

Food Grade Ethanol Production Process of Sorghum Stem Juice Using Immobilized Cells Technique

Tri Widjaja¹, Ali Altway¹, Arief Widjaja¹, Umi Rofiqah¹ & Rr Whiny Hardiyati Erlan¹

¹Department of Chemical Engineering, Sepuluh Nopember Institute of Technology, Indonesia

Correspondence: Tri Widjaja, Department of Chemical Engineering, Sepuluh Nopember Institute of Technology, Surabaya 60111, Indonesia. E-mail: papatri2003@yahoo.com

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Abstract

One form of economic development efforts for waste utilization in rural communities is to utilize stem sorghum to produce food grade ethanol. Sorghum stem juice with 150 g/L of sugar concentration was fermented using conventional batch process and cell immobilization continuous process with *K-carrageenan* as a supporting matrix. The microorganism used was Mutated *Zymomonas Mobilis* to be compared with a mixture of *Saccharomyces Cerevisiae* and *Pichia Stipitis*, and a mixture of Mutated *Zymomonas Mobilis* and *Pichia Stipitis*. Ethanol in the broth, result of fermentation process, was separated in packed distillation column. Distillate of the column, still contain water and other impurities, was flown into molecular sieve for dehydration and activated carbon adsorption column to remove the other impurities to meet food grade ethanol specification. The packing used in distillation process was steel wool. For batch fermentation, the fermentation using a combination of *Saccharomyces Cerevisiae* and *Pichia Stipitis* produced the best ethanol with 12.07% of concentration, where the yield and the productivity were 63.49%, and 1.06 g/L.h, respectively. And for continuous fermentation, the best ethanol with 9.02% of concentration, where the yield and the productivity were 47.42% and 174.27 g/L.h, respectively, is obtained from fermentation using a combination of *Saccharomyces Cerevisiae* and *Pichia Stipitis* also. Fermentation using combination microorganism of *Saccharomyces Cerevisiae* and *Pichia Stipitis* produced higher concentration of ethanol, yield, and productivity than other microorganisms. Distillation, molecular sieve dehydration and adsorption process is quite successful in generating sufficient levels of ethanol with relatively low amount of impurities.

Keywords: adsorption, distillation, fermentation, food grade ethanol, immobilized cells, sorghum

1. Introduction

Sorghum (*Sorghum bicolor* L.) is a plant belongs to the family *Gramineae* the same with rice, corn, wheat, and others. *Sorghum bicolor* L included in genus *Sorghum*, order *Cyperales*, class *Liliopsida*, division *Magnoliophyta*, superdivision *Spermatophyta*, subkingdom *Traechobionta*, and kingdom *Plantae*.

Issues of batch fermentation using *Saccharomyces Cerevisiae* immobilization of sorghum stem juice into ethanol has been studied by Shen (Shen et al., 2011). Sorghum stem juice with initial sugar concentration 218.1 g/L in a temperature of 33°C, pH 4.5 has resulted 40.05% of yield and 0.0486 g/g.h of productivity.

Fermentation generally used is batch process. In batch process, low productivity of ethanol produced due to inhibition of the ethanol formed in fermenter will poison the microorganisms. The presence of ethanol inhibition will decrease the growth and production of microorganisms slowly or even stop it (Minier and Goma, 1982). When the concentration of ethanol from fermentation broth reached 12% (v/v) the specific growth rate of microorganisms will decrease, so that many sugar solution cannot be fermented perfectly. Continuous fermentation is the solution that can be used to increase the rate of ethanol production (Cheng and Wang, 2008). Frequently continuous fermentation used is cell immobilization technique in a packed bed bioreactor. In addition to reduce ethanol concentration, the productivity of ethanol in batch fermentation is limited because of high concentration of ethanol in the fermented broth decrease the ethanol production rate. This inhibition is a well-known feature of many fermentation processes and commonly referred to product inhibition. Continuous fermentation solves the inhibition problem in batch process, which can increase the ethanol production rate. However, this process cannot be performed for high-density cell culture, which which has low ethanol concentration and makes the reduction of residual sugar not significant. (Widjaja et al., 2014).

Substrate concentration has an important role in ethanol manufacture. The increasing of concentration sugar concentration has some advantages. It will increase the final ethanol concentration and reduce the cost of distillation. The addition of glucose to the fermentation substrate can increase the concentration of ethanol produced, but adding glucose continuously will decrease the critical concentration of ethanol produced. The decline of ethanol concentration in the concentration of substrate is possible inhibitor (Goksungur and Zorlu, 2000).

The process of fermentation followed by distillation, molecular sieve dehydration, and adsorption processes which are expected to increase the ethanol concentration. The initial process is done on the fermentation of glucose-containing material that ethanol can be obtained. The fermented broth contains ethanol and other impurities. The impurities can be separated from fermented broth using distillation, molecular sieve dehydration and adsorption process to produce food grade ethanol. The adsorption process usually uses activated carbon.

There are several characteristics of microorganism for fermentation, such as, having the ability to grow and multiply rapidly in a suitable substrate, able to produce enzyme to convert glucose into alcohol quickly, having a high power of glucose, fructose, galactose and maltose fermentation, having a high resistance due to high concentration of alcohol, as well as resistance to other microbes. Based on Barros's study, *Saccharomyces Cerevisiae* is the best microorganism for alcohol industry (Barros et al., 1986). But, based on Gunasekaran and Raj's study, *Zymomonas Mobilis* and *Pichia Stipitis* are the best candidate microorganisms for alcohol industry (Gunasekaran and Raj, 1999).

The aim of this study is to produce ethanol with high productivity and no impurities by using continuously fermentation (packed bed reactor) integrated with distillation and adsorption processes, and using cell immobilization technique which κ -carrageenan as a supporting matrice. And to find which microorganisms can produce higher concentration of ethanol, yield, and productivity.

2. Materials and Method

2.1 Materials

Materials used were: sorghum stem juice, fermipan, Mutated *Zymomonas Mobilis*, *Saccharomyces Cerevisiae*, *Pichia Stipitis*, PDA, κ -carrageenan (Fluka brand), KH_2PO_4 , $(\text{NH}_4)_2\text{SO}_4$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, KCl, NaCl, kid-tartrate, Na-metabisulfite, glucose, DNS, aquadest, molecular sieve, and activated carbon. The equipments were: packed bed bioreactor, peristaltic pump, spectrophotometer, Gas Chromatography (GC), incubator shaker, autoclave, analytical balance, hot plate, stirrer, flask, glass beaker, measuring cups, wire loop, separator funnel, flask and distillation and adsorption columns. Operating conditions and equipment dimension used in research is shown in Table 1

Table 1. Operating Conditions and Equipment Dimensions Research

Remarks	Operating Conditions
Process	Continuous fermentation
Sugar concentration	150 g/L
pH	4-5
Temperature	29-30°C (ambient temperature)
Carrier of immobilization	κ -karaginan
Weight of bead	250 gram
Concentration of κ -karaginan	2%
Feed rate	10 mL/min
Dillution rate	2,45 jam ⁻¹
Periode of taking sample	6 h during 90 h
<i>Fermentor</i>	
Type	<i>Packed bed</i>
Volume of liquid in the reactor	245 mL
Volume of bead in the reactor	314 mL
Height of Reactor	52 cm
Diameter of Reactor	3,7 cm
<i>Distillation dan Adsorption Column</i>	
Diameter of Distillation Column	5 cm
Height of Distillation Column	60 cm

Volume of Distillation Flash	1000 mL
Volume of Adsorption Column	1000 mL
Diameter of Adsorption Column	5 cm
Height of Adsorption Column	60 cm

2.2 Methods

Sorghum stem juice was sterilized by using an autoclave at 121°C and 15 psia for 15 minutes, then cooled until room temperature. 5.19 g $(\text{NH}_4)_2\text{SO}_4$; KH_2PO_4 1.53 g; and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.55 g were added as nutrient to sorghum stem juice that was sterilized (Cazetta et al., 2007).

2.2.1 Making Starter and Bead

Before fermentation and extraction process, the starter and immobilized cells were needed to be made. Added 3 oses of microorganism and the nutrients (1 g $(\text{NH}_4)_2\text{SO}_4$; KH_2PO_4 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g; 10 g of yeast extract) into 100 mL of sorghum stem juice. After that breed it in an incubator shaker at 30°C for 15 hours. For making immobilized celss, dissolved 10 grams of carrageenan in 450 mL of aquadest, then heated it at 70°C until start to become gel (heating for 15 minutes). Cool carrageenan solution to 50°C. Mix 50 ml of a starter with 450 ml carrageenan solution so the concentrations of the mixture become 2%. Shape 50 mL of the mixture in 1000 mL of 3.5% KCl solution, and to get the beads. The beads harden within 15 minutes. Wash the beads with 0.85% of NaCl solution. To improve cell growth, beads were added to production medium (sap of feed) and then incubated in an incubator shaker for 24 hours. Beads were stored at 4°C.

2.2.2 Fermentation Process

There are 2 kinds of fermentaion, continuous and batch fermentation. For continuous fermentation, the first thing to do was adding the beads of immobilization cells into the fermenter.

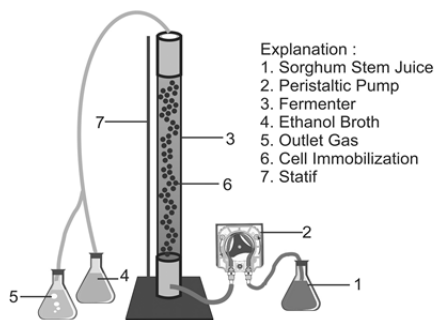


Figure 1. Set-Up of Packed-Bed Continuous Fermentation in a Packed-Bed Reactor without Recycle

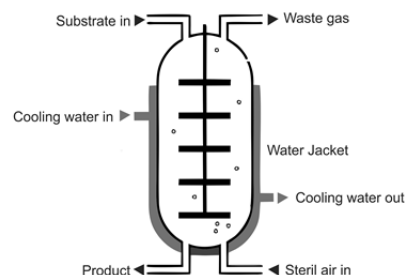


Figure 2. Set-up of Batch fermentation

Sterile sorghum stem juice was flowed by a peristaltic pump into the fermenter (bioreactor packed bed), Take fermentation product (broth) as a sample every 6 hours for 90 hours. Analyze residual glucose concentration in every sample (broth) using DNS (dinitro salicylic) method.

2.2.3 Separation Process

Separation process in this research is by using distillation process. Fermented broth was continued to distillation process by checking the condition of distillation equipment first and make sure that all the valves were closed. Then a two-neck flask filled with fermented broth. The cooling water were flawn into the condenser. Turn on the hot plate to heat the two neck flask. Set the hot plate temperature until reach the specified temperature. Distillation process is done by using a structured form of steel wool packing with 2-3 in of diameter. It can be carried away by the liquid because it only works best when arranged evenly. Another advantage of the steel wool is the price quite cheap than other packing.

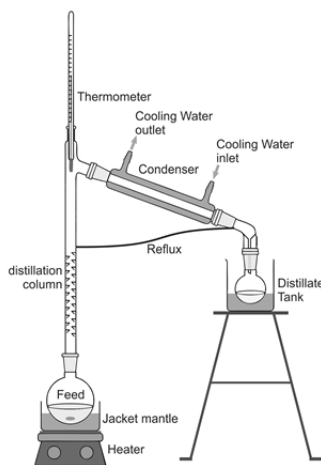


Figure 3. Set-Up of Distillation Equipment

The distillation products was stored and purified by using molecular sieve dehydration and adsorption process. Distillation is done twice, distillation 1 and distillation 2. Distilate from the first distillation was flown into the second distillation. In the second distillation, the distilate was refluxed. This process is carried out to obtain a large ethanol.

2.2.4 Purification Process

To remove the water contenned, it used molecular sieve dehydration process. Molecular sieve has fine pores where the size is very standardized and uniform. Check adsorption equipments first and make sure all the valves were closed. Fill two-neck flask with distillate. Flow cooling water into the condenser. Turn the hot plate on to heat two neck flask. Set hot plate temperature steam appropriate with gas rate that has been specified.

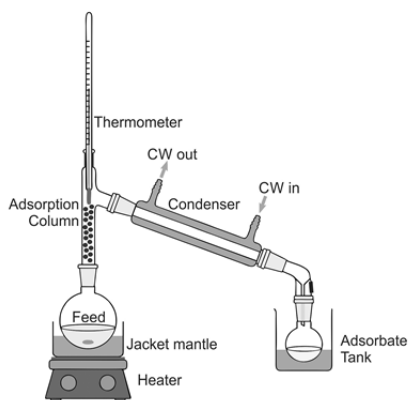


Figure 4. Set-Up of Molecular Sieve Dehydration

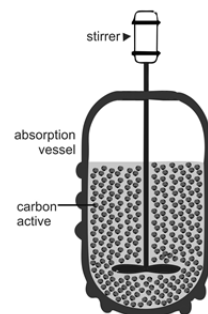


Figure 5. Set-up of Carbon Active Adsorption

The pores can selectively capture molecules passing by large-small molecular size. The ability of molecular sieve in capturing high enough H_2O molecule, ie up to 20-25% of the weight of molecular sieve itself. Type of sieve used in the purification of ethanol is the type 3A with ± 3 angstrom pore size. After molecular sieve dehydration process, it was continued to carbon active adsorption process. Store products that produced from adsorption process and then analyze it by GC method.

3. Results

3.1 Fermentation Performa

The fermentation process was done in two processes, which were batch and continuous process. There were two different microorganisms used in each process. The usage of different microorganisms was to determine which microorganisms would produce higher ethanol concentration, which have larger yield and productivity. The ethanol concentration was found by Gas Chromatography (GC) analysis.

The main material used in this study was sorghum stem juice with 150 g/L of sugar concentration. This concentration was the best concentration of ethanol production in continuous process (Widjaja et al., 2011). The result from previous study could be applied in batch fermentation process. Continuous fermentation used Mutated *Zymomonas Mobilis* with *k-carrageenan* as the supporting matrix produced ethanol with 3.654% of concentration where the yield and the productivity were 10.42% and 38.27 g / L.h, respectively. The ethanol concentration of fermentation used the combination of *Saccharomyces Cerevisiae* and *Pichia Stipitis* was 9.02% where the yield and the productivity were 28.831% and 70.638 g/L.h, respectively. The ethanol concentration that produced using combination of Mutated *Zymomonas Mobilis* and *Pichia Stipitis* was 3.741 % where the yield and the productivity were 49.188% and 72.307 g/L.h, respectively. These results indicate in continuous fermentation process, the best result obtained from *Saccharomyces Cerevisiae* and *Pichia Stipitis*.

In the conventional batch fermentation, the best results obtained from combination of *Saccharomyces Cerevisiae* and *Pichia Stipitis*. Batch fermentation using *Saccharomyces Cerevisiae* and *Pichia Stipitis* produced ethanol concentration of 12.07% where the yield and the productivity were 63.49% and 1.06 g / L.h, respectively. In other side, batch fermentation with Mutated *Zymomonas Mobilis* produced ethanol concentration of 5.05%, with yield and productivity were 26.51% and 0.44 g / L.h, respectively. Batch fermentation used combination of Mutated *Zymomonas Mobilis* and *Pichia Stipitis* produced ethanol concentration as much as 4.402 %, with the yield and the productivity were 34.732% and 0.386 g / L.h, respectively.

The results can be seen in Table 2. This means that the usage of combination between *Saccharomyces Cerevisiae* and *Pichia Stipitis* were more suitable for stem sorghum juice fermentation.

Table 2. Ethanol concentration, yield and productivity in batch and continuous fermentation with different microorganisms

Fermentation Process	Mutated <i>Zymomonas Mobilis</i>			<i>Saccharomyces Cerevisiae</i> + <i>Pichia Stipitis</i>			Mutated <i>Zymomonas Mobilis</i> + <i>Pichia Stipitis</i>		
	Ethanol	Yield	Productivity	Ethanol	Yield	Productivity	Ethanol	Yield	Productivity
	Concentration (%)	(%)	(g/L.h)	Concentration (%)	(%)	(g/L.h)	Concentration (%)	(%)	(g/L.h)
Batch	5.05	26.51	0.44	12.07	63.488	1.058	4.402	34.732	0.386
Kontinyu	3.654	28.831	70.638	9.02	47.421	174.273	3.741	49.188	72.307

From Figure 6 it is known that the growth of Mutated *Zymomonas Mobilis* was higher than the combination of *Saccharomyces Cerevisiae* and *Pichia Stipitis* and the combination of Mutated *Zymomonas Mobilis* and *Pichia Stipitis*. At 10th hours, in the growth peak of microorganisms, the value of Optical Density (OD) of Mutated *Zymomonas Mobilis* was 0.4499 while the combination of *Saccharomyces Cerevisiae* and *Pichia Stipitis* was 0.3110, and at 18th hours the OD value of the combination of Mutated *Zymomonas Mobilis* and *Pichia Stipitis* was 0.284. Numbers of Mutated *Zymomonas Mobilis* was higher than the combination of *Saccharomyces Cerevisiae* and *Pichia Stipitis*, and the combination of Mutated *Zymomonas Mobilis* and *Pichia Stipitis*. With that large number, it was able to change the substrate into ethanol quickly and much more.

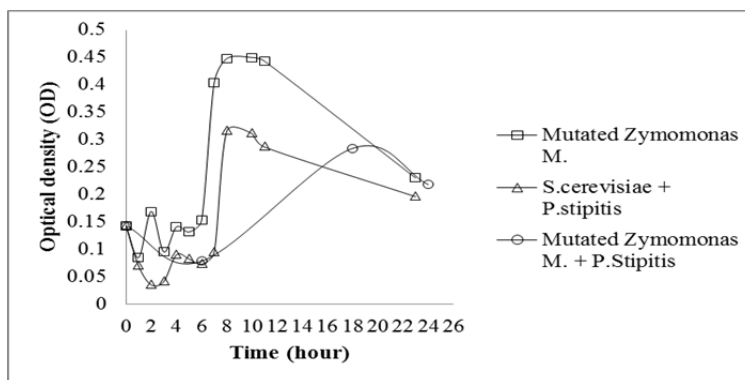


Figure 6. Growth curves of Mutated *Zymomonas Mobilis* and Combination of *Saccharomyces Cerevisiae* and *Pichia Stipitis*, and Combination of Mutated *Zymomonas Mobilis* and *Pichia Stipitis*

The combination between microorganisms was to determine which microorganisms could produce higher ethanol concentration. From the analysis was known that the combination of *Saccharomyces Cerevisiae* and *Pichia Stipitis* could produce larger yields of ethanol in fermentation process which occurs at pH 4-5 (Antoni et al., 2012). But from growth curves above, Mutated *Zymomonas Mobilis* was better than others, eventhough concentration of ethanol, yield and productivity were smaller than using the combination of *Saccharomyces Cerevisiae* and *Pichia Stipitis*.

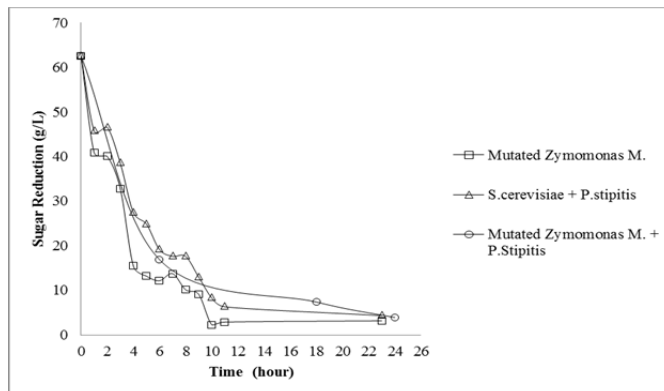


Figure 7. Sugar reduction curves of Mutated *Zymomonas Mobilis* and Combination of *Saccharomyces Cerevisiae* and *Pichia Stipitis*, and Combination of Mutated *Zymomonas Mobilis* and *Pichia Stipitis*

From Figure 7 it is known that in 10th hour, sugar concentration was begin to decline steadily. The concentration of sugar in the hours to 10 to Mutated *Zymomonas Mobilis* of 2.306 g/L, while for the combination of *Saccharomyces Cerevisiae* and *Pichia Stipitis* was 8.320 g/L and for the combination of Mutated *Zymomonas Mobilis* and *Pichia Stipitis* was 3.945 g/L.

3.2 Ethanol Food Grade

This research is expected to produce ethanol food grade with concentration between 90-94%, whereas the continuous fermentation process was carried out in an integrated manner with distillation process, dehydration by using molecular sieve and activated carbon adsorption, the ethanol concentration obtained only 83%. It needs to be done to improve distillation of ethanol in order to achieve food grade ethanol concentration around 90-94%. For batch fermentation, substrate dilution needs to be done with the maximum reducing sugar concentration of 100 g/L as if it is above 100 g/L substrate become inhibitory to microorganisms.

Broth Fermentation was flown to a distillation column to separate ethanol from water. Furthermore, the dehydration process was carried out using molecular sieve and using activated carbon adsorption to remove the water and impurities.

Table 3. Composition of Ethanol produced

Component	Before Purification	After Purification
Water (%)	64.169	16.935
Ethanol (%)	35.778	83.064
Amyl Alcohol (%)	0.036	0
Acetic acid (%)	0.0164	0

GC chromatogram of ethanol before and after purification process are shown in figure 8 and 9. In Figure 8, the resident time of water is 2.330 with 16.935% of concentration. For ethanol, the resident time and the % concentration are 5.468 and 35.778, respectively. For acetic acid, the resident time and the % concentration are 11.258 and 0.0164, respectively. But amyl alcohol has resident time 12.336 and 15.109 with 0.00152 % and 0.03450 % of concentration, respectively. From Figure 9 it is known that water has resident time 2.322 with 16.935 % of concentration and ethanol has resident time 4.681 with 83.064 % of concentration. There is no impurities after purification.

Table 4. Specification of ethanol food grade

Level of ethanol	:	90 – 94%
Acidity (as acetic acid)	:	Not more than 0.003%
Alkalinity (as NH ₃)	:	Not more than 3 mg/kg
Fusel oil	:	Passes test
Ketones, isopropyl alcohol	:	Passes test
Heavy metals (as Lead)	:	Not more than 0.5 mg/kg
Methanol	:	Passes test
Nonvolatile residue	:	Not more than 0.003%
Solubility in water	:	Passes test
Substances darkened by sulphuric acid	:	Passes test
Substances reducing permanganate	:	Passes test

(Food Chemicals Codex, 5th ed.2003).

Food grade ethanol specifications are shown on Table 4. The ethanol concentration must be 90-94%, the ethanol must be free from fusel oil, ketones isopropyl alcohol, methanol. The acidity is not more than 0.003%, and the alkalinity is not more than 3 mg/kg. From this research, it was known that the process of fermentation and integrated with the distillation and adsorption produced ethanol with food grade specification.

4. Discussion

Fermentation using combination microorganism of *Saccharomyces Cerevisiae* and *Pichia Stipitis* produced higher ethanol concentration, yield, and productivity than others. From entire fermentation process, the usage of combination microorganisms of *Saccharomyces Cerevisiae* and *Pichia Stipitis* produced higher concentration of ethanol. Some advantages of *Saccharomyces Cerevisiae* in the ethanol fermentation process is rapidly proliferating, resistant to high temperatures, and quick to adapt. While *Pichia Stipitis* has the ability to decompose glucose and xylose to ethanol. The yield and the productivity of ethanol produced by a mixture of *Saccharomyces Cerevisiae* and *Pichia Stipitis* higher compared to using one of these types, that were 41% and 77 g/L.h, respectively (Rouhollah et al., 2007).

Batch fermentation gave better value of yield than continuous fermentation, but continuous fermentation provide better productivity than batch fermentation. Ethanol that formed in conventional batch fermentation became an inhibitor that would be toxic to microorganisms growth so ethanol productivity decreased. In other hand, in continuous fermentation ethanol that formed would go out as an output and there was no accumulation in the fermenter that would interfere the growth of microorganisms. Continuous fermentation was one of methods to improve yield and productivity of fermentation process due to the fact that the presence of ethanol inhibition would decrease the growth and production of microorganisms or even stop it (Minier and Goma, 1982). Food grade ethanol specifications with high concentration of ethanol is obtained by further purification process in the process of distillation, dehydration, and adsorption.

Ability and resistance of microorganisms in the fermentation process are affected by the value of yield and productivity of ethanol itself. Microorganisms converted sugar into ethanol in the fermentation process. The fermentation process occurred in relatively acidic condition with pH around 4-5. This study sought a microorganism which were suitable for relatively acidic operating condition and could produce high concentration of ethanol. Figure 6 shown the performance of microorganisms in the growth curve of microorganisms. The number of microorganisms were analyzed using Optical Density (OD) analysis, where the number of microorganisms were expressed in the amount of OD.

Mutated *Zymomonas Mobilis* able to live in low pH between 4 – 5 (Alfena et al, 2009). Mutated *Zymomonas Mobilis* is resistant to acidic conditions. The optimum pH for fermentation using Mutated *Zymomonas Mobilis* is at pH 4 – 5. All these microorganisms can work at the same pH range, ie 4-5. Commercial-scale ethanol fermentation mostly was done by fungi, one of the fungus is *Saccharomyces Cerevisiae* (Judoamidjojo et al., 1992). However, *Saccharomyces Cerevisiae* apparently has some shortcomings, which are not resistant to high concentrations of ethanol produced. *Pichia Stipitis* was expected able to cover the shortcoming of *Saccharomyces Cerevisiae*.

Zymomonas Mobilis has properties that are resistant to high concentration of ethanol produced in the fermentation process when compared with *Saccharomyces Cerevisiae* because this bacterium has hopanoid structure or lipid complex membrane, causing the membrane become more stable and has a good density, so that other molecules are difficult to penetrate these cells, including ethanol.

Distillation, molecular sieve dehydration and adsorption process are quite successful in generating sufficient levels of ethanol with high and relatively low amount of impurities. The fermentation process that was integrated

with an advanced purification process, distillation, dehydration, and adsorption using activated carbon, could produced ethanol concentration of 83% without impurities. It needs to improve distillation technique and fermentation process to get ethanol with high concentration.

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