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Time-course Cellulase Production by B2S8 Bacterial Isolate and Its Utilization in Bioethanol Fermentation from Corn Stover

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Abstract

Cellulase enzymes play crucial roles in hydrolyzing cellulose into simple sugars, which potentially serve as substrates for bioethanol production. These enzymes can be sourced from cellulolytic bacteria, which are characterized by exhibiting different growth and enzyme production patterns. This study was aimed to determine the cellulase activity of B2S8 bacteria previously isolated from compost products, followed byoptimization oftheir application for bioethanol fermentation. The research was conducted in two stages: examination of the effect of incubation time on bacterial growth and enzymatic activity within 144 h divided into 19 levels, and the second stage was to optimize fermentation conditions using a factorial randomized block design with two factors: crude cellulase enzyme concentration (7, 9, and 11% *v/v*) and fermentation temperature (30, 35, and 40 °C). The results of the first stage showed that incubation time significantly influenced the optical density of bacterial culture measured at 660 nm (OD_{660}), pH, and enzyme activity. The highest enzyme production was observed at 72 h, with OD_{660} 2.4, pH 7, endoglucanase activity 0.097 IU/mL, exoglucanase activity 0.018 IU/mL, soluble protein 0.0363 mg/mL, and specific activities of 2.906 IU/mg and 0.018 IU/mg of endoglucanase and exoglucanase, respectively. The following optimization study revealed that the combination of 9% (*v/v*) enzyme concentration and fermentation at 35 °C yielded the highest ethanol content (0.50 ± 0.07% *v/v*), with a resulting pH of 4.85 ± 0.07, total soluble solids of 3.4 ± 0.07 °Brix, and reducing sugars value of 0.802 ± 0.03 °Brix. In this work, isolate B2S8 demonstrated promising cellulase production and effective application in bioethanol fermentation, which indicating its potential for further development in bioconversion processes.

Keywords: B2S8 Isolate, Bioethanol, Cellulolytic Bacteria, Corn Stover, Growth Characteristics

Introduction

Bioethanol is one of the alternative energy sources needed in the present and future. Due to the vast availability of raw materials for bioethanol production, the production is likely to expand in the future significantly. One of the raw materials from farm-waste biomass for making bioethanol can be used as cellulosic biomass, such as rice straw, corn cobs, corn stover, and others.

One type of biomass waste that is very abundant in Indonesia is agricultural waste in the form of corn stover [1]. Corn stover consists of several parts, namely corn stalks, leaves, and straw. According to the Indonesian Central Bureau of Statistics (BPS), corn production in Indonesia exceeded 15 million tons, which is expected to generate a substantial amount of corn waste [2]. Corn stover waste has great potential as a raw material for producing bioethanol, as it contains components that can be utilized in this process, one of which is lignocellulose [3]. Lignocellulosic biomass is a type of biomass composed of complex bonds of lignin, cellulose, and hemicellulose [3]. Corn stover itself contains 8.2% lignin, 42.6% cellulose, and 21.3% hemicellulose.

Delignification is a necessary process to break the bonds between cellulose and lignin and hemicellulose because lignocellulosic biomass is extremely difficult to bio-transform using microorganisms and enzymes [4]. Delignification aims to break down the crystal structure of cellulose, reduce the content of hemicellulose and lignin, and increase the porosity of the material [4]. Delignification using a low concentration NaOH solution is the most common method. After the delignification process, corn straw was found to contain cellulose, hemicellulose, and lignin of 65.46%, 14.58%, and 8.66%, respectively [4]. The following saccharification process would convert cellulose into simple sugars, after which the fermentation process converts them into bioethanol.

Some organisms, like bacteria and fungi, are known to possess cellulase enzymes which able to degrade cellulose into glucose to obtain energy. Numerous microorganisms possess the ability to degrade cellulose, but only a few are capable of synthesizing multiple enzymes that can hydrolyze the crystalline structure of cellulose in vitro [5]. Cellulolytic bacteria with a high level of efficiency usually possess one or more enzymes of the three types of cellulases needed to degrade the microcrystalline structure of cellulose into glucose [6]. The cellulase enzymes are the key enzymes in the bioconversion of organic waste. Cellulase can be produced by microbes in the bacterial, actinomycete, and fungal groups. Cellulase is an enzyme that hydrolyzes the β-1,4-glucosidic bonds present in cellulose, which is a major component of the plant cell wall [7]. Cellulase-producing microorganisms from bacteria, instead of fungi, are the preferred option due to their rapid growth, which reduces the time required for cellulase production [8], so cellulases from bacteria have better potential than those from fungi for industry [9]. Several bacteria that are known to produce cellulase are *Salmonella thypimurium*[10]; *Bacillus pumilus* EWBCM1 [11]. *Paenibacillusdendritiformis* KFX-0 and *Bacillus sub*tilis KFY-40 [7]; *Clostridium thermocellum* [12, 13]; *Cytophagahutchinsonii*, *Acinetobacter baumannii* [10]. Cellulolytic bacteria are usually found in cellulose-rich environments, such as soil, garbage, or landfill sites [7].

B2S8 bacterial isolate was previously isolated from compost products produced by Bintang Tani Sejahtera (BTS) in Karangmelok Village, Bondowoso Regency, East Java-Indonesia, were found to have the potential to be used as cellulase enzyme producers [16]. Meanwhile, *S. cerevisiae* strain ATCC9763 has a fast growth rate, high bioethanol yield, resistance to high glucose concentration, and resistance to high salt concentration. The optimum fermentation pH is low, and the optimum fermentation temperature is around 25–30 °C, thus having the potential to be used in the saccharification process of cellulosic substrates and bioethanol production.

Enzymatic activity is usually characterized as the rate at which substrate concentration decreases or the rate at which products are generated under optimal conditions. Several factors, such as pH, substrate concentration, temperature, presence of inhibitors, incubation time, and enzyme concentration, influence cellulase enzyme activity [12]. The pH significantly affects the structure and biological activity of the enzyme. At optimum pH, the ionic interactions present in its structure will stabilize the enzyme, enabling it to recognize and bind to the substrate [17]. Meanwhile, the optimal temperature and pH value for the synthesis of cellulase enzymes by the B2S8 isolate were previously found at 36.9 °C and pH 6.9, respectively [18].

In the industry, *Saccharomyces cerevisiae* is usually used to produce bioethanol through fermentation [19]. Enzymatic hydrolysis and fermentation are usually combined as the Simultaneous Saccharification and Fermentation (SSF) technique, which is conducted concurrently in a single bioreactor. The optimum conditions for the SSF process were found to be at 72 h with a pH of 4.8 and a temperature of 30 °C [20].

Hence, this study was aimed to determine the optimal incubation time for cellulase activity during the growth of B2S8 bacterial isolates using standard carboxymethyl cellulose (CMC) liquid media, with incubation times ranging from 0 to 144 h, to identify the optimal time for producing cellulase enzymes. This was followed by an investigation of bioethanol production from crude cellulose of corn stover through SSF and evaluating the effects of cellulase enzyme concentration from B2S8 bacterial isolate and fermentation temperature in the presence of *Saccharomyces cerevisiae* ATCC 9763 to determine the optimal conditions for maximum bioethanol yield.

Materials and Methods

Materials

The materials used in this study were corn stover of *Zea mays* type saccharata obtained from Tabanan Regency, Bali-Indonesia, and B2S8 cellulolytic bacterial isolate property of Bioindustry Laboratory, Faculty of Agricultural Technology, Udayana University [18], *Saccharomyces cerevisiae* ATCC 9763 obtained from Bogor Agricultural University Culture Collection (IPBCC), yeast extract (Himedia), sodium hydroxide, dextrose (Lihua Starch), buffered peptone (Merck KGaA, Germany), $CaCl_2$, NH_4NO_3, $FeCl_3 \cdot 6H_2O$, dinitro salicylic reagent (DNS) solution (Sigma-Aldrich), sodium citrate buffer pH 4, and pH 7, carboxymethyl cellulose (Merck KGaA, Germany), KH_2PO_4, NaCl, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, $CaCl_2 \cdot 2H_2O$, sodium potassium tartrate (Merck, Germany), Folin-Ciocalteu Reagent (Merck KGaA, Germany), filter paper (Whatman no.1), distilled water, and 70% alcohol.

Experiment design

Randomized Block Design (RBD) was utilized as the experimental design for the

investigation of the cellulase enzyme activities during growth in this work. This experiment was a simple complete random design. The factor was the difference in enzyme-treatment incubation time, which consisted of 19 levels: 0, 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 42, 48, 60, 72, 84, 96, 120, and 144 h, repeated twice, which resulted in 38 experimental units. For the following experiments, an RBD factorial experiment with two factors was also employed to evaluate the generation of bioethanol from crude cellulose of corn stover, where the initial concentration which consist of: 7, 9, and 11% (*v/v*) of crude cellulase enzyme concentration combined with three levels of fermentation temperature of 30, 35, and 40 °C were and repeated twice resulted in total of eighteen experimental units.

Preparation of Corn Stover Powder

The dried corn stover was first washed and then cut into small pieces and dried using an oven at 60 °C for 48 h, crushed using a blender to reduce the size, and sieved using a 60 mesh sieve [4].

Delignification of Corn Stover Powder

The corn stover powder was then soaked in 4% (w/v) NaOH solution with a 1:10 (b/v) ratio for 8 h at room temperature (25 °C). The corn stover powder slurry was then filtered and washed with distilled water until its pH reached 7.0 (neutral), followed by oven-drying at 60 °C until the water content reached 10% [4].

Liquid Media and Inoculum Preparation

Preparation of the liquid media for the cell multiplication to characterized the B2S8 cellulolytic bacteria consisted of dissolving KH_2PO_4 (1.36 g/L), $(NH_4)_2SO_4$ (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), NaCl (2 g/L), yeast extract (1 g/L), $CaCl_2 \cdot 2H_2O$ (0.01 g/L), CMC (10 g/L) diluted in one liter of distilled water at adjusted pH of 6.8 – 7.0 place din Duran Bottle, heated while stirring until dissolved [21], followed by sterilization at 121 °C for 15 min.

The rejuvenated B2S8 bacterial isolate was then added to each Erlenmeyer flask containing 100 mL of sterile cellulose medium (1% CMC), followed by incubation at room temperature for 48 h and shaking at 150 rpm. The cell multiplication was carried out by inoculating 7 mL of the rejuvenated cells into a 1000 mL Erlenmeyer flask containing 700 mL of cellulolytic medium and incubating at room temperature for 72 h with agitation at 150 rpm. The cells were then precipitated at 5000 rpm for 20 min at 4 °C and washed with 0.85% NaCl twice, and the optical density at 660 nm (OD_{660}) was then adjusted to 5 [22].

Preparation of *S. cerevisiae* inoculum for ethanol fermentation was carried out using yeast-peptone-D-glucose (YPD) broth media in 250 and 1000 mL Erlenmeyer flasks. The 100 mL YPD broth media was prepared by mixing 1% yeast extract, 2% peptone, and 2% D-glucose into distilled water, followed by sterilization at 121 °C and 1 atm pressure for 15 min, followed by cooling to room temperature. The amount of 1 mL of *S. cerevisiae* culture was added to the media and incubated at 37 °C for 24 h. The incubation was carried out in a rotary water bath shaker at 120 rpm. The culture was then added to 750 mL of YPD broth in a 1000 mL Erlenmeyer flask and similarly incubated. The *S. cerevisiae* cells were harvested by precipitation at 5000 rpm for 5 min at 4 °C. The obtained *S. cerevisiae* cells were washed twice using 0.85% NaCl, and the optical density was similarly adjusted at 660 nm (OD_{660}) [10,23].

Cellulase Enzyme Activity-TestUpon Growth

CMC was used as an inducer and carbon source in the media to test the cellulase

enzyme activity upon growth, which aimed to determine the optimal time to achieve the highest cellulase enzyme activity. A total of 19 Erlenmeyer flasks, each containing 100 mL of sterile 1% CMC liquid media, were repeated twice, which resulted in 38 experimental units. An amount of 1% of the previously cultured cells (OD_{660}=5) was added to the sterile media in the Erlenmeyer flask at room temperature, followed by incubation at 37 °C with pH value adjusted between 6.9–7 in a water bath shaker, followed by observation of cellulase enzyme activity for 0–144 h [11]. After each of the 19 incubation times, the supernatant was collected by centrifugation at 10,000 rpm for 5 min at 4 °C [12] and tested for cellulase enzyme activity (endoglucanase and exoglucanase), soluble protein, and cell growth (OD_{660}) [23].

Production and Preparation of Crude Cellulase Enzyme for Bioethanol Production

The production of crude cellulase by the B2S8 isolate in liquid media prepared with the same composition as in the previous report [18] was optimized at 37 °C and pH 6.9.

Bacterial cell propagation was carried out by preparing sterilized liquid media in 750 mL in two 1000 mL Erlenmeyer flasks. The already rejuvenated bacterial culture was then added to each Erlenmeyer flask, followed by incubation at 37 °C for three days, and the cultures with multiplied B2S8 cells were deemed ready for use in cellulase enzyme production.

The production of crude cellulase enzyme was carried out using sterilized liquid media that contained KH_2PO_4 (1 g/L); K_2HPO_4 (1 g/L); $MgSO_4 \cdot 7H_2O$ (0.2 g/L); $CaCl_2$ (0.02 g/L); NH_4NO_3 (1 g/L); $FeCl_3 \cdot 6H_2O$ (0.05 g/L); peptone (2 g/L); corn stover crude cellulose powder (10 g) addition of distilled water until 1000 mL Erlenmeyer. Enzyme production was carried out in a 1000 mL Erlenmeyer flask containing 700 mL of media with 5% (*v/v*) of previously harvested bacterial culture, followed by incubation at 37 °C for 3 days [18] in a water bath shaker and agitated at 150 rpm. The supernatant was collected by centrifugation for 10 min at 4 °C at a speed of 14000 rpm. The supernatant, which contained crude cellulase enzyme, was collected for enzyme activity tests, namely endo-β-glucanase and exo-β-glucanase. The cellulase enzyme was deemed ready for the next experiment.

Simultaneous Saccharification and Fermentation

The saccharification and fermentation processes were carried out simultaneously in a single fermentor. The initial stage was carried out by transferring 20 g of crude cellulose powder from corn stover into a 250 mL Erlenmeyer flask with the addition of crude cellulase enzyme that was previously prepared and *S. cerevisiae* ATCC9763 inoculum as much as 5% (*v/v*) at a cell concentration of OD_{660} 5 as a fermentation agent. Before the SSF process was carried out, the pH of the fermentation medium was adjusted to 5.0 using citrate buffer, and then distilled water was added until the volume became 100 mL, and then the pH was re-adjusted to 5 by adding 0.1 M Na-citrate [8]. The SSF process with the three variations in fermentation temperature was carried out for 4 days [16].

Distillation

The distillation process of SSF samples refers to the procedure used by Pisitsungkakarn and Neamyou [21]. After the fermentation process, the fermentation residue was filtered, while the filtrate was distilled at 78–80 °C and then stopped when the ethanol was separated, and the ethanol content was analyzed.

Analytical Procedure

The growth of the B2S8 isolate was observed by measuring cell density (OD_{660}) [23], and the effects of the growth, such as pH [19], soluble protein [24], and cellulase enzyme activity (endoglucanase and exoglucanase) [25]. While for the bioethanol production experiment from corn stover substrate, the following analyses were conducted before and after SSF was carried out: total soluble solids (TSS), total reduced sugar [19], pH [6], and ethanol [23].

Endoglucanase activity was tested using a reaction mixture containing 1 mL of crude cellulase enzyme solution with 1 mL of 1% CMC solution in sodium citrate buffer (pH 5.4), followed by incubation at 50 °C for 15 min. The reaction was stopped by adding 3 mL of dinitrosalicylic acid and then boiled for 5 min. Cellulase activity was expressed in international units (IU/mL), where one unit was defined as the amount of enzyme required to break down 1 µmol of cellulose into reducing sugars per minute under test conditions. Glucose levels, which result from the hydrolysis of cellulose by the cellulase enzymes, were based on the absorbance value at a wavelength of 540 nm [25].

The exoglucanase activity (FPase) was tested using a reaction mixture containing 1 mL of crude enzyme solution (supernatant) with 2 pieces (1 × 1.5 cm) of Whatman No.1 paper and 1 mL of 50 mM sodium citrate buffer (pH 5.0), incubated at 50 °C for 1 h. The reaction was stopped by adding 3 mL of dinitrosalicylic acid and then boiling for 5 min [26]. The following formula was used to calculate enzyme activity:

$$\text{Enzyme activity (U/mL)} = \frac{CxVx\frac{1000}{180}}{vxt}$$

The C denotes the mass concentration of the analyte (mg/mL), which is estimated from its absorbance; V is the total reaction volume (mL), 180 represents the molecular weight of glucose (g/mol), v is the volume of the sample used (mL), and t denotes the total reaction time (min). The glucose concentration was converted into units of IU/mL:

1 IU = 1 µmol/minute of glucose produced
= 0.18 mg/minute glucose
MWG = Molecular Weight of Glucose (180 g/mol)
Ve = Volume of Enzyme Crude Extract (mL)
t = Incubation Time (min)

Data Analysis

The objective data were statistically analyzed using Analysis of Variance (ANOVA). When a significant effect was observed ($p < 0.05$), further analysis was conducted using Tukey's Honest Significant Difference (HSD) test. All statistical procedures were performed using Minitab version 19.

Results and Discussion

Growth of B2S8 Isolate

The observation of bacterial growth and cellulase enzyme activity was carried out at the start (0 h), from 1 h to 144 h. The B2S8 isolate of cellulolytic bacteria was observed to be able to grow well on 1% CMC medium, which served as an inducer component. Bacterial growth curves observed at 660 nm were used to determine the harvest time for cellulase enzymes. The growth curve was obtained by plotting the incubation time against OD_{660} as cell density. The OD_{660} illustrates population density, with the OD increment indicating the rate of microbial growth [27]. The amount of

bacteria in the media is deemed proportional to the absorbance obtained from the spectrophotometer measurement with a wavelength of 660 nm. The growth curves obtained can be seen in Fig. 1.

The growth of the B2S8 isolate observed through OD_{660} values shows a typical growth phase characteristic of cellulolytic microorganisms. At 0–72 h, there was a significant increase in OD_{660} value, indicating an exponential phase in which microbes actively divided and utilized the substrate optimally. The growth peak was reached at 72 h with an OD_{660} approaching 2.5, followed by a gradual decline until 144 h. The decrement in OD_{660} indicates that the bacterial culture is entering the stationary phase and progressing toward the death phase due to reduced nutrient availability and the increased concentration of the metabolites. The transition of the stationary phase from the exponential phase resulted in possible toxic excess metabolite accumulations as well as the depletion of nutrients.

Endoglucanase and Exoglucanase Activity During Growth

Cellulase enzyme activity test was carried out during growth at 0 h, 1 h, and 144 h. As can be seen in Fig. 1, the highest cellulase activity was found to be at 72 h of incubation time with an average value of 2.4 IU/mL, where the cellulase activity increased significantly from 24 to72 h. However, the rate of increase in cellulase activity after 72 h was found to be not significant. According to Wijanarka and Kusdiyantini [27], the production of enzymes, which are primary metabolites, occurs from the cell growth phase to the end of the exponential phase or the beginning of the stationary phase, which is concurrent with this result. The high enzyme activity suggests that at 6–60 h, the incubation time has entered the final logarithmic phase or the beginning of the static phase.

Endoglucanase activity exhibited a concurrent trend with cell growth, reaching the maximum value of 0.097 ±0.011 U/mL at 72 h, followed by a decreasing trend. This indicates that endoglucanase production by the B2S8 isolate is growth-associated and is closely related to the active growth phase of the microorganism.

In contrast, the exoglucanase activity was found to remain low throughout the incubation period, with the observed maximum value of approximately 0.02 U/mL. These conditions indicate that isolate B2S8 is more dominant in producing endoglucanase instead of exoglucanase. This pattern aligns with recent findings, which reported that many cellulolytic isolates from soil and lignocellulosic waste exhibited endoglucanase dominance as the initial step in cellulose degradation, while exoglucanase activity is relatively low and requires synergy with β-glucosidase to achieve more efficient cellulose hydrolysis [28]. For better industrial applications in the future, the co-culture approach or genetic engineering of the B2S8 isolate could be served in enhancing the balance of cellulase enzyme production to improve the efficiency of lignocellulosic biomass conversion.

The activity of endoglucanase and exoglucanase enzymes was observed to have peak activity, namely the 72^{nd} h (0.097 IU/mL) and 0.018 IU/mL), respectively, which is shown in Table 1 and 2. Cellulase activity decreased when growing cellulolytic bacteria from the 84^{th} to the 144^{th} h, where 72 h is the time at which the highest enzyme production occurred. The highest production time is concurrent with the phase of bacterial growth. This finding is supported by research, according to Shankar and Isaiarasu [11],

which stated that the maximum amount of enzyme production was obtained with an incubation time of 72 h (0.5740 ± 0.002 IU/mL) and the minimum amount of enzyme production was obtained in a period of 120 h (0.0653 ± 0.002 IU/mL).

Endoglucanase (CMCase) Activity: Endoglucanase was a component of cellulases that was always found in cellulolytic microorganisms. The decrease in viscosity of soluble substrates caused by this enzyme is due to its high affinity for cellulose derivatives and random reaction with amorphous cellulose fibers, it has high activity on CMC substrates [29].

The types of enzymes and the available substrates had strong effects on the ability of microorganisms to produce cellulose enzymes from the substrate. The B2S8 isolate was stated to have the ability to degrade the specific substrate used in this study, which was the CMC substrate. The glucose level resulting from the cellulase enzyme-facilitated hydrolysis on the CMC substrate was determined using the Nelson-Somogyi method.

The highest CMCase activity, which can be seen in Fig. 1, shows that the activity was obtained at an incubation time of 72 h. A very significant increase in enzyme activity was observed during the incubation period of 0 to 60 h. At 0 h, the enzyme activity was at0.037 IU/mL and increased until the 60th h, reaching the value of 0.101 IU/mL. At 72 h, the maximum value of enzyme activity was reached, namely 0.106 IU/mL. After passing 72 h and entering the 84, 96, 120, and 144 h incubation, the CMCase enzyme activity was gradually decreased from 0.101 IU/mL to 0.083 IU/mL.

The design of the experimental condition in this study is based on previous studies conducted by Kusumaningrum et al. [18], where B2S8 isolates were incubated at a temperature of 37 °C and a pH of 6.9. In these conditions, the activity of the CMCase was able to be maximized. The cellulolytic index value describes the ability of cellulolytic microbial isolates to excrete CMCase, where the greater the cellulolytic index value, the greater the ability to produce CMCase [8].

Microorganisms have varying growth periods, where, in the metabolic activity, the microorganisms have several phases in their growth. This is reflected in the fermentation process at the window between 31 h and 72 h, which is in the exponential production phase for endoglucanase, following a short lag phase. This finding is in accordance with the report by Nkohla et al. [26], which shows that the maximum production of endoglucanase enzyme was achieved after 48 h of fermentation. Upon the active growth phase, where the microorganisms grew rapidly, followed by a decrement in metabolic activity after the peak growth phase had passed.

This declining phase is referred to as the death phase. These growth phases are highly influential on the enzymes produced by microorganisms to aid substrate and nutrient consumption. According to the research conducted [11], the optimum incubation time is 72 h, as enzyme activity reaches its peak. The increase in the viscosity of the liquid medium and the decrease in the viscosity of the current medium during the optimal growth phase also supported that the optimal growth time for the B2S8 isolate is 72 h.

Exoglucanase (FPase) Activity: The conducted Analysis of Variance indicated that the incubation period of the B2S8 isolate showed a highly significant influence on cellulase enzyme activity, with the mean enzyme activity values presented in Fig. 1.

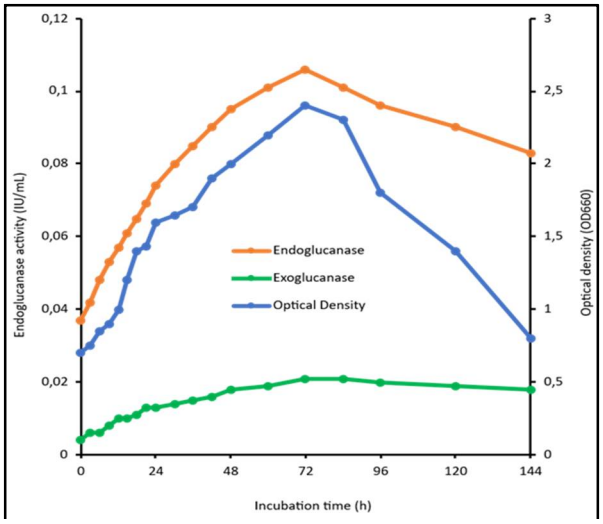

Figure 1. **Time course of B2S8 growth curve, endoglucanase activity, and exoglucanase activity during growth at 37 °C, pH 6.9, using CMC liquid media with shaking at 150 rpm**

Fig. 1 shows that the B2S8 isolate exhibited the highest mean exoglucanase (FPase) enzyme activity, reaching only 0.021 IU/mL, with the lowest concentration produced being 0.004 IU/mL. The growth media composition used for the cultivation of the B2S8 isolate might have greatly influenced the FPase activity produced, which may be useful for hydrolyzing cellulose, thereby increasing the availability of carbon sources during bacterial growth in enzyme production media. Isolates that exhibit cellulase activity on filter paper substrates, namely synthetic cellulose, a mixture of amorphous and crystalline cellulose, demonstrate synergism between CMCase and FPase, enabling the reduction of cellulose to glucose. This is in line with the report from Sohail et al. [29], these enzymes are not capable of efficiently breaking down complex crystalline cellulose individually, but when functions together synergistically, they can greatly enhance the rate of cellulose degradation.

Dissolved Protein Levels During Growth

The Analysis of Variance revealed that the incubation period of the B2S8 bacterial isolate had a high effect on the soluble protein content, with the mean values presented in Fig. 2.

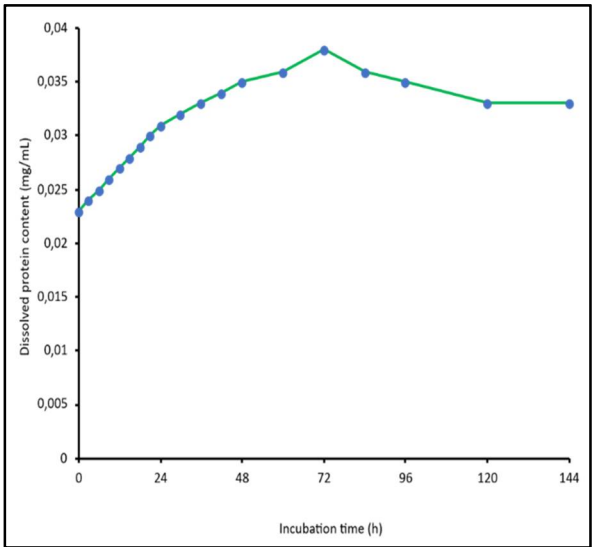

Figure 2. **Curve of dissolved protein content during growth of B2S8 isolate**

The dissolved protein measured in the enzyme filtrate indicated the cellulase enzyme proteins. Fig. 2 shows that the growth time of the B2S8 isolate at 72 h resulted in the highest average dissolved protein content value of 0.038 mg/mL, where the lowest dissolved protein content in cellulolytic bacterial growth was observed at time 0, specifically 0.023 mg/mL. This indicated that the duration of incubation had a high impact on the growth of isolate B2S8, as reflected by the differences in dissolved protein concentration of the crude cellulase enzyme upon incubation. When associated with the previous part results, the growth time was found to have a high effect on the protein content produced by the cellulolytic bacteria isolate.

CMCase and FPase Specific Activity

CMCase Specific Activity: The analysis of variance revealed that the time-length for bacterial growth produced a very significant effect on the specific activity of CMCase. The

mean value of CMCase specific activity is presented in Table 1.

The highest mean value of CMCase specific activity was found to be 2.906 IU/mg obtained from the 72 h of incubation period, as shown in Table 1, where the lowest value was 0.860 IU/mg, obtained from the 0 h incubation time. This might be due to the fact that the decrease in CMCase activity is higher than the decrease in dissolved protein levels, thereby reducing the enzyme activity.

The value of CMCase specific activity was determined from the value of CMCase activity with dissolved protein in crude enzyme filtrate. The exponential phase of CMCase synthesis, which was observed between 31 and 72 h of incubation, subsequent to a short lag phase, and then the CMCase production phase.

FPase Specific Activity: According to the results of the Variance test, the length of time for bacterial growth concurrently affects the specific activity of FPase. The average value of FPase specific activity can be seen in Table 2.

Table 2 presents that the maximum specific activity of FPase, reaching 0.018 IU/mg, was achieved after 72 h of incubation, whereas the minimum activity of 0.004 IU/mg was recorded at the initial (0 h) incubation period. This could be hypothesized due to the decrease in the activity on the filter paper, which is greater than the decrease in the dissolved protein content, resulting in a lower specific activity.

Table 1. **Mean values of endoglucanase specific activity analysis results.**

Incubation Time (h)	Endoglucanase Specific Activity (IU/mg)	Incubation Time (h)	Endoglucanase Specific Activity (IU/mg)	Incubation Time (h)	Endoglucanase Specific Activity (IU/mg)
0	0.860 ± 0.022^{b}	21	1.515 ± 0.077^{b}	72	2.906 ± 0.192^{a}
3	1.010 ± 0.037^{ab}	24	1.535 ± 0.256^{ab}	84	2.366 ± 0.654^{ab}
6	1.174 ± 0.021^{ab}	30	1.550 ± 0.536^{ab}	96	2.064 ± 0.627^{ab}
9	1.260 ± 0.160^{ab}	36	1.580 ± 0.524^{ab}	120	2.028 ± 0.060^{ab}
12	1.280 ± 0.063^{ab}	42	1.280 ± 0.063^{ab}	144	1.200 ± 0.275^{ab}
15	1.420 ± 0.130^{ab}	48	1.820 ± 0.616^{ab}		
18	1.480 ± 0.004^{ab}	60	2.167 ± 0.959^{ab}		

Note: Mean values followed by the same letter indicate a highly significant difference at the 1% level of error ($p < 0.01$).

Table 2. **Mean values of exoglucanase specific activity analysis results.**

Incubation Time (h)	Specific Activity of Exoglucanase (IU/mg)	Incubation Time (h)	Specific Activity of Exoglucanase (IU/mg)	Incubation Time (h)	Specific Activity of Exoglucanase (IU/mg)
	0.004 ± 0.001^{g}	21	0.013 ± 0.000^{bc}	72	0.018 ± 0.000^{a}
3	0.006 ± 0.001^{fg}	24	0.013 ± 0.001^{bc}	84	0.016 ± 0.000^{ab}
6	0.007 ± 0.000^{fg}	30	0.014 ± 0.001^{bc}	96	0.013 ± 0.000^{bc}
9	0.008 ± 0.000^{efg}	36	0.015 ± 0.000^{ab}	120	0.001 ± 0.000^{def}
12	0.010 ± 0.000^{cde}	42	0.016 ± 0.001^{ab}	144	0.009 ± 0.001^{cde}
15	0.011 ± 0.001^{cd}	48	0.016 ± 0.000^{ab}		
18	0.012 ± 0.000^{cd}	60	0.016 ± 0.001^{ab}		

Note: Different letters behind the mean value show a very significant difference at the 5% error rate ($p < 0.05$).

Bioethanol Production from Corn Stover using Crude Cellulase Enzyme of Isolate B2S8 and S. cerevisiae by the SSF Method

At the end of the bioethanol production from corn straw using the SSF method, the following factors were analyzed: pH, reducing sugars, total soluble solids (TSS), and ethanol content.

pH: The Analysis of Variance revealed that the effect of enzyme concentration and SSF temperature resulted in a highly significant influence ($p \leq 0.01$), whereas the interaction showed no significant influence ($p \geq 0.05$), as presented in Table 3.

Data from Table 3 showed a decrement in pH value at each enzyme concentration and fermentation temperature, ranging from 4.35 to 4.85. Based on the data in Table 3, it can be concluded that pH value variations were found among different types of microbes, with a tendency for pH to decrease as fermentation time increased. The pH factor is one of the main parameters that could play a crucial role in the SSF process. According to Dinana and Anggun [30], throughout the fermentation process, the production of CO_2 and various organic acids occurred, affecting the observed pH reduction, which could be attributed to the formation of acetic, pyruvic, and lactic acids, whereas other acids, such as butyric and long-chain fatty acids, exert only a slight influence on the decrease in substrate pH [31].

Generally, enzymes are most active within a narrow pH range. Cellulase enzyme is usually active at pH 4.5–5.5 [32]. A decrease in pH can coherently cause enzyme performance to decrease, thereby inhibiting the process of glucose production, resulting in a coherently small level of ethanol produced. The growth of *S. cerevisiae* is strongly influenced by pH, where microbes usually have an optimal pH for growth and a high concentration of bioethanol is produced. According to Orij et al. [33], the pH range for microbial growth of *S. cerevisiae* is between pH 3.5 and 8.5, and where the fermentation kinetics are not affected within the pH range of 3.5 to 6.0. The *S. cerevisiae* yeast grows optimally in a temperature range of 30–35 °C. These suggested that the pH obtained in this study is in the optimal range, allowing the SSF process to proceed effectively.

Sugar Content Reduction: The Analysis of Variance revealed that enzyme concentration and incubation temperature affected the reduction of sugar content upon SSF. The results revealed a highly significant effect ($p \leq 0.01$), whereas the interaction exhibited a significant influence ($p \leq 0.05$) on the reducing sugar content during the SSF process. The mean values of reducing sugar obtained from the SSF process are presented in Fig. 3.

Table 3. **Mean values of pH on the SSF Processes.**

Cellulase Enzyme Concentration (%) v/v	Incubation Temperature (°C)			
	30	35	40	Mean
7	4.65 ± 0.07	4.75 ± 0.07	4.45 ± 0.07	4.58 ± 0.07^{b}
9	4.55 ± 0.07	4.85 ± 0.07	4.55 ± 0.07	4.76 ± 0.08^{a}
11	4.55 ± 0.07	4.70 ± 0.00	4.35 ± 0.07	4.45 ± 0.10^{c}
Mean	4.61 ± 0.14^{ab}	4.65 ± 0.16^{a}	4.50 ± 0.16^{b}	

Notes: Values in the same row or column followed by different letters are significantly different at the 5% level of significance ($p \leq 0.05$).

From Fig. 3, the reducing sugar content shows an increasing trend during the SSF process at 30 °C and 40 °C, resulting in a significant increment. Enzymatic activity increases at that temperature, should be allowing it to efficiently saccharify cellulose into glucose, where the reduced sugar content decreased at 35 °C. After the fermentation process ended, a higher glucose content and a higher TSS value were also obtained. According to Loelovich and Morag [34], the higher the concentration of enzymes added in the saccharification process, the higher the soluble solids would be obtained due to the greater opportunity for enzymes to bind to and convert the cellulose content in the substrate into simple molecules, specifically glucose. The components measured as total soluble solids can include organic acids, sucrose, reducing sugars, and proteins, which may greatly affect the °Brix value [35].

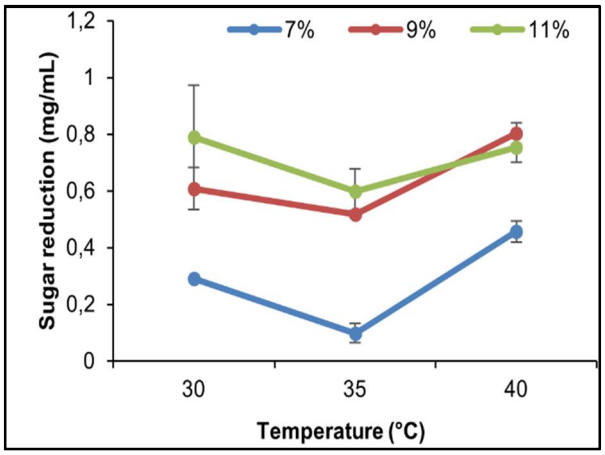

Figure 3. **Mean value of reducing sugar content with different enzyme concentrations and incubation temperature**

According to Widyaningrum and Parahadi [36], ethanol production is positively correlated with the extent of reducing sugar consumption by microbes; the higher sugar utilization would promote greater ethanol formation, and vice versa. The rise in *S. cerevisiae* cell numbers would also contribute to a marked reduction in reducing sugar content. This is possibly due to the *S. cerevisiae* effectively utilizing reducing sugar as a source of nutrients, namely carbon and nitrogen, which are contained in fermentation media, and as raw material to produce ethanol in the saccharification process, which is also converted into organic acids, although in small amounts.

Total soluble Solids: According to the Analysis of Variance results, the enzyme concentration and incubation temperature showed and exerted of highly significant influence ($p \leq 0.01$), while their interaction had no significant effect ($p \geq 0.05$) on the changes in TSS during SSF, which were recorded, and their mean values are depicted in Fig. 4.

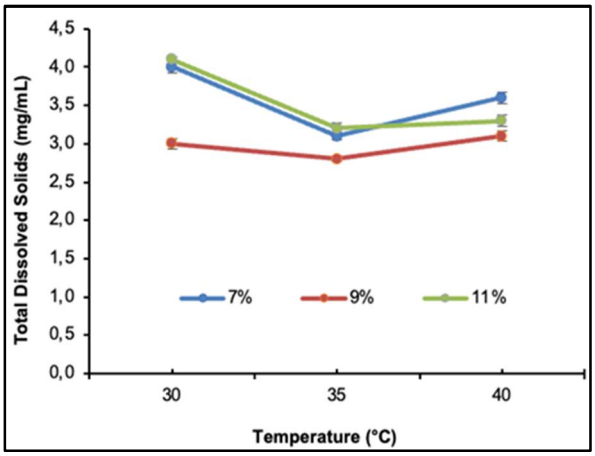

Figure 4. **Mean value of total soluble solids (°Brix) with different enzyme concentrations and incubation temperature**

Fig. 4 shows the differences in TSS values that were evident among the various enzyme concentrations. The average TSS, which was recorded at the 9% (*v/v*) enzyme level, reached 4.76, which was slightly higher than that observed at 7% (*v/v*), which is 4.76, and at 11% (*v/v*) enzyme concentration is 4.45. The high value of TSS was possibly due to the less than optimum conversion of glucose into ethanol. Temperature treatment could also be speculated to result in the decrement of TSS value with each increase in SSF temperature.

The highest average value of TSS fermentation was obtained from the 35 °C incubation temperature treatment at 4.65, followed by the 30 °C at 4.61, with the lowest value obtained from the 40 °C at 4.53.

Total soluble solids could be deemed as a good representation of the amount of cellulose that is successfully converted into glucose, and conversely, in the form of cellulose that is not saccharified during incubation. The observed decline in total soluble solids value can be explained by the metabolic activity of yeast, where glucose is converted into ethanol during fermentation. This expected glucose depletion subsequently contributes to the overall reduction in total soluble solids. Yeast requires substrates and nutrients for survival. While substrates and nutrients in the media are decreasing would result in decrement of TSS in the media. The decrement in total soluble solids during fermentation is possibly due to the sugars contained convertedin to alcohol, and the remaining organic acids, sucrose, and lactose dissolved in water are accounted for as TSS [37].

Based on the research by Loelovich and Morag [35], which stated that the higher the concentration of enzymes added in the saccharification process, the soluble solids obtained were also increased. This is concurrent with Sukamto et al. [38], who reported that the dissolved total solids value increases as the concentration of glucoamylase enzyme was added. This is possibly due to the greater chance for enzymes to interact and convert cellulose in the substrate into simple molecules such as cellobiose and glucose. The components measured as total soluble solids can be organic acids, sucrose (disaccharides), reducing sugars, and proteins that greatly affect the °Brix value [39].

Ethanol Content: The results of the Analysis of Variance indicated that both enzyme concentration and incubation temperature significantly affected ethanol production ($p \leq 0.01$), while their interaction showed no significant effect ($p \geq 0.05$). These findings are illustrated in Fig. 5.

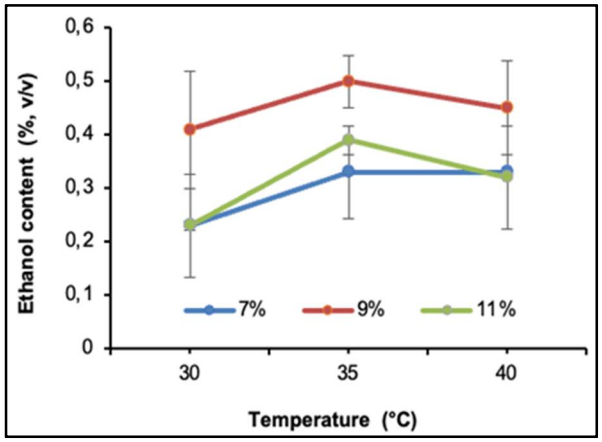

Figure 5. **Mean ethanol value content at different enzyme concentrations and incubation temperatures**

Fig. 5 shows that the highest average ethanol content was achieved at a 9% (*v/v*) enzyme concentration and a 35 °C temperature of 0.50%. The lowest ethanol content was observed at both 7% and 11% (*v/v*) of enzyme concentrations at 30 °C temperature of 0.23% (*v/v*).

Temperature treatment in SSF also showed an increase in ethanol content with each increment in fermentation temperature variables; however, at high temperatures, the fermentation result decreased, although higher than at low temperatures. The average ethanol content of SSF results in a 40 °C temperature treatment is 0.34%, meanwhile, in a 30 °C temperature treatment, it is 0.28%. The highest value of ethanol content is observed at 35 °C incubation temperature, which resulted in 0.40%. The fermentation process has an optimum temperature at which the highest ethanol content is produced. However, if the

temperature is not suitable, the number of microbes might also decrease and enter the death phase. At the optimum incubation temperature, the higher the level of reducing sugar and TSS, the higher the level of ethanol produced.

These results are consistent with previous studies indicating that increased ethanol production is associated with greater microbial sugar consumption, leading to significant reductions in reducing sugar and total soluble solids during fermentation.

According to Salihu et al. [40], *S. cerevisiae* has an optimum temperature of 35 °C and pH 4.5, producing 81% ethanol yield. The low amount of ethanol content in this study could suggest that glucose, which was used as substrate was did not fully utilized for the formation of ethanol. This could be that during the SSF process, glucose is also utilized to maintain cell metabolism and to form biomass, as well as pyruvic acid, which is produced through the glycolysis process, which cannot yet be fully converted into ethanol by *S. cerevisiae*, but it can form organic acid compounds. Organic acid compounds produced could be in the form of acetic, lactic, and pyruvic acids. In addition, the concentration of ethanol may decrease due to less glucose because the fermentation rate is fast enough, resulting in a lack of glucose, so that some *S. cerevisiae* yeast tends to consume bioethanol; besides, the further reaction of bioethanol might be oxidized to acetic acid [41].

Conclusion

This study demonstrates that incubation time is the key determinant of cellulase production by the B2S8 isolate. The peak growth phase occurred between 60 and 72 h, with 72 h yielding the highest crude cellulase activity (endoglucanase 0.106 IU/mL; exoglucanase 0.021 IU/mL), alongside increased protein concentration and specific activities. In the simultaneous saccharification and fermentation of corn stover, enzyme concentration and temperature significantly influenced pH, total soluble solids, reducing sugars, and ethanol yield. The optimal condition, 9% (*v/v*) enzyme concentration at 35 °C, produced the highest ethanol content (0.50 ± 0.07% (*v/v*). Ethanol production using crude cellulase enzyme from B2S8 bacteria is relatively low. In the future, further purification of the cellulase might be beneficial to increase its activity. Overall, both incubation time in cellulase production and process parameters during saccharification–fermentation critically affected the bioethanol conversion efficiency.

Use of AI tools declaration

The author states that they did not use Artificial Intelligence (AI) tools in creating this article.

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Conflict of Interest

The authors declare no conflict of interest relating to the study.

References

1. J. C. Assaf, Z. Mortada, S. A. Rezzoug, Z. Maache-Rezzoug, E. Debs and N. Louka, *Processes,* 12 (2024) 1. https://doi.org/10.3390/pr12051001
2. BPS. *Badan Pusat Statistik.* 2024, https://www.bps.go.id/id/pressrelease/2024/10/15/2378/pada-2024--luas-panen-jagung-pipilan-diperkirakan-sebesar-2-58-juta-hektare-dan-produksi-jagung-pipilan-kering-dengan-kadar-air-14-persen-sebesar-15-21-juta-ton-.html.
3. T. M. Mata, S. Rodrigues, N. S. Caetano and A. A. Martins, *Energy Rep.,* 8 (2022) 468. https://doi.org/10.1016/j.egyr.2022.01.059
4. I. B. W. Gunam, Y. Setiyo, N. S. Antara, I. M. M. WIjaya, I. W. Arnata and I. W. W. P. Putra, *Rasayan J. Chem.,* 13 (2020) 1022. http://dx.doi.org/10.31788/RJC.2020.1325573
5. Z. E. Elsababty, S. H. Abdel-Aziz, A. M. Ibrahim, A. A. Guirgis and G. E. Dawwam, *J. Genet. Eng. Biotechnol*., 20 (2022) 34. https://doi.org/10.1186/s43141-022-00317-4
6. F. Li, Y. Xie, X. Gao, M. Shan, C. Sun, Y. D. Niu and A. Shan, *Electron. J. Biotechnol.,* 48 (2020) 29. https://doi.org/10.1016/j.ejbt.2020.09.001
7. Y. Zhang, B. Tang and G. Du, *J. Microbiol. Biotechnol.,* 27 (2017) 514. https://doi.org/10.4014/jmb.1608.08048.
8. A. Balla, A. Silini, H. Cherif-Silini, A. C. Bouket, A. Boudechicha, L. Luptakova, F. N. Alenezi and L. Belbahri, *Catalysts,* 12 (2022) 1. https://doi.org/10.3390/catal12070769
9. A. T. Nottingham, L. C. Hicks, A. J. Q. Ccahuana, N. Salinas, E. Baath and P. Meir, *Biol. Fertil. Soils,* 54 (2018) 219. https://doi.org/10.1007/s00374-017-1247-4
10. I. S. Fratty, D. Shachar, M. Katsman and S. Yaron, *Front. Cell. Infect. Microbiol.,* 12 (2022) 01. doi 10.3389/fcimb.2022.967796
11. T. Shankar and L. Isaiarasu, *Middle-East J. Sci. Res.,* 8 (2011) 40. https://idosi.org/mejsr/mejsr8(1)11/7.pdf
12. S. Naresh, B. Kunasundari, A. A. N. Gunny, Y. P. Teoh, S. H. Shuit, Q. H. Ng and P. Y. Hoo, *Trop. Life Sci. Res.,* 30 (2019) 123. https://doi.org/10.21315/tlsr2019.30.1.8
13. Y. J. Liu, S. Liu, S. Dong, R. Li, Y. Feng and Q. Cui, *Biotechnol. Biofuels,* 11 (2018) 1. https://doi.org/10.1186/s13068-017-1009-4
14. L. Tian, P. M. Conway, N. D. Cervenka, J. Cui, M. Maloney, D. G. Olson and L. R. Lynd, *Biotechnol. Biofuels,*12 (2019) 1. https://doi.org/10.1186/s13068-019-1524-6
15. I. Borthakur, R. P. Devi, S. Karthikeyan, D. Ramesh and D. Murugananthi, *J. Pure Appl. Microbiol.,* 18 (2024) 2188. https://doi.org/10.22207/JPAM.18.4.08
16. Z. Arifin, I. B. W. Gunam, N. S. Antara and Y. Setiyo, *J. Rekayasa Manaj. Agroind.,* 7 (2019) 30. https://doi.org/10.24843/JRMA.2019.v07.i01.p04
17. L. Mazzei, F. Musiani and S. Ciurli, *J. Biol. Inorg. Chem.,* 25 (2020) 829. https://doi.org/10.1007/s00775-020-01808-w
18. A. Kusumaningrum, I. B. W. Gunam and I M. M. Wijaya, *J. Rekayasa Manaj. Agroind.,* 7 (2019) 243. https://doi.org/10.24843/jrma.2019.v07.i02.p08
19. A. M. M. Napitupulu, L. Suhendra and I. B. W. Gunam, *IOP Conf. Ser.: Earth Environ. Sci.,*913 (2021) 1. https://doi.org/10.1088/1755-1315/913/1/012026

20. E. Derman, R. Abdulla, H. Mabawi, M. K. Sabullah, J. A. Gansau and P. Ravindra, *Fermentation,* 8 (2022) 1. https://doi.org/10.3390/fermentation8070295
21. S. S. -H. Pisitungkakarn and P. Neamyou, *Energies,* 15 (2022) 1. https://doi.org/10.3390/en15134688.
22. P. Gupta, K. Samant and A. Sahu, Int. *J. Microbiol., 1* (2012) 1. https://doi.org/10.1155/2012/578925
23. I. B. W. Gunam, I. G. A. Sujana, I. M. M. Wijaya, Y. Setiyo, I. W. Arnata and I. W. W. P. Putra, *J. Trop. Life Sci.*,13 (2023) 341. https://doi.org/10.11594/jtls.13.02.12
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.,* 193 *(*1951) 265. https://pubmed.ncbi.nlm.nih.gov/14907713/
25. M. A. Dar, K. D. Pawar and J. P. Jadhav, *Int. Biodeterior. Biodegrad.,* 98 (2015) 73. https://doi.org/10.1016/j.ibiod.2014.11.016
26. A. Nkohla, K. Okajyeto, A. Olaniran, U. Nwodo, L. Mabinya and A. Okoh, *J. Biotech. Res. Edmond.,* 8 (2017) 33. https://search.proquest.com/openview/0244548073217c8a6facb02a3bb87e9a/1?pq-origsite=gscholar&cbl=136084
27. K. L. B. Wijanarka and E. Kusdiyantini, *J. Phys. Conf. Ser.,* 1217 (2019) 1. https://doi.org/10.1088/1742-6596/1217/1/012178
28. V. Juturu and J. C. Wu, *Renew. Sustain. Energy Rev.,*33 (2014) 188. https://doi.org/10.1016/j.rser.2014.01.077
29. M. Sohail, N. Barzkar, P. Michaud, S. T. Jahromi, O. Babich, S. Sukhikh, R. Das and R. Nahayadi, *Molecules,* 27 (2022) 1. https://doi.org/10.3390/molecules27123783
30. F. A. Dinana and S. P. Anggun, *Ho Chi Minh City Open Univ. J. Sci- Eng. Technol.,* 12 (2021) 42. doi:10.46223/Hcmcoujs.Tech.En.12.1.1901.2022
31. I.-I. Hanidah, R. Safitri and T. Subroto, *J. Penelit. Pangan,* 1 (2017) 27. https://doi.org/10.24198/jp2.2016.vol1.1
32. M. J. Taherzadeh and K. Karimi, *Int. J. Mol. Sci.,* 9 (2008) 1621. https://doi.org/10.3390/ijms9091621
33. R. Orij, M. L. Urbanus, F. J. Vizeacoumar, G. Giaever, C. Boone, C. Nislow, S. Brul and G. J. Smits, *Genome Biol.,* 13 (2012) 1. https://doi.org/10.1186/gb-2012-13-9-r80
34. M. Loelovich and E. Morag, *BioResource,* 7 (2012) 1040. https://doi.org/10.15376/biores.7.1.1040-1052
35. I. R. Laila, A. A. Tulsi, B. Susilo and D. F. Al-Riza. *Food Res.,* 1 (2025) 110, https://doi.org/10.26656/fr.2017.9(S1).060
36. T. Widyaningrum and M. Parahadi, *J. Trop. Biodivers. Biotechnol.,* 5 (2020) 01. doi: 10.22146/jtbb.52189
37. F. D. P. Valim, E. A. Oliveira, E. S. Kamimura, V. D. Alves and R. R. Maldonado, *Indian J. Microbiol.,* 56 (2016) 476. https://doi.org/10.1007/s12088-016-0601-9
38. Sukamto, O. E. Cahya, Sudiyono and Suprihana, *Eur. J. Agric. Food Sci.,*7 (2025) 8. http://dx.doi.org/10.24018/ejfood.2025.7.1.891
39. A. Mehboob, S. Iram and R. Fatima, *Pak. J. Anal. Environ. Chem.,* 26 (2025) 141. https://doi.org/10.21743/pjaec/2025.06.12
40. U. Y. Salihu, U. G. Usman, A. Y. Abubakar and G. Mansir, *Dutse J. Pure Appl. Sci.,* 8 (2022) 9. https://dx.doi.org/10.4314/dujopas.v8i4b.2
41. A. Hayat, S. Rafiq, M. Rehman, F. Kausar, Z. M. Khalid and P. Anwar, *Pak. J. Anal. Environ. Chem.,* 22 (2021) 180. http://doi.org/10.21743/pjaec/2021.06.1