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Studies on Cellulase Production by *Trichoderma reesei* (QM 9414)

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Trichoderma reesei QM 9414를 이용한 섬유소 분해 효소 생산조건에 관한 연구

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ABSTRACT

In order to increase the productivity of cellulolytic enzymes, medium composition and culture conditions were studied. When cellulose powder (Avicel) supplemented with rice straw was used as carbon source, productivity of β-glucosidase was increased by about 3 times compared with the runs with only cellulose powder as a carbon source. In this case no negative effects on the production of CMC enzyme activity and filter paper activity was found. For the production of cellulolytic enzymes using *T. reesei* QM 9414, casitone was found to be a good nitrogen source compared with other sources studied, such as peptone, yeast extract, tryptone, and casein. The highest cellulase activity was attained when 0.3% glucose and 0.01% Tween 80 were supplemented to the standard medium of Reese. An adequate oxygen transfer rate was also found to be important to the cellulase fermentation and about 50 mmole of oxygen/liter/hour supported good cellulase biosynthesis during cellulase fermentation.

INTRODUCTION

Efficient utilization of cellulosic materials as a renewable resource is drawing a great deal of attention in the scientific community. Reese, *et al.* (1950) proposed the C_1—C_x cellulase component concept.

Thereafter many researchers have suspected that coenzyme is a limiting factor in enzymatic hydrolysis of cellulose, and this stimulated many studies on characterization and role of C_1 enzyme in hydrolysis of cellulose using *Trichoderma reesei* that is known to be most active for hydrolysis of natural cellulose (Siu, 1953; Wood and McCroe, 1972; Halliwell and Griffin, 1973; Berghem, 1973, 1975; Wood, 1968; Mandels, 1975). However, it was found that substrate pretreatments could lower the requirement of C_1 enzyme for enzymatic hydrolysis of cellulose. Also found was that

cellobiose, which is produced mainly by the action of β-1, 4 glucan cellobiohydrolase (C_1 enzyme) on cellulose, inhibited β-1, 4 glucan cellobiohydrolase competitively. In addition, when the cellulase enzyme from *T. reesei* is used, more than half of the sugar produced by enzymatic hydrolysis of cellulose is cellobiose, and the higher the concentration of sugar produced, the slower the hydrolytic reaction rate (Sternberg, 1976).

All of these findings imply the importance of inhibition effects of β-glucosidase, which hydrolyses cellobiose to glucose and of β-1, 4 glucan cellobiohydrolase which hydrolyses cellulose to cellobiose. Mandels(1975) and Sternberg (1976) showed that during cellulase production using *T. reesei*, filter paper hydrolytic activity and CMC hydrolytic activity increased when the pH was controlled at 3 and β-glucosidase activity was almost completely lost when pH fell below 3. Sternberg tried to overcome this problem using buffered medium and control pH above 3.5. In this case β-glucosidase activity was increased but both CMC hydrolytic activity and filter paper hydrolytic activity were decreased (sternberg, 1976). Katz detected mostly glucose but hardly detected cellobiose in the enzymatic hydrolysate of cellulose when *T. reesei* enzyme was supplemented with *Aspergillus luchuensis* enzyme which was known to have high β-glucosidase activity (Katz and Reese, 1968). It was reported that β-glucosidase was inhibited by glucose (Gong *et al.*, 1977; Berghem and Patterson, 1974). In our study, medium compositions and culture conditions were studied to increase the productivity of cellulolytic enzyme using *T. reesei* QM9414. Particularly, improvement of β-glucosidase productivity without causing negative effects on the productivity of CMC hydrolytic activity and filter paper hydrolytic activity was achieved, when cellulose powder was supplemented with rice straw as a carbon source.

MATERIALS AND METHODS

T. reesei QM9414 supplied by Dr. M. Mandels, U.S. Army Natick Research and Development Command, was used. After growth at 29°C for seven days, cultures were stored at 4°C (Menezes *et al.*, 1973). A loopful of spores was transferred from the stock culture to a fresh agar slant medium, then the agar slant was incubated at 28°C~30°C for about seven days until good sporulation occurred. The spores from the tube were then suspended in 5ml of cellulose medium (Mandels and Weber, 1969) in 500ml Erlenmyer flask. This culture was incubated for about five days in rotary shaker (New Brunswick Scientific) under the standard conditions (27°C to 30°C and 300 rpm with 50mm throw), and this culture was used as inoculum. The fermentation in shake-culture started with transfer of 5ml inoculum to 100ml cellulose medium. Compositions of basal medium are as follows: KH_2PO_4, 0.2%; $(NH_4)_2SO_4$, 0.14%; Urea, 0.03%; $MgSO_4 \cdot 7H_2O$, 0.03%; $CaCl_2$, 0.03%; Cellulose, 1.0%; Proteose peptone, 0.1%; $FeSO_4 \cdot 7H_2O$, 5.0 ppm; $MnSO_4 \cdot H_2O$, 1.56 ppm; $ZnSO_4 \cdot 7H_2O$, 1.4 ppm; and $CoCl_2$, 2.0 ppm in distilled water (Mandels and Weber, 1969). After harvest, culture broth was

centrifuged at 300 rpm for 20 minutes, then the supernatant was assayed for the soluble protein by the method of Lowry, *et al.* (1975), and for the enzyme activities by using the same method reported by Mandels *et al.* Hydrolytic activities of carboxymethylcellulose (CMC) activity and filter paper (FP)activity were measured by Somogyi-Nelson Method (1952). Cellobiase activity was measured using glucostat enzyme(Kwon, 1977). All enzyme activities are expressed in International Unit per unit volume (ml) of culture broth.

RESULTS AND DISCUSSION

The effect of nitrogen source

Casitone was found very good as a protein source. This result is shown in Table 1. During fermentation of cellulose using *T. reesei* proteolytic enzymes are known to be produced in the culture broth, and extracellular proteolytic enzymes may cause the deactivation of celluloytic enzymes in the culture broth (Selby and Maitland, 1967).

Table 1. The effect of nitrogen source on the production of cellulase.

Protein	Source (%)	Final pH	FP Activity (IU/ml)	CMC Activity (IU/ml)	Cellobiase (IU/ml)
Peptone	0.1	4.03	0.88	0.255	0.138
Yeast extract	0.1	6.52	0.58	0.214	0.128
Tryptone	0.1	6.14	0.59	0.245	0.134
Casitone	0.1	6.40	2.05	0.208	0.134
Casein	0.1	6.66	1.70	0.379	0.125

*Conditions: $(NH_4)_2SO_4$ 2.0g/l, Carbon soruce 1% $CaCl_2$ 0.1g/l, KH_2PO_4 0.14g/l, $MgSO_4$ $7H_2O$ 0.1g/l, with tap water to 1 liter

*Carbon source: Mixtures of rice straw (0.7%) and Avicel (0.3%) 8 day-old culture

The effect of glucose

It was reported that soon after the addition of glucose during fermentation of cellulose using *T. reesei*, the pH of culture broth rapidly fell to as low as pH 2.2 (Sternberg, 1976). 0.3% of glucose added before inoculation was found to be optimal (Table 2).

Table 2. The effect of glucose on the cellulase production.

Time added (hour)	Glucose (%)	Final pH	CMC Activity (IU/ml)	FP Activity (IU/ml)	Soluble Protein (mg/ml)
0	0.0	7.16	0.296	0.98	0.945
0	0.1	7.39	0.290	0.74	0.998
0	0.3	6.96	0.340	1.13	1.200
54	0.2	7.30	0.200	0.57	0.945
54	0.3	7.27	0.128	1.13	0.998
54	0.5	7.10	0.276	0.69	0.998
140	0.2	7.23	0.250	0.74	0.966
140	0.5	6.90	0.276	0.78	1.082

*Conditions: 13 day-old culture 1% rice straw

The effect of surfactant Tween 80

Reese and Maguire found that Tween 80 doubled the cellulase yield in *Trichoderma reesei*, thus 0.2% Tween 80 is routinely added to the growth medium (Reese and Maguire, 1976). In the case of using rice straw as carbon source, maximum productivity of FP activity and soluble protein was obtained at 0.3 % of Tween 80 (Table 3).

Table 3. The effect of Tween 80 on the cellulase production.

Time added (hour)	Tween 80 conc. (%)	Final pH	FP Activity (IU/ml)	CMC Activity (IU/ml)	Soluble Protein (mg/ml)
0	0	7.13	0.18	0.116	0.777
0	0.1	7.03	0.98	0.170	0.924
0	0.2	6.96	1.09	0.220	1.060
0	0.3	6.93	1.58	0.290	1.113
96	0.1	7.12	0.80	0.220	0.840
96	0.2	7.43	0.64	0.190	0.861
96	0.3	7.53	0.51	0.180	0.714
150	0.1	6.98	0.67	0.256	0.914
150	0.2	6.86	0.51	0.300	0.998
150	0.3	6.85	0.43	0.386	0.945

*Conditions: 10 day-old culture 1% rice straw

The effect of rice straw/Avicel ratio

When carbon source was supplied in the form of mixture of rice straw and Avicel at various ratios, the final pH value of culture broth increased with the increase in rice straw to Avicel ratio (RS/Avicel ratio). The maximum productivities of FP activity and soluble protein was obtained when RS/Avicel ratio was 1(Table 4). This medium was designated as RS/Avicel medium.

Table 4 shows that the final pH of culture broth was 6.42 when RS/Avicel ratio was 1.

Table 4. The effect of rice straw/Avicel ratio on the cellulase production.

Rice straw (g/100ml)	Avicel (g/100ml)	Soluble protein (mg/ml)	FP Activity (IU/ml)	CMC Activity (IU/ml)	Final pH
0.0	1.0	0.80	0.55	0.296	2.90
0.2	0.8	0.80	0.98	0.275	3.20
0.5	0.5	1.00	1.50	0.375	6.42
0.8	0.2	0.84	0.80	0.385	6.80
1.0	0.0	0.92	0.85	0.400	6.71

*6 day-old culture

The effect of pH on cellulase activity during fermentation

Table 5 shows that pH and activity changes during fermentation on RS/Avicel medium. The pH value decreased during the first 48 hours, but increased thereafter and final pH of the culture broth was 6.4. Minimum pH value was

Table 5. Changes in pH and cellulase activity during fermentation on RS/Avicel medium (RS/Avicel ratio was 1.0).

Culture Time (hour)	pH	Cellobiase (IU/ml)	CMC Activity (IU/ml)	FP Activity (IU/ml)
0	5.6	—	—	—
48	3.5	0.081	0.41	1.50
95	5.8	0.124	0.42	1.58
125	6.4	0.136	0.31	0.91

observed at 48th hour after incubation, but its value was far above 3. On the other hand, pH decreased more rapidly when Avicel was used as carbon source, and there after its value maintained below 3 throughout the fermentation process. Final pH in this case was 3.0 (Table 6). Productivity of cellobiase was about three times higher in RS/Avicel medium than that in the medium containing Avicel as the only carbon source (Avicel medium). The productivity of FP activity was better in RS/Avicel medium.

Sternberg (1976) reported that productivities of FP activity and CMC activity was slow in buffered medium where pH was controlled above 3.5 and below 4.5 with 0.011 M citrate buffer, and that productivity of β-glucosidase increased in buffered medium by about six times compared with that of unbuffered midium. However, productivity of FP activity and CMC activity decreased in buffered medium compared with that in unbuffered medium. Based on these results rice straw as carbon source prevents the pH drop below 3.5 during fermentation and the increase in pH to about 6.5 did not cause the decrease in productivities of FP activity and CMC activity. Therefore it appears that pH within the range of 3.5—6.0 provided satisfactory conditions for cellulase biosynthesis.

Table 6. Changes in pH and cellulase during fermentation on Avicel medium (1% Avicel)

Culture Time (hour)	pH	Cellobiase (IU/ml)	CMC Activity (IU/ml)	FP Activity (IU/ml)
0	5.60	—	—	—
48	2.72	0.032	0.040	0.68
95	2.74	0.048	0.404	1.13
125	2.99	—	0.236	1.15

The cellobiase activity was, however, less than FP activity in these cases (Table 5) studied. In the case of saccharification of 10% BW 200 with culture filtrate of *T. reesei* QM 9414, after incubation at pH 5.0 at 50°C in shake flask produced glucose and cellobiose (Sternberg, 1976). Katz showed that it was necessary to use high concentration of *T. reesei* cellulase with supplemented β-glucosidase from *A. luchuensis* in order to produce highly concentrated glucose solution. By doing so, he was able to produce sugars consisting of

nearly all glucose. From these results cellobiase may be suspected as a limiting factor for the hydrolysis of cellulosic materials using *T. reesei* QM9414 cellulolytic enzymes at high concentration of cellulose.

The effect of $CaCO_3$

It is known that calcium carbonate is an effective buffering and neutralizing agent when acid is produced (Pirt, 1975; Stanier *et al*, 1970). But Table 7 showed that $CaCO_3$ was not effective in preventing pH decrease even at 0.3% concentrations of $CaCO_3$ during fermentation process.

Table 7. The effect of $CaCO_3$ on the final pH and productivity of cellulase.

$CaCO_3$ (%)	Final pH	FP Activity (IU/ml)	CMC Activity (IU/ml)	Cellobiase (IU/ml)
0.01	2.91	0.59	0.207	0.064
0.05	2.90	0.80	0.243	0.046
0.1	3.01	0.61	0.194	0.056
0.2	2.92	1.09	0.255	0.046
0.3	3.17	0.95	0.194	0.052

*100 hour-old culture
*Natick medium = $CaCO_3$

7. The effect of aeration

When about 50 mM O_2/l/hour of oxygen transfer rate is maintained, the highest productivity of FP activity was obtained (Table 8).

Table 8. The effect of aeration on the cellulase productivity

Working Volume (ml)	Soluble Protein (mg/ml)	FP Activity (IU/ml)	CMC Activity (IU/ml)	O.T.R. (mMO_2/l/hour)
30	1.176	1.38	0.536	75
50	1.453	1.90	0.520	50
75	1.575	1.48	0.486	40
100	1.453	0.83	0.476	30

*7 day-old culture

적 요

T. reesi QM9414 균주를 사용하였을 때 glucose는 0.3% Tween 80은 0.3%의 농도에서 cellulase의 productivity가 높았으며 nitrogen source로는 casitone이 좋은 결과를 나타냈다. 볏짚을 탄소원으로 하였을때 culture broth의 final pH는 7부근이었으며 볏짚과 Avicel을 반반씩 섞어 탄소원으로 하였을때 cellulase의 productivity가 높게 나타났는데 특히 cellobiase의 productivity가 현저하게 증가하였으며 이 경우 fermentation 동안에 pH가 3.5 이하로 떨어지지 않고 final pH는 6부근으로 나타났다. 그리고 최적 oxygen transfer rate는 50 mM O_2/l/hour임을 찾아냈다.

REFERENCES

1. Berghem, Lars E. R and L. G. Patterson. 1973. *Eur. J. Biochem.* **37**, 1
2. Berghem, Lar E.R. 1975. *Eur. J. Biochem* **53**, 55
3. Berghem, Lar E. R. 1974. *Eur. J. Biochem.* **46**, 295
4. Gong, C., M.R. Ladicsh and G.T. Taso. 1977. *Biotech. Bioeng* **19**, 959
5. Halliwell, G. and M. Griffin. 1973. *Biochem. J.* **135**, 587
6. Katz, M. T. E. and Resse. 1968. *Appl. Microbiol.* **16**, 419
7. Kwon, T. I. 1977. Purification and properties of β-1,4-glucosidase of *Trichoderma koningii* cellulase. M.S. thesis, The Korea Advanced Institute of Science. p. 9
8. Mandels, M and J. Weber. 169. *Adv. Chem. Ser.* **95**, 393
9. Mandels, M. 1975. *Biotech. Bioeng. Symp.* **5**, 99
10. Mandels, M., R. Andreotti and C. Roche. *Biotech. Bioeng.* **6**, 21
11. Mandels, M. 1975. *Biotech. Bioeng. Symp.* **5**, 81
12. Menezes, H. C. D., T. J. B. Menezes and H. V. Boas, Jr. *Biotech. Bioeng.* **15**, 1123
13. Prit, S. J. 1975. Principle of microbe and cell cultivation. John Wiley & Sons, N.Y. p. 144
14. Reese, E. T., R. G. H. Siu and H. S. Levinson. 1950. *J. Bact.* **59**, 485
15. Reese, E. T. and A. Maguire. 1976 *Biotech. Bioeng. Symp.* **6**, 36
16. Selby, K. and C. C. Maitland 1967. *Biochem. J.* **104**, 716
17. Siu, R. G. H. 1963. in E. T. Reese, ed., "Advanced in Enzymic Hydrolysis of Cellulose and Related Materials", pp. 257, Pergamon Press, London, England
18. Somogi, M. 1952. *J. Biol. Chem.* **195**, 19
19. Stanier, R. Y., M. Doudoroff and E. A. Adelberg. 1970 · The Microbial World, 3rd ed., pp. 83, Englewood Cliffs, New Jersey.
20. Sternberg, D. 1976. *Biotech. Bioeng. Symp.* **6**, 42.
21. Sternberg, D. 1976. *Biotech. Bioeng. Symp.* **6**, 51.
22. Sternberg, D. 1976. *Appl. Environ. Microbiol.* **31**(5), 648.
23. Wood, T.M. 1968. *Biochem. J.* **109**, 217.
24. Wood, T. M. and S. L. McCroe. 1972. *Biochem. J.* **128**, 1183.