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Optimization of Enzyme Dosage and Sulfuric Acid (H₂SO₄) Concentration on Newspaper Waste Hydrolysis and Fermentation of its Hydrolyzate in Single and Consortium

Ratu Safitri^{1, a)}, Tia Setiawati¹, Mohamad Nurzaman¹, Roostita Balia²,
Franky Pangaribuan¹ and Jetty Nurhayati¹

¹Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran,
Jl. Raya Bandung Sumedang Km. 21, Jatinangor, Jawa Barat 45363, Indonesia

²Faculty Of Animal Husbandry Jl. Raya Bandung Sumedang Km. 21, Jatinangor,
Jawa Barat 4536, Indonesia

^{a)}Corresponding author: ratusafitrie@yahoo.com

Abstract. Research of the optimum concentration of sulfuric acid (H₂SO₄) on acid hydrolysis and enzyme at the optimum dosage of enzyme hydrolysis of newspaper waste in producing hydrolyzate at optimum levels in reducing sugars and fermentation of its hydrolyzate was conducted. The purpose of this study was to obtain the optimum enzyme dosage and concentration of sulfuric acid hydrolysis of waste paper to produce the best reducing sugar hydrolyzate and microbes to obtain the most effective use of microbial fermentation process. Descriptive and experimental method were used in this research. Descriptive method was applied for evaluating enzyme and acid hydrolysis, preparation hydrolyzate and starter of culture as well as fermentation. The fermentation was conducted by experimental methods using Completely Randomized Design (CRD) with three replications. This study parameter included reducing sugar content, ethanol content, microbial population, Dextrose Equivalent (DE) and pH. Our finding showed that the highest reducing sugar content on sulfuric acid hydrolysis (H₂SO₄) was at a concentration of 6, whereas the liquefaction α -amylase enzyme was at dosage 0.52 $\mu\text{L} \cdot \text{g}^{-1}$. The optimum dosage for the hemicellulase enzyme hydrolysis was at 0.001 $\text{g} \cdot \text{g}^{-1}$, whereas the saccharification cellulase and amyloglucosidase enzyme were 0.83 $\mu\text{L} \cdot \text{g}^{-1}$ and 0.56 $\mu\text{L} \cdot \text{g}^{-1}$. The most effective and optimum microbial fermentation process was obtained with a consortium of *Zymomonas mobilis* and *Pichia stipitis* that produces the highest ethanol content by 6.523 % at 72 h with fermentation efficiency by 45.77 %. Consortium *Zymomonas mobilis* and *Pichia stipitis* also reached the highest end of exponential population phase with 27.5×10^{10} CFU at 36 h with the highest maximum growth rate by 2.48. Additionally the effectiveness was also supported by the lowest reducing sugar content at 9.09 % with the highest growth yield by 1.689 and the end of Dextrose Equivalent (DE) by 56.140 at the lowest pH of 3.34.

Keywords: Acid hydrolysis, consortium, fermentation, hydrolysis enzyme.

INTRODUCTION

Bioethanol is a renewable fuel with zero emissions; meaning that its use can reduce the greenhouse gas (CO₂), thereby reducing the effects of global warming. Principally, ethanol is produced through hydrolysis and fermentation process. Hydrolysis is a chemical reaction that solves a molecule into two molecules to convert polysaccharides into simple monomers. Hydrolysis process can be carried out enzymatically and non-enzymatically. In contrast, fermentation is the process of breaking down sugars into alcohol and carbon dioxide caused by the activity of microbial cells that grow and thrive in it. Fermentation occurs due to the activity of microbes that cause fermentation in the corresponding substrate. In general, the simultaneous saccharification and fermentation (SSF) are considered as the most effective fermentation method. SSF is a bioethanol production process where the

hydrolysis and fermentation are completed in one reactor unit. The use of microbial consortium in the process of fermentation is known to be more efficient than using a single starter. The use of starch as a material of bioethanol is still limited, due to its common utilization as a staple food. Other alternative materials that can be used as raw material for the production of cellulosic bioethanol is glucose because of its constituent monomers that are together with starch. Newsprint is one resource that has a high cellulose, derived from hardwood raw material that has short fibers. Furthermore, the chemical composition of the hardwood as raw material for newsprint, namely; the cellulose content of 45 % (\pm 3 %), hemicellulose 30 % (\pm 3 %), lignin 20 % (\pm 4 %) and extractive 5 % (\pm 3 %).

This research was conducted by the hydrolyzate fermentation process for optimization newsprint by microbial fermentation using single and mixed culture. To be able to produce bioethanol at a high level, the information on which microbes with the greatest ability to ferment sugars into ethanol is crucially required. A single culture of fermentative microbes is *Saccharomyces cerevisiae* strain of palm wine, *Zymomonas mobilis* and *Pichia stipitis*. The mixed culture of microbes was used from a consortium of *Zymomonas mobilis* and *Pichia stipitis* and a consortium of wine strains of *Saccharomyces cerevisiae* and *Pichia stipitis*.

MATERIAL AND METHODS

Preparation of Hydrolyzate for Fermentation

Acid and enzymes hydrolyzed newspaper waste. Six percent of sulfuric acid (H_2SO_4) was added to newspapers waste and incubated at 121 °C in an autoclave for 120 min [1]. The substrate pH was set to 6 and then α -amylase enzyme was added as a dose of 1 mL \cdot g⁻¹ or 0.52 mL \cdot g⁻¹. The mixture was then incubated at 104 °C with an agitation speed of 500 rpm for 1 h (1 rpm = 1/60 Hz). The substrate was set back to the pH of 6; hemicellulase enzymes were then added as much as one dose (0.001 g \cdot g⁻¹), incubated at 55 °C with an agitation speed of 500 rpm for 360 min. Furthermore, the substrate was set to pH of 4.8 and then a cellulase and amylogucosidase enzyme was added as much as one dose (0.83 mL \cdot g⁻¹) and one dose (0.56 mL \cdot g⁻¹). Moreover, the mixture was incubated for 48 h at 60 °C to 62 °C with an agitation speed of 130 rpm [2], Substrate was 20 % regulated from the sugar reduction. Then additional nutrients such as ammonium sulfate [(NH₄)₂SO₄] and peptone were added as much as 0.4 % and 1 % of the total substrate volume (w/v), respectively. Furthermore, the substrate was added to a 100 mL Erlenmeyer flask. Fermentation substrate was sterilized in an autoclave at 121 °C, 1 atm for 15 min (1 atm = 101 325 Pa).

Hydrolyzate Fermentation by Single and Mixed Culture

In the microbial fermentation by a single starter, 10 % of each microbial starter was added to the fermentation substrate. In the fermentation by the Consortium 1, incorporated as much as 5 % (v/v) of starter for both *Zymomonas mobilis* and *Pichia stipitis*. The fermentation by the Consortium 2 included 5 % (v/v) of starter for both *Saccharomyces cerevisiae* (a strain of wine) and *Pichia stipites*. Each starter was added to the fermentation substrate and incubated while shaken (shake-incubation) at 28 °C for 72 h with an agitation speed of 150 rpm. During incubation, samples were taken every 12 h [(0; 12; 24; 36; 48; 60; and 72) h]. Each sample was placed in a different Erlenmeyer flask. Parameters measured were the sugars reduction levels and the ethanol content by the DNS and dichromate oxidation method, respectively. Dextrose Equivalent (DE) was also used as a parameter.

RESULT AND DISCUSSION

Preparation of Hydrolyzate for Fermentation

In this study, newsprint waste was prepared by acid and hydrolysis enzyme. Acid hydrolysis results showed the highest sugars reduction levels was obtained in 120 min of incubation time with 6 % of sulfuric acid concentration 4.47 % with the highest DE values about 5.48. During liquefaction process, the α -amylase enzyme will convert the starch into glucose and dextrin. The optimal dose use of α -amylase was at a dose of 1 mL or 0.52 mL with the highest reducing sugars levels at 6.25 and DE value of 7.66 %. After experiencing liquefaction process, the liquefaction hydrolyzate then entered the stage of hemicellulase hydrolysis. Hemicellulase enzyme enables to convert hemicellulose into xylose. In the present study, a dose of one hemicellulase enzyme reduced 15.23 % of sugar content with 18.66 DE. Furthermore, hydrolyzate entered saccharification stage where cellulase enzymes to

convert cellulose to glucose and also the rest of the glucoamylase enzyme that converts dextrin into glucose. From this process, 70.42 % of sugars reduction was obtained with 86.28 DE. Our study showed that reducing sugar levels increased proportionally with hydrolysis process.

Hydrolyzate Fermentation by Single and Mixed Culture

Fermentation occurs due to the activity of microbes causing fermentation in the corresponding substrate [3]. Microbial biomass for *Saccharomyces cerevisiae* strain palm *Zymomonas mobilis* and *Pichia stipitis* before fermentation was 1.97×10^{10} CFU, 2.28×10^{10} CFU and 1.81×10^{10} CFU, respectively.

Sugar Content Reduction

Sugar content reduction during fermentation of bioethanol newspaper paper was analyzed statistically using analysis of variance (ANOVA) by SPSS. ANOVA showed that microbes as well as affect the average levels of reducing sugars produced during the fermentation process of bioethanol from newsprint (Fig. 1).

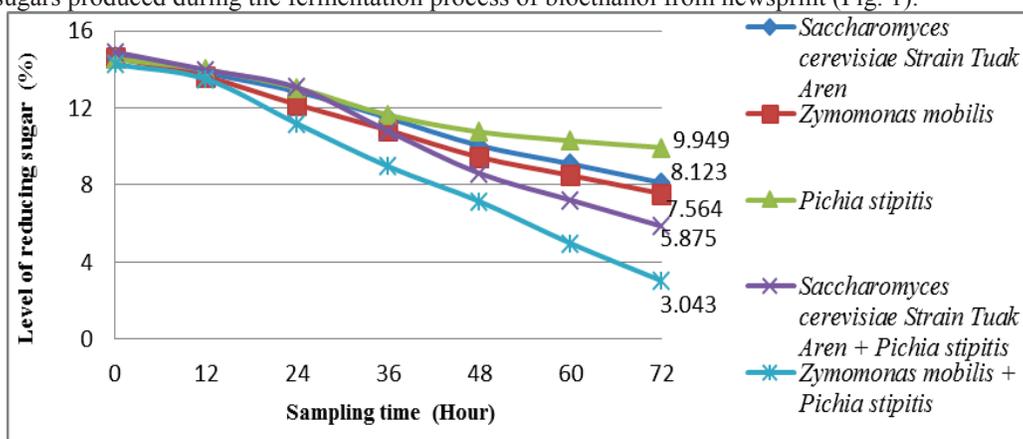


FIGURE 1. Sugar content reduction level after fermented by microbes

Each microbial fermentation extends the condition of the substrate, which indicated by the reduction of sugar levels with increasing of fermentation time. The conversion of glucose into pyruvic acid via the Embden-Meyerhof-Parnas, the pyruvic acid produced will be decarboxylated to acetaldehyde which later will be dehydrogenated into ethanol. This process is catalyzed by enzymes which are produced by microbes [4]. Duncan test table showed that the lowest sugar reduction levels (3.04 %) were obtained in consortium *Zymomonas mobilis* and *Pichia stipitis* in 72 h fermentation.

The effective and optimum fermentative microbes were the one who gain lowest sugar reduction levels. Effective sugar reduction indicated among single starter microbes in fermentation, such as *Zymomonas mobilis* cooperated with *Pichia stipitis*. In the fermentation process, *Zymomonas mobilis* work optimally to break glucose, while *Pichia stipitis* has an advantage in breaking down sugar in the form of xylan.

Dextrose Equivalent (DE)

Dextrose Equivalent is the ratio between the percentage of sugars reduction obtained by hydrolysis from the total amount of carbohydrates contained in the substrate. The average value of DE during the fermentation process of ethanol was statistically analyzed using ANOVA. The results showed that microbial species and the time influence DE generated during bioethanol fermentation process. Fig. 2 shows DE value during the fermentation process of bioethanol from newsprint.

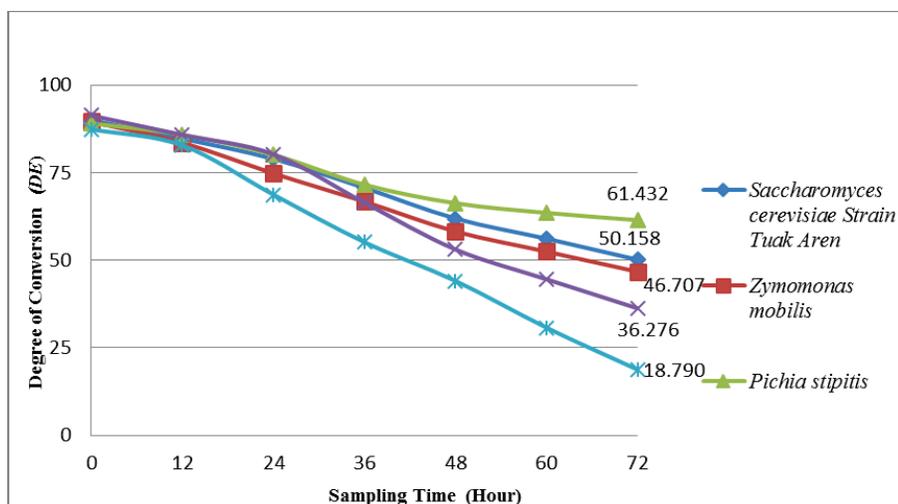


FIGURE 2. Mean of Dextrose Equivalent during Fermentation by microbes

As expected, the result showed that DE decreased proportionally with fermentation time. The decreased of DE followed the decreased levels of reducing sugars. The lowest DE indicated the effective and optimum fermentative microbial. Duncan test showed that the lowest mean of DE (18.79) was obtained by a consortium of *Zymomonas mobilis* and *Pichia stipitis* in 72 h of fermentation.

Ethanol during Fermentation

Percentage of ethanol produced during fermentation from newsprint waste was statistically analyzed with ANOVA. ANOVA showed that both microbes species and time significantly affect the ethanol produced during the fermentation (Fig. 3).

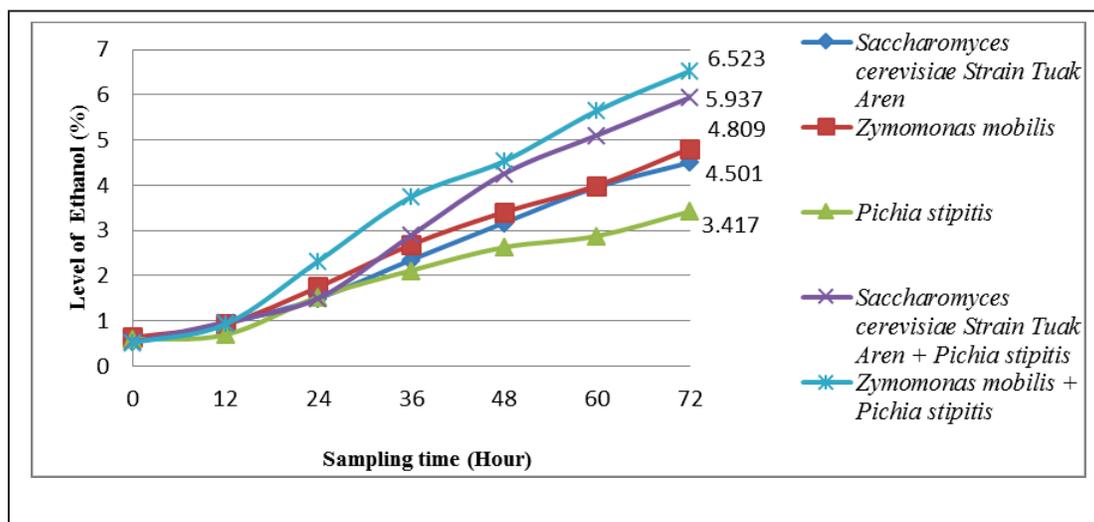


FIGURE 3. Ethanol levels during fermentation by microbes

Figure 3 showed that ethanol level increased over time. The increasing ethanol was caused by the activities of each fermentative microbes in turning glucose into ethanol. This probably due to the time length of the fermentation of ethanol production greatly affects the levels of ethanol produced. The longer the fermentation time, the higher levels of ethanol produced [5]. Moreover, Duncan test showed that the consortium of *Zymomonas mobilis* and *Pichia stipitis* produce the highest levels of ethanol (6.52 %) with 45.77 % of fermentation efficiency. These results

indicated that consortium of *Zymomonas mobilis* and *Pichia stipitis* is the most effective and optimum fermentative microbes. Perhaps, mixed culture of *Zymomonas mobilis* and *Pichia stipitis* is able to use the hydrolyzate from bagasse, resulting in a yield of bioethanol which is higher than the use of single culture [6]. Microbial consortia are synergistic and able to produce various enzymes so that the fermentation runs effectively.

Analysis on several parameters showed that consortium of *Zymomonas mobilis* and *Pichia stipitis* was the best treatment with the acquisition of the highest ethanol and reduction of sugar consumption. Figure 4 showed the relationship of reducing sugar content, ethanol production and the population of the consortium *Zymomonas mobilis* and *Pichia stipitis*.

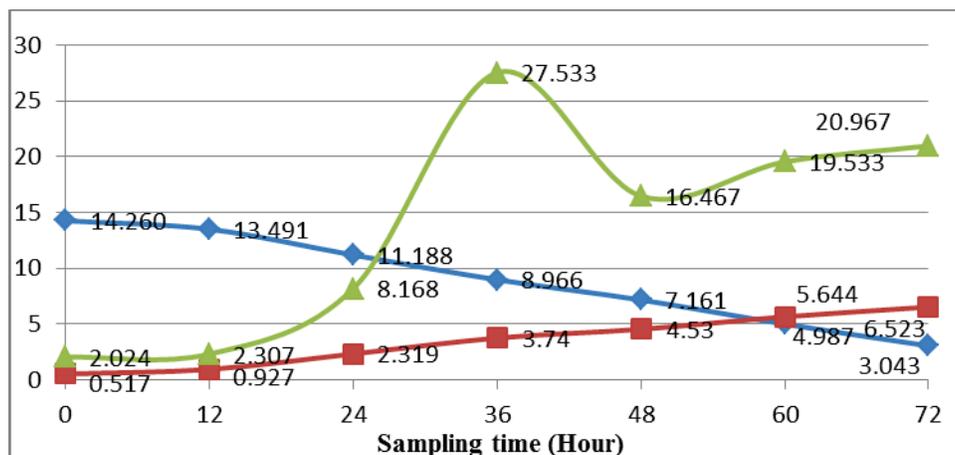


FIGURE 4. Relationship between sugar reduction (Blue), ethanol level (red) and the population (green) of the consortium *Zymomonas mobilis* and *Pichia stipitis*

Based on Fig. 4, a consortium *Zymomonas mobilis* and *Pichia stipitis* increased levels of ethanol at the 12th hour by 0.92 %. Increased levels of ethanol are in line with the decrease in the reducing sugar content 13.49 % due to the increase in the population of microorganisms. Furthermore, reducing sugar content in the consortium declined treatment and is accompanied by increased levels of ethanol. The condition is supported by a consortium of the microbial population after the exponential phase and then entered the stationary phase that lasts a long time. This consortium was the optimum microbes in the fermentation from newspaper waste at 6.52 % of ethanol content with 45.77 % of fermentation efficiency. This result was in coherence with Nan [6] who observed that *Zymomonas mobilis* has many advantages, for instance, it grew faster than yeast and its ethanol tolerance (as high as 20 %). Additionally, it ferments the glucose, fructose or sucrose substrate into bioethanol and enable to work synergistically with yeast *Pichia stipitis* that can ferment glucose and xylose into bioethanol.

CONCLUSION

It can be concluded that the best reducing sugar of newspaper waste fermentation for bioethanol is gain by a consortium of *Zymomonas mobilis* and *Pichia stipitis*. It contains about 3.043 % of reducing sugar at 72 h fermentation process. The best dextrose equivalent (DE) value of newspaper waste fermentation for bioethanol is gain by a consortium of *Zymomonas mobilis* and *Pichia stipitis*. It contains 18.790 at 72 h fermentation process. The highest ethanol content gain by a consortium of *Zymomonas mobilis* and *Pichia stipitis* with value about 6.523 % and the efficiency of fermentation about 45.771 %.

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