목재공학15(4) : 18~25(原著)
Wood Sci. & Tech. 15(4) : 18~25. 1987

Microbial Conversion of Woody Waste into Sugars and Feedstuff(Ⅱ)*1

Production of Cellulolytic Enzymes from *Aspergillus fumigatus* and Saccharification of Popla Wood

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微生物에 의한 木質資源의 糖化 및 飼料化에 關한 硏究(Ⅱ)*1

Aspergillus fumigatus KC-1으로부터 섬유소 분해 효소의 생산 및 현사시나무의 효소가수분해

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要 約

섬유성 물질을 자화(資化)하는 미생물을 자연계로 부터 분리하여 효소생산 및 당화조건을 검토하였다. 분리 균주 256주 중 효소생산에 가장 유효하다고 인정되는 *Aspergillus fumigatus* KC-1을 우수 균주로 선발하였다. 본 균은 alkaline peroxide로 전처리한 현사시 목분 1%를 탄소원으로하여 45°C에서 진탕 배양시 4~5일째 효소생성(여지, Avicel, 탈지면, CMC, Salicine 및 Xylan 당화활성)이 최고치에 달했다. 효소의 최적 pH는 4.5, 최적온도는 60°C였다.

본 균의 효소에 의한 현사시 목분의 가수 분해시 1% NaOH와 20% 과초산으로 탈리그닌한 목분의 가수 분해율이 가장 높았고, 최종 산물로 glucose와 약간의 cellobiose 및 xylose가 검출되었다. 따라서 본균의 cellulase는 cellulose를 쉽게 glucose로 당화하는데 매우 유효한 효소로 판단되었다.

현사시 목분의 효소분해에 대한 탈리그닌 정도, 기질의 크기 및 농도의 영향도 아울러 검토하였다.

Summary

The cellulolytic activities of *Aspergillus fumigatus* KC-1 was investigated, which showed the most active producer of cellulase among the 256 strains of cellulose-decomposing microorganisms screened in our laboratory. All the examined cellulolytic activities (filter paper-, Avicel-, cotton-, CMC-, salicin- and xylansaccharifying activity) in a culture of *A. fumigatus* KC-1 grown on 1% popular sawdust pretreated with peroxide alkaline reached a maximum within 4–5 days. The optimum pH and temperature for the enzymatic activity was found to be pH 4.5 and 60°C respectively.

*1. 接受 9月 28日 Received September 28, 1987.
Report Ⅰ in this Journal 14(3) : 23-29, 1986.

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This work was funded by the grant of Korea Science and Engineering Foundation(KOSEF).

The sawdust of poplar wood delignified with 1% NaOH and 20% peracetic acid succesively recorded the highest hydrolysis rate in the tests of enzymatic saccharification. The major end product of hydrolysis of poplar wood with the cellulolytic enzymes obtained from *A. fumigatus* KC-1 was glucose with small amount of cellobiose and xylose. It can be concluded from these results that *A. fumigatus* KC-1 is an advantagous source of a cellulase that is capable of hydrolyzing cellulose to glucose rapidly. The influence of degree of delignification, substrate size and its concentration on the rate of hydrolysis of poplar wood was also discussed.

Key words; cellulase activities, *Aspergillus fumigatus* KC-1, properties of enzyme, enzymatic saccharification, poplar wood.

1. Introduction

The utilization of lignocellulosic materials by means of hydrolytic conversion processes has been a topic of widespread interest. Particularily it has been reported that the high yields in enzymatic hydrolysis can only be obtained if suitable pretreatment methods are applied prior to the enzymatic degradation step.

Various processes have been proposed altering or destroying the lignin and thereby making the wood more digestible.[2, 6] In previous work, we have reported that pretreatment with alkaline perioxide was highly suitable for upgrading the enzymatic hydrolysis of poplar wood.[9]

To gain further insight into the use of pretreated woody waste for the microbial conversion, 256 strains of cellulose-decomposing microorganisms from various natural sources were collected and their cellulolytic activities were investigated. Among the isolates, *Aspergillus fumigatus* KC-1 has showed the most active producer of cellulase.[5] Consequently the cellulolytic activities, properties of this enzyme of *A. fumigatus* will be presented in this communication. In addition, the saccharification of poplar wood by this enzyme will be also reported.

2. Materials and Methods

2.1 Organism and culture medium;

Aspergillus fumigatus KC-1 was employed for the present study. It was isolated form a compost sample collected in Kitami, Hokkaido, Japan by the first author of this communication and maintained on potato-dextrose agar slant.[3] Optimum culture medium for this fungus shown in Table 1 was developed by Chung et al.[5] This microorganism was cultivated at 45°C in a shaken flask.

2.2 Enzyme assays;

Five ml portions of culture broth were taken out at regular intervals, and mycelium and insoluble materials were removed by centrifugation (12,000 x g, 10 min). The supernatant as crude enzyme solution was used for the enzymatic assays. Unless otherwise stated, the supernatant from *A. fumigatus* cultures was assaysed at the pH 5.0 with 0.05 M citrate buffer and at 50°C.

Filter-paper (FP) saccharifying activity:[13]

Reaction mixture consisting of 0.25 ml of appropriately diluted enzyme solution 0.5 ml of buffer and 25 mg of a filter paper (Whatman No. 1, 0.5 x 6 cm) was incubated for 1 hr. The reducing sugar liberated was determin-

ed by 3, 5-dinitrosalicyclic acid (DNS) method.[14]

Avicel saccharifying activity;[19]

Reaction mixture consisting of 0.5 ml of diluted enzyme solution and 0.5 ml of a suspension of Avicel (Asahi Chem. Co. Ltd.) in buffer was incubated and stirred for 1 hr. After centrifuation, reducing sugar in the supernatant was determined.

Cotton saccharifying activity:[12]

Fifty mg of absorbent cotton was added to a mixture of 1.0 ml of diluted enzyme solution and 1.0 ml of buffer and incubated. After a 24-hrs incubation, the reducing sugar liberated was determined.

CMC saccharifying activity:

Reaction mixture consisting of 0.25 ml of diluted enzyme solution and 0.25 ml of a solution of 1% carboxymethyl cellulose (CMC; P.S. 0.6–0.7, DP = 450–500, Wako Pure Chem. Ind. Ltd.) in buffer was incubated for 30 min and the reducing sugar was determined.

β-Glucosidase activity:

Salicin hydrolyzing activity was measured with a 0.5% salicin solution in buffer in the same as for CMC-saccharifying activity.

Xylan saccharifying activity:

The activity of xylanase was determined by the release of xylose from xylan (Nakarai Chem. Ltd.).

All the enzyme activity units were defined as μmol of glucose liberated per min except the cotton- and xylan-saccharifying activity. One unit of cotton saccharifying activity was taken to be mg of glucose liberated per 24 hrs, whereas one unit of xylanase activity μmol of xylose per min. Protein was determined by the method of Lowry et al[11], using bovine serum albumin as the standard.

2.3 Properties of enzyme:

Optimum pH and temperature of this enzyme were examined. For the determination of pH optimum, the activity of enzyme was measured in the standard assay mixture containing 1 ml buffer and 0.5 ml of dilute enzyme solution. The buffer used included glycin-HCl (pH 2.0), citric acid-sodium citrate (pH 3.0–6.5), Na-phosphate (pH 7.0–8.0) and glycine-NaOH (pH 9.0–10.0). The effect of temperature on the enzyme activity was tested at elevated temperature of 20–70°C with the standard assay mixture described above.

2.4 Enzymatic saccharification of poplar wood:

Poplar wood hammer-milled and sieved as substrate was delignified with alkaline peroxide or NaOH + peracetic acid according to the methods described by Kim et al[9] or Toyama and Ogawa[20] respectviely. These sawdusts pretreated were added to the crude enzyme solution (concentration = 0.025%) to a final concentration of 1% (w/v) and hydrolyzed at 60°C. Crude enzyme was obtained from the culture broth as mentioned in 2.2 and used with the addition of merthiolate sodium (0.05%). Hydrolyzates after the saccharification were spotted on a Kieselgel 60 plate (Merck) and developed with the solvent system of n-butanol: isopropanol: water (3:12:4, v/v).[8] Reducing sugar was detected using the anisaldehyde reagent method.[10]

3. Results and Discussion

3.1. Production of cellulolytic enzymes by *A. fumigatus* KC-1

The appearance of the extracellular cellu-

lolytic activities in a culture of *A. fumigatus* KC-1 grown on 1% poplar sawdust pretreated with alkaline peroxide (Tab. 1) is shown in Fig. 1

Table 1. Optimal medium for cellulase production by *A. fumigatus* KC-1

Component	Content (g/l)
$(NH_4)_2SO_4$	2.8
KH_2PO_4	2.0
Urea	0.3
$CaCl_2$	0.3
$MgSO_4 \cdot 7H_2O$	0.3
Trace element solution[a)]	1 ml
Tween 80	1
Proteose peptone	1
Sawdust (25°C/100hr)[b)]	10.0

a) Composition of the mixture(%): $FeSO_4 \cdot 7H_2O$, 0.5; $MnSO_4 \cdot H_2O$, 0.16; $ZnSO_4 \cdot 7H_2O$, 0.14; $CoCl_2$, 0.2.

b) Dilignified with 1% H_2O_2(pH 11.5)

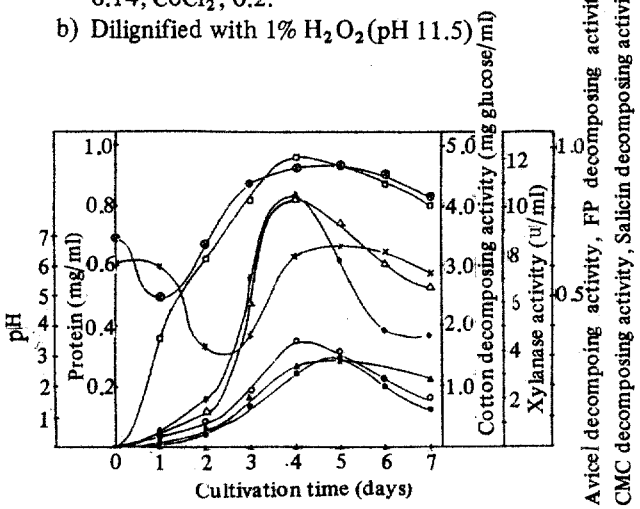

Fig. 1. Time course of growth and cellulase activities production in optimal medium by *A. fumigatus* KC-1.
-×- : pH, -⊗- : Protein,
-△- : Cotton decomposing activity,
-●- : Avicel decomposing activity,
-◆- : CMC decomposing activity,
-▲- : Salicin decomposing activity,
-○- : FP decomposing activity,
-□- : Xylanase activity.

Saccharifying activity for xylan appeared after one day of cultivation and increased rapidly thereafter with a marked growth. The extracellular CM-cellulase approached a maximum within 4 days. Cotton decomposing activity of the culture filtrate developed simultaneously with CM-cellulase activity and amounted to 4.0 mg reducing sugar/ml. It was also found that all the other cellulolytic enzyme activities tested (FP, Avicel and salicin decomposing activities) reached a maximum at 4–5 days of cultivation under optimum condition. The extracellular protein concentration reached 0.9 mg/ml in the stationary phase. The value of pH in the culture broth fell to about 3 in the early stage of cultivation. However, pH began to rise and remained constant (pH 6.0) in the stationary phase. The fact that cellulolytic enzyme activities of *A. fumigatus* KC-1 reached a maximum within 4–5 days, indicates that its cellulolytic enzyme system is very efficient for the practical use and that it might be a good source of enzyme for saccharification of cellulasic materials.

Cellulase and other glycan-decomposing enzymes produced in the optimum medium were summarized in the Table 2. Interestingly the sawdust of poplar wood delignified with NaOH and peracetic acid (socalled Toyama's saw dust in this communication) was not necessarily the most suitable substrate for the cellulase production, although the lignin in this substrate was almost removed by these chemicals (Table 2). In contrast, the sawdust delignified with alkaline peroxide showed the comparable enzyme activites to that of Toyama's substrate, although the removal of lignin in this substrate was about 60%. From these results it can be seen that the total removal of lignin alone does not result in the achievement of high cellulase

production. It is well known that increase in the enzymatic susceptibility of the cellulose is not strictly a function of the extent of delignification.[7, 16, 17]

3.2 Optimal pH and temperature on the enzyme activity

The activity of the saccharifying enzyme was determined at different pHs (Fig. 2). The

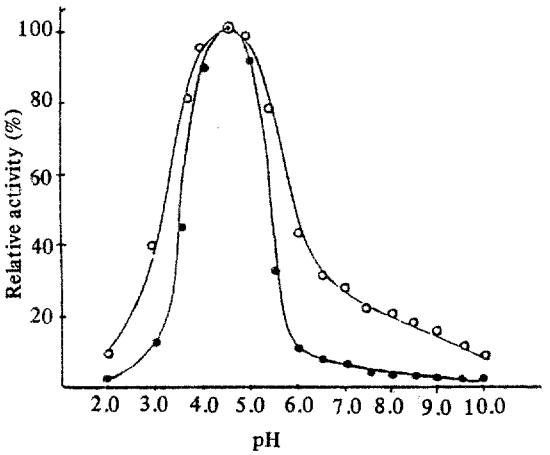

Fig. 2. Effect of pH on cellulase activities from *A. fumigatus* KC-1.
—○—: FP decomposing activity.
—●—: Cotton decomposing activity.

supernatant of the culture broth as a crude enzyem solution was used in this experiement. The optimum pH for the enzyme activity was found to be 4.5. This enzyme was considered to be an acid-durable one. The value of optimum pH in the strain of *A. fumigatus* KC-1 was appeared similar to those found by Bae et al[1] and Chung,[4] who examined the optimum pH in the strains of *Trichoderma* sp. and *Penicillium verruculosum* F-3 respectively. The effect of temperature on the enzyme activity was examined at pH 4.5. The activity increased with temperature up to 60°C, especially over 50°C.

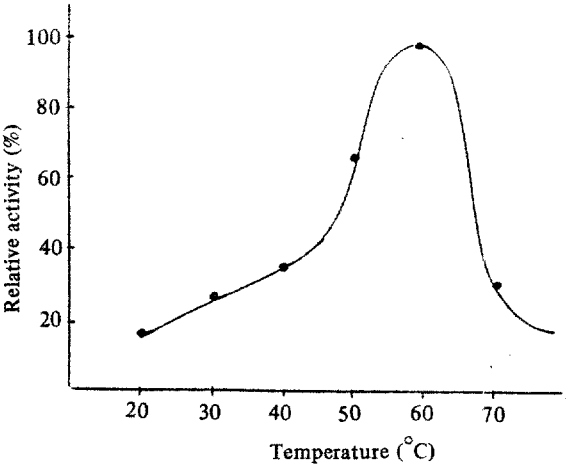

Fig. 3. Effect of temperature on cellulase activity from *A. fumigatus* KC-1.

The maximum activity was seen at 60°C. The optimum temperature of cellulolytic enzyme from our fungus was somewhat higher than those other cellulolytic fungi reported.[20]

3.3 Saccharification of poplar sawdust by crude enzyme

Various kinds of poplar sawdust were added to the enzyme solution from *A. fumigatus* KC-1 to a final concentration of 1% (w/v). Saccharifications have been carried out for at least 24 hrs at temperature 60°C. The pH of the reaction mixture was adjusted to 4.5. The rate of saccharification calculated with the following equation:[18]

$$\text{Conversion rate}(\%) = \frac{\text{reducing sugar produced}}{\text{weight of substrate}} \times \frac{162}{180} \times 100$$

All the sawdust of poplar wood pretreated with alkaline peroxide showed the higher rate of enzymatic decomposition than that of untreated wood (Fig. 4); the poplar wood treated with 1% H_2O_2 (pH 11.5) in 25°C/ 100 hrs showed the two-fold increase of decomposition rate of cellulose. However, the sawdust deligni-

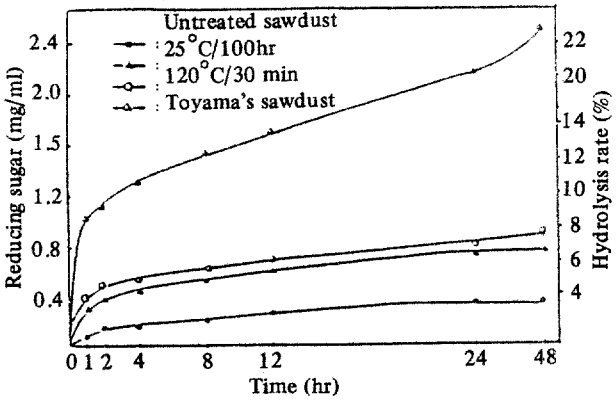

Fig. 4. Saccharification of four different substrates.

. Conversion rate (%) =

$$\frac{\text{Produced reducing sugar}}{\text{Weight of substrate}} \times \frac{162}{180} \times 100$$

fied with 1% NaOH and 20% peracetic acid successively (Toyama and Ogawa, 1972) recorded the highest hydrolyis rate in the present experiments. Under the extended reaction time of hydrolysis (48 hrs.), the rate of saccharification resulted even in the seven-fold increase in comparison with that of control. The depolymerization of cellulose, swelling of substrate and the removal of lignin by these chemicals during delignification may be reponsible for the increased hydrolysis rate.

An attempt was also made to evaluate the influence of substrate size and its concentration

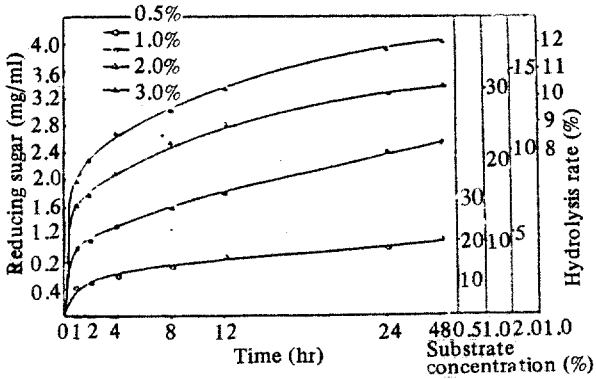

Fig. 5. Effect of concentration of substrate (Toyama's sawdust) on saccharification.

on the rate of hydrolysis of poplar wood. The effects of different substrate concentrations on the rate of hydrolysis and sugar production from poplar wood are shown in Fig. 5. The rate of hydrolysis increased with increasing concentration of substrate up to 1.0%. However, its rate was decreased above 2.0% of substrate concentration, although the absolute amount of reducing sugar liberated was increased with the increase of substrate concentration. Fig. 6

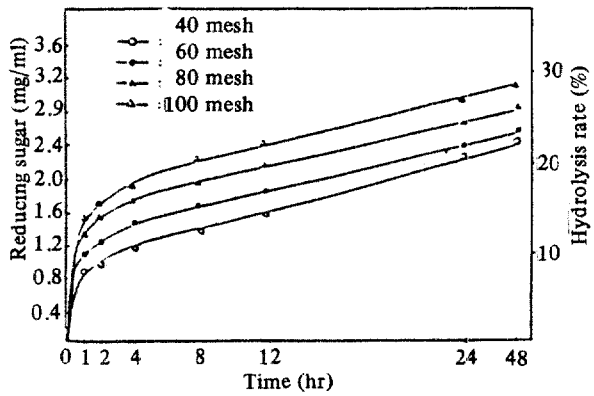

Fig. 6. Effect of substrate(Toyama's sawdust) size on saccharification.

shows that particle size of substrate (wood) has a significant effect on the rate of hydrolysis; the finer the substrate particles, the higher the rate of hydrolysis. This accelerating hydrolysis rate was possibly due to the increase of surface area available with enzyme. It appears likely that the extent of saccharification is also governed by particle size, concentration of substrate besides the degree of depolymerization and delignification of substrate.

The products obtained by hydrolysis of different substrates were analysed by thin layer chromatography (TLC). The major and product of hydrolysis of poplar wood was glucose with small amount of cellobiose. The TLC showed also the trace of xylose (Fig. 7). The formation of glucose as the major end-product due to high

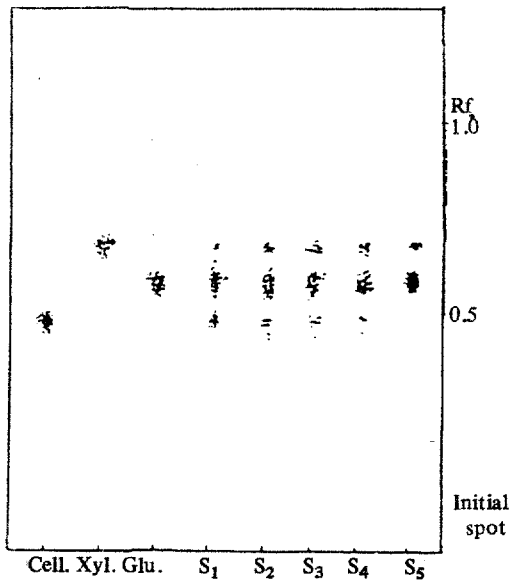

Fig. 7. Thin-layer chromatogram of sugar produced from cellulosic biomass. Chromatoplate, kieselgel G; Solvent system, n-butanol-isopropanol-water (3 : 12 : 4, v/v); Development, ascending technique at room temperature; Detection, anisaldehyde reagent.

S_1: 1hr, S_2: 6hr, S_3: 12hr, S_4: 24hr, S_5: 48hr.

β-glucosidase activity of *A. fumigatus* KC-1 is significant for further processing of the hydrolysate. It can be concluded from these results that *A. fumigatus* KC-1 is an advantagous source of a cellulase that is capable of hydrolyzing cellulose to glucose rapidly. The culture supernatant can be also used directly for cellulose saccharification. Furthermore, this enzyme preparation may be able to degrade other insoluble materials such as hemicellulose because of its high xylanase activity (Table. 2).

Further studies for the nutritive value of this substrate for ruminant animals,[15] for the production of single-cell protein (SCP) from this poplar wood and for the fermentation with the strain of *Candida tropicalis* Y 405 468 are undertaken in our lab.

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Table 2. Effect of various substrates on cellulase activities production by *A. fumigatus* KC-1

Substrate (%)	pH	Protein (mg/ml)	Cellulase activities (U/ml)						Degradation of sawdust[d]
			F.P.	Avicel	Salicin	CMC	Cotton[a]	Xylan	
Untreated sawdust	7.10	0.43	0.11	0.08	0.19	0.11	0.97	7.26	–
25°C/100hr[b]	6.50	0.84	0.32	0.27	0.27	0.82	3.87	9.88	++
120°C/30min[b]	6.85	0.83	0.26	0.23	0.24	0.65	4.32	11.55	+
Toyama's sawdust[c]	6.70	0.92	0.25	0.24	0.29	0.78	3.91	13.22	+++
KC-flock W200	6.10	0.59	0.23	0.21	0.24	0.73	3.38	9.05	+++

a) units; mg glucose produced/ml/24hr
b) Delignified with alkaline peroxide (1% H_2O_2, pH 11.5)
c) Delignified with 1% NaOH/1hr followed with 30% peracetic acid.
d) -: No degradation, +++: complete degradation.

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