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Studies on the Microbial Decomposition of Cellulosic Materials

Part I. Isolation of Cellulase-producing Microorganisms and Characterization of the Enzyme Activities

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纖維素分解의 微生物學的 硏究

第一報. 纖維素分解 微生物의 分離 및 酵素特性硏究

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抄 錄

纖維素를 利用하기 위한 基礎硏究로서 纖維素分解能이 優秀한 菌株를 分離하여 同定한 結果 *Trichoderma spp.*로 추정하였으며 사용된 섬유소 基質은 볏짚을 溶劑로 前處理한 것과 여기에 산, 加熱處理를 더 加한 것을 使用하였으며 이 微生物에 依하여 전처리 및 산 加熱處理에서 생성되는 섬유소 분해 酵素를 시험한 結果 0.1% H_2SO_4, 120°C, 한시간동안 처리한 것을 基質로 하였을 때 酵素活性이 높았다. 또한 pH 5.0, 5日 培養에서 酵素活性이 增加하였으며 요소첨가시 20%, 인산칼륨첨가시 21 %, 육류추출물첨가시 25%, 감귤피첨가시 19%의 增加를 나타냈다. 48時間 酵素反應을 시켰을 때 前處理基質보다 酸, 加熱處理基質이 酵素活性이 높았으며 24時間後에는 活性이 완만하였다.

Introduction

Cellulosic materials are organic substances abundantly produced by plants and accumulated every year on the earth as main components of the plant bodies. The celluloses can be utilized as nutrient sources when they are hydrolyzed by cellulase enzymes to produce sugars. Therefore, the utilization of the natural cellulosic materials as energy sources has been paid much attention by many scientists. A lot of information has been accumulated in the development of new strains of microorganisms pocessing high cellulase activities and in the methods of sugar production

from the cellulosic materials.

A variety of microorganisms have been surveyed for their cellulase activities and studied for the efficiency of sugar production. Many fungal species have been studied for their potent cellulase activities : *Aspergillus niger* by King et al.[1,2,3], *Asp. saitoi* by Masumura and Maejima[4], *Trichoderma viride* by Mandels and Reese[5], Sternberg[6], Chung[7], Nevalainen and Palva[8], and Chang and Usami[9], *Thermonospora curvata* by Stutzenberger[10], *Neurospora* by Kuroda[11], *Myriococcum albomyces* by Chung[12], *Rhizopus* by Cete et al.[13], and *Pellicula filamentosa* by Tanaka and Takegawa[14]. Han and Anderson[15] and Grant et al.[16] studied microbial decomposition of rye grass straw cellulose. The cellulases produced by different species of fungi[17], and the enzymes and acids[18], were compared in the decomposition rate of celluloses. New strains of microorganisms were isolated from natural environments by Bae et al.[19], Sung[20], Ueda et al.[21], Cho et al.[22], and Murao et al.[23] A mutant pocessing higher cellulase activity was induced by Mandels etal.[24], and studied the effects of environmental factors on the enzyme activity.

Research on cellulose decompositions by microorganisms has been focused on cellulase production in order to develope a methods of utilizing celluloses as nutrients for humans and livestocks[25,26], and to solve the economic problems arising in the clothing and canning industries[28]. Agricultural waste materials can be also studied for their utilization by treating them with cellulase-producing microorganisms. Silicic acid and lignin included in the materials have been known to limit the hydrolyzing action of cellulases on the materials[29]. In addition, phenol compounds, tannin, and leucoanthocyanin compounds are also reported to inhibit cellulase activities in nature[29].

Rice is the most important crop in Korea, but the rice straw, a byproduct of rice farming, is not much used for economic purposes. Most straw is left to be naturally decomposed and then filled into the farm soils in most farms. For the economic utilization of rice straw wastes by microorganisms, a microbe hydrolizing the straw celluloses was isolated from environmental materials in this study. The raw rice straw was chemically and thermally treated to remove the enzyme-limiting substances from the straw, and then reacted with the cellulase produced by the organism in order to increase sugar production from the straw substrate. The cellulase activities were characterized under various conditions by using the pre-and/or acid-treated rice straws as substrate. The rate of cellulase production by the organism was also studied by adding nitrogen compounds, phosphates, and natural organic substances into the culture medium.

Materials and Methods

1. Isolation of microorganisms

The potent cellulose-decomposing microorganism used in this study was isolated from 30 samples of various compost heaps and soils collected around *Cheong-ju*. One gram of the sample was suspended in 100 ml of sterile saline solution. The suspension was diluted to 10^{-2} to 10^{-4} and then the dilutions were plated on the isolation plate. After 3 to 7 days incubation at 30°C, the separately developed colonies were isolated and examined for their cellulase activities.

2. Culture media

The medium used for the isolation of cellulose-decomposing microorganisms is consisted of 0.3% $NaNO_3$, 0.3% $(NH_4)_2SO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.01% $FeSO_4$ $7H_2O$, 1.5% agar, and 1% pulp powder as a carbon source in 100 ml of distilled water. The pH of the

medium was adjusted to 6.0.

For storage of the isolted organisms, the medium is composed of 0.5% pulp powder, 0.5% CMC (carboxylmethyl cellulose), 0.2% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, and 0.05% $MgSO_4 \cdot 7H_2O$ in 100 ml of wheat bran extract. The wheat bran extract was made by boiling 100g wheat bran in 1 liter of distilled water for 15 minutes and by filtering the mixture through a double sheet of cotton gauze.

The medium used for selecting the potent rice straw-decomposing organisms is composed of 1% rice straw powder, 0.2% $NaNO_3$, 0.2% $(NH_4)_2SO_4$, 0.2% KH_2PO_4 0.05% $MgSO_4 \cdot 7H_2O$ in 100m*l* of the wheat bran extract. This medium was also used for preparation of the crude cellulase enzyme solution of the organism.

3. Selection and identification of the potent cellulase-producing microorganisms

The organisms isolated from the isolation medium were inoculated into 50ml of the selection medium in 500m*l* shaking flasks and incubated at 30°C for 5 days. The potent cellulose-decomposing organisms were selected by examining the cellulase activity of the spent culture solution by the Somogi-Nelson method[33,34].

The selected organisms were identified in accordance with the guidelines described by Bisby[30], Chang et. al.[31], Nomura et al.[32], Sung[20], and Lee et al[35].

4. Chemical and thermal treatments of the straw substrate

The raw straw materials were preliminarily treated with several kinds of chemicals to remove the cellulase-inhibiting substances from the straw. They were first crushed into about 20 mesh powder and then put into the benzene and acetone mixture (2 : 1) at 65°C for 24 hours. The straw celluloses were washed with distilled water, soaked in 0.8% acetone at 2 to 3°C for 24 hours, washed again, and dried at room temperature. The dried materials were soaked again in 1N NaOH solution at room temperature for another 24 hours, and then washed with distilled water until the washed water became neutral. Parts of the pretreated straw materials were additionally treated with HCl or H_2SO_4 at various concentrations and thermally treated at 120°C for different period of time. Before the acid treated straw materials were used as substrate, they were washed as previously described.

5. Conditions of enzyme production

The cellulase production of the microorganism was experimented under various conditions of cultivation time and pH of the media. Several nitrogen compounds, phosphate salts, and organic nutrients were also examined for their effects on the enzyme production. The crude cellulase enzymes were from the cultures for 6 day periods of cultivation and at various pH values from pH 2.0 through 8.0.

6. Preparation and measurement of the crude cellulase

The *Trichoderma* spp. was cultivated in 500 m*l* of the selection medium, pH 5.0, at 30°C for various periods of time. After centrifugation of the culture at 6,000 rpm for 15 minutes, the supernatant was used as the crude enzyme solution, 500*mg* of the pretreaed and/or acid-treated straw cclluloses were suspended in 4m*l* of 0.05M sodium acetate buffer, pH 5.0, and mixed with 1m*l* of the crude enzyme solution. The mixtures were reacted in a 50°C water bath for 2 hours and then filtered through a Toyo No.2 filter paper. The filtrates were measured for the cellulase activities by examining the amount

of reduced sugars according to the Somogi-Nelson method[33,34].

Results and Discussion

1. Isolation and identification of the potent cellulase-producing microorganisms

Table 1. Cellulase activity of isolated microorganisms

Isolates	Sugar production (mg/ml)
CB-3	1.01
CB-8	0.97
CB-11	1.21
CB-15	0.83
CB-20	1.14
CB-23	1.02
CB-27	0.74
CB-31	0.96

38 strains of microorganisms were isolated from 30 samples. Among them, 8 isolates were selected as the potent cellulase producing microorganism and their cellulase activities are shown in Table 1. The characteristics of the CB-11 were examined by cultivating them on Czapeck Agar medium at 30°C for 6 days. According to the guidelines described by Bisby[30], Chang et al.[31], Nomura et al.[32], Sung[20], and Lee et al.[35], the isolate was primarily identified as *Trichoderma* spp. The typical morphology of the isolate is shown in Fig. 1a and 1b.

2. The effects of chemical and thermal treatments of the substrate on cellulose decomposition

The crude cellulase enzymes were obtained by cultivating the *Trichoderma* in two different selection media, one of which contained the rice straws pretreated with solvents and the other contained the pre-and acid-treated straws. The activities of these enzymes were examined by using the chemically and thermally treated straw powder as substrate. The activities of two crude enzymes on the same straw substrate are shown in Tables 2 and 3, respectively. In both cases, the straw saubstrate treated with 0.1% HCl or 0.1% H_2SO_4 were more vulnerable to the cellulase enzyme than the others. The activities of the cellulase which had been produced in the media containing the pretreated rice straw (Table 2) were not much affected by the substrates prepared by different thermal trea-

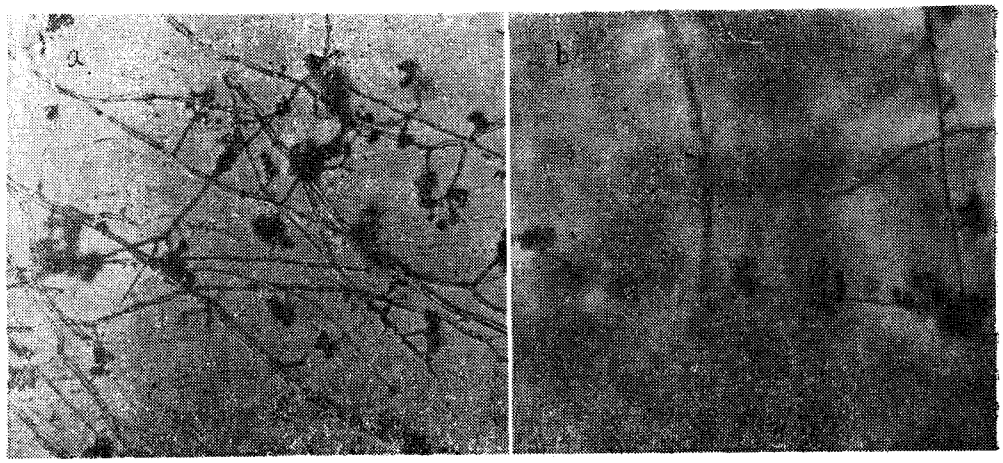

Fig. 1. Photomicrographs of the *Trichoderma* spp. isolated. Typical mycelial morphology (a) of the organism and its sporulation (b) are seen.

Table 2. Activities of cellulasse produced in the medium containing pretreated rice straw

Thermal treatment	Sugar production (mg/ml) from the substate treated with							
	HCl (%)				H_2SO_4(%)			
	0.01	0.05	0.1	0.5	0.01	0.05	0.1	0.5
120°C, 30min	0.43	0.79	1.02	0.44	0.31	0.42	0.6	0.44
120°C, 1hr.	0.46	0.75	1.04	0.44	0.67	0.43	0.75	0.55
127°C, 1hr.	0.54	0.75	1.02	0.44	0.33	0.45	0.75	0.72

Table 3. Activities of cellulase produced in the medium containing pretreated and acid-treated rice straw

Thermal treatment	Sugar production(mg/ml) from the substrate treated with							
	HCl (%)				H_2SO_4 (%)			
	0.01	0.05	0.1	0.5	0.01	0.05	0.1	0.5
120°C, 30min	0.6	1.1	1.2	0.38	0.66	1.38	1.54	0.42
120°C, 1hr	0.84	1.06	1.5	0.7	1.24	1.5	1.85	0.62
127°C, 1hr	1.25	1.27	1.0	0.46	1.42	1.7	1.24	0.46

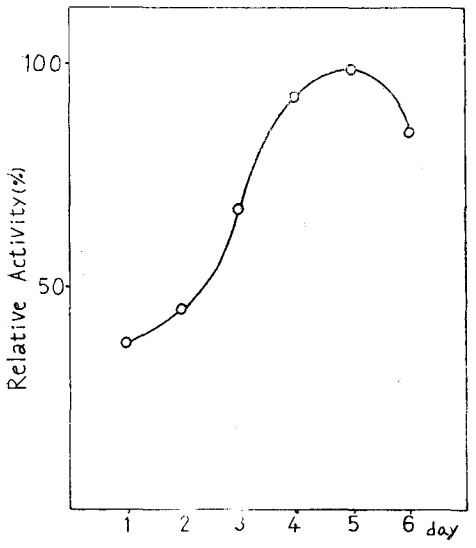

Fig. 2. Time course of cellulase production

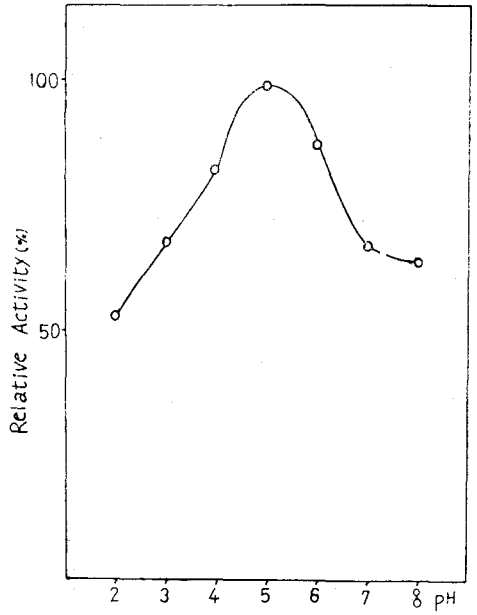

Fig. 3. Effect of pH on cellulase production

ments. On the other hand, the enzyme which had been produced in the media containing the pre-and acid -treated rice straw (Table 3) was more active to the substrate treated with 0.05% acids and at 127°C rather than to the substrate treated with 0.1% acids and at 120°C. In the sugar production from the same straw substrate, the enzyme produced in the media containing pre-and acid-treated straw (Table 3) was more active than the

enzyme provided in the pretreated straw media (Table 2).

Han and anderson[15] and Grant et al.[15] reported on the effects of acid treatment of the substrate for the cellulase activities. They showed that treatment with 1 to 2.5% H_2SO_4 at 121°C for 15 to 30 minutes was most effective for the production of reduced sugars, but these results were obtained by treatment of the substrate with only acid, without pretreatment. In our study, the substrate was pretreated with solvents and then additionally treated with acids and by heat. For the remaining experiments in this study, the rice straws which had been pretreated and additionally treated with 0.1% H_2SO_4 and at 120°C for 1 hour were used as a medium ingredient for production of cellulase and as a substrate for enzme activity test.

3. Environmental conditions for cellulase production

(1) Effects of cultivation time

The activities of the cellulase enzyme were examined, during the time when the *Trichoderma* was cultivated in the enzyme-producing media at 30°C for 6 days. As seen in Fig. 2, the fifth day culture showed the strongest cellulase activity. These results are similar to those of Lee et al.[35], but the enzyme activities of our study were a little more retarded in the early stages of cultivation.

(2). Effects of pH

The pH effects on the cellulase production are shown in Fig. 3. The optimum pH appeared to be 5.0 in this study, but different values of the optimum pH were reported to be 6.0 by Lee et al.[35], 4.2 by Bae et al.[36], and 5.0 by Sung[37] Kim and Choi[38] and Lee et al.[35] reported that the cellulase activities of *Asp. niger* were maximum at pH 5.0 and 5.5, respectively. The pH of *Asp. saitoi*[39] was reported to be 3.0 to 4.0. The optimum pH for cellulase production and its activity appeared to depend upon the species of organisms and culture conditions.

Table 4. Effect of nitrogen compounds on cellulase production

Nitrogen compounds (0.5%)	O.D (580nm)*	Cellulase activity (%)
Control	2.05	100
NH_4Cl	1.61	79
NH_4NO_3	1.47	72
$(NH_4)_2SO_4$	2.25	110
$(NH_2)_2CO$	2.46	120
KNO_3	2.43	119

* Optical density (O.D) measured at 580nm was corrected by the dilution factors.

Table 5. Effect of phosphate salts on cellulase production

Phosphate salts (0.5%)	O.D (580nm)*	Cellulase activity(%)
Control	2.04	100
KH_2PO_4	2.45	120
K_2HPO_4	2.24	110
NaH_2PO_4	2.34	115
$(NH_4)_2HPO_4$	2.34	115

* OD's measured at 580 nm corrected by the dilution factors.

(3) Effects of nitrogen compounds

Several nitrogen compounds were added into the selection media, using normal selection medium as a control. After 5 days cultivation of the *Trichoderma* in each medium, the activities of the cellulase produced were examined and the results are seen in Table 4. The cellulase activities were increased by 20% and 19% by addition of 0.5% $(NH_2)_2CO$ and KNO_3, respedtively, over the control. Guto et al.[40] reported that different sources of nitrogen did not affect cellulase production. Cellulase activities increased by addition of $(NH_4)_2SO_4$ or NH_4NO_3 in the experiments with *Asp. niger*.[31,35]. Masumura and Maejima[4] reported that the addition of 1% $(NH_4)_2SO_4$ increased the enzyme production, however the addition of 3% of the chemical had a proportionately

smaller effect. Chung[12] insisted that additional inorganic nitrogen compounds had no effect on the enzyme production.

(4) Effects of phosphate salts

A few phosphate salts were examined for their effects on the cellulase production, by cultivating the organism for 5 days in the selection media, to which 0.5% of different phosphate salts had been additionally added. As seen in Table 5, about 20% increase of cellulase production was obtained by addition enzyme provided in the pretreated straw media (Table 2).

Han and Anderson[15] and Grant et al.[16] reported on the effects of acid treatment of the substrate for the cellulase activities. They showed that treatment with 1 to 2.5% H_2SO_4 at 121°C for 15 to 30 minutes was most effective for the production of reducing sugars, but these results were obtained by treatment of the substrate with only acid, without pretreatment. In our study, the substrate was pretreated with solvents and then additionally of KH_2PO_4. This result is in agreement with the results of *Trichoderma viride* [9,35] This effect is thought to be a buffering action of the salt in the media.

Table 6. Effect of organic materials on cellulase production

Organic materials (0.1%)	OD (580nm)*	Cellulase activity (%)
Control	1.94	100
Polypepton	2.05	106
Casein	2.01	104
Yeast extract	1.62	84
Nutrient broth	1.74	90
Albumin	2.01	104
Meat extract	2.43	125

* OD is measured at 580 nm corrected by the dilution factors.

Table 7. Effect of natural organics on cellulase production

Natural organics	OD (580nm)*	Cellulase activity (%)
Control	2.03	100
Rice bran	2.23	110
Orange peel	2.42	119
Corn cob	1.72	85
Perilla cake	2.33	115
Defatted soybean	2.15	106

* OD is measured at 580nm corrected by the dilution factors.

(5) Effects of organic materials

The effects of organic materials on cellulase production are seen in Table 6. Addition of the meat extract most increased the enzyme production by 20%, compared to the control. Many kinds of organic compounds have been reported to increase the cellulase production; corn steep liquor in *Asp. niger* by Lee et al.[35], vitamin-free casamino acid in *Trichoderma viride* by Lee et al.[35], thiamin and biotin in *Thermonospora curvata* by Stutzenberger[10], polypeptone and casamino acid in *Myricoccus albomyces* by Chung[12], and fat-free soybean and corn steep liquor *Neurospora* spp.[11].

(6) Effects of natural organic substances

Several organic substances were pulverized and added to the selection media. After 5 day cultivation of the *Trichoderma*, the cellulase activites were examined. The results are seen in Table 7. The orange peel increased cellulase production by 19%, compared to the control. These results are in agreement with those of Sodoyama[41], Lee et al.[35] and Choi[42]. Sodoyama found that the organic acids contained in the orange peel increased enzyme production.

4. Cellulase activities with reaction time

The crude cellulase enzyme produced by the *Trichoderma* was examined by reacting it with the substrates for 2 hours in this study, according to the Somogi-Nelson method. The reaction time was extended to 48 hours in order to understand the characteri-

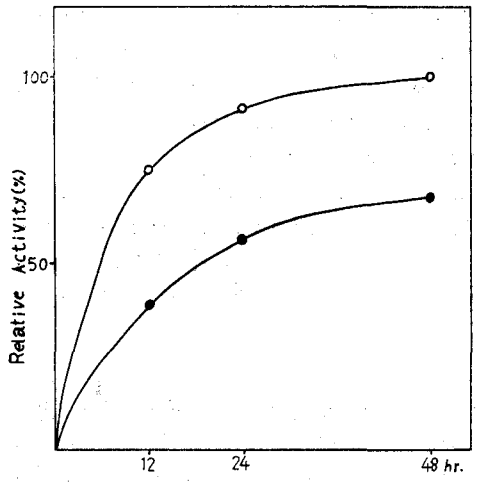

Fig. 4. The cellulase activities on the differently prepared straw substrates during the extended period of reaction time. The reaction mixture consisted of 50*mg* straw powder, 1m*l* of crude enzyme solution, and 4m*l* of 0.05M acetacte buffer (pH5.0) was incubated at 50°C. Pre-and acid-treated straw powder(○), pretreated straw powder(●).

stics of the enzyme activities. Changes in the enzyme activities for 48 hours of reaction time are shown in Fig. 4. The activities of the enzyme on two kinds of straw substrates increased proportionlly with the reaction time for 48 hous, but the activities of the same enzyme varied depending upon the substrate. When the rice straws which had been pre-and acid-treated were used as the substrate, the enzyme activities were much higher than when only pre-treated substrates were re cted. The cellulase activities of *Trichoderma viride* were studied by Mandels and Sternberger[27] and Weber and Mandels[43]. Mandels and Sterberg reported that the enzyme activities on several cellulose compounds increased up to the reaction time of 12 hours and then gradually decreased. Weber and Mandels obtained the maximum activity of the enzyme, when the reaction time was 48 hours. These differences are thought to be attributed to the different substrates.

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Abstract

For the utilization of natural cellulosic materials by microorganisms, a potent cellulase-producing microorganism was isolated and identified as *Trichoderma* spp. Rice straw used as a substrate in this study was preliminarily treated with chemical solvents and/or additionally treated with acids and by heat, and then examined with the cellulase produced by the organism. Better results in sugar production by decomposing the straw cellulose were obtained, when the cellulase was produced by cultivating the organism in the selection medium, pH 5.0, for 5 days, and when the pretreated straw substrate was additionally treated with 0.1% H_2SO_4 sulfuric acid at 120°C for 1 hour. The enzyme production was increased by about 20%, when 0.5% urea 0.5% phosphate, 0.1% meat extract, or 5% orange peel was added into the culture medium. For the practical purposes, the sugar production from the rice straw by the cellulase-producing microorganism can be improved by extending the reaction time of the enzyme up to 24 hr or longer.

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