support for the immobilize cells was also provided.

2 LITERATURE REVIEW

2.1 Overview

This research was conducted to investigate the production of ethanol through immobilized fermentation of *S. cerevisiae*. Ethanol has been extensively studied as a kind of renewable resource due to its characteristic that burns more cleanly in air than petroleum, producing less carbon (soot) and carbon monoxide. Ethanol-fuelled vehicles produce lower carbon monoxide and carbon dioxide emissions, and the same or lower levels of hydrocarbon and oxides of nitrogen emissions. Therefore, it is one of the best tools we have to fight air pollution from vehicles. There is no fuel available that matches the ethanol's ability to improve overall environmental quality compared to gasoline. From its biodegradable nature to reductions in greenhouse gas and tailpipe emissions, ethanol provides a tool to address environmental concerns without requiring an entirely new way for goods and people to get from one place to another. Hence, the best method to be used that give the maximum yield of ethanol production with the minimized disadvantages is the immobilization technique. Although there are several disadvantages have been mentioned by the previous researchers, but from the positive perspective, immobilization offers more advantages compared to other techniques.

2.2 Introduction

A microbial fermentation for ethanol production by using immobilized fermentation method was intensively studied previously. However, there are only several researchers use immobilization as the preferred method to be carried out probably due to time constraints and additional cost is needed. Therefore, most of the research studies were focussing on the production of ethanol by using batch and continuous culture. Hence, previous work on production of ethanol was explained below. Besides, the detail information about world ethanol demand, ethanol producers, yeast *Saccharomyces cerevisiae*, general growth profile possess by a bacteria and immobilization technique were described as below.

2.3 Global Ethanol Demand Reviews

Nowadays, the prime focus of ethanol production throughout the world has led to the continuous demand for its energy consumption factor. Due to the problem that facing by the current energy produced from fossil fuel, it has created debates among the experts since it can give negative impact on the environment and the emerging doubts concerning its sustainability for the future. Therefore, many countries have started to use ethanol as their main energy source that can be applied in various kind of industrial and daily application. One of the largest country that consume and produce ethanol as their main energy source is United States.

In the U.S., the Energy Independence and Security Act of 2007 requires that 36 billion gallons of renewable fuels, largely ethanol, be used in the nation's motor fuel supply by 2022. According to the Renewable Fuels Association, the U.S. ethanol industry has current capacity to produce more than 8.5 billion gallons of ethanol and an additional 5.1 billion of new capacity are under construction and will come on-line within the next several years. 20.4 billion gallons of ethanol production expands the quantity of gasoline available to consumers around the world (Urbanchuk, 2008). Although the environmental costs associated with producing ethanol are significant, it have been ignored by most investigators in terms of energy and economics (Pimentel *et al.,* 2007). Since ethanol has lower energy content than gasoline, there is not a one-to-one substitution of ethanol for gasoline. Reflecting this, if ethanol were not available, the world's oil refiners would have to "find" an additional 13.4 billion gallons (320 million barrels) of gasoline to make up the shortfall.

On the other hand, according to the EIA and referring to Figure 2-1, renewable fuels in the US in 2009 make up 1.6% of the energy used, with 13 billion gallons of ethanol produced in last year of 2008. For better or for worse, ethanol is part of our future. Not only has the government mandated a 36 billion gallon target by the EPA for 2022, but the EPA has recently approved the use of E-15 blended gasoline (15% ethanol) for cars that are as old as 2001. Essentially, the majority of cars and trucks on the road can now run on E-15, which is better for the environment as burning ethanol produces less greenhouse gas emissions, but the downside is you need more of it, as ethanol contains less energy than gasoline (Biofuels and Global energy, 2011).

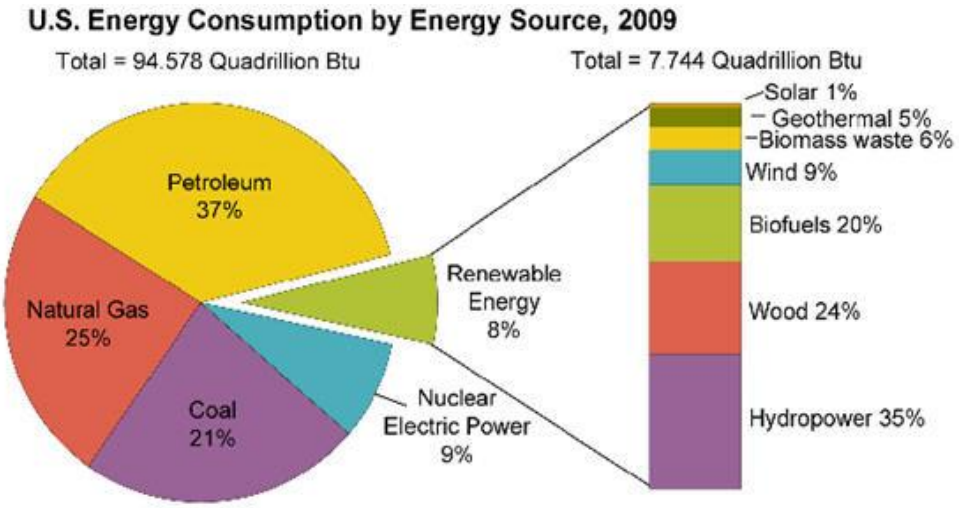


Figure 2-1: U.S Energy consumptions in 2009 (Biofuels and Global energy, 2011)

From the overall production in all over the world, Table 2-1 reported that ethanol production is said to reach 13.1 billion gallons in 2007 and is projected to total nearly 20 billion gallons in year 2009. This represents a near doubling of ethanol production in just five years. More than 7 countries reported annual production in excess of 100 million gallons with the two largest producers which are the U.S. and Brazil that accounting for nearly 78 percent of global ethanol production. The growth in global ethanol production has been the result of increased interest in biofuels as a result of sharply rising oil and gasoline prices. Global ethanol production is expected to continue expanding as world crude oil prices increase to new record levels and remain high (Urbanchuk, 2009).

Table 2-1: World fuel ethanol production by certain country (Urbanchuk, 2009)

World Fuel Ethanol Production by Country (Millions Gallons)			
Country	**2007**	**2008**	**2009**
USA	6,499	9,000	10,600
Brazil	5,019	6,472	6,578
Europe	570	734	1,040
China	486	502	542
Canada	211	238	291
Thailand	79	90	435
Colombia	75	79	83
India	53	66	92
Australia	26	26	57
Other	82	128	247
WORLD	**13,101**	**17,335**	**19,965**

2.4 Current Ethanol Production Technology and Lignocellulosic Ethanol Biorefinery

Virtually all biotechnological ethanol fuel currently manufactured at the industrial scale is produced through fermentation of relatively easily accessible sugars by the yeast *S. cerevisiae*. Beyond the relatively limited level of worldwide investment made to this date to finance industrial biotechnology projects (Kircher, 2006), it is the various robustness of the cell and process economics limitations that currently restrict the profitability of lignocellulosic ethanol, and thus still to this date hinders the financing and development of efficient value chains to convert lignocellulose into commodity chemicals.

Most of the biotechnological ethanol currently derived from maize is produced from the dry grind process, a process which was designed to maximize capital return per hectolitre of maize-derived ethanol, as opposed to extracting the full value of the maize kernel. In nominal terms, the variable costs of dry grind ethanol have exponentially decreased from approximately 0.925 \$ L^{-1} in the 1980s to a value that remained essentially unchanged between 1998 (0.251 \$ L^{-1}) and 2002 (0.253 \$ L^{-1}) (Shapouri, 2005). When plotted versus time, the ethanol manufacturing variable cost curve reveals that the present yeast-based technology has already reached its productivity limit (Figure 2-2), since its shape suggests that the current strategy of incremental improvements has reached a threshold of marginal return on investments.

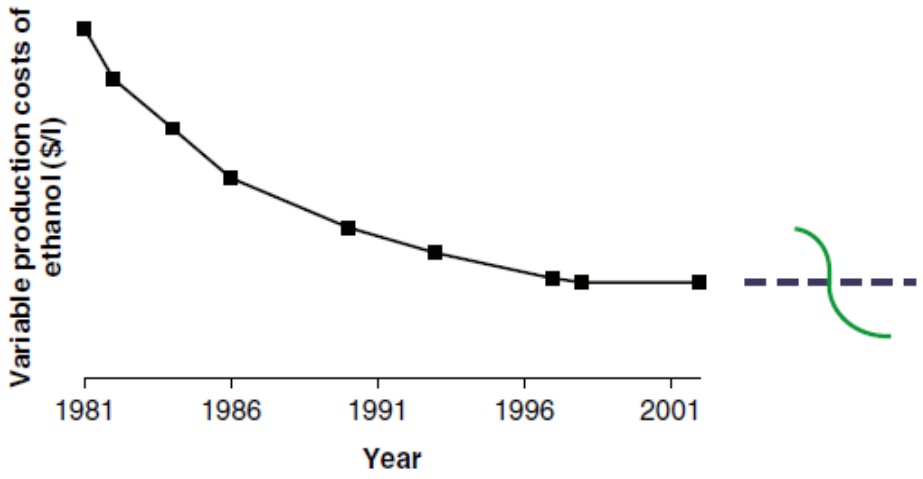


Figure 2-2: Evolution of the variable cost of ethanol production from maize treated by the dry-grind process and hypothetical S-curve in technological improvement of lignocellulosic ethanol.

Interestingly, over recent years dry mills have already experienced reduced energy, labour, and maintenance inputs, in addition to economies of scale (Shapouri, 2006). As a result, these 20 year data suggest that a novel technological discontinuity is now needed in order to further improve the economic performance of ethanol manufacturing. Considering that the net costs of raw materials constitute the most significant cost of biotechnological ethanol, it appears now increasingly urgent to implement alternative raw materials, such as lignocellulosic materials, and to generate by-products of higher value and deeper market than, for example, Distillers Dried Grains with Soluble. All these various components can be captured by the materialization of the biorefinery concept, which closely mimics the petrochemical refinery economic model.

Although yeast based technologies might to this date represent the optimal solution for the batch process conversion into fuel ethanol, this technological approach might not always be the most appropriate. For example, it cannot be excluded that a compact plant operating with growth-arrested microbial cells might be more efficient at exploiting the resources of agricultural or industrial environments where land is scarce, of low fertility or where infrastructures are insufficiently developed. Consequently, only a portfolio of technological options would give the flexibility that is necessary to enable the optimal use of such numerous raw material opportunities, and thus constitutes the most adequate response to address the challenges of an imminent energy crisis.

2.5 Ethanol Producers

Ethanol can be produced by fermentation of sugars from various waste agricultural materials. Whichever system for ethanol production is chosen, the attention must be paid to the overall economics and energy consumption. The economic evaluation of different materials for ethanol production was thoroughly studied previously (Meo, 1984; Maiorella *et al.*, 1984; Greg and Saddler 1995).

In recent years, metabolic engineering for microorganisms used in fuel ethanol production has shown significant progress. The most commonly used ethanol producer is *Saccharomyces cerevisiae* but recently, microorganisms such as *Zymomonas mobilis*, *Escherichia coli* and a few others also have been targeted through metabolic engineering for ethanol production as illustrated in Table 2-2 (Jeffries *et al.*, 2004).

Saccharomyces cerevisiae, the most widely utilised in industry, is considered the best organism because of its high yield and robustness. However, its application was found to be limited because of its inability to utilise C5 sugars. This is necessary since feedstock for the ethanol production is undergoing a shift from using food crops (corn and sugar cane) to cheaper lignocellulosic materials, therefore a more robust strain is required to ferment the C5 and C6 sugars that are produced from cellulose and hemicellulose hydrolysis. Apart from this, the preferred strain must be able to withstand the toxicity effect arising from lignin degradation (Jamai *et al*., 2001).

Efficient ethanol production requires a rapid fermentation leading to high ethanol concentrations; therefore a yeast strain must have a good specific growth rate and good specific ethanol production rate at high osmotic activities and ethanol concentration. Therefore, *Saccharomyces cerevisiae* is considered to be the best organism due to its high yield and robustness. Traditionally, baker's yeast (*Saccharomyces cerevisiae*), has long been used in the brewery industry to produce ethanol from hexoses (six-carbon sugars). Due to the complex nature of the carbohydrates present in lignocellulosic biomass, a significant amount of xylose and arabinose (five-carbon sugars derived from the hemicellulose portion of the lignocellulose) is also present in the hydrolysate. Therefore, the ability of the fermenting microorganisms to use the whole range of sugars available from the hydrolysate is vital in the fermentation process. Yeast cells are especially attractive for cellulosic ethanol processes because they have been used in biotechnology for hundreds of years, are tolerant to high ethanol and inhibitor concentrations and can grow at low pH values to reduce bacterial contamination.

There are some basic requirements that must be fulfilled for selecting a microorganism as an ethanol producer, such as (Dien *et al*., 2003):

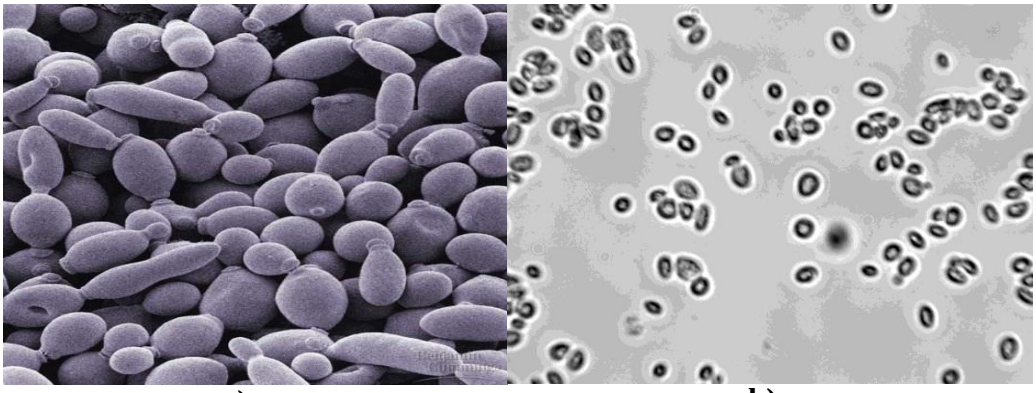
- Ethanol yield must be more than 90% of theoretical
- Ethanol tolerance must exceed >40 gL^{-1}
- Ethanol Productivity > 1 $gL^{-1}h^{-1}$
- Robust, require inexpensive medium formulation with various substrate.
- High resistance to inhibitors and osmotic stress (high sugar concentration)
- Able to grow in acidic environment and higher temperatures
- Elimination of by-product formation (Kunz, 2008)

Table 2-2: Lists of the ethanol producer comprised with its advantages and disadvantages

Microorganism	Advantages	Disadvantages	References
Saccharomyces cerevisiae	• Considerable high ethanol yield • Fast growth • Robust • Stable • High tolerance to inhibitor	• Growth related production • Susceptible to high ethanol concentration • Limited substrate • Contamination	Karagoz *et al*., (2008); Kunz (2008); Balat *et al*., (2008)
Zymomonas mobilis	• Produce less biomass • Higher yield (5-10% more ethanol per g glucose) • High tolerance towards ethanol (up to 120 gL^{-1}). • High productivity • Safe	• Ferments only glucose, sucrose and fructose • Low tolerance to acetic acid • Unstable	Amin and Verachtert, (1982); Delgenes *et al*., (1996); Kesava *et al*., (1996); Dien *et al*., (2003).
Klebsiella oxytoca	• Able to grow in acidic pH and higher temperature. • Utilizes wide range of sugars (C5-C6)	• Yields many by-products • Low ethanol yield	Dien *et al*., (2003)
Recombinant *Escherichia coli*	• Able to ferment various sugars • Produce less biomass compare to *S. cerevisiae*	• Low tolerance to pH (6.0-8.0) • Less hardy cultures • Problems with biomass and public perceptions	Dien *et al*., (2003).
Candida tropicalis	• Thermotolerant • Able to ferment various carbon sources (alcohols and sugar) • Ability to tolerate lignin like-polyphenols	• Lower productivity compared to • *S. cerevisiae.* • Lower tolerance towards ethanol • Slow adaptation to anaerobic condition	Jamai *et al*., (2001); De Deken (1966).
Pichia Stipitis	• Able to ferment C5-carbon source	• Low yield	Balat *et al*., (2008)

2.6 Yeast (Saccharomyces cerevisiae)

S. cerevisiae was adopted as a model system for laboratory study in the 1930s, as investigators developed genetic tools to understand its life cycle and differentiation (Hall and Linder, 1993). Extensive studies also have been carried out on the fermentation process of ethanol by others organisms, however, *S. cerevisiae* remained the organism of choice, which is the same species used for bread making and some wines or beers (Walker *et al*., 1990; Converti *et al*., 2003; Moreira *et al*., 2005). Figure 2-3 demonstrates the characteristic of this kind of yeast that was examined under a Staining Electron microscope with higher magnification and resolution. The schematic diagram of *S. cerevisiae* is also presented in Figure 2-4.



a) **b)**

Figure 2-3: The shape of *S. cerevisiae* a) patented by Fleischmann's (Thorp, 2009) and b) examined under Staining Electron microscope (SEM) using 650x magnification with scale bar of 20 µm (Konig *et al.,* 2009).

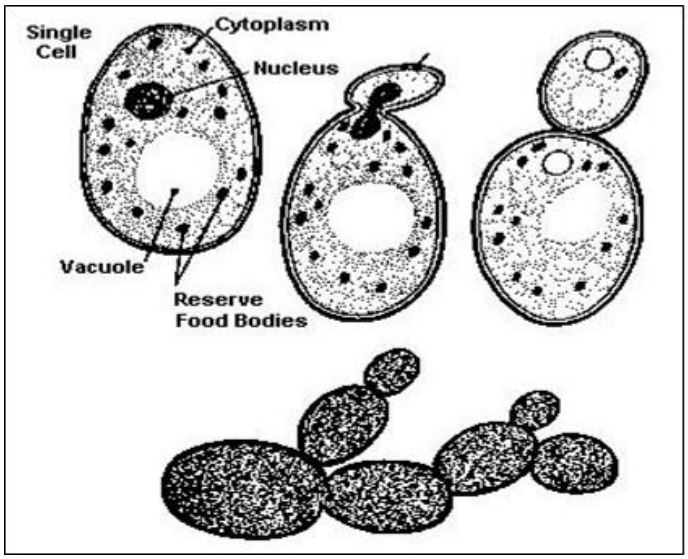


Figure 2-4: Schematic diagram of *S.cerevisiae* (Wan Salwanis, 2013)

S. cerevisiae is a unicellular microorganism which belongs to the fungi group. Its typical shapes are either spherical or oval, with clear internal cell structures. Observation under electron microscope, shows that *S. cerevisiae* is consists of vacuole, mitochondria, cytoplasm and nucleus (Werner-Washburne *et al*., 1993). Normally, most the size of yeasts can vary from 3-5 μm in diameter, although some can reach 7 μm. *S. cerevisiae* is commonly grow by budding, but very rarely it can also multiply by binary fission for instance as in *Saccharomyces pombe*. Due to the surface of the yeast is normally negatively charged, thus it can be easily immobilized on solid surfaces of opposite charge. Generally, yeast is hydrophobic therefore it prefers hydrophobic surfaces, and attaches weakly to hydrophilic surfaces such as glass (Fortman *et al*., 2008).

The advantages of *S. cerevisiae* for industrial ethanol production include its long history of safe use, a high ethanol tolerance (up to 15% ethanol), and high final ethanol concentration (10–12%). Moreover, its sugar conversion ratios are close to the theoretical maximum (95%) and its tolerance of low pH helps prevent the growth of spoilage organisms. The robustness of yeast-based processes is limited by the sensitivity of *S. cerevisiae* to temperatures higher than 35^{o}C, to the contamination of lactic acid bacteria, and to glucose repression phenomena that regulate its central metabolism. Moreover, the growth of *S. cerevisiae* is inhibited by a variety of compounds generated during the saccharification of lignocellulosic material and it cannot ferment xylose or arabinose. These two pentoses represent a significant portion, respectively 20% and 5% of the sugars in lignocellulosic biomass (Aristidou and Penttila, 2000). Furthermore, actively growing yeasts produce ethanol up to 33 times faster than stationary cells (Bellissimi & Ingledew, 2005) preventing the use of growth-arrested yeasts.

Although *S. cerevisiae* was successfully employed in industry, further improvement is still desirable with respect to increased productivity, stability and tolerance towards stressors. Improvements are still evolving and relying on the random mutagenesis, classical breeding and genetic crossing of two strains and a successful strain with the previously mentioned qualities will be selected for the optimisation Kunz, 2008). Recent studies were also directed to the thermotolerant strains, because high temperature is needed to withstand the requirement of high temperature (45-50^{o}C) for the saccharification process in the simultaneous saccharification and fermentation (SSF) process (Edgardo *et al*., 2008; Ylitervo *et al*., 2011).

2.7 Immobilization

2.7.1 Importance of Immobilization

Immobilized cell system has been suggested as an effective means for improving ethanol fermentation, since it is possible to increase the concentration of cells within the reactor volume, hence increasing the product formation and process productivity, thus minimizing production costs (Santos *et al*., 2008). The immobilization of cells leads to higher cell densities with consequent increases in reaction rates and productivity. As a result, shorter residence time and smaller reactor size can be employed. For industrial purposes, an important choice criterion is the carrier cost which, combined with the interest in by-products recycling, has been leading to an increasing search for cheap and available potential cell carriers (Santos *et al*., 2008).

Immobilized microbial cell systems offer advantages over cell suspension systems in terms of ethanol productivity and the stability of cell activity (Sho *et al*., 2001). Cell immobilization via gel entrapment techniques is widely utilized in biotechnology at a laboratory level and also in some selected cases on an industrial scale (Brodelius *et al*., 1987). However, gel carriers are the hindrance of substrate diffusion to the immobilized cells and of metabolites leaving the gel-entrapped biomass (Maryse *et al*., 1996). In order to reduce such carrier-induced additional diffusion barriers, highly porous matrices are, therefore, necessary. Another main factor that influences the immobilization behaviour of the cells and their productivity is thought to be the surface characteristics of the carrier including pore size, hydrophilicity and magnetism (Shinonaga *et al*., 1992; Passarinho *et al*., 1989).

The benefits of the immobilization process in microbial fermentations are as follows (Kargi, 2001; Karagoz *et al*., 2008):

- Produces high cell concentrations that maintain high productivity throughout the operation period, and avoid wash out during high dilution rate.
- Provides protection for the cells from the harsh environment and is highly beneficial to shear sensitive organisms when operating at high dilution rates.
- The supports are stable and resistant to extreme environments such as pH, temperature, and toxic metabolites.

- ❖ The immobilized cells are stable and can be re-used, thus reducing the cost and time for preparation.
- ❖ Attachment of the cells to the polymer matrix changes the physiological properties of the biocatalyst, which normally are more stable compared with free cells, to overcome the inhibition during fermentation.
- ❖ Facilitates the development of a microenvironment which reduces the barrier in mass transfer, increases cell-cell interaction, and also improves the biocatalyst performance.

Despite all the advantages of the immobilization techniques, major disadvantage of the immobilization process is the additional resistance of flow created by the matrix (Erhan *et al.,* 2004). Aerobic processes will suffer from oxygen limitation due to its low solubility which might be the rate limiting factor in such processes. However, the use of highly porous structures may or may not improve the mass transfer of substrates and the products from the matrix cavity (Margaritis and Kilonzo, 2005), thus further increases the productivity. There are several immobilization techniques that usually applied by researchers as presented in Figure 2-5.

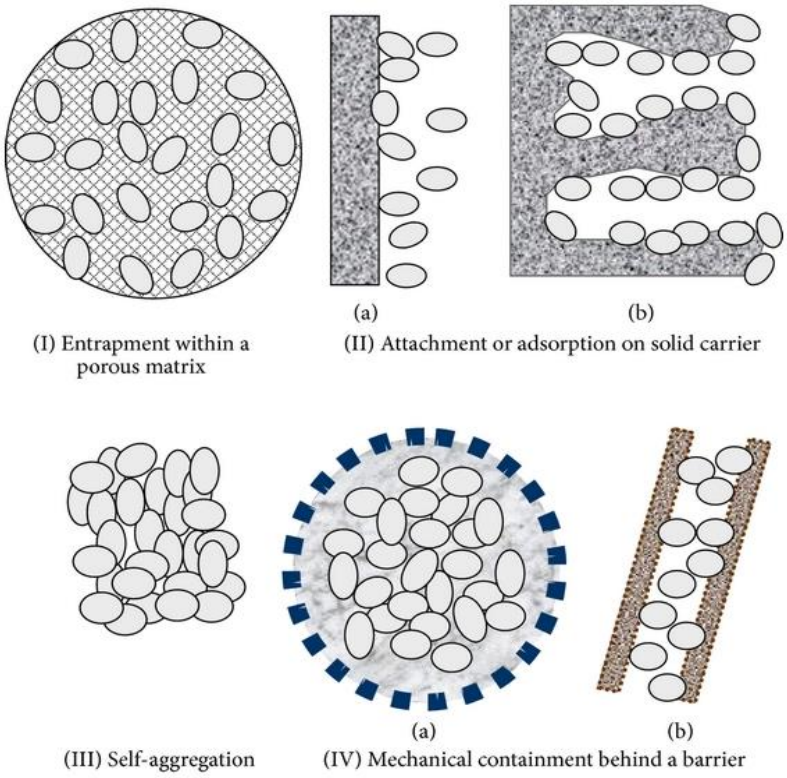


Figure 2-5: Basic method of cell immobilization. (Lacroix, 1996)

2.8 Effect of Immobilizations on Yeast Morphology and Physiology

Numerous authors have reported that the immobilization technique has significantly or slightly altered the physiology and the metabolic capacity of yeast compared to free cells. Many changes in the morphology of yeast cells entrapped in immobilized medium were described by different authors. The well-documented review of Martynenko and Gracheva (2003) quotes studies dealing with the physiological activity and morphological changes of immobilized cells in the special case of Champagne process. It was said that the adaption of yeast cells to immobilization was accompanied by vacuolization. Also the thickness of the cell wall increased and ribosomes became scarcely visible. A study also demonstrated that the shape of the cells was also altered due to cytomechanical effects that change the physical appearance from an ellipsoid shape to a rod-shape (Jirku, 1994). Apart from this, the content of the cell walls, such as surface proteins, lipids and amino acid sugars, is different from the free cells. The mechanism of these changes was still unclear and was attributed to either the difference between the microenvironment and the bulk environment, or was caused by a modification of cellular physiology induced by the immobilization (Norton and D'Amore, 1994). It was also shown the specific rate of cell division was very low: the yeasts proliferated at the periphery of the bead, while the number of cells in the core remained constant.

The term "physiological activity" could describe various important parameters: fermentation potential, stress tolerance, aging, growth or reproduction abilities. The physiology of immobilized cells is affected by the microenvironment and the supply of nutrients and metabolic products (internal and external mass transfer) (Junter *et al.*, 2002; Pajić-Lijakovic *et al.*, 2007; Gonga *et al.*, 2010). Cellular stress at the stage of immobilization may also have a significant impact on the physiological state, and according to Smart (2001) this physiological history may determine a cell's efficiency during the technological process. The general aim is to maintain the greatest viability and metabolic activity of the cells, allowing to the process to be carried out with high efficiency for the longest time possible. Continuous system technologies in beer production require immobilized yeast cells to be kept for several months in bioreactors. In the case of yeast plankton populations, over time the linear dimensions of cells increase, there is a longer generation time and their metabolic activity decreases.

It has been shown that free and immobilized yeast cells differ in chemical composition and ploidy (Verbelen *et al.,* 2006). Immobilized cells, in comparison with free cells, have a higher content of glycogen, trehalose, structural polysaccharides (glucans and mannan), fatty acids and DNA. Immobilization also causes changes in the proteome of a cell, in the level of gene expression, and has a significant impact on the quantitative composition and organization of the cytoplasmic membrane and cell wall structures (Brányik *et al.,* 2008; Parascandola *et al.,* 1997). Many studies have reported an increase in metabolic activity (increased rate of sugar uptake and productivity of selected metabolites) in immobilized cells (Junter *et al.,* 2002; Angelova *et al.,* 2000; Talebnia and Taherzadeh 2007; Plessas *et al.,* 2007; Li *et al.,* 2007; Behera *et al.,* 2011).

Adsorption of *Saccharomyces carlsbergensis* on porous glass and *S. cerevisiae* on ceramic support resulted in increased production of ethanol and reduced production of CO_2 (Kourkoutas *et al.,* 2004). In yeast entrapped in alginate matrices, a slight decrease was noticed in intracellular pH due to increased enzymatic activity. This promotes the permeability of membranes, which in turn leads to an increase in proton transport and ATP use, stimulating glycolysis processes (Galazzo and Bailey 1990). Higher efficiency in the pentose phosphate pathway and of glycolytic flux may also be explained through the increased activity of alcohol dehydrogenase and by more efficient regeneration of the NADH and NADPH cofactors by Brányik *et al.* (2008).

2.9 Principle of Bacteria Growth

The increase in the cell size and cell mass during the development of an organism is termed as growth. It is the unique characteristics of all organisms. The organism must require certain basic parameters for their energy generation and cellular biosynthesis. The growth of the organism is affected by both physical and nutritional factors. The physical factors include the pH, temperature, osmotic pressure, hydrostatic pressure, and moisture content of the medium in which the organism is growing. The nutritional factors include the amount of carbon, nitrogen, sulphur, phosphorous, and other trace elements provided in the growth medium. Bacteria are unicellular (single cell) organisms. When the bacteria reach a certain size, they divide by binary fission, in which the one cell divides into two, two into four and continue the process in a geometric fashion. The bacterium is then known to be in an actively growing phase. To

study the bacterial growth population, the viable cells of the bacterium should be inoculated on to the sterile broth and incubated under optimal growth conditions. The bacterium starts utilising the components of the media and it will increase in its size and cellular mass.

The dynamics of the bacterial growth can be studied by plotting the cell growth (absorbance) versus the incubation time or log of cell number versus time. The curve thus obtained is a sigmoid curve and is known as a standard growth curve. The increase in the cell mass of the organism is measured by using the Spectrophotometer. The Spectrophotometer measures the turbidity or optical density which is the measure of the amount of light absorbed by a bacterial suspension. The degree of turbidity in the broth culture is directly related to the number of microorganism present, either viable or dead cells, and is a convenient and rapid method of measuring cell growth rate of an organism. Thus the increasing the turbidity of the broth medium indicates increase of the microbial cell mass. Generally, the growth curve has four distinct phases as illustrated in Figure 2-6.

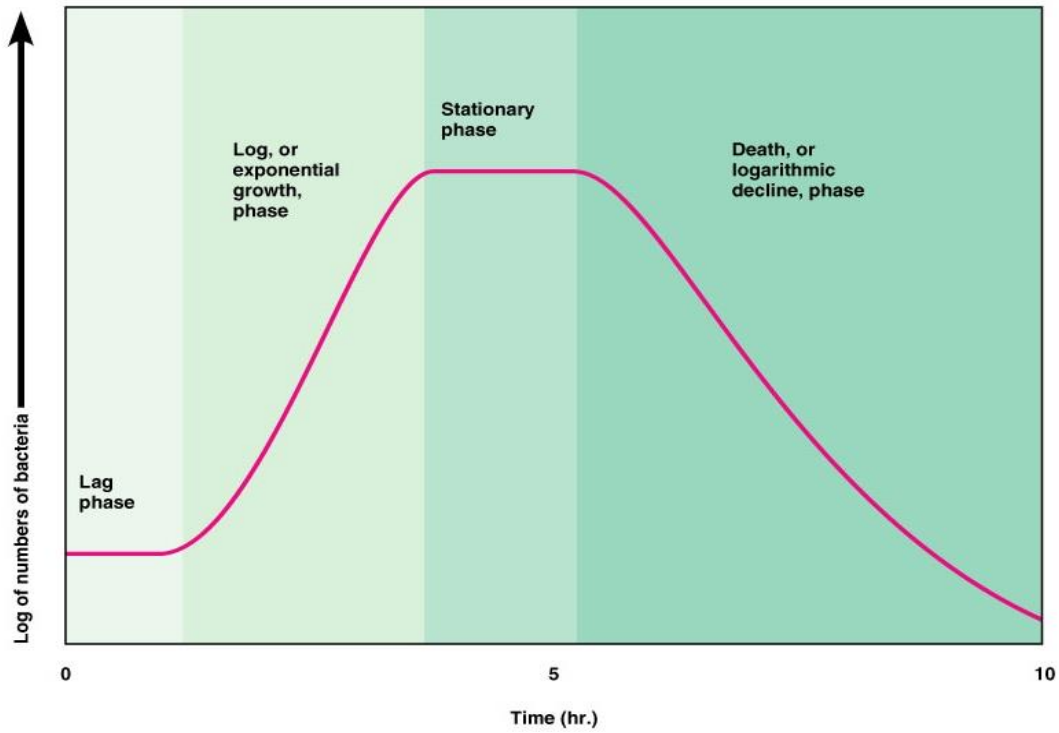


Figure 2-6: General kinetic growth curve of bacteria (Benjamin, 2004).

2.9.1 Lag phase

When a microorganism is introduced into the fresh medium, it takes some time to adjust with the new environment. This phase is termed as Lag phase, in which cellular metabolism is accelerated, cells are increasing in size, but the bacteria are not able to replicate and therefore no increase in cell mass. The length of the lag phase depends directly on the previous growth condition of the organism. When the microorganism growing in a rich medium is inoculated into nutritionally poor medium, the organism will take more time to adapt with the new environment. The organism will start synthesising the necessary proteins, co-enzymes and vitamins needed for their growth and hence there will be a subsequent increase in the lag phase.

2.9.2 Exponential or Logarithmic (log) phase

During this phase, the microorganisms are in a rapidly growing and dividing state. Their metabolic activity increases and the organism begin the DNA replication by binary fission at a constant rate. The growth medium is exploited at the maximal rate, the culture reaches the maximum growth rate and the number of bacteria increases logarithmically (exponentially) and finally the single cell divide into two, which replicate into four, eight, sixteen, thirty two and so on (That is 2^0, 2^1, 2^2, 2^3.....2^n, n is the number of generations) This will result in a balanced growth. The time taken by the bacteria to double in number during a specified time period is known as the generation time. The generation time tends to vary with different organisms. *E.coli* divides in every 20 minutes, hence its generation time is 20 minutes, and for *Staphylococcus aureus* it is 30 minutes.

2.9.3 Stationary phase

As the bacterial population continues to grow, all the nutrients in the growth medium are used up by the microorganism for their rapid multiplication. This was result in the accumulation of waste materials, toxic metabolites and inhibitory compounds such as antibiotics in the medium. This shifts the conditions of the medium such as pH and temperature, thereby creating an unfavourable environment for the bacterial growth. The reproduction rate will slow down, the cells undergoing division is equal to the number of cell death, and finally bacterium stops its division completely. The cell number is not increased and thus the growth rate is stabilized. If a cell taken from the

stationary phase is introduced into a fresh medium, the cell can easily move on the exponential phase and is able to perform its metabolic activities as usual.

2.9.4 Decline or Death phase

The depletion of nutrients and the subsequent accumulation of metabolic waste products and other toxic materials in the media will facilitates the bacterium to move on to the death phase. During this, the bacterium completely loses its ability to reproduce. Individual bacteria begin to die due to the unfavourable conditions and the death is rapid and at uniform rate. The number of dead cells exceeds the number of live cells. Some organisms which can resist this condition can survive in the environment by producing endospores.

2.10 Factors Affecting Ethanol Production

Apart from severe inhibition caused by ethanol accumulation, CO_2 production has been reported to repress the growth of yeast, which later reduces the ethanol production. Figure 2-7 summarizes some of the possible stresses that have negative impacts on the growth of *S. cerevisiae* and ethanol production.

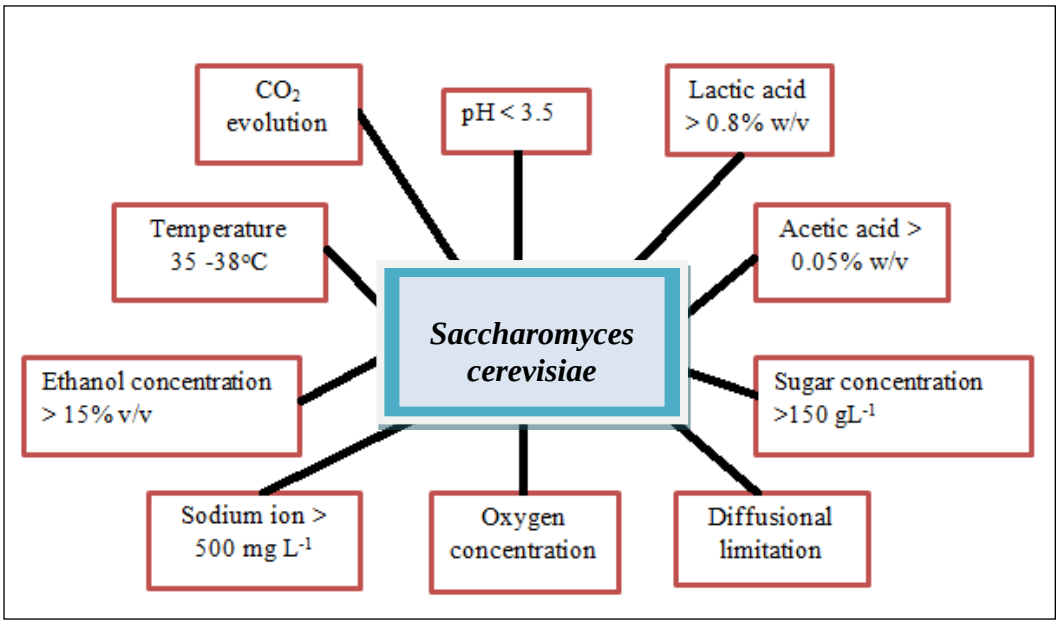


Figure 2-7: Potential environmental stresses on *S. cerevisiae* during ethanol fermentation in both free and immobilized cells (Bai *et al.*, 2008).

Ethanol is produced using various operating modes, with typical processing modes of batch, fed-batch and continuous processes. Switching from batch processes to continuous processes needs further study in order to avoid losses and the instability of production. Currently, there are less than 30% of bioethanol plants operating using continuous modes, while the rest still employ either batch or fed batch modes (Brethauer and Wyman, 2009).

Ethanol production by *S. cerevisiae* is affected not only by fermentation conditions (temperature, pH and sugar concentration) but also by the intrinsic factors e.g., culture medium, dissolved O_2, immobilization and other micronutrients. A previous research was conducted in order to investigate the influence of key factors on ethanol production by *S. cerevisiae*, a laboratory strain *S. cerevisiae* IFST-072011 was used in this study. Several fermentation runs were carried out varying temperature, pH, sugar concentration, aeration, immobilization and supplementation of metal ions employed. Experimental data on several fermentation runs showed that reducing sugar concentration ranged between 5-6%, temperature of 30°C and pH between 5.0 and 6.0 were optimum for maximum yield of ethanol by *S. cerevisiae* IFST-072011.The strain produced 86.9 g L^{-1} ethanol by free cells using the initial reducing sugar concentration 5.50% at 48 h under shaking condition. Maximum yield of ethanol 94.8 g L^{-1} was produced by immobilized cells using the reducing sugar concentration 5.50% at 48 h. Ethanol production was higher by immobilized cells in shaking conditions than free cells with same culture conditions. Influence of boron, chromium, copper and magnesium was investigated on ethanol production. Only chromium was found to show slight stimulatory effect on ethanol production (Fakhrudin, 2012).

2.11 Effect of Ethanol Accumulation on the Growth of Yeast

The production of ethanol was generally limited to 12-13% (v/v) because of it strong inhibition on growth and ethanol production. The toxicity of ethanol on growth has been reported in many studies, both in batch and continuous processes (Medawar *et al*., 2003). Ethanol affects the solubility of the cell membrane, in which the membrane's permeability is increased by the presence of ethanol. This causes excessive transport of inert and cell components in and out of cell, subsequently altering the cell constituents

that are often fatal to the cell. The rapid accumulation of ethanol due to high cell loading can increase cell death (Wan Salwanis, 2013).

In a study on the effect of inhibition by ethanol (by increasing the initial ethanol concentration in the medium from 0 gL^{-1} to 85.9 gL^{-1}), not only it prolonged the lag phase but also reduced the final cell concentration (Medewar *et al*., 2003). No sign of growth was detected at 91 gL^{-1} of ethanol, suggesting that it was the critical ethanol concentration that inhibits the growth of *Brettanomyces intermedius* in the shake flask culture. Hilge-Rotmann and Rehm (1991) reported that no sign of ethanol inhibition was observed below an ethanol concentration of 100 gL^{-1} for *S. cerevisiae* that immobilized in calcium alginate beads.

2.12 Previous Work on Ethanol Production within Free and Immobilized Cells

Based on the previous researches, one of the strategies to reduce production costs and to make ethanol fuel economically competitive with fossil fuels could be the use of wild yeast with osmotolerance, ethanol resistance and low nutritional requirements. Therefore, kinetic study of ethanol fermentation using *Saccharomyces cerevisiae* yeast strain in a batch system has been intensively investigated to determine different glucose and ethanol concentrations, pH values and temperature in order to obtain the optimum fermentation conditions. From the results obtained, it can be stated that this type of strain showed osmotolerance (its specific growth rate, μ_{max}) remained unchanged at glucose concentrations between 100 and 200 gL^{-1} as well as ethanol resistance (it was able to grow at 10% v/v ethanol). The optimal conditions for ethanol production were pH 3.5, 30°C and initial glucose concentration 150 gL^{-1}. In this case, maximum ethanol concentrations of 58.4 gL^{-1}, ethanol productivity of 1.8 $gL^{-1}h^{-1}$ and ethanol yield of 0.41 gg^{-1} were obtained (Benigno *et al.*, 2010).

In addition, there was a study on the ethanol production by free and immobilized *Saccharomyces cerevisiae* GC-IIB31 under stationary culture. Cane molasses in different concentration was used as sugar source for maximum conversion of reducing sugar into ethanol. The substrate was optimized after maintaining different levels of sugar concentrations (12-21%), medium pH (4.0-5.5), incubation temperatures (25-30°C), volume of fermentation medium (200-350 mL) and reuse of immobilized yeast

cells. Immobilized yeast cells gave significant results up to four consecutive batches. Rate of ethanol production was maximal with the free cells. The results indicated that 2 g vegetative cells of yeast on utilizing molasses at 15% sugar level with medium pH 4.5 at 30°C and 300 mL fermentation volume in 500 mL Erlenmeyer flasks gave maximum ethanol production with both free and immobilized yeast cells. Maximum ethanol production by immobilized yeast cells was obtained in the 4th batch after which it declined markedly (Mariami *et al.,* 2009).

Besides, a research was conducted to investigate the immobilization of *Saccharomyces cerevisiae* for bioethanol production from corn meal hydrolysates has been performed. For this purpose the biocompatible polymers such as polyvinyl alcohol (PVA) and Ca-alginate were assessed. The parameters of ethanol fermentation, such as inoculum concentration in different carriers and the choice of a convenient carrier for the efficient ethanol production were studied. The maximum ethanol concentration of 10.05% (w/w) was obtained in the fermentation of corn meal hydrolysates by 5% (v/v) of inoculum concentration of the yeast immobilized in Ca-alginate using a method of electrostatic droplet generation. The repeated batch fermentation with the yeast immobilized in Ca-alginate indicated that alginate gels degraded after the second fermentation cycle. PVA carrier exhibited better mechanical properties and stability; however lower ethanol concentrations were achieved during the fermentation.

Apart from that, the effect of different initial reducing sugar concentration on ethanol production in batch fermentation of Chuntian No. 2 sweet sorghum cultivar stalk juice by immobilized *Saccharomyces cerevisiae* CICC 1308 was also previously investigated. With the increase of initial sugar concentration, a significant inhibition on the maximum specific growth rate was clearly observed; however, there was no significant influence on the maximum specific ethanol production rate. Hinshelwood model was successfully applied to describe the effects of different initial reducing sugar concentration on the kinetic parameters of ethanol fermentation by immobilized *Saccharomyces cerevisiae* CICC 1308, using sweet sorghum stalk juice as the substrate. The kinetic parameters of fermentation process were obtained. The experimental results showed that Hinshelwood model could describe very well when related to the dynamics of ethanol fermentation with immobilized *Saccharomyces cerevisiae* growing on sweet sorghum stalk juice containing initial reducing sugar concentration of 85-156 gL^{-1} (Hui *et al.,* 2011).

2.13 Analysis Method for Ethanol Production

Generally, an effective ethanol production was been measured by the increasing of its concentration in the medium relatively to the fermentation duration and its controlled parameters. Most commonly method that was used to identify metabolites of ethanol is by using Gas Chromatography (GC). An ethanol assay was conducted by the injection of supernatant into a gas chromatography (Varians 450, California). The sample was filtered through a filter disc prior to the injection. The filtrate was injected into a column coupled with a flame ionization detector (FID). The column temperature was initially maintained at 120°C; 2 minutes later, the oven temperature was increased at a rate of 10°C/minute until it reached 180°C while the FID and injection port temperatures were both kept at 200°C. Nitrogen was used as the carrier gas and the combustion gas were the mixture of hydrogen and air (Wan Salwanis, 2013). The concentrations of ethanol in the samples were determined based on a standard calibration curve from pure ethanol dilution in range from 2 to 10 gL^{-1} concentration. Isopropyl alcohol was used as the internal standard at 10 gL^{-1}. Prior to analyze, the samples were separated by centrifugation at 5000 rpm for 5 minutes and the supernatants were filtered. Ethanol will be identified and quantified by comparison of retention times and peak areas with authentic samples (Ethanol 99%, Sigma Aldrich, Malaysia).

Previous studies have stated that gas chromatography is the best equipment/method to ensure the precision and accuracy of the result obtained. A method employing gas chromatography for the determination of ethanol in beer has been collaboratively tested by the Analysis Committee of the Institute of Brewing. It was judged that precision values were independent of concentration over the range 0.93 to 6.05% (v/v) ethanol.

Besides, other research has also been conducted to prove GC as the best method employed to get a better data collected. A gas chromatography with flame ionization detection method (GC–FID) with direct injection, using a capillary column, was validated to determine ethanol, acetaldehyde, methanol, and acetone in different human matrices, such as whole blood, vitreous humour, and urine, with clinical and forensic interest. A good peak resolution was achieved, with linear correlation between concentration and peak areas for all the compounds in all the matrices. The inter- and

intra-day precisions of the method were always under 15% and 10%, respectively. The accuracy of the method, calculated as the percentage of the target concentration, was within the acceptable limits. This method is easy to perform, making it suitable for the routine of clinical biochemistry and forensic laboratories (Tangerman, 1997).

Apart from that, samples taken were also analyzed for determination of glucose concentration through DNS method, colony forming unit (CFU), biomass concentration, optical density at 600 nm using UV-Vis Spectrophotometer and viewing of *S. cerevisiae* attachment on immobilized supports by Scanning Electron Microscope (SEM). The relationship between each analysis was thoroughly studied to determine which supports capable to give maximum ethanol production during fermentation process.

2.14 Summary

This subtopic was described the overview of ethanol production, characteristic of *S. cerevisiae*, immobilization technique, growth profile and analysis methods that were carried out for ethanol production. The four-phase pattern of growth profile and why it needs to be performed was explained as above. A growth profile for bacteria is crucial to be studied first before conduct any laboratory works to ensure the success of the research and to get the exact duration of time to achieve exponential phase since the bacteria at this phase will be employed in fermentation process due to the highest cells produced at that time. The previous works by researchers on ethanol production have been intensively studied before this research was conducted.

3 MATERIALS AND METHODS

3.1 Overview

This fermentation was performed to study the effect of immobilized yeast (*Saccharomyces cerevisiae*) on the production of ethanol. The main raw materials for the fermentation are the media preparation consists of agar medium, growth medium and production medium for *S. cerevisiae,* Phosphate Buffer Saline (PBS) solution and saline solution was also been prepared for the cell washing procedure to remove the cell debris in the seed culture. All medium and apparatus used were been autoclaved first before they can be used in fermentation process so that factor of contamination can be avoided. This section also presents the experimental setup for the fermentation along with the setup of analysis that was performed including culture preparation through the germination of stock culture and inoculum, immobilization procedure through shake flask seeding technique and analysis of data using GC, SEM and DNS method.

3.2 Introduction

In this chapter, the usage of chemicals, detail explanation regarding the procedure and methods employed, the types and availability of immobilized support were clearly stated. In addition, the experimental setup for the research was illustrated through Figure 3-1.

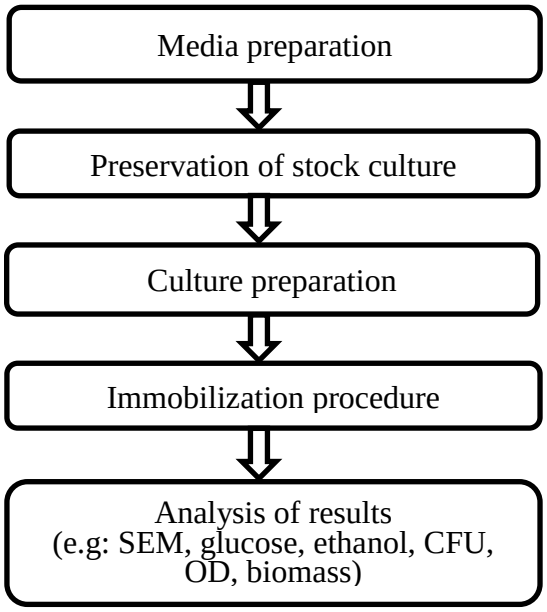


Figure 3-1: Flow chart process of experiment

3.3 Materials

3.3.1 Chemicals

Glucose, bactopeptone, yeast extract, nutrient agar, K_2HPO_4, KH_2PO_4, KCl, $MgSO_4$, NaCl, H_2SO_4, NaOH, $KNaC_4H_4O_6 \cdot 4H_2O$, 3,5-dinitrosalicylic acid, isopropyl alcohol, glycerol and glutaraldehyde were obtained from FKKSA Laboratory, UMP that were purchased from Sigma-Aldrich, Malaysia and were of biological and chemical grade.

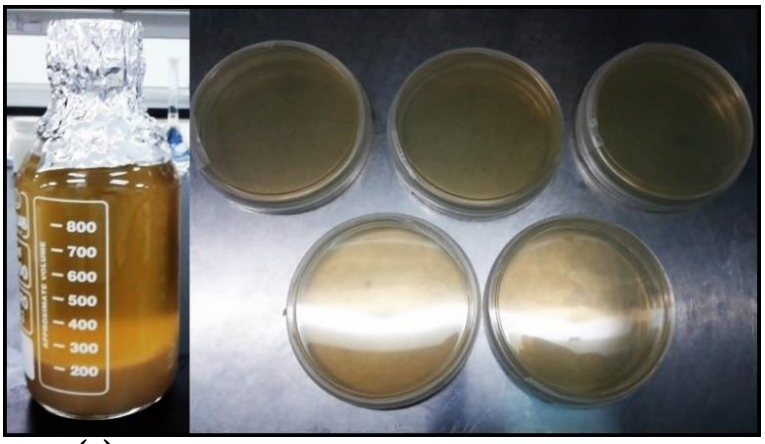
3.3.2 Immobilized Cells and Supports

S. cerevisiae was obtained from the commercial Baker yeast available in the market. Immobilized supports such as muslin cloth and membrane were available in FKKSA Laboratory, UMP that were purchased from Sigma-Aldrich Malaysia and were of chemical grade. Meanwhile, sugarcane stalk was retrieved from natural resources.

3.4 Media preparation

3.4.1 Agar Medium for S. cerevisiae

The preparation of nutrient agar and agar plates was shown as in Figure 3-2. 2.3% (w/v) agar powder was added to the growth medium which contained 2% (w/v) glucose, 2% (w/v) bactopeptone and 1% (w/v) yeast extract, and adjusted to pH 5.5 using 0.1 M H_2SO_4 and 0.1 M NaOH solution. The mixture was stirred vigorously on a heating mantle to dissolve all the components. The glucose was autoclaved separately from the other components and added to the medium after the temperature had reduced to 50-60°C. The solution was inverted several times to mix it and were left to solidify in the petri dishes at room temperature and stored at 4°C until further use.



(a) **(b)**

Figure 3-2: Preparation of a) nutrient agar and b) agar plates.

3.4.2 Growth Medium for S. cerevisiae

The medium for growth as presented in Figure 3-3 was prepared according to the procedure described in Section 3.4.1 without the addition of agar.

(a) **(b)**

Figure 3-3: Preparation of a) growth medium for *S. cerevisiae* b) seed culture.

3.4.3 Production Media for Ethanol from S. cerevisiae (Batch Fermentation)

1 L of production medium for the batch operation of ethanol production was prepared as demonstrated in Figure 3-4. The medium consisted of 15% (w/v) glucose, 2% (w/v) peptone, and 1% (w/v) yeast extract. The pH was adjusted to 5.5 using 0.1 M H_2SO_4 and 0.1 M NaOH prior to sterilisation. The glucose was autoclaved separately at 121°C for 20 minutes from the other components and added to the medium after the temperature had reduced to 50°C.

(a) **(b)**

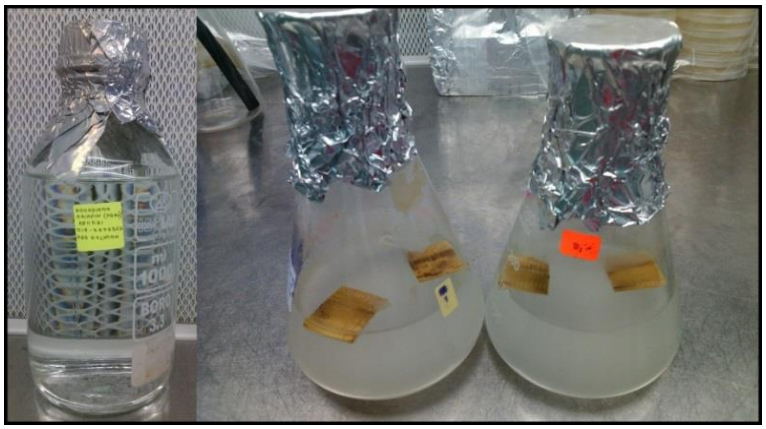
Figure 3-4: Preparation of a) media for ethanol production with 150 gL^{-1} amount of glucose b) medium containing immobilized supports.

3.4.4 Phosphate Buffer Saline (PBS) Solution

PBS solution was prepared as in Figure 3-5 and according to the composition listed in Table 3-1. The solution was autoclaved at 121°C for 20 minutes prior to use.

Table 3-1: Composition of PBS solution

Chemicals	Amount	
1M K_2HPO_4	0.802	mL
1M KH_2PO_4	0.198	mL
5M KCl	1.000	mL
0.1M $MgSO_4$	1.000	mL
Distilled water	97.000	mL
NaCl	0.850	g

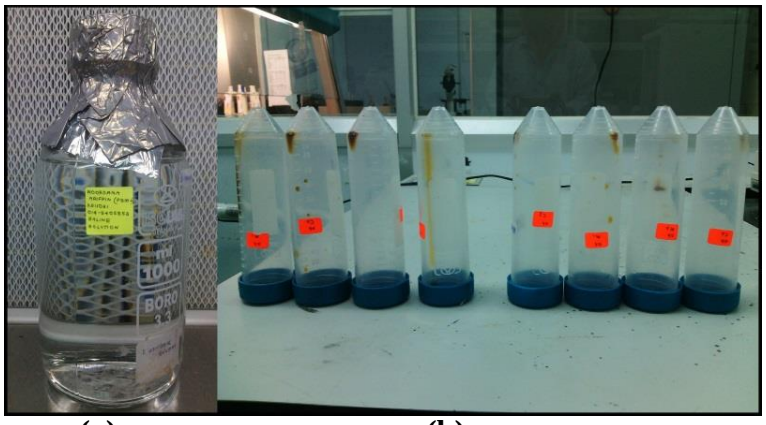


(a) **(b)**

Figure 3-5: Preparation of a) PBS solution for b) bacterial suspension medium.

3.4.5 Saline Solution

Saline solution was prepared as shown in Figure 3-6. 0.85% (w/v) NaCl was dissolved in a 1 L of distilled water. Then, the solution was autoclaved at 121^{0}C for 20 minutes for further use.



(a) **(b)**

Figure 3-6: Preparation of a) saline solution for cell washing and b) removal of cell debris.

3.5 Preservation of Stock Culture

Cells were cultured in the medium broth for 18 hours and were streaked later on agar plate for 24 hours. Single colony of *S. cerevisiae* was streaked from cultured plate and was put into the medium broth again for 18 hours to ensure that only a single colony of bacteria was retrieved for stock cultures. Figure 3-7 showed that for long term

preservation, the microorganism was kept in the 20% (v/v) glycerol stock and stored in -80°C in the freezer for further subsequent microbial works.

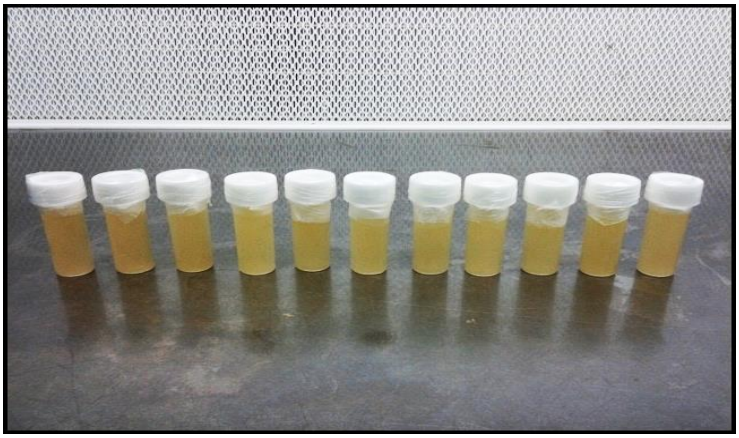


Figure 3-7: Preparation and preservation of stock culture in sterilized glycerol solution.

3.6 Culture Preparation

3.7.1 Germination of Stock Culture and Inoculum

A loopful of refrigerated stock culture was transferred onto a petri dish containing medium agar as illustrated in Figure 3-8 and incubated at 30^{o}C for 24 hours. A colony of germinated yeast cells was transferred to a 250 mL shake flask containing 100 mL of growth medium (without agar), then placed in an incubator shaker at 30^{o}C and 200 rpm for 18 hours. The cells were then centrifuged at 5000 rpm for 5 minutes, washed once with 0.85% (w/v) NaCl, and re-centrifuged for 3 minutes (Jamai *et al*., 2001). The supernatant was discarded and the pellet was suspended in phosphate buffer solution (PBS) by vortexing to form bacterial suspension solution. The total cell concentration was adjusted to an absorbance of approximately ~1.0 at 600 nm using a spectrophotometer and it would give approximately ~2 x 10^{7}/mL of viable cells.

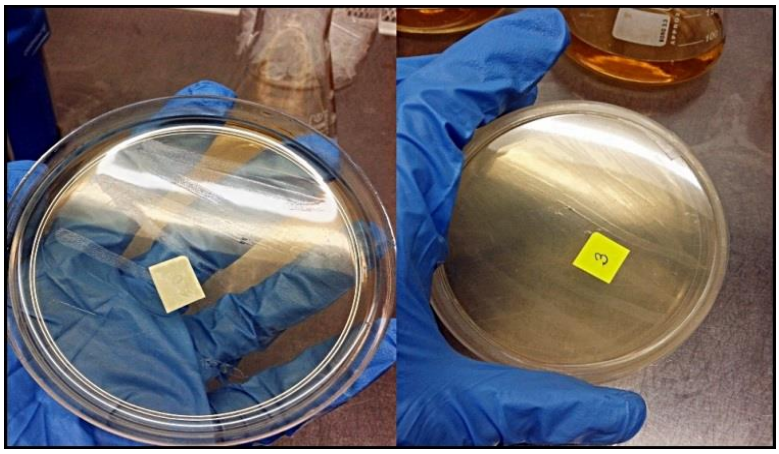


Figure 3-8: Germination of yeast cells on agar medium from stock culture

3.7 Immobilization Procedure

3.7.1 Shake Flask Seeding Technique

10% (v/v) of the yeast inoculum was inoculated into the production media of ethanol with the 15% (w/v) glucose, giving a final volume of 100 mL as shown in Figure 3-9 (a). The sterilised immobilized materials that have been immersed in the bacterial suspension solution for 24 hours of incubation were taken out and washed once with saline solution to remove unbounded cells and cell debris. Then, they were transferred into the 250 mL shake flask as expressed in Figure 3-9 (b) containing 100 mL of production media for ethanol, and then were incubated in the incubator shaker at 30^{o}C and 200 rpm for 10 hours.

(a)

(b)

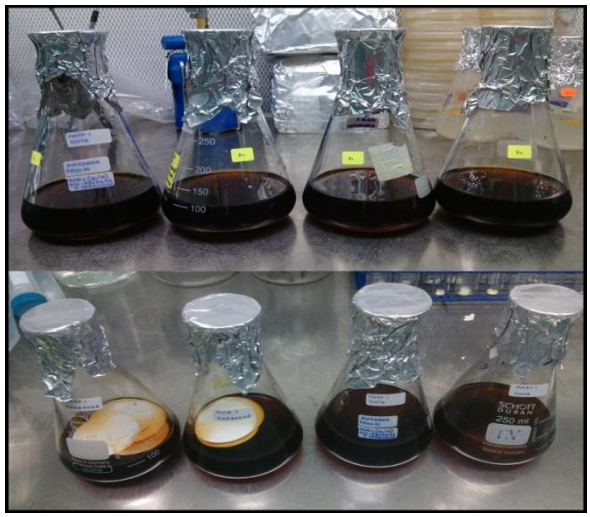


Figure 3-9: Preparation of production media for a) batch fermentation and b) immobilized fermentation

3.8 Analysis of data

3.8.1 Cells Optical Density, Biomass and Colony Forming Unit (CFU)

Cells optical density was observed from the samples using UV-Vis Spectrophotometer. Biomass concentration was determined by centrifuging 1 mL of cell suspension at 5000 rpm for 15 minutes. The supernatant was decanted off, washed once using 1 mL of 0.85% (w/v) NaCl and re-centrifuged for 3 minutes. The supernatant fluid was discarded and the pellet was suspended in 1 mL of distilled water, then transferred into aluminium dishes and dried for 24 hours in a 105^0C oven before weighing. The yeast cells were collected from the growth medium and were quantified using a plating method. The samples were diluted from 10^{-3} up to 10^{-5} dilution using sterilized distilled water. 100 µL of aliquots was transferred onto an agar plate and incubated at 30^0C for 24 hours. The number of colonies form on the agar surface was counted and measured as CFU/mL.

3.8.2 Dinitrosalicyclic Acid (DNS) Method

The quantification of residual sugar was conducted using the DNS method (Miller, 1959), and using glucose as a standard. The DNS method works based on the principle that 3,5–dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicyclic acid and an equivalence is established between the amino-nitrosalicylic acid produced and the sugar present. The DNS solution was prepared by dissolving 300 g of potassium sodium tartrate tetrahydrate and 16 g of sodium hydroxide into 500 mL of distilled water by heating gently. When the solution was clear, 10 g of 3,5-dinitrosalicylic acid was added slowly and was let to cool in room temperature. Then, the solution was making up to 1 L with distilled water. 3 mL of DNS solution was pipetted into test tubes containing 3 mL of sample, and then heated for exactly 10 minutes in boiling water bath (100^0C). The test tubes were then cooled under running tap water and brought to ambient temperature. 1 mL of 40% (w/v) potassium sodium tartrate was pipetted and mixed properly into each test tube. The optical density was measured at 575 nm using a UV-Vis spectrophotometer against a reagent blank. The concentration of reducing sugars was determined by using a calibration curve obtained for glucose concentrations at range 20-200 gL^{-1}. This analysis was performed triplicate to obtain precise data by its mean value.

3.8.3 Gas Chromatography (GC-FID)

An ethanol assay was conducted by using GC to determine the concentration of ethanol in samples. The 0.1 µL supernatant was injected into a gas chromatograph (Varians 450, California). The sample was filtered through a 0.20 µm filter disc prior to injection. The filtrate was injected into a column coupled with a flame ionization detector (FID). The column temperature was initially maintained at 120°C; 2 minutes later, the oven temperature was increased at a rate of 10°C/minute until it reached 180°C while the FID and injection port temperatures are both kept at 200^0C. Nitrogen was used as the carrier gas and the combustion gas was the mixture of hydrogen and air (Wan Salwanis, 2013). The concentrations of ethanol in the samples were determined based on a standard calibration curve of ethanol. Ethanol concentrations ranging from 2 to 10 gL^{-1} were used to obtain the standard curve. Isopropyl alcohol was used as the internal standard at 10 gL^{-1}.

3.8.4 Scanning Electron Microscope (SEM)

Determination of the *S. cerevisiae* size was carried out by using Scanning Electron Microscope (SEM). Observation of the attachment of yeast cells on immobilized cloth was also conducted using SEM after 10 hours of fermentation duration. The immobilized cloth sample was fixed in the well-mixed solution of 2% of glutaraldehyde and 0.1M of phosphate buffer saline (PBS) and was stored at 4°C until required. Prior to SEM analysis, the sample was dehydrated in an ethanol series; 30%, 50%, 70%, 80%, 90%, 100% (each step for 15 minutes excepting 100% ethanol treatment was for 1 hour). The dehydrated sample in 100% ethanol was critical-point dried with liquid CO_2 prior to viewing with SEM and then, the sample was kept in desiccator to remove the extra moisture.

4 PRODUCTION OF ETHANOL THROUGH IMMOBILIZED *SACCHAROMYCES CEREVISIAE*

4.1 Overview

This section presents the result of fermentative study for ethanol production by using immobilized *S. cerevisiae* through immobilized supports such as muslin clothes, sugarcane stalk and membrane. 150 gL^{-1} of glucose was used as a substrate supplied to the cells. The effect of glucose utilization with the increasing of ethanol production was intensively studied in this research.

4.2 Introduction

In this chapter, the ethanol concentration through different kinds of immobilized supports was determined. In order to determine the best type of support that will produce maximum concentration of ethanol, the effect between the utilization of glucose with the productivity of ethanol need to be investigated first. Therefore, six different analyzes were conducted during the fermentation process to study the relationship between glucose consumption on ethanol concentration. Then, the best immobilized support can be determined along with the highest concentration of ethanol produced. The determination of best producer will be based on their performance in releasing ethanol that was analyzed by Gas Chromatography (GC-FID).

4.3 Kinetic Growth Profile of S. cerevisiae

Study was conducted on the ethanol production through duplicate microbial fermentation by *Saccharomyces cerevisiae* cultured in a condition of pH 5, 200 rpm at 30^0C using a seeding flask technique. Referring to Figure 4-1, the growth profile of *S. cerevisiae* was followed the typical trend of growth curve for yeast (Asaduzzaman, 2007). It was supported with the cell optical density and biomass concentration that exhibited the same pattern of growth which comprised of four main phases: lag phase (first 2 hours), exponential phase (2 to 10 hours) and prolong the duration of fermentation, the curve will showing the stationary and death phase.

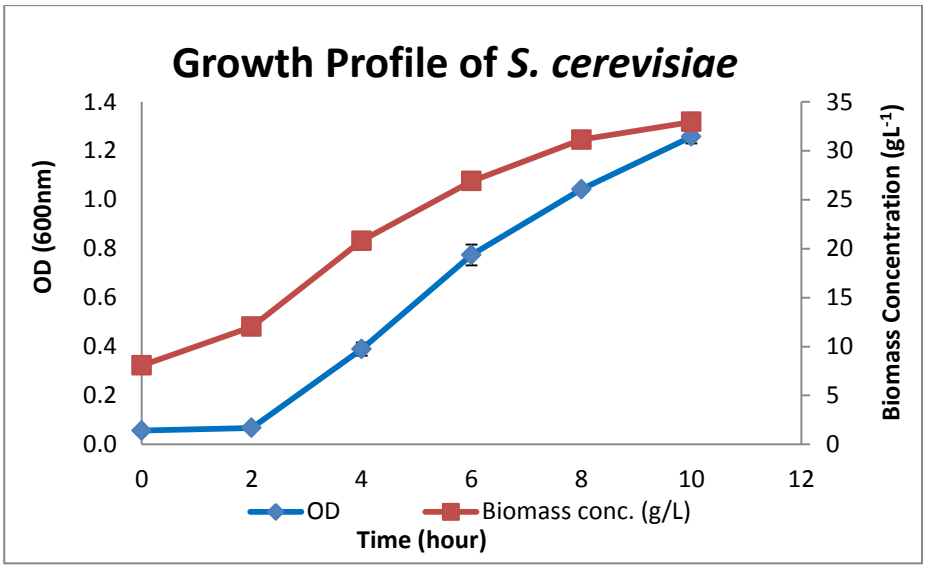


Figure 4-1: Growth profile of *S. cerevisiae* and its biomass concentration for 10 hours fermentation fixed at 30^0C and 200 rpm

The pattern of growth was demonstrated that the first 2 hours of fermentation was the adaptation period for yeast cells to the condition in the medium. Immediately after inoculation of the cells into fresh medium, the population remains temporarily unchanged. Although there was no apparent cell division occurred, the cells might be growing in volume or mass, synthesizing enzymes and proteins for the utilization of the available substrates and increasing in metabolic activity but there was no increased in the cell number. The length of the lag phase was apparently dependent on a wide variety of factors including the size of the inoculum, time necessary to recover from physical damage or shock in the transfer, time required for synthesis of essential coenzymes or division factors and time required for synthesis of new (inducible) enzymes that were necessary to metabolize the substrates present in the medium.

The gradient of curve was started to decrease at subsequent time when the exponential phase of growth has taken place. It is a pattern of balanced growth when all cells were divided regularly by binary fission and were growing by geometric progression. The constant rate of cell division occurred depending upon the composition of the growth medium and the conditions of incubation. The rate of exponential growth for yeast cells were expressed as generation time, also the doubling time of the bacterial population. Therefore, it can be said that there was a rapid exponential increased in population, which doubles regularly until a maximum number of cells were reached. Population growth was limited by one of three factors which were the exhaustion of

available nutrients, accumulation of inhibitory metabolites or end products and exhaustion of space, in this case called a lack of "biological space". However, the cells grew most rapidly and consumed more nutrients in this stage. With prolong of fermentation period exceeding 10 hours, this growth will then lead to stationary phase when depletion of nutrient slowed down the metabolism for cell division. During this stage, viable cells counted cannot be determined whether some cells are dying and an equal number of cells are dividing, or the population of cells has simply stop growing and dividing.

Lastly, if incubation continues with additional period of fermentation, a death phase follows, in which the viable cell population declines. The number of viable cells is decreasing geometrically, essentially the reverse of growth during the log phase. This observation is due to the continuing depletion of nutrients and build-up of metabolic wastes; resulting the microorganisms to die at a rapid and uniform rate. Previous study has reported that researcher took the bacteria at exponential phase to undergo fermentation since they were capable to effectively enhance the productivity of the process when compared to other phases (Wan Salwanis, 2013).

4.4 Effect of Immobilization on Cell Optical Density, Biomass Concentration and Colony Forming Unit (CFU)

Immobilization of *S. cerevisiae* through medium of muslin cloth, sugarcane stalk and membrane have been proved to succeed in increasing the biomass concentration along with the cells optical density as presented in Figure 4-2. Immobilized cells in cloth were capable to exceed over 27.3% optical density (compared to batch) which is 1.602 corresponding to the highest concentration of biomass produced at 39.6 gL^{-1}. This result was due to the factor that immobilized cells has an effective ability to eliminate inhibition caused by high concentration of substrate and product, capable to improve the metabolic activity of cells and increasing glucose uptake, thus enhancing the productivity and yield of ethanol production (Williams and Munnecke, 1981). However, sugarcane stalk and membrane exhibited less deviation relative to batch in term of optical density and biomass concentration when they showed decreasing at 34.6% and 49.9% of cells absorbance, thus lowering the biomass concentration to 30.5 gL^{-1} and 20.0 gL^{-1} respectively.

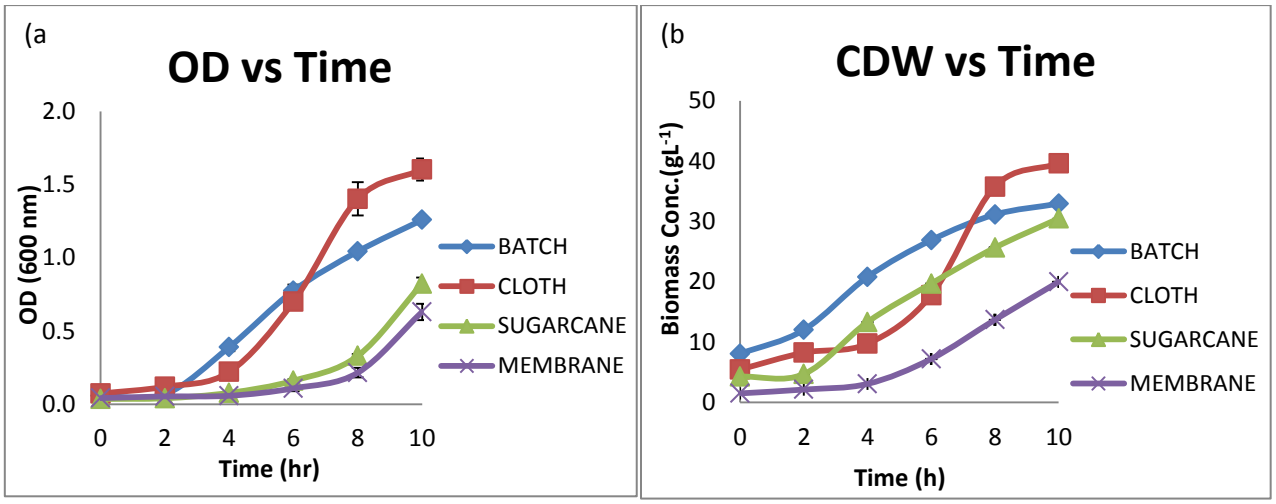


Figure 4-2:(a) Cells OD at 600 nm and (b) biomass concentration using different types of immobilized supports for 10 hours fermentation fixed at 30ºC and 200 rpm

Data tabulated in Table 4-1 observe the difference of cells in term of weight for each immobilized supports after comparing them at the beginning and the end of fermentation process. This analysis indicated the amount of cells attach on muslin cloth, sugarcane and membrane. Meanwhile, Figure 4-3 shows the observation of the three different types of immobilized supports after 10 hours of fermentation that was dried for 24 hours at 60°C. The results obtained showed that cloth was able to achieve the highest cells attachment up to 0.45g. The less increment of cells absorbance and concentration possessed by sugarcane stalk and membrane over batch operation was might due to several factors. These probably cause by the factors of the hardened surface, coarse roughness and stalk thickness of sugarcane that makes the cells difficult to attach on the immobilized support. Besides, the nature of membrane materials such as its pore size distribution, dimension, porosity, thickness or hydrophobic and hydrophilic of the membrane might contribute to the deviation as mentioned above.

Table 4-1: Data on the attachment of cells on immobilized supports after 10 hours of fermentation fixed at 30^{0}C and 200 rpm

Experiments	Weight of cells (g)		
	CLOTH	SUGARCANE	MEMBRANE
Initial weight of immobilized supports	0.9999	1.1294	1.0085
Final weight of immobilized supports	1.4474	1.3679	1.0553
Difference of cells attach on immobilized supports	0.4475	0.2385	0.0468

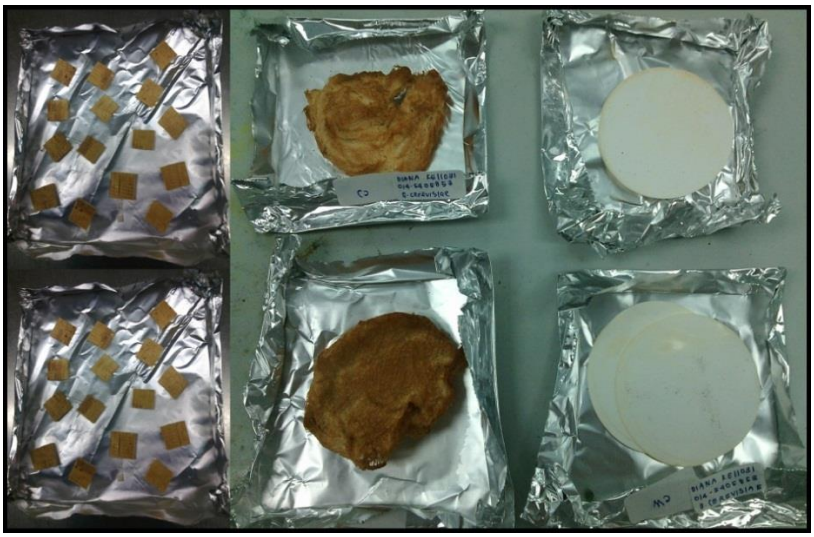


Figure 4-3: Observation on immobilized supports after 10 hours fermentation. (Process conditions: pH 5.0; 30 ºC; 200 rpm; initial glucose concentration 150 gL^{-1})

Graph shown in Figure 4-4 express the immobilized fermentation exhibit a positive trend of growth curve. The immobilized muslin cloth has an ability to increase 17.9% of the colonies forming over batch fermentation and gave approximately up to 2.57 x 10^8/mL of viable cells. However, immobilized cells in sugarcane stalk and membrane were unable to surpass the number of colonies in batch operation, thus indicate a slow growth pattern as observed in the fgure below.

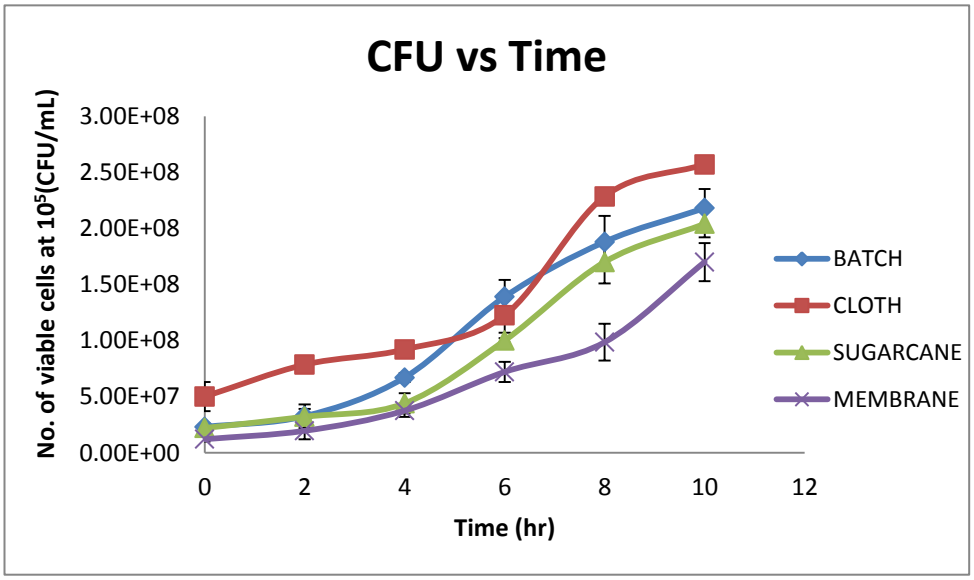


Figure 4-4: The number of immobilized cells during 10 hours of fermentation by *S. cerevisiae* with various types of immobilized supports. (Process conditions: pH 5.0; 30ºC; 200 rpm; initial glucose concentration 150 gL^{-1})

Sugarcane normally has been used as a feedstock in the production of ethanol. Although it contain small amounts of sugar extracted from the carrier itself that can make it possible to surpass the batch operation, the results presented in Figure 4-4 has proved that colonies forming unit of yeast cells was not able to surpass batch operation but the viable cells was closed to the amount of cells in batch process. This observation might due to the hardness of sugarcane stalk that does not undergo any acid or alkaline pre-treatments to give out the sugars content, therefore apart from the difficulties of cells attachment on it, the lack of sugar content was one of the factor contribute to the small amount of viable cell over batch process.

This situation was consistent with a research by Jianliang *et al. (*2010) regarding immobilized cells applied through bagasse carrier which is derived from natural resources of sweet sorghum stalk that is the same as sugarcane stalk which also a type of natural resources. Bagasse is found to have a large amount of stalk cells which are vacuous and porous. Yeast cells can penetrate into the porous stalk cells and be embedded. Meanwhile, electrostatic interactions between cells and carrier surfaces enable the immobilization process to occur (Kolot, 1981). However, in order to boost the attachment of yeast cells, the surface of bagasse carrier can be modified by cellulose hydrolysis, which can cut the cellulose chain into small pieces and make the surface much coarser. In other words, hydroxyls and phenolic sites would form as cellulose was decomposed into oligosaccharides, which increased the level of surface charge. Therefore, the immobilized cell concentrations were proved to increase from 0.87 gg^{-1} to 1.35 gg^{-1} of dry cell weight as the hydrolysis time increased.

Apart from the carrier surface factor, the sizes of carriers can affect the specific ethanol production rate. Therefore, all the immobilized supports used in this study were fixed at 1cm x 1cm x 1cm. The previous paper indicated that the larger carrier could obtain higher concentration of immobilized cells (Yu *et al.,* 2007). However, as the size increases, the mass transfer at the interior of the carrier may become more difficult, and influence the fermentation productivity. Jianliang *et al. (*2010) have reported that as the size of carrier increased, the ethanol productivity will decrease. Though the carrier bagasse with the biggest size had the highest concentration of immobilized cells, the specific productivity rate of ethanol was the lowest. It was thought to be due to the hindrance of substrate diffusion to the immobilized cells and of metabolites leaving the

carriers. Based on the higher concentration of immobilized cells in larger carriers, it is supposed that larger carriers have the potential to get higher productivity. So, modifying the carriers in order to increase the mass transfer ability is necessary. The pretreatment for the bagasse can be a solution to overcome this problem.

4.5 Glucose Utilization and Ethanol Production by Immobilized S. cerevisiae

Batch fermentations in shake flasks for ethanol production were carried out in duplicate with initial reducing sugar concentrations of 150 gL^{-1} and controlled at constant temperature of 30°C. Figure 4-5 demonstrate the profile of ethanol and glucose concentration which relative to duration of fermentation in different types of immobilized supports. It was expected that the ethanol concentration would increase and gradually reach a steady state value. The steady state value was not well defined due to the fluctuating ethanol concentration but the expected trend was achieved. The result presented in graph below suggested that immobilized cells were consumed the available sugar during the 10 hours of fermentation in order to produce ethanol. The variations of glucose utilization with time were illustrated by the depletion of glucose concentration. This trend was observed across all immobilization media and in the batch process. For the continuous runs, there are instances where glucose concentration was fluctuated. This scenario could be the consequence of variation in the fermentation broth flow rate and the interference of an invading microorganism thus decreasing the activity of *S. cerevisiae* or a combination of both (Charlimagne *et al.*, 2012).

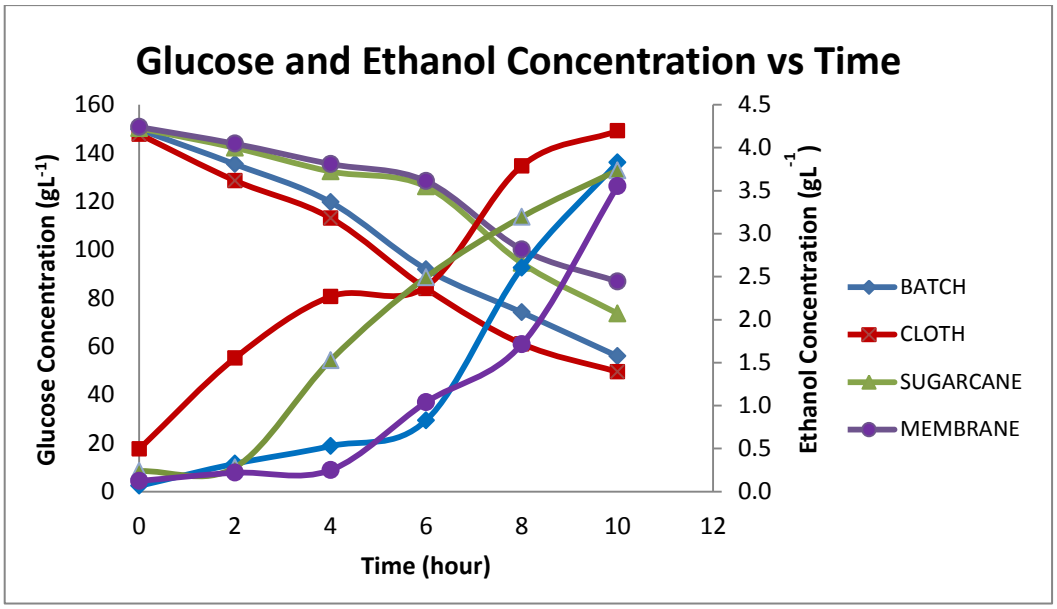


Figure 4-5: Profile of glucose and ethanol concentration by *S. cerevisiae* immobilized in various supports for 10 hours fixed at 30^0C and 200 rpm

When the initial reducing sugar concentrations was 150 gL^{-1}, the ethanol concentrations of 4.20 gL^{-1}, 3.74 gL^{-1} and 3.55 gL^{-1} were obtained by the end of fermentation with immobilized *S. cerevisiae* in muslin cloth, sugarcane stalk and membrane. It was indicated that the ethanol concentrations increased with the consumption of reducing sugar which was consistent with the report by Gulnur Birol *et al.* (1998). Immobilized cloth was proved in having a capability to produce the maximum ethanol concentration by the increment of its ethanol productivity equivalence to of 9.62% respectively over batch. In fact, during 10 hours of fermentation, glucose uptake was rapidly increased from 1.5% to 67.0% when compared to other immobilized supports. An increase in the ethanol concentration, especially in the immobilized sugarcane stalk was probably caused by the fermentation of small amounts of sugar extracted from the carrier that make it possible to surpass the batch operation.

Referring to Figure 4-5, an accumulation of ethanol started when cells entered the exponential phase, after being in the lag phase for two hours. The ethanol continued to accumulate until 10 hours of fermentation, giving the highest concentration of 4.195 gL^{-1} and overall productivity of 0.42 $gL^{-1}h^{-1}$ that was observed in the immobilized muslin cloth which act as the best immobilized support for *S. cerevisiae*. According to the previous study, with the prolong of fermentation duration, the curve plotted will

shows that the ethanol concentration will drop rapidly after achieving the maximum concentration, and gradually reduced until it was exhausted at certain period of fermentation. The reduction was due to the assimilation of the ethanol as a consequence of the glucose depletion.

The production pattern of ethanol follows the growth associated product, in which the production speeds during the exponential growth. Wan Salwanis (2013) reported that by prolonging the cultivation time resulted in decreased ethanol productivity, which was due to the exhaustion of glucose in the culture. The *S. cerevisiae* altered its metabolic pathway from glucose catabolism to ethanol assimilation and consumed ethanol to maintain its cellular activity. At this stage, the number of viable cells reduced from 10^8 to 10^7 CFU/ml as a result of the deprived condition with poor nutritional values.

Yeast metabolism is rather complex, and varies significantly between strains. This study indicated that the assimilation of ethanol as an alternative carbon source only occurred after the exhaustion of glucose, but, occasionally, ethanol can be metabolized simultaneously with glucose in aerobic conditions when the sugar flux uptake in the cell is much lower than the respiratory capacity (Ramon-Portugal *et al.*, 2004). This happened when the energy requirements for cell maintenance exceeded the energy generation from the breakdown of glucose. As a result, ethanol was used as a substitute for glucose and was assimilated by the yeast in order to gain more energy for cell regulation.

4.6 Improvement of Yield against Batch Fermentation

The relationship between the immobilization medium and yield factor of ethanol production which respect to biomass, glucose and ethanol concentration were investigated. These factors were independently analysed and the significance of the immobilization media is determined at three different types of immobilization medium as mentioned before. The yield of production for each immobilized support was then recorded as shown in Table 4-2. Immobilization with cloth generally resulted in approximately 35.6% higher of $Y_{X/S}$, but showing a slightly decreasing of $Y_{P/X}$ at 8.3% which respect to the batch operation. However, $Y_{P/S}$ has shown that immobilized cloth

gave the highest attainable percentage yield calculated at 0.08, thus improving the productivity of ethanol.

Apart from that, as tabulated in Table 4-2 that referring to the membrane support, it is actually boost the production of ethanol, where more glucose was channelled for the ethanol production rather than the biomass. This scenario might due to the factor of the membrane that having high porosity surface as yeast cells carrier, which facilitates the transmission of substrates and products between carrier and medium. Still it is necessary to make more detailed studies to clarify the mechanism of *S. cerevisiae* cells attachment and deattachment from the support particle. Meanwhile when compared to batch operation, immobilized membrane support was capable to give the yield of product form over biomass produced, $Y_{P/X}$ at 33.3% higher compared to batch production. It is also proved in increasing the yield of product form over substrate consumed, $Y_{\Delta P/\Delta S}$ that respect to the beginning until the end of fermentation process which is at 0.05 and it is consistent with sugarcane stalk support.

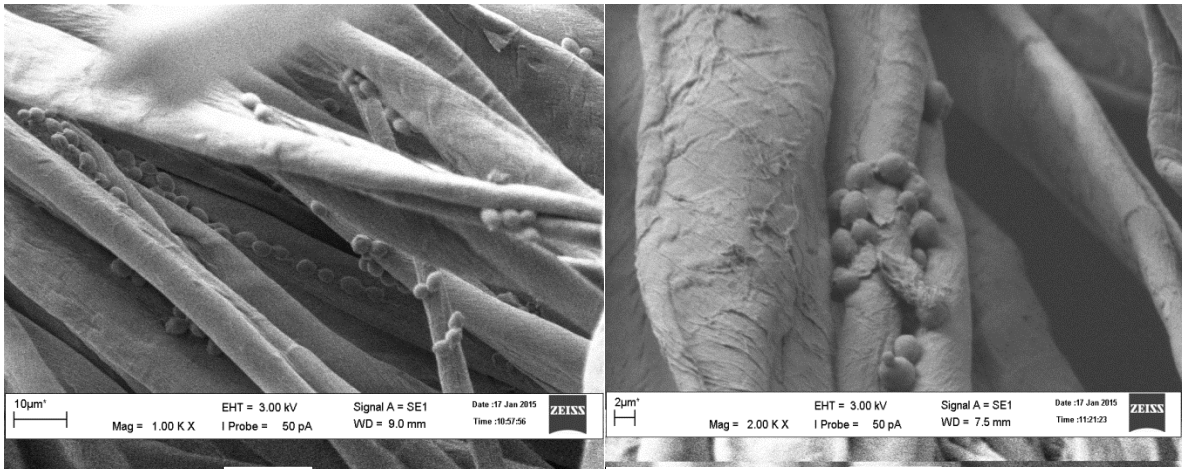
Table 4-2: Data on the yield of production depending on different immobilized supports after 10 hours of fermentation fixed at 30^0C and 200 rpm

Types of immobilized supports	**Yield of production after 10 hours of fermentation**					
	$Y_{X/S}$	**$Y_{\Delta X/\Delta S}$**	**$Y_{P/X}$**	**$Y_{\Delta P/\Delta X}$**	**$Y_{P/S}$**	**$Y_{\Delta P/\Delta S}$**
Batch	0.59	0.27	0.12	0.15	0.07	0.04
Cloth	0.80	0.35	0.11	0.11	0.08	0.04
Sugarcane	0.41	0.34	0.12	0.13	0.05	0.05
Membrane	0.23	0.29	0.18	0.19	0.04	0.05

4.7 Morphology of Immobilized Cells within Muslin Cloth

Cell immobilisation was presented by optical micrographs using Scanning Electron Microscope (SEM) as presented in Figure 4-6. The investigations were carried out to elucidate the mode of adsorption of cells on the solid matrix. Referring to the Figure 4-6 (a) has shown that yeast cells were densely and homogenously adhered onto the surface of the carrier, as a result of entrapment into the muslin cloth thread and physical adsorption by electrostatic forces or covalent binding between yeast cell membrane and the carrier as illustrated in Figure 4-6 (b). As it is demonstrated by the uniform cell growth onto the surface of muslin cloth, the cells immobilisation was

effective, suggesting possible recycling of cells in repeated batch runs, also taking into account that cells grow even after 10 hours of fermentation.



(a) **(b)**

Figure 4-6: Optical microphotograph of *Saccharomyces cerevisiae* cells at a) magnification of 1000x b) magnification of 2000x that immobilised into muslin cloth after 10 hours of fermentation.

4.8 Summary

Efficient ethanol production requires a rapid fermentation leading to high ethanol concentrations; therefore a yeast strain must have a good specific growth rate and good specific ethanol production rate at high osmotic activities and ethanol concentration. Therefore, *Saccharomyces cerevisiae* is considered to be the best organism due to its high yield and robustness. However, the main study was focussed on the types of immobilized supports that capable to give a good housing for cells attachment on it. Enhanced growth of cells was observed with the muslin cloth, but the productivity of ethanol increased with increased fermentation for all four types of supports. But, muslin cloth had showed the highest ethanol producing within 10 hours of fermentation.

5 CONCLUSION

5.1 Conclusion

This project focuses on the biological attempt for the production of ethanol through fermentation process using immobilized *S. cerevisiae* with different types of supports (e.g: muslin cloth, sugarcane stalk and membrane). Experiments carried out with immobilized cells proved that both growth and ethanol production were occurring simultaneously. The effect of hydrophilicity on the cell adhesion and cell growth cannot be defined based on this study, as cell-surface interactions were mediated by many other factors as discussed previously. However, an increased hydrophilicity contributed to an enhanced wetness of the muslin cloth, thus providing a better supply of medium to the immobilized cells compared to the other types of supports. Therefore, this study can be concluded that the production of ethanol increases with the depletion of glucose concentration throughout fermentation duration. Yield of production calculated depending on the different supports proved that the immobilization medium significantly affects the performance of a batch fermentation process. Analysis conducted shown, the best support that is effectively enhanced the ethanol productivity in terms of yield and its concentration through immobilization method was muslin cloth. The rapid utilization of reducing sugar over batch operation in cloth medium was encouraging the increment of yield and productivity of ethanol.

5.2 Recommendation

Muslin cloth is a promising immobilization medium as its performance was statistically enhanced the fermentation process due to the highest cells attachment on it. The results demonstrated that the muslin cloth could be an interesting support for cell immobilisation, with possibility of application for improving ethanol productivities. The prepared immobilised cloth showed higher fermentation activity compared to free cells. Moreover, from the research conducted, it was able to promote adhesion and proliferation of *S. cerevisiae* within its cavity, subsequently producing stable ethanol concentration. The advantage of muslin cloth in terms of material strength and reusability should also be credited. The economic advantage of cloth may also be considered as reducing the cost of immobilized fermentation. Therefore, it is recommended to be used by other researchers in the future work.

6 REFERENCES

Angelova, M.B., Pashova, S.B. and Slokoska, L.S. (2000). Comparison of antioxidant enzyme biosynthesis by free and immobilized Aspergillus niger cells. *Enzyme Microb Tech*. 26(7):544-549.

Anxo, M.M., Lorenzo, P., Jose, A.V., Jesus, M., and Maria, P.G. (2008). Alcoholic chestnut fermentation in mixed culture. Compatibility criteria between *Aspergillus oryzae* and *Saccharomyces cerevisiae* strains. *Bioresourc. Technol*., 99: 7255-7263.

Aristidou A. and Penttila M. (2000). Metabolic engineering applications to renewable resource utilization. *Curr. Opin. Biotech.,* 11:187–198.

Assaduzzaman, M. (2007). Standardization of yeast growth curves from several curves with different initial sizes. Chalmers University of Technology and Goteborg University. 2-5.

Bai, F.W., Anderson, W.A., and Moo-Young, M. (2008). Ethanol fermentation technologies from sugar and starch feedstocks. *Biotech. Adv.,* (26) 89–105: 91.

Balat, M., Balat, H. and Oz, C. (2008). Progress in bioethanol processing and progress in energy and combustion science. 34:551-573.

Baptista, C.M.S.G., Coias, J.M.A., Oliveira, A.C.M., Oliveira, N.M.C., Rocha, J.M.S., Dempsey, M.J., Lannigan, K.C. and Benson, B.S. (2006). Natural immobilization of microorganisms for continuous ethanol production. *Enzyme Microb. Technol.,* 40: 127-131.

Behera, S., Mohanty, R.Ch, Ray, R.Ch. (2011). Ethanol production from mahula (*Madhuca latifolia* L.) flowers with immobilized cells of *Saccharomyces cerevisiae* in *Luffa cylindrica* L. sponge discs. *App. Energy*. 88:212–215.

Bellissimi, E. and Ingledew W.M. (2005). Metabolic acclimatization: preparing active dry yeast for fuel ethanol production. *Pro. Biochem.,* 40:2205–2213.

Birol, G., Doruker, P., Kirdar, B., Onsan, Z.I. and Ulgen, K. (1998). Mathematical description of ethanol fermentation by immobilized *Saccharomyces cerevisiae*, *Pro. Biochem.,* 33, pp.763–771.

Brányik, T., Vicente, A.A., Dostalek, P. and Teixeira J.A. (2008). A review of flavour formation in continuous beer fermentations. *J Inst Brew*. 114:3–13.

Brodelius, P. and Vandamme, E.J. Immobilized cell systems. In: Rehm HJ, Reed G,editors. (1987). Biotechnology. 2nd ed., 7a. Weinheim: Verlag Chemie; p. 405–64.

Buckee, G.K and Mundy, A.P. (1993). Determination of ethanol in beer by Gas Chromatography (Direct Injection). 99, pp. 381-384.

Demirbas, M.F. (2006). Global renewable energy resources. *Energy Sources*. 28: 779-792.

Dien, B.S., Cotta, M.A. and Jeffries, T.W. (2003). Bacteria engineered for fuel ethanol production: Current Status. *App. Microb. Biotech.,* 63: 258-266.

Edgardo, A., Carolina, P., Manuel, R., Juanita, F. and Jaime, B. (2008). Selection of thermotolerant yeast strain *Saccharomyces cerevisiae* for bioethanol production. enzyme and microbial technology. 43:120-123.

Fakruddin, Md., Abdul Quayum, Md., Monzur, M.A and Naiyyum, C. (2012). Analysis of key factors affecting ethanol production by *Saccharomyces cerevisiae*. 11:4: 248-252.

Fortman, J.L, Chhabra, S., Mukhophadhyay, A., Chou, H., Lee, T.S., Steen, E. and Keasling, J.D. (2008). Biofuel alternatives to ethanol: Pumping the microbial well. *Trends in Biotech.,* 26 (7) 375-381.

Galazzo, J.L., Bailey, J.E. (1990). Growing Saccharomyces cerevisiae in calcium-alginate beads induces cell alterations which accelerate glucose conversion to ethanol. *Biotechnol Bioeng.* 36(4):417-26.

Gonga, G. H., Houa, Y., Zhaoa, Q., Yua, M. A., Liaob, F., Jiangc, L. and Yanga, X. L. (2010). A new approach for the immobilization of permeabilized brewer's yeast cells in a modified composite polyvinyl alcohol lens-shaped capsule containing montmorillonite and dimethyldioctadecylammonium bromide for use as a biocatalyst. *Pro. Biochem.,* 45:1445–1449. doi: 10.1016/j.procbio.2010.05.021

Greg, D. and Saddler J. (1995). Bioconversion of lignocellulosic residue to ethanol, proces flowsheet development. *Biomass Bioenergy,* 9: 287-302.

Haq, I. and S. Ali. (2007). Kinetics of improved invertase production by *Saccharomyces cerevisiae* in batch culture. *Pak. J. Bot.,* 39: 907-912.

Hilge-Rotmann, B. and Rehm, H. (1991). Relationship between fermentation capability and fatty acid compositions of free and immobilized *Saccharomyces cerevisiae*. *App. Microb. Biotech.,* 34:502-508.

Hui, J., Ronghou, L. and Yiliang, H. (2011). Kinetics of batch fermentations for ethanol production with immobilized *Saccharomyces cerevisiae* growing on sweet sorghum stalk juice.pp:1-9.

Jamai, K., Sendide, K., Ettayebi, K., Errachidi, F., Hamdouni-Alami, O., Tahri-Jouti, M.A., McDermott, T. and Ettayebi, M. (2001). Physiological difference during ethanol fermentation between calcium alginate-immobilized *Candida tropicalis* and *Saccharomyces cerevisiae*. *FEMS Microb. Lett.*, 204:375-379.

Jeffries T.W. and Jin Y.S. (2004). Metabolic engineering for improved fermentation of pentoses by yeasts. *Appl Microb. Biotech.,* 63 (5): 495–509.

Jianliang Yu, Guojun Yue, Jing Zhong, Xu Zhang, Tianwei Tan., 2010. Immobilization of *Saccharomyces cerevisiae* to modified bagasse for ethanol production. Journal Renewable Energy. 35, 1130-1134.

Junter, G.A., Coquet, L., Vilain, S. and Jouenne, T. (2002). Immobilized-cell physiology: current data and the potentialities of proteomics. *Enzyme Microb Tech.*; 3:201–212. doi: 10.1016/S0141-0229(02)00073-X

Kamm, B., Kamm, M., Schmidt, M., Hirth, T. and Schulze, M. (2006). Lignocellulose-based chemical products and product family trees. In *Biorefineries – Industrial Processes and Products*, Volume 2, edition by Kamm B, Gruber PR and Kamm M. Wiley-VCH, Weinheim Germany pp. 97–150.

Karin Kovarova, K. and Thomas, E. (1998). Growth kinetics of suspended microbial cells: From single-substrate-controlled growth to mixed-substrate kinetics. *Microb. Mol. Bio. Rev.,* 62, 646-666.

Kaur, M. and Kocher, P. (2002). Ethanol production from molasses and sugarcane juice by an adapted strain of *S. cerevisiae. Ind. J. Microbiol.,* 42: 255-257.

Kircher, M. (2006). White biotechnology: ready to partner and invest in. *Biotechnol J.* 1:787–794.

Kolot, F.B. (1981). Microbial carriers-strategy for selecction (part 2). *Process Biochem* ;16:30–3.

Kourkoutas, Y., Bekatorou, A., Banat, I.M., Marchant, R. and Koutinas, A.A. (2004). Immobilization technologies and support materials suitable in alcohol beverages production. *Food Microbiol*. 21:377–397.

Kunz, M. (2008). Bioethanol: Experiences from running plants, optimization and prospects. *Biocat. and Biotrans.,* 26(1-2): 128-132.

Lacroix, C., Sodini, I. and Corrieu, G. (1996). Progress in biotechnology 11, immobilized cells: Basics and applications, Wijffels, R.H., Buitelaar, R.M., Bucke, C., Tramper, J., eds., *Elsevier Science B.V.*, 600-608.

Leupold, U. (1993). The origin of *Schizosaccharomyces pombe* genetics. In the early days of yeast genetics, Hall, M. N. and Linder, P. eds. *New York, Cold Spring Harbor Press.*, pp. 125-131.

Li, G.Y, Huang, K.L., Jiang, Y.R. and Ding, P. (2007). Production of (R)-mandelic acid by immobilized cells of *Saccharomyces cerevisiae* on chitosan carrier. *Process Biochem*. 42:1465–1469.

Liu, Y. (2006). A simple thermodynamic approach for derivation of a general Monod equation for microbial growth. *Biochem. Eng. J., 31:102–105*.

Maiorella, B.I., Blanch, H.W. and Wilke, C.R. (1984). Biotechnology report. economic evaluation of alternative ethanol fermentation processes. *Biotechnol. Bioeng.,* 26: 1003-1025.

Mariami, I., Manzoori, K., Sikander, A. and Ikram, U.H. (2009), Enhanced production of ethanol from free and immobilized *S.cerevisiae* under stationary condition. *Pak. J. Bot.,* 41(2): 821-833, 2009**.**

Maryse, G. and Dravko, D. (1996) Wood blocks as a carrier for *Saccharomyces cerevisiae* used in the production of ethanol and fructose. *Chem. Eng. J*., 61:233–40.

Meo, M. (1984). Economic evaluation of ethanol fuel production from agricultural crops and residues in California. *Res. Conserv.* 11: 1-25.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analy. Chem.,*22: 426-428.

Montealegre Charlimagne, M., Emerson, R., Dionisio, Sumera Lawrence, V., Jay, R.T., Adolacion and Rizalinda, L. De Leon (2012). Continuous bioethanol production using *Saccharomyces cerevisiae* cells immobilized in Nata De Coco (Biocellulose), 35: 4-5.

Nigam, J.N., Gogoi, B.K. and Bezbaruah, R.L. (1998). Alcoholic fermentation by agar immobilized yeast cells. *World J. Microbial. Biotechnol.,* 14: 457-459.

Norton, S. and D'Amore, T. (1994). Physiological effects of yeast cell immobilization: applications for brewing. *Enzyme Microb. Tech*., 16:365–375.

Ortiz-Muniz, B., Carvajal-Zarrabal, O., Torrestiana-Sanchez, B. and Aguilar-Uscanga, M.G. (2010). Kinetic study on ethanol production using *Saccharomyces cerevisiae* ITV-01 yeast isolated from sugar cane molasses, 10:1361–1367.

Pajić-Lijakovic, I., Bugarski, D., Plavsic, M. and Bugarski, B. (2007) Influence of micro environmental conditions on hybridoma cell growth inside the alginate-poly-l-lysine microcapsule. *Pro. Biochem.,* 42:167–174. doi: 10.1016/ j.procbio. 07.023.

Parascandola, P., De Alteriis, E., Sentandreu, R. and Zueco, J. (1997). Immobilization and ethanol stress induce the same molecular response at the level of the cell wall in growing yeast. *Microbiol Lett.* 150(1):121-6.

Passarinho P.C.L.V., Vieira A.M.S, Cabral J.M.S, Novais J.M. and Kennedy J.F. (1989). Effect of carrier matrix on fermentative production of ethanol by surface immobilized yeast cells. *Chem Technol Biotechnol. J.,* 44:183–94.

Patil, S.G. (1991). Novel additives activate alcoholic fermentation. *Biotechnol.,* 34: 217-222.

Pimentel D, Patzek and Cecil G, (2007). Ethanol production: energy, economic, and environmental losses. pp. 189:25-4.

Plessas, S., Bekatorou, A., Koutinas, A.A., Soupioni, M., Banat, I.M, and Marchant, R. (2007). Use of Saccharomyces cerevisiae cells immobilized on orange peel as biocatalyst for alcoholic fermentation. *Bioresour Technol*. 98(4):860-5.

Prescott, S. and Dunn's, C. (1987). Production de grasa por glutinis. In: Garcia, J. and V. Palasi. *Indust. Microb.*, 4th Ed. Espanola de Aguilar, Madrid. pp. 835-859.

Qi, W., Ma, J., Yu, W., Xie, Y., Wang, W. and Ma, X. (2006). Behaviour of microbial growth and metabolism in Alginate-Chitosan-Alginate (ACA) microcapsules. *Enz. and Microb. Tech.,* 38:697-704.

Reddy, Y. H. K., Reddy, L. P. A. and Reddy, O. V. S. (2008): Wine production by yeast biocatalyst prepared by immobilization on watermelon (Citrullus vulgaris) rind pieces and characterization of volatile compounds. *Process Biochem.,* 43: 748-752.

Santos, D.T., Sarrouh, B.F., Rivaldi, J.D., Converti, A., & Silva, S.S. (2008). *Food Engineering J.,* 86: 542–548.

Shapouri, H. and Gallagher, P. (2005). USDA's 2002 ethanol cost of production survey. Report,http://www.ncga.com/ethanol/pdfs/031506 USDA Cost Of Production.pdf, United States Department of Agriculture, USA.

Shapouri, H., Gallagher, P. and Graboski, M.S. (2002). USDA's ethanol cost of production survey. Report, http://www.usda.gov/oce/ reports/energy/aer-808.pdf, United States Department of Agriculture, USA.

Shapouri, H., Salassi, M. and Fairbanks J.N. (2006). The economic feasibility of ethanol production from sugar in the United States. Report, http://www.usda.gov/oce/Ethanol Sugar Feasibility-Report3.pdf, Office of Energy

Policy and New Uses, Office of the Chief Economist, United States Department of Agriculture and Louisiana State University, USA.

Shinonaga M, Kawamura Y, Yamane TN. (1992) Immobilization of yeast cells with cross-linked chitisan beads. *Ferment Bioeng. J.*, 74:90–4.

Shiotani, T. and Yamane, T. (1981). A horizontal packed-bed bioreactor to reduce CO_2 gas holdup in the continuous production of ethanol by immobilized yeast cells. *App. Microb. and Biotech. European J.*, 96-101.

Sho S, Susumu T, Haruo T, Noboru Y. (2001). Development of novel carrier using natural zeolite and continuous ethanol fermentation with immobilized *Saccharomyces cerevisiae* in a bioreactor. *Biotechnol. Lett.,* 23:2001–4.

Smart, K. A. (2001). The management of brewing yeast stress. *Proc. Congr. Eur. Brew. Conv.,* 21:306–315.

Talebnia, F. and Taherzadeh, M.J. (2007). Physiological and morphological study of encapsulated *Saccharomyces cerevisiae*. *Enzyme Microb Technol*. 41:683–688.

Tangerman, A. (1997). Highly sensitive gas chromatographic analysis of ethanol in whole blood, serum, urine, and fecal supernatants by direct injection method. *Clinical Chem.,* 43: 1003–9.

Tao, F., Miao, J.Y., Shi, G.Y. and Zhang K.C. (2005). Ethanol fermentation by an acid-tolerant *Zymomonas mobilis* under non-sterilized condition. *Proc. Biochem.,* 40: 183-187.

Urbanchuk, J.M. (2009). Impact of ethanol on world oil demand and prices, 63,245-321.

Verbelen, J.P., De Schutter, D.P., Delvaux, F., Verstrepen, K.J. and Delvaux, F.R. (2006). Immobilized yeast cell systems for continuous fermentation applications. *Biotechnology Lett.,* 28:1515-1525.

Verbelen, P.J., De Schutter, D.P., Delvaux, F., Verstrepen, K.J. and Delvaux, F.R. (2006). Review Immobilized yeast cell systems for continuous fermentation applications. *Biotechnol Lett*. 28(19):1515-25.

Walker, G.M., Maynard, A.I. and Johns, C.G.W. (1990). The importance of magnesium ions in yeast biotechnology. *Ferment. Technol. J.,* 28: 233-240.

Wan Salwanis, W. M. Z. (2013). Bioprocess Intensification: Production of bioethanol from *Saccharomyces cerevisiae* W303 in monolithic microreactor, pp 77.

Werner-Washburne, M., Braun, E., Johnston, G.C. and Singer, R.A. (1993). Stationary phase in the yeast *Saccharomyces cerevisiae. Microbiological Rev.,* 57(2):383-401.

Werpy, T., Petersen, G., Aden, A., Bozell, J. and Holladay, J. (2004). Top value added chemicals from biomass. National Renewable Energy Laboratory, USA.

Williams, D. and Munnecke, D.M. (1981). The production of ethanol by immobilized yeast cells. *Biotechnol. Bioeng.,* 23: 1813-1825.

Ylitervo, P., Franzen, C.J. and Taherzadeh, M.J. (2011). Ethanol production at elevated temperatures using encapsulation yeast. *Biotech. J.,* 156 (1) 22-29.

Yu, J.L., Zhang, X. and Tan, T.W. (2007). A novel immobilization method of *S. cerevisiae* to sorghum bagasse for ethanol production. *J Biotechnol* ;129:415–20.

7 APPENDICES

A1: SUMMARY DATA FOR CELL OPTICAL DENSITY

Table 7-1: Data of optical density obtained in 10 hours of fermentation fixed at 30^0C and 200 rpm

Time (hr)	Optical Density (Absorbance 600nm)								ln(OD)			
	BATCH	SE (±)	CLOTH	SE (±)	SUGARCANE	SE (±)	MEMBRANE	SE (±)	BATCH	CLOTH	SUGARCANE	MEMBRANE
0	0.0565	0.0025	0.0730	0.0120	0.0355	0.0025	0.0415	0.0115	-2.8735	-2.6173	-3.3382	-3.1821
2	0.0675	0.0015	0.1190	0.0040	0.0405	0.0025	0.0530	0.0060	-2.6956	-2.1286	-3.2065	-2.9375
4	0.3895	0.0265	0.2205	0.0015	0.0775	0.0015	0.0575	0.0075	-0.9429	-1.5119	-2.5575	-2.8560
6	0.7748	0.0423	0.6995	0.0425	0.1605	0.0085	0.1085	0.0205	-0.2552	-0.3574	-1.8295	-2.2210
8	1.0430	0.0100	1.4020	0.1140	0.3300	0.0130	0.2150	0.0330	0.0421	0.3379	-1.1087	-1.5371
10	1.2585	0.0285	1.6025	0.0755	0.8225	0.0425	0.6305	0.0555	0.2299	0.4716	-0.1954	-0.4612

A2: SUMMARY DATA FOR CELL DRY WEIGHT

Table 7-2: Data of cell dry weight obtained in 10 hours of fermentation fixed at 30^0C and 200 rpm

Time (hr)	Cell Dry Weight (g)								Biomass Concentration, C_X (gL^{-1})			
	BATCH	SE (±)	CLOTH	SE (±)	SUGARCANE	SE (±)	MEMBRANE	SE (±)	BATCH	CLOTH	SUGARCANE	MEMBRANE
0	0.0807	0.0046	0.0547	0.0037	0.0429	0.0118	0.0151	0.0013	8.0700	5.4700	4.2900	1.5050
2	0.1203	0.0048	0.0826	0.0172	0.0470	0.0162	0.0215	0.0018	12.0250	8.2550	4.6950	2.1450
4	0.2081	0.0079	0.0975	0.0252	0.1333	0.0138	0.0308	0.0027	20.8100	9.7450	13.3300	3.0800
6	0.2691	0.0037	0.1779	0.0118	0.1971	0.0042	0.0722	0.0142	26.9100	17.7900	19.7050	7.2200
8	0.3115	0.0037	0.3579	0.0298	0.2567	0.0187	0.1371	0.0160	31.1500	35.7850	25.6650	13.7100
10	0.3296	0.0085	0.3964	0.0043	0.3050	0.0021	0.2000	0.0105	32.9600	39.6400	30.4950	19.9950

A3: SUMMARY DATA FOR COLONY FORMING UNIT

Table 7-3: Data of Colony Forming Unit (CFU) obtained in 10 hours of fermentation fixed at 30^0C and 200 rpm

Time (h)	CFU/ml at Dilution factor of 10^{-5}							
	BATCH	SE (±)	CLOTH	SE (±)	SUGARCANE	SE (±)	MEMBRANE	SE (±)
0	2.30E+07	3.00E+06	5.00E+07	1.30E+07	2.20E+07	3.00E+06	1.20E+07	2.00E+06
2	3.25E+07	1.05E+07	7.85E+07	6.50E+06	3.20E+07	7.00E+06	1.95E+07	7.50E+06
4	6.70E+07	4.00E+06	9.20E+07	6.00E+06	4.40E+07	9.00E+06	3.75E+07	5.50E+06
6	1.39E+08	1.50E+07	1.23E+08	1.55E+07	1.00E+08	2.00E+06	7.20E+07	9.00E+06
8	1.88E+08	2.30E+07	2.29E+08	3.50E+06	1.70E+08	1.90E+07	9.85E+07	1.65E+07
10	2.18E+08	1.70E+07	2.57E+08	4.00E+06	2.04E+08	1.20E+07	1.70E+08	1.70E+07

A4: SUMMARY DATA FOR GLUCOSE UTILIZATION AND CONSUMPTION USING DNS METHOD

Standard Curve of Glucose Concentration

Table 7-4: Data of standard curve for DNS method obtained in 10 hours fermentation fixed at 30^0C and 200 rpm

Glucose Conc. (gL^{-1})	OD			Average OD	DNS Standard Error (±)
	RUN 1	RUN 2	RUN 3		
0.0	0.000	0.000	0.000	0.000	0.000
20.0	1.391	1.387	1.373	1.384	0.005
40.0	1.555	1.551	1.555	1.554	0.001
60.0	1.860	1.857	1.857	1.858	0.001
80.0	2.018	2.013	2.051	2.027	0.012
100.0	2.208	2.217	2.201	2.209	0.005
120.0	2.218	2.257	2.219	2.231	0.013
140.0	2.357	2.321	2.354	2.344	0.012
160.0	2.387	2.366	2.387	2.380	0.007
180.0	2.419	2.420	2.428	2.422	0.003
200.0	2.387	2.373	2.368	2.376	0.006

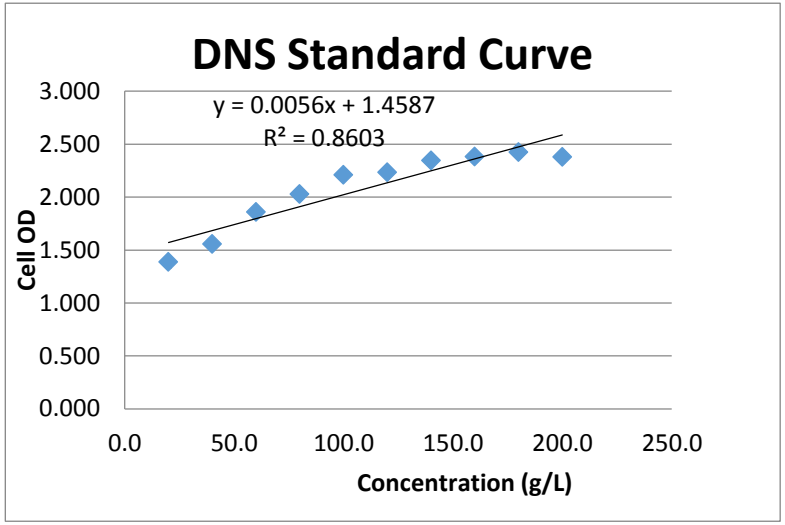


Figure 7-1: Graph of DNS standard curve

DNS Method Analysis for Fermentation Samples

Table 7-5: Data of DNS method analysis for samples obtained in 10 hours fermentation fixed at 30^0C and 200 rpm

Time (hr)	Glucose Concentration, C_S (gL^{-1})				Glucose Consumption (gL^{-1})			
	BATCH	CLOTH	SUGARCANE	MEMBRANE	BATCH	CLOTH	SUGARCANE	MEMBRANE
0	149.76	147.79	150.23	150.77	0.24	2.21	-0.23	-0.77
2	135.41	128.63	142.20	143.86	14.59	21.38	7.80	6.14
4	119.70	113.15	132.38	135.47	30.30	36.85	17.63	14.53
6	91.96	83.92	126.13	128.39	58.04	66.08	23.88	21.61
8	74.10	61.13	94.52	100.11	75.90	88.88	55.48	49.89
10	56.01	49.52	73.68	86.84	93.99	100.48	76.32	63.16

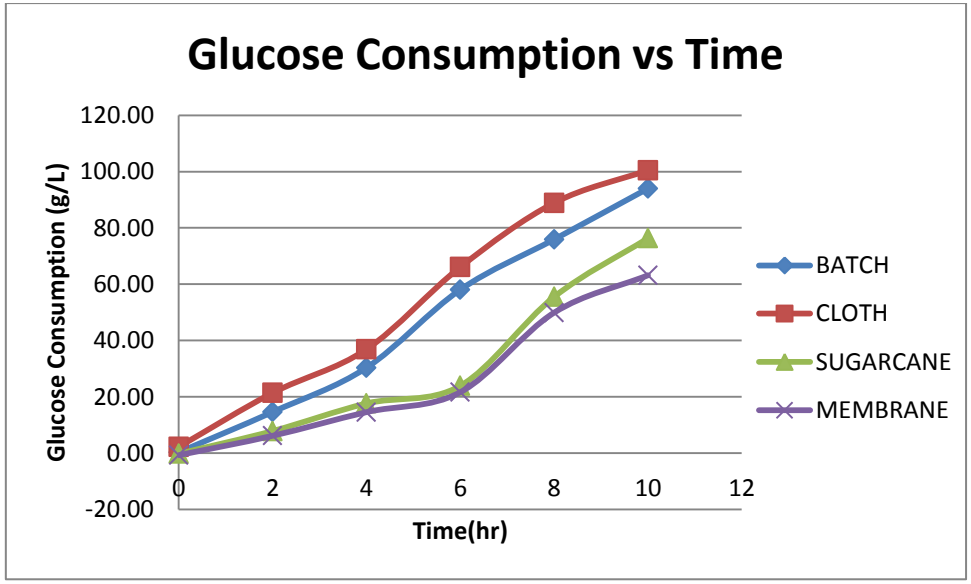


Figure 7-2: Profile of glucose consumption which relative to 10 hours of fermentation

A5: DATA FOR ETHANOL CONCENTRATION

Table 7-6: Data of ethanol concentration for standard and fermentation samples obtained in 10 hours fermentation fixed at 30^{0}C and 200 rpm

Concentration of Ethanol (gL^{-1})	Area (pA*s)
0	6.23785
2	95.57068
4	185.65588
6	284.36929
8	406.43677
10	516.04095

Time (hr)	Area (pA*s)				Ethanol Concentration, C_P (gL^{-1})			
	BATCH	CLOTH	SUGARCANE	MEMBRANE	BATCH	CLOTH	SUGARCANE	MEMBRANE
0	3.35031	24.88917	11.96368	6.23785	0.067	0.495	0.238	0.124
2	16.30022	77.99863	14.15238	11.02528	0.324	1.553	0.282	0.219
4	26.63147	113.9607	76.73087	12.58249	0.530	2.269	1.527	0.250
6	41.66095	119.8363	125.26227	52.26181	0.829	2.385	2.493	1.040
8	130.8702	190.3475	160.49098	86.14237	2.605	3.789	3.195	1.715
10	192.2658	210.7518	187.71721	178.45325	3.827	4.195	3.737	3.552

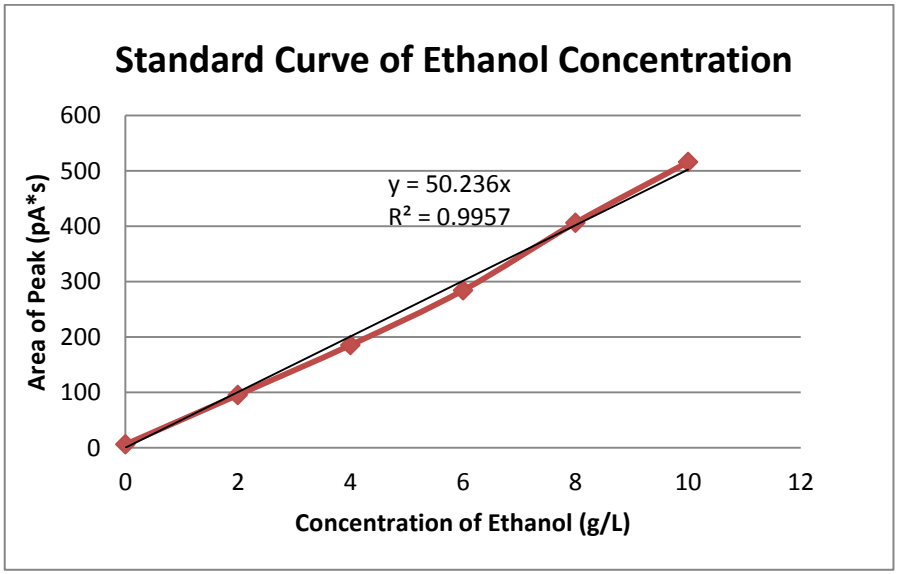


Figure 7-3: Graph of ethanol concentration standard curve

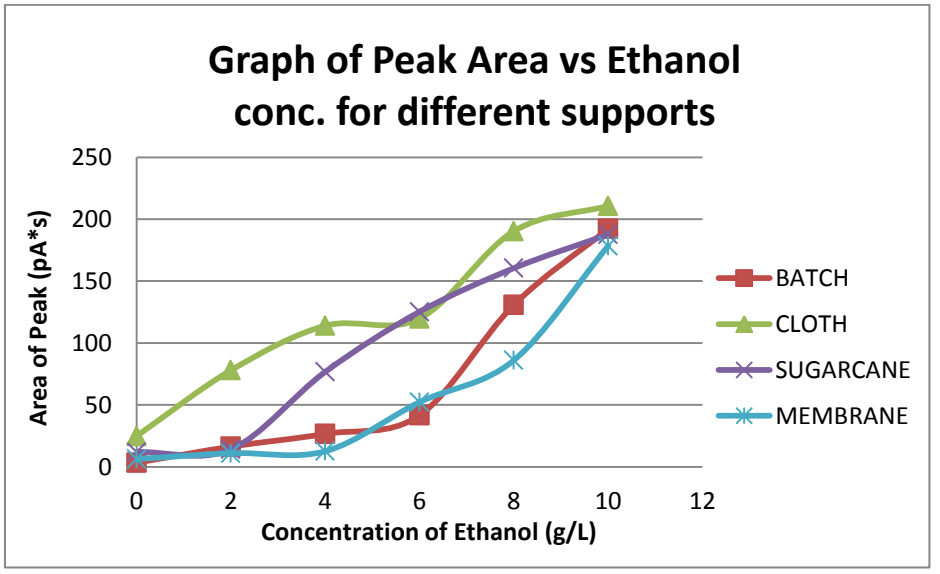


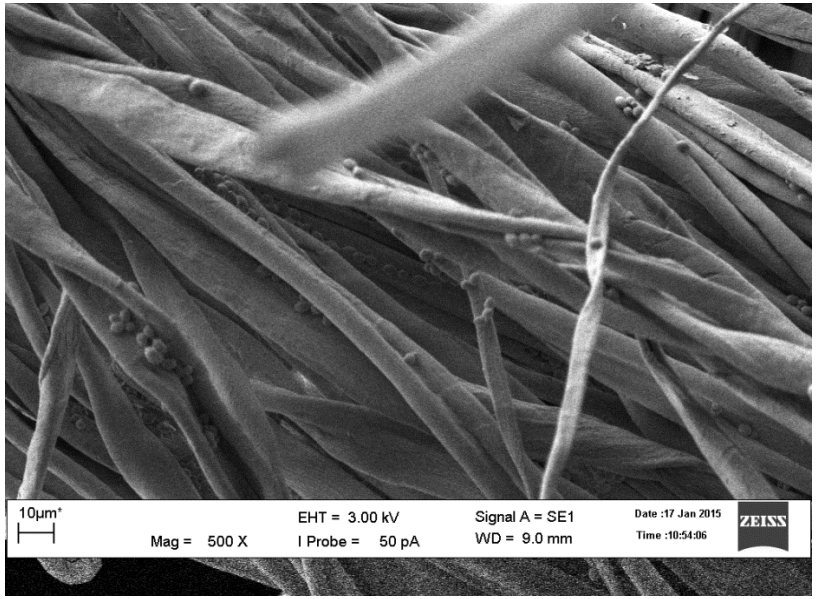
Figure 7-4: Graph of peak area which respect to ethanol concentration for different immobilized supports

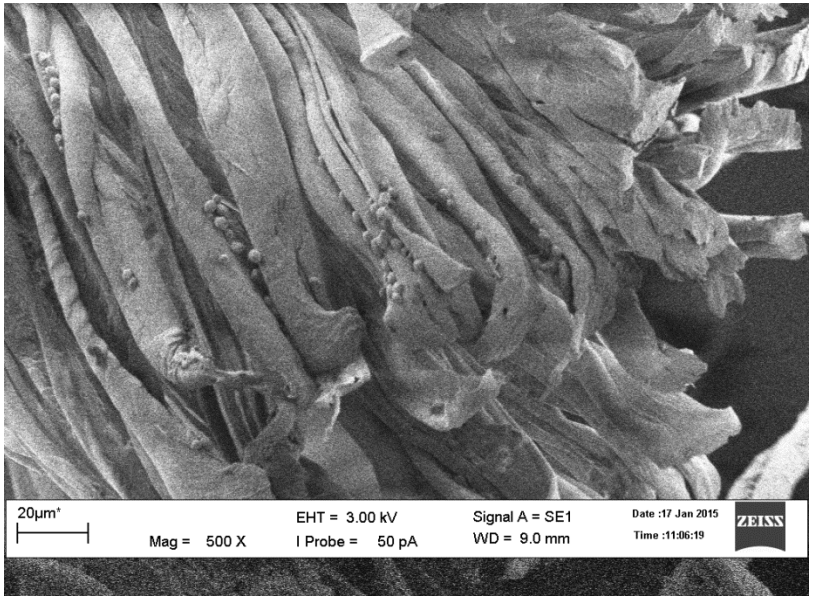
A6: DATA FOR IMMOBILIZATION METHOD

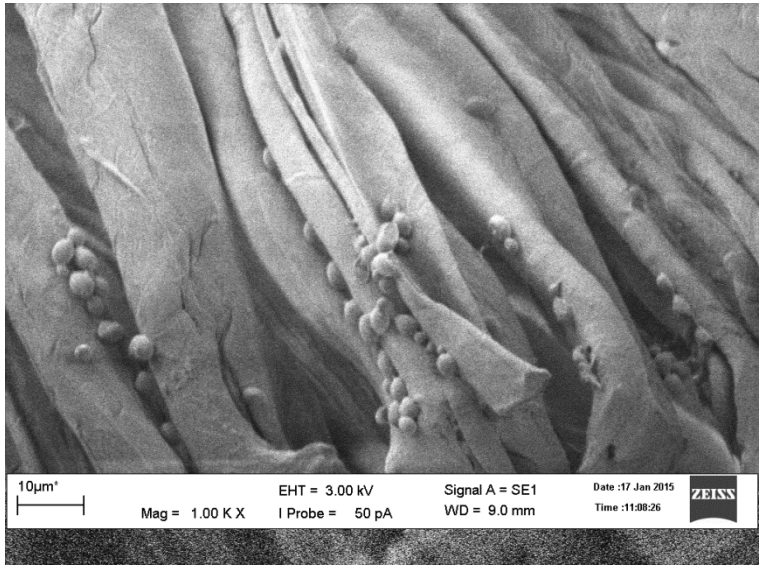
Table 7-7: Data of optical density for bacterial suspension solution before and after the immobilization method

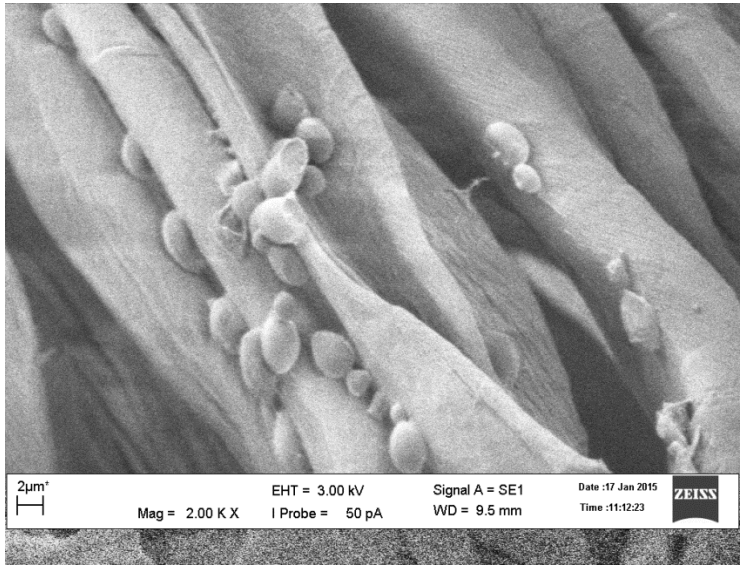
Experiments	Optical Density at 600 nm							
	BATCH		CLOTH		SUGARCANE		MEMBRANE	
	RUN 1	RUN 2	RUN 1	RUN 2	RUN 1	RUN 2	RUN 1	RUN 2
PBS Initial OD before immobilization	1.075	0.981	1.320	1.320	1.043	1.057	1.335	1.335
PBS Final OD after immobilization	-	-	0.837	0.932	0.842	0.905	1.053	1.199
Difference of cells attach on immobilized supports	-	-	0.483	0.388	0.201	0.152	0.282	0.136
Average of cells attach on immobilized supports	-		0.436		0.177		0.209	

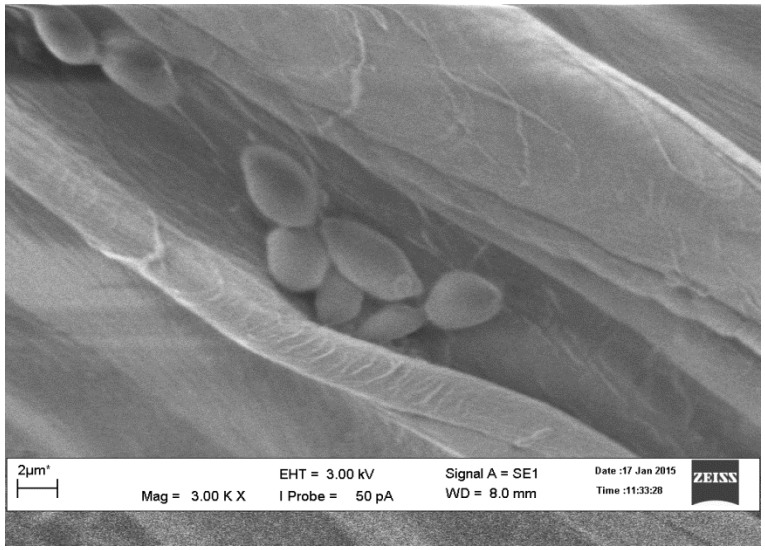
A7: IMAGES ON ATTACHMENT OF CELLS USING SEM

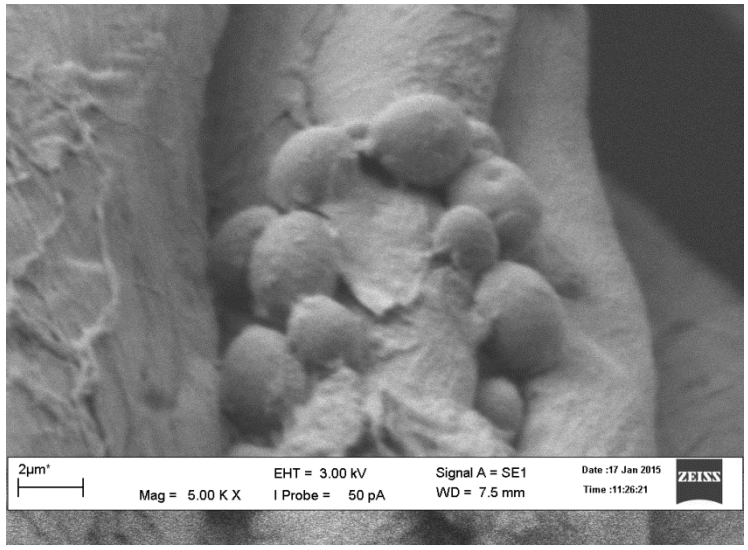





10µm*
Mag = 1.00 K X
EHT = 3.00 kV
I Probe = 50 pA
Signal A = SE1
WD = 9.0 mm
Date :17 Jan 2015
Time :11:08:26
ZEISS


2µm*
Mag = 2.00 K X
EHT = 3.00 kV
I Probe = 50 pA
Signal A = SE1
WD = 9.5 mm
Date :17 Jan 2015
Time :11:12:23
ZEISS


2µm*
Mag = 3.00 K X
EHT = 3.00 kV
I Probe = 50 pA
Signal A = SE1
WD = 8.0 mm
Date :17 Jan 2015
Time :11:33:28
ZEISS


2µm*
Mag = 5.00 K X
EHT = 3.00 kV
I Probe = 50 pA
Signal A = SE1
WD = 7.5 mm
Date :17 Jan 2015
Time :11:26:21
ZEISS