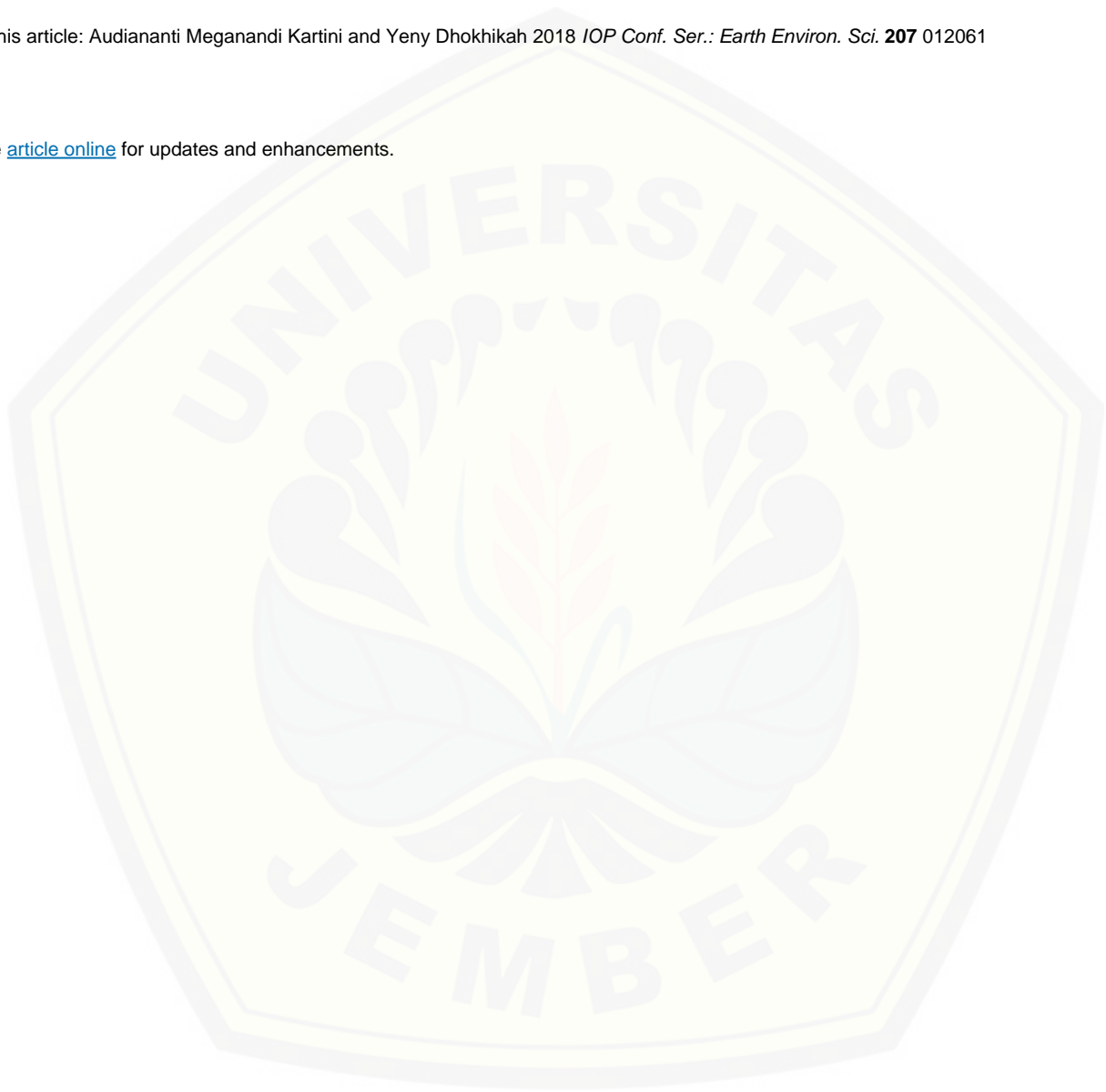


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Bioethanol Production from Sugarcane Molasses with Simultaneous Saccharification and Fermentation (SSF) Method using *Saccaromyces cerevisiae*-*Pichia stipitis* Consortium

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Abstract

The sugar-making industry from sugar cane has a byproduct of sugar cane as molasses. One alternative to reduce the amount of molasses waste is by reusing molasses as biomass for bioethanol production. Molasses can be reused as bioethanol that have economic value because of their high sugar content. The process of making bioethanol from molasses is done by Simultaneous Saccharification and Fermentation (SSF) method using 10% substrate (100 gr / L molasses) and 20% substrat (200 gr / L molasses). The process of hydrolysis of sugarcane molasses is carried out biologically using *Aspergillus niger* and *Trichoderma viride*, while the fermentation process uses the consortium *Saccaromyces cerevisiae*-*Pichia stipitis*. Ethanol production using the SSF methods had the highest bioethanol concentration of 14.38% v/v for 20% substrat and 10% consortium usage at 72 hours fermentation.

Keywords: Molasses, Bioethanol, Simultaneous Saccharification and Fermentation (SSF)

1. Introduction

Indonesia has a large population with sugar consumption reaching 4.2-4.7 million tons / year (PTPN X, 2015). The sugar-making industry from sugar cane in Indonesia has a byproduct of sugar cane or molasses. The high amount of molasses produced by sugar companies can be an environmental problem. Molasses waste decreases the dissolved oxygen content in water and disrupts the lives of aquatic organisms. The principle of Reuse, Reduce, and Recycle is still the best way to manage and handle waste with various problems including reducing the amount of molasses waste (Sarris *et al*, 2014).

Molasses waste is one of the wastes that can be reused as raw material which if processed will have high economic value. Most people use molasses as a raw material for making syrup, but the content of non-sugar impurities is very dangerous for health. The use of molasses in Indonesia is more widely used as raw material for making monosodium glutamate (MSG), but molasses has other features because the sucrose content ranges from 48 - 55% which has the potential to be used as raw material for ethanol production (Prescott and Dunn, 1959).

Ethanol is an alternative energy source that has several advantages, including renewable and environmentally friendly properties due to its low carbon dioxide emissions (Jeon, 2007). Ethanol can be used as a mixture of gasoline and can be used as fuel (McKetta, 1983). Ethanol production can be done by fermentation techniques (Hemamalini, 2012).

This large amount of molasses and high sugar content allows molasses to be converted into environmentally friendly fuel products. The role of the Indonesian government is expected to emerge as a policy maker for the use of ethanol as a national fuel mixture, this is needed to save the budget for crude oil purchases and support the national economy from ethanol production



2. Methods

2.1 Tools and Materials

The materials that will be used in this research are molasses, aquades, acetic acid buffer, sodium acetate, Potato Dextrose Broth (GDP), Potato Dextrose Agar (PDA), *A. niger* culture, *T. viride*, *S. cerevisiae* and *P. stipitis*. The tools used are 2 liter reactor, erlenmeyer, petridish, shaker, analytic balance sheet, test tube, thermometer, indicator pH paper, spectrophotometer, gas chromatography-mass spectrometry (GC-MS).

2.2 Preparation

Sugarcane molasses are obtained from the Glenmore Sugar Factory in Banyuwangi, East Java. *A. niger* and *T. Viride* were bred at the Microbiology Laboratory of FMIPA, University of Jember. *S. cerevisiae* CC 3012 has been bred in the Food And Nutrition Culture Collection (FNCC), PAU Food and Nutrition, Gajah Mada University. *P. stipitis* has been bred in the Laboratory of Microbiology, Department of Biology, Airlangga University.

Breeding in Potato Dextrose Broth (PDB) was done by agitation of 180 rpm shakers and incubated based on the growth velocity of $\frac{1}{2}$ of V_{max} (log phase) *A. niger* and *T. viride* which were 3 days, while $\frac{1}{2}$ of V_{max} *S. cerevisiae* CC 3012 and *P. stipitis* is 24 hours.

2.3 Hydrolysis and Fermentation (SSF)

Inoculum *A. niger* and *T. viride* 3 days (w/v) incubation and the consortium *S. cerevisiae* CC 3012-*P. stipitis* (2:1) (v/v) 24 hour incubation was added to the sample in the reactor then incubated for 6 days. Every 24 hours measurement of temperature, pH and reducing sugar is carried out. During the process of hydrolysis and fermentation in the reactor, microbial growth is observed every 24 hours. Periodic temperature and pH measurements were taken during the hydrolysis and fermentation processes to maintain stable pH conditions at 5. Reduction sugar test was carried out by the Nelson Samogyi Method. The samples were measured for bioethanol content after commencement after addition of *A. niger* and *T. viride* isolates and *S. cerevisiae* CC 3012-*P. stipitis* counts from 24, 48, 72, 96 and 120 hours with the Gas Chromatography Method.

3. Results And Discussion

3.1 Characteristics of Molasses Substrate

Characteristics of C, N and P substrate molasses in this study need to be known to determine the characteristics and potential of nutrients contained in molasses (Table 3.1).

Table 3.1 C: N: P Mollases

Measurement Time	C:N:P Mollases (%)		
	C	N	P
Before hydrolysis	22.27	1.4	0.19
After hydrolysis	25.70	1.2	0.31

C: N: P molasses at the stage before hydrolysis in this study is 22.27: 1.4: 0.19 (%), whereas after going through the hydrolysis stage, the C: N: P ratio is known to be 25.70: 1.2: 0.31. The ideal comparison of C: N: P for biomass for microorganism growth is 100: 5: 1 (%). In this study the ideal comparison is quite good. The initial C / N ratio in this study was 15.90%, while the C / N ratio after hydrolysis was 21.41%. These results are close to the ideal ratio of 20-30% (Napon *et al.*, 2013).

3.2 Reduction Sugar Levels in SSF

The SSF process that combines the process of hydrolysis and fermentation has an effect on the reducing sugar produced. Hydrolysis aims to break down polysaccharides into monosaccharides so that they can be fermented directly by a yeast consortium.

3.2.1 Reduction Sugar Levels in SSF with 8% and 10% Consortium

The advantage of the SSF process is that hydrolysis and fermentation are carried out in one container or reactor so that it can run efficiently. The results of SSF reduction sugar using 10% substrate (100 gr

/ L molasses) and 20% substrat (200 gr / L molasses) with the addition of 8% yeast consortium found in Figure 3.1.

In SSF treatment with a consortium concentration of 8% there was an increase in reducing sugar from 0 hours to 48 hours in all treatments, but thereafter decreased. The increase in reducing sugar at 0 to 48 hours was caused by hydrolysis of sucrose molasses to glucose. During the hydrolysis process, an average sugar level increase of 1.12 mg / g for the substrate 10% and 1.33 mg / g for the substrate 20% on average.

In SSF treatment with the addition of 10% yeast *S. cerevisiae-P consortium. stipitis* also has a different amount of reducing sugar. The process of increasing and decreasing the reduction sugar in the treatment of 10% yeast has the same trend as the treatment of 8% of the consortium. These results can be seen in Figure 3.2.

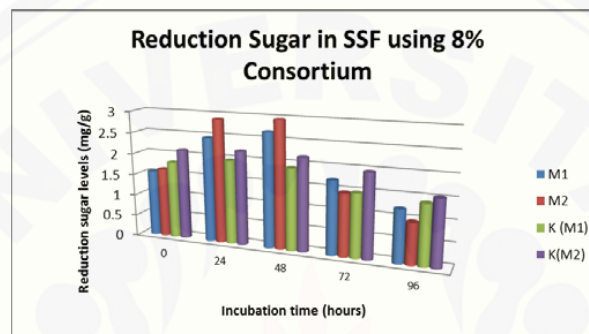


Figure 3.1. Reduction Sugar Levels in SSF using 8% consortium.

Description: M1 = 10% substrate concentration (100 g / L)

M2 = 20% substrate concentration (200 gr / L)

K = Control

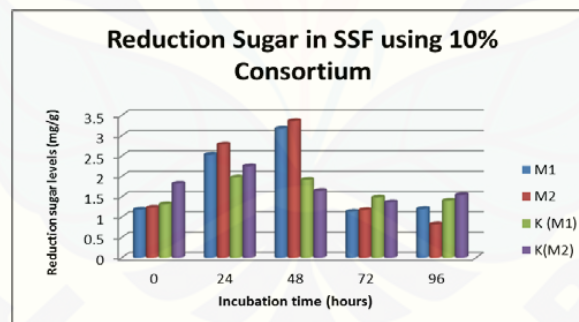


Figure 3.2 Reduction Sugar Levels in SSF using 10% consortium.

Description: M1 = 10% substrate concentration (100 g / L)

M2 = 20% substrate concentration (200 gr / L)

K = Control

During SSF hydrolysis process using 10% consortium, there was an increase in the average sugar level of 1.99 mg / g for the substrate 10% and 2.13 mg / g for the substrate 20%. Reduction in reducing sugar occurs in all treatments at 48 to 96 hours. The reduction in reducing sugar is proportional to the amount of ethanol produced, the longer the fermentation, the greater the ethanol produced (Hemamalini et al., 2012). The decrease in the amount of reducing sugar during the fermentation process is caused by sugar being used by microbes as a carbon source in fermentation. In the fermentation method simultaneously gives an effect on the reduction in the amount of reducing sugar in all treatments of SSF samples with 8% and 10% of the consortium.

In the process of hydrolysis of this study, *T. viride* plays a role in destroying high levels of cellulose and has the ability to synthesize several essential factors to dissolve parts of cellulose which are strongly bound to hydrogen bonds. In addition, *T. viride* produces cellulase complete with all the

components needed for total hydrolysis of crystalline cellulose with high sugar content produced (Esterbauer *et al.*, 1991).

A. niger plays a role in producing the enzyme hemicellulase which can hydrolyze hemicellulose complex into glucose, and also produce high cellulase. The advantages of *A. niger* from other fungi that is besides being able to produce cellulolytic enzymes also produce xyloglucanase (Pham *et al.*, 2010). Based on the advantages of these two fungi, the combination at the same time is able to provide better performance in reducing sugar. This is shown from the reduced sugar production which continues to increase until 48 hours when hydrolysis.

3.3 Microbial Growth

3.3.1 Microbial Growth During Hydrolysis

The stages of hydrolysis and fermentation in this study were carried out biologically (enzymatic hydrolysis using a combination of mold *T. viride* and *A. niger*). The results of growth observations of *T. viride-A. niger* using 10% substrate in this study found in Figure 3.3 and growth observations of *T. viride-A. niger* using 20% substrate found in Figure 3.4.

The results of growth observations of *T. viride* and *A. niger*, both on the usage of 10% substrate and 20% substrate, in this study showed that the combination of the two fungi experienced a significant increase at 48 hours. The significant increase in cell growth at 48 hours was a log (exponential) phase in this study. In this log phase the growth of microorganisms is the fastest.

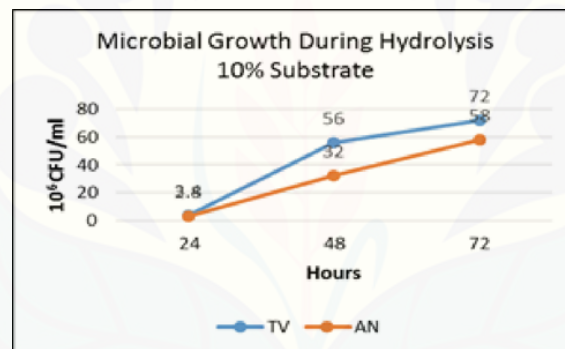


Figure 3.3. Microbial Growth During Hydrolysis with 10% Substrate..

Description: TV = *Trichoderma viride*

AN = *Aspergillus niger*

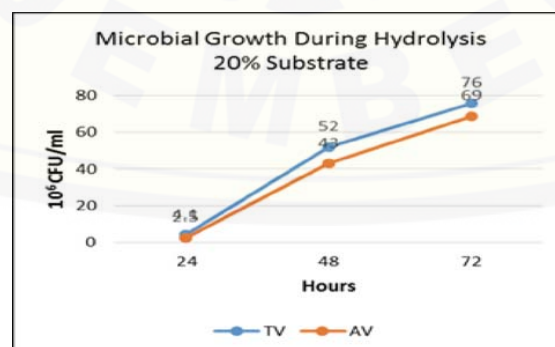


Figure 3.4. Microbial Growth During Hydrolysis with 20% Substrate..

Description: TV = *Trichoderma viride*

AN = *Aspergillus niger*

This is due to the large amount of substrate catabolism used for growth, enzyme synthesis and synthesis other compounds (Ernes et al., 2014), along with the high sugar content produced at the hydrolysis stage, but at 72 hours *T. viride* and *A. niger* began to enter the stationary period because the substrate that can be hydrolyzed decreases so that the substrate catabolism process also decreases, this is inversely proportional to reducing sugar produced. During the exponential phase the sugar levels continue to increase until the 48th hour. This is because the process of cellulose catabolism becomes simple sugars due to the cellulose enzyme activity of *T. viride* and *A. niger*.

3.3.2 Microbial Growth During Fermentation

The stages of hydrolysis and fermentation in this study are carried out biologically (enzymatic hydrolysis). The microorganisms used in the fermentation process are *S. cerevisiae*-*P. stipitis* consortiums. The results of growth observations of *S. cerevisiae*-*P. stipitis* consortium in this study found in Figure 3.5

The results of observations of *S. cerevisiae*-*P. stipitis* consortium growth in this study shows that *S. cerevisiae*-*P. stipitis* as a consortium experienced a significant increase in cell growth at 24 hours incubation since the consortium was inoculated into the reactor. Significant increase in cell growth at 48 hours was indicated as a log (exponential) phase in *S. cerevisiae* and *P. stipitis*. But on the 72 hour of *S. cerevisiae*-*P. stipitis* consortium no longer has a significant increase in cell numbers. This is because microorganisms enter the stationary phase where the number of cells is relatively fixed due to the limited number of substrates so that living cells are the same as dead cells (Wardani and Pertiwi, 2013).

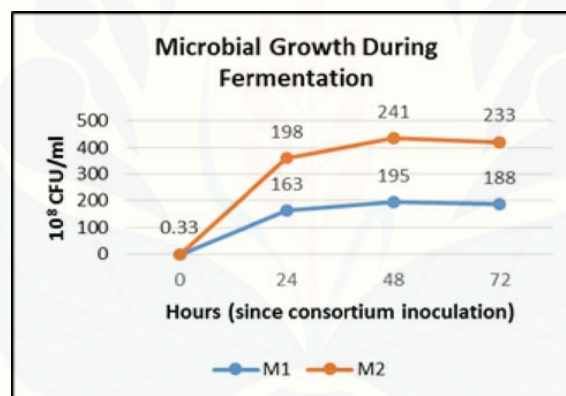


Figure 3.5. Microbial Growth During Fermentation
Description: M1 = 10% substrate concentration (100 g / L)
M2 = 20% substrate concentration (200 gr / L)

The growth of fermentation microbial cells is inversely proportional to the reduction in sugar content. The higher number of *S. cerevisiae* and *P. stipites* cells, the lower the reducing sugar level. This is because the simple sugars produced during hydrolysis are utilized by *S. cerevisiae*-*P. stipitis* consortium as a carbon source and converted to ethanol. In this fermentation stage, *S. cerevisiae* produces ethanol from hexose sugar contained in the substrate (Bayrakci and Kocar, 2014), while *P. stipitis* ferments hexose and pentose to ethanol (Ganguly et al., 2012).

3.4 pH and Temperature Analysis of Reduced Sugar and Bioethanol

In this study, the acetate buffer pH 5 in the inoculum was adjusted to the optimum conditions of microorganisms. The average pH range in this study was pH 5. The initial temperature of the substrate before treatment was 30°C, while the temperature range after pretreatment in this study increased to 31°C and continued to increase up to 32.5°C at the end of the fermentation stage.

There was no significant increase in pH during the hydrolysis process and in the fermentation process, but the temperature continued to increase during the process of hydrolysis and fermentation. The decrease in pH that occurs during the fermentation process because the microorganisms used in addition to producing bioethanol also produce CO₂ and organic acids (Wignyanto et al., 2001). While

the increase in temperature that occurs in the hydrolysis and fermentation stages proves the occurrence of microbial activity in the reactor.

pH stabilization is used acetate buffer pH 5. pH 5 is the optimum pH of fungal growth, especially yeast *S. cerevisiae* and *P. stipitis*. According to Apriwinda (2013), yeast can grow at a pH range of 2.5-8.5 and can grow optimally at pH 4.0-5.0. In addition, growing yeast at low pH can prevent bacterial contamination. In fermentation, pH control is very important because optimum pH must be maintained during fermentation. Conditioning pH to be stable at 5 is to maintain yeast activity in fermentation more optimally. pH also affects enzyme activity and lignin degradation process (Patel *et al.*, 2009).

3.5 Ethanol Levels in the SSF Method

Microbes as fermenting agents are certainly very influential on the length of fermentation. In alcoholic fermentation generally yeast is used because yeast can convert sugar to alcohol in the presence of enzymes. In this study, the microbes used were *S. cerevisiae* - *P. stipitis* consortium. According to O'leary *et al.* (2004), *S. cerevisiae* is a yeast commonly used in alcoholic fermentation. *S. cerevisiae* has several advantages that can produce high ethanol yields compared to other microbes that can also form alcohol.

3.5.1 Ethanol Yield in SSF using 8% *S. cerevisiae*-*P. stipitis* Consortium.

The results of ethanol yield through the SSF stage are much greater than the control treatment because in the control treatment the microbial fermentation is not added. The results of the bioethanol yield of the SSF method using 8% consortium are shown in Figure 3.6.

The highest Ethanol yield of SSF using 8% consortium and 10% substrate treatment in the experiment was done at 72 hours with result 8.2% (v / v). The highest ethanol yield of SSF using 8% consortium and 20% substrate was done at 72 hours with result 9.83% (v / v). The level of ethanol produced with a substrate amount of 20% is higher than the level of ethanol produced with a substrate amount of 10%. The results of this study indicate that the highest ethanol content on average is formed at 72 hours incubation with pH 5 and temperature 32°C.

According to Wahlbom and Hahn-Hägerdal (2002), *S. cerevisiae* can produce ethanol optimally in anaerobic facultative state at temperature 30-35 °C and pH range 4-5. Ethanol produced by *S. cerevisiae*-*P. stipitis* consortium tends to be higher. This is because the ability of *S. cerevisiae* which is able to ferment hexose sugar and *P. stipitis* is capable of fermenting xylose and other pentose sugars both aerobically and anaerobically (Ho *et al.*, 1998)

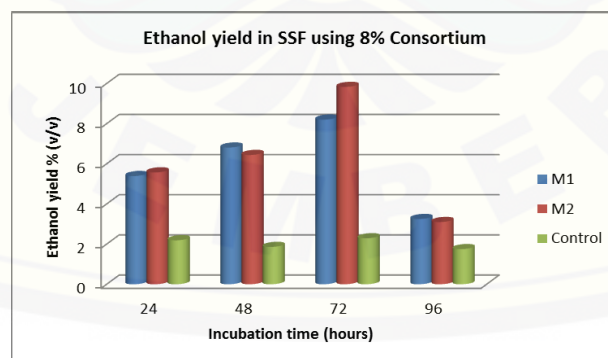


Figure 3.6. Ethanol Yield in SSF using 8% Consortium
Description: M1 = 10% substrate concentration (100 g / L)
M2 = 20% substrate concentration (200 gr / L)
K = Control

P. stipitis is able to ferment under aerobic and anaerobic conditions, and has the highest natural ability to ferment xylose, turning it into ethanol without producing xylitol (Nigam, 2002). The ability to ferment xylitol is not owned by *S. cerevisiae*. *P. stipitis* completes the performance of *S. cerevisiae* so that the consortium can produce ethanol better than using a single microorganism.

3.5.2 Ethanol Yield in SSF using 10% *S. cerevisiae*-*P. stipitis* Consortium.

The ethanol concentration obtained in this study is quite high. This is because in the fermentation process occurs the process of solving sucrose into monosaccharides or reducing sugars which can be utilized by the *S. cerevisiae*-*P. stipitis* for biological activities. During fermentation there is a decrease in the concentration of reducing sugar because it is used by the yeast consortium as a carbon source in its metabolic process. The results of bioethanol content using 10% consortium using the SSF method are shown in Figure 3.7.

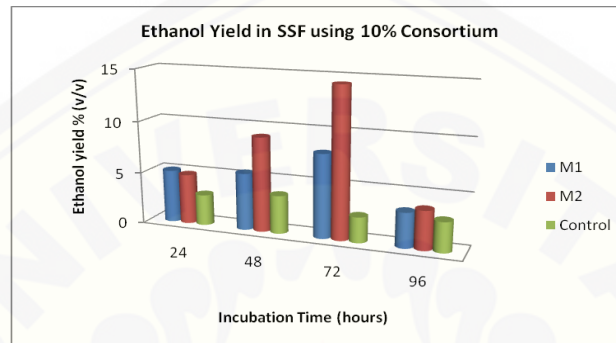


Figure 3.7. Ethanol Yield in SSF using 10% Consortium
Description: M1 = 10% substrate concentration (100 g / L)
M2 = 20% substrate concentration (200 gr / L)
K = Control

The 10% yeast consortium usage treatment produced ethanol reached 8.04% for the substrate 10% and 14.38% for the substrate 20% at 72 hours incubation. The concentration of ethanol produced was much greater compared to the control treatment that produced the highest ethanol with a level of 3.67%. The ethanol content obtained with the treatment of 10% yeast consortium yeast decreased at 96 hours. A decrease in ethanol in excess glucose concentration occurred as an inhibitory effect of substrates and products.

There was a decrease in ethanol levels at 96 hours up to 3.36% (v / v) ethanol yield for SSF using 10% substrate and 3.72% (v / v) ethanol yield for SSF using 20% substrate. This shows a relationship between fermentation stage and bioethanol produced. This fermentation time is also related to yeast cell growth. From the observations of microbial growth, the log (exponential) phase of the consortium is up to 48 hour incubation. Whereas at 72 hours incubation, the stationary phase begins to enter. In the stationary phase the microbes are in the stag phase, where nutrients have begun to run out and the number of cells tends to be stable until it enters the phase of death. During the phase there will be accumulation of toxins so that it can paralyze other microbes (Griffin, 1996).

4. Conclusion

Based on the research that has been done, the conclusions obtained are ethanol yield from sugarcane molasses using the SSF methods has the highest bioethanol concentration of 14.38% (v / v) for substrate 20% usage at 72 hours fermentation.

5. Acknowledgments

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