EXTRACELLULAR CELLULASE PRODUCTION FROM A WHITE-ROT FUNGUS *PORODAEDALEA PINI*

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ABSTRACT

The present study investigated the conditions for extracellular cellulase production of a white-rot fungus *Porodaedalea pini* and compared the enzyme activities between the two culture methods using a fermentor or agitating flasks. The fungus was cultured in the modified Norkrans medium for 24 days, and Avicelase, CMCase, β-glucosidase (BGL), cellobiose dehydrogenase (CDH), and protease activities were assayed every 2 days. BGL activity by flask culture was higher than that by fermentor culture. Avicelase activity by flask culture was slightly higher than that by fermentor culture. The highest activity of CMCase was recognized on the 24th day of the incubation in the flask culture method, whereas the highest activity was found on the 20th day in the fermentor method. CDH activity by flask culture showed much higher value than that by fermentor culture. Protease activity in the both methods did not correspond with change in the protein concentration during culture for 24 days. The flask culture method was proved to produce higher respective cellulases, especially CDH, compared to fermentor method.

Keywords: White-rot fungus; Cellulase; β-Glucosidase; Cellobiose dehydrogenase

INTRODUCTION

After the Kyoto protocol in 1997, many countries in the world have been tackled the reduction of greenhouse gas (GHG) emission. Especially, reduction of CO_2 accounting for about 90% of GHG is immediately required through the globe [1]. In order to grapple with this issue, researches and development for bio-ethanol production are actively undergone around the world. However, most of the raw materials for ethanol production are currently derived from cereals, such as corn and sugar cane, leading to new problems, such as competition to acquire the materials with food supply [2]. Hence, the ethanol production from wood biomass is notified now because the system does not compete with food supply in terms of acquisition of raw materials.

Cellulose in the materials is required to be degraded into fermentable sugars to convert the wood biomass into bio-ethanol. There are two main methods for conversion of the materials into fermentable sugars (saccharification): acid-hydrolysis saccharification and enzymatic saccharification. Acid-hydrolysis saccharification has some merits to hydrolyze cellulose in short duration and to degrade all kinds of polysaccharides, while some demerits to be costly in the facilities durable to strong acids and to have an environmental problem for treating wastes after hydrolysis. In addition, acid-hydrolysis saccharification results in low sugar yield because of further

degradation of produced sugars by strong acids [3]. On the other hand, enzymatic saccharification has some merits that saccharification conditions are rather moderate, the saccharification is less problematic in waste treatment, and that the saccharification results in higher sugar yield because of no further degradation of released sugars. However, enzymatic saccharification has some demerits that it requires rather longer reaction time and huge amount of enzymes [4]. The background for enzymatic saccharification of wood biomass, therefore, requires the better enzymes with higher saccharification efficiency.

Cellulose-degrading microorganisms are widely distributed in the nature, whereas limited microorganisms can degrade crystalline cellulose completely. Among such microorganisms, moulds, such as *Trichoderma ressei*, and white-rot fungi, such as *Phanerochaete chrysosporium* have powerful cellulases. These microorganisms have common features to secrete some extracellular cellulases with different properties to degrade crystalline cellulose, resulting in complete degradation of crystalline cellulose by additive effects of those cellulases [5].

A white-rot fungus *Porodaedalea pini* belongs to Hymenochaetaceae in Basidiomycetes. *P. pini* is known to have powerful enzymes because it thins the wide range of cell walls after degrading most of lignin in secondary walls and compound middle lamellae [6]. In addition, this fungus has been investigated for its culture conditions to produce extracellular cellulases, and it has been suggested that the fungus produces highly active β–glucosidase compared to other cellulases assayed, such as Avicelases and CMCase [7].

The present study assayed four extracellular cellulases, i.e. Avicelase, CMCase, β–glucosidase (BGL), and cellobiose dehydrogenase (CDH), and protease from *P. pini* in time-course experiments, and investigated the activity patterns of these enzymes in order to establish efficient production system for highly active cellulases of this fungus. Moreover, the two culture methods, flask culture and fermentor culture were compared to select better one for respective cellulases.

MATERIALS AND METHODS

Fungal material. A white-rot fungus *Porodaedalea pini* (Brot.) Murrill. WD1174 was used for the experiments. This fungus was obtained from the Institute of Forestry and Forest Products, Tsukuba, Japan. The fungus was precultured in Petri dishes (9 cm i.d.) containing potato-dextrose-agar medium (BD Difco, Becton, Dickison and Company, Sparks, MD, USA) at 25ºC in the dark.

Flask-agitating culture. The fungus was cultured in 2 L Erlenmeyer flasks containing modified Norkrans medium [8] as a basal medium. A flask contained the following culture components: modified Norkrans medium components (30.00 mM glucose, 16.65 mM asparagines, 18.37 mM KH_2PO_4, 5.28 mM $MgSO_4 \cdot 7H_2O$, 4.28 mM NaCl, 2.25 mM $CaCl_2$, 0.05 mM $FeCl_3 \cdot 6H_2O$, 770 nM $ZnSO_4$, 40 nM thiamine hydrochloride), 27.8 mg Avicel (Funacel II, Funakoshi, Tokyo, Japan), additional solution (139.8 mg glucose, 693.3 mg $MgSO_4 \cdot 7H_2O$, and 1,042.8 mg urea in 9.7 mL H_2O), 56.25 mL bovine calf serum (BCS) (HyClone, South Logan, Utah, USA), and 1,250 mL H_2O. The medium solution in flasks except for additional solution and BCS was autoclaved for 30 min at 121ºC. Additional solution was filter-sterilized with Millex-GV filter (0.22 μm, Merck Millipore, Darmstadt, Germany) and added to the flasks. The pre-sterilized BCS was added to the autoclaved culture solution in a laminar flow cabinet.

Mycelial disks (6 mm in diameter) were prepared by punching out fungal pre-cultures in Petri dishes, and then the 30 disks per a flask were inoculated to the culture medium. Total 3 flasks

containing culture medium were agitated by a shaker (NR-150, TAITEC Corp., Koshigaya, Japan) at 100 rpm in the dark for 24 days. The culture experiment was repeated 3 times.

Fermentor culture. The components of the culture medium were same as those of flask-agitating culture, and total 2.5 L culture medium was prepared. The 60 mycelial disks were inoculated to the culture medium. The culture of the fungus was carried out with a fermentor (MBF, EYELA, Tokyo, Japan) under aeration and agitation at 100 rpm in the dark at 25ºC for 24 days. The fermentor culture was repeated 2 times.

Preparation of crude enzyme solution. Each 30 mL of culture solution was collected from each flasks every second day for flask-agitating culture. Each 30 mL of culture solution was sampled 3 times every other day for fermentor culture. The collected culture solution was filtered through Miracloth (Calbiochem, Darmstadt, Germany), and then the filtrate was centrifuged at 1,500 x *g* for 15 min at 4ºC. After the supernatant was moved into dialysis tubes, the tubes were placed in measuring cylinders, and then the space between cylinder and tube was packed with polyethylene glycol (mean molecular weight 20,000 ± 5,000, Wako Pure Chemical Industries, Osaka, Japan), which resulted in dehydration and concentration of culture solution. The obtained solution was moved to another dialysis tube and dialyzed against 20 mM succinate buffer (pH 5.5) overnight. The obtained solution was used as crude enzyme solution for enzyme assays.

Enzyme assay. Protein concentration of the crude enzyme solution was determined by Bradford method [9] with using chicken ovalbumin as a standard.

Avicelase and CMCase activities were determined as the amount of total sugars released from the substrates by the crude enzyme preparation. A reaction mixture (1 mL) contained the crude enzyme solution (0.5 mL) and 0.5% (w/v) Avicel (Funacel II, Funakoshi) or carboxymethyl cellulose (CMC) sodium salt (Wako Pure Chemical Industries) aqueous solution (0.5 mL). The reaction mixture was incubated for 6 h at 37ºC under reciprocal agitation at 45 rpm. After incubation, reaction mixture was boiled for 5 min. The reaction mixture was then centrifuged at 1,500 x *g* for 15 min at 4ºC. The amount of total sugars released in the reaction mixture was determined by phenol-sulfuric acid method [10]. One katal (kat) was defined as the amount of enzyme preparation to release 1 mol of total sugars from Avicel or CMC per second.

β-Glucosidase (BGL) activity was determined as the amount of *p*-nitrophenol (*p*NP) released from *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) (Wako Pure Chemical Industries) by the enzyme preparations. A reaction mixture (3 mL) contained the crude enzyme solution (1 mL), 4 mM *p*NPG, and 50 mM acetate buffer (pH 5.0). The reaction mixture was incubated for 30 min at 50ºC under reciprocal agitation at 45 rpm. After incubation, ice-cold 0.5 M Na_2CO_3 solution (1 mL) was added to the reaction mixture. The amount of *p*NP released in the reaction mixture was determined by the absorbance at 405 nm [11]. One kat was defined as the amount of enzyme preparation to release 1 mol of *p*NP from *p*NPG per second.

Cellobiose dehydrogenase (CDH) activity was determined by monitoring the reducing activity of cytochrome c (bovine heart sample, C-2037, Sigma-Aldrich, St. Louis, MO, USA) by the enzyme preparation. CDH activity was assayed at 30ºC in a 100 mM sodium acetate buffer (pH 4.2) containing 1.5 mM cellobiose and 0.15 mM cytochrome c. The activity was determined as the time-course change in absorbance at 550 nm [12]. One kat was defined as the amount of enzyme preparation to reduce 1 mol of cytochrome c per second.

Protease activity was measured by hydrolyzing Azocoll (Wako Pure Chemical Industries). Azocoll (10 mg) was incubated with 1 mL of the enzyme preparation at 30ºC under reciprocal agitation at 60 rpm for 1 h. After incubation, 700 μL of the colored supernatant was removed, and then the pH was adjusted to 5.0 by adding 300 μL of 500 mM sodium acetate buffer (pH 5.0). The absorbance of the obtained solution was measured at 550 nm [13].

RESULTS AND DISCUSSION

Fig.1 shows the time-course changes in Avicelase specific activity for flask-agitating and fermentor cultures. In flask-agitating culture, each replicate did not show any common and constant trends in the activity (Fig.1A). In fermentor culture, the activity of 2nd replicate increased dramatically after 18th day of culture and showed the maximum (16.8 nkat/mg) on 20th day (Fig.1B). The maximal activity of 2nd replicate was about 4 times higher than that of 1st replicate.

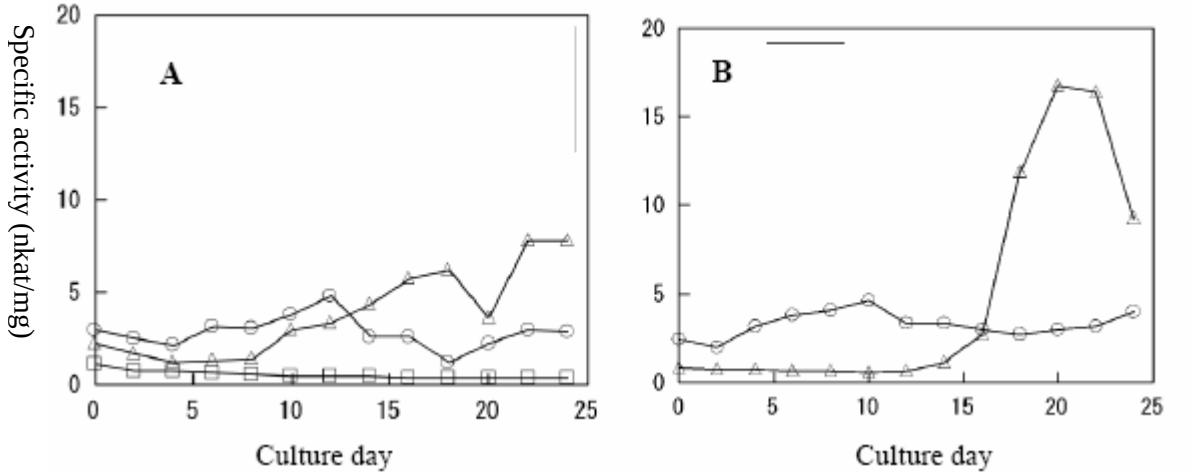

Figure 1: Time-course changes in average Avicelase specific activity
A: Flask-agitation culture; B: Fermentor culture
○: 1st replicate; △: 2nd replicate; □: 3rd replicate

Time-course changes in CMCase specific activity are shown in Fig. 2. In flask-agitating culture, the activity mostly ranged from 0 to 6 nkat/mg, while in the 2nd replicate the activity showed more than 10 nkat/mg and the maximum on the 24th day of culture (Fig. 2A). In the case of fermentor culture, the specific activity in both replicates exhibited lower values (0~4 nkat/mg). However, the pattern of activity change was different between 1st and 2nd replicate, the former showed the maximum on the 4th day, whereas the latter on the 20th day (Fig. 2B).

Fig. 3 shows time-course changes in BGL specific activity. Although the flask-agitating culture did not show any common trends among 3 replicates, some distinct higher peaks of the activity were observed on 8th and 20th day of culture for the 1st replicate, and on 4th, 10th, and 20th day for the 2nd replicate (Fig. 3A). In contrast, the 3rd replicate did not show any distinct peaks but very low activity through the time-course experiment. On the other hand, the fermentor culture gave the highest activities (31.5 and 147.2 nkat/mg) on 12th and 18th day in the 1st and 2nd replicate, respectively (Fig. 3B).

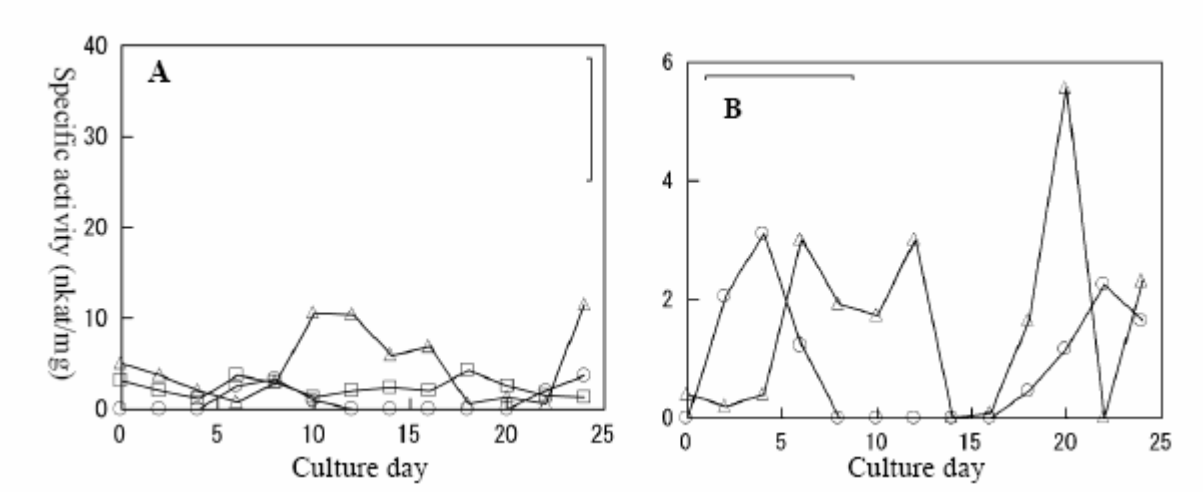

Figure 2: Time-course changes in average CMCase specific activity
A: Flask-agitation culture; B: Fermentor culture
◯: 1st replicate; △: 2nd replicate; □: 3rd replicate

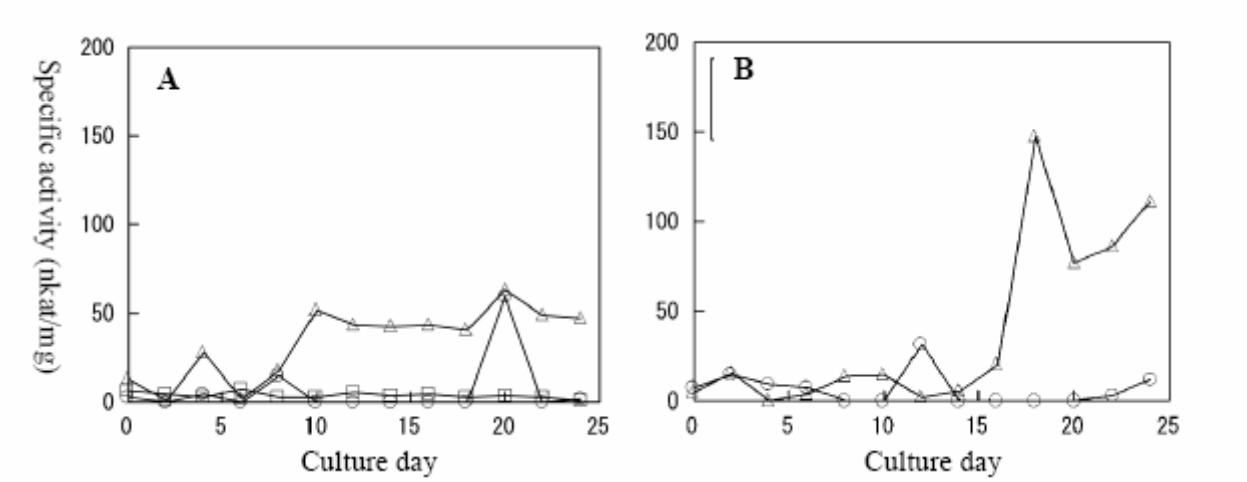

Figure 3: Time-course changes in average BGL specific activity
A: Flask-agitation culture; B: Fermentor culture
◯: 1st replicate; △: 2nd replicate; □: 3rd replicate

Time-course changes in CDH specific activity are shown in Fig. 4. In flask-agitation culture, the activity in the 1st and 2nd replicates increased after 12th day of culture, reached the maximum on 20th day, and decreased thereafter, though all replicates did not any common trends through the time course (Fig. 4A). In the case of fermentor culture, the maximal activity (80.1 nkat/mg) in the 1st replicate was about 20 times higher than that (4.3 nkat/mg) in the 2nd replicate. In addition, the activity in the 1st replicate was much higher than that in the 2nd replicate through the time-course experiment (Fig.4B).

In the present study, BGL and CDH activities exhibited rather higher values compared to Avicelase and CMCase in both flask-agitation and fermentor culture. Habu et al. [11] reported that addition of BCS to the culture medium significantly enhanced CDH activity as well as BGL for a white-rot fungus *Phanerochaete chrysosporium*. Hence, addition of BCS to the culture medium is considered to have also enhanced both activities for *P. pini* in this study.

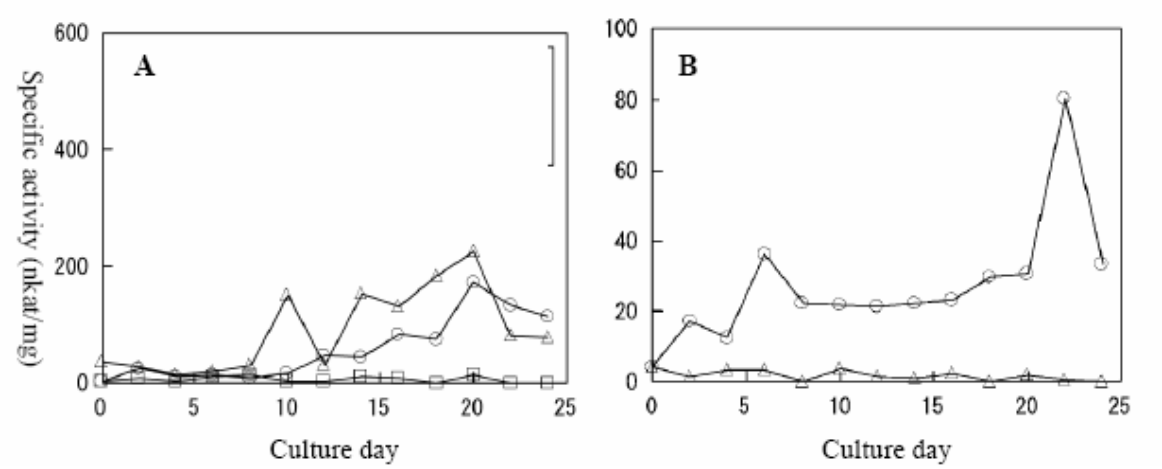

Figure 4: Time-course changes in average CDH specific activity
A: Flask-agitation culture; B: Fermentor culture
○: 1st replicate; △: 2nd replicate; □: 3rd replicate

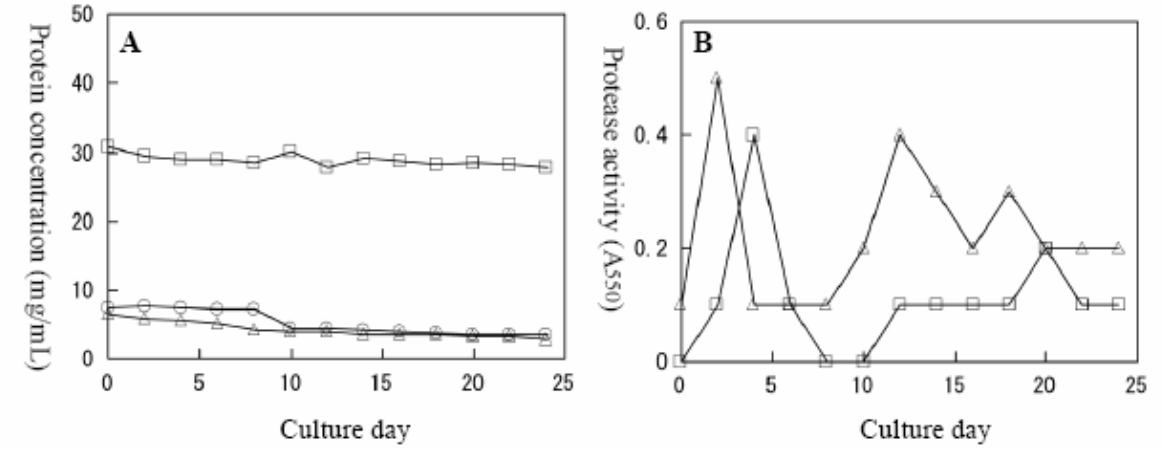

Figure 5: Time-course changes in protein concentration (A) and protease acitivity (B) for falsk-agitation culture
○: 1st replicate; △: 2nd replicate; □: 3rd replicate

Fig. 5A shows time-course changes in protein concentration for flask-agitation culture. In the 1st replicate, protein concentration decreased to almost 3 mg/mL from 8th to 10th day of culture and then decreased gradually. Protein concentration in the 2nd and 3rd replicates decreased gradually. It was suggested that marked decrease in protein concentration for the 1st replicate might be caused by protease from the fungus. Hence, time-course change in protease activity was measured in the 2nd and 3rd replicates (Fig.5B). Dosoretz et al. [14] have measured time-course changes in protease activity and protein concentration in the culture medium of a white-rot fungus *P. chrysosporium* in order to examine the effects of protease on the ligninolytic enzymes from the fungus. They confirmed that protein concentration in the culture medium had decreased dramatically concomitant with the increase in protease activity. On the other hand, significant decrease in protein concentration was not observed for flask-agitation culture in this study, although maximal activities of protease were recognized on 2nd day of 2nd replicate and on 4th day of 3rd

replicate, respectively (Fig.5). Hence, protease seems to less affect decrease in protein concentration for flask-agitation culture.

Protein concentration of 1st replicate also gradually decreased in fermentor culture. Thus, time-course change in protease activity was measured for 2nd replicate in fermentor culture (Fig.6). Protease activity slightly increased after 12th day of culture, while protein concentration decreased drastically after 12th day and showed almost constant lower values after 18th day. Protein concentration was dropped down to 1/24 from 12th to 18th day of culture, whereas protease activity increased about 1.2 times during the same period. These results suggest that protease caused decrease in protein concentration to some extent in fermentor culture of *P. pini*.

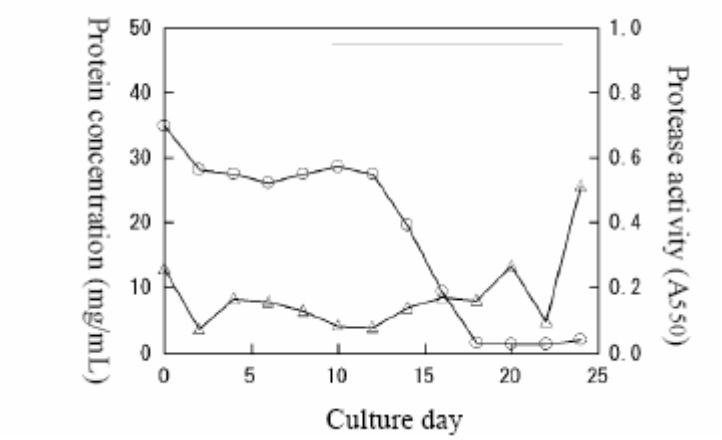

Figure 6: Time-course changes in protein concentration and protease acitivity for fermentor culture.
○ Protein concentration; △ : Protease activity

The maximal specific activities of each cellulase in respective cultures are summarized in Table 1 for both flask-agitation (9 cultures in total) and fermentor culture (6 cultures in total). The maximal specific activities of Avicelase, CMCase, BGL, and CDH for flask-agitation culture were 18.8, 31.8, 154.3, and 571.7 nkat/mg, respectively, indicating that all cellulase activities in flask-agitation culture were higher than those in fermentor culture. Especially, CMCase and CDH in flask-agitation culture exhibited about 6 and 7 times higher values compared to those in fermentor culture.

Table 1: Maximal specific activities (nkat/mg) of each cellulase in respective cultures for flask-agitation and fermentor culture

Cellulase	Flask-agitation	Fermentor
Avicelase	18.8	16.8
CMCase	31.8	5.5
BGL	154.3	147.2
CDH	571.7	80.1

CONCLUSIONS

The conditions for extracellular cellulase production from a white-rot fungus *P. pini* were investigated, and each cellulase (Avicelase, CMCase, BGL, and CDH) activity was compared between flask-agitation and fermentor culture in the present study. As the results, it was indicated that flask-agitation culture has a potential to produce cellulases with higher activities, especially CDH, compared to fermentor culture.

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