Bioethanol production by batch and repeated batch using immobilized yeast cells on sugarcane bagasse fiber and analysis of spent immobilized yeast as a potential animal feed supplement

Ms. Apinya Sowatad

ID. 5929701

A Thesis Submitted in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biotechnology
Department of Food Biotechnology
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Department	: Food Biotechnology
Faculty	: Biotechnology
Academic year	: 2017

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..... Date 3/9/2018

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ABSTRACT

Delignified sugarcane bagasse from sugar industry was used as a carrier for *Saccharomyces cerevisiae* SC90 immobilization. The proficiency of the cell immobilization of *S. cerevisiae* SC90 on delignified sugarcane bagasse was determined through the ratio of dry weight of immobilized cells and dry weight of carrier (g/g) together with scanned electron microscope (SEM). *S. cerevisiae* SC90 demonstrated the highest immobilization on day 1 when diluted molasses (231 g/L total sugar concentration) was used as a substrate. The efficiency of ethanol production by the immobilized cells was compared with the suspended cells in the repeated batch process under the shake flask. The immobilized cell represented a higher ethanol production than suspended system in all 5 consecutive batches. The maximum ethanol yield ($Y_{P/S}$) of the immobilized cells was 0.42±0.02 g/g (82.48% theoretical yield) in 3 L packed bed bioreactor. With 3L bioreactor, the ethanol production efficiency could be maintained for 5 consecutive batches. The composition of spent yeast cells on delignified sugarcane bagasse was analyzed and compared with the non-delignified sugarcane bagasse. The result represented higher protein content in spent yeast cells on delignified than non-delignified sugarcane bagasse while yeast added up protein content to be served as an animal feed.

Keywords: Ethanol; Sugarcane bagasse; Molasses; Immobilization; *Saccharomyces cerevisiae*

ACKNOWLEDGEMENTS

First, I wish to express my sincere thanks to my advisor Asst. Prof. Dr. Tatsaporn Todhanakasem for giving me advice and encouragement. Without her valuable guidance and financial support, this thesis would not have been possibly done. Moreover, I would like to thank Faculty of Biotechnology, Assumption University including instructors for giving me knowledge and facilities support for this research.

Also, I would like to thank the Thailand Research Fund (TRF) and KSL Green Innovation Public Company Limited for financial granted support.

In addition, I would like to express gratitude to my committee chair advisor, Dr. Viyada Kunathigan and Dr. Pattanop Kanokratana whose spend their time attending in my presentation and giving recommendation for errors and future development of ethanol production. Also, I am thankful to all technicians in Assumption University for giving instruction about laboratory work and helping me to solve problem.

Last but not the least, I would like to thank my family for supporting me throughout writing this thesis and my life in general.

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INTRODUCTION

There is a huge concern on the economic, environmental effects and depletion of nonrenewable fuel sources over the usage of petroleum therefore, bioethanol has been considered as a potential energy source to replace the petroleum. As the ethanol demand increases, there is a need to search for less expensive technology to reduce the cost of production. In Thailand, bioethanol production from molasses base ethanol provides high net energy for vehicle since sugarcane is a prime economic crop in Thailand [1]. Therefore, in order to make the process competitive, it is essential to produce ethanol at low cost in the short time. Bioethanol has been widely produced under batch or fed batch process using free suspended cells. Others have been used cell immobilization based on cell entrapment in gels such as carrageen and calcium alginate [2-4]. However, the process instability is the main drawback among these types of cell immobilizations by the gel degradation, limitation of nutrient and gas transfer, nutrient and metabolite mass transfer. Recently, natural cell immobilization has been emerged as an effective biocatalyst as it eliminates the limitations that usually appear through the other chemical immobilization techniques. The system does not only increase the productivity and yield of ethanol, it also provides the cell recycle system and facilitates the product recovery. This also minimizes the bioethanol production cost in various bioprocesses [5-7].

Cell immobilization is occurred by the natural adsorption of microorganisms on the carrier. Carriers support growth of microorganisms and protect cells from toxic inhibitors, high sugar concentration substrate and high ethanol product [8-10]. Cell immobilization on the carrier could lead to a higher biomass concentration and enhanced the biological stability which improve the ethanol production rate by reducing the fermentation time. The presence of high biomass concentration also minimize the risk of contamination in the process [11]. Another advantage of cell immobilization is cell recycle system in which also reduces cost requirement for inoculum development and further reduces the production cost as a whole. Natural carriers have been studied in the past including wood block, guava piece, porous cellulose, apple cutting, loofa sponges, sorghum bagasse, saw dust, watermelon shell, silk cocoons and delignified agricultural materials [12-17,11]. Since sugarcane is a prime economic crop in

Thailand, sugarcane bagasse is a remarkably by product. This brought sugarcane bagasse as a specific interest to be used as a natural carrier. The spent cell immobilization adsorbed on these agricultural byproducts can be further modified to animal feed supplements at the end of the fermentation process.

S. cerevisiae have been widely used for bioethanol production because it can use various type of substrate, tolerate to wide range of pH and ethanol concentration [13]. It has been found to be immobilized on different types of natural carriers including sorghum bagasse, sugarcane pieces, wood blocks, silk cocoons, and loofa sponges [12,18,13,14,11] Yeast cell immobilization on agricultural materials illustrated higher ethanol production yield almost 10 times higher than suspended cultures without any significant loss in ethanol production [18]. Immobilized yeast could faster fermentation by providing higher cell densities per unit fermentation volume with the in-situ removal of cells reduces the cost of product recovery. In addition, the spent agricultural carrier such as delignified lignocellulose can be used as protein enriched animal feed [19,20]. Pretreatment process increase digestibility of lignocellulose by removing lignin. Thus, ruminal microorganisms can easily access the nutritional part of the delignified lignocellulose [21]. Hence, yeast immobilization for bioethanol production have represented as a promising technology of industrial scale ethanol production using various sources of glucose rich substrates with high economic benefits.

The aim of this work is to study on the potential of using immobilized *S. cerevisiae* on sugarcane bagasse for the ethanol production using molasses as a substrate. Sugarcane bagasse and molasses are natural, abundant from sugar industry, therefore, we decided these sugarcane materials in the process. Delignified sugarcane bagasse provides benefits in term of mechanical strength, light weight, high surface area and eventually as animal feed. *S. cerevisiae* SC90 was immobilized on delignified sugarcane bagasse using molasses as a substrate to optimize the immobilization and fermentation processes. Immobilization efficiency of yeast was analyzed on the immobilization yield and visualized through scanning electron microscope (SEM). The ethanol production by the immobilized yeast was determined under batch and repeated batch processes to compare with the suspended culture using ethanol yield ($Y_{P/S}$) and percent conversion of sugar to ethanol (%) as the kinetic parameters. The spent immobilized yeast absorbed on delignified bagasse was determined on its nutritional value to be applicable as an animal feed supplement.

Objectives

- To study the efficiency of immobilized *S. cerevisiae* SC90 on delignified sugarcane bagasse
- To study and compare the repeatability of free suspended and immobilized *S. cerevisiae* SC90 in batch and repeated batch process
- To analyze protein and fiber content of spent immobilized yeast
- To determine economic feasibility of the overall process

Expected Result

The fermentation efficiency will be remained for a certain number of batch to minimize the production cost through the cell recycle. Moreover, spent immobilized yeast will be effectively used as animal feed by containing specific level of fiber and protein to reach the standard of animal feed. Thus, Overall cost of production should be reduced through this process.

LITERATURE REVIEW

In Thailand, energy consumption continuously increases while the domestic oil production and reserves is limited, forces Thailand to be 2nd rank of oil importer. To solve the problem of an increase of global oil price and reduce the petroleum consumption, alternative biofuels are promoted by the government and causes ethanol to become the leader of alternative fuel in Thailand [22]. However, the complexity of ethanol production process causes high production cost of ethanol including the transformation process of some bioresource such as lignocellulosic feedstocks which requires pretreatment process for fermenting microorganisms to convert them into ethanol. This product also requires dehydration process to be utilized for gasoline. So, the ethanol production cost is 40 % higher than crude oil price which is not economically feasible to use as a fuel [23]. Thus, cost-effective technologies have to be investigated to reduce the cost of production. Selecting appropriate feedstock is one of important factors to design the cost-effective process.

Molasses is chosen to be a raw material for ethanol production because it is by-product from sugar industry which sugar cannot be extracted out anymore, thus raw material cost can be eliminated. Moreover, molasses can produce higher ethanol yield than cassava as 1 ton of molasses can produce 260 L ethanol (95%) by volume while only 180 L of 95% ethanol could be obtained from 1 ton of cassava. Besides, molasses is obtained from sugar-based plant so, it can be directly used in fermentation without the additional process to convert starch into fermentable sugar [24].

Table 2 volume of thanol obtained from 1 ton of grain, molasses and cassava [24]

Raw material	Volume of ethanol per 1 ton of raw material
Grain e.g. rice, corn	375 L
Molasses	260 L
Cassava	180 L

According to El-Gendy et al., sugarcane molasses is a dark viscous liquid with pH value of 5-5.5 which has high nutritive value for fermenting microorganisms without furfural which is toxic to most fermentable microorganisms. It contains fermentable

sugars~55% (wt%) and 5% (wt%) non-fermentable sugars including 68.36% sucrose, 18.50% glucose, and 13.14% maltose [25].

Table 3 Chemical composition of sugarcane molasses [25]

Composition	Quantity
Carbon	64 %wt
Nitrogen	6 %wt
Phosphorus	0.3 %wt
Sodium	0.33 %wt
Potassium	5.5 %%wt
Calcium	0.7 %wt
Copper	2.2 ppm
Zinc	3.91 ppm
Manganese	4.74 ppm
Iron	78.37 ppm
Magnesium	1370 ppm
Non-nitrogenous compounds (e.g., citric acid, oxalic acid)	2-8 %wt
Ash	10-16 %wt

The composition of molasses depends on soil and climate, variety and maturity of the cane and the processing conditions in the factory [25]. Apart from sugarcane, molasses can be produced from grapes, sugar beets, sorghum or other plants. There are several steps to produce molasses including cutting the sugarcane plants, boiling, straining, skimming and reboiling [26]. Before fermentation, molasses is diluted with water to reduce its viscosity and prevent incomplete sugar conversion and is sterilized to prevent contamination from other microorganisms [27].

Recently, cell immobilization has received increasing interest in ethanol production as it facilitates cell recycle and product purification. Plus, it can protect cells from high substrate or product concentration. There are many methods of cell immobilization including entrapment, cross linking, encapsulation. These methods use organic polymers.(alginate, carrageenan, agar, agarose, chitosan, etc.) and synthetic polymers (acrylamide, polyurethane, polyvinyl, resins, etc.) as a carrier. Sree et al. demonstrated

the use of calcium alginate as a yeast carrier to produce ethanol via repeated batch. The maximum amount of ethanol produced by immobilized yeast was lower than free cells in the first batch. However, after fourth cycles, the amount of ethanol production was decreased from 72 g/L to 25 g/L while that of immobilized yeast was increased from 44 to 72 g/L [28]. Calcium alginate was also used to immobilize *Zymomonas mobilis* for ethanol production from paper sludge. This was reported by Yamashita et al. The experiment was conducted by using 200 g/L initial paper sludge as a substrate. The fermentation was carried out 48 hours. The maximum ethanol of 18 g/L was obtained and immobilized *Z. mobilis* was attempted to be repeated for 4 cycles [29].

Despite the fact that they allow high mechanical strength, they contain some disadvantages such as cell leakage from gel degradation, limitation of mass and gas transfer, low loading capacity, high cost of raw materials [30]. Cell immobilization on natural carrier is based on a physical interaction between cells and carrier surfaces via weak forces including van der Waals forces, ionic and hydrophobic interactions and hydrogen bond [31]. Since the carrier can be easily modified from agricultural waste, the cost of production is minimized. Apart of these, the carrier is biodegradable, so it does not create any environmental problem [32].

Cell immobilization has been applied in batch and repeated batch system. A batch culture operation can be characterized by no addition or withdrawal of nutrient medium of culture until the process is finished. However, the addition of small amount of acid, base and some defoaming agent are exceptable to maintain pH and suppress foaming. Typically, some factors such as pH and dissolved oxygen might be not controlled. The process is started when the small number of microbial cells from starter or sub-culture is inoculated into sterile medium and will be terminated near the end of the growth phase or during the stationary phase [33]. The advantages of batch process are that the process parameters are easier to be controlled and measured. Particularly, it has less chance of getting contamination [34]. In contrast, batch operation has high capital cost and operational costs and has a problem of secondary sludge disposal [35]. Plus, it is time consuming and low biological conversion as the cells need to adapted to new environment and utilize the nutrient in the medium to grow before producing the metabolites [33]. To minimize these costs in the industrial bioprocess, the culture is recycled from batch to batch which is called repeated batch. As the cells are recycled, they are ready to convert substrate into product without adaptation, so it minimizes the

fermentation time and has a good depletion of medium at the end of fermentation. Also, high cell density in the culture results in high productivity [36]. Ethanol Production by repeated batch and continuous fermentations using *S. cerevisiae* immobilized in a fibrous bed bioreactor was developed by Chen et al. The ethanol productivity achieved from immobilized yeast was 41.93% higher than that free cells by which 91.36 g/L ethanol concentration was obtained from 200 g/L initial sugar concentration and the ethanol productivity can be maintained at least 22 cycles for repeated batch [37].

S. cerevisiae is eukaryotic microorganisms which belongs to Fungi kingdom. It can be mostly found in diploid form which has ellipsoid shape with 5-6 µm in diameter. Another form is haploid which has more spherical with a diameter of 4 µm. With haploid form at exponential phase, it takes approximately 90 minutes for doubling time in YPD media and approximately 140 minutes in synthetic media. *S. cerevisiae* grows well in a neutral or slightly acidic pH environment under aerobic condition at the optimum temperature of 28-30°C [38]. *S. cerevisiae* utilize glucose as a main carbon source via the glycolytic pathway and the Krebs cycle to energy in the form of ATP. Though, it can utilize other hexose such as fructose and saccharide such as sucrose which will be hydrolyzed by extracellular invertase into glucose and fructose and mannose with mannase enzyme [39]. *S. cerevisiae* refers to facultative anaerobe which can grow with or without oxygen. In aerobic condition, glucose will be converted to CO_2, water and energy via undergoes mitochondrial electron transport chain and oxidative. In contrast, by-products such as ethanol, glycerol and CO_2 will be produced instead and the cells will obtain energy from glycolysis only. As the result, the cells will grow slowly during fermentation and the energy from glucose utilization will be used only for maintain cell viability [40]. Apart from carbon source, some elements including N, P, S, Fe, Cu, Zn and Mn which are essential for yeast's growth, are added into growth media usually in ammonium ions and urea form for nitrogen and inorganic phosphates and sulfates for phosphorus and sulfur [41].

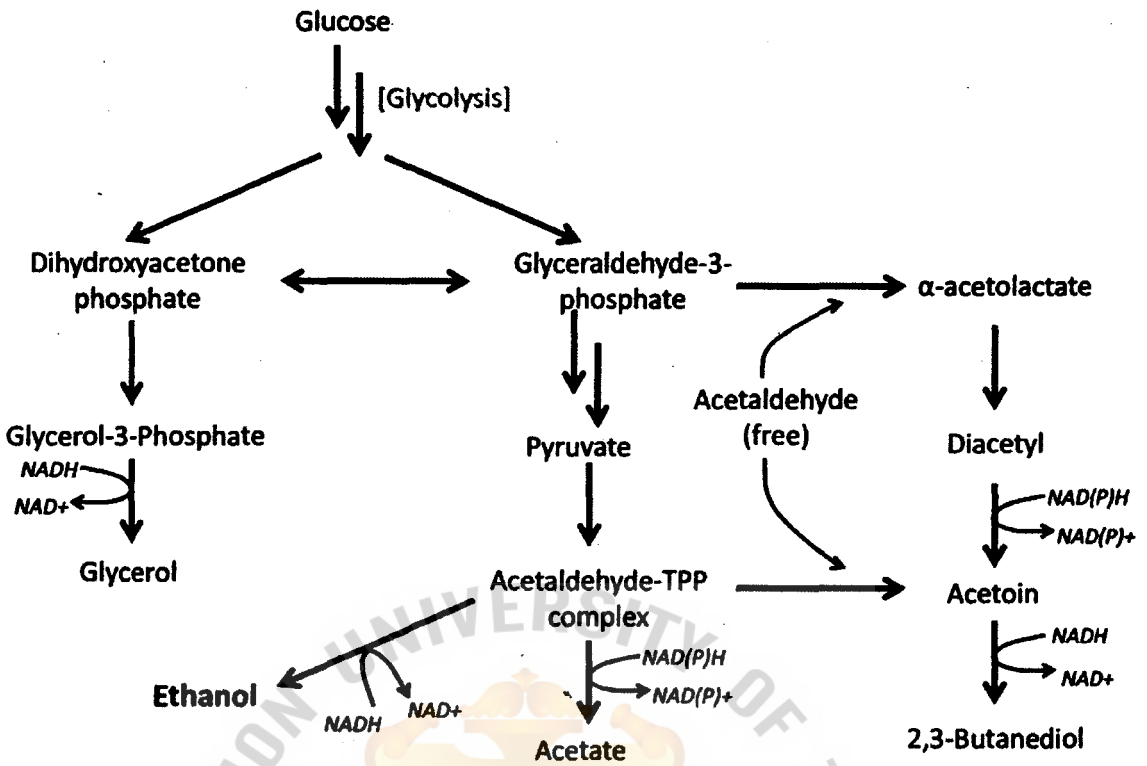


Figure 1 Ethanol biosynthesis pathway in *S. cerevisiae* [42] .

There are many microorganisms that has been used for ethanol production such as *Zymomonas mobilis* which has a higher yield and has a much higher specific ethanol productivity than *S. cerevisiae*, *Escherachia coli* which as the ability to ferment a wide spectrum of sugars, no requirements for complex growth factors, and prior industrial use and many cellulose-to-ethanol biotransformation including *Clostridium thermocellum*, *Aspergillus sp.*, or *Trichoderma viride*. However, *S. cerevisiae* has many advantages against them. First, it can ferment a wide spectrum of sugar while *Z. mobilis* can ferment only glucose, fructose and sucrose. Second, *S. cerevisiae* can grow in wider range of pH and hardier than *E. coli*. Furthermore, *S. cerevisiae* is generally recognized as safe (GRAS) as a food additive for human consumption while some *E. coli* strains are pathogenic which is danger to human health. Apart of this, *S. cerevisiae* can be used as animal feed after fermentation. In contrast, there is no data of using *E. coli* cell mass residual as an animal feed. Third, fermentation process by using *S. cerevisiae* is faster than cellulose-to-ethanol microorganisms with a higher yield. Another disadvantage of cellulose-to-ethanol microorganisms is the formation of various by-products [27]. Apart of this, yeast have a potential to immobilize on varies types of carriers such as wood block, porous cellulose, apple cutting, loofa

sponges, sorghum bagasse, sugar beet pulp, silk cocoon, and sugarcane bagasse [32,12,15,13,14,17,42,11]. Vučurović and Razmovski reported that ethanol production from sugar beet thick juice by using yeast immobilized on sugar beet pulp can be carried out at least seven cycles without significant decrease in ethanol yield. The ethanol concentration of 52.26 ± 2.0 g/l and ethanol yield of 0.446 ± 0.017 g/g was obtained in the seventh fermentation batch with initial sugar of 120 g/l [42]. Rattanapan et al. has developed TSC-immobilized cell system for repeated batch by using thin-shell silk cocoon to be a carrier for yeast cells. By using 240 g/L molasses as a substrate, the result showed that the ethanol concentration produced by immobilized cells was 11.5% higher than that produced by suspended cells with high stability through five cycled repeated batch [14].

Sugarcane bagasse is an agricultural waste obtained from sugar industry. It is composed of cellulose (32-44%), hemicellulose (27-32%) and lignin (19-24%) [43]. Sugarcane bagasse has a potential to be a carrier for cell immobilization because of its high porosity, high water retention, high surface area, high mechanical strength, maximum loading and low toxicity which facilitates the absorption of yeast on the surface [32]. Anita et al reported that using immobilized yeast cells on sugarcane bagasse gave the ethanol yield three times higher than free cells. Also, the maximum cell density achieved for immobilized cells was 1.3 times higher than free cells system [32].

After fermentation, spent yeast with sugarcane bagasse will be utilized as animal feed. Spent yeast contains high protein (30-60 %), carbohydrates (15 – 60%) and vitamin B and D [44]. In addition, yeast enhances ruminant performance by stimulating the cellulolytic bacterial population and balance the pH in the rumen which result in increase of lactate, vitamin B and ammonia usage, and enhance fiber digestion. Nevertheless, by promoting competition between methanogenics and acetogenetics bacteria, yeast helps to reduce the production of methane [45]. Maamouri et al. reported that supplementing 2.5 g of yeast per cow per day can increase the milk production by 1.1 kg/cow. Plus, it also increases fat and protein content by 52.8 and 41.7 g/cow/day, respectively [46].

Generally, sugarcane bagasse has been used as ingredient in ruminant diets because of its abundant and low cost. However, low palatability and digestibility (25 – 35 %) of bagasse due to the physical and chemical complexity of carbohydrates (cellulose,

hemicellulose and lignin) prevents more extensive utilization as an animal feed [47]. To increase the accessibility of cellulose and hemicellulose which is the main carbohydrate source, removal of lignin has been done under the process called pretreatment. Alkaline pretreatment is the process of removing lignin by using alkaline agent such as sodium, potassium, calcium and ammonium hydroxide. The process can be carried out at ambient temperature, though it requires long time to finish the process. Alkalis increases the surface area which is caused by swelling of lignocellulose. Also, Black liquor residue which was obtained after the process should be treated before disposed of [43].

In this study, sugarcane bagasse will be modified by pretreatment process to remove lignin, disrupt the crystalline structure of cellulose, increase the surface area and porosity and improve digestibility of enzyme [48]. However, physical properties of sugarcane bagasse after pretreatment might be changed which will affect the cell adhesion and ethanol production. Therefore, the immobilization efficiency by using delignified sugarcane bagasse as a carrier need to be investigate and the fermentation efficiency by using immobilized yeast on delignified sugarcane bagasse will be analyzed and compared with free suspended cells. Plus, evaluation of the potential of using spent immobilized yeast as an animal feed and economic feasibility of the overall process will be determined.

MATERIALS AND METHODS

Yeast strain and culture preparation

S. cerevisiae SC90 (TISTR 5606) was obtained from Thailand Institute of Scientific and Technological Research (TISTR). The culture was stocked in Yeast Extract–Peptone–Dextrose medium (YPD) medium containing 20 g/L glucose, 10 g/L (w/v) yeast extract, and 20 g/L peptone, pH 4.8. Starter culture was prepared by transfer a single colony to 5 ml YPD broth, pH 4.8 and incubating at 30°C under the shaking condition at 120 rpm for 12 hours until the OD_{600} was approximately 1.0. The seed culture was prepared with 50 ml YPD broth and grew under the same condition.

Carrier and medium preparations

Sugarcane bagasse and molasses were obtained from Khon Kaen Sugar Industry (KSL) Green Innovation Public Company Limited, Thailand. Sugarcane pieces were obtained and cut into small pieces of 1 cm length. Sugarcane bagasse was delignified using 1:10 (w/v) ratio of potassium hydroxide in 0.01 % (v/v) acetic acid at 70 °C for 1 hour. The process was repeated 3 times. Then the bagasse was neutralized by rinsing with water and dried in oven at 60°C until the dry weight was constant [49]. The concentration of total sugar in molasses was measured by using Fehling titration method [50] and the molasses was diluted to a specific sugar concentration with distilled water before used. The subsequently diluted molasses was supplemented with 1 g/L $(NH_4)_2SO_4$ and 1 g/L KH_2PO_4 with pH 4.8. Molasses and delignified bagasse were sterilized at 121°C, 15 min.

Determination on the optimum condition for the cell immobilization and prepare of cell immobilization on the supports

The total of 10 % (v/v) of the starter culture (OD_{600} approximately 1.0) was inoculated into flask containing 10% (w/v) delignified sugarcane bagasse in the total volume of medium (50 ml). YPD and molasses (with total sugar concentration at 40, 94, 140, 185 and 231 g/L) were applied as immobilized media. The immobilization under each specific condition was cultured at 30°C and 80 rpm for 1-3 days. The optimum immobilization time and condition were evaluated under the quantitative and qualitative analysis.

The quantitative analysis was based on the dried weight of immobilized cells per dried weight of sugarcane bagasse (w/w). The suspended culture was rinsed out and the immobilized cells on sugarcane bagasse were washed twice using sterile distilled water and oven dried at 70°C until the weight was stable. The immobilization efficiency in each specific medium condition and time was calculated as dried weight immobilized cell (g) per dried weight of sugarcane bagasse (g). The experiments were performed triplicate, the results were statistically analyzed by ANOVA with Duncan's multiple range test. The null hypothesis was accepted or reject with 95% confidence interval ($p = 0.05$).

The qualitative analysis of cell immobilization submerged in YPD and molasses supplement with $(NH_4)_2SO_4$ and KH_2PO_4 using a scanning electron microscope (SEM) (JCM-6000, Japan). The immobilized cells on the sugarcane bagasse on day 1 was oven dried at 70°C and followed by freeze dried at -35°C for 3.5 hours, -5°C for 8 hours, 15°C for 8 hours and 35°C for 3 hours (FD8-Economic Series, Thailand). The sample was prepared for SEM by fixing the specimen with tape and then sputtered with gold in sputter-coater under high vacuum condition. Each sample was examined at 2000-fold magnification using SEM.

Batch and repeated batch fermentation

S. cerevisiae SC90 suspended and immobilized cultures were fermented for five consecutive batches using molasses as a substrate for ethanol production. Suspended culture and immobilized culture of *S. cerevisiae* SC90 were compared on the fermentation efficiencies under the shake flask condition contained 50 ml molasses supplement with $(NH_4)_2SO_4$ and KH_2PO_4 (molasses 125 g/L of reducing sugar and supplemented with 1 g/L $(NH_4)_2SO_4$ and 1 g/L KH_2PO_4), pH 4.8 in 125 ml Erlenmeyer flask. The flask of immobilized cell contained additional 10% (w/v) of delignified bagasse inoculated with 10 % (v/v) of the starter culture (OD_{600} approximately 1.0). The flask of suspended culture contained only fermentation medium with 10 % (v/v) of the starter culture (OD_{600} approximately 1.0). Flasks were shaken at 80 rpm at 30°C for 24 hours and proceeded further under the non-shaking condition for another 24 hours (JSSI-100C, Korea). The duration of each batch was 48 h. The experiment was monitored by removing 5 mL sample at the end of 48 hours in order to analyze on the concentration of reducing sugar (g/L) and ethanol (g/L). Repeated batches of immobilized cell were operated by discarded the supernatant and replaced with the fresh molasses to continue the fermentation in the following batch. The suspended cultures were repeated by centrifuged the cell culture in 5,000 rpm centrifugation machine (PLC-012, Taiwan) and resuspended in molasses under the sterile condition and pursued the fermentation in the following batch. The repeated batch was operated in the total of 5 consecutive cycles. The yield ($Y_{P/S}$) and percentage of theoretical yield of ethanol (%) based on ethanol production from glucose consumed. These parameters were compared among suspended and immobilized cultures. The experiments were done in triplicates. The result was analyzed using ANOVA with level of significance at $p<0.05$. Mean comparison was performed by the Duncan's multiple range test.

The scale up was carried out in 3L stirred tank bioreactor (GBJX-5C, Zhenjiang) under the packed bed containing immobilized cell bed. The temperature and pH were control at 30°C and pH 4.8 respectively. Two liters of molasses with initial concentration of 125 g/L of reducing sugar supplemented with 1 g/L $(NH_4)_2SO_4$ and 1 g/L KH_2PO_4 was added with 10% (w/v) delignified bagasse and inoculated with 10% (v/v) of the seed

culture. The fermentation was conducted in the duration of 48 hours while the first 24 hours was operated with the control agitation speed of 80 rpm and without the agitation for the last 24 hours. Ten milliliter samples were collected at the end of 48 hours for ethanol and sugar analysis. The fermentation was performed in the total of 5 consecutive batches by aspirated out the supernatant and refilled the fermenter with fresh molasses. Ethanol yields ($Y_{P/S}$) and percentage theoretical yield (%) were observed among each batch. The experiment performed in duplicates, the results were analyzed by ANOVA using Duncan's multiple range test for mean comparison with the significant level of $p < 0.5$.

The ethanol produced was analyzed by gas chromatography (GC) (HP Innowax Agilent 6890N), using an Innowax column (29.8 m × 0.25 mm × 0.25 mm) with a flame ionization detector (FID). The column temperature was 150°C, the program run time was 5.5 minute, the ethanol retention time was about 1.9 minute, the carrier gas was nitrogen (16 kPa), the injector temperature was 175°C, the detector temperature was 250°C, the flow rate was 40 ml/min; the split ratio was 1:50, and the velocity of H_2 flow was 60 ml/min, with a sample quantity of 1 ml. One part of the supernatant was filtered through a 0.22 mm cellulose acetate filter prior to GC analysis. Standard ethanol solutions were prepared at 0.1%, 0.3%, 1%, 5% and 10% (v/v), using 95% absolute ethanol. Total sugar concentration was monitored using Fehling titration method [50]. The 20 ml of sample was mixed with 70 ml of distilled water and 5 ml of conc. HCl. The solution was heated at 70°C for 10 minutes. The sample was neutralized by using NaOH solution, then the solution was prepared to the total volume of 100 ml before taken into the burette. The 10 ml of distilled water, 5 ml of Fehling A and 5 ml of Fehling B were mixed in a 200 ml conical flask. After the solution was boiled for 2 minutes, 4 drops of methylene blue were added into the solution before titrated with the prepared sample until the blue color was disappeared. The standard invert sugar was also prepared for standardized Fehling solution. 4.75 g of sucrose was dissolved in 70 ml of distilled water. 5 ml of HCl was added and the solution was heated for 10 minutes. The solution was neutralized with NaOH solution to pH 7 and diluted to the volume of 500 ml with distilled water. 25 ml of the solution was diluted to the total volume of 100 ml using distilled water. Titration of standard invert sugar was done followed the procedure described above.

Analysis of delignified sugarcane bagasse for potential to be animal feed

The spent immobilized yeast adsorbed on sugarcane bagasse was collected after 5 cycles of batch fermentations by discarded the supernatant. Delignified sugarcane bagasse with immobilized cell was oven dried at 60°C for 24 hours. Chemical composition of immobilized yeast cells on delignified sugarcane bagasse was analyzed by Betagro Science Center Co., Ltd using the standard methods; ash (AOAC (2012) 942.05), energy (Compendium of Methods for Food Analysis (2003, Food Composition and Nutrition Labeling Chapter, p 2-9), fiber (Inhouse Method: TI-C00-040 based on AOAC (2012) 978.10), fat (Inhouse Method: TI-C00-015 based on AOAC (2012) 920.39), moisture (ISO 6496 : 1999) and protein (Inhouse Method : TI-C00-016 based on ISO 5983-2 : 2005). The chemical composition of spent immobilized yeast on delignified sugarcane bagasse was compared with the non-delignified sugarcane bagasse [51].

Economic of feasibility analysis

Variable cost of overall process in 3 L packed bed bioreactor was analyzed based on cost of raw material, electricity and water and compared with ethanol price.

RESULT AND DISSCUSSION

Sugarcane bagasse was chosen to be a carrier due to the high water retention, high water content, high water absorption index and low lignin content [32]. These properties could facilitate the adhesion, and supported the growth of microorganisms because they required the water content of 30-80 % for solid-state fermentation and the high water retention offers sugarcane bagasse to absorb more water [32,52]. Sugarcane bagasse has initial water content of 7.77 % (w/w), however, its water content can be reached 84.27 % (w/w) after submerging with water [32,52]. Sugarcane bagasse showed higher water absorption (8.58 g/g) index than other materials such as corn cobs (3.77 g/g), sugar beep pulp (6.59 g/g), loofa sponge (7.76 g/g), and coffee husk (8.30 g/g). This represents the ability of sugarcane bagasse in absorbing high quantity of water than other materials [53]. Lignocellulosic is a main composition for agricultural waste including the sugarcane bagasse. Lignocellulosic materials were composed of cellulose, hemicellulose and lignin. Lignin covers the structures of cellulose and hemicellulose to create a smooth surface that impedes the adhesion of the cells to the material [54]. Thus, removing lignin is a promising technique to increase the microbial adhesion or immobilization to the agricultural waste.

Scanned electron microscope (SEM) illustrated in Figure 2 illustrated the surface structure of delignified sugarcane bagasse. The surface of delignified sugarcane bagasse was rough and has some pores due to lignin removal from alkaline pretreatment.

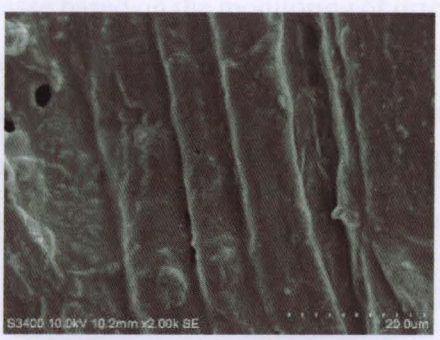


Figure 2 The scanned electron microscope (SEM) represented the surface structure of delignified sugarcane bagasse. The pictures were taken with 2000x magnification on day 1.

Apart of water retention to induce the cell immobilization, the rough surface structure could also induce the cell immobilization. The immobilization abilities of *S. cerevisiae* SC90 on the delignified sugarcane bagasse cultured in the rich medium (YPD) and molasses at different sugar concentration were determined for 3 days based on the dry weight of immobilized cells adsorbed on sugarcane bagasse per dried weight of sugarcane bagasse (g/g) (Figure 3).

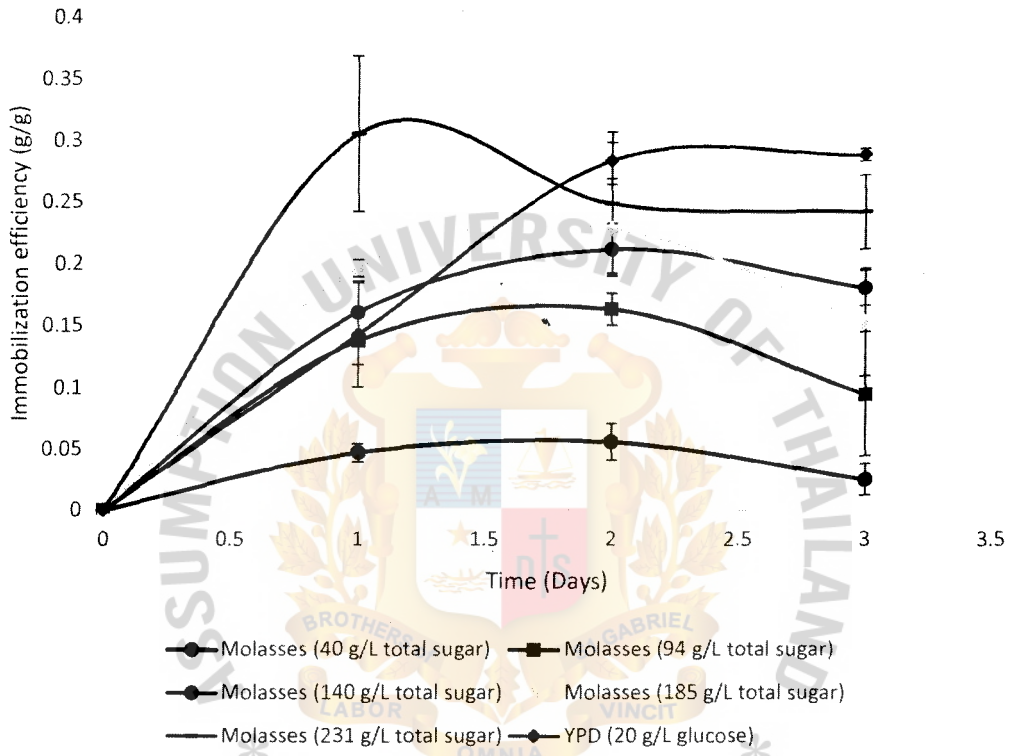
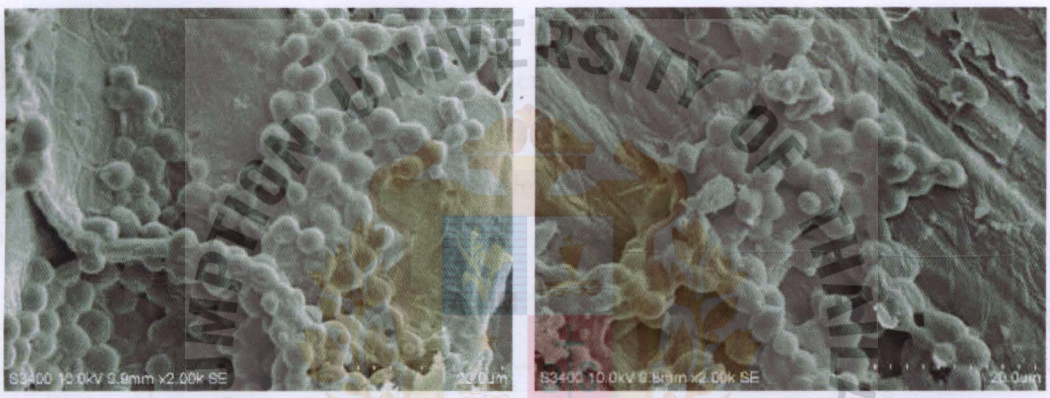


Figure 3 The figure represented immobilization efficiency of *S. cerevisiae SC90* on delignified sugarcane bagasse based on the monitoring of dried immobilized yeast cells per dried weight of sugarcane bagasse (g/g) using Yeast powder dextose medium (YPD) and molasses containing 40 - 231 g/L of total sugar concentration as substrates. The data were analyzed among 3 days of cultivation. Error bar indicates the standard deviation (n=3). Data was compared in each specific enzyme among all conditions [Appx. C-1 – C-3].

According to the dry weight basis, molasses was chosen to be used as a cell immobilization medium. The result showed that immobilized yeast using molasses with 231 g/L total sugar concentration was optimum with the highest weight of 0.31 ± 0.06 g/g when compared with other concentrations of molasses as well as YPD. The

immobilization was also optimized in a short period of time within one day. The efficiency of yeast immobilization was related to the concentration of reducing sugar in molasses, however the excess amount of reducing sugar could inhibit the growth of microorganism [55]. Since molasses was the optimized culture medium for cell immobilization, it would be more benefit in term of avoiding the lag phase time for medium adaptation when immobilization and fermentation media are the same. Therefore, diluted molasses containing 231 g/L of total sugar concentration was selected to be used as an immobilized medium and fermentable medium. Yeast immobilization on delignified bagasse on day 1 when cultured in YPD and molasses were also visualized in Figure 4 (a) and 3 (b).



A B

Figure 4 The scanned electron microscope (SEM) represented the immobilization of *S. cerevisiae SC90* on the delignified sugarcane bagasse when cultured in Yeast Peptone Dextrose (YPD) (A) and immobilization of *S. cerevisiae SC90* on the delignified sugarcane bagasse when cultured in molasses containing 231 g/L total sugar concentration (B). The pictures were taken with 2000x magnification on day 1

Yeast cells homogeneously adhered to the surface of delignified bagasse in the similar pattern when cultured in YPD and molasses. The low level of continuous shaking during the immobilization could lead to better immobilization performance [56]. The adhesion of yeast on delignified agricultural wastes were reported to be depend on electrostatic interactions between the support and the negatively charge cell surface through the physical adsorption [18]. The removal of lignin made the cells accessible to cellulose that contains a large number of hydrophilic groups in the form of positive charge with the absorption of negative charge cells [57]. In the determination of

immobilization condition, the process optimization was 1 day at 30°C with 80 rpm in molasses containing 231 g/L of total sugar concentration.

Yeast immobilization on delignified sugarcane bagasse (IM) and suspended culture or free cell (FC) toward the production of ethanol using 231 g/L of total sugar in molasses supplemented with $(NH_4)_2SO_4$ and KH_2PO_4 as a substrate were compared through the five-cycle of repeated batch process under the shake flask. Ethanol yield ($Y_{P/S}$) and percent theoretical yield were calculated at the end of 48 hours fermentation period (Table 3).

Table 4 The table showed the fermentation efficiency based on ethanol yield ($Y_{P/S}$) and percent of theoretical yield (%) of five cycle repeated batch fermentation using suspended cells or free cell system (FC) and immobilized yeast cells (IM) under shake flask with total volume of 50 ml. The fermentation was set at 30 °C for 48 hours. Data represents the mean of three replicates. Based on ANOVA, a, b, and c had the three highest activity levels. The different letters represent a significant difference of $p < 0.05$ [Appx. C-4 – C-5].

Batch		**$Y_{P/S}$ (g/g)**	**Percent of theoretical yield (%)**
1	FC	0.36 ± 0.02^{cd}	70.58
	IM	0.40 ± 0.01^{abc}	78.43
2	FC	0.33 ± 0.02^{d}	64.71
	IM	0.42 ± 0.03^{ab}	82.35
3	FC	0.34 ± 0.04^{d}	66.67
	IM	0.42 ± 0.03^{ab}	82.35
4	FC	0.39 ± 0.04^{abcd}	76.47
	IM	0.43 ± 0.05^{a}	84.31
5	FC	0.37 ± 0.06^{bcd}	72.55
	IM	0.43 ± 0.03^{a}	84.31

The ethanol yield ($Y_{P/S}$) obtained from immobilized yeast was continuously increased and maximized to the level of 0.43 g/g (84.31% theoretical yield) in the fourth and fifth batch. The high fermentation rate of immobilized yeast over suspended cells could be resulted by high cell density and viability of cells on the carrier. The cell density of immobilized yeast could be maintained in the system while some cell mass could be lost during media replacement in suspended cell system [32]. The carrier also acted as a protective barrier against extreme environmental condition which causes by pH, temperature, toxic chemicals in medium, high concentration of substrate whereas the suspended cells might utilize some sugar for survival instead of producing ethanol [58]. The proteins and minerals released from the carrier into the media favored the yeast growth and enhance the ethanol productivity [54]. The maximum bioethanol yield obtained from immobilized yeast on sugarcane bagasse three times higher than free cells which related to the maximum concentration of total cells, obtained in the immobilized cells system, was about 1.3 times higher than the concentration of cells in a free cells system [32]. By using silk cocoon as a carrier and blackstrap molasses as a substrate, the ethanol concentration was maintained through five cycles batches in the immobilized cells and ethanol yield was also higher than suspended culture in all batches. This could produce the maximum ethanol yield of 91% theoretical yield [14]. Immobilized yeast on corn stem ground tissue was investigated to increase ethanol yield and decrease amount of residual sugar in ethanol fermentation comparing to suspended cell system [42].

We also carried five-repeated cycles fermentation in 3L stirred tank bioreactor (GBJX-5C, Zhenjiang) using molasses with 231 g/L total sugar concentration as substrate. *S. cerevisiae* SC90 was immobilized on delignified sugarcane bagasse for 24 hours with 80 rpm agitation and further fermented for 24 hours with no agitation.

The result from table 4 represented fermentation efficiency of immobilized *S. cerevisiae* SC90 which could be remained at least 3 consecutive batches with the ethanol yield ($Y_{P/S}$) 0.41-0.42 g/g and 80-82% theoretical yield.

Table 5 The table represented the fermentation efficiency of immobilized yeast cells on delignified sugarcane bagasse under five cycle repeated batch fermentation that was carried out in 3 L stirred tank bioreactor. The fermentation was set at 30 °C, pH 4.8 for 48 hours. Data represents the mean of three replicates. Based on ANOVA, a,

b, and c had the three highest activity levels. The different letters represent a significant difference of $p < 0.05$ [Appx. C-6].

Batch	$Y_{P/S}$ (g/g)	Percent of theoretical yield (%)
1	0.41 ± 0.04^{a}	80.39
2	0.42 ± 0.02^{a}	82.35
3	0.41 ± 0.04^{a}	80.39
4	0.34 ± 0.03^{b}	66.67
5	0.38 ± 0.02^{ab}	74.51

The ethanol yield was significantly dropped in the fourth batch to 0.34 g/g (67% theoretical yield) expecting that it was resulted from cell detachment from the support. However, the ethanol yield was risen up again in the fifth batch to 0.38 g/g (75% theoretical yield) that may cause by the resuming of cell growth and colonization on delignified sugarcane bagasse. By comparing the result obtained from shake flask and fermenter, the ethanol yield ($Y_{P/S}$) from fermenter was higher than obtained from shake flask under the well-controlled condition in the fermenter. The future prospects for the bioreactor is to be designed properly to facilitate the translocation of carrier in and out of bioreactor and mass transfer between immobilized cells and substrate [59]. Stirred tank bioreactor gave an advantage in good homogenization between substrate and carrier. Although the larger scale of fermentation and high compaction of delignified sugarcane bagasse in the molasses might require high speed agitation to obtain well mixed condition which would result in cell damage due to high sheared force of the impeller. Stirred tank bioreactor was successfully developed into packed bed bioreactor to be used in glycerol production by immobilized *S. cerevisiae* using sintered glass Raschig rings as a carrier packed in the stainless steel basket [60]. This could prevent direct contact between immobilized cell and impeller. In addition, sieve plate baffles in bioreactor was designed by Ramakrishna et al. (1988) to reduce the compaction problem of the carrier and increase the cross-sectional area of immobilized cells to be easily contact with the substrate [61]. .

In this research we also analyzed on the potential of using spent immobilized yeast cells on delignified sugarcane bagasse as an animal feed supplement. Ruminants could digest only 25-35 % (w/w) of lignocellulosic feed because of its complex structure [19]. Therefore, various methods were tested to increase digestibility of sugarcane bagasse [43]. By using as NaOH and lime as alkaline treatment, amount of lignin in sugarcane bagasse could be lost by 41.2 % and cellulose component in delignified sugarcane was also increased by 8.9 times. This enhanced the digestibility of sugarcane bagasse for ruminants as the ruminal enzyme could easily access the polysaccharide parts of the bagasse. [19]. Table 5 summarized and compared the chemical composition of delignified sugarcane bagasse covered by immobilized yeast, delignified and non-delignified sugarcane bagasse [51].

Table 6 Composition analysis of spent immobilized yeast cells adsorbed on delignified sugarcane bagasse comparing to delignified sugarcane bagasse and non-delignified sugarcane bagasse [51].

Composition	Amount of compound in non-delignified sugarcane bagasse (per 100 g) [51]	Amount of compound in delignified bagasse (per 100 g)	Amount of compound in Spent immobilized cells on delignified bagasse (per 100 g)
Ash	2.3 – 13.7 g	2.8 g	8.71 g
Energy	363.05 - 444.25 Kcal	353.99 Kcal	333.40 Kcal
Fiber	30.2 – 61.3 g	59.53 g	30.93 g
Fat	0.3 – 3.9 g	0.59 g	0.68 g
Moisture	0.8 – 17.2 g	9.44 g	8.79 g
Protein	0.8 – 4.9 g	1.29 g	8.01 g

The result showed that delignified sugarcane bagasse had crude protein higher than delignified and non-delignified sugarcane bagasse. The crude protein was obtained from immobilized yeast cells. Therefore, the spent immobilized yeast cells with the delignified sugarcane bagasse can be further used as protein enriched (SCP production) animal feed. Molasses contained mineral and trace elements including vitamins of B complex which increase yield of milk production in cow and improve wool quality in

sheep. The remaining sugar from molasses that adhered on the fiber could enhance the digestive activity of ruminants as it was utilized by ruminal microorganisms [62]. However, further in situ investigation is necessary to analyze digestibility and toxicity in the animals.

Economic feasibility was analyzed based on the cost-benefit analysis which is classified into cost analysis and benefit analysis. In cost analysis, there are two factors which is investment cost and operating cost. Investment cost is expenses for land, plant equipment, labor, warehouse, site development etc. Operating cost is divided into fixed cost and variable cost. Fixed cost expenses the labors, insurance, maintenance and repair. Variable costs are feedstock cost, water, gas, electricity, waste disposal and other utilities. The benefit analysis is also separated into financial benefit and economic benefit. Financial benefits refer to the revenue from selling ethanol product while economic benefit describes the effect of ethanol price to other fuel product such as MTBE or gasoline [63]. In this research, variable cost was focused since the ethanol production was done in laboratory scale. Total ethanol concentration after five repeated batch was approximately 0.62 L which it could be sold at 15.67 Baht as selling price of ethanol is 25.12 Baht/L. However, the production cost in laboratory scale was very high (4536.68 Baht). Variable cost of ethanol production in Figure 6 summarized the percentage of variable cost of ethanol production in 3 L packed bed bioreactor.

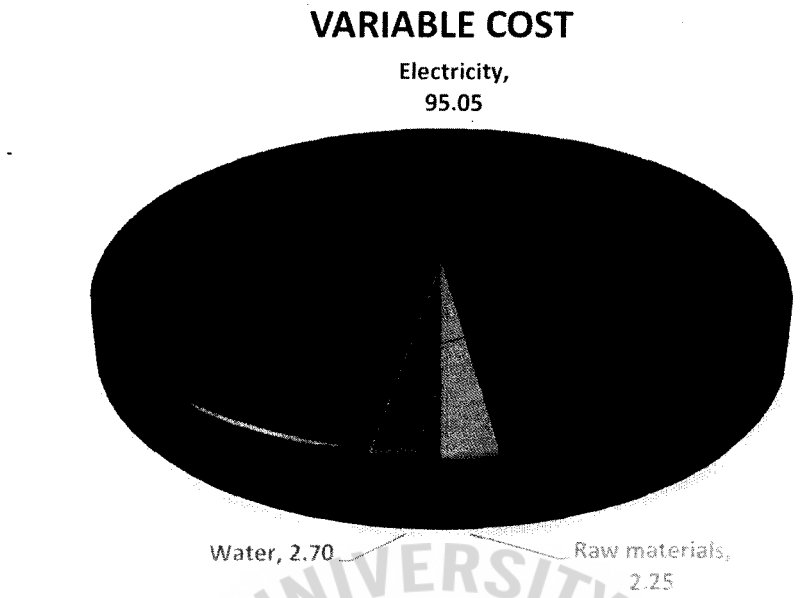


Figure 5 Percentage of variable cost from ethanol production using immobilized yeast in 3 L bioreactor [Appx. C-9]

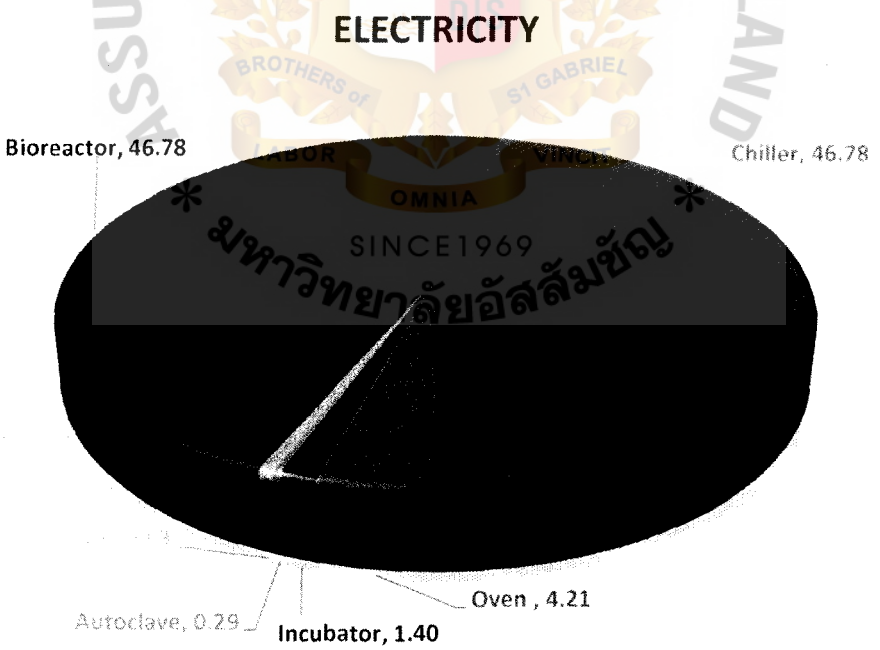


Figure 6 Percentage of electricity used in the ethanol fermentation process [Appx. C-8]

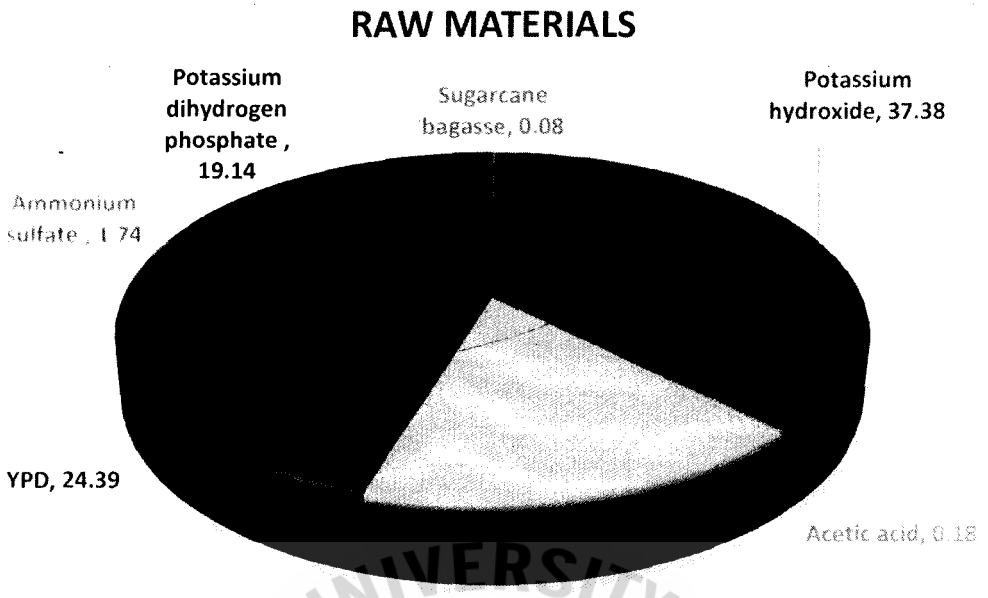


Figure 7 Percentage of raw material cost in the ethanol fermentation process [Appx. C-7]

Most expense of variable cost was electricity used by bioreactor and chiller (Figure 7). The equipment was used to control the fermentation condition. The cost of cooling process could be eliminated by using thermotolerant microorganisms which could grow at 40 – 45 °C with 28 to 67.8 g/L of ethanol production [64]. In figure 8, potassium hydroxide became the major component of raw material cost followed by molasses as the amount of alkalis affected the quality of pretreatment process. Moreover, the chemical recovery system should be constructed as the black liquor obtained from pretreatment process should be concentrated and treated before disposed of. Though, the capital investment could be reduced by co-operated with paper mills which already imply the chemical recovery system [65].

CONCLUSION

Batch and repeated batch of immobilized *S. cerevisiae* SC90 on delignified sugarcane bagasse has been demonstrated in this study. The results of this study mainly demonstrated the potential applications of immobilized yeast cells on delignified sugarcane bagasse in the ethanol production from molasses under the repeated batches. Immobilized biocatalysts represented high fermentation ability and high stability among five repeated batches. In addition, immobilization reduced the complexity of the cell separation to inoculate to the following batches. Also, the spent yeast immobilized on delignified sugarcane bagasse had the composition that could be served as animal feed. The immobilization and fermentation technologies described in this study could be further applied with other agricultural supports with other industrial fermentations to reduce the cost of production and maximized the productivity.

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APPENDIX A

Media Preparation

Yeast Extract Dextrose medium (YPD)

- 20 g glucose
- 10 g yeast extract
- 20 g peptone

Adjust pH to 4.8 by H_2SO_4 before sterilized at 121°C, 15 minutes

Diluted Molasses

Total sugar in molasses was measured by Fehling titration method. The total sugar in molasses was diluted out using distilled water to get specific sugar concentration. Dilute molasses was supplement with 1 g/L $(NH_4)_2SO_4$ and 1 g/L KH_2PO_4 and pH of media was adjusted to 4.8 using H_2SO_4 before sterilized at 121°C, 15 minutes

Chemical Preparation

(1) Standard invert sugar solution

Accurately weigh 4.75 g of sucrose, transfer with 70 mL of water. Add 5 mL of hydrochloric acid and heated at 70°C for a period of 10 min. The solution was neutralized with 6 N sodium hydroxide aqueous solution to pH 7, and dilute to volume of 100 ml with water.

Transfer a 25 mL portion of the solution above to a 100 mL volumetric flask, neutralize with 6 N sodium hydroxide aqueous solution to pH 7, and dilute to volume with water.

Use the solution as standard invert sugar solution for the standardization of Fehling's Solution.

(2) 1% Methylene Blue solution

Dissolve 1 g of methylene blue in water to make 100 mL.

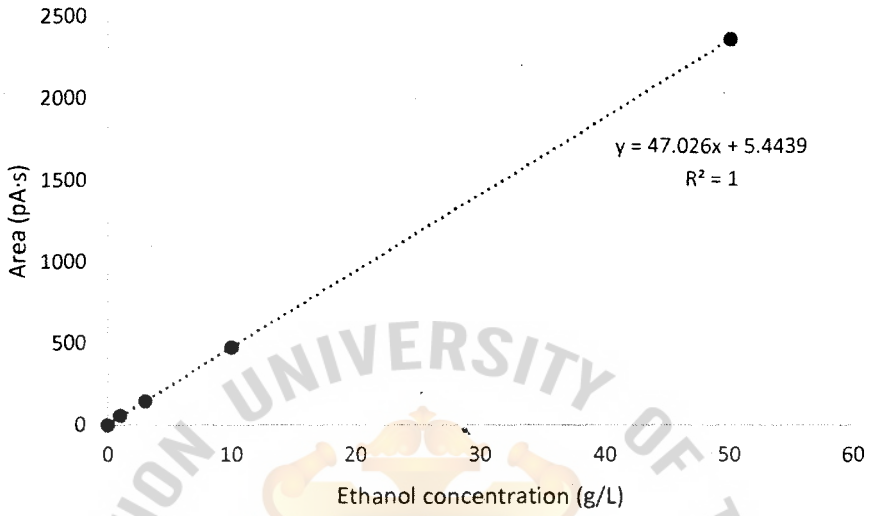
(3) Fehling's Solution

Solution A: Dissolve 34.639 g of copper sulfate ($CuSO_4 \cdot 5H_2O$) in water to make exactly 500 mL, leave it for two days, and then filter.

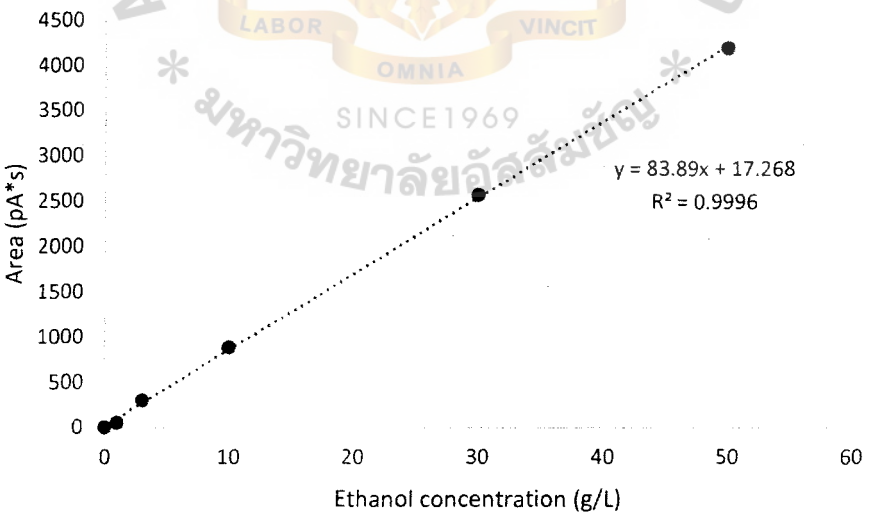
Solution B: Dissolve 173 g of potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) and 50g of sodium hydroxide in water to make exactly 500 mL, leave it for two days, and then filter.

APPENDIX B

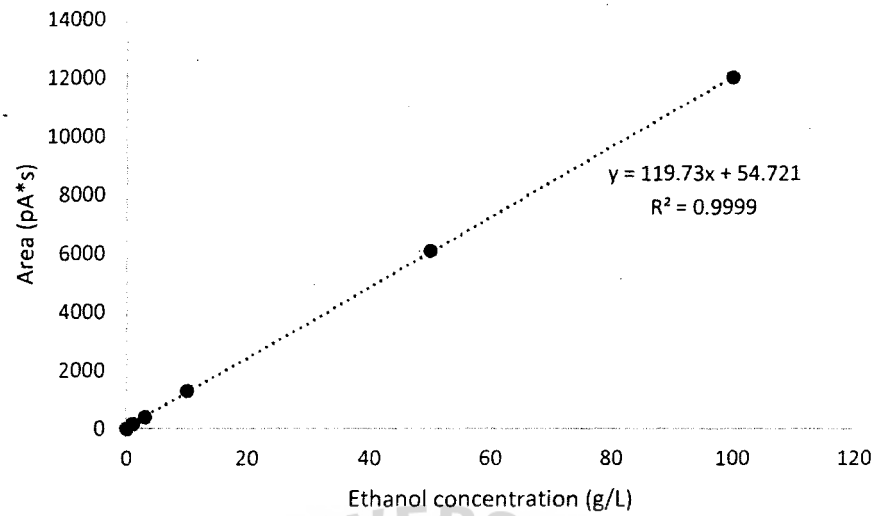
Ethanol standard curve



Appx. B-1 The ethanol standard curve for ethanal production from free cell and immobilized cell system under shake flask condition



Appx. B-2 The ethanol standard curve of ethanol production from immobilized cell system in 3L packed bed fermenter, first replicate



Appx. B-3 The ethanol standard curve of ethanol production from immobilized cell system in 3L packed bed fermenter, second replicate

APPENDIX C

Appx. C-1 The table represented immobilization efficiency of *S. cerevisiae SC90* on delignified sugarcance bagasse based on the monitoring of dried immobilized yeast cells on delignified sugarcane bagasse per dried weight of sugarcane bagasse (g/g) using Yeast powder dextrose (YPD) and diluted molasses (40 g/L total sugar concentration)

Medium	Day	Rep	Total DW	DW of flask (g)	DW of bagasse (g)	DW of immobilized cell (g)	DW of immobilized cell/ DW of bagasse (g/g)	Average	SD
YPD	1	1	44.89	43.59	1.09	0.20	0.19	0.14	0.04
		2	41.77	40.56	1.09	0.11	0.10		
		3	41.69	40.46	1.09	0.15	0.14		
	2	1	45.40	44.03	1.08	0.29	0.27	0.28	0.01
		2	40.81	39.45	1.07	0.30	0.28		
		3	45.48	44.14	1.03	0.31	0.30		
	3	1	45.64	44.31	1.02	0.30	0.29	0.29	0.01
		2	86.22	83.61	2.03	0.58	0.29		
		3	95.38	92.80	2.01	0.57	0.29		
40 g/L total sugar of molasses	1	1	45.31	44.21	1.06	0.04	0.04	0.05	0.01
		2	41.38	40.30	1.02	0.05	0.05		
		3	41.32	40.25	1.02	0.05	0.05		
	2	1	44.72	43.61	1.07	0.04	0.04	0.06	0.01
		2	45.42	44.31	1.04	0.07	0.07		
		3	42.00	40.91	1.03	0.06	0.06		
	3	1	41.39	40.32	1.02	0.04	0.04	0.03	0.01
		2	45.68	44.66	1.01	0.02	0.02		
		3	40.99	39.94	1.03	0.02	0.02		

Appx. C-2 The table represented immobilization efficiency of *S. cerevisiae SC90* on delignified sugarcance bagasse based on the monitoring of dried immobilized yeast cells on delignified sugarcane bagasse per dried weight of sugarcane bagasse (g/g) using diluted molasses (94 g/L and 140 g/L total sugar concentration)

Medium	Day	Rep	Total DW	DW of flask (g)	DW of bagasse (g)	DW of immobilized cell (g)	DW of immobilized cell/ DW of bagasse (g/g)	Average	SD
94 g/L total sugar of molasses	1	1	41.87	40.72	1.03	0.13	0.12	0.14	0.02
		2	41.59	40.34	1.08	0.17	0.16		
		3	40.27	39.10	1.04	0.13	0.13		
	2	1	40.51	39.32	1.02	0.17	0.16	0.16	0.01
		2	43.62	42.40	1.04	0.18	0.18		
		3	45.43	44.17	1.09	0.16	0.15		
	3	1	41.20	40.02	1.03	0.16	0.15	0.09	0.05
		2	40.62	39.52	1.03	0.07	0.07		
		3	41.30	40.17	1.07	0.06	0.06		
140 g/L total sugar of molasses	1	1	41.20	39.98	1.04	0.18	0.18	0.16	0.03
		2	41.26	40.12	1.01	0.13	0.13		
		3	40.79	39.60	1.01	0.18	0.17		
	2	1	43.65	42.39	1.03	0.23	0.22	0.21	0.02
		2	43.73	42.50	1.04	0.20	0.19		
		3	44.26	43.03	1.00	0.23	0.23		
	3	1	45.47	44.21	1.07	0.19	0.18	0.18	0.01
		2	41.21	39.98	1.04	0.19	0.18		
		3	41.42	40.19	1.06	0.16	0.15		

Appx. C-3 The table represented immobilization efficiency of *S. cerevisiae SC90* on delignified sugarcance bagasse based on the monitoring of dried immobilized yeast cells on delignified sugarcane bagasse per dried weight of sugarcane bagasse (g/g) using diluted molasses (185 g/L and 231 g/L total sugar concentration)

Medium	Day	Rep	Total DW	DW of flask (g)	DW of bagasse (g)	DW of immobilized cell (g)	DW of immobilized cell/ DW of bagasse (g/g)	Average	SD
185 g/L total sugar of molasses	1	1	40.81	39.58	1.03	0.20	0.19	0.20	0.01
		2	41.34	40.07	1.06	0.20	0.19		
		3	45.80	44.52	1.07	0.22	0.20		
	2	1	45.08	43.84	1.03	0.20	0.19	0.23	0.04
		2	41.17	39.90	1.04	0.24	0.23		
		3	45.29	44.02	1.00	0.26	0.26		
	3	1	40.30	39.04	1.05	0.21	0.20	0.15	0.04
		2	44.87	43.72	1.03	0.12	0.11		
		3	44.87	43.68	1.04	0.15	0.15		
231 g/L total sugar of molasses	1	1	41.02	39.72	1.05	0.25	0.23	0.31	0.06
		2	45.16	43.77	1.03	0.36	0.35		
		3	44.98	43.61	1.04	0.34	0.33		
	2	1	45.20	43.89	1.00	0.31	0.31	0.25	0.06
		2	41.19	39.91	1.08	0.20	0.19		
		3	41.33	39.99	1.07	0.27	0.25		
	3	1	45.29	43.95	1.05	0.29	0.28	0.24	0.03
		2	41.27	39.96	1.07	0.24	0.23		
		3	40.97	39.73	1.02	0.23	0.22		

Appx. C-4 The table showed the ethanol yield ($Y_{P/S}$) and percent of theoretical yield (%) of five cycle repeated batch fermentation using suspended cells or free cell system (FC) of *S. cerevisiae SC90* under shake flask with total volume of 50 ml. The fermentation was set at 30 °C for 48 hours.

Cell system	Batch	Rep	Glucose consumption (g/L)	Ethanol production (g/L)	$Y_{P/S}$	Mean	SD	Percent conversion of sugar into ethanol (%)	Mean	SD
FC	1	1	208.46	69.75	0.33	0.36	0.02	64.71	69.28	4.93
		2	187.07	71.13	0.38			74.51		
		3	190.48	67.23	0.35			68.63		
	2	1	235.10	77.98	0.33	0.33	0.02	64.71	64.71	3.92
		2	216.62	76.65	0.35			68.63		
		3	222.80	68.27	0.31			60.78		
	3	1	148.28	56.31	0.38	0.34	0.04	74.51	65.36	6.89
		2	163.42	50.16	0.31			60.78		
		3	169.85	58.47	0.34			66.67		
	4	1	214.64	74.14	0.35	0.39	0.04	68.63	75.82	7.92
		2	198.21	75.94	0.38			74.51		
		3	173.34	75.40	0.43			84.31		
	5	1	193.22	77.48	0.40	0.37	0.02	78.43	71.90	5.66
		2	210.83	73.73	0.35			68.63		
		3	208.32	73.75	0.35			68.63		

Appx. C-5 The table showed the ethanol yield ($Y_{P/S}$) and percent of theoretical yield (%) of five cycle repeated batch fermentation using suspended cells or free cell system (FC) of *S. cerevisiae SC90* under shake flask with total volume of 50 ml. The fermentation was set at 30 °C for 48 hours

Cell system	Batch	Rep	Glucose consumption (g/L)	Ethanol production (g/L)	$Y_{P/S}$	Mean	SD	Percent conversion of sugar into ethanol (%)	Mean	SD
IM	1	1	198.16	82.04	0.41	0.40	0.02	80.39	79.08	4.08
		2	197.93	82.29	0.42			82.35		
		3	211.77	80.97	0.38			74.51		
	2	1	225.30	92.31	0.41	0.42	0.02	80.39	83.01	4.53
		2	226.15	93.43	0.41			80.39		
		3	203.28	90.53	0.45			88.24		
	3	1	182.07	72.84	0.40	0.42	0.03	78.43	81.70	5.66
		2	184.23	73.87	0.40			78.43		
		3	174.97	79.19	0.45			88.24		
	4	1	182.79	86.88	0.48	0.43	0.05	94.12	84.31	9.80
		2	218.24	83.55	0.38			74.51		
		3	193.44	83.85	0.43			84.31		
	5	1	218.33	86.59	0.40	0.43	0.03	78.43	83.66	4.93
		2	197.22	89.15	0.45			88.24		
		3	195.37	83.52	0.43			84.31		

Appx. C-6 The table represented the fermentation efficiency of immobilized *S. cerevisiae SC90* on delignified sugarcane bagasse under five cycle repeated batch fermentation that was carried out in 3 L stirred tank bioreactor.

Batch	Rep	Dup	Glucose consumption (g/L)	Ethanol production (g/L)	$Y_{P/S}$	Mean	SD	Percent conversion of sugar into ethanol (%)	Mean	SD
1	1	1	215.98	82.07	0.38	0.41	0.04	74.51	81.37	8.08
		2	204.88	77.86	0.38			74.51		
	2	1	188.41	82.90	0.44			86.27		
		2	185.19	85.19	0.46			90.20		
2	1	1	196.32	80.49	0.41	0.42	0.02	80.39	82.84	2.94
		2	198.35	81.32	0.41			80.39		
	2	1	210.33	90.44	0.43			84.31		
		2	212.59	93.54	0.44			86.27		
3	1	1	197.45	86.88	0.41	0.41	0.04	86.27	81.37	8.08
		2	206.69	95.08	0.46			90.20		
	2	1	214.90	81.66	0.38			74.51		
		2	210.56	80.01	0.38			74.51		
4	1	1	228.05	72.98	0.32	0.34	0.03	62.75	66.67	5.31
		2	230.04	75.91	0.33			64.71		
	2	1	207.83	68.58	0.33			64.71		
		2	205.53	78.10	0.38			74.51		
5	1	1	235.52	87.14	0.37	0.38	0.02	72.55	74.51	3.92
		2	228.68	93.76	0.41			80.39		
	2	1	222.60	82.36	0.37			72.55		
		2	225.08	83.28	0.37			72.55		

Appx. C-7 Raw materials cost for ethanol production for 5 consecutive batches using immobilized yeast in 3 L packed bed bioreactor

Raw Materials	Cost per unit (Baht)	Quantity per unit (kg or L)	Amount of usage in the process (kg or L)	Cost per process (baht)	Percentage (%)
Sugarcane bagasse	400	1000	0.2	0.08	0.10
Potassium hydroxide	4784.64	2.5	0.02	38.28	47.25
Acetic acid	6067.42	1	0.00003	0.18	0.22
Molasses	3500	1000	5	17.50	21.60
YPD	124.86	1	0.2	24.97	30.82
Ammonium sulfate	80.00	0.45	0.01	1.78	1.74
Potassium dihydrogen phosphate	980.00	0.50	0.01	19.60	19.14
			Total	102.39	100.00

Appx. C-8 Electricity usage of equipments in ethanol production for 5 consecutive batches using immobilized yeast in 3 L packed bed bioreactor

Equipment	Brand	Voltage (V)	Frequency (Hz)	Power (kW)	Time (hours)	Unit	Percentage (%)
Oven	Memmart ULP400	230	60	1.8	24	43.2	4.21
Incubator	JSR-JSSI-100C	220	50	1.2	12	14.4	1.40
Autoclave	Hirayama HA300 MII	220	50/60	2	1.5	3	0.29
Water bath	Memmart WNB 14	230	50/60	1.8	3	5.4	0.53
Bioreactor	GBJX-5 C	220	50/60	2	240	480	46.78
Chiller		221	50/61	2	240	480	46.78
			Total	10.8		1026	100.00

Appx. C-9 Total variable cost for ethanol production for 5 consecutive batches using immobilized yeast in 3 L packed bed biorector

Operational cost	Cost (Baht)	Percentage (%)
Water	123.21	2.70
Electricity	4332.46	95.05
Raw materials	102.39	2.25
Total	**4558.06**	**100.00**

APPENDIX D

Calculation

Fehling titration

$$Total\ sugar\ (\%gm) = \frac{5.128}{Dilution\ factor \times Fehling\ factor \times titrate\ value}$$

$$Fehling\ factor = \frac{20.36}{A}$$

A = Volume of the standard invert sugar solution required (ml)

Fermentation efficiency

$Glucose\ consumption\ (g/L) = G_I - G_F$

G_I = Glucose concentration at time 0

G_F = Glucose concentration after 48 hours fermentation

$Ethanol\ production\ (g/L) = EtOH_F - EtOH_I$

$EtOH_I$ = Ethanol concentration at time 0

$EtOH_F$ = Ethanol concentration after 48 hours fermentation

$$Ethanol\ yield\ (g/g) = \frac{Glucose\ consumption\ (g/L)}{Ethanol\ production\ (g/L)}$$

$$Percent\ conversion\ of\ sugar\ to\ ethanol\ (\%) = \frac{Ethanol\ yield\ (g/g)}{Theoretical\ yield\ (0.51\ g/g)} \times 100$$

$$Approx.\,Ethanol\ production\ per\ batch\ (L) = EtOH \times V_M \times \rho_{EtOH}$$

EtOH = Ethanol production (g/L)

VM = Volume of molasses per batch = 2 L

ρ_{EtOH} = Density of Ethanol = 789 g/L

$$Total\ approx.\,Ethanol\ production\ per\ batch\ (L) = EtOH_{B1} + \cdots + EtOH_{B5}$$

$EtOH_{B1}$= Approx. Ethanol production from 1st batch

$$Variable\ cost\ (Baht) = R\ + E\ + W$$

R = Raw material cost (Baht)

E = Electricity (Baht)

W = Water (Baht)