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The superiority of *Zymomonas mobilis* in producing bioethanol from *Coffea arabica* peel using cellulolytic enzyme treatment

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ABSTRACT

As petroleum supplies are being depleted, there is a growing need for bioethanol as an alternative energy source. With the aid of crude cellulose enzymes from *Bacillus subtilis* IFO 13719, coffee arabica peels can be used as one of the waste materials to manufacture bioethanol. Microorganisms that can convert sugar into bioethanol, specifically *Saccharomyces cerevisiae* and *Zymomonas mobilis*, are also necessary for the synthesis of bioethanol. The purpose of this study is to demonstrate *Zymomonas mobilis's* superiority in generating bioethanol from fermented Arabica coffee peels treated with the crude cellulose enzyme *B. subtilis*. The concentrations of the *B. subtilis* crude cellulase enzyme at 0%, 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, and 17.5% were all independent variables in the fully randomized experimental design. The amount of sugar and ethanol is the dependent variable. The DNS method was used to measure the sugar concentration, and an alcohol meter was used to determine the ethanol content. Data analysis using the SPSS 16 software to examine DMRT and one-way ANOVA. The results showed that the maximum sugar content (1.0 g/mL) was obtained with the cellulase enzyme *B.subtilis* 2.5 % and the highest ethanol content (4.46 %) was obtained with the crude enzyme *B. subtilis* concentration 10% and fermented using *Z. mobilis*.

Keywords: *Bacillus subtilis,* blend cellulase enzyme, *Coffea arabica* peel, *Zymomonas mobilis*

INTRODUCTION

Fossil energy is part of the needs of society in any country, including Indonesia. According to the Minister of Energy and Mineral Resources of the Republic of Indonesia, energy consumption in Indonesia in 2020 is natural gas 11.53%, coal 13.44%, fuel 26.36%, biofuel 21.21%, biogas 0.02%, LPG 8.24%, and electricity 19.19% (Minister of Energy and Mineral Resources of the Republic of Indonesia, 2021) . Therefore, alternative renewable energy that is environmentally friendly is needed (Gielen et al., 2019; Johnsson, Kjärstad, & Rootzén, 2019). One form of the right solution to save fossil energy sources is to use bioethanol. Bioethanol is ethanol made from biomass contain sugar, starch, or cellulose components. The use of ethanol as an alternative fuel is also able to reduce fuel consumption by up to 13.42% and increase thermal efficiency by 14.67%, meaning that it saves fuel and is able to improve the performance of the vehicle (Paloboran et al.,2016). One of the wastes that can be utilized for the manufacture of bioethanol is coffee arabica peel. Arabica peel contains 21.30% sugar and 11.60% hemicellulose (Corro et al., 2013; Said & Purnama, 2020). There are several stages in the process of making bioethanol, namely pretreatment, hydrolysis, fermentation, and distillation. Pretreatment is the process of breaking the lignin bonds that cover cellulose and hemicellulose. Pretreatment aims to break down the lignocellulosic structure containing lignin, cellulose, and hemicellulose so that the enzymes to be used can delignify cellulose more easily and make the interaction between cellulose and enzymes work (Febriana, 2020).

Biodegradation of waste in the environment by microbes involves a series of enzymatic activities. Utilization of enzymes

produced from bacteria, more widely used when compared with enzymes from plants or animals because the growth of bacteria is fast and easy. One example of a bacterium that produces cellulase enzymes is *Bacillus subtilis* (Nababan et al., 2019). *Bacillus subtilis* has higher cellulase activity compared to other bacteria that can hydrolisis celulosa into glucosa (Reddy et al., 2016).

Bacillus subtilis converts cellulose to glucose, which is then utilized in the fermentation process to create bioethanol. The fermentation process of sugar is carried out by microorganisms like *Saccharomyces cerevisiae* and *Zymomonas mobilis*. Compared to other microbes employed in the bioethanol production process, *S. cerevisiae* has a number of benefits, such as being more adaptable, readily available, environmentally friendly, and resistant to high alcohol levels. (Azizah et al., 2012). *Zymomonas mobilis* is the most frequently used microorganism in ethanol conversion from lignocellulosic biomass. The advantages of this microorganism are that it has a higher sugar absorption and produces a higher ethanol yield, a higher ethanol concentration of up to 16%, and does not require oxygen control during fermentation (Susilo et al., 2017). This study aims to prove the superiority of *Zymomonas mobilis* in producing bioethanol from *Coffea arabica* peel fermentation on *B. subtilis* crude cellulose enzyme treatment.

METHOD

Preparation sample

Arabica coffee peel obtained from coffee plantations in Sidorejo Village, Kemalang District, Klaten Regency, Central Java is then dried and made into powder. *Bacillus subtilis*, *Zymomonas mobilis*, and *Saccharomyces cerevisiae* cultures were acquired from the University of Georgia's Food & Nutrition Culture Collection (FNCC).

Zymomonas mobilis and *Bacillus subtilis* cultures were grown on NA media (0.42 g of NA media and 15 mL of distilled water), and then they were incubated for 24 hours at room temperature (Zhang, K. & Feng, 2010). *Saccharomyces cerevisiae* culture were grown on PDA media.

Preparation of nutrient solutions

Powdered urea 10 g of powder (NH_4), up to 3 g per liter KH_2PO_4 per liter, 0.5 g powder, 3 g powder, and H_2SO_4 per liter 0.5 g/L calcium chloride, $7H_2O$ powder, and MgSO4 per liter Weighing H_2O L was done with an analytical balance. In a 200 mL beaker, combine the aforementioned ingredients and pour in 100 mL of distilled water. A magnetic stirrer is used to fully mix all of the contents (Argo et al., 2014).

Production of the cellulase enzyme

Five grams of Arabica coffee peel powder are put to each 250 mL Erlenmeyer flask, along with 25 mL of nutritional solution. After sterilizing the media in an autoclave at 121 degrees Celsius, 1 atmosphere of pressure, and 15 minutes, a *B. subtilis* culture is added, and the mixture is cultured for eight days at 37 degrees Celsius (Argo et al., 2014). 100 mL of an 80% tween solution was added to each incubation result, and the mixture was agitated at 150 rpm for 120 minutes at 4 °C. The solution should be centrifuged for 10 minutes at 3000 rpm. As a crude enzyme, the resultant supernatant was utilized (Safaria et al., 2013).

Coffea arabica peel fermentation using crude enzymes *B. subtilis*

An Erlenmeyer flask was filled with 200 grams of powdered Arabica coffee peel, followed by three additions of 1,600 milliliters of distilled water and boiling. The substrate was separated from the boiled water by filtering the boiled results. 48 bottles (100 mL fermentation bottles) containing the substrate were separated. Using a measuring pipette, the crude enzyme *B. subtilis* was aseptically added three times (to a total of 48 fermentation bottles) at a 10% concentration in accordance with the treatment (0%, 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, and 17.5%). A sterile glass stirrer was then used to stir the mixture. The fermentation bottles were sealed with sterile cotton and covered with

aluminum foil before being incubated for 24 hours at 37°C. The sugar content of the hydrolysis results was determined using the DNS technique (Jackson & Jayanthy, 2014).

Saccharomyces cerevisiae with *Zymomonas mobilis* treatment

The results of hydrolysis using crude enzymes (24 bottles) combined with 10% *S. cerevisiae* culture which was cultured for 72 hours (Widyaningrum & Parahadi, 2020). 24 bottles of hydrolysis results with crude enzymes plus 10% *Zymomonas mobilis* culture and fermented for 96 hours (He et al., 2014). Following distillation of the fermentation product produced by *Saccharomyces cerevisiae* and *Zymomonas mobilis*, an alcohol meter is used to measure the bioethanol that results (Widyaningrum & Parahadi, 2020).

Data analysis

The results of reducing sugar measurements and bioethanol levels were analyzed using the SPSS 16 software to examine DMRT and one-way ANOVA.

RESULTS AND DISCUSSION

Sugar content of the *Coffea arabica* peel before and after NaOH pretreatment according to the study, we discovered different sugar levels in the *Coffea arabica* peel both before and after pretreatment with NaOH (Table 1).

Table 1. *Coffea arabica* peel substrate sugar content

Replication	Reducing sugar content	
	Before *Pretreatment* (g/mL)	After *Pretreatment* (g/mL)
1	0.32	1.01
2	0.32	0.93
3	0.31	0.91
Average	0.32	0.95

To determine whether cellulose could be delignified or broken down, sugar levels were examined both before and after pretreatment; the results showed that sugar levels were higher following pretreatment. Table 1 shows that the sugar levels decreased by 0.32 g/mL with pretreatment and by 0.95 g/mL with posttreatment. The findings indicated a rise in the content of sugar. To improve the quantity of sugar that will subsequently be turned into bioethanol, pretreatment can hydrolyze cellulose and hemicellulose into simple sugars and remove lignin from lignocellulose (Hendrasarie & Mahendra, 2020). After crude enzyme treatment, the sugar content of *Coffea arabica* peel substrate is as Figure 1.

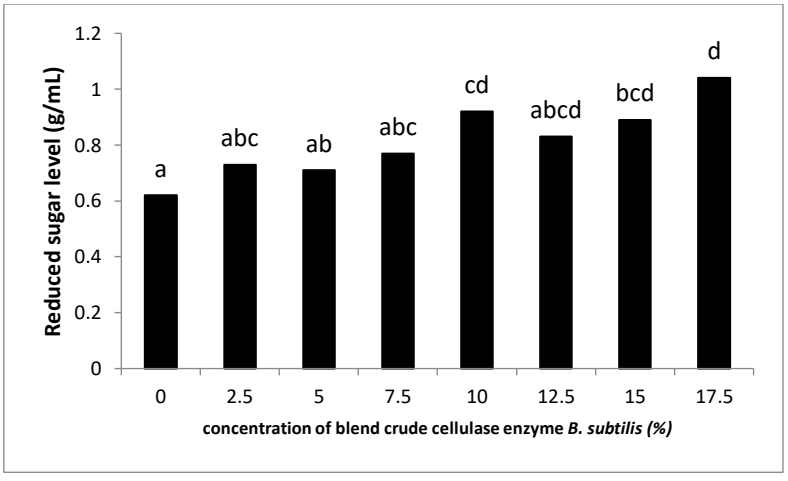

Figure 1. Reduced sugar levels after treatment with concentration of blend crude cellulase enzyme *B. subtilis*. The Duncan test indicates that there is no significant difference ($p > 0.05$) between the identical letter in each column.

The results of the study show that following crude enzyme treatment, the sugar content of the *Coffea arabica* peel substrate varies from 0.52 g/mL to 1.04 g/mL (Figure 1). The sugar level of all treatments was higher than that of the control. Since all computed values are higher than control sugar levels, all therapies utilizing crude enzymes have an impact on sugar levels. Treatment 12.5% crude enzyme (1.04 g/mL), which has the highest sugar content. Based on the results of measuring the reducing sugar content of the coffee peel after the crude enzyme *B. subtilis* treatment, the highest average reducing sugar content was 1.04 g/mL in the 17.5 % and the lowest average sugar content of the peel *Arabica coffee* of 0.71g/mL in treatment with a crude concentration of *B. subtilis* enzyme (5%).

The addition of crude *B. subtilis* enzyme in each treatment had the effect of reducing sugar levels where the sugar concentration the higher the crude *B. subtilis* enzyme concentration the higher the sugar content. The greater the concentration of enzymes, the more substrates are broken down and hydrolyzed, the more simple sugars are produced. The increase in reducing sugar levels after the addition of crude *B. subtilis* enzymes (Rahmawati, 2010). This means that the higher the enzyme concentration, the greater the amount of hydrolyzed substrates and the production of simple sugars.

Safaria et al., (2013) research finds the resulting increase in hydrolysis time using enzymes occurs due to the high interaction between cellulase enzymes and substrates. The interaction between the cellulase enzymes and the cellulose substrate will form an enzyme-substrate complex that produces glucose as an ethanol product so that the sugar content increases. The increase in sugar content after fermentation using crude enzymes showed that *B. subtilis* degraded cellulase into simple sugars so that it showed a higher reducing sugar content than before treatment with *B. subtilis. Bacillus subtilis* has higher cellulase activity compared to other bacteria (Mulyasari et al., 2015).

Measurement of sugar content using the DNS (Dinitrosalicylic acid) method. The resulting reducing sugar will change the color of the DNS reagent from yellow to reddish-orange. The darker the resulting color indicates that the more reducing sugar is formed (Jennifer & Thiruneelakandan, 2015). Figure 2 shows the sugar content of *Coffee arabica* peel after fermentation with *S. cerevisiae*.

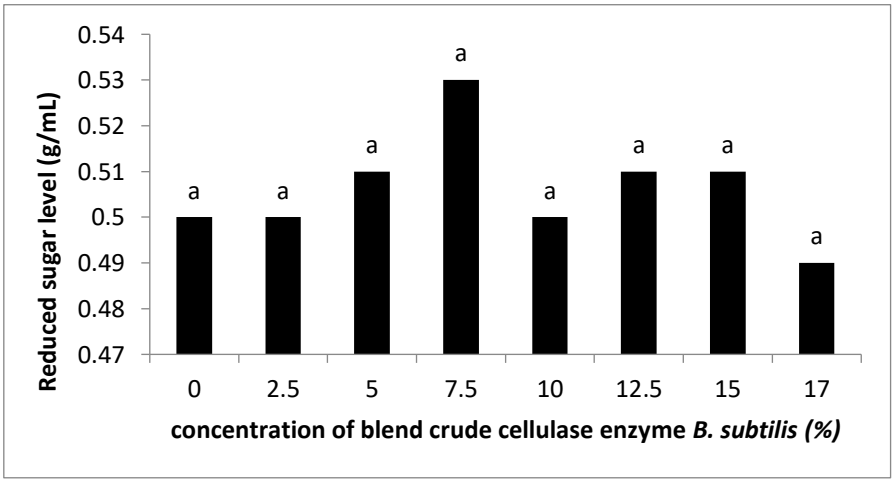

Figure 2. Reduced sugar levels after treatment with *Saccharomyces cerevisiae.* The Duncan test indicates that there is no significant difference ($p > 0.05$) between the identical letter in each column.

In Figure 2, the highest average reducing sugar content was 0.53 g/mL at a crude *B. subtilis* enzyme concentration of 7.5% and the lowest reducing sugar content was obtained at a

crude *B. subtilis* enzyme concentration of 17.5%, namely 0.49 g/mL, but based on the ANOVA test (0.05%) the sugar content was not significantly different. The results of measuring the reducing sugar content after the fermentation process using *Saccharomyces cerevisiae* produced the highest average sugar content of 1.00 g/mL at crude enzyme concentration of 2.5% and the lowest average sugar content was 0.49 g/mL with a crude enzyme concentration of 15 %. In treatment with a concentration of 17.5%, there was a significant decrease from the sugar content of 1.04 g/mL to 0.50 g/mL.

The addition of *S. cerevisiae* has an effect on reducing sugar levels where the sugar concentration decreases so that when the reducing sugar content is measured the results will decrease when compared to the reduced sugar level before *S. cerevisiae* treatment. This is because *S. cerevisiae* requires substrate for growth, both to reproduce and to maintain cell life. Sugar is used by *S. cerevisiae* to produce bioethanol as a primary metabolite (Wiratno et al., 2014). This means that glucose has been converted into bioethanol (Saragih et al., 2023). The change of glucose into bioethanol due to the activity of the invertase enzyme from *Saccharomyces cerevisiae*.

The amount of ethanol in the coffee arabica peel substrate after fermentation with *S. cerevisiae* was measured, with the results presented in Figure 3.

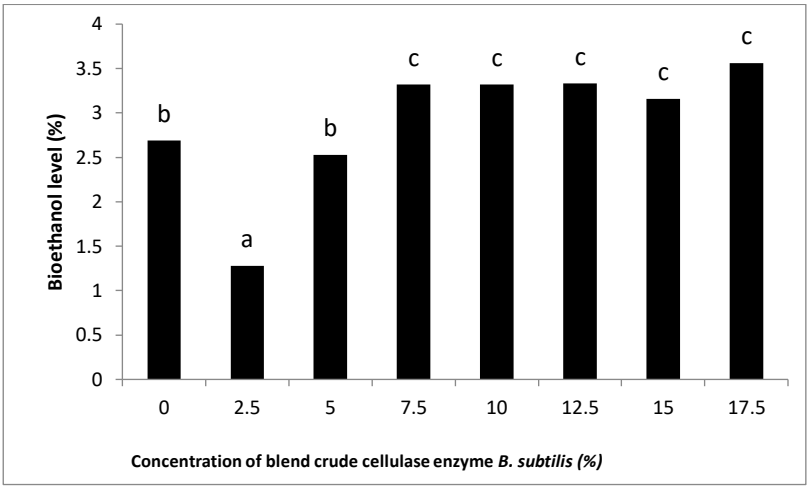

Figure 3. Levels of bioethanol following *S. cerevisiae* treatment. The Duncan test indicates that there is no significant difference ($p > 0.05$) between the identical letter in each column.

The results of measurements of bioethanol content obtained the highest average bioethanol content in the treatment of the crude enzyme *B. subtilis* concentration of 17.5% (3.56%) and the lowest average bioethanol content of 1.28% in the treatment of the crude enzyme *B. subtilis* 2 concentrations 5%. In alcoholic fermentation using *S. cerevisiae* , this occurs due to the presence of the zymase enzyme which helps break down sugar into ethanol so that the ethanol content increases while the amount of reducing sugar decreases because *Saccharomyces cerevisiae* utilizes sugar to maintain *Saccharomyces cerevisiae* as a carbon source and the media used to produce ethanol (Bilyartinus & Siswanto, 2021). In the control that was not supplemented with crude *B. subtilis* enzyme, it was seen to produce higher bioethanol levels compared to the treatment with the addition of 2.5% cellulase enzyme after the treatment of *S. cerevisiae* administration, this may be because the control already contained high sugar levels that were not different from the sugar levels in the treatment of cellulase enzyme administration (Figure 2), so that after being treated with *S. cerevisiae*, it produced high bioethanol levels and exceeded the administration of 2.5% cellulase enzyme. The amount of sugar in coffee arabica peel after *Zymomonas mobilis* treatment can be seen in Figure 4.

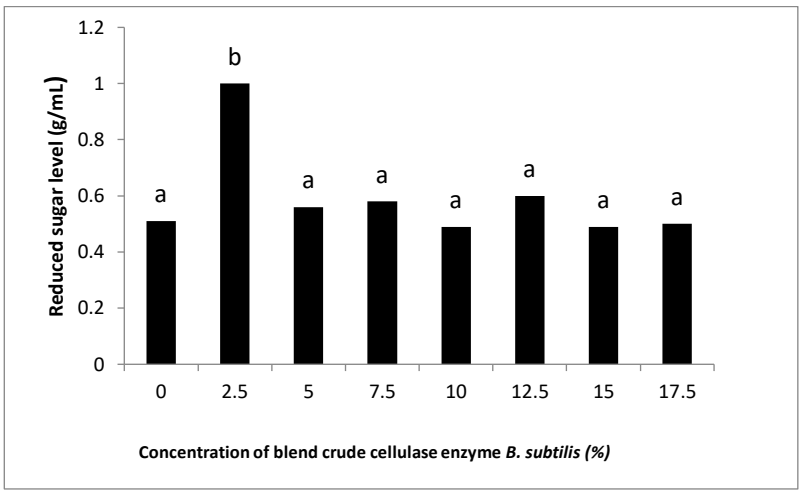

Figure 4. Reduced sugar levels after treatment with *Zymomonas mobilis.* The Duncan test indicates that there is no significant difference (p > 0.05) between the identical letter in each column.

The results of measuring the reducing sugar content of Arabica coffee peel after being fermented using *Z. mobilis*, the highest average sugar content was obtained at a crude concentration of *B. subtilis* enzyme 2.5% (1.0 g/mL), and the lowest average was obtained at 10% concentration of crude enzyme *B. subtilis* (0.49 g/mL). The sugar content produced before and after fermentation with *Z. mobilis* experienced the greatest decrease in the crude enzyme concentration of *B. subtilis* 2.5% from 1.0 g/mL to 0.49 g/mL. The decrease in reducing sugar levels after the fermentation process is due to the sugar content being utilized by *Z. mobilis* for growth (Irvan et al., 2016; Sandika et al., 2017). The sugar level dropped from 1 to 0.49 g/mL before and after fermentation with *Z. mobilis. Zymomonas mobilis* used the sugar in the fermentation medium to create new cells and ethanol, which resulted in a drop in sugar content in the subsequent fermentation. (Irvan et al., 2016). *Zymomonas mobilis* produces bioethanol as its primary metabolite by feeding on sugar (Rahmadani et al., 2017). In addition to being converted into bioethanol, sugar provides bacteria with nourishment for survival and procreation.

Levels of bioethanol in the peel of *Coffee arabica* after *Zymomonas mobilis* treatment can be seen in Figure 5.

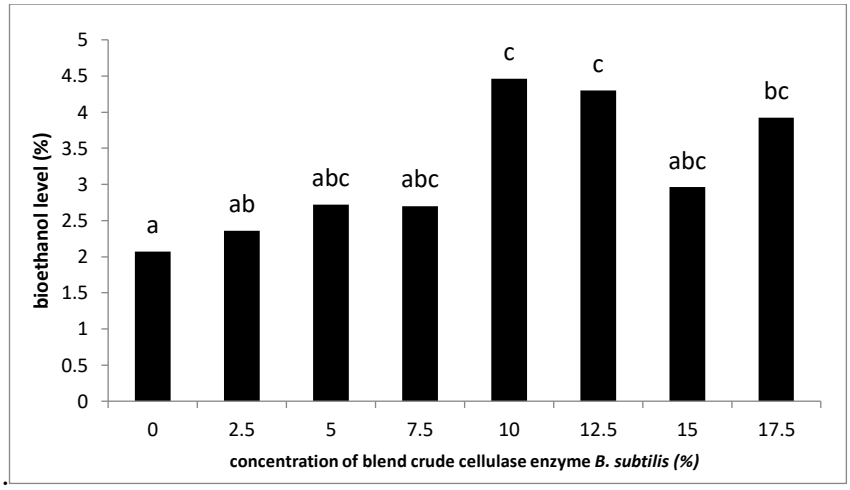

Figure 5. Levels of bioethanol following *Zymomonas mobilis* treatment. The Duncan test indicates that there is no significant difference (p > 0.05) between the identical letter in each column.

The crude cellulase enzyme concentration treatment had the highest average bioethanol content (4.46%) following *Z. mobilis* treatment, while the control group had the lowest average bioethanol content (2.07%). This is due to *Z. mobilis's* ability to use the Entner-Doudoroff metabolic pathway to break down glucose, fructose, or sucrose as a carbon source. *Z. mobilis* breaks down glucose quickly to generate enough energy for its growth because this metabolic pathway only generates one mole of ATP per mole of glucose or fructose.. Because it only produces one ATP molecule, *Z. mobilis* decomposes glucose quickly to meet ATP needs (Kusumaningati et al., 2013). In the control, it showed the lowest ethanol yield, because the crude enzyme *B. subtilis* was not added, so that the breakdown of glucose became bioethanol cannot occur optimally (Widyaningrum & Parahadi, 2020).

CONCLUSION

This study indicates that crude enzymes *Bacillus subtilis* affect the sugar and bioethanol content of fermented *Coffea arabica* peel. The maximum sugar content was 1 g/mL in the treatment of crude enzyme *B. subtilis* 2.5%. Crude enzymes *Bacillus subtilis* affect the bioethanol level of *Coffea arabica* peel. When *Zymomonas mobilis* was used to ferment the crude enzyme *B. subtilis* 10%, the highest amount of bioethanol was 4.46%. Compared to *Saccharomyces cerevisiae* , *Zymomonas mobilis* generated fermented *Coffea arabica* peel with a substantially greater bioethanol content.

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