Culture of *Trichoderma* sp. with Biochar to Produce High-activity Cellulase in a Laboratory

Seongwoo Myeong and Jeonghee Yun *

Biochar (BC) was used in *Trichoderma* sp. culture to produce high-activity cellulase on a laboratory scale. The biochar was added into the flask before being applied to the fermenter to identify the enhancement effect and to determine the best amount of addition and the most suitable incubation period. Cellulase production was performed with a working volume of 4 L, and enzymatic hydrolysis was conducted to evaluate the saccharification ability of the enzyme. During incubation, the activities of three enzymes (Endoglucanase (EG), β-glucosidase (BGL), and cellobiohydrolase (CBH)) were measured for three days, and the cellulase activity was determined using a filter paper unit (FPU). In flask scale, EG, BGL, and CBH activities were increased by 1.4, 2.1, and 1.8 folds, respectively, and the incubation period was shortened by adding BC. In the fermenter scale, EG, BGL, and CBH activities were noticeably enhanced by 12.1, 5.8, and 7.2 folds, respectively, and FPU was 42.1 (9.8 folds). Additionally, the conversion rates of cellulose and steam exploded softwood and hardwood were 109.4%, 75.4%, and 87.3%, which were similar to a commercial enzyme (Cellic CTec Ⅱ). This study demonstrated that biochar could be used to produce high-activity cellulase in a shorter period and suggests a novel method for effective cellulase production.

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Contact information: Department of Forest Products and Biotechnology, Kookmin University, Seoul 02707, Republic of Korea; Corresponding author: yunjh@kookmin.ac.kr*

INTRODUCTION

Fossil fuels have been the primary source of energy over the past century; however, because of unsustainability and environmental issues resulting from combustion, exploring alternative energy sources has been of great interest since the last decade (Sun and Cheng, 2002; Rodionova *et al*. 2017). As an alternative to fossil resources, cellulose from plant biomass, such as agricultural feedstock (rice, wheat, corn, and sugarcane bagasse) and lignocellulose (forest wood residue and wood chips), has been the focus of attention (Kurian *et al*. 2013). However, biofuel production from agricultural feedstock is limited because of ongoing arguments with the food industry (Van Dyk and Pletschke 2012). Therefore, cellulose from lignocellulosic biomass has been considered a suitable alternative to fossil fuels.

Cellulose, the most abundant biopolymer, is the major constituent of plant cell walls and has a structure of linearly arranged β-D-glucose with β-(1→4)-glycosidic bonds (Gardner and Blackwell 1974; Gandla *et al*. 2018). Sugars depolymerized from cellulose can be converted into other valuable products, such as biofuels and biochemicals (Adsul *et al*. 2014). Hydrolyzing cellulose to glucose can be achieved using chemicals or biocatalysts, but the former has disadvantages, such as difficulty in recovering sugar, production of toxic waste, and production of inhibitors of post fermentation process (furfural, HMF, vanillin) (Sukumaran *et al*. 2009; Visser *et al*. 2015; Takano and Hoshino 2018). Therefore, cellulase is needed as a catalyst for the green method of cellulose conversion.

Cellulase accounts for 20% of the technological catalyst market and is used in the textile, food, detergent, and animal industries (Bhati *et al*. 2021). Despite such wide applications, the cost of cellulase production is the main debated topic, and a way to overcome this bottleneck is to reduce the production time and invent novel advanced strategies at the reactor scale in the production process (Klein-Marcuschamer *et al*. 2012; Singh *et al*. 2021).

Cellulase is produced by various microorganisms, particularly *Trichoderma*, *Aspergillus,* and *Penicillium* sp. (Gao *et al*. 2008; Singh *et al*. 2021b). Many studies have been conducted on genetic engineering (Xue *et al*. 2016; Luo *et al*. 2020; Chen *et al*. 2020; Pant *et al*. 2022) and artificial mutation (Dong *et al*. 2019; Singh *et al*. 2020; Dong *et al*. 2022; Lv *et al*. 2023) to produce improved cellulase from microbes. Additionally, there are various other strategies for culture technologies. Cellulase is produced using two basic culture technologies: submerged fermentation (SmF) and solid-state fermentation (SSF). The SmF process is performed using a substrate submerged in liquid, whereas SSF is performed in conditions with near-to-absence of liquid and microbes growing on the substrate's surface (Singh *et al*. 2021a; Korsa *et al*. 2023). Although more concentrated enzyme production is possible by SSF, the SmF system is preferred on the industrial scale because the process is conducted under controlled conditions, and the product is easily recovered (Singh *et al*. 2021b). The main factors of SmF are nutrition (carbon and nitrogen sources), pH, temperature, incubation period, dissolved oxygen, and aeration (Reihani and Khosravi-Darani 2019). Therefore, many studies have aimed to optimize the culture conditions to improve cellulase production in SmF (Ramanathan *et al*. 2010; Matkar *et al*. 2013; Infanzón-Rodríguez *et al*. 2020). Otherwise, to improve cellulase activity in SmF, other novel aspects were investigated, which fuse the merit of SSF in the SmF system. Following a related strategy, this study aimed to investigate the role of microbe habitat by using biochar in the SmF system to enhance cellulase production.

Biochar is a carbon-rich porous material produced through thermal combustion under oxygen-limited conditions from various biomasses, such as agriculture and wood residue (Wang and Wang 2019; Joseph *et al*. 2021). Biochar is widely used in wastewater treatment, soil remediation, carbon sequestration, and pollutant adsorption because of its large specific surface area, porosity, and abundant functional groups (Mohanty *et al*. 2018; Wang and Wang 2019; Zhang *et al*. 2021). In addition, biochar could improve soil's biological activity. The biochar's surface has been found to positively affect bacterial and fungal growth and enzyme activity in soil because it is highly porous and comprises easily degradable carbon and nitrogen (Zhang *et al*. 2021). Many studies have indicated that biochar could enhance cellulase activity in soil or sludge treatment (Awad *et al*. 2018; Du *et al*. 2019; Yin *et al*. 2023), but research on the use of biochar in cellulase production is limited.

In this study, biochar was used in *Trichoderma* sp. culture to improve cellulase activity. Previously, to determine the effect of biochar on cellulase activity in the liquid state, different biochar contents were added to *Trichoderma* sp. culture in a flask scale. Three cellulolytic enzyme activities and protein concentrations were measured during incubation every three days. Cellulase production with biochar was conducted in a 7 L fermenter with a working volume of 4 L for 17 d. For a flask scale, enzyme and protein assays were performed every three days, and cellulase activity was determined using a filter paper unit (FPU). Finally, to evaluate the hydrolysis ability of the produced enzyme, enzymatic hydrolysis of steam-exploded biomass was conducted.

EXPERIMENTAL

Cellulase Production

Microorganism and precultures

For inoculation, *Trichoderma* sp. (Kim *et al*. 2018a) was grown on malt extract agar (MEA; malt extract: 20 g/L, glucose: 20 g/L, peptone: 1 g/L, and agar: 20 g/L) plates at 25 °C

until the surface was dense green, and stored at 4 °C. The preculture was performed in a 250-mL baffled flask with 100-mL potato dextrose broth (PDB). The medium was sterilized at 121 °C for 20 min, and 10 loops of 1-cm diameter fungal-agar plugs of *Trichoderma* sp. grown on the MEA plate were inoculated. The incubation was done at 26 °C and 150 rpm for 5 d.

Biochar used in Trichoderma sp. culture

The biochar (BC, Kookmin university, Republic of Korea) was generated by pyrolyzing wood chips with oxygen limited condition. To determine the physical properties of BC, Brunauer-Emmett-Teller (BET) analysis was performed. The average pore size and surface area were found to be 1.89 nm and 170 m^2/g, respectively.

Identifying the effects of biochar on cellulase activity at the flask level

To identify whether biochar positively affects cellulase activity, a control group was prepared using *Trichoderma* sp. culture without biochar, whereas the experimental groups contained 1%, 2%, and 3% (w/v) biochar (BC, Kookmin University, Republic of Korea), respectively. All groups were prepared at 100 mL in a 250-mL baffled flask and triplicated. The medium for cellulase activity evaluation was based on previous study (Choi 2021). It included yeast extract: 15 g/L, KH_2PO_4 5 g/L, K_2HPO_4: 5 g/L, $MgSO_4 \cdot 7H_2O$: 3 g/L, Avicel 3% (w/v), initial pH of 5.0, and biochar was added to the experimental groups. Every flask was sterilized at 121 °C for 30 min, and 5% (w/w) of preculture was inoculated. The flasks were incubated at 30 °C, 150 rpm, for 21 d. To determine the change in enzyme activity and protein concentration, 1 mL broth was centrifuged at 10,000 rpm, 10 min, and 4 °C for every 3 d, and the supernatant was used for the assay.

Cellulase production

Cellulase was produced in a 7 L fermenter (working volume 4 L). The medium composition was yeast extract: 10 g/L, KH_2PO_4: 5 g/L, K_2HPO_4: 5 g/L, $MgSO_4 \cdot 7H_2O$: 3 g/L, Avicel: 20 g/L, and an initial pH of 5.0. Additionally, 1% (w/v) biochar was added to the experimental group and sterilized at 121°C for 30 min. Each fermenter was cooled to room temperature (25°C), and 5% (v/v) preculture was inoculated. Incubation was performed at 31.3 °C, 150 rpm, and aeration of 2 L/min for 17 d. The sampling procedure was the same flask level. Cellulase production proceeded as follows: After 17 d, the broth was filtered using filter paper (Whatman No.1, England), and the filtrate was concentrated using Amicon® Stirred Cells (Catalog No. UFSC40001, Milipore Corp., Germany) equipped with 10-kDa cutoff polyethersulfone membrane (PM 10 membrane, Milipore Corp., Germany) up to 1/60 of the initial volume. The produced cellulase activity was evaluated by measuring the filter paper unit (FPU)/mL.

Assay Method

Enzyme activity

EG activity was analyzed using the Somogyi-Nelson method (Nelson 1944). Carboxymethylcellulose (CMC, 2%) diluted in 0.1M sodium citrate buffer (pH 5.0) was used as the substrate. The mixture of 45 μL CMC and 5 μL enzyme was incubated at 60 °C for 30 min. Then, 50 μL of copper reagent was added and kept in boiling water for 10 min to halt the reaction. Subsequently, 50 μL of Nelson reagent and 850 μL of distilled water were added to the mixture. Finally, the supernatant was measured at an absorbance of 650 nm.

For the BGL and CBH assays, 10 mM p-nitrophenyl-β-D-glycopyranoside (pNPG, Sigma-Aldrich, USA) and p-nitrophenyl-β-D-cellobioside (pNPC, Sigma-Aldrich, USA) diluted in pH 5.0 0.1 M sodium citrate buffer were used. A 200 μL mixture containing 160 μL buffer, 20 μL substrate, and 20 μL enzyme was incubated at 65°C for 15 min. Then, 50 μL Na_2CO_3 was added and absorbance was measured at 405 nm (Joo *et al*. 2009).

For the quantitative assay, specific activity (SA) was measured as shown in Eq. 1.

$$\text{SA } (^{\text{U}}/_{\text{mg}}) = \frac{\text{Enzyme activity (U/mL)}}{\text{Protein concentration (mg/mL)}} \quad (1)$$

The protein concentration assay was as follows: FPU was measured using the method described by NREL TP-510-42628 (2008). First, 1.0 mL of pH 5.0 0.1M sodium citrate buffer, 0.5 mL enzyme, and 0.05 g Whatman No.1 filter paper (Whatman, UK) were added to a conical tube. The tubes were incubated at 50 °C for 1 h. 3,5-Dinitrosalicyclic acid (DNS) reagent (3.0 mL) was added, and the mixture was boiled for 5 min. The mixture was centrifuged at 13,000 rpm at 4 °C for 10 min and measured at an absorbance of 540 nm. The absorbance was compared with a standard curve to determine the amount of each product. One unit (U) of each enzyme activity was defined as the amount of enzyme that released 1 μmol glucose (EG, FPU) or p-nitrophenol (BGL, CBH) per min.

Protein assay

Protein concentration was analyzed to verify the fungus growth. A Bradford's method-based protein assay kit (Bio-Rad, USA) was used for protein assay. A mixture of 400 μL distilled water, 100 μL protein assay reagent, and 10 μL enzyme was incubated at room temperature for 5 min and absorbance was measured at 595 nm. The absorbance was put in the Bovine serum albumin standard curve. All assays were done in duplicates.

Enzymatic Hydrolysis

Biomass pretreatment

Biomass was hydrolyzed to determine the saccharification ability of the produced cellulase. Softwood *(Larix leptolepis*, Hongcheon-gun, Gangwon-do, Republic of Korea) and hardwood (*Quercus variabilis*, Goesan-gun, Chungcheongbuk-do, Republic of Korea) were used as substrates for enzyme hydrolysis. Steam-explosion pretreatment was performed in a batch pilot unit (Youlim high-tech, Republic of Korea) using the Masonite technique. The operating pressure was 25 kgf/cm^2 for 13 min. The pretreated biomass obtained from the cyclone was cooled to 40 °C. The solids were used for hydrolysis, and their compositions were analyzed, as shown in Table 1. The analysis was conducted using the modified method described in NREL TP-510-42618 (2012), TAPPI T222 om-2 (2002), and T249 cm-09 (2009).

Table 1. Composition of Steam-Explosion Pretreated Biomass Feedstock

Steam-Exploded biomass	Component Ratio (%)					Total
	Glucan	Xylan	Arabinan	Lignin	Extractive	
Larix leptolepis[1)]	46.8	7.1	0.4	29.7	18.3	102.3
Quercus variabilis[2)]	61.8	4.1	0.2	17.1	17.8	101

[1)] 25 kgf/cm^2, 7 min; [2)] 25 kgf/cm^2, 13 min

Enzymatic hydrolysis

The enzyme hydrolysis was performed in glass tubes with 5 mL volume. The aforementioned biomasses and cellulose were used as substrates for 7% (w/v). Before hydrolysis, the biomass moisture contents of biomass, except cellulose, were adjusted from 75 to 80% and sterilized at 121 °C for 30 min. Cellic CTec2® (Novozyme, Denmark), as a comparison enzyme, and the produced enzyme were treated 60 FPU/g*glucan. Additionally, 100 mg/g*glucan of Polysorbate 80 (Tween 80, Sigma-Aldrich, USA), 0.05 to mL of 2% sodium azide, and pH 5.0 0.1 M sodium citrate buffer (pH 5.0 0.1 M sodium acetate for comparison enzyme) were added in each glass tube. The reaction was conducted for 72 h at 250 rpm and 50 °C. After hydrolysis, the mixtures were boiled for 30 min to protein denaturation and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was used in reduced sugar determination. The analysis was performed with the mixture of buffer 120 μL,

DNS reagent (Miller 1959) 150 µL, and supernatant 30 µL. The solution was boiled for 5 min, and absorbance measured at 540 nm. The assay was done in duplicates.

The conversion rate was calculated as shown in Eq. 2.

$$\text{Conversion rate (\%)} = \left(\frac{\text{Reduced sugar (mg)}}{\text{glucan of substrate (mg)}}\right) \times 100 \quad (2)$$

RESULTS AND DISCUSSION

Identifying the Effect of the Biochar on Cellulase Activity

The change of enzyme activities and protein concentration resulting from different amounts of biochar in the flask scale and each of the maximum values are shown in Fig. 1 and Table 2.

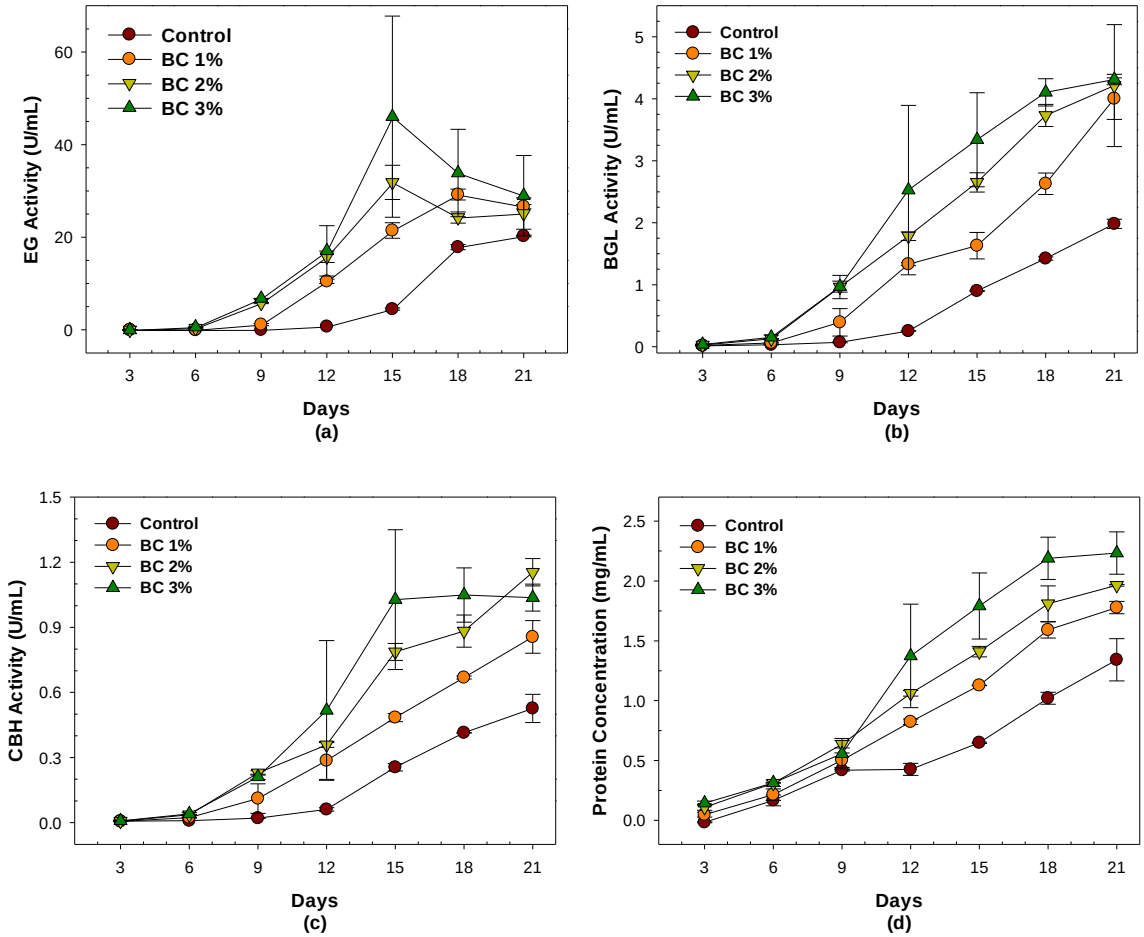


Fig. 1. Enzyme activity and protein concentration of *Trichoderma* sp. with different amounts of biochar in a shake flask. (a) EG activity, (b) BGL activity, (c) CBH activity, (d) Protein concentration

Table 2. Maximum Enzyme Activity and Protein Concentration of *Trichoderma* Sp. with Different Biochar Concentrations in Flask Scale

	Control[1)]	BC 1%[2)]	BC 2%	BC 3%
EG (U/mL)	20.2 ± 0.20	29.2 ± 1.18	31.8 ± 3.71	46.0 ± 21.70
BGL (U/mL)	2.0 ± 0.08	4.0 ± 0.34	4.2 ± 0.98	4.3 ± 0.08
CBH (U/mL)	0.5 ± 0.07	0.8 ±0.08	1.2 ± 0.06	1.0 ± 0.06
Protein (mg/mL)	1.3 ± 0.18	1.8 ± 0.05	2.0 ± 0.01	2.2 ± 0.18

[1)] Culture of *Trichoderma* sp. without BC, [2)] w/v

For EG, maximum activity (Table 2) increased by 1.4, 1.6, and 2.3 folds compared to control along with BC concentration (BC 1%, 2%, and 3%, respectively). Furthermore, peak days of EGactivity differed for different BC amounts. The peak of the EG activity of BC 1% was at day 18, whereas the peaks for BC 2% and 3% were at day 15, and the control was at day 21 (Fig. 1). However, with the addition of higher BC concentrations (2% and 3%), a higher standard deviation was found on day 15 (3.71 and 21.70, respectively). It could be interpreted that excessive use of BC could result in different growth patterns in each flask. Therefore, although the activity value during incubation differed, it could be observed that the BC addition could enhance the EG activity and reduce the production cost by shortening the culture period.

BGL and CBH activities were also increased by adding BC, but the amount of BC that represented the maximum activity value differed. The maximum BGL activity increased proportionately with BC concentration. However, the CBH activity was the highest at 2% BC. For BGL, the maximum activity of 1%, 2%, and 3% BC were 2.1, 2.1, 2.2 folds greater than the control and CBH were 1.6, 2.2, and 2.0, respectively. This indicates that the biochar's effect on the activity value differed depending on the enzyme type. Additionally, it was shown that BGL and CBH activities were noticeably enhanced by the addition of just 1% BC, and no remarkable difference was observed with higher BC concentrations (2% and 3%). Therefore, for cost-effective enzyme production, BGL and CBH with higher activity could be obtained using 1% BC.

Protein concentration also increased with the concentration of BC: 1.3, 1.5, and 1.7-fold compared with the control (1%, 2%, and 3% BC, respectively). This indicates that adding BC increases enzyme activity and fungus growth. Based on these results, it was observed that the microorganism and cellulase activity could be enhanced by the BC treatment. While studies applying biochar to cellulase production are limited, diverse research reports indicate that the treatment of BC enhances cellulase activity. Du *et al*. (2019) reported an 8.5-fold increase in cellulase activity when 10% rice straw BC was added during sewage sludge composting. Additionally, Yin *et al*. 2021 noted a 56% increase in cellulase activity by adding 10% bamboo BC to chicken manure composting, and Duan *et al*. (2022) reported increased microbial and cellulase activity in sheep manure composting treated with apple tree branch biochar. Furthermore, in a study applying biochar to cellulase production, Saeed *et al*. (2023) used BC as a catalyst in SSF to produce fungal β-glucosidase (BGL) from coconut waste, resulting in a 1.2-fold increase in BGL activity. Research investigating the causes of enhanced microbial growth and enzyme activity due to biochar treatment has also been reported. It has been stated that biochar, owing to its high porosity and extensive surface area, can serve as a habitat for microorganisms, thereby promoting microbial growth by improving aeration (Akdeniz 2019, Jindo *et al*. 2012, Sanchez-Monedero *et al*. 2018). Based on these findings, one might speculate about reasons for the increased activity of cellulase and microorganisms with the addition of biochar in this study. However, further research is required to precisely elucidate the underlying mechanisms.

To determine whether the cause of the high activity was simply due to an increased amount of enzyme or increased activity per enzyme, the SA was measured, and the results are shown in Table 3.

Table 3. Maximum Specific Activity (U/Mg) of *Trichoderma* Sp. with Different Amounts of Biochar Treatment in Flask Scale

	EG		BGL		CBH	
	Protein[1)]	U/mg	Protein	U/mg	Protein	U/mg
Control	1.34	15.08	1.34	1.48	1.34	0.39
1% BC	1.59	18.38	1.78	2.25	1.78	0.48
2% BC	1.41	22.58	1.96	2.15	1.96	0.59
3% BC	1.79	25.70	2.23	1.93	2.19	1.02

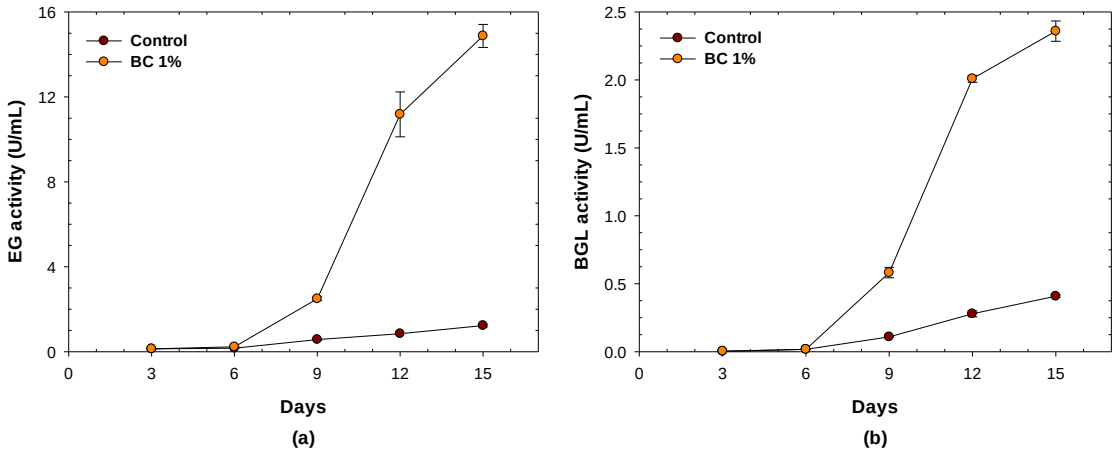
[1)] Protein concentration at maximum enzyme activity

The SA of each enzyme increased with the addition of BC. This indicates that the high activity value was not due to an increase in the amounts of enzymes but rather due to an increase in activity per enzyme. Additionally, it was noticeable that BGL activity was highest at 1% BC, whereas those of EG and CBH increased with increasing BC concentration.

Trichoderma sp. is mostly used for cellulase production because it produces all classes of cellulolytic enzymes (EG, BGL, and CBH) and has a high FPU value and relatively low BGL and CBH activities (Lv *et al*. 2022). Accordingly, Zhao *et al*. (2020) reported that cellulase from *T. viride* has lower BGL activity, and Lv *et al*. 2022. (2022) reported that cellulase produced by *T. reesei* has low BGL and CBHII activities. To overcome the low activity of BGL and CBH, various strategies, such as blending with cellulase produced from other fungi, have been steadily reported (Copete-Pertuz *et al*. 2019). In this study, 3% BC treatment exhibited the highest activity values for all enzymes; however, the substantial variability observed made it impractical for reliable enzyme production. Additionally, 2% BC treatment demonstrated increased activity compared to 1%; yet, the difference was not prominently noticeable. Consequently 1% BC was selected for cellulase production because the SA of BGL showed the maximum value, and CBH also increased remarkably.

Cellulase Production in the Fermenter Scale

Cellulase was produced at the fermenter level. Figure 2 shows the enzyme activity and protein concentration in the fermenter with or without the biochar. The control group showed no noticeable increase in enzyme activity.



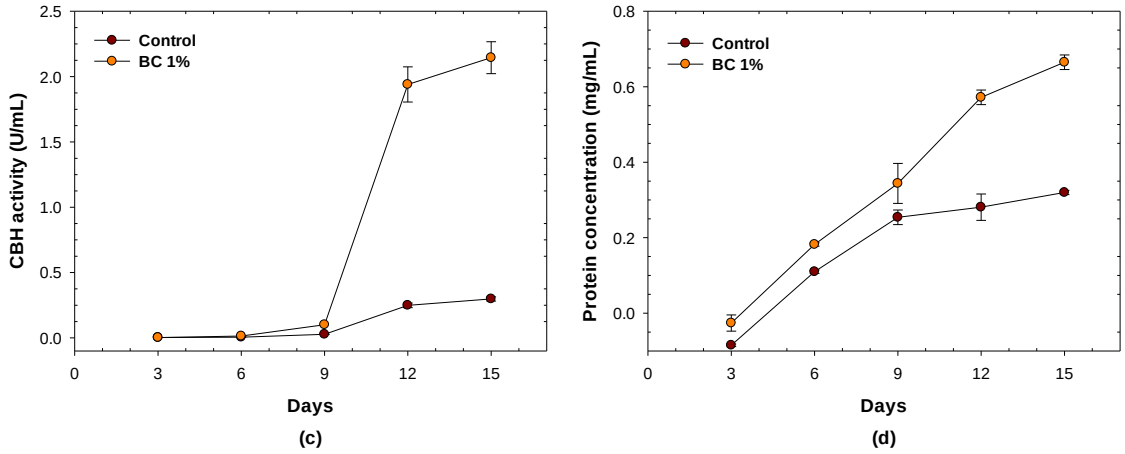


Fig. 2. Enzyme activity and protein concentration of *Trichoderma* sp. at the fermenter level (a) EG activity, (b) BGL activity, (c) CBH activity, (d) Protein concentration

The protein concentration in the control increased similarly with 1% BC until 9 d, but after that, there was no additional rise. This indicates that the growth of *Trichoderma* sp. was negligibly low after the 9th day, which could be a reason for the low activity. For the 1% BC, all enzymes showed remarkably increased activity values between day 9 and 12, and the protein concentration steadily increased during incubation. This indicated that biochar could increase enzyme activity and fungal growth in the fermenter.

Table 4 shows the maximum enzyme activity and protein concentration. Activities of the three enzymes in the fermenter were also increased, but the increase rate differed. To compare the increase ratio, the index of the ratio of the maximum value of 1% BC per control (B/C) was calculated. For the fermenter, the B/C of EG, BGL, and CBH were 12.1, 5.8 and 7.2, but the flask was only 1.4, 2.0, and 1.6, respectively. Protein concentrations also increased, and B/C was 2.1 and 1.3 in the fermenter and flask, respectively. The FPU values are also shown in Table 4. 1% BC was 9.8-fold higher than the control. This means that biochar is appropriate as an effective cellulase enhancer as it could significantly improve poor cellulase activity and fungal growth in a larger scale. Therefore, the addition of biochar could be a way to produce high-quality cellulase.

Table 4. Maximum Enzyme Activity and Protein Concentration of *Trichoderma* Sp. in a 7 L Fermenter

	EG (U/mL)	BGL (U/mL)	CBH (U/mL)	Protein (mg/mL)	FPU/mL
Control[1)]	1.229	0.408	0.298	0.320	4.273
1% BC	14.873	2.359	2.145	0.665	42.050

[1)]Culture of *Trichoderma* sp. without biochar

Table 5 shows the SA and B/C of the flask and fermenter. For the control group, the SA of EG and BGL decreased as the volume increased. Conversely, the SA of 1% BC in the fermenter was higher than that in the flask. This suggests that the addition of biochar was more efficient in a larger scale. B/C also showed a similar pattern, which was bigger in a large scale. Consequently, BC is beneficial in cellulase production as it could increase cellulase activity and seems to be more advantageous in scale up process.

Table 5. Maximum Specific Activity (U/Mg) of *Trichoderma* Sp. by the Addition of Different Amounts of Biochar in the Flask And Fermenter

		EG (U/mg)	[1)]B/C	BGL (U/mg)	B/C	CBH (U/mg)	B/C
Flask	1% BC	18.365	1.2	2.251	1.5	0.392	1.3
	Control	15.083		1.477		0.481	
Fermenter	1% BC	22.365	5.8	3.547	2.8	3.226	3.5
	Control	3.841		1.275		0.931	

[1)] Maximum activity of 1% BC per control

Therefore, the addition of biochar could be one strategy for overcoming the shortages of the SmF system. SmF is suitable for cellulase production in a larger scale because of its easy system handling. However, lower enzyme activity than SSF and scale-up are the main bottlenecks of the process (Singhania *et al*. 2010; Prévot *et al*. 2013). Many studies have aimed to find strategies to overcome these problems by changing the enzyme production system. Weber and Agblevor (2005) reported the lower power consumption of cellulase production in the SmF from *T. reesei* using a microbubble dispersion-equipped air sparger, which enhanced cell mass productivity and similar enzyme activity with a low agitation system. de Lima *et al*. (2022) provided a cellulase production design from *Trichoderma sp*. utilizing bioprocess optimization using techno–economic analysis and life cycle assessment, which reduced enzyme production cost and improved activity in a larger scale. To obtain the both advantages of SmF and SSF, Cunha *et al*. (2012) reported that the EG activity from *A. niger* via sequential solid-state and submerged (SoSF–SmF) cultivation showed a 3-fold improvement compared with SmF. Additionally, Intasit *et al*. (2021) found that cellulase with the cocultivation of *T. reesei* and *A. turbingensis* in SoSF–SmF showed higher activity than in SmF. To enhance the low activity, Lan *et al*. (2013) improved cellulase activity from *T. viride* with immobilized cells, which exhibited higher FPU activity than single cell culture. da Silva Delabona *et al*. (2016) found that glycerol-induced *T. harzianum* cultivation could increase FPU by approximately 2-fold compared with the control. Zhao *et al*. (2018) reported that the enzyme from the cocultivation of recombinant *T. reesei* and *A. niger* showed 12.17 FPU/mL, which was 1.3-fold higher than the monoculture of recombinant *T. reesei*. In this study, cellulase from *Trichoderma* sp. with biochar application was shown to improve cellulase activity and efficiency in a larger scale. It was considered sufficient to supplement the inadequacies of the SmF system. Therefore, the addition of biochar to *Trichoderma sp*. cultivation was suggested as a novel method of cellulase production in the SmF system.

Enzymatic Hydrolysis

Figure 3 shows the results of cellulose hydrolysis of steam-exploded *L. leptolepis* (SE-La), and *Q. variabilis* (SE-Qu) by commercial enzyme (Cellic CTec II) and produced enzyme. For all cellulolytic materials, the produced enzyme showed a conversion rate similar to that of the commercial enzyme. The conversion rates of commercial enzymes of cellulose, SE-La, and SE-Qu were 109.4%, 81.5%, and 95.3%, whereas the conversion rates of the produced enzyme were 109.4%, 75.4%, and 87.3%, respectively. This indicates that the produced enzyme had the same saccharification ability of lignocellulosic materials as the commercial enzyme with the same FPU treatment. Furthermore, the hydrolysis rates of cellulose and SE-La with produced enzyme without BC were 94.1% (Kim *et al*. 2018b) and 29.1% (Choi, E. 2021), respectively. It was shown that the conversion ability was improved by BC treatment.

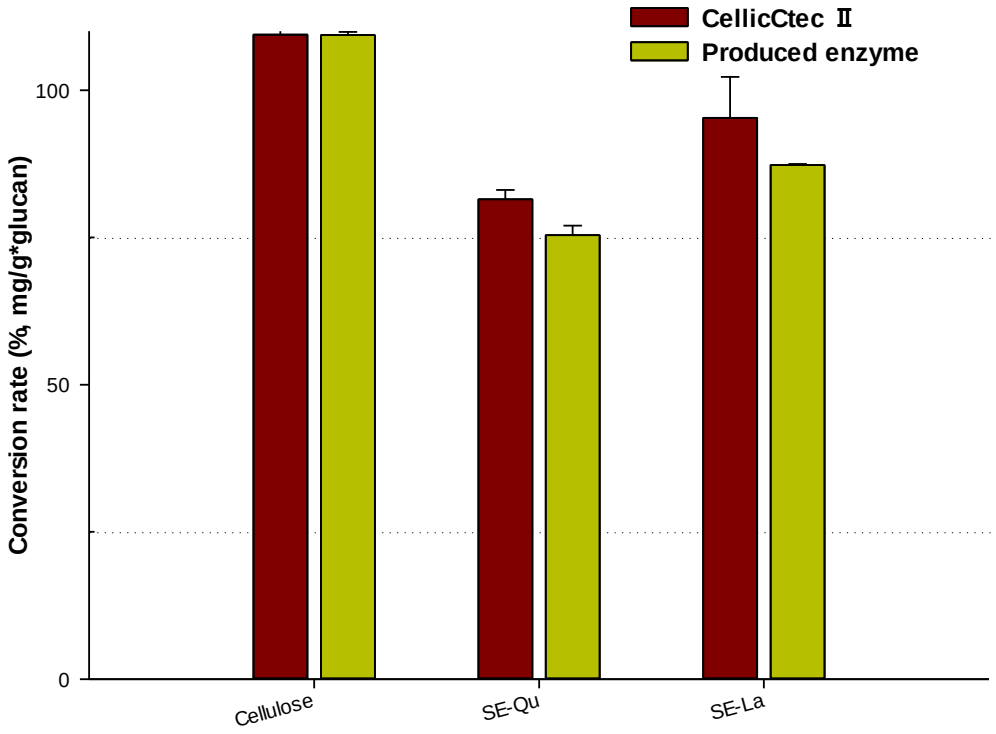


Fig. 3. Comparison of the hydrolysis ability of commercial and produced enzyme

Woody biomass residue from hardwood and softwood is a sustainable and abundant material that provides cheap sources for hydrolysis, such as branches, chips, and sawdust (Deng *et al*. 2023). However, it is quite recalcitrant in comparison to nonwoody materials, such as agricultural residue, because woody biomass typically contains more lignin, which defends the approach of microbes and enzymes (Álvarez *et al*. 2016). Therefore, many studies focused on the conversion of woody biomass using commercial enzymes or produced enzymes. Fujii *et al*. (2009) estimated the hydrolysis ability of the produced enzyme (*Acremonium cellulolyticus*) against woody biomass, *Eucalyptus* and Douglus fir, and conversion rates for both were above 69%, which was higher than the commercial enzyme (Accellase 1000), which was below 60%. Novy *et al*. (2021) customized the enzyme mixture of Celluclast 1.5 L and cellulase from *T. reesei* and treated it with pretreated lodgepole pine, which represented approximately the same conversion rate as Cellic CTec 3 (80%). Aksenov *et al*. (2020) observed that the conversion rate of bleached hardwood and softwood pulp with cellulase from *Penicillium verruculosum* and the reduced sugar yield were approximately 60%. Lai *et al*. (2020) demonstrated the glucose yield of organosolv-pretreated poplar sawdust using different surfactants with a commercial enzyme (SAE0020), and the maximum yield was 82.5%. To improve the bioconversion of poplar sawdust, Chu *et al*. (2019) estimated the saccharification rate using steam-explosion pretreatment with different phenolic compounds, and the maximum glucose conversion rate by a commercial enzyme (Cellic CTec 2) was 72.5%. Li *et al*. (2019) observed the enzymatic hydrolysis ability of bleached hardwood chemical pulp (eucalyptus and poplar pulp) and the one-dried blenched pulp, showing a 73.1% conversion yield. Takada and Saddler (2021) suggested that nonpelletized steam-treated softwood with mild refining showed the highest enzymatic hydrolysis yield of cellulose compared with pelletized softwood (approximately 50% and 10%, respectively). In this study, the hydrolysis ability of the produced enzyme was similar against SE-La and SE-Qu in comparison to the commercial enzyme, and its conversion rate was comparatively competitive with the aforementioned studies.

CONCLUSIONS

1. In this study, biochar was added to *Trichoderma* sp. culture to produce high-activity cellulase in a laboratory scale. Before enzyme production, the change of cellulase activity (EG, BGL, and CBH) with different amounts of biochar in the flask scale was observed to determine whether biochar had the ability as an enzyme activity enhancer and the amount for application in the fermenter scale. The results showed that the maximum activities of EG, BGL, and CBH were increased by 1.4, 1.6, and 2.1 folds, respectively, in comparison to nontreatment with only 1% biochar addition. Additionally, a decrease in the incubation period was observed with biochar addition.
2. Cellulase was produced in a 7 L fermenter with 1% biochar treatment. The maximum activities of EG, BGL, and CBH were 12.1, 5.8, and 7.2 folds higher, respectively, than the control. The increased rate caused by adding biochar in the fermenter was higher than in the flask scale, indicating that biochar was more effective in a larger scale. Additionally, biochar increased FPU, which improved by 9.8 fold compared with the control.
3. The hydrolysis ability of the produced enzyme was evaluated via the saccharification of cellulose, steam-exploaded softwood, and hardwood. The conversion rates were 109.4%, 87.3%, and 75.4%, respectively, which were nearly the same as of the commercial enzyme. Consequently, the addition of biochar could be a novel strategy for enhanced cellulase production.

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