Continuous Ethanol Production with Immobilized Ca-Alginate Cells Technique in Packed Bed Bioreactor using Bacteria: Z. mobilis and Mutated Z. mobilis

Raden Darmawan\textsuperscript{a}, Tri Widjaja\textsuperscript{a}, Tontowi Ismail\textsuperscript{b}, Setiyo Gunawan\textsuperscript{a} and Surya Putra Rosa\textsuperscript{b}

\textsuperscript{a}Department of Chemical Engineering
\textsuperscript{b}Department of Chemistry
Sepuluh Nopember Institute of Technology, Surabaya 60111 Indonesia

\textsuperscript{*}Corresponding Author’s E-mail: rdarmawan@chem-eng.its.ac.id

Keywords: Ethanol, Hydroxylamine, Immobilized Cells, Packed bed bioreactor, Z. mobilis

Abstract

\textit{Zymomonas mobilis} possesses many advantages for ethanol production including higher substrate conversion and high ethanol tolerance. \textit{Zymomonas mobilis} mutated by hydroxylamine has ability to achieve a faster conversion and more resistant to acidic conditions. These bacteria were used in fermentation with immobilized cells technique. Immobilized cell technique is a cell-entrapment technique in order to keep the cells stay in the bioreactor so that it is able to optimally produce ethanol. The cells were immobilized using Ca-alginate as the supporting matrices. This research aims to find out the influence of glucose concentration using both \textit{z.mobilis} and mutated \textit{z.mobilis} on the performance of ethanol production in packed bed bioreactor in order to enhance the concentration, yield, and ethanol productivity, respectively. The ethanol production used molasses as raw material by fermentation process in the packed bed bioreactor. The experiment was started by making starter, molasses pretreatment, culture growth, making production medium, and making immobilized Ca-Alginate cells. Immobilized cells have spherical-shape with diameter of 2 mm and weight of 220 g. Having prepared beads, the fermentation was performed in the packed bed bioreactor by flowing molasses using peristaltic pump under the flow rate condition of 0.154 L/hr (dilution rate of 0.75 hr\textsuperscript{-1}). Ethanol product (broth) was analyzed by gas chromatography (GC) method. It was shown from the result that maximum ethanol production conducted using mutated \textit{z.mobilis} gave the following concentration, yield, and productivity: 73.26 g/L, 40.96 %, 54.95 g/L.hr, respectively. While the result of ethanol production conducted using \textit{z.mobilis} gave the following concentration, yield, and productivity: 62.17 g/L, 36.85 %, 46.63 g/L.hr, respectively.

1. Introduction

Previous studies on strain of \textit{zymomonas mobilis} have known that these microorganisms can convert glucose efficiently and rapidly to ethanol with significantly higher specific rates of glucose uptake and ethanol production than useful yeasts (K.J.Lee et al, 1981). The microorganisms used to produce ethanol in this study is mutation \textit{Zymomonas mobilis}, the mutated bacteria \textit{Zymomonas mobilis} using hydroxylamine mutagen selected in acid medium to form a characteristic resistant to acid. A3 is the excess of \textit{Zymomonas mobilis}, has a high temperature tolerance, ability to attain a faster conversion, more resistant to high levels of ethanol produced in the fermentation process when compared \textit{Zymomonas mobilis} is not mutated (Putra, S,R. and Chrisnawati, A., 2008).

It is well known that immobilizing cells is a process that is used to halt the action of enzymes and catalysts. Immobilization is performed due to microorganisms density is closed to that of water, and therefore, there is the possibility they could bond in supporting matrice while in the product stream. The advantages in using immobilized cell than those of the other free cells are the ease to separate the product, a highly volumetric productivity, an increase in the process control and a decrease in the contamination, a lessen separation cost, and prevention of wash out occurring in product stream.
Of the various immobilized techniques, trapping cell in calcium-alginate gel system is the simplest method and it does not have poisonous property. Then immobilized cell technique with Ca-alginate was developed by Goksungur and Zorlu which involved drop-wise cell suspension in sodium alginate that hardened. The immobilized cell technique with Ca-Alginate and κ-Carrageenan as supporting matrix was also learned by Grote et al who fermented sugar cane juice to high concentrations of ethanol. It was more ethanol tolerant and had faster specific rates of glucose uptake and ethanol production than the other strains of zymomonas mobilis which had been conducted by Skotnicki et al. This method was also easy to apply with various cells for example bacteria, cyanobacteria, algae, and yeast fungi. A variety of bioreactors, such as agitated bioreactor (continuously stirred tank reactor), fluidized bed reactor, and packed bed reactor could be used in this experiment, but the authors recommended that the packed bed should be used as a result of a low cost, easier operability compared to others, and automatic industrial operations.

The aim of this experiment was to study the effect of total sugar concentration using Ca-Alginate immobilized cells of Zymomonas mobilis and mutated Zymomonas mobilis on packed-bed bioreactor.

2. Experimental
2.1 Materials

In this study, using materials such as Molasses, zymomonas mobilis from Biochemical Laboratory of Chemical Engineering Department, Faculty of Industrial Technology, ITS Surabaya-Indonesia, mutated zymomonas mobilis was purchased from Biochemical Laboratory of Chemistry Department, Faculty of Mathematical and Science, ITS Surabaya-Indonesia, PDA (pottato dextrose agar), Na-Alginate, yeast extract, H$_2$SO$_4$, (NH$_4$)$_2$SO$_4$, KH$_2$PO$_4$, MgSO$_4$.7H$_2$O, Na$_2$HPO$_4$, CaCl$_2$, NaCl, NaOH, DNS liquid and aquadest.

2.2. Methods

The following experimental steps consisted of pretreatment media, immobilized cells preparation, fermentation process and analytical methods.

Pretreatment media

The incubated mutated Zymomonas mobilis from a sloping breeding media was taken using a sterile wire ose, was embedded as much as 6 ose into 100 ml nutrition media (yeast extract 10 g, (NH$_4$)$_2$SO$_4$ 1,0 g, KH$_2$PO$_4$ 1,0 g and MgSO$_4$.7H$_2$O 0,5g) with a certain total sugar concentration. These organisms were then cultivated in an incubator shaker on temperature of 30°C for 36 hours. Also, the observation of total bacteria found in the media was counted using haemacytometer neubaeur and the logarithmic phase of bacterial growth was detected using a microscope.

Immobilized cells preparation

Ca-Alginate: A nutrient media containing (NH$_4$)$_2$SO$_4$ 5,19 gram, KH$_2$PO$_4$ 1,53 gram, MgSO$_4$.7H$_2$O 0,55 gram and yeast extract 1 gram + breeding was put into incubator shaker for 24 hours. Then 50 ml of this nutrient media was mixed with 50 ml of alginate solution. Then 100 ml of alginate-cell mixture was added in 1000 ml solution of 2% CaCl$_2$ until a solid solution formed. After 30 minutes solid formed was washed with 0.85% NaCl to decrease excessive ions. To increase cell growth, immobilized cell in production media was incubated in an incubator shaker for 24 hours.

A production media was made by mixing 1 liter of molasses with (NH$_4$)$_2$SO$_4$ 5,19 gram, KH$_2$PO$_4$ 1,53 gram and MgSO$_4$.7H$_2$O 0,55 gram, then this solution was added with H$_2$SO$_4$ until pH 4.6. if not used directly, the solid in 2% of yeast extract was kept in temperature of 4°C.

Fermentation process

Reactor type used in this experiment was a packed-bed bioreactor with Ca-Alginate beads of 2% (w/v). During fermentation process, it was operated on temperature of 30°C and pH 4 – 5. Certain total sugar concentrations of immobilized cell beads produced was put into a packed bed bioreactor tray. Then the sterilized molasses containing a total sugar concentration according to the variables (115 g/L, 160 g/L,
190 g/L) was fed into packed bed bioreactor with a volumetric rate of 0.06 L/hr and dilution rate of 0.4 hr⁻¹. A schematic diagram of a continuously packed bed bioreactor is shown in Figure 1.

Fig. 1. Continuously Packed Bed Bioreactor

At the beginning of experiment for continuous fermentation, a packed column was filled with Ca-Alginate beads until they were fully filled up. Furthermore, molasses was pumped down by a peristaltic pump through silicon tube to the packed bed bioreactor with a flow rate of 0.06 L/hr and a dilution rate of 0.4 hr⁻¹, and ethanol as a product was flowing out from the liquid effluent stream on the upper of the bioreactor. Meanwhile, a batch fermentation was conducted using a batch stirred tank reactor equipped with jacket and agitator. The agitator’s speed rotation was kept constant. During the experiments, the continuous and batch fermentation pH of 4-5 was adjusted. A Gas Chromatography method was used to analyze the ethanol products and the Optical Density method was used to analyze the free cells from batch fermentation. Table 1 shows parameters and conditions for continuous fermentation and batch fermentation.

Table 1. Parameters and conditions of the experiments

<table>
<thead>
<tr>
<th>Information</th>
<th>Continuous Fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioreactor</td>
<td>Packed bed</td>
</tr>
<tr>
<td>Microorganism</td>
<td>Z.mobilis and Mutated z.mobilis</td>
</tr>
<tr>
<td>Reactor Volume (ml)</td>
<td>510</td>
</tr>
<tr>
<td>Bead</td>
<td>Ca-Alginate</td>
</tr>
<tr>
<td>Bead weight (g)</td>
<td>220</td>
</tr>
<tr>
<td>Dilution rate</td>
<td>0.75</td>
</tr>
<tr>
<td>Residence time (hr)</td>
<td>1.3</td>
</tr>
</tbody>
</table>

**Analytical Methods**

Concentration of glucose was determined using a high performance liquid chromatography with cosmosil sugar-D column and the UV detector at wavelength of 540 nm, using the reagent 3,5 -dinitrosalicylic acid (Adney,B. and J.Baker, 1996), (Miller, G.L., 1959). Counting cells were carried out using haemocytometer. The product ethanol concentration was analyzed using a Gas Chromatography.
3. Results and Discussion

This study aims to find out the influence of total sugar concentration and various of microorganisms using immobilized Ca-alginate cell technique on the performance of ethanol production in packed bed bioreactor in order to enhance the concentration, yield, and ethanol productivity.

The microorganisms used in this study were *zymomonas mobilis* and mutated *zymomonas mobilis*. The mutated *z. mobilis* is *z. mobilis* bacteria having undergone a mutation process using hydroxylamine mutagen selected in acid medium to form a characteristic resistant to acid. In addition mutated *z. mobilis* has a high temperature tolerance, ability to attain a faster conversion, more resistant to high levels of ethanol produced in the fermentation process when compared to the un-mutated *z. mobilis* (Putra, S.R. and Chrisnawati, A.,2008). The following figure shows behavior of bacteria growth in molasses.

![Fig. 2. Growth curve of z. mobilis](image)

![Fig. 3. Growth curve of mutated z. mobilis](image)

From the figure 2 shows that the bacterial growth phase of *z. mobilis* takes a long time to reach the logarithmic phase about 21 hours. However, the figure 3 shows that the bacterial growth phase of mutated *z. mobilis* takes a shorter time to reach the logarithmic phase than *z. mobilis* about 15 hours. It means that mutated *z.mobilis* has faster adaptation period than *z. mobilis*, thus, more ethanol products can be produced.

The Effect of Total Sugar Concentration and Types of Bacteria Used (*z. mobilis* and mutated *z.mobilis*) on The Concentration, Yield and Ethanol Productivity

The Ratio between ethanol concentrations and various total sugar concentrations using both *z. mobilis* and mutated *z. mobilis* is shown in Figure 4a. The concentration of ethanol produced for 2% of Ca-Alginate bead using *z. mobilis* were 48.43 g/L; 52.32 g/L; and 62.17 g/L, respectively; while the ethanol concentrations produced using mutated *z. mobilis* were 44.61 g/L; 49.99 g/L; and 73.26 g/L, respectively. The highest ethanol concentration obtained for *z. mobilis* with 190 g/L of total sugar concentration was 62.17 g/L and the highest ethanol concentration obtained for mutated *z. mobilis* with 190 g/L of total sugar concentration was 73.26g/L. At the total sugar concentration of 190 g/L, the higher ethanol production was achieved for both *z. mobilis* and mutated *z. mobilis* compared to other concentrations. This is due to the fact that at the 190 g/L sugar concentration, the quantity of the substrate of total sugar is more abundant and thus, a higher concentration of ethanol can be produced. Whereas the highest ever concentration of ethanol in this study was obtained by using mutated *z. mobilis* with the total sugar concentration of 190 g/L. This was caused by the fact that the mutated *z. mobilis* was more resistant to acidic conditions than *z. mobilis*, so that it can produce higher ethanol concentrations.

Yield is defined as the ratio between ethanol produced and total sugar consumed during fermentation. Figure 4.b shows the relationship between ethanol yields (%) and total sugar concentration in molasses (g/L). According to figure 4.b the ethanol yield for *z. mobilis* are: 40.58%; 33.81%; and 36.86%
while the ethanol yield for mutated \textit{z. mobilis} are: 40.65\%; 34.18\%; and 40.96\%. The highest ethanol yield achieved for \textit{z. mobilis} and 115 g/L of total sugar was 40.58 \% and then the highest ethanol yield reached for mutated \textit{z. mobilis} and 190 g/L of total sugar was 40.96\%.

In fermentation, productivity is defined as grams ethanol product/liter/hours. Figure 4.c shows the relationship between ethanol productivity (g/L.hr) and concentration of total sugar in molasses (g/L). The results from the experiments showed that the ethanol productivities produced for \textit{zymomonas mobilis} are 36.32 g/L.hr; 39.24 g/L.hr; and 46.63 g/L.hr, then the ethanol productivities produced for mutated \textit{zymomonas mobilis} are 33.46 g/L.hr; 37.49 g/L.hr; and 54.95 g/L.hr.. Ethanol productivity is directly proportional to ethanol concentration because ethanol productivity is ethanol concentration divided by residence time.

According to these results, mutated \textit{z.mobilis} were found to have larger to concentration, yield and ethanol productivity than \textit{z.mobilis}. This was because of the differences in resistant to acidic conditions. Mutated \textit{z. mobilis} has more resistant to acidic conditions than \textit{z. mobilis} especially in high concentration of total sugar.

4. Conclusion

The mutated \textit{Zymomonas mobilis} has been found to be more resistant to acidic conditions and able to produce higher concentration, yield and productivity of ethanol than \textit{Zymomonas mobilis}. It was shown from the result that the maximum ethanol production conducted using mutated \textit{z.mobilis} gave the following concentration, yield, and productivity: 73.26 g/L, 40.96 \%, 54.95 g/L.hr, respectively. While the result of ethanol production conducted using \textit{z.mobilis} gave the following concentration, yield, and productivity: 62.17 g/L, 36.85 \%, 46.63 g/L.hr, respectively.

Acknowledgements

This project was granted by Penelitian Guru Besar 2010 by Institution Research and Public Services of ITS (LPPM-ITS), under contract No.: 0892/I2.7/PM/2010 July 1, 2010 and by Hibah Bersaing by Directorate Research and Public Services, Directorate General of Higher Education (DIKTI) Department of National Education Republic of Indonesia under contract No.: 042/SP2H/DP2M/III/2010 March 1 2010 for financial support. The authors would like to thanks Teddy Apri Riantiarno, Yanu Pamungkas, Nur Fauziah Arini, Winda Savitri, E. Topan Ardiansyah and Natalia Hariani for their contribution to this experiment.
Nomenclature
D dilution rate \( \text{(hour}^{-1}\text{)} \)
F flow rate \( \text{(L/hour)} \)
V bioreactor volume \( \text{(Liter)} \)
r retention time \( \text{(hour)} \)

REFERENCES