Cellulase production by B2S8 bacterial isolates using corn stover as a substrate

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Abstract. The aim of this study was to determine the substrate concentration and fermentation time to produce high activity of cellulase enzyme. Crude cellulase production in different substrate concentrations and fermentation times used a factorial randomized block design (RBD) consisting of two factors. The first factor was the substrate concentration which consisted of 4 levels: 2%, 4%, 6%, and 8% (w/v). The second factor was the fermentation time which consisted of four levels: 24 h, 48 h, 72 h, and 96 h. This study used B2S8 cellulolytic bacterial isolates which had the highest value of cellulase activity and the highest rate of cellulose degradation in previous studies. To test the enzyme activity, this study used carboxymethyl cellulose (CMC), citrate buffer, and cellobiose. Protein levels were analyzed using the Lowry method. The results showed that the highest cellulase activity was under 8% (w/v) substrate concentration and fermentation time 96 h which resulted in endoglucanase activity was 0.1320 IU/mL, exoglucanase activity was 0.0320 IU/mL, *β*-glucosidase activity was 0.0172 IU/mL, dissolved protein level was 0.1719 mg/mL, specific endoglucanase activity was 0.9804 IU/mg, specific exoglucanase activity was 0.2730 IU/mg, and specific activity of *β*-glucosidase was 0.1260 IU/mg. The ability of B2S8 bacteria shows great potential for cellulase enzyme production and can be applied in lignocellulose hydrolysis.

1 Introduction

The abundant availability, especially in the form of agricultural, plantation and forestry waste, makes this material as a potential energy source through the conversion process of physical, chemical, and biological properties [1]. Lignocellulosic is biomass derived from plants with components of lignin, hemicellulose and cellulose [2, 3]. Cellulose is a crystalline structure that has a dense bundle of microfibrils, which prevents the penetration of hydrolytic enzymes and their enzymatic conversion into glucose [4]. Corn stover is from corn plant waste consisting of stems and leaves as lignocellulose sources which are abundant in nature. According to the Indonesian Corn Farmers Association Yasmin [5], Indonesian corn production reached 19 million tons in July 2018. Due to the large amount of production, the agricultural waste from corn is also high. However, the corn straw is mostly used as animal feed and the rest is thrown away. Utilizing corn stalk waste as the cellulose source can increase the utility value of agricultural waste. The corn plant itself contains 42.6% cellulose, 21.3% hemicellulose, and 8.2% lignin so that the high potential of corn stover waste has the opportunity to be an alternative cellulose source in cellulase enzyme production which are useful for various industrial needs [2].

Enzymes can be produced by groups of bacteria, mold, and yeast. One type of enzyme which has an important role in bioconversion of organic wastes is the cellulase enzyme. Cellulase-producing microorganisms from groups of bacteria are the main choice since they have fast growth so that the time needed to produce cellulase is shorter [6]. Bacteria which can produce cellulase enzymes include *Clostridium, Cellulomonas, Bacillus, Erwinia, Acetobibrio, Microbispora*, *Streptomyces*, *Sclerotium rolfisii,* and *P. chrysosporium*. Meanwhile, from the fungi category, they include *Trichoderma*, *Aspergillus*, *Schizophyllum*, and *Penicillium* sp. [7, 8].

Gunam et al. [9] reported that the concentration of 2% (w/v) corn straw substrate added with delignification treatment with 4% (w/v) NaOH solution will produce optimal cellulase activity at 30°C within 7 days of fermentation time using *Aspergillus niger* FNU 6018. Gautam et al. [10] reported that the treatment of agricultural waste substrate with 1−6% concentration had produced the best cellulase enzyme activity with 5% substrate concentration on the 7th day of fermentation. According to Abubakar et al. [11], 6% and 8% (w/v)

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substrate concentrations in orange peel had produced the highest enzyme activity using *Aspergillus niger* with a fermentation time of 96 h. There are several factors that influence the production of cellulase enzymes, namely temperature, pH, substrate concentration, fermentation time, strain used, and inoculum concentration [12]. The optimum temperature and pH in the production of cellulase enzymes with the bacterial isolate B2S8 were 36.9°C and pH 6.9 respectively [13]. The bacteria used in cellulase production were the bacterial isolate B2S8, because this local bacterium has the ability to degrade cellulose of corn crop waste from surrounding areas relatively better [14]. The concentration of inoculum added to the fermentation medium was 5% (v/v) [15]. Based on the above, the purpose of this study was to determine the effect of corn stover substrate concentration and fermentation time on the crude cellulase enzyme activity produced by isolate B2S8.

2 Materials and methods

2.1 Materials and chemicals

The materials used in this study were corn stover from Sanur Village, aged 3 months old, used dark brown stems and leaves, B2S8 cellulolytic bacterial isolates obtained from previous studies (stored in the Bioindustry Laboratory, Faculty of Agricultural Technology, Udayana University), carboxymethyl cellulose (CMC) (Merck), K_2HPO_4, KH_2PO_4, NaCl, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 7H_2O$, $CaCl \cdot 2H_2O$, yeast extract, NaOH, $CaCl_2$, pH buffer 4, pH buffer 7, NH_4NO_3, $FeCl_3 \cdot 6H_2O$, peptone, dinitrosalicylic acid (DNS), $CuSO_4$ (Merck), Na_2CO_3, sodium potassium tatrate (Merck), Folin-Ciocalteu reagents (Merck), sodium citrate buffer pH 4.8, filter paper (Whatman No.1), distilled water, and 70% alcohol.

2.2 Research design

The study used randomized block design (RBD) as the experimental design. It was a factorial experiment with two factors. The first factor was the substrate concentration which consisted of four levels: 2%, 4%, 6%, and 8% (w/v). The second factor was the derivation duration which consisted of four levels: 24 h, 48 h, 72 h, and 96 h. From the above factors, 16 treatment combinations were obtained, each treatment was grouped into two based on the processing time, so that 32 experimental units were obtained.

2.3 Reduction in materials size and delignification

Lignocellulosic waste from corn stover was weighed and dried with an oven, up to 10% moisture content. After drying, it was crushed with a grinder until it became a powder which passed 60 mesh sieve, then the lignocellulosic powder was obtained with a uniform size [7, 16]. Corn stover powder was weighed 100 g, then transferred into a container and poured with 4% NaOH solution (soaked for 8 h at room temperature). The delignification treatment of corn stover powder at room temperature was aimed at cost efficiency. After filtering, it was washed to obtain the neutral state and dried in an oven at 60°C to reach 10% water content [3, 7, 17].

2.4 Making liquid media

Preparing the liquid media for cellulolytic bacterial confirmation tests consisted of: KH_2PO_4 1.36 g, $(NH_4)_2SO_4$ 1 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, NaCl 2 g, yeast extract 1 g, $CaCl_2 \cdot 2H_2O$ 0.01 g, CMC 5 g, in one liter of distilled water at a pH of 6.8-7 which was then mixed and heated to boiling. While stirring until dissolved, put into a 1 L duran bottle. After dissolving, the media was sterilized by autoclaving at 121°C for 15 min [18, 19].

2.5 Rejuvenation and propagation of cellulolytic bacterial cells

The B2S8 bacterial isolate was rejuvenated by inoculating 1 mL of the isolate into each of 5 Erlenmeyer flasks containing 100 mL of cellulose medium with 1% CMC. It was incubated at room temperature for 48 h and shaken out with a shaker at 150 rpm. Then, cell multiplication was carried out by inoculating the cell culture into a 1000 mL Erlenmeyer flask containing 700 mL of cellulose medium with 1% CMC. Incubated at room temperature for 72 h and shaken at 150 rpm. Finally, it was centrifuged at 5000 rpm at 4°C for 20 min and washed twice using 0.85% NaCl solution and then adjusted to $OD_{660nm} = 5$ [18].

2.6 Production of crude cellulase at different substrate concentrations

The use of corn stover in producing crude cellulase as a substrate and as an inducer to generate crude cellulase enzymes. Sixteen Erlenmeyers containing 100 mL of different liquid media with a mixture of corn stover substrate with concentrations of 2%, 4%, 6%, and 8% (w/v) were grouped based on their processing time twice so that 32 units were obtained. After the sterile media was cooled at room temperature, it was added by culture cell pellets which had been adjusted for OD_{660nm} of 5. They were then incubated at 37°C in a waterbath shaker for 24 h, 48 h, 72 h, and 96 h [11]. After incubation, it was centrifuged at 10000 rpm for 5 min at 4°C [20]. The production of cellulase enzymes in liquid media, the optimum temperature and pH growth of B2S8 isolates were 37°C and 6.9, respectively [13]. Corn stover substrate was also used by bacteria as a carbon source to produce glucose [21]. Furthermore, the obtained crude enzymes were tested for endoglucanase activity (CMCase), exoglucanase (FPase), β-glucosidase and dissolved protein levels [22].

2.7 Endoglucanase (CMCase), exoglucanase (FPase), and β-glucosidase activity test

Endoglucanase activity was tested by a reaction mixture containing 1 mL of crude cellulase with 1 mL of 1% CMC solution in a sodium citrate buffer (pH 4.8) incubated at 50°C for 15 min. The reaction was stopped by adding 3 mL of dinitrosalicylic acid and boiled for about 5 min [18]. Cellulase activity was expressed in international units, namely IU/mL. One unit is the number of enzymes needed to break 1 µmol cellulose into reducing sugars per minute under test conditions. Glucose levels are produced from the hydrolysis of cellulose with the cellulase enzyme based on the absorbance value at a wavelength of 540 nm [19, 20].

Exoglucanase activity was tested by a reaction mixture containing 1 mL of crude enzyme (supernatant) with 2 pieces (1x1.5 cm) of Whattman No.1 paper and 1 mL of sodium citrate buffer (pH 4.8), incubated at 50°C for 1 h. The reaction was stopped by adding 3 mL of dinitrosalicylic acid and then boiled for 5 min [19].

β-glucosidase activity was tested by a reaction mixture containing 1 mL of crude cellulase with 1 mL of 1% cellubiose solution in a sodium citrate buffer (pH 4.8), incubated at 40°C for 10 min. The reaction was stopped by adding 2 mL of Na_2CO_3 [19].

2.8 Measurement of dissolved protein level

Zero point fifteen milliliters of crude enzyme sample was placed in a test tube, added with 3 mL of biuret solution, and let it be for 10 min. After that, it was added with 0.3 mL of Folin solution which was vortexed and let it be for 30 min. Quantitative analysis was performed with UV-visible spectrophotometer at a wavelength of 750 nm [22].

2.9 Statistical analysis

Results were expressed as mean ± standard deviation (n = 2). Statistical analysis of data was performed using Minitab 17 statistical software. One-way analysis of variance (ANOVA) followed by Duncan's post hoc test was carried out to determine significant statistical differences. Differences were considered as significant with $p<0.05$. The best treatment viewed by highest produces crude cellulase enzyme activated.

3 Results and discussion

3.1 Endoglucanase activity test

The results of variance analysis showed that the treatment factor of substrate concentration and fermentation time as well as the interaction between treatments had a very significant effect ($p<0.05$) on the endoglucanase enzyme activity (Figure 1).

Figure 1 showed that 8% (w/v) substrate concentration and 96 h fermentation time produced the highest endoglucanase activity average value by 0.1320 IU/mL of crude extract and they were significant differences ($p < 0.05$). However, 2% (w/v) substrate concentration and 24 h fermentation time produced the lowest average value by 0.0327 IU/mL. This showed that the higher the concentration of corn stover substrate, the higher the increase in enzyme activity produced. It was due to the active side of the enzyme will trap more substrate, so that more glucose is produced. Likewise, the longer the fermentation time, the higher the enzyme activity will tend to be. This occured because the active side of enzyme required time to hydrolyze all cellulose into glucose products. However, under optimum conditions, increasing the substrate concentration and fermentation time will cause the active side of the enzyme to be filled with the substrate or be saturated with the substrate so that the number of reaction results or the resulting activity also did not increase or even decrease [23]. This is in accordance with the research of Sharma et al. [12] on cellulase production using natural media and its application in thermo enzymatic hydrolysis of biomass. pre-chemical at a substrate concentration of 8% which obtained the highest cellulase activity of 0.53 IU/mL. Damisa et al. [24] reported that a maximum cellulase activity by 0.18 IU/mL after 96 h fermentation time. Also the research of Menezes et al. [25] concerned the evaluation of endoglucanase and *β*-glucosidase production by bacteria and yeast isolated from eucalyptus plantations in the Minas Gerais Cerrado which obtained the highest endoglucanase activity by 0.214 IU/mL at 1% CMC substrate treatment and 96 h fermentation time.

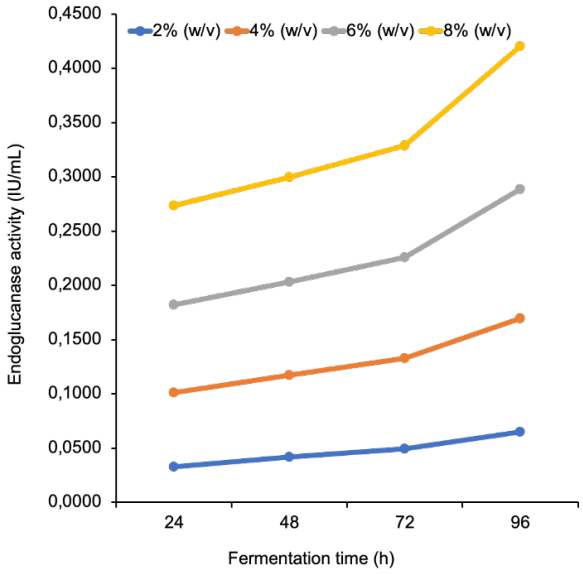

Fig. 1. Average values of endoglucanase activity (IU/mL).

3.2 Exoglucanase (FPase) activity test

Variance analysis results showed that the treatment of substrate concentration and fermentation time as well as the interaction between treatments had a very significant effect ($p < 0.05$) on the exoglucanase activity (Figure 2).

Figure 2 showed that 8% substrate concentration and 96 h fermentation time produced the highest

exoglucanase activity average by 0.0302 IU/mL. However, 2% substrate concentration and 24 h fermentation time produced the lowest exoglucanase enzyme by 0.0091 IU/mL. It was not significantly different from exoglucanase activity namely 0.0098 IU/mL at 2% concentration and 48 h fermentation time. The treatment of substrate concentration and fermentation time significantly affected the exoglucanase activity which showed a significant difference ($p < 0.05$). Under optimum conditions, the increase in substrate concentration and fermentation time would cause the active side of enzyme to be saturated with the substrate so that the number of reaction or the resulting activity did not increase [23].

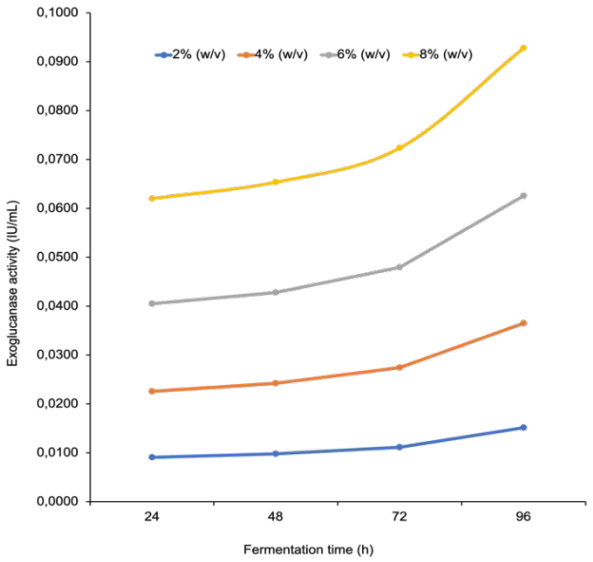

Fig. 2. Average values of exoglucanase activity (IU/mL).

It is similar with the study conducted by Sadhu et al. [26] who obtained the highest exoglucanase activity of 1.117 IU/mL using 8% CMC substrate concentration. It was also in line with the study of Gautam et al. [27] who examined the production of cellulase from solid waste residues using *Aspergillus niger* and *Trichoderma* sp. and obtained the highest exoglucanase activity of 1.64 IU/mL at 96 h fermentation.

Furthermore, it was in accordance with the research conducted by Lugani et al. [28] about optimizing cellulase production using *Bacillus* sp. Y3, and getting the results of exoglucanase activity of 6.84 IU/mL with 96 h fermentation treatment at 37°C.

3.3 β-glucosidase activity test

The results of variance analysis showed that the treatment of substrate concentration and fermentation time as well as the interaction between treatments had a significant effect ($p < 0.05$) on the activity of the *β*-glucosidase (Figure 3).

Figure 3 showed that 8% substrate concentration and 96 h fermentation time produced the highest average *β*-glucosidase activity of 0.0060 IU/mL. However, 2% concentration and 24 h fermentation time resulted in the lowest activity of 0.0015 IU/mL. This showed that higher substrate concentration caused higher enzyme activity in degrading cellubiosa, so that it resulted in higher sugar production. The fermentation time is closely related to the enzyme production and other metabolisms to some extent. Too long fermentation causes nutrient depletion, thereby it suppresses the physiological condition of microorganisms resulting in decreased enzyme production [27].

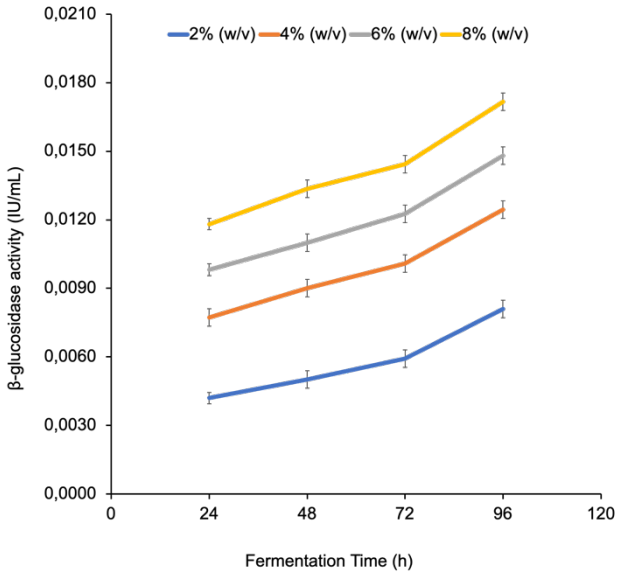

Fig. 3. Average values of *β*-glucosidase activity (IU/mL).

Under optimum conditions, the increase in substrate concentration and fermentation time would cause the interaction between the enzyme and the substrate which had a lot of formed glucose to decrease. This decrease was caused by the accumulation of previously formed products and caused inhibition of the cellulase enzyme. Cellulase enzyme inhibitors are products of cellulose hydrolysis, namely glucose and cellobiose. Selobiosa inhibits the exoglucanase enzyme while glucose inhibits the *β*-glucosidase enzyme. This statement is also supported by Kristinsen et al. [29] that an increase in glucose and cellobiose concentrations in fermentation media causes inhibition of enzyme activity.

This study is inline with research by Sadhu et al. [26] who obtained the highest exoglucanase activity of 1.041 IU/mL using 8% CMC substrate concentration. It also similar with the study of Jasani et al. [30] concerning the isolation, the optimization and production of cellulase by *Aspergillus niger* from agricultural waste which obtains the highest *β*-glucosidase activity of 0.30 IU/mL with 96 h fermentation treatment. Abubakar et al. [11] who examined the production and cellulase activity of *Aspergillus niger* using orange peel as the substrate which obtained the highest activity of 16.63 U/min using 8% rice bran substrate in 96 h fermentation treatment. This difference in results was due to several factors such as the strain and substrate of fermentation media which could affect the activity of cellulase enzymes [12].

3.4 Dissolved protein level test

Variance analysis results showed that the treatment of substrate concentration, fermentation time, and the interaction between treatments had a significant effect ($p < 0.05$) on dissolved protein levels (Table 1).

Table 1 showed that 8% substrate concentration and 96 h fermentation time produced the highest average protein content of 0.1719 mg/mL. However, the lowest protein content was 0.0384 mg/mL at 8% substrate concentration and 24 h fermentation time. The treatment of substrate concentration and fermentation time which significantly affecting the level of dissolved protein showed a significant difference ($p < 0.05$). In this study the levels of dissolved protein in crude enzyme filtrate were assumed to be cellulase enzyme proteins. These results indicate that the higher the concentration of corn stover substrate and the duration of fermentation, the higher the level of dissolved protein obtained, so that the optimum concentration of corn stover substrate and fermentation time can be achieved.

Table 1. Average values of dissolved protein levels (mg/mL).

Substrate concentration (%)	Fermentation time (h)			
	24	48	72	96
2	0.0384 ± 0.0005^{m}	0.0452 ± 0.0005^{l}	0.0571 ± 0.0005^{k}	0.0691 ± 0.0015^{j}
4	0.0716 ± 0.0010^{j}	0.0831 ± 0.0005^{i}	0.0979 ± 0.0010^{h}	0.1124 ± 0.0010^{f}
6	0.1088 ± 0.0010^{g}	0.1192 ± 0.0015^{e}	0.1326 ± 0.0010^{d}	0.1463 ± 0.0005^{c}
8	0.1373 ± 0.0005^{d}	0.1481 ± 0.0005^{c}	0.1607 ± 0.0010^{b}	0.1719 ± 0.0005^{a}

Different letters behind the average value indicated a significant difference ($p < 0.05$).

Sa'adah et al. [31] stated that the longer the fermentation time causes the higher protein content. This is because at that time the microbial growth has reached its maximum point. The longer the fermentation time, the soluble protein levels produced will tend to increase. Gunam et al. [7] reported that the higher the substrate and the longer the fermentation time, the more substrate hydrolysis will occur, so that the protein content increases, and indicates that the cellulase enzyme produced also increases. This is in accordance with Fadel's research [32] which the physiology of cellulase production and the β-glucosidase enzyme from *Aspergillus niger* are grown on fermentation media which produce the highest average of dissolved protein, namely 106.7 mg/mL at 96 h fermentation treatment.

3.5 Endoglucanase specific activity

Based on the result of variance analysis, it showed that the treatment of substrate concentration, fermentation time, and the interaction between treatments had a significant effect ($p < 0.05$) on the specific activity of endoglucanase. The average value specific activity can be seen in Table 2.

Table 2 showed that the highest average of endoglucanase specific activity was 0.9804 IU/mg obtained from the treatment interaction of 2% substrate concentration and 24 h fermentation time. This result was not significantly different from the treatment of 2% substrate concentration and 96 h fermentation time and from the treatment of 4% substrate concentration and 24 h fermentation time where the activities were 0.9486 IU/mg and 0.9704 IU/mg, respectively. However, the lowest value was 0.6422 IU/mg obtained from the treatment of 8% substrate concentration and 72 h fermentation time. These results were not significantly different from the treatment of 8% substrate concentration with 24 and 48 h fermentation time when the activities were 0.6783 IU/mg and 0.6531 IU/mg, respectively. This occurred because the increase in enzyme activity was smaller than that in dissolved protein content, resulting in high comparison values as specific activities.

Table 2. Average values of endoglucanase specific activity (IU/mg).

Substrate concentration (%)	Fermentation time (h)			
	24	48	72	96
2	0.9804 ± 0.0347^{a}	0.9202 ± 0.0041^{bc}	0.8752 ± 0.0037^{cd}	0.9486 ± 0.0115^{ab}
4	0.9704 ± 0.0327^{a}	0.9106 ± 0.0079^{bc}	0.8503 ± 0.0045^{de}	0.9151 ± 0.0139^{bc}
6	0.7646 ± 0.0011^{fg}	0.7179 ± 0.0037^{gh}	0.6978 ± 0.0004^{hi}	0.8115 ± 0.0016^{ef}
8	0.6783 ± 0.0023^{hij}	0.6531 ± 0.0021^{ij}	0.6422 ± 0.0000^{j}	0.7678 ± 0.0015^{f}

Different letters behind the average value indicated a significant difference ($p < 0.05$).

The results it can be seen that the treatment of all substrate concentrations and 72 h fermentation generated smaller value of the specific activity than the value of the treatment in all concentrations with 96 h fermentation. It was due to the increase of endoglucanase activity in the treatment of all substrate concentrations and 96 h fermentation time was higher than the increase of endoglucanase activity in the treatment of all substrate concentrations and 72 h fermentation time. Nevertheless, the increasing levels of dissolved protein in all concentrations and 96 h fermentation were smaller than those in the treatment of all concentrations and 72 h fermentation so that the resulting activity was lower.

The specific activity is the number of units of endoglucanase activity per milligram of dissolved protein [26]. The higher the specific activity value of the enzyme, the purer the enzyme produced by the

microorganisms. Conversely, the lower the specific activity value of the enzyme, the more other proteins will be produced by the microorganisms during their growth [33].

3.6 Exoglucanase specific activity

Based on the result of variance analysis it showed that the treatment of substrate concentration, fermentation time, and the interaction between treatments had a significant effect ($p < 0.05$) on the specific activity of exoglucanase (Table 3).

Table 3 showed that the highest average specific exoglucanase activity value was 0.2730 IU/mg obtained from the treatment interaction of 2% substrate concentration and 24 h fermentation time. However, the lowest value of 0.1519 IU/mg was obtained from 8% substrate concentration treatment and 72 h fermentation time. This result was not significantly different from the treatment of 8% substrate concentration with 24 and 48 h fermentation time and from 6% substrate concentration treatment with 48 and 72 h fermentation time, in which the successive activities were 0.1601 IU/mg, 0.1523 IU/mg, 0.1561 IU/mg, and 0.1536 IU/mg, respectively. It occured because the increase in enzyme was smaller that in the level of dissolved protein, resulting in a high ratio value as a specific activity. In the results, it can be seen that in the treatment of all substrate concentrations and 72 h fermentation, the specific activity value was smaller than the value of the treatment in all concentrations with 96 h fermentation. It was because the increase of exoglucanase activity in the treatment of all substrate concentrations with 96 h fermentation was higher than that in the treatment of all substrate concentrations with 72 h fermentation time.

Table 3. Average values of exoglucanase specific activity (IU/mg).

Substrate concentration (%)	Fermentation time (h)			
	24	48	72	96
2	0.2730 ± 0.0091^{a}	0.2148 ± 0.0060^{b}	0.1982 ± 0.0011^{c}	0.2203 ± 0.0073^{b}
4	0.1916 ± 0.0004^{c}	0.1744 ± 0.0019^{e}	0.1664 ± 0.0000^{efg}	0.1880 ± 0.0031^{cd}
6	0.1692 ± 0.0000^{ef}	0.1561 ± 0.0006^{fgh}	0.1536 ± 0.0000^{gh}	0.1775 ± 0.0017^{de}
8	0.1601 ± 0.0018^{fgh}	0.1523 ± 0.0005^{h}	0.1519 ± 0.0019^{h}	0.1758 ± 0.0004^{de}

Different letters behind the average value indicated a significant difference ($p < 0.05$).

On the other hand, the increasing levels of dissolved protein in all concentration treatments with 96 h fermentation time were smaller than those in the treatment of all concentrations with 72 h fermentation so that the resulting activity was lower. The growth ability of microorganisms depends on pH, temperature, incubation time, and substrate concentration. Therefore, the optimum conditions will increase the growth of microorganisms which have a direct impact on the high and low specific enzyme activities. Increasing the value of specific activities is in accordance with the increase in enzyme activity. The higher the specific activity is, the higher the purity level of the enzyme. Enzyme specific activity can be calculated based on the value of obtained enzyme activity divided by protein content [34].

3.7 Specific activity of β-glucosidase

Based on the result of variance analysis, it showed that the treatment of substrate concentration, fermentation time, and the interaction between treatments had a very significant effect ($p < 0.05$) on the specific activity of *β*-glucosidase (Table 4).

Table 4 showed that the highest average specific activity value of *β*-glucosidase was 0.1260 IU/mg obtained from the treatment interaction of 2% substrate concentration and 24 h fermentation time.

Table 4. Average values of *β*-glucosidase (IU/mg).

Substrate concentration (%)	Fermentation time (h)			
	24	48	72	96
2	0.1260 ± 0.0057^{a}	0.1100 ± 0.0072^{bc}	0.1049 ± 0.0077^{cde}	0.1179 ± 0.0029^{b}
4	0.1097 ± 0.0038^{c}	0.1088 ± 0.0046^{cd}	0.1030 ± 0.0028^{cde}	0.1094 ± 0.0024^{c}
6	0.0927 ± 0.0015^{fg}	0.0922 ± 0.0020^{fg}	0.0920 ± 0.0021^{fg}	0.1009 ± 0.0029^{de}
8	0.0879 ± 0.0015^{g}	0.0902 ± 0.0022^{g}	0.0899 ± 0.0018^{g}	0.0998 ± 0.0019^{ef}

Different letters behind the average value indicated a significant difference ($p < 0.05$).

However, the lowest value of 0.0899 IU/mg was obtained from 8% substrate concentration treatment and 72 h fermentation time. This result was not significantly different from the treatment of 8% substrate concentration with 24 and 48 h fermentation time and from 6% substrate concentration treatment with 24, 48, and 72 h fermentation time in which successive activities were 0.0879 IU/mg, 0.0902 IU/mg, 0.0927 IU/mg, 0.0922 IU/mg, and 0.0920 IU/mg, respectively. This occured because the increase in enzyme activity was smaller than that in the level of dissolved protein, resulting in high comparison values as its specific activity. In the results, it can be seen that in the treatment of all substrate concentrations and 72 h fermentation, the specific activity value was smaller than that in the treatment of all concentrations with 96 h fermentation. This was because the increase of endoglucanase activity in the treatment of all substrate concentrations with 96 h fermentation time was higher than the increase of *β*-glucosidase activity in the treatment of all substrate

concentrations with 72 h fermentation. Meanwhile, the increasing levels of dissolved protein in the treatment of all concentrations with 96 h fermentation were less than those in the treatment of all concentrations with 72 h fermentation so that the produced specific activity was lower. Enzyme specific activity can be used as an indication of the presence or absence of enzyme inhibitors or enzyme repressors in enzyme production media. When the protein content is high, but the specific activity is low, it shows that in addition to the presence of other proteins, the concentration of inhibitors in the medium of enzyme production is high. High protein levels can also be caused by measured proteins including structural proteins of bacteria and proteins contained in the medium [21].

4 Conclusions

Based on the research results which has been done, it can be concluded that the treatment of substrate concentration and fermentation time on the production of crude cellulase enzymes have a very significant effect on the enzyme activity of endoglucanase, exoglucanase, and β-glucosidase. The 8% substrate concentration treatment and 96 h fementation time were the best treatment to produce crude cellulase enzymes. The B2S8 bacterial isolate has the potential to be used for the production of cellulase enzymes. Further research that needs to be done includes optimization of cellulase enzyme production and purification.

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References

1. L.R. Lynd, P.J. Weimer, W.H. Van-Zyl, I.S. Pretorius, Microbiol. Mol. Biol. R. **56**, (2002)
2. N. Sarkar, S.K. Ghosh, S. Bannerjee, K. Aikat, Renew. Energ. **37**, (2012)
3. I.B.W. Gunam, Y. Setiyo, N.S. Antara, IM.M. Wijaya, I.W. Arnata, I W.W.P. Putra, Rasayan J. Chem. **13**, (2020)
4. S. Pandey, R. Tiwari, S. Singh, L. Nain, A.K. Saxena, J. Microbiol. Biotechnol., **24**, (2014)
5. P.A. Yasmin, Corn Production until July 2018 Reaches 19 Million Tons. Available at: https://finance.detik.com/berita-ekonom i-bisnis/d-4148996/produksi-jagung-hingga -juli-2018-capai-19-juta-ton, accessed July 2018.
6. M. Alam, M. Manchur, M. Anwar, J. Biol. Sci. **7**, (2004)
7. I.B.W. Gunam, W.R. Aryanta, I.B.N.S. Darma, J. Biol. **15**, (2011)
8. P. Sakpetch, A. H-Kittikun, A. Chandumpai, Walailak J Sci & Tech., **14**, (2017)
9. I.B.W. Gunam, N.S. Antara, A.A.M.D. Anggreni, Y. Setiyo, I P.E. Wiguna, I M.M. Wijaya, I W.W.P. Putra, Chemical pretreatment of lignocellulosic wastes for cellulase production by *Aspergillus niger* FNU 6018. AIP Conf. Proc. **2155**, (2019)
10. S.P. Gautam, P.S. Bundela, A.K. Pandey, Jamaluddin, M.K. Awasthi, S. Sarsaiya, Int. J. Environ. Sci. **1**, (2010)
11. F.A. Abubakar, O.B. Oloyede, Int. J. Sci. Res. Manag., **1**, (2013)
12. S. Sharma, V. Sharma, A. Kuila, 3 Biotech **6**, (2016)
13. A. Kusumaningrum, I.B.W. Gunam, I M.M. Wijaya, J. Rekayasa Manaj. Agroindustri. **7**, (2018)
14. M. Nababan, I.B.W. Gunam, I M.M. Wijaya, J. Rekayasa Manaj. Agroindustri. **7**, (2018)
15. N. Purba, I.B.W. Gunam, I M.M. Wijaya, J. Rekayasa Manaj. Agroindustri. 8, (2019)
16. I.B.W. Gunam, I.A.P.K. Dewi, N.S. Antara, A.A.M.D. Anggreni, Y. Setiyo, 6th International Conference on Climate Change, IOP Conf. Series: Earth and Environmental Science **824**, (2021)
17. C.S. Gajula, R. Konakalla, C.A. Kumar, R. Rudravaram, L.N. Mangamoori, Curr. Trends Biotechnol. Pharm., **5**, (2011)
18. P. Gupta, K. Samant, A. Sahu, Int. J. Microbiol., 578925, (2012)
19. A. Dar, K. Pawar, J.D. Jadhav, R.S. Pandit, Int. Biodeter. Biodegr. **98**, (2015)
20. M. Duza, S. Mastan, Int. J. Pharm. Biosci. **10**, (2013)
21. S. Deviani, H. Yuli, J. Christine, JOM FMIPA. **1**, (2014)
22. O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randall, J. Biol. Chem. **193**, (1951)
23. A. Poedjiadi, F.M.T. Supriyanti, Fundamentals of Biochemistry (Revised Edition). UI Press, Jakarta. (2006)
24. D. Damisa, E.I. Sule, S. Moneme, African J. Biotechnol. **11**, (2012)
25. F.S.R. Menezes, G.D.C. Gizelly, D.O.L. Matheus, L.N. David, Q.G.M. Tamira, L.D.L. Marcelo. Ambiente e Agua - Interdiscip. J. Appl. Sci. **14**, (2019)
26. S. Sadhu, P.K. Ghosh, G. Aditya, T.K. Maiti, J. King Saud Univ. Sci. **26**, (2014)
27. S.P. Gautam, P.S. Bundela, A.K. Pandey, J. Khan, M.K. Awasthi, S. Sarsaiya, Biotechnol. Res. Int. (2011)
28. Y. Lugani, S. Rajesh, S.S. Balwinder, J. Bioprocess. Biotech. **5**, (2015)
29. J.B. Kristensen, C. Felby, J. Henning, Biotechnol. Biofuels. **2**, (2009)
30. H. Jasani, U. Nimita, D. Darshan, J. Pure Appl. Microbiol. **10**, (2016)

31. Z. Sa'adah, S.N. Ika, Abdullah, Cellulase enzyme production by *Aspergillus niger* uses a straw substrate with a solid fermentation system. Thesis. Faculty of Engineering, Diponegoro University, Semarang (2009)
32. M. Fadel, J. Biol. Sci. **1**, (2001)
33. H.F. Rohmah, S. Ratna, P. Artini, L.A.S. Siti, Pros. Sem. Nas. Masy. Biodiv. Indon. **5**, (2019)
34. J. Ngangi, J. Pelealu, J. Warouw, L. Mandey, Int. J. Sci. Eng. Investig. **2**, (2013)