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論　文（Original article）

Cellulase production by *Trichoderma reesei* in fed-batch cultivation on soda-anthraquinone pulp of the Japanese cedar

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Abstract

Cellulase production using the soda-anthraquinone (AQ) pulp of Japanese cedar as the carbon source was carried out by *Trichoderma reesei* strain PC-3-7 (ATCC 66589). To avoid the difficulty of stirring the pulp, a fed-batch cultivation system was adopted, characterized due to starting with a low pulp concentration and pulp feeding according to the base supply for pH control. In a laboratory fermenter, the cellulase productivity by fed-batch cultivation on pulp was comparable to that of the batch cultivation on commercial cellulose powder. The fed-batch cultivation system was applied to cellulase production in a 500-L stirred fermenter for large-scale production. The protein concentration, cellulase activity and β-glucosidase activity in the culture medium reached 14.5 mg/mL, 13.6 U/mL and 11.4 U/mL, respectively at 144 h. The hydrolysis experiments performed with soda-AQ pulp as a substrate indicated that the enzyme induced by the pulp hydrolyzed the pulp more efficiently than that induced by the cellulose powder.

Key words :cellulase production, on-site enzyme production, pulp, bioethanol, *Trichoderma reesei*, biorefinery

1. Introduction

Applying lignocellulose hydrolysis to sugars is a critical first step for many potential biomass applications, including the production of bioethanol and other chemicals (Sheehan and Himmel 1999). However, the high enzyme cost limits the industrial use of enzymes for producing soluble sugars, which makes economical enzyme production key to developing enzyme-based biorefinery from lignocellulosic biomass. Since the major cost factor in cellulase production has been estimated as the carbon source (Ryu and Mandels 1980), cellulase production using the lignocellulosic biomass as the carbon source instead of commercial cellulose offers significant scope to reduce the enzyme cost. A series of further costs such as the purchase of the carbon source, storage and transportation can also be omitted using the lignocellulose pretreated on-site. The Forestry and Forest Products Research Institute (FFPRI) developed the bioethanol production process from Japanese cedar (*Cryptomeria japonica*); comprising soda-anthraquinone (AQ) pulping and enzymatic saccharification (Nojiri et al. 2008). A pilot plant had been constructed in Kitaakita city, Akita prefecture to verify this process (Forestry Agency 2013). In this plant, enzyme was planted for on-site production via the submerged cultivation of *T. reesei*; using soda-AQ pulp as the carbon source to reduce the enzyme cost.

Many reports have appeared on cellulase production by the submerged cultivation of *T. reesei* using pretreated lignocellulosic biomass as the carbon source. The utilization of these materials is often accompanied by mechanical milling to avoid the difficulty of stirring the culture medium (Gallo et al. 1978, Kawamori et al. 1986, Doppelbauer et al. 1987, Shin et al. 2000, Liming and Xueliang 2004); however, the necessary pulverization of the pretreated material increases the production cost. To maintain sufficient stirring of the culture medium without further treatment, the initial concentration must be reduced and the material fed gradually according to the consumption by the microorganism. Accordingly, when lignocellulose is used as the carbon source, the process of fed-batch cultivation must be adopted for efficient cellulase production.

The fundamental problem of a fed-batch cultivation process to produce cellulase is how to maintain conditions for optimal enzyme productivity (Bailey and Tähtiharju 2003). For example, overfeeding the pulp would result in stagnation of microorganism growth and enzyme production due to insufficient stirring of the culture medium. Conversely, underfeeding would also decrease the enzyme productivity due to exhaustion of the carbon source. Accordingly, it is important to manage the pulp concentration in the culture medium by appropriate feeding to ensure efficient enzyme production by fed-batch cultivation.

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In the present work, the pulp feeding protocol based on the pH control was verified using 3-l fermenters to establish the cellulose production process by fed-batch cultivation using soda-AQ pulp as the carbon source. The enzyme productivity was compared to the value obtained by batch cultivation using commercial cellulose powder as the carbon source, while the fed-batch cultivation was applied in cellulose production in a 500-L fermenter for large-scale production.

2. Methods

2.1 Fermentation substrate

Soda-AQ pulp and commercial cellulose powder (Solka Floc 40, International Fiber Corporation, USA) were used as fermentation substrates. The pulp was made from Japanese cedar (*Cryptomeria japonica*) chips obtained in Akita prefecture, Japan. The wood chips were cooked under the following conditions in a pilot plant designed by the FFPRI (Forestry agency 2013). The pulping liquor was 3.5-4.1% (w/w) NaOH containing 1,4-dihydro-9,10-dihydroxyanthracene disodium salt, which was equivalent to 0.1% (w/w) on wood, while the liquor-to-wood ratio was 5:1 (w/w). The cooking temperature was maintained at 168-170°C for 3 h. After soda-AQ cooking, the solid pulp was collected and bleached with sodium hypochlorite (about 3% (w/w) on pulp) for 24 h, whereupon the bleaching pulp was fed to a screen and washed. The contents of α-cellulose, hemicellulose (holocellulose – α-cellulose) and lignin were 77, 19 and 2.3% (w/w), respectively.

2.2 Microorganisms

The cellulose production strain, *Trichoderma reesei* PC-3-7 (also designated ATCC66589), was obtained from the American Type Culture Collection, and grown on potato dextrose agar (Difco, Sparks, MD) plates at 28°C for 10 days. The spore plates were stored at 4°C until use.

2.3 Cellulase production in a laboratory jar fermenter

The procedures and media composition used to produce cellulase were essentially from Schell et al. (1990), while the seed culture was typically grown in two stages. In the first-stage culture, a 5 × 5 mm size spore plate was used to inoculate a baffled 300-mL Erlenmeyer flask containing a 100 mL working volume of a medium of Schell et al. (1990), except for 4.2% (w/v) $(NH_4)_2SO_4$ and 2% (w/v) glucose as the sole carbon source. The flasks were incubated in a temperature-controlled orbital shaker operating at an agitation rate of 200 rpm at 28°C. After 48 h, the first-stage culture was transferred at a 5% (v/v) inoculum level to the baffled 500-mL shake flask containing a 200 mL working volume of the nutrient medium containing 1% (w/v) soda-AQ pulp or cellulose powder as the sole carbon source. This second-stage flask was incubated for 36-48 h prior to inoculating the production vessel at 5% (v/v).

Batch cultivation was carried out in a 3-L jar fermenter (MDN-3L, B. E. MARUBISHI, Tokyo, Japan) with a working volume of 1.5 L medium containing 106 g of cellulose powder as the sole carbon source supplemented with 0.6% (v/v) Adekanol LG-109 (ADEKA, Tokyo, Japan) as antifoam. The pH was maintained between 4.6 and 4.8 using automatic base and acid additions of 5.0 N ammonia solution and 4.0 M phosphoric acid solution, respectively. The agitation speed and aeration rate were kept at 275 rpm and 1.5 L/min, respectively. The culture was grown for 6 days at 28°C.

In the case of the fed-batch cultivation, 15 g of pulp was used as the carbon source instead of cellulose powder. During the cultivation, the count number corresponding to the run-time of the base supply pump was monitored, while the time course of the count number of the base supply pump was used as an indicator for feeding new pulp. When the base supply frequency declined, sterile pulp (7.5 g) was fed into the fermenter, while the agitation speed and aeration rate were kept at 275 rpm and 1.5 L/min, respectively. The culture was grown for 6 days at 28°C.

2.4 Cellulase production in a pilot plant scale tank

First-stage culture was followed by laboratory-scale cellulase production. After 48 h, the first-stage culture was transferred at a 5% (v/v) inoculum level to two baffled 3-L shake flasks containing a 1.5 L working volume of a nutrient medium containing 15 g pulp as the sole carbon source. The second-stage flasks were then incubated for 36-48 h prior to inoculating the production tank at 1% (v/v).

A 500-L stirred-tank fermenter with a working volume of 300 L medium containing 2.7 kg pulp as the sole carbon source was used for cellulose production. The pH was maintained between 4.7 and 4.8 using automatic base and acid additions of 4.4 N ammonia solution and 8.0 M phosphoric acid solution, respectively. During the cultivation, the data points for temperature, pH, tank level, and the run-time of base supply pump were recorded every 5 min and the pump data was used as an indicator for feeding new pulp. When the base supply pump did not work for more than 15 min, sterile pulp (1.3-1.4 kg) was fed into the fermenter. The agitation speed and aeration rate were kept at 207 rpm and 300 L/min, respectively. The culture was grown for 6 days at 28°C.

2.5 Analytical methods

Samples were drawn daily from the fermenters, and centrifuged, and the supernatant was analyzed for enzyme activity and protein content. The cellulase activity was measured using the procedure by Ghose (1987) and expressed in terms of international cellulase activity units as the amount of enzyme that forms 1 μmol reducing sugars as glucose per minute during the hydrolysis reaction. β-Glucosidase was assayed by monitoring the release of *p*-nitrophenol from 1 mM *p*-nitrophenyl-β-D-glucoside in 1.0 mL of 50 mM sodium acetate buffer (pH 4.5) at 50°C. The test tubes containing 0.9 mL reaction mixture were incubated with 0.1 mL of diluted enzyme solution at 50°C for 10 min and 1.0 mL of 0.2 M of sodium carbonate was added to stop the reaction. The post-hydrolysis concentration of *p*-nitrophenol was determined by the absorbance of 400 nm, while the unit of activity was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per minute. The protein concentration in the culture medium was assayed by the Bradford protein assay using γ-globulin as the standard (Bio-Rad, Hercules, CA).

2.6 Adjusting enzyme activity and protein content to account for the change in culture volume

In cases involving significant changes in culture volume, reported performance parameters (such as volumetric productivity and yield) will be subject to major errors if no corrections are made to account for the changed working volume (Hayward et al. 2000). Therefore, measured enzyme activities and protein contents were corrected for volume change by multiplying by the culture volume ratio at the time of the sample (V_t) to the initial culture volume, according to

$$E_c = (V_t / V_0)\, E_t$$

And where E_c is the corrected enzyme activity or protein content (U/mL or g/L); E_t is the enzyme activity or protein content at time t (U/mL or g/L); V_t is the volume at time t (mL or L); and V_0 is the initial volume (mL or L). In cellulase production using a 500-L fermenter, the tank level monitored during the cultivation was used to correct the enzyme activity and protein content. In a laboratory fermenter, V_t was calculated by adding the total volumes of base and acid added for pH control and pulp feeding (density assumed at 1.0 g/mL) and subtracting the total volumes withdrawn during sampling and the evaporation of the water (the rate of which was assumed to be constant during the cultivation period) at time t.

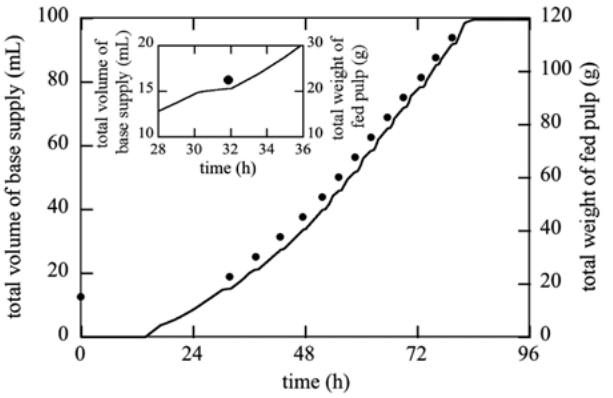

Fig. 1. Time course of base supply and pulp feeding during the fed-batch cultivation in a 3-L laboratory fermenter. A 3-L fermenter (working volume: 1.5 L) was inoculated with 75 mL of a 2-day flask culture of the fungus and cultivated at 28°C. The pH was maintained between 4.6 and 4.8 during the whole culture. The agitation speed and aeration rate were kept at 275 rpm and 1.5 L/min, respectively. The culture was grown for 6 days at 28°C. When the frequency of the base supply (—) declined, sterile pulp was fed into the fermenter (●).

2.7 Estimation of enzymatic hydrolysis

The hydrolysis experiments were performed with 5% (w/w) soda-AQ pulp as a substrate and different enzyme dosage at 50°C, pH 4.8 for 72 h. Culture supernatant of 6 days cultivation medium using pulp or cellulose powder as the carbon source was used as an enzyme. Reducing sugars were determined by the dinitrosalicylic acid method (Ghose 1987) using D-glucose as the standard. The enzymatic hydrolysis yield was calculated as: yield (%) = reducing sugar (g) × 0.9 × 100/polysaccharides in substrate (g).

3. Results and Discussion

3.1 Fed-batch cultivation in a laboratory fermenter

During the cellulose production cultivation of *T. reesei*, the pH of the medium is maintained using automatic base and acid additions to the optimal enzyme production pH. In *T. reesei*, a strong tendency for the pH of their culture medium to decrease during growth on carbohydrate substrates is apparent. After exhaustion of the carbon source, a marked increase in pH is observed. Therefore the decrease in the base addition rate can be used as an indicator of imminent exhaustion of the carbon source in the medium, while pulp feeding based on the base addition rate is expected to maintain the pulp concentration within a constant range.

The pulp feeding protocol based on the pH control was verified using 3-L fermenters. Fig. 1 shows the time course of the base and pulp supplies relative to the cultivation. The

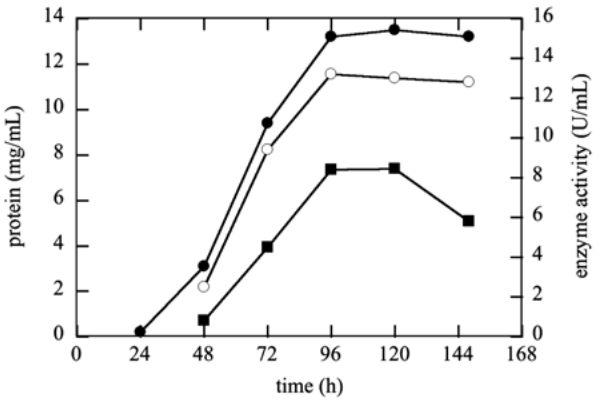

Fig. 2. The time course of enzyme production of the fed-batch cultivation using soda-AQ pulp as a carbon source in a laboratory fermenter. The culture was done for 6 days at 28°C using 7.5% pulp. Protein concentration (●), cellulase activity, (○) and β-glucosidase activity (■) on 6 days is shown, respectively.

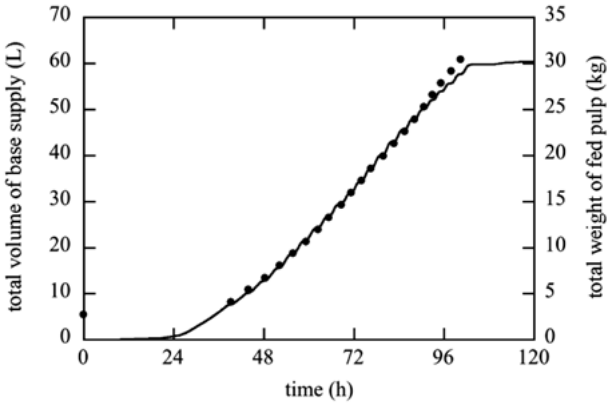

Fig. 3. Time course of base supply and pulp feeding during the fed-batch cultivation in a 500-L fermenter. A 500-L stirred-tank fermenter (working volume: 300 L) was inoculated with 3 L of a 2-day flask culture of the fungus and cultivated at 28°C. The pH was maintained between 4.7 and 4.8 during the whole culture. The agitation speed and aeration rate were kept at 207 rpm and 300 L/min, respectively. The culture was grown for 6 days at 28°C.When the base supply (—) did not work for more than 15 min, sterile pulp was fed into the fermenter (●).

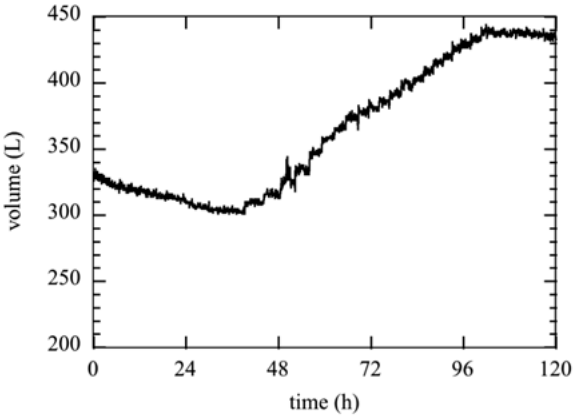

Fig. 4. Time course of the culture volume of fed-batch cultivation using soda-AQ pulp in a 500-L fermenter.

cultivation was started with 1.0% (w/v) pulp concentration to maintain sufficient agitation of the culture medium. The supply of the base began at 14 h to maintain the pH of the culture medium and the supply rate gradually decreased at 30 h (Fig. 1 inside), suggesting that fungus had almost consumed the pulp in the medium. At 32 h, the first pulp was fed into the fermenter, which caused an increase in the base supply (Fig. 1 inside). New pulp was then fed into the fermenter whenever the supply rate of the base decreased. The last pulp was fed at 80 h (Fig. 1) and the total pulp reached 7.5% (w/v) of the initial culture medium.

The time course of enzyme production by the fed-batch cultivation is shown in Fig. 2. The enzyme production continued for 2 days corresponding to the feeding phase (32-80 h), while the protein concentration, cellulase and β-glucosidase activity in the culture medium reached 13.2 mg/mL, 13.2 U/mL and 8.4 U/mL, respectively at 96 h. The calculated volumetric productivity of the cellulase activity by the fed-batch cultivation on the pulp was compared to that by the batch cultivation on cellulose powder (Table 1). The volumetric productivity in the feeding phase (48-72 h) closely resembled the maximum value observed during batch cultivation on cellulose powder (Table 1). It is also interesting to note how regular the pulp feeding intervals were (Fig. 1), suggesting that the pulp consumption by microorganism was stably maintained during the feeding phase. These results suggested that the pulp feeding protocol based on the pH control maintained an optimal enzyme production condition by microorganism, resulting in high cellulase productivity comparable to batch cultivation.

Table 1. Volumetric productivities of cellulase activity in batch and fed-batch cultivation using cellulose powder and soda-AQ pulp.

carbon source	substrate conc. (g/L)	cultivation mode	volumetric productivity (U/[L·h])			
			0 - 48 h	48 - 72 h	72 - 96 h	0 - 96 h
cellulose powder	70.8	batch	72	280	210	150
soda-AQ pulp	75.0	fed bacth	52	290	160	140

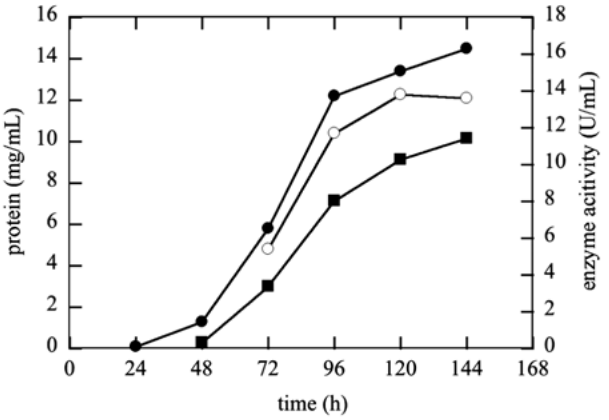

Fig. 5. The time course of enzyme production of the fed-batch cultivation using soda-AQ pulp as a carbon source in a 500-L fermenter. The culture was done for 6 days at 28°C using 10.1% pulp. Protein concentration (●), cellulase activity, (○) and β-glucosidase activity (■) on 6 days is shown, respectively.

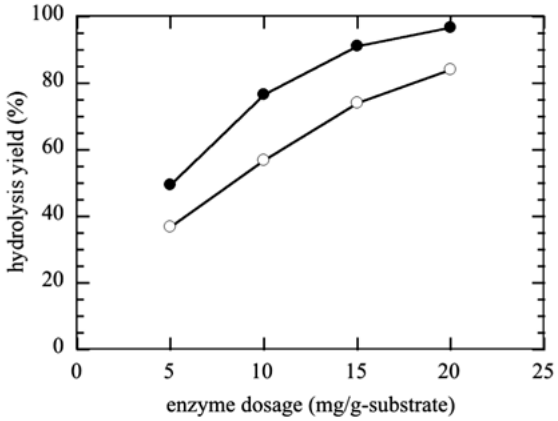

Fig. 6. The results of soda-AQ pulp hydrolysis by different enzyme dosages. Soda-AQ pulp (5% w/w) was hydrolyzed at 50°C, pH 4.8 for 72 h by the culture supernatant using pulp (●), or cellulose powder (○) as the carbon source.

3.2 Cellulase production in a 500-L fermenter

A pulp feeding protocol based on pH control was employed for the cellulose production in a 500-L pilot plant scale fermenter. Fig. 3 shows the time course of the base and pulp supplies relative to the cultivation. The cultivation was started with 0.9% (w/v) pulp concentration and pulp was fed into the fermenter whenever the supply rate of the base decreased. At 101 h, the last pulp was fed and the total pulp fed to the cultivation reached 10.1% (w/v) of the initial culture medium. Fig. 4 shows the time course of the culture volume of fed-batch cultivation. A significant change in culture volume occurred during the aerobic cultivation run. The reduction in volume was presumably attributable to the water in the culture media evaporating, while the increase in volume, which occurred from 36 to 102 h, resulted from the base supplied for pH control and pulp feeding. The high water content of the pulp (about 77% w/w) in particular boosted the increase in culture volume. However the pulp feeding intervals were pretty regular (Fig. 3), suggesting that the pulp consumption by microorganism was stably maintained; regardless of the change in culture volume. The time course of enzyme production is shown in Fig. 5. The enzyme production continued until the pulp feeding was terminated, and the protein concentration, cellulase activity and β-glucosidase activity in the culture medium reached 14.5 mg/mL, 13.6 U/mL and 11.4 U/mL, respectively at 144 h. These results were comparable to those achieved through fed-batch cultivation in a laboratory fermenter and showed the potential for *T. reesei* to produce cellulase on a large scale using soda-AQ pulp as the carbon source.

T. reesei PC-3-7 was examined for cellulase production on cellulosic biomass and achieved 24 mg/ml of protein production in a 5-L fermenter cultivation with 4% alkali-treated bagasse as the carbon source for 7 days (Kawamori et al. 1986). Although they kept the agitation speed at 450 rpm during the cultivation, we had to keep the agitation speed at 207 rpm, the upper limit of the 500-L tank agitation ability. In the study of fed-batch cultivation in a 3-L fermenter with agitation speed at 275 rpm, we found that dissolved oxygen density in culture medium dropped to 0% just after feeding of the pulp (data not shown). Investigation of cultivation condition is showing that increasing aeration improves the cellulase production (Hayward et al. 2000). Therefore if the agitation speed of the 500-L tank could be higher, cellulase productivity would be improved.

3.3 Hydrolysis of soda-AQ pulp by on-site cellulase

The supernatant of the cultivation medium using soda-AQ pulp or cellulose powder as the carbon source was used to hydrolyze the soda-AQ pulp (Fig. 6). When the enzyme dosages per gram substrate were changed from 5 to 20 mg, the enzymatic hydrolysis yields rose from 49.5 to 96.7% for the broth using pulp and 36.9 to 84.0% for the broth using cellulose powder as the carbon source. These results indicated that the enzyme induced by the pulp hydrolyzed the pulp more efficiently than that induced by the cellulose powder, as previously reported (Nojiri et al 2008). The reducing sugars converted from the pulp by on-site cellulase could be used to produce bioethanol and other valuable materials. This research may be meaningful for economical enzyme production, which is key to developing enzyme-based biorefinery from lignocellulosic biomass. Furthermore, the method described in this work could be applied to produce secondary metabolites by other fungi (e.g. *Aspergillus*, *Penicillium*) with similar pH behavior.

Acknowledgements

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ソーダ・アントラキノン蒸解スギパルプを用いた半回分培養法によるセルラーゼ生産

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要 旨

林野庁委託事業により北秋田市に建設した木質バイオエタノール製造実証施設において製造したソーダ・アントラキノン蒸解スギパルプを炭素源とした酵素生産技術を確立するため、セルラーゼ生産菌 *Trichoderma reesei* による酵素生産培養法を検討した。3-L 容ジャーファーメンターを用いた実験では、培養液の十分な撹拌を維持するため、培養開始時のパルプ濃度を 1% とし、セルラーゼ生産菌によるパルプの消費に伴いパルプを随時投入する半回分培養法を採用した。培養液の pH 制御に用いるアンモニア水の添加量をパルプ投入の指標とすることにより、市販のセルロース粉末を炭素源とした回分培養と同程度の酵素生産性を達成した。さらにこの半回分培養法を木質バイオエタノール製造実証施設に設置された 500-L 容タンクを用いたオンサイト酵素生産運転に適用することにより実験室レベルと同様の結果が得られることを実証した。本研究はパルプを炭素源とした酵素生産培養の大規模化につながる成果であり、リグノセルロース系バイオマスを原料とするバイオリファイナリーの実用化に向けて大変有用であると考えられる。

キーワード：セルラーゼ生産、オンサイト酵素生産、パルプ、バイオエタノール、トリコデルマリーセイ、バイオリファイナリー

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