Biotechnol. Bioprocess Eng. **1998**, 3, 44-47

Optimization of Cellulase Production in Batch Fermentation by *Trichoderma reesei*

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Maximum cellulase production was sought by comparing the activities of the cellulases produced by different *Trichoderma reesei* strains and *Aspergillus niger*. *Trichoderma reesei* Rut-C30 showed higher cellulase activity than other *Trichoderma reesei* strains and *Aspergillus niger* that was isolated from soil. By optimizing the cultivation conditions during shake flask culture, higher cellulase production could be achieved. The FP (filter paper) activity of 3.7 U/ml and CMCase (Carboxymethylcellulase) activity of 60 U/ml were obtained from shake flask culture. When it was grown in 2.5 L fermentor, where pH and DO levels are controlled, the enzyme activities were 133.35 U/ml (CMCase) and 11.67 U/ml (FP), respectively. Ammonium sulfate precipitation method was used to recover enzymes from fermentation broth. The dried cellulase powder showed 3074.9 U/g of CMCase activity and 166.7 U/g of FP activity with 83.5% CMCase recovery.

Key words: *Trichoderma reesei*, Cellulase, CMCase, FP activity, β-glucosidase

INTRODUCTION

Celluase have many industrial applications. The largest application is the hydrolysis of cellulose for the production of glucose, which can be used for fuel, food and chemical production. Other applications of cellulase can be found in many other fields, such as food, feed, textile, detergent, and pulp industries. Recently, one of the most important usage of cellulase is the application in enzymatic deinking process of waste paper in pulp and paper industry [1-3]. There has been many studies concerning cellulase production with *Trichoderma* species, especially with *Trichoderma reesei*. In this study three *Trichoderma reesei* strains and one *Aspergillus niger* were cultured for cellulase production. The medium optimization for the selected strain was carried out in flask culture. The effect of medium pH change on cellulase production was considered and medium composition that can alleviate this influence was suggested. When it was grown in pH-controlled fermentor, much higher cellulase production could be achieved.

MATERIALS AND METHODS

Microorganisms

Trichoderma reesei QM9414 and *Trichoderma reesei* Rut-C30 were purchased from KCTC (Korean Collection for Type Cultures). *Trichoderma reesei* QM-m is a UV-mutant of *Trichoderma reesei* QM9414, and *Aspergillus niger* 6 was isolated from soil. All the strains were stored on potato dextrose agar slants at 4°C.

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Medium Composition and Cultivation

Medium compositions were based on Mandels' medium [4]. Several modifications were made in medium composition to study the effect of medium composition on cellulase production (Table 1). Fungal cultivations were performed using 250 ml Erlenmeyer flasks with 50 ml of medium and grown in a shaking incubator (KMC-8480SF, Vision Scientific Co.) at 200 rpm at 28°C for 4 to 5 days.

Fermentor Operation

The production of cellulase by *Trichoderma reesei* Rut C-30 was carried out in a 2.5 L fermentor (KF-2.5L, Korea Fermentor Co.) with an operating volume of 1.4 liter. Temperature was controlled at 28°C and pH was maintained at 4.0 by adding 2 N NH_4OH. The agitation speed (200-500 rpm) and aeration rate (1-2 L/min) were controlled to maintain sufficient dissolved oxygen level. Antifoaming agent (Antifoam 204, Sigma Chemical Co.) was added whenever it was necessary.

Enzyme Assays

CMCase and filter paper (FP) activities were measured as recommended by IUPAC [5]. β-glucosidase activity was determined by estimating the reducing sugar liberated from 1% of salicin (2-[hydroxymethyl]phenyl β-D-glucopyranoside, Sigma Chemical Co.) suspended in 0.1 M acetate buffer (pH 4.8) after 10 min of reaction at 50°C. The concentration of reducing sugar was determined by DNS (Dinitrosalicylic acid) method by using glucose as a standard sugar. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per minute under above condition.

Table 1. Cellulase production medium

Compounds (g/l)	medium I	medium II	medium III	medium IV	medium V
Wheat bran	10	10	10	10	10
Avicel	10	10	10	20	30
$(NH_4)_2SO_4$	1.5	1.5	1.5	2.5	6.0
Peptone	1.5	3.0	3.0	3.0	2.5
Yeast extract	0.2	0.5	0.5	0.5	0.5
KH_2PO_4	4.0	4.0	4.0	4.0	3.0
K_2HPO_4	-	3.0	-	3.0	-
$MgSO_4 \cdot 7H_2O$	0.3	0.3	0.3	0.3	0.3
$CaCl_2 2H_2O$	0.3	0.3	0.3	0.3	0.3
Tween-80	1.0	1.0	1.0	1.0	1.0

Enzyme Extraction

The fermentation broth was centrifuged at 4000 rpm for 10 min to remove solid materials including fungal mycelium. The clear supernatant was treated with various concentrations of ethanol and ammonium sulfate, respectively. The wet crude enzyme precipitate was collected by filtration and was vacuum dried at 40°C for 24 hours. Crude cellulase was prepared as homogeneous fine powder by grinding the dried powder.

RESULTS AND DISCUSSION

Strain Selection and Production of Cellulase in Shake Flask Culture

Mandels' medium [4] is a widely used medium for cellulase production. Mandels' medium with 1% Solka Floc was first used for strain selection and cellulase production study. As the concentrations of carbon and nitrogen sources in Mandels' medium are insufficient for high yield of enzyme synthesis, the cellulase production was relatively low. It has been reported that high concentration of nutrient is necessary for high cellulase productivity [6, 7].

Wheat bran is a cheap agricultural by-product. It is a rich nutrient and it can stimulate fungal growth. Avicel is one of the best carbon sources for cellulase production. It was reported that combination of Avicel with wheat bran can enhance CMCase production [8]. When Mandels' medium (trace element solution was omitted) with 2% Avicel plus 1% wheat bran was used (Table 2), CMCase productivity was improved much. However, it was observed that the final pH decreased to 3.0. Dorval [7] reported that the drop of medium pH below 3.0 caused inactivation of cellulase and affected fungal growth, too. As low pH is a limiting factor for the production of CMCase under this condition, it is necessary to maintain pH higher than 3.0.

There are several ways to prevent pH decrease too much. First, by increasing the concentration of KH_2PO_4 in the medium, buffer capacity of medium could be enhanced. In this study the concentration of KH_2PO_4 in the medium was increased from 0.2% to 0.4%, while the concentration of Avicel was decreased from 2% to 1% (medium I, Table 1). The results were shown in Table 2. The final medium pH was maintained higher and CMCase activity increased. However, pH was still near 3.0. As medium pH was not maintained at a value higher than pH 3.0, it was found that increasing the concentration of KH_2PO_4 only in the medium has a limit in maintaining medium pH.

The KH_2PO_4 and K_2HPO_4 buffer system has a larger capacity than KH_2PO_4 buffer system. In medium II both KH_2PO_4 and K_2HPO_4 were included in buffer solution while the concentration of nitrogen was slightly increased: 1% wheat bran and 1% Avicel were used as carbon sources and 0.4% KH_2PO_4 and 0.3% K_2HPO_4 were used (Table 1). In this experiment the final pH was maintained higher and CMCase activity increased a lot (Table 2). The CMCase activity was measured on various medium compositions for different microorganisms. As shown in Table 2, *Trichoderma reesei* Rut C-30 was found to be the best in CMCase production. As a result, *Trichoderma reesei* Rut C-30 was selected for further study.

It is known that the higher C/N ratio of medium composition results in pH decrease and lower C/N ratio of medium composition results in pH increase. Consequently, increasing nitrogen concentration is another way to prevent pH decreasing during cell cultivation. The influence of various peptone concentrations on CMCase production was investigated. Medium III, which does not contain peptone, was used as a control and the result was shown in Table 3. It shows that the pH increased with peptone concentration. However, 0.3% peptone in medium III showed the highest CMCase activity.

Besides pH, cellulase production was also strongly affected by culture temperature and agitation speed. It was found that cellulase production at 28°C (CMCase 36.0 U/ml) was higher than 30°C (19.0 U/ml) and at agitation speed 150 rpm (CMCase 56.2 U/ml) was higher than 200 rpm (38.2 U/ml). This result was consistent with Lejeune's [9] and Merivuori's [10] reports.

Under optimal culture condition for the production of cellulase (28°C, 150 rpm), the effect of medium II, III,

Table 3. Effect of the concentration of peptone on cellulase production

Concentration of peptone (%)	0.1	0.2	0.3	0.4
CMCase activity (U/ml)	18.3	31.8	36.8	36.0
pH	2.6	2.7	3	3

Trichoderma reesei Rut C-30 grown at 30°C at 200 rpm

Table 2. CMCase production by different strains in various media compositions

	Mandels' medium (1% Solka Floc)		Mandels' medium (1% wheat bran+2% Avicel)		Medium I		Medium II	
Strains	CMCase	pH	CMCase	pH	CMCase	pH	CMCase	pH
[1]QM9414	1.84	3.64	9.18	2.60	19.14	2.91	32.0	6.48
[2]QM-m	1.11	2.70	9.13	2.69	11.04	2.72	27.9	5.17
[3]Rut C-30	0.30	7.22	8.05	2.69	22.35	3.01	37.7	5.63
[4]Asp 6	0.75	3.32	4.41	3.90	4.61	2.88	1.17	2.56

[1,2,3]: *Trichoderma reesei*, [4]: *Aspergillus niger*

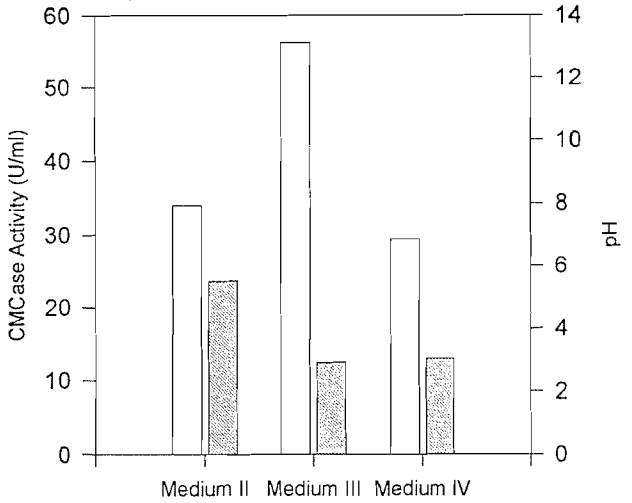

Fig. 1. CMCase production by *Trichoderma reesei* Rut C-30 on various media. (□) CMCase, (▨) pH.

and IV were compared again (Fig. 1). Under optimal cellulase production condition for temperature and agitation speed, medium III was the best in cellulase production: 60 U/ml of CMCase and 3.7 U/ml of FP activities were obtained.

Cellulase Production in 2.5 L Fermentor

In the fermentor equipped with pH controller, buffer solution is not necessary. 2 N NH_4OH was used to control the pH above 3.0 and also used as a nitrogen source. Much greater cellulase production was achieved in the fermentor when higher cellulose concentration was used. In the fermentor experiment, Avicel concentration was increased to 3%, KH_2PO_4 concentration was decreased to 0.3% (medium V in Table 1). The result was shown in Fig. 2. After two days cellulase was synthesized rapidly. The concentration of product cellulase was much higher than that obtained from shake flask culture. After 130.5 hours of cultivation, CMCase activity was 133.35 U/ml and FP activity was 11.67 U/ml. However, β-glucosidase activity was very low. The lower activity of β-glucosidase compared to other enzyme components has been reported as a common feature of *Trichoderma* strains [11, 12].

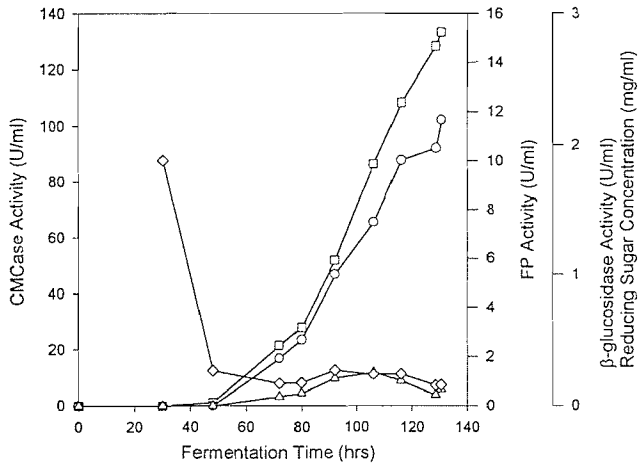

Fig. 2. Cellulase production by *Trichoderma reesei* Rut C-30 in 2.5 L fermentor. (□) CMCase, (○) FP, (△) β-glucosidase, (◇) Reducing sugar.

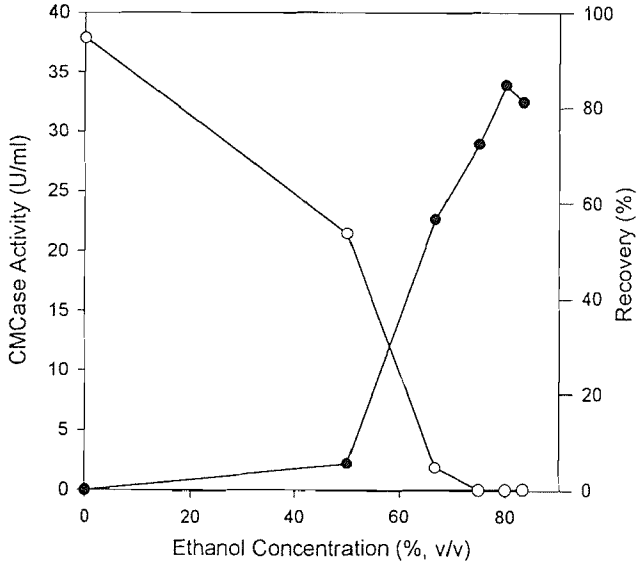

Fig. 3. Ethanol precipitation curve. (○) Supernatant CMCase, (●) Recovery.

Cellulase Recovery

Generally an organic solvent or a salt is used to extract enzymes from enzyme containing solution. In this study ethanol and ammonium sulfate were applied respectively to precipitate cellulases. Ethanol precipitation curve was shown in Fig. 3. The maximum recovery of CMCase was 84.8% at ethanol concentration of 80% (v/v). $(NH_4)_2SO_4$ precipitation curve was shown in Fig. 4. The maximum recovery of CMCase was 99.8% at 60% (w/v) ammonium sulfate. As a result, $(NH_4)_2SO_4$ precipitation method was used to extract cellulase from fermentation broth. After centrifugation, 875 ml of clear broth (CMCase activity 133.35 U/ml, FP activity 11.67 U/ml, β-glucosidase activity

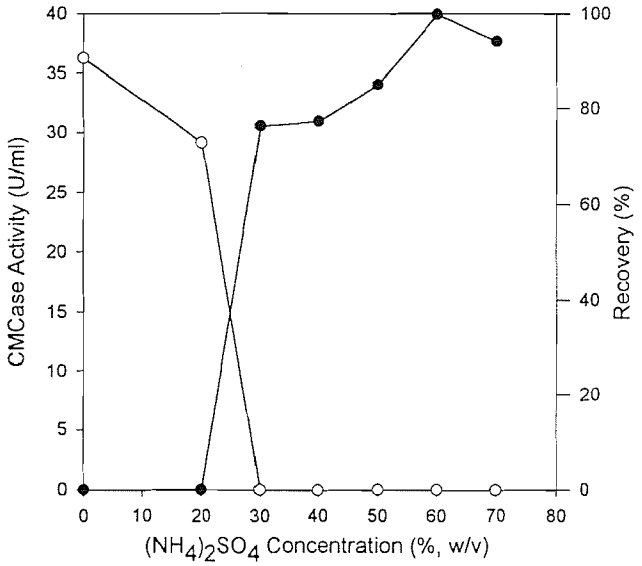

Fig. 4. Ammonium sulfate precipitation curve. (○) Supernatant CMCase, (●) Recovery.

0.34 U/ml) was collected from 1400 mL of fermentation broth. Cellulase powder (31.7 g) was obtained by $(NH_4)_2SO_4$ precipitation followed by vacuum drying. The activities of prepared powder was CMCase activity of 3074.9 U/g and FP activity of 166.7 U/g. The recovery of CMCase and FP activities were 83.5% and 51.9% respectively. The cellulase powder has light yellow color and it was soluble in water.

Acknowledgment This Research work was financially supported by the Project Developments for the Clean Production Technology of the Korea Ministry of Trade and Commerce.

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