

Acceleration of ethanol production from paper mill waste fiber by supplementation with β -glucosidase

Zhao Xin, Qu Yinbo and Gao Peiji

Institute of Microbiology, Shandong University, Jinan, Shandong, China

Ethanol production through simultaneous saccharification and fermentation (SSF) is discussed, in which paper mill waste fiber (WF) from the ammonium sulfite pulp process was used. The process was improved by the supplementation of the cellulase from *Penicillium decumbens* JU-A10 with the β -glucosidase-rich cellulase from *Aspergillus niger* L22. When the ratio of β -glucosidase activity (β GA) to filter paper activity (FPA) of the mixed cellulase was increased to 1.0, and FPA ≥ 2.0 IU ml⁻¹, the maximum ethanol concentration of 5.4% was obtained. Furthermore, the degradation of cellulose and WF was increased by 33.9 and 15.2%, respectively, and the ethanol yield was increased by 42.1% when compared to those in the SSF using *P. decumbens* JU-A10 cellulase only.

Keywords: β -Glucosidase (EC 3.2.1.21); ethanol; *Penicillium decumbens*; *Aspergillus niger*; *Saccharomyces cerevisiae*; paper mill waste fiber

Introduction

Cellulose comprises some 40% of plant tissue, and is expected to be a renewable energy resource. It can be hydrolyzed to produce D-glucose, which is available for use as a feedstock for the production of, for example, ethanol, single cell protein, and antibiotics.¹

In the process of enzymatic hydrolysis and fermentation of cellulosic materials, the maximization of cellulose hydrolyzation to produce glucose is one of the most important steps. But the cellulase from only a single strain of microorganism is usually not satisfactory, especially when its β -glucosidase activity (β GA) is low. A low β GA always causes the accumulation of cellooligosaccharides,² which may cause feedback inhibition to the whole hydrolysis process and lead to low production rates of glucose and ethanol.

In most of the published research, ethanol production from cellulose is based on the use of expensive cellulosic materials, such as pure cellulose powder or ball-milled cellulose as the raw material. This is not economical for the industrial process.

In our laboratory, paper mill waste fiber (WF) and spent liquor from an ammonium-sulfite pulp process

(SASL) were used to carry out studies on cellulase and ethanol production.

The present paper reports the addition of crude β -glucosidase from *Aspergillus niger* L22 to the cellulase from *Penicillium decumbens* JU-A10 and its influence on ethanol production using WF as the substrate.

Materials and methods

Materials

SASL and WF were taken from Taian Paper Mill, Shandong, China.

Organisms

The stock cultures of *P. decumbens* JU-A10, *A. niger* L22, and *S. cerevisiae* M1300 were all maintained on potato-dextrose agar slant. The former two strains were from our laboratory, and the third was from the Shandong General Ethanol Factory.

Culture conditions

Diluted SASL with 3% (w/v) of WF was used as the medium for cellulase production by *P. decumbens* JU-A10 in shake flasks (180 rev min⁻¹) at 28°C for 60 h.

A. niger L22 was cultured on solid medium which was composed of WF and Mandel's inorganic salts³ at 30°C for 3 days. The water extract of the culture material was used as a crude β -glucosidase preparation.

Address reprint requests to Dr. Xin at the Institute of Microbiology, Shandong University, Jinan, Shandong, 250100, China.
Received 12 December 1991; revised 7 June 1992

Table 1 Influence of different ratios of β GA/FPA in cellulase to ethanol production in SSF process

No. ^a	1	2	3	4	5	6
FPA (IU ml ⁻¹)	2.9	2.5	2.2	1.6	1.3	0.5
β GA (IU ml ⁻¹)	0.9	2.0	2.2	3.2	4.1	5.4
β GA/FPA	0.3	0.8	1.0	2.0	3.2	11.8
m_{EtOH} (v/v, %)	3.8	4.7	5.4	4.4	3.9	3.1
A (w/w, %)	69.0	73.3	79.5	67.0	60.0	52.5
B (w/w, %)	66.7	NT ^b	89.3	NT	NT	55.1
C (w/w, %)	47.0	58.5	66.7	55.0	47.8	38.4

^a No. 1 is *P. decumbens* JU-A10 cellulase, No. 6 is *A. niger* L22 cellulase, Nos. 2-5 are JU-A10 + L22 mixed cellulases

^b NT = not tested

FPA = filter paper activity; β GA = β -glucosidase activity; m_{EtOH} = ethanol concentration in culture broth; A = degradation of WF; B = degradation of cellulose; C = ethanol yield

tion. The *P. decumbens* JU-A10-*A. niger* L22 mixed cellulase (JU-A10 + L22) was prepared by mixing the *P. decumbens* JU-A10 cellulase and *A. niger* L22 cellulase in various proportions (Table 1). Non-aerated SSF was used for ethanol production, at 37°C for 96 h, using 20% (w/v) WF as the substrate.

Analytical methods

Both filter paper activity (FPA) and β GA were assayed according to the method of T. K. Ghose.⁴ One unit was defined as the amount of enzyme (milliliters spent broth) required to liberate one micromole of reducing sugar per minute. All assays were carried out at pH 4.8 and 50°C. Ethanol was determined by gas chromatography (Shanghai-103 Type). The content of reducing sugar was determined by the DNS method.⁴ The content of cellulose was determined by the "nitric acid-ethanol" method.⁵ Paper chromatography (isopropanol:acetic acid = 9:1)⁶ was used for the quantitative determination of xylose, glucose, and cellobiose in the SSF culture medium.

Calculations

$$A = [1 - (W_r/W)] \times 100\%$$

$$B = [1 - (W_r \times R_c)/(W \times W_c)] \times 100\%$$

$$C = (m_{\text{EtOH}}/M_{\text{EtOH}}) \times 100\%$$

where

A = degradation of WF (%)

B = degradation of cellulose (%)

C = ethanol yield (%)

W_r = weight of residues from SSF (g)

W = total weight of WF used in SSF (g)

R_c = content of cellulose in the solid residues from SSF (%)

W_c = content of cellulose in WF (%)

m_{EtOH} = concentration of ethanol in SSF broth (%)

M_{EtOH} = theoretical ethanol concentration in SSF (%)

Results and discussion

Using *P. decumbens* JU-A10 only

This strain produced cellulase very quickly when grown on WF and SASL, and it had a high level of FPA and low β GA.⁷ After the culture broth was centrifuged to remove the mycelia and spores, the supernatant was incubated at 35°C for 90 h and samples were taken for the determination of their FPA and β GA. Under these conditions, the FPA and β GA of the cellulase changed slightly as time went on (Figure 1). Thus the *P. decumbens* JU-A10 cellulase was stable under the SSF culture conditions.

The test was also carried out with the culture broth, which still contained mycelia and spores. The cellulase broth was incubated under the same conditions as previously, and now the FPA changed a little but the β GA increased from 0.9 to 2.7 IU ml⁻¹ in the first 50 h (Figure 2). Upon microscopic examination, no integral cells were found in the samples taken after 50 h SSF. This means that the cells were autolytic under these conditions, possibly due to a lack of nutrient and oxygen. It was found that *P. decumbens* JU-A10 excreted very little β -glucosidase into the culture broth (Figure 2). This may be because its β -glucosidase is cell-bound or exists in the periplasmic space. When the cells were autolysed, the β -glucosidase was released to the culture medium and β GA concentration rose significantly in the broth (Figure 2).

For the *P. decumbens* JU-A10 cellulase in the SSF process, the change of β GA is shown in Figure 3. The change of β GA was the same as that shown in Figure 2, but in the first 25 h the FPA fell from 2.0 to 1.0 IU ml⁻¹, rose to its original activity. The likely explanation is that at the beginning of the SSF process, when the cellulose substrate (WF) was added to the cellulose liquid, some cellulase components such as cellobiohydrolase (EC 3.2.1.9) were adsorbed^{8,9} to the cellulose substrate (WF), so the FPA in the suspension was

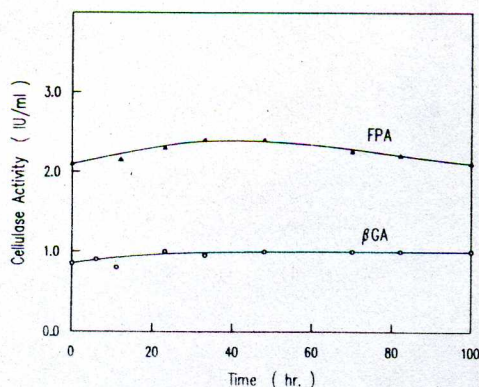


Figure 1 Change in FPA and β GA of *P. decumbens* JU-A10 cellulase in cell-free broth incubated at 35°C

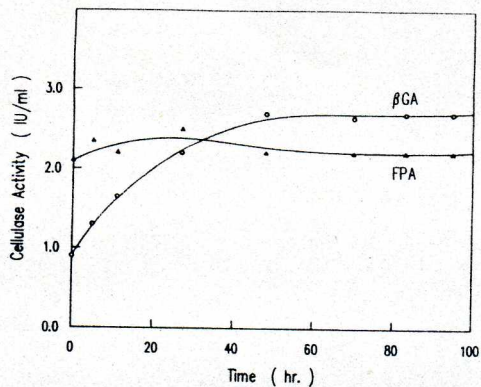


Figure 2 Change in FPA and β GA of *P. decumbens* JU-A10 cellulase in cell-containing broth incubated at 35°C

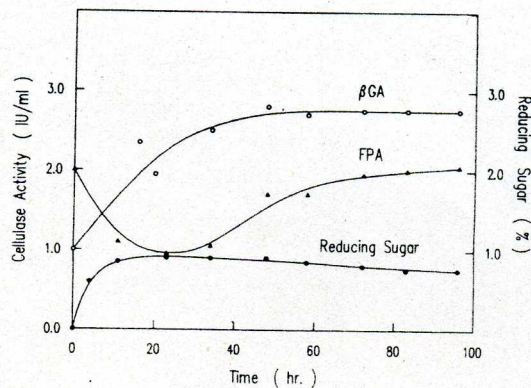


Figure 3 Change in FPA and β GA of *P. decumbens* JU-A10 cellulase, and reducing sugar concentration, in SSF at 35°C

reduced. As the SSF proceeded, the WF was gradually hydrolyzed to sugars and the adsorbed cellulase was released into the suspension, so that the FPA in the suspension rose to its original level. The results also indicate that the β -glucosidase was not adsorbed by the cellulose substrate.

For a rapid SSF process, an initial high level of β GA was required. However, it took too much time (about 50 h) for *P. decumbens* JU-A10 to produce a high level of β GA (about 3.0 IU ml⁻¹).

SSF using mixed cellulase

Mixed cellulase with different ratios of β GA/FPA from *P. decumbens* JU-A10 and *A. niger* L22 were used to carry out SSF. The final ethanol concentrations were measured, as was the content of cellulase in the

raw WF and the solid SSF residues (Table 1). The results showed that when the β GA/FPA ratio of the mixed cellulase was increased to 1.0 and its FPA ≥ 2.0 IU ml⁻¹, the maximum ethanol concentration and degradation of cellulose and WF were obtained. They increased by 42.1, 33.9, and 15.2%, respectively, when compared to the results obtained from the SSF using *P. decumbens* JU-A10 cellulase only.

Figure 4 shows the comparison of ethanol production in SSF processes using *P. decumbens* JU-A10, *A. niger* L22, and JU-A10 + L22 mixed cellulases separately. It is obvious that by using JU-A10 + L22 mixed cellulase, the highest production, concentration, and rate of ethanol were obtained, when compared to those using *P. decumbens* JU-A10 or *A. niger* L22 cellulases separately.

Determination of reducing sugars in the culture medium in the SSF process

The glucose, xylose, and cellobiose concentration remaining in the SSF culture medium were qualitatively determined by using paper chromatography. Figure 5D shows the results from samples taken after 60 h SSF using *P. decumbens* JU-A10 cellulase only. Cellobiose and xylose, but no glucose, remained in the culture medium. Figure 5E and F show the results of samples taken after 48 and 60 h SSF using JU-A10 + L22 mixed cellulase. In the 48 h SSF medium, there xylose and glucose were left. As the SSF went on, all the glucose was consumed, but xylose remained in the 60 h SSF

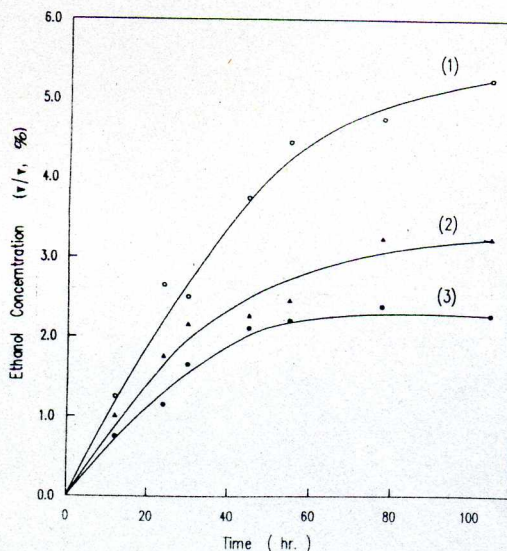


Figure 4 Comparison of ethanol production by *S. cerevisiae* M1300 during SSF using cellulase from JU-A10 + L22 (1), *P. decumbens* JU-A10 (2), and *A. niger* L22 (3)

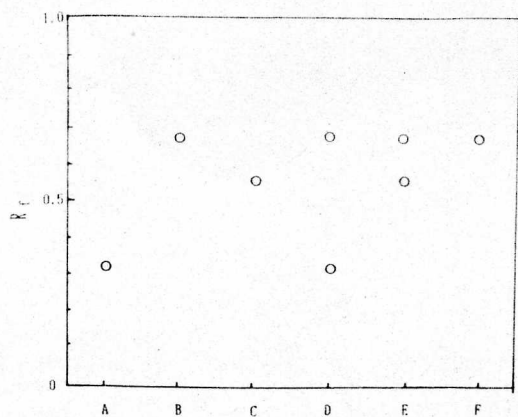


Figure 5 Analysis of reducing sugar in the culture medium in the SSF process using paper chromatography. (A) Pure cellobiose; (B) pure xylose; (C) pure glucose; (D) 60 h SSF using JU-A10 cellulase; (E) 48 h SSF using JU-A10 + L22 mixed cellulase; (F) 60 h SSF using JU-A10 + L22 mixed cellulase

medium. This shows that xylose could not be used, and that almost all the cellobiose and glucose entered the fermentation pathway in the SSF.

All these results indicate that:

1. The use of mixed cellulase of *P. decumben* JU-A10 and *A. niger* L22 in SSF provided enough β -glucosidase to prevent the accumulation of cellobiose which might otherwise cause feedback inhibition of the cellulose hydrolyzation process.

2. Since xylose remained after the SSF, it is expected that more ethanol would be produced through the fermentation of xylose.

3. It has also been shown that for rapid cellulose hydrolysis, it is very important to have an initial high level of β -glucosidase for the full utilization of natural cellulosic materials in a large scale.

References

- 1 Hendy, N. A., Wilke, C. R. and Blanch, H. W. *Enzyme Microb. Technol.* 1984, **6**, 73-74
- 2 Stockton, B. C. in *Biotechnol. Lett.* 1991, **13**(1), 57-62
- 3 Yin-bo, Q., Xin, Z., Pei-ji, G. and Zu-nong, W. *Appl. Biochem. Biotechnol.* 1991, **28/29**, 363-368
- 4 Ghose, T. K. *Pure Appl. Chem.* 1987, **59**, 257-268
- 5 In: *Chemical Analysis of Paper Industry* (Chinese), 1981, 44-52
- 6 In: *Guides of Biochemical Techniques* (Chinese), 1987, 142-14
- 7 Xin, Z., Yin-bo, Q., Pei-ji, G. and Zu-nong, W. *Indust. Microbiol.* 1989, **5**, 15-19
- 8 Rabinovich, M. L. *et al. Biochemistry* 1984, **27**(1-6), 55-59
- 9 Wilke, C. R. *et al. Enzymatic Hydrolysis of Cellulose. Theory and Application*, Noyes Data Corporation, New Jersey, 1983