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Effect of hydrolysis temperature of *Bacillus subtilis* crude enzyme on sugar content and bioethanol production from date seeds

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ABSTRACT

This study aims to determine the effect of *Bacillus subtilis* crude enzyme temperature on reducing sugar content and bioethanol production from ajwa date seeds (*Phoenix dactylifera* L.). The study was conducted using an experimental method with a completely randomised design (CRD) of five temperature treatments, namely 34°C, 37°C, 40°C, 43°C, and 46°C, at pH 7 each with three replicates. Ajwa date seeds that had undergone pretreatment were hydrolysed using crude *B. subtilis* enzyme, then fermented with *Zymomonas mobilis*. The reducing sugar content was measured using the DNS method, while the bioethanol content was measured through distillation and an alcohol meter. The results showed that an increase in crude enzyme temperature had a significant effect on the reducing sugar and bioethanol content. A temperature of 46°C produced the highest reducing sugar content before fermentation at 3.89 g/mL, sugar content after fermentation at 2.47 g/mL, and the highest bioethanol content at 3.11%. Statistical analysis through regression and ANOVA showed a significant relationship between temperature and the two dependent variables, with R^2 values of 0.9248, 0.9514, and 0.9542, respectively. In conclusion, the temperature of *B. subtilis* crude enzyme is an important factor in optimising bioethanol production from ajwa date seeds. This study provides an alternative use of biomass waste for environmentally friendly renewable energy.

Keywords: *Bacillus subtilis*, bioethanol, date seeds, fermentation, temperature

INTRODUCTION

The main source of energy for many industries is fossil fuels such as petroleum, natural gas, and coal. These fuels still dominate global energy production and contribute around 1.5 trillion dollars (Al-Yasiri, 2022; Islam et al., 2026). On the other hand, fossil fuel production and reserves in Indonesia are declining by around 10% each year, while oil consumption is increasing by 6% annually. This imbalance between energy availability and demand could trigger an energy crisis in the coming decades if not anticipated immediately (Rahman et al., 2021). In response to this, one solution that is being developed is the use of biofuels such as bioethanol (Ghazali & Mustafa, 2025; Perdana, 2024; Widyaningrum et al., 2016).

Bioethanol is a liquid fuel produced by fermenting glucose derived from starchy or cellulosic materials. Bioethanhazalol has characteristics similar to premium petrol and offers advantages as a renewable energy source, with minimal pollution and easy production from local materials containing sugar and starch (Novelia et al., 2022). Starch and cellulose are the main carbon sources in bioethanol production. Cellulose itself is a linear biopolymer of glucose found in the stems, seeds, skin, and fruit of plants (Broda et al., 2022). One natural material rich in cellulose is the ajwa date seed (*Phoenix dactylifera* L). Besides being rich in carbohydrates and nutrients, date seeds also contain 42% cellulose, 18% hemicellulose, and 11% lignin. This content is higher when compared to the fruit which contains 4%

cellulose, 4.5% hemicellulose, and 3.5% lignin (Alkhoori et al., 2022; Bouaziz et al., 2020; Desmagrini et al., 2021; Kiesler et al., 2024). Despite their great potential, date seeds in Indonesia are still widely discarded as waste, especially after Ramadan. In fact, their high cellulose content makes them a potential raw material for bioethanol production. The process of producing bioethanol from cellulose-based biomass such as date seeds requires an initial stage of hydrolysis, which is the breakdown of cellulose into fermentable reducing sugars.

This hydrolysis can be carried out with the help of crude cellulase enzymes, which function to break down cellulose into glucose molecules (Febryanti et al., 2025; Nurusman et al., 2023). One potential microorganism that produces crude cellulase enzymes is *Bacillus subtilis*, a gram-positive bacterium that has high enzymatic activity and is easy to cultivate industrially (Fouda et al., 2024; Nargotra et al., 2016) The sugar produced by hydrolysis then becomes the substrate for the fermentation process in bioethanol production. This fermentation process can be carried out with the help of microorganisms such *as Zymomonas mobilis,* a facultative anaerobic Gram-negative bacterium that is efficient in producing ethanol from carbohydrates (Ajit et al., 2017; Frohwitter et al., 2024).

One important factor in fermentation is temperature, as it affects the activity of microorganisms. Several studies have shown that cellulase enzymes from *Bacillus subtilis* have varying optimal working temperatures. (Sholihati et al., 2015) reported an optimum temperature of 30°C with an activity of 5.6609 × 10^{-3} U/mL, while Islam et al. (2019) mentioned that the optimum temperature range was 40–50°C. Theoretically, enzyme activity will increase with rising temperatures until it reaches its optimum point, and will then decrease again due to denaturation (Eğilmez & Haspolat, 2024; Sari, 2021). The differences in the results of these studies indicate that there is still inconsistency in the data regarding the optimum temperature of Bacillus subtilis cellulase enzymes. This necessitates further research to test the optimum conditions suitable for specific raw materials, in this case, Ajwa dates, which are rich in cellulose.

To date, there have been few studies that specifically utilise date seed waste as a raw material for bioethanol production using crude cellulase enzymes from *Bacillus subtilis* and systematically examine the effect of temperature variations on fermentation yields. Thus, this study not only contributes to enriching the literature on the utilisation of biomass waste, but also provides an alternative solution for environmentally friendly renewable energy that supports national energy independence.

The problem in this study is how the temperature *of Bacillus subtilis* crude enzyme affects the sugar and bioethanol content produced from date seed fermentation. The purpose of this study is to determine the effect of *Bacillus subtilis* crude enzyme temperature on the sugar and bioethanol content produced from date seed fermentation.

METHOD

This study employed an experimental method. The main material used was ajwa date seeds (*Phoenix dactylifera* L) obtained from the Cokisku production house in the Special Region of Yogyakarta. The microorganisms used in this study were *Bacillus subtilis* and *Zymomonas mobilis* obtained from the microbiology laboratory of the Faculty of Medicine, Gadjah Mada University. This study used a completely randomised design (CRD) with five temperature treatments, namely 34°C, 37°C, 40°C, 43°C, and 46°C. Each treatment was repeated three times. The independent variable in this study was temperature, while the dependent variables were the reducing sugar content and the bioethanol content produced.

Growth of *Bacillus subtilis* and *Zymomonas mobilis*

The slant medium was prepared by dissolving 11.2 g of NA in 400 mL of distilled

water, then sterilised in an autoclave at 121°C for 15 minutes and placed in test tubes. After solidification, *B. subtilis* and *Z. mobilis* were cultured using an ose and incubated for 48 hours at 37°C (Braga et al., 2021).

Sample pretreatment

Date seeds were cleaned, washed, dried for 2 days under the sun, then oven-dried for 3 days at 60°C. Date seeds were ground using a blender, sieved, and weighed at 800 grams. The powder is soaked in a 0.5 M NaOH solution for 24 hours, then neutralised using HCl to a pH of 7 and dried at room temperature for 5 days (Catulisti et al., 2025). The reduction sugar content is measured before and after treatment using the DNS (Dinitrosalisylate) method (Limahelu et al., 2021).

Preparation of nutrient solution

Nutrient solutions are used to support the activity and growth of microorganisms during the research process. These solutions are prepared by dissolving urea (6 g), $(NH_4)_2SO_4$ (20 g), KH_2PO_4 (6 g), $MgSO_4 \cdot 7H_2O$ (1 g), and $CaCl_2 \cdot H_2O$ (1 g) into 200 mL of distilled water until homogeneous (Bonnet et al., 2020).

Crude enzyme production

A total of 5 g of Nutrient Broth was dissolved in 250 mL of distilled water, sterilised in an autoclave (121°C, 15 minutes), then inoculated with *Bacillus subtilis* and incubated for 48 hours at 37°C. The 10 % culture was mixed with 40 g of date seed powder and 50 mL of sterile nutrient solution, then incubated for 6 days at 37°C. Next, 200 mL of Tween-80% solution was added and stirred for 120 minutes at room temperature (150 rpm), then centrifuged for 10 minutes (3000 rpm) to obtain crude enzyme extract (Raul et al., 2014).

Hydrolysis process

The pretreated date seed powder was mixed with distilled water (1:10; 250 g:2.5 L), boiled until boiling, then filtered. A total of 100 mL of filtrate was placed in 15 bottles (100 mL), crude *B. subtilis* enzyme 15% was added, then stirred homogeneously. The bottles are covered with cotton and aluminium foil, then incubated for 24 hours at treatment temperatures of 34°C, 37°C, 40°C, 43°C, and 46°C, at pH 7 with three replicates each (Widyaningrum & Parahadi, 2020).

Fermentation process

Ajwa date palm seed filtrate was added with 10% *Zymomonas mobilis* culture, at pH 7 under anaerobic conditions, then fermented for five days. After that, reducing sugar levels were analyzed using the DNS method to determine the difference in sugar levels before and after *Z. mobilis* treatment (Nurusman et al., 2023).

Measurement of reducing sugar content

The measurement of reducing sugar content was carried out using the DNS method. A total of 750 µL of sample was mixed with 750 µL of DNS solution in a reaction tube, then mixed using a vortex until homogeneous. The mixture was heated in a water bath at 90°C for 5 minutes. After cooling, 500 µL of 40% Rochelle salt solution is added to the solution. The absorbance value is measured using a UV-Vis spectrophotometer at a wavelength of 540 nm (Widyaningrum & Parahadi, 2020).

Bioethanol content measurement

Ethanol content is measured through a distillation process. The solution was heated to 78°C to evaporate the ethanol, then the vapour was passed through a pipe in water to condense into ethanol liquid. The substrate was measured for volume and placed in a distillation apparatus. The distillation product was collected in a 100 mL measuring cylinder, then an alcohol meter was dipped in to determine the ethanol content. The bioethanol content was calculated using the formula:

$$\frac{\text{Destilation Vol}}{\text{Substrate Vol}} \text{ x Alcoholmeter Measurement Result}$$

(Hermanto, 2021).

RESULTS AND DISCUSSION

Reducing sugar content of date seeds after treatment with crude *Bacillus subtilis* enzyme

Measurements of reducing sugar content after treatment with crude *Bacillus subtilis* enzyme at various temperatures are shown in Figure 1. The results indicate that increasing the crude enzyme temperature from 34°C to 46°C affects the increase in reducing sugar content. A temperature of 34°C produced an average sugar content of 1.35 g/mL, while at 46°C the sugar content increased significantly to 3.89 g/mL. The increase in sugar content with increasing temperature indicates that cellulase enzyme activity increased to an optimum point before denaturation occurred, in line with the theory of (Eğilmez & Haspolat, 2024; Sari, 2021). This optimum temperature of 46°C is consistent with the literature, which states that cellulase enzymes from *B. subtilis* have an optimum temperature range of 40–50°C (Islam et al., 2019).

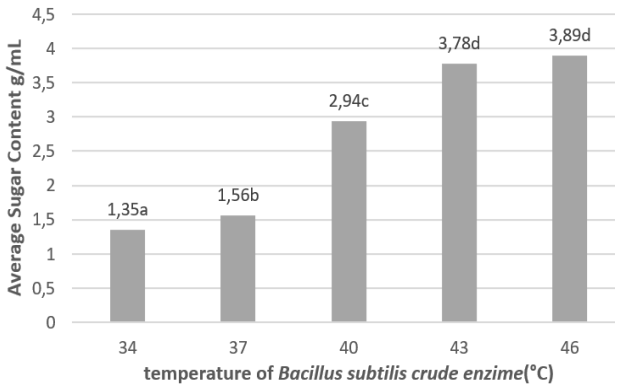

Figure 1. Diagram of the average reducing sugar content of date seeds after treatment with crude *Bacillus subtilis* enzyme.

Based on statistical analysis, which includes regression testing and ANOVA testing, it is consistently confirmed that temperature treatment greatly affects the level of reducing sugar. The regression test shows a very strong linear relationship between temperature (x) and sugar content (y) with the equation y = 0.2433x - 7.0293 and a high determination value (R^2 = 0.9248). Meanwhile, the ANOVA Test results show a significance value of 0.00 (< 0.05), which means there is a significant difference. These two results validate that temperature is the main factor regulating the enzymatic reaction. Furthermore, a 5% DMRT test was conducted to identify which treatment groups had significant differences, marked with different letters in Figure 1.

Reducing sugar content of date seeds after fermentation using *Z. mobilis*

The highest reducing sugar content of date seeds after fermentation using *Zymomonas mobilis* was recorded at 2.47 g/mL in treatment P5 (temperature 46°C), while the lowest average was 1.13 g/mL in treatment P1 (temperature 34°C) (Figure 2). The reducing sugar content decreased after fermentation with *Zymomonas mobilis.* This decrease occurred because the sugar in the fermentation medium was continuously utilised by *Zymomonas mobilis* for growth and bioethanol production. *Zymomonas mobilis* requires a carbon source, such as sugar, as an essential nutrient that provides energy for these bacteria. The more reducing sugars are utilised, the higher the concentration of bioethanol produced, and vice versa (Suryawanshi et al., 2018). In addition, one of the factors that influence optimal fermentation is pH, where the average pH measurement after fermentation with *Z. mobilis* shows a value of 6. This is in accordance with the theory that *Zymomonas mobilis* can ferment optimally in the pH range of 4.0–7.0 (Wei et al., 2024).

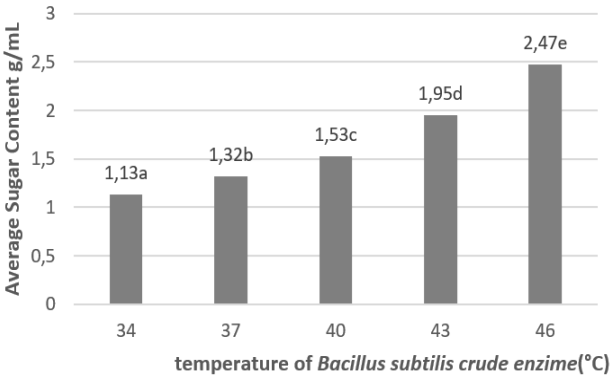

Figure 2. Diagram of the average reducing sugar content of date seeds after fermentation with *Zymomonas mobilis.*

Based on regression and ANOVA tests to determine the effect of *Bacillus subtilis* crude enzyme temperature on sugar levels between treatments after fermentation with *Zymomonas mobilis.* The regression test results showed the equation y = 0.1103x – 2.7333 with a determination value of R^2 = 0.9514. This value indicates that the higher the hydrolysis temperature, the higher the residual reducing sugar content after fermentation. The significance of the ANOVA test was 0.00 (< 0.05), indicating a significant difference between each temperature treatment on the residual reducing sugar content after fermentation. The temperature in the hydrolysis process plays an important role in affecting the reducing sugar content produced, which is then utilised by *Zymomonas mobilis.* Therefore, the analysis was continued with Duncan's test at a 5% confidence level, where the results showed that higher temperatures (40°C, 43°C, and 46°C) had significant differences in all treatments, in line with research (Suraningsih & Widyaningrum, 2022) which stated that sugar levels decreased because they were used by *Z. mobilis* for its growth.

Ethanol content of date seeds after fermentation using *Z. mobilis*

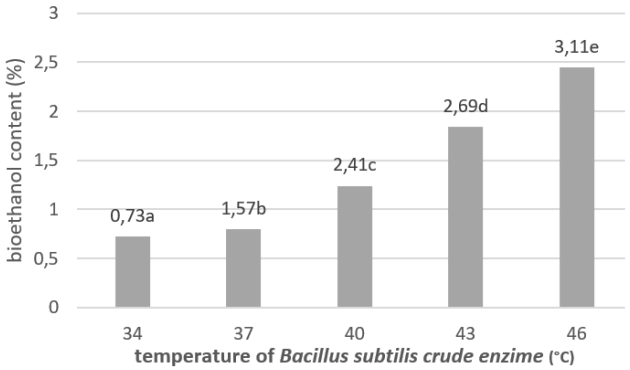

Figure 3. Diagram of average bioethanol content in date seeds.

The test results showed the highest bioethanol content of 3.11% at 46°C and the lowest at 0.74% at 34°C. The low ethanol yield (3.11%) was due to sequential biochemical barriers (Figure 3). Structural barriers and toxic by products prevented enzymes from fully converting plant polymers into fermentable sugars, which further hampered yeast metabolism and fermentation efficiency (Kumar et al., 2025).

The increase in bioethanol content was in line with the increase in the treatment temperature of *Bacillus subtilis* crude enzyme, driven by the optimal temperature that increased the metabolism of *Zymomonas mobilis* and enzyme activity (Bilyartinus & Siswanto, 2021). Statistical analysis confirmed these findings: Regression testing produced the equation y = 0.196x – 5.734 with a very high determination value (R^2 = 0.9542), indicating that 95.42% of the variation in bioethanol content was influenced by temperature.

CONCLUSION

Based on the results of this study, it can be concluded that the temperature of Bacillus subtilis crude enzyme has a significant effect on the reducing sugar content and bioethanol production from Ajwa date seeds. Increasing the temperature to 46°C increases cellulase enzyme activity, resulting in higher reducing sugar production, which is then utilised by Zymomonas mobilis in fermentation to produce bioethanol with a maximum concentration of 3.11%. This indicates that controlling the hydrolysis temperature is an important factor in optimising bioethanol production from cellulosic biomass. As a suggestion, further research could explore combinations of other factors such as incubation time, enzyme concentration, or type of fermentation microorganism to improve production efficiency, as well as considering the characteristics of other local raw materials to make the research results more applicable and economical.

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