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Fuel Ethanol Production from Papaya Waste using Immobilized *Saccharomyces cerevisiae*

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Liquid biofuels such as bioethanol is gaining much interest as it can be produced from various biomass feedstocks. Papaya peels, one of the major agricultural waste in Malaysia has immense potential to be used as a promising bioethanol feedstock. Thus, the main objective of this research is to optimize the production of bioethanol from *Carica* papaya peels using immobilized yeast cells. At first, the papaya skin was dried and powdered prior to hydrolysis at 120˚C for 15 minutes using 0.2 M H_2SO_4. Then *Saccharomyces cerevisiae* Type II strain was immobilized using 12% polyvinyl alcohol and 1% sodium alginate using entrapment technique. These immobilized beads were later employed for the production of bioethanol from dried papaya peels. Most significant parameters such as temperature, agitation speed, pH and fermentation time were optimized by employing batch fermentation and bioethanol produced was quantified using Gas Chromatography-Mass Spectrometry. A bioethanol yield of 0.514 g/L was obtained from papaya peels at the optimized conditions of 30˚C, 200 rpm, pH 5 and 48 h of fermentation. In short, since the sugars can be easily released from papaya skin, this can be considered as a potential feedstock for bioethanol**.**

Keywords: Papaya, *Saccharomyces cerevisiae*, Immobilization, Fermentation, Bioethanol

I. INTRODUCTION

Malaysia is a developing country in Southeast Asia region that well known globally for its richness of sources that mainly exploited for energy production. In this country, majority of the energy produced was consumed for transportation and industrial sectors (Tye *et al.*, 2011). Among the existed sources, petroleum, coal and natural gas are the main choice that employed to meet the energy demand for the entire nation (Mahy, *et al.* 2013)

However, for the past several decades, it was realized that the petroleum reserve in Malaysia showed decreasing trend in its production (Shaikh, *et al.*, 2017). Furthermore, the utilization of this petroleum sources especially for transportation sector contributes to the tremendous increase of air pollution in Malaysia (Aditiya, *et al.*, 2016). One of the most viable alternatives to solve the problem is the production of biofuels specifically bioethanol that can be employed in vehicles engine whether on its own or blended with petrol fuel. This fuel

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will reduce the air pollution level because it will burn completely in air and emit small amount of carbon dioxide to environment (Ahmad, *et al.*, 2016).

Bioethanol can be produced from varieties of renewable sources such as sugar, starch, lignocellulosic biomass as well as marine organisms. Papaya peels (fruit waste) that generated from agricultural activities has a high potential to be converted into many useful and high value-added product including bioethanol (Gebregergs *et al.*, 2016). In this case, Malaysia holds a promising potential for sustainable production of bioethanol derived from papaya peels as this country is blessed with biodiversity richness (Ozturk *et al.*, 2017). The high sugars content in papaya peels make it served as superior feedstock for bioethanol production as it can be easily converted into simple sugars by invertase enzyme present in *S. cerevisiae*.

Currently, about 50% of world ethanol supply is produce through free cell fermentation, but the productivity is very low in conventional batch processes. Alternately, employing cell immobilization method can increase the fermentation productivity. When compared with free cells, cell immobilization has several advantages such as prevention of microorganism leakage, high cell loading, high yield, low risk of contamination and also protect microorganism from environmental stress (Balat & Balat, 2009).

Entrapment in PVA-alginate is the most widely used procedure for *S. cerevisiae* immobilization. Fermentation of sugar source from papaya peels for production of fuel ethanol by using immobilized *S. cerevisiae* must considers various factors including pH, temperature, PVA alginate concentration, inoculum size and bead diameter. Hence, this study is aimed to optimize the production of bioethanol from papaya peels using immobilized yeast cells (*S. cerevisiae* Type II).

II. MATERIALS AND METHODS

A. Collection and Preparation of Papaya Peels

Carica papaya peels were collected from local market in Sabah. The papaya peels were prepared based on method studied by Vaitheki and Deepa (Vaitheki & Deepa 2016). The peels were washed with distilled water until it clean and dust free. Then, it was oven dried at 70 ˚C for 24 h. After drying, the dried peels were ground using a blender to a powder form. The powdered papaya peels were then stored in a sealed plastic bag until further use. Figure 1 shows the papaya peels that were prepared.

Figure 1. Papaya peels, A) Oven dried peels and B) Powder form of papaya.

B. Thermal Pretreatment

The 10 g of papaya peel powder was added in 100 mL of distilled water with a solid to liquid ratio of 10% (w/v). Then, it was autoclaved at 121°C for 15 min. The thermal pretreated samples were cool down at room temperature. The extract was then filtered using a muslin cloth to get a pure solution. This papaya hydrolysate was used for acid hydrolysis process.

C. Acid Hydrolysis

Acid hydrolysis was done on the papaya hydrolysate. First, 10 mL of 0.2 M sulphuric acid (H_2SO_4) was added to 100 mL of pretreated papaya hydrolysate. Then, the solution was autoclaved at 121°C for 15 min. The solution was cool at room temperature and then centrifuge at 10,000 rpm for 15 min. The pellet was discarded and pH of the hydrolysate was adjusted to 5. The sugar content of the hydrolysate was analyzed using dinitrosalicyclic acid (DNS) method.

D. Quantification of Reducing Sugar Glucose

Dinitrosalicyclic acid (DNS) assay was used for quantitative determination of the reducing sugar. DNS reagent and 100 mL of 0.1% of glucose stock solution was prepared. DNS reagent of 1 mL was added to 1 mL of distilled water in the test tubes before covered with aluminium foil. Then, the test tubes were placed in boiling water for 5 min and the absorbance was measured at 575 nm.

Standard curve was constructed based on the absorbance reading against the glucose concentration. For glucose determination in sample, 1mL of sample and 1 mL of DNS reagent was added into the test tube. Then, test tube was kept in boiling water bath for 5 min after covered with aluminium foil. After that the absorbance was measured at wavelength of 575 nm. Absorbance reading was compared with the standard curve and concentration of the glucose in the sample was determined.

E. Immobilization of S. cerevisiae into PVA-alginate

For cell immobilization, method of entrapment was used. The beads were prepared based on a study conducted by Zain (Zain, 2009). The optimized bead size and PVA-to-alginate concentrations were used in the immobilization of *S. cerevisiae* Type II. Based on research done by Adriana *et al.* and Zhan *et al.*, the optimum bead size for maximum ethanol production is 3 mm and the optimum PVA-to-alginate concentration is 12% of PVA and 1% for Na-alginate (Adriana *et al.*, 2010; Zhan *et al.*, 2013). First, 100 mL solution of 12% PVA and 1% of Na-alginate was prepared by heating at 70°C. PVA (12 g) and Na-alginate (1 g) was added to distilled water. The solution was continuously stirred at 70°C and then autoclaved at 121°C for 15 min.

Next, 100 mL of 5% (w/v) boric acid (H_3BO_3) and 2% (w/v) calcium chloride ($CaCl_2$)

was prepared and autoclaved. The pellet obtained from previous stage was transferred into PVA-alginate solution. The PVA-alginate solution was drop gently into the 100 mL solution of H_3BO_3 and $CaCl_2$ using a syringe needle (21G x 3 cm) to form beads. The beads formed from the entrapment method were stirred for 30 to 50 min. Then, the beads were stored at 4 °C for 24 h before wash with 7.0% (w/v) H_3BO_3 solution for 30 min and another 30 min in sodium sulphate (Na_2SO_4) solution. Finally, the beads were kept at 4°C in distilled water until further use.

F. Optimization of Fermentation for Bioethanol Production

A total of 12 experiments were done with its respective parameters. The parameters selected were pH (3-5), temperature (25°C-35°C), agitation rate (150-250 rpm), and fermentation time (48h-72 h). The baseline is set at pH 5, temperature at 30°C, agitation rate at 200 rpm, and fermentation time of 48 h. Batch fermentation was carried out for the optimization process (Zain, 2009). The 100 mL of papaya hydrolysate and 10 g of PVA-alginate beads were added and incubated in the shaker at a different speed, temperature, pH and time. At the end of the fermentation process, each sample was centrifuged at 10,000 rpm for 15 min. The supernatant was used for distillation process. Distillate obtained was analyzed for bioethanol determination using GC-MS.

G. Determination of Bioethanol

The bioethanol content from the sample was analysed using GC-MS which equipped with a thermal conductivity detector (TCD) and HP-5 MS column (0.25 mm x 30 mm x 0.25 μm ID). The sample (1.0 μl) was injected into the GC-MS in split with a split ratio of 100:1. Helium gas with 99.995% purity was used as the carrier gas and the flow rate was set as 10 ml/min. The initial temperature of the oven used was 40°C which increased at a rate of 10°C/min until 100°C. Acetone was used as a solvent.

III. RESULTS AND DISCUSSIONS

A. Papaya Peel Samples

In the preparation of papaya peels, it was oven dried at 70°C. This is to prevent contamination from occur in order to obtain a higher bioethanol yield. Then, the peels were ground into a powder form which is useful for acid hydrolysis process as it has a larger surface area. There are few studies done to determine the effect of sample preparation on bioethanol production. It is reported that the oven dried banana peels produced higher ethanol yield compared to a fresh papaya peels (Claassen *et al.*, 2008).

Moreover, previous study also reported that an oven dried mango peels contain higher ethanol yield (Okuda *et al.*, 2007). It is stated that a maximum ethanol yield is produced using powdered mixed fruit waste compared to freshly blended waste (Vaitheki & Deepa, 2016). Thus,

it was proven that oven dried and powdered sample can yield higher ethanol compared to the fresh sample.

B. Immobilization of S. cerevisiae Type II on PVA-alginate

S. cerevisiae Type II which harvested at exponential phase was mixed with sodium alginate and PVA for immobilization process. From the process, diameter of PVA-alginate beads formed were 2 mm to 3 mm as shown in Figure 2 (A). Smaller diameter beads are used because larger surface area is available for substrates diffusion into the small beads (Vaitheki & Deepa, 2016). Besides, beads with small diameter can reduce the resistance of mass transfer so more substrate could be converted into ethanol by yeast cells (Najafpour *et al*., 2008). The optimum bead size to gain higher yield of bioethanol production is 3 mm in which *S. cerevisiae* immobilized into PVA-alginate beads for bioethanol production from papaya waste (Zain *et al*., 2011).

PVA is the largest water-soluble polymer which is cheap, has higher durability, chemical stability and non-toxic to viable yeast cells (Khoo & Ting, 2001). However, it is highly hydrophilic so it has to be cross-linked either chemically or physically to make it insoluble (Kerchove & Elimelech, 2007). Concentration of PVA plays an important role in size of pores and strength of the beads. PVA-alginate beads using 12% of PVA and 1% of Na-alginate can produced highest yield of bioethanol (Nunes *et al*., 2010). It can be seen that using a 12% of PVA, amount and size of macropores will decreased while the thickness of pore wall will increase. Nunes *et al.* (2010) also reported that bioethanol yield will decrease when the concentration of PVA is increased more than 12% (Nunes *et al*., 2010). This is due to the small pores formation that limits the diffusivity of the substrate. Furthermore, PVA-alginate is used as immobilization matrix for *S. cerevisiae* and the beads are dropped into a mixed solution of 5% of H_3BO_3 and 2% of $CaCl_2$ (Wu & Wisecarver, 2012).

Concentration of 5% H_3BO_3 is selected as the best concentration for beads formation because the excess borate ions resulting from higher H_3BO_3 concentration could create an acidic microenvironment in the beads which will affect the activity of the *S. cerevisiae* in the beads. Concentration of calcium chloride which is more than 2% could leads to excessive gelation of Na-alginate formed on the surface and will limit the diffusivity of substrate. Research conducted by Zain *et al.* (2011) reported that higher concentration and yield of bioethanol are obtained at optimum conditions of 12% (w/v) PVA and 1% (w/v) Na-alginate matrix which is prepared with 4% (w/v) H_3BO_3 and 2% (w/v) $CaCl_2$ solution for 30 min (Zain *et al*., 2011). Thus, in this study, 12% (w/v) PVA, 1% of Na-alginate, 5% of H_3BO_3 and 2% of $CaCl_2$ was chosen for the immobilization of *S. cerevisiae* Type II into PVA-alginate beads.

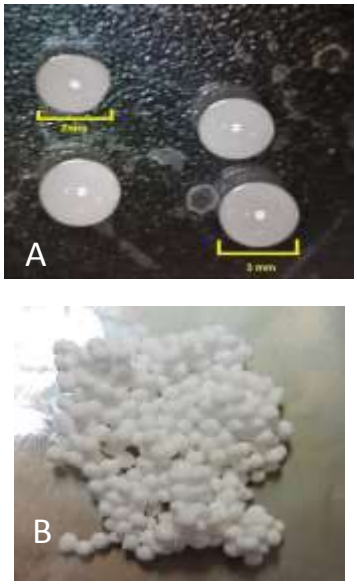

Figure 2. A) Immobilized *S. cerevisiae* Type II in PVA-alginate beads under Dino-Lite microscope and B) Round shape beads for Fermentation process.

C. Optimization of Fermentation for Bioethanol Production

Fermentation processes of papaya hydrolysate were optimized by varying the pH, temperature, agitation speed and fermentation time. Presence of bioethanol in the sample after fermentation was determined using GC-MS. In Figure 3, the chromatogram of standard ethanol (95%) showed a peak at retention time of 4.56 min. The peak with the same retention time can be seen in all samples. Therefore, peak seen in the chromatogram indicate that ethanol in present in the sample.

D. Effects of pH on Bioethanol Production

The study is carried out to determine the significant influences of pH on ethanol fermentation at pH 4, 5 and 6. Based on the results obtained (Figure 4), the maximum bioethanol concentration and yield produced is at pH 5 with 0.079 g/L and 0.036 g/g respectively. At pH 4 to 5, the yield of bioethanol was in an increasing trend from 0.027 g/g to 0.036 g/g. However, from pH 5 to 6, bioethanol yield showing a decreasing trend where bioethanol yield is drop to 0.016 g/g at pH 6. It can be deduced that, the production of bioethanol increased until it reaches pH 5 and drops beyond pH 5. Hence, pH 5 is considered as the optimum pH for fermentation process (Woldu *et al.*, 2015; Togarepi *et al.*, 2012).

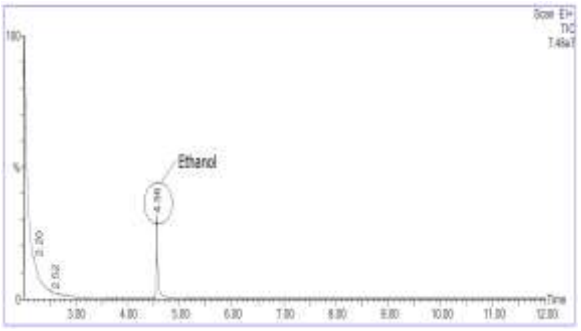

Figure 3. Chromatogram of standard ethanol peak at retention time of 4.56 min

Highest bioethanol concentration and yield produced at pH 5 because the enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (AD) work best at mild acidic condition at pH range of 5.0 to 5.5 (Oh *et al.*, 2000). Lin *et al.* which studied the factors influencing ethanol fermentation using immobilised *S. cerevisiae* also reported that maximum bioethanol concentration is obtained at pH 5 (Lin *et al.*, 2012). Lower bioethanol production at pH 4 is due to high concentration of acetic acid produced instead of ethanol (Kasemets & Nisamedtinov, 2007; Gaudy & Gaudy, 2000).

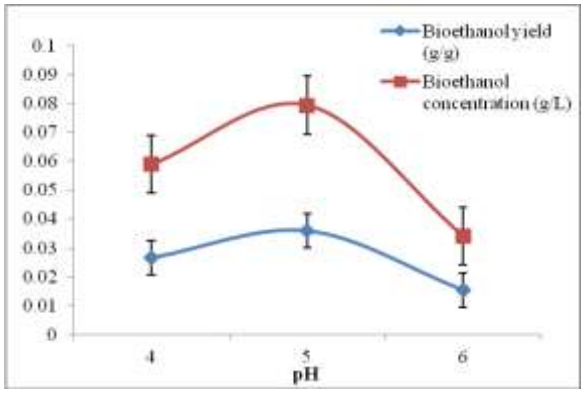

Figure 4. Effects of pH on bioethanol concentration and yield

E. Effects of Temperature on Bioethanol Production

In this study, the influence of temperature on fermentation process by *S. cerevisiae* Type II is studied with regards to bioethanol production. Three different temperature; 25°C, 30°C and 35°C produced 0.074 g/L, 0.194 g/L and 0.149 g/L bioethanol concentration respectively. Based on Figure 5, the maximum bioethanol concentration and yield produced is at 30°C with 0.194 g/L and 0.088 g/g. At 35°C, bioethanol concentration and yield is decreased. Higher temperature results in changing transport activity or saturation level of soluble compound and solvents in the *S. cerevisiae* Type II cells, which increase the accumulation of toxins inside the cells (Togarepi *et al.*, 2012). Moreover, high temperature also indirectly causes denaturation of ribosomes, PDC and AD (Najafpour *et al.*, 2008). The changes occur to *S. cerevisiae* Type II at 35°C cause the fermentation to decline.

It is reported that maximum bioethanol concentration is obtained at 30°C and begins to decline above 30°C (Lin *et al.*, 2012). Besides, optimization of bioethanol production using *S. cerevisiae* into Ca-alginate beads also reported highest ethanol productivity is observed at 30°C (Liu & Shen, 2008). In this study, bioethanol production is higher at 30°C because the *S. cerevisiae* immobilised into PVA-alginate beads are fully activated. The activated *S. cerevisiae* Type II consumed maximum amount of glucose available effectively in the papaya hydrolyste and then converted it into pyruvate through glycolysis and finally it is converted into ethanol by PDC and AD (Oh *et al.*, 2000). Both PDC and AD is enzyme that work best at 30°C and begin to denature at temperature higher than 30°C. Thus, at 30°C both PDC and AD will convert the pyruvate released from glycolysis pathway into ethanol at maximum rate. *S. cerevisiae* Type II also has high tolerance to ethanol at 30°C.

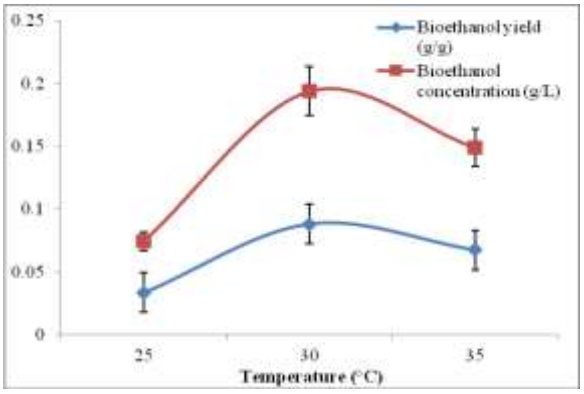

Figure 5. Effects of temperature on bioethanol concentration and yield

F. Effects of Agitation Speed on Bioethanol Production

After optimization of pH and temperature, fermentation is conducted to determine the effect of agitation speed. Fermentation process is carried out at pH 5, 30°C, 48 h, and at

different rate of agitation (150, 200 and 240 rpm) to determine the optimum agitation speed needed to produce higher bioethanol yield from papaya hydrolysate. Application of agitation speed on bioethanol production using immobilised *S. cerevisiae* into PVA-alginate beads is important. Without agitation, beads will remain at the bottom of the fermentation flask which could decrease the availability of substrate for the immobilised *S. cerevisiae* cells. From Figure 6, the maximum bioethanol concentration and yield produced is at 200 rpm which is 0.217 g/L and 0.098 g/g. Bioethanol production increase as the agitation speed increase from 150 to 200 rpm and then decrease as the speed increases to 250 rpm. Bioethanol yield produced at 150 rpm and 250 rpm are 0.0578 g/g and 0.054 g/g respectively. Hence, the optimum agitation speed for maximum bioethanol production is using 200 rpm.

Agitation speed of 200 rpm is beneficial to the growth and performance of the *S. cerevisiae* cells by improving the mass transfer characteristics with respect to substrates, products and oxygen (Ibrahim *et al.*, 2014). The agitation speed at 200 rpm results in a better mixing of the papaya hydrolysate and also helps in maintaining a concentration gradient between the interior and exterior of the cells. Such concentration gradient works in both directions through better diffusion which maintain a satisfactory supply of sugars and other nutrients to the *S. cerevisiae* (Rodmai *et al.*, 2008). Besides, it is also facilitates the removal of gases and other by-products of catabolism from the microenvironment of the cells (Lin *et al.*, 2012). The satisfactory supply of glucose from papaya hydrolysate to *S. cerevisiae* in PVA-alginate beads cause maximum amount of sugar converted to ethanol.

However, bioethanol production is decline as the agitation speed increase to 250 rpm. When speed is increased, it caused increase in the sheer force and turbulence in the cultivation medium containing PVA-alginate beads. The high shear force between beads damaged the PVA-alginate matrix and causes leakage of *S. cerevisiae* into the fermentation medium (Vijay & Rintu, 2013). The sheer force also causes damage to the *S. cerevisiae* cells which causes the fermentation rate to decline (Thai *et al.*, 2015). Meanwhile, bioethanol production at 150 rpm is low compared to agitation speed at 200 and 250 rpm. This phenomenon may be explained because 150 rpm is considered as low speed, thus low amount of sugar was transferred into beads. Thus, low amount of bioethanol was produced at 150 rpm.

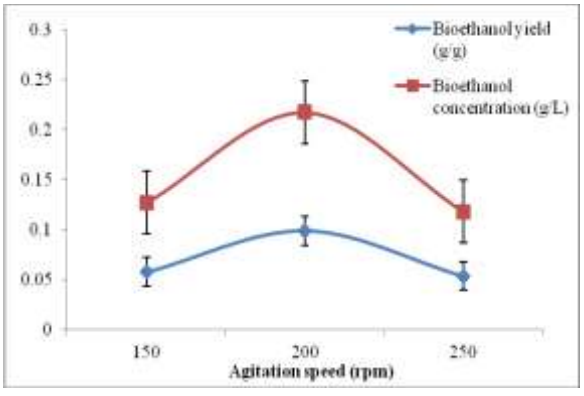

Figure 6. Effects of agitation speed (rpm) on bioethanol concentration and yield

G. Effects of Time on Bioethanol Production

In this study, different fermentation time (24, 48 and 72 h) is used in the optimization process. According to Figure 7, bioethanol concentration is increased until 48 h and then decline after 48 h. High concentration and yield of bioethanol is achieved at 48 h with 0.514 g/L and 0.233 g/g which is higher compared to 24 and 72 h of fermentation time. Moreover, bioethanol concentration at 48 h is two times higher compared to 24 h of fermentation time. At 72 h, the bioethanol yield has dropped drastically to 0.0648 g/g. It can be deduced that the optimum fermentation time is 48 h.

Bioethanol production is decreased at 72h has high concentration of bioethanol produced at 48 h inhibit the ethanol fermentation. High bioethanol concentration produced will induce the thinning of yeast cell membrane (Henderson *et al.*, 2013). The ethanol-induced changes in the membrane thickness of yeast cells could interfere with membrane associated protein function which involved with sugar transport. At 72 h, bioethanol production is also affected by mass transfer limitation and large amount of ethanol produced at 48 h may be deposited around the pore wall of the beads. The deposition of ethanol will reduce the pore size and limit the entering of glucose into the beads and also limit the ethanol release from the beads.

Bioethanol production is lower at 24 h of fermentation process because glucose available in the medium is not fully utilised by *S. cerevisiae*. It is reported that highest ethanol production is observed at 48 h when optimization of fermentation process done on sweet sorghum (Nadir *et al.*, 2009).

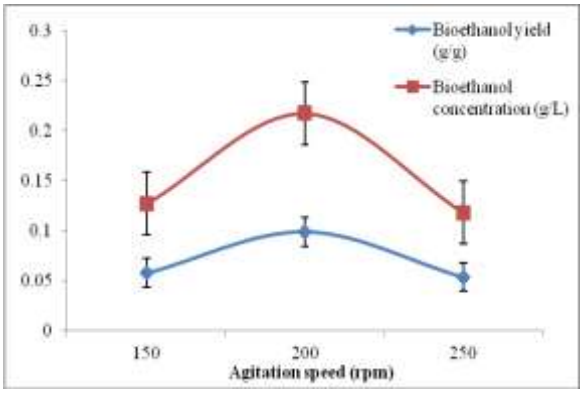

Figure 7. Effects of agitation speed (rpm) on bioethanol concentration and yield.

IV. SUMMARY

In conclusion, optimization process was done using immobilized *S. cerevisae* PVA-alginate beads for bioethanol production. From the studies, it was found that the highest bioethanol concentration and yield was obtained at pH 5, 48 h 30°C and 200 rpm. The maximum bioethanol concentration and yield produced is 0.514 g/L and 0.233 g/g. It can be concluded that papaya peels have high potential to be used as feedstock for bioethanol production using immobilized *S. cerevisiae* Type II.

V. ACKNOWLEDGMENT

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