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Potency of *Aspergillus* sp. in Hydrolysis Process to Produce Ethanol from Vegetable and Fruit Wastes at Wonokromo Market, Surabaya

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Abstract. Vegetable and fruit wastes are potential to be used as a feedstock in ethanol production that consist of several steps, including pretreatment, hydrolysis, fermentation, and destilation. Hydrolysis process that commonly used is acid hydrolysis because it is easier to be done, but it produces end product that can inhibit fermentation process. Thus, mold utilization is the best alternative in hydrolysis process, one of the potential mold is *Aspergillus* sp. This research was aimed to determine *Aspergillus* sp. potency in hydrolysis process to produce ethanol from vegetable and fruit wastes at Wonokromo market Surabaya with various treatments of optimum *Zymomonas mobilis* inoculum size and fermentation duration. *Aspergillus* sp. in this research was obtained from mold exploration result of Alas Purwo National Park that were succesfully tested its cellulolytic ability and resulting cellulases enzyme such as endoglucanase, exoglucanase, and β-glycosidase. Fermentation process was conducted using *Zymomonas mobilis* bacteria in several treatments, inoculum size (0, 5, 10, and 15%) and fermentation duration (0, 2, 4, 6, and 8 days). Each treatment was done in two replications, so there were 40 units of trial which is analyzed using ANOVA test. The observed parameter was ethanol concentration. The result showed that *Aspergillus* sp. was a potential ability to produce cellulase enzyme in hydrolysis process to produce ethanol from vegetable and fruit wastes at Wonokromo market Surabaya. Optimum ethanol concentration 9,5% (v/v) was obtained from interaction between 10% inoculum size and 6 days fermentation duration.

Key-Words: *vegetable and fruit wastes, wonokromo market surabaya, enzymatic hydrolysis, ethanol, Aspergillus sp., Zymomonas mobilis*

1. Introduction

Hydrolysis process that common used to produce bioethanol is acid hydrolysis because more easier than enzymatic hydrolysis, but it has weakness to produce end product that can inhibit fermentation process [1]. Thus, the best alternative in hydrolysis process to produce bioethanol is enzymatic hydrolysis. Substrate that contain glucose can enter directly in fermentation process. But, substrate that contain cellulose, for example vegetable and fruit waste used enzymatic hydrolysis of cellulose to glucose is carried out by cellulase enzymes. It is often become a problem in lignocelluloses biomass utilization that abundant in nature. The cost of this production will be expensive if it is done in hydrolysis process with commercial enzyme. An appropriate, efficient and cheap method is needed in mold utilization that can produce cellulase enzymes. This method was not maximum utilized yet and in fact rarely to do.

Aspergillus sp. with code (H_5) collection from Microbiology Laboratorium, Department of Biology, Faculty of Science and Technology, Airlangga University that obtained from exploration product in Alas Purwo National Park. It was succesfully tested its cellulolytic ability to degradation of cellulose with produced cellulase enzymes. This enzyme were a complex enzymes such as endoglucanase, exoglucanase, and β-glycosidase. Endoglucanase enzyme was brokedown amorf cellulose became short chain cellulose and then continued with exoglucanase enzyme was brokedown short chain cellulose became cellobiose, the last β-glicosidase enzyme brokedown cellobiose become glucose [2]. After that process, glucose was converted became to ethanol in fermentation process using *Zymomonas mobilis* bacteria.

Utilization of organic wastes in Wonokromo market, Surabaya with acid hydrolysis had been resulted ethanol [3]. Another researches to produce bioethanol from orange wastes using *Zymomonas mobilis* bacteria had been produced 11,64% ethanol in 6 days [4], from tomato wastes had been produced 9,68% ethanol in 6 days too [5]. The differences of in substrate and hydrolysis process method can affect the final result in bioethanol production. Therefore, the researcher will do the research to find out the potency of *Aspergillus* sp. in hydrolysis process to produce ethanol from vegetable and fruit wastes at Wonokromo market, Surabaya.

2. Materials and Methods

2.1. Pretreatment Process

Two kilograms of vegetable and fruit wastes contain cellulose were taken at Wonokromo market, Surabaya and was washed. Pretreatment had been done by physical, mechanical and chemical process. In physical pretreatment process, the wastes were dried under the sunlight (±1-2 days). After that, by mechanical

pretreatment, the wastes were cut finely less than 2cm in size, mashed, sifted with 40 mesh filter and stored in dry place. Delignified vegetable and fruit wastes were treated with 2% (wt) NaOH in 1:6 (b/v) comparison at room temperature (30°C-32°C) for 24 hours to remove hemicellulose and had been sterilized with autoclave at 120°C for 1 hour. After that, it was washed with water until neutral pH (7) and furnaced at 65°C until obtained a constant weight [6]. This extract was used to make growth curve, starter, hydrolysis process and fermentation process.

2.2. Enzymatic Hydrolysis process Using *Aspergillus* sp.

There are many steps in this process, the first was cultivating *Aspergillus* sp., the second was enzyme production, and the last was harvesting and testing the enzyme activity [6]. The culture in Potato Dextrose Agar (PDA) medium. Production of enzyme was using 150 ml/bottle an Andreoti medium and added 1,5 gr (1%) wastes extract that finished in pretreatment process with pH 5, closed with cotton fat and sterilized with autoclave at 121°C for 20 minutes [7]. Then, spore suspension from *Aspergillus* sp. be old in 7 days with 10% (v/v) consentration were moved to fermentation medium and shaked on rotary shaker with 120 rpm speed for 7 days [6].

Enzyme activity was harvested and tested in the last fermentation for 7 days. Crude enzyme sentrifuged at 4000 rpm speed for 16 minutes, a filtrate were taken and filtered using paper filter until the solution clearly, were ready to analysis and its called a hydrolizates. In this research, a parameter that searched was an endoglucanase enzyme activity tested using 1% CMC substrate. The result had been measured in Unit/ml. One unit in endoglucanase enzyme activity as same as equals one micromolecule glucose that resulted from treatment of enzyme with 1% CMC substrate for 1 minute [6].

2.3. Stock Culture

Nutrien Agar (NA) medium was used for the isolation of *Zymomonas mobilis*, incubated at 30°C for 24 hours. To enrichment a number of cells, the medium was added 20g/L glucose, 10 g/L yeast extract, 1 g/L $(NH_4)_2SO_4$, 1 g/L K_2HPO_4, and 0,5 g/L $MgSO_4.7H_2O$) [8].

2.4. Measurement of Bacterial Growth Curve

One ose of *Z. mobilis* was inoculated into 50 ml Erlenmeyer flask containing 5 ml of sterile vegetable and fruit wastes hydrolizates that has been set at pH 4 by adding 30% HCl solution, then incubated at 30°C for 24 hours (Activation I). A total of 1 ml of activation I inoculated again into 50 ml Erlenmeyer flask containing 9 ml of vegetable and fruit wastes hydrolizates, incubated at 30°C for 24 hours (Activation II). A total of 5 ml of activation II inoculated again into 100 ml Erlenmeyer flask containing 50 ml of vegetable and fruit wastes hydrolizates, were incubated at 30°C and incubated at 30°C for 24 hours (Activation III) [9,10].

Performed dilutions were from 10^{-1} to 10^{-9} dilution. One ml of medium cultures were taken and put into test tubes containing 9 ml of sterile aquades, mixed with vortex mixer, one ml was taken and put into the others test tubes until 10^{-9}. The graph of growth curve was made by measuring the absorbance of *Zymomonas mobilis* bacteria on vegetable and fruit wastes extract. *Zymomonas mobilis* was measured at 600 wavelength with intervals of once every one hour during 24 hours. The graph of growth curve measured by absorbance values and fermentation duration [11]. Determination of regeneration or doubling time (Td) conducted during the incubation period until log phase with the highest value (Td) became the age of starter [12].

2.5. Starter Preparation of *Zymomonas mobilis*

Z. mobilis was inoculated into 50 ml Erlenmeyer flask containing 5 ml of sterile vegetable and fruit wastes hydrolizates that has been set pH to 4 by adding 30% HCl solution, then incubated at 30°C for 24 hours (Activation I). A total of 1 ml of activation I inoculated again into 50 ml Erlenmeyer flask containing 9 ml of vegetable and fruit wastes hydrolizates, incubated at a temperature of 30°C for 24 hours (Activation II). Five milliliters of activation II was inoculated again into 100 ml Erlenmeyer flask containing 50 ml of vegetable and fruit wastes hydrolizates, were incubated at 30°C until the hour in which log phase of *Z. mobilis* occur (in accordance with the growth curve) (Activation III) [9,10].

2.6. Fermentation Process and Measurement of Ethanol Concentration

Starter was added with a concentration in accordance with the study design (0, 5, 10, and 15%) into the fermenter bottle (100 ml) containing 50 ml of vegetable and fruit wastes hydrolyzates, incubated with the duration according to the study design (0 day, 2 days , 4 days, 6 days and 8 days) at room temperature (30°C-32°C). Fermentation process performed on anaerobic conditions. Anaerobic fermentation was done by using Hungate techniques, namely by passing nitrogen gas into the fermenter. Fermenter was closed with rubber

stoppers and then closing the gas flowing nitrogen for 2 minutes. After fermentation is complete, the bottle cap was removed, covered with cotton fat and Pasteurized at a temperature of ± 80 °C for 10 minutes [13]. The aim of Pasteurization process was to turn off the bacteria contained in the sample so that the fermentation process can be stopped [5]. Ethanol concentration measured by using spesific gravity method [14]. Reducing sugar concentration was measured using Somogyi-Nelson method [15].

2.7. Statistical Analysis

This research consists of two factors treatments, inoculum size (0, 5, 10, 15%) and fermentation duration (0, 2, 4, 6, and 8 days). The experiments were carried out in two replications, thus there were 40 units of trial. The experimental data were analyzed according to Analysis of Varians (ANOVA) procedure followed by Tukey test with a hypotesa (H_0) There was no interaction between inoculum size of *Zymomonas mobilis* bacteria and fermentation duration in ethanol produced (H_1) There was interaction between inoculum size of *Zymomonas mobilis* bacteria and fermentation duration in ethanol produced [16].

3. Results and Discussion

3.1. Potency of Aspergillus sp. in Hydrolysis Process

Aspergillus sp. were used in hydrolysis process that was 7 days when it in the end of the log phase and entered early stationer phase [17,18,19]. It was aligned with the research that the highest of celullase enzyme activity was obtained at the time of stationer phase [20]. Harvesting was done at the end of the fermentation at 7th days and analysed the enzyme activity using 1% CMC substrate thus it commonly called as an endoglucanase enzyme [21].

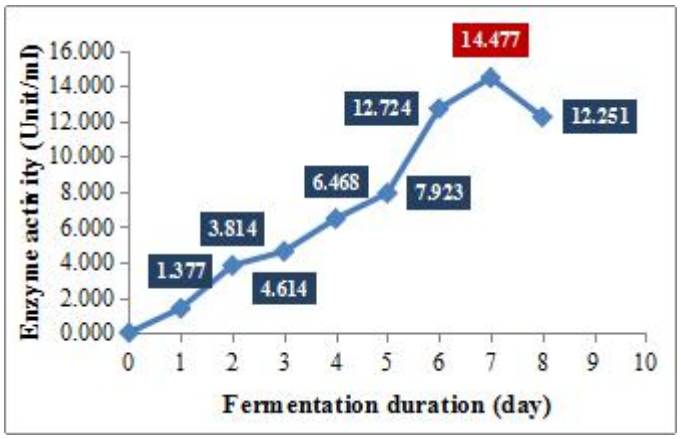

Figure 1. Graph of endoglucanase enzyme activity using *Aspergillus* sp.

The value of enzyme activity is affected by several factors, such as humidity, temperature, substrate, and a pH [22]. Enzyme activity on 1st day to 7th days has increased and reached a maximum on 7th days as well as a decline in the 8th days (Fig.1). It was aligned with the other research which the enzyme production during fermentation can be reached within a certain period, then decline rapidly or slowly [6]. Thus, harvesting time must be known exactly to get the maximal activity.

Fermentation medium was adding nutrients that it will be exhausted during the fermentation process to produce a maximal enzyme activity, then with the depletion of nutrients will lead to the production of the enzyme activity and *Aspergillus* sp. growthwas decrease. Thus, it can be inferred, the proper enzymes harvesting time use *Aspergillus* sp. with vegetable and fruit waste substrate was the 7th days with an endoglucanase enzyme activity value 14,477 Units/ml. One unit of enzyme activity cellulase was defined as the amount of enzyme which produced one micromol reducing sugar (glucose) every minute of it.

Andreoti medium was used in hydrolysis process with pH 5 because it was the optimum pH for *Aspergillus* sp. growth [6]. Decreasing pH facilitated the release of cellulase enzyme, while an increasing pH caused a release of cellulase enzyme outside cell will be hampered. At the end of the hydrolysis, pH 5 changes to pH 6. The speed of an enzymatic reaction increased with increasing pH. Enzyme activity was affected by pH due to the ionic nature of the amino group and a carboxyl group which is easily influenced by pH.

The temperature in this research was room temperature (30°C-32°C) because it was an optimum temperature for genus Aspergillus. Above the optimum temperature, reaction rate dropped sharply because of the

enzyme as a protein will be denaturated, while the temperature is too low the enzyme cannot work. In addition, the humidity can also affect enzyme activity, related to *Aspergillus* sp growth. The Genus Aspergillus can grow well on the relational humidity 80%. It shaked using rotary shaker that the aim was balancing an availability of oxygen in the medium, a high speed wobble in an Erlenmeyer flask will caused spore sporulated homogeneously. Thus, it was not sporulated on the surface [23].

The amount of substrate or vegetable and fruit wastes extract that had pretreatment by as much as 1.5 grams of extract incorporated into 150 ml Andreoti medium or 1% of the total medium. It was corelated with the other research which a substrate that is too high can cause fermentation medium be rather concentrated and it can be given a problems in air circulation, decreasing the level of its homogenity and the spread of *Aspergillus* sp. [6]. The size of substrate can also influence, more smaller substrate, more easily the enzyme contacted with substrate [22]. Thus, in the hydrolysis process to be aware of pH, temperature, humidity, and the optimum substrate for *Aspergillus* sp. in resulting a maximum enzyme activity.

Before *Aspergillus* sp. inoculated, there was 2,14% reducing sugar resulted. After hydrolysis process, it increased to 10,80%. This result was showed using *Aspergillus* sp. was not different with using acid hydrolysis or commercial enzyme hydrolysis that commonly used in many researches. For example, in ethanol production from spirogyra with commercial enzyme hydrolysis resulted 10,05% reducing sugar [24]. Another research with acid hydrolysis process from market wastes resulted 9,98% reducing sugar [3] and with enzyme hydrolysis process had more advantages than chemical hydrolysis that it was not degradation of reducing sugar resulted. The reducing sugar accomplished as secondary data in ethanol production beside the temperature, pH and substrate because a glucose that resulted in hydrolysis process will be canged become ethanol in fermentation process using *Zymomonas mobilis* bacteria. To know that *Aspergillus* sp. was potential or not in hydrolysis process as an alternative method can be seen in ethanol produced.

3.2. Ethanol Production from Vegetable and Fruit Wastes at Wonokromo Market Surabaya

The sample of wastes used in this research was a vegetable and fruit wastes in Wonokromo market, Surabaya with five kinds of vegetable waste (ipomoea, chickpea, lettuce, mustard greens and green Cosmos caudatus), and five kinds of fruit bins (banana, apple, orange, guava, and papaya) containing cellulose. The final result of the pretreatment process was obtained from 2000 gr to 187 gr vegetable and fruit wastes extract. Then, this extract used as the substrate to produce bioethanol through of hydrolysis, fermentation and distillation processes.

Strater was made in fermentation process that was collection of microorganisms that are ready inoculated into fermentation medium. This is adaptation step of microorganism to adapt in new environmental medium. The growth curve gives an overview of the environmental factors that can influence the growth of microorganism such as substrate, ambient temperature, and pH.

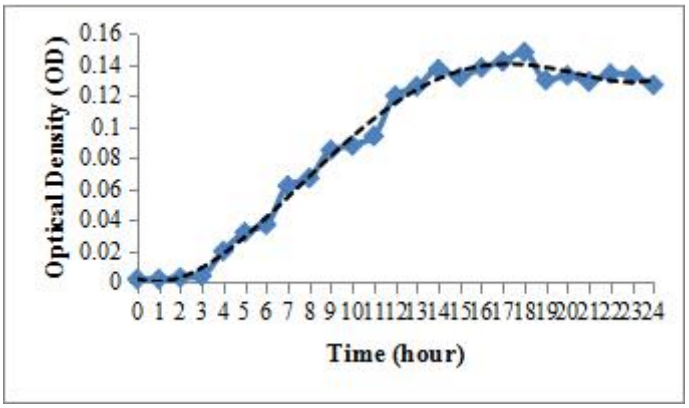

Figure 2. *Zymomonas mobilis* bacteria growth curve in vegetable and fruit wastes media.

Lag phase in that curve (Figure. 2) happened at 0 to 3rd hours. The growing cells looked and continued uphill at 3rd to 19th hours. It was also commonly phase that called exponential phase with multiplicated the number of cells, cell activities increased, an was an important phase in *Zymomonas mobilis* growth. After multiplicated was occur, cell activities will be increased too and being stagnant graph that called a stationary

phase at 19th to 24th hours. On this research wasn't found death phase because until 24th hours the graph showed a stagnant.

Then, by making this growth curve can be determined the optimal age for a starter. Age of starter was determined by counting the starter rate growth specific (μ) and the doubling time (Tg) based on the number of data cells and incubation time on growth curves [12]. The starter of *Zymomonas mobilis* used by a growth curve that was 6,5 hours at μ=0,476 generation/hours with the quickest time (doubling time)=80,56 minutes.

Table 1. The average of ethanol concentration (%) in vegetable and fruit wastes at Wonokromo market, Surabaya extract using *Zymomonas mobilis* bacteria.

Inoculum size of *Z. mobilis* bacteria	**Ethanol concentration (%) in duration**				
	0 day	**2 days**	**4 days**	**6 days**	**8 days**
0%	0,00 ^{h}	0,00 ^{h}	0,10 ^{h}	0,20 ^{h}	0,20 ^{h}
5%	0,00 ^{h}	0,30 ^{h}	2,70 ^{f}	4,40 ^{e}	6,60 ^{c}
10%	0,00 ^{h}	2,30 ^{g}	5,00 ^{d}	9,50 ^{a}	9,10 ^{b}
15%	0,00 ^{h}	2,40 ^{fg}	5,40 ^{d}	9,40 ^{ab}	9,20 ^{ab}

*Explanation: numbers followed by the same alphabets in each coloumn and row indicate that the relationship was not highly different according Tukey test (α=0,05).

Based on Anova test results, F count greater than F table that means H_0 is rejected and the H_1 is accepted. So there was interaction between inoculum size of *Zymomonas mobilis* and fermentation duration in ethanol produced. Since H_1 was accepted, then followed by the Tukey test at 95% confidence level to know what was a real difference between inoculum size of *Zymomonas mobilis* and fermentation duration occured. According to a Tukey test, the results obtained that the inoculum size of *Zymomonas mobilis* and fermentation duration had a noticeable difference. Thus, the results of the interaction can be seen in the Table 1.

In Figure 3, had seen the inoculum size of 0% as a control, the graph showed in stagnant position, it was mean that no one fermentation product resulted because no added *Zymomonas mobilis* bacteria. On 4th to 8th days, 0,2% (v/v) ethanol resulted. It was expected that vegetable and fruit wastes generally contained ethanol naturally. According to the literature, in a natural conditions, without added of bacteria, reducing sugar was converted into acetaldehide and some other metabolites [26]. So, more vegetables and fruit wastes, more higher the ethanol concentration resulted, but still under 1% ethanol.

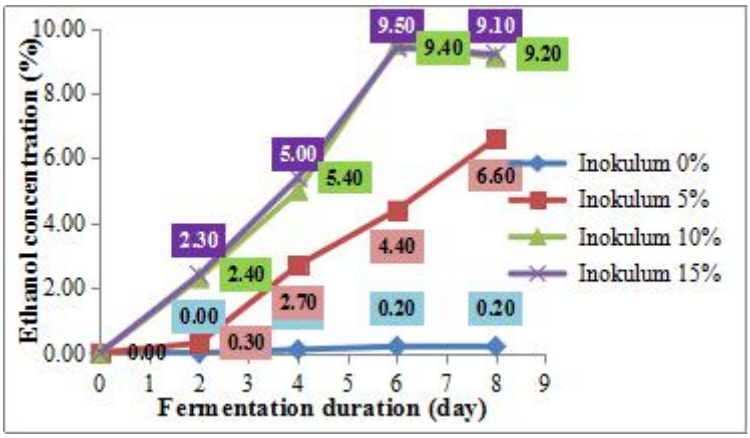

Figure 3. Graph of ethanol concentration (%) in fermentation process using *Zymomonas mobilis* bacteria.

Increasing of ethanol concentration was occurred along with the fermentation duration. For example, 5% of inoculum size increasing at 0 day to 8th days in fermentation process (Fig.3). That process can be continued by using glucose as an energy source in the glycolysis cycle which produced pyruvate. Pyruvate in anaerobic catabolism will produced ethanol and CO_2 as the result. Thus, it is might be 5% inoculum size concentration will obtained more greater ethanol concetration with a longer fermentation duration, because on the 8th days was

produced only 6.6% (v/v) ethanol. It can be concluded that using 5% inoculum size in production scale was less effective for ethanol production because it took a long fermentation.

The graph showed that ethanol production with 10% and 15% inoculum size were increased on 0 day to 6th days and then decreased at 8th days. More longer the fermentation duration, nutrients in the medium were decreased due to the number of cells that can lead to increased competition and eventually entered in death phase. The accumulation of ethanol from metabolism of microorganisms results can inhibit the fermentation activity and cell division, resulting in the amount of ethanol [27].

Optimum ethanol concentration 9,5% (v/v) was obtained from interaction between 10% inoculum size and 6 days fermentation duration. It was as same as equals in other researches, used tomato waste extract 9,68% (v/v) ethanol was obtained from 6 days by using *Zymomonas mobilis* bacteria [5] and used orange wastes extract 11,64% (v/v) ethanol was obtained from 6 days too [4]. The differences process in hydrolysis process used an acid hydrolysis and commercial enzyme hydrolysis [4,5]. It can be concluded that the enzymatic hydrolysis using *Aspergillus* sp. as same as potential for ethanol production. In fact enzymatic hydrolysis process was more effective used on vegetable and fruit wastes substrate that contained cellulose and more specific enzymes in work.

Based on statistical tests, 10 % and 15% inoculum size were not significantly different (^{a} notation). However, for producing ethanol on production scale by using 15% inoculum size was less effective because 9,40% (v/v) ethanol was obtained from 6 days and 9,20% (v/v) ethanol was obtained from 8 days (^{ab}notation) was not significantly different with the optimum ethanol concentration 9.50% (v/v) was obtained from 6 days (^{a}notation). Thus it can be concluded that if 10% inoculum size already able to produce the optimum ethanol, 15% inoculum size no needed which was inefficient. Using an inoculum size too high can caused increasing viability cell [28].

4. Conclusion

The result showed that *Aspergillus* sp. with 14,477 Unit/ml endoglucanase enzyme activity was a potential ability to produce cellulase enzyme in hydrolysis process to produce ethanol from vegetable and fruit wastes at Wonokromo market Surabaya. Optimum ethanol concentration 9,5% (v/v) was obtained from interaction between 10% inoculum size and 6 days fermentation duration.

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