Isolation of a Cellulolytic Enzyme Producing Microorganism, Culture Conditions and Some Properties of the Enzymes

Takashi YAMANOBE, Yasushi MITSUISHI
and Yoshiyuki TAKASAKI

Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Higashi 1-1-3, Yatabe-machi, Tsukuba-gun, Ibaraki 305, Japan

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A fungus was isolated from soil as a cellulolytic enzyme-producing microorganism. This wild strain, named fungal strain Y-94, secreted a large amount of cellulolytic enzyme components consisting of 0.8 units of avicelase, 9.2 units of carboxymethyl cellulose (CMC)-hydrolyzing enzyme (CMCase) and 2.0 units of β-glucosidase per ml of culture broth on cultivation in Mandels medium for 7 to 10 days at 30°C, and also produced in the culture filtrate various other polysaccharide-hydrolyzing enzymes such as xylanase, β-1,3-glucanase, amylase, *etc.* Higher productivity of the cellulolytic enzymes (avicelase, 6.5 units/ml; CMCase, 82 units/ml; β-glucosidase, 25 units/ml; and filter paper units (F.P.U.),* 5.0 units/ml) was obtained by altering the medium composition. The maximum production of the cellulolytic enzymes was observed when this strain was cultured in a medium containing 4% cellulose powder, 1% peptone, 0.6% KNO_3, 0.2% urea, 1.2% KH_2PO_4, 0.16% KCl, 0.12% $MgSO_4 \cdot 7H_2O$, 0.001% $ZnSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 6H_2O$, 0.001% $CuSO_4 \cdot 7H_2O$ and 0.1% Tween 80 or polyethylene glycol 1000, pH 4.0, under the same culture conditions.

Many microorganisms[1～17] which produce various cellulolytic enzymes have already been isolated by a number of investigators.

Several microorganisms, such ones belonging to the genera *Trichoderma* and *Aspergillus*, have been selected and used in attempts to develop an enzymatic saccharification process for various kinds of cellulosic materials, but these attempts have not yet been successful since the performance and productivity of the cellulolytic enzymes produced by these microorganisms are insufficient for perfect saccharification of cellulose.

The saccharification ability of an enzyme is very important for the production of glucose from cellulosic materials. It is well known that this ability is influenced by the composition of the enzyme components, especially crystalline cellulose-hydrolyzing enzyme (avicelase or FPase)** and β-glucosidase. No single strain capable of producing large amounts of both enzymes at the same time has yet been found. With this background, the present authors tried to find a microorganism that secretes large quantities of a cellulolytic enzyme system sufficient for the aim of the present research, that is, the complete conversion of cellulosic materials to glucose.

* Synthetic cellulase activity generated through the synergistic actions of cellulase components, avicelase, CMCase and β-glucosidase, toward cellulose molecules.

** FPase (F.P.A.) represents the enzymatic activity of the hydrolysis of Whatman No. 1 filter paper ($1 \times 6\,cm^2$) into reducing sugar. This enzymatic activity has been defined in accordance with *J. Ferment. Technol.*, **1976**, 267, and expressed in International Units (F.P.U.). It is generally assumed that avicelase, FPase, F.P.A. and F.P.U. show almost equal enzymatic activity although these technical terms are used in a complicated way now to express the synthetic cellulase activity generated through the synergistic actions of cellulase components.

This paper deals with the isolation of a cellulolytic enzyme producing microorganism and examination of the cultural conditions for the fungal isolate and some properties of its cellulase components.

MATERIALS AND METHODS

Chemicals. Cellulose powder was obtained from Toyo Kagaku Sangyo Co., Ltd. Microcrystalline cellulose (Avicel SF) was purchased from Asahi Kasei Co., Ltd. Salicin, laminarin and carboxymethyl cellulose (CMC) were obtained from Nakarai Chemicals Ltd. Pectin (from citrus fruit), soluble starch, dextran and curdlan were purchased from Wako Pure Chemical Industries, Ltd. Xylan (from oat spelts) was obtained from Sigma Chemical Co., Ltd. Xyloglucan (from tamarind kernels (trade name, Glyloid 3S)) was purchased from Dai-Nihon Pharmaceutical Co., Ltd. Solka Floc BW-200 was obtained from James River Co., Ltd. KC-Flock was purchased from Sanyo Kokusaku Pulp Co., Ltd. Schizophyllan was denoted by Taito Co., Ltd. Other chemicals used were of the purest grade commercially available.

Compositions of media. The composition of the medium used for the enrichment culture was virtually the same as that described by Mandels and Sternberg,[18] except that a filter paper strip (Toyo filter paper No. 50, $1 \times 4\,cm^2$) was used as a carbon source. The same medium was also used for the production of the enzymes except that cellulose powder was used as a carbon source. Malt extract-dextrose or potato-dextrose was used as the medium for isolating the microbial colonies which grew on the agar plates.

Cultivation conditions. Cultivation was carried out as follows. Four milliliters of a liquid medium in a test tube was sterilized, and then one loopful of cells or conidia of the isolated strain was inoculated. After 7 to 10 days cultivation at 30°C on a reciprocal shaker, the culture broth was centrifuged and the enzyme activities in the supernatant were assayed.

Enzyme assay.

Microcrystalline cellulose-hydrolyzing enzyme (avicelase). Avicelase activity was assayed in 1 ml of a reaction mixture containing 0.25 ml of 0.1 M acetate buffer (pH 5.0), 0.5 ml of 0.5% Avicel SF and 0.25 ml of appropriately diluted enzyme solution. The mixture was incubated at 50°C. After 10 minutes incubation, the reducing sugar formed was determined as glucose by the Somogyi–Nelson method.[19]

Carboxymethyl cellulose (CMC)-hydrolyzing enzyme (CMCase) and β-glucosidase. These two enzyme activities were assayed in the same manner as described for the avicelase assay except that 1% CMC and 1% salicin solution were used as the substrates for CMCase and β-glucosidase, respectively.

Filter paper-hydrolyzing activity (FPase, F.P.U.).[18,20,21] To 50 mg ($1 \times 6\,cm^2$ strip) of Whatman No. 1 filter paper were added 1 ml of 0.1 M acetate buffer (pH 5.0) and 0.5 ml of enzyme solution. The mixture was incubated at 50°C for 1 hour, and then the reducing sugar liberated was measured by the Somogyi–Nelson method[19] or the 3,5-dinitrosalicylic acid method.[22]

One unit of each enzyme was defined as the amount of the enzyme which liberated 1 μmol of reducing sugar per minute.

Other glycan-hydrolyzing enzymes. The procedure for measuring the activities of other glycan-hydrolyzing enzymes was essentially the same as in the case of the cellulose-hydrolyzing enzyme described above.

Paper chromatography. The hydrolysates were spotted onto Toyo No. 50 filter paper and then separated with a solvent system of pyridine–1-butanol–water (4 : 6 : 3, v/v) by the ascending technique at room temperature. Spots of sugar were detected by the dipping method using silver nitrate reagent.

Determination of protein. Protein was determined by the method of Lowry *et al.*[23] using bovine serum albumin as the standard or by measuring the absorbance at 280 nm.

RESULTS

Isolation of microorganisms producing potential cellulolytic enzymes

Samples of soil, each weighing about 0.1 g, obtained from various locations, were placed in individual test tubes of liquid medium containing a filter paper strip as the sole source of carbon. After incubation with continuous shaking for about two weeks at 30°C, the cultures showing good growth and degradation of the filter paper were picked up and subcultured in fresh medium for several days. Enrichment cultivations were repeated several times under the same culture conditions. After that, the culture which most strongly degraded the filter paper was appropriately diluted with sterile water and then placed on non-selective agar plates containing potato-dextrose or malt extract-dextrose agar. Some of the many colonies formed on the plates were randomly picked out and transferred to liquid medium in

order to test for cellulolytic enzymes. After 10 days cultivation, their avicelase, CMCase and β-glucosidase activities were compared with each other. Among them, several strains were found to extracellularly produce strong cellulolytic enzyme components in the culture medium. On further examination of their cellulolytic enzyme productivities, a fungus, strain Y-94, which was isolated by one of the authors (T. Yamanobe) from soil from the northeastern district of Japan in the middle of August, 1982, was finally selected as the best cellulase-producing fungus.

This strain was deposited, on January 12, 1983, with the Fermentation Research Institute, Agency of Industrial Science & Technology, Tsukuba, Ibaraki-ken, Japan, under Deposit No. FERM P-6867, and also deposited under the Budapest Treaty on March 1, 1984, and given Deposit No. FERM BP-495.

Growth profile and morphological characteristics of strain Y-94

On malt extract dextrose agar, colony growth proceeded rapidly, a diameter of 70 mm being reached in seven days at 30°C. The color of a colony was initially white which then changed to a yellowish white. In a later stage of culture (older than 5 days), the underside of the colony became a rosy brown to reddish-brown color. Aerial hyphae arose loosely from the colony surface and sometimes formed rope-like aggregates (Fig. 1). A photomicrograph of the isolated fungal strain Y-94 is shown in Fig. 1. On potato dextrose agar or Czapek agar, growth proceeded in a similar way except for a smaller increase in the height of aerial hyphae. The pH range for growth was 3.5 to 6.0, with an optimum around 4.0. The temperature range for growth was 15° to 43°C, with an optimum around 30°C. Hyphae measured 0.5 to 2.5 μm in diameter and were colorless. They were septate and had a smooth surface. Even though strain Y-94 was cultivated with various kinds of media that are often employed for the cultivation of fungi, it produced no conidia. No further study on the identification of strain Y-94 could be performed because of this inability to produce conidia. Therefore, we would like to delay our judgement as to the identification of strain Y-94 until the following paper. This newly isolated strain bore very little resemblance in morphological and physiological characteristics, that is, spore-forming ability, rope-like aggregate-forming property of aerial hyphae, whole appearance of a colony, and the productivity and properties of cellulolytic enzymes produced, *etc.*, to other typical cellulolytic strains, several strains belonging to the genera *Trichoderma*,[8,11] *Aspergillus*,[12,13] and penicillium[4~7] *etc.*, which had already been reported by other investigators.

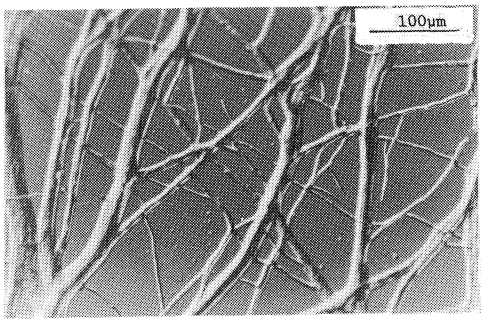

FIG. 1. Aerial Mycelia of Fungal Strain Y-94 (Photomicrograph).

Cells were cultured on potato dextrose agar for 5 days at 30°C.

Examination of culture conditions

Although the selected fungal strain, Y-94, produced a good composition of cellulolytic enzyme components, avicelase, CMCase and β-glucosidase, the enzyme yields were not as high as expected when it was grown in the screening medium. We therefore examined various culture conditions in order to enhance its enzyme productivity.

1. *Effect of cellulose concentration.* Strain Y-94 was able to assimilate many kinds of carbon sources. Among the carbon sources tested, however, cellulose powder was most effective for the production of enzymes (Table I). As shown in Table II, the activities of cellulase components and β-glucosidase per unit vol-

TABLE I. EFFECT OF THE CARBON SOURCE ON THE CELLULOLYTIC ENZYME PRODUCTION BY STRAIN Y-94

Carbon source	Enzyme activity (units/ml)		
	Avicelase	β-Glucosidase	CMCase
Cellulose powder	0.8	1.8	8.9
Microcrystalline cellulose	0.6	1.3	4.5
Defatted Napier grass[a]	0.3	1.2	6.1
Xyloglucan[b]	0.5	1.2	5.2
Solka Floc BW-200	0.3	1.2	4.4
KC-Flock	0.3	0.7	1.9
α-Cellulose[c]	0.2	0.4	1.7
Hemicellulose A[d]	0.02	0.2	1.3
Pectin	0.02	0.1	0.4
Soluble starch	0.02	0.1	0.3
Glucose	0.0	0.0	0.0
Lactose	0.0	0.0	0.0
Xylose	0.0	0.0	0.0

The medium used was composed of 0.75% cellulose powder, 0.15% peptone, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and minor elements. The initial pH of the medium was adjusted to 4.5, and cultivation was carried out with shaking in 200-ml flasks at 30°C for 7 days.

[a] Residue of an ether extract of air-dried Napier grass.

[b] From tamarind kernels.

[c] 24% KOH precipitate of holocellulose, obtained by delignifying air-dried Napier grass.

[d] Chilled water precipitate of the neutralized substance in the 24% KOH extract, obtained from the residue of Napier grass which was extracted with benzene–ethanol (1 : 1) and hot water (90°C), and delignified with 10% peracetic acid.

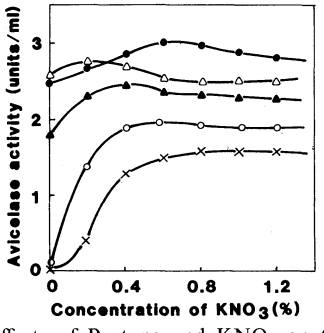

FIG. 2. Effects of Peptone and KNO_3 on Cellulolytic Enzyme Production by Strain Y-94.

Various concentrations of peptone and KNO_3 were added to the medium which, apart from these components, was the same as the control medium in Table III. The culture conditions were also the same as in Table III. Concentrations of peptone in the medium: △, 1.2%; ●, 1.0%; ▲, 0.6%; ○, 0.2%; ×, 0.0%.

TABLE II. EFFECT OF THE CELLULOSE CONCENTRATION OF THE MEDIUM ON CELLULOLYTIC ENZYME PRODUCTION BY STRAIN Y-94

Cellulose (%)	Enzyme activity (units/ml)			Final pH
	Avicelase	β-Glucosidase	CMCase	
1.0	2.3	4.2	11.2	6.5
2.0	2.4	4.3	10.5	6.5
3.0	2.5	4.8	10.5	6.6
3.5	3.2	5.5	13.8	6.5
4.0	3.4	6.2	14.4	6.6
4.5	2.9	5.8	14.2	6.7
5.0	2.8	5.6	14.1	6.5
5.5	2.8	5.8	14.1	6.5
6.0	2.7	5.6	13.6	6.5
7.0	1.2	4.8	12.8	6.6

Various concentrations of cellulose powder were added to the medium for the enzyme production. Each enzyme activity was assayed after 8 days cultivation.

TABLE III. EFFECTS OF VARIOUS NITROGEN SOURCES ON CELLULOLYTIC ENZYME PRODUCTION BY STRAIN Y-94

Nitrogen source (1%)	Enzyme activity (units/ml)		
	Avicelase	β-Glucosidase	CMCase
Control	2.5	11.3	29.3
Bacto-peptone	2.2	9.8	23.9
Polypepton	2.2	10.5	24.6
NZ amine	2.2	8.5	26.5
NZ case	2.1	8.3	25.2
Proteose peptone	2.1	7.5	23.5
Polypepton S	1.0	4.8	14.9
Albumin egg	1.1	6.7	14.1
Urea	1.1	7.0	13.3
Meat extract	0.98	6.0	10.2
KNO_3	0.88	5.2	9.0
NH_4NO_3	0.85	4.8	8.6
$(NH_4)_2SO_4$	0.42	3.5	4.2
NH_4Cl	0.36	2.0	5.9
$(NH_4)_2HPO_4$	0.37	6.0	5.0

The medium used for the examination was prepared by the addition of 1% of each nitrogen source instead of the several kinds of nitrogen sources contained in the control medium. The control medium consisted of 4% cellulose powder, 1% Bacto-peptone, 0.6% KNO_3, 0.2% urea, 1.2% KH_2PO_4, 0.16% KCl, 0.12% $MgSO_4 \cdot 7H_2O$ and minor elements.

The initial pH of the medium was adjusted to 4.0.

The strain was cultured at 30°C for 6 days in a shaking flask.

ume increased along with an increase in the cellulose concentration of the medium. However, the enzyme productivity decreased gradually when the cellulose concentration of the medium exceeded 4%. Therefore the optimum concentration of cellulose as a carbon source was set at about 4%.

2. *Effect of nitrogen source.* In order to determine a suitable nitrogen source, several kinds were tested using the medium containing 4% cellulose powder. Among the tested sources, peptone or polypepton as an organic nitrogen source and KNO_3 as an inorganic nitrogen source gave better productivity of the cellulolytic enzymes. Even better productivity of the enzymes was obtained by supplying both KNO_3 and peptone than by supplying them singly. As shown in Table III and Fig. 2, effective production of avicelase, CMCase and β-glucosidase was attained in the medium containing 1.0% peptone and 0.6% KNO_3 as the nitrogen sources.

3. *Effect of other nutrients.* The effects on the production of avicelase, CMCase and β-glucosidase of several kinds of phosphate salts and their concentrations in the medium were examined. KH_2PO_4 showed an optimum concentration of around 1.2% for the production of these cellulase components.

Various mineral salts were tested as to their effects on the production of avicelase, CMCase and β-glucosidase. The addition of Zn^{2+}, Mn^{2+} and Cu^{2+} ions to the medium promoted

TABLE IV. MEDIUM FOR ENZYME PRODUCTION BY STRAIN Y-94

Cellulose powder	4.0%
Peptone	1.0
KNO_3	0.6
Urea	0.2
KH_2PO_4	1.2
KCl	0.16
$MgSO_4 \cdot 7H_2O$	0.12
Tween 80 or PG[a] 1000	0.1
Minor elements	
$ZnSO_4 \cdot 7H_2O$	10.0 μg/ml
$MnSO_4 \cdot 6H_2O$	10.0
$CuSO_4 \cdot 7H_2O$	10.0
pH	4.0

[a] Polyethylene glycol.

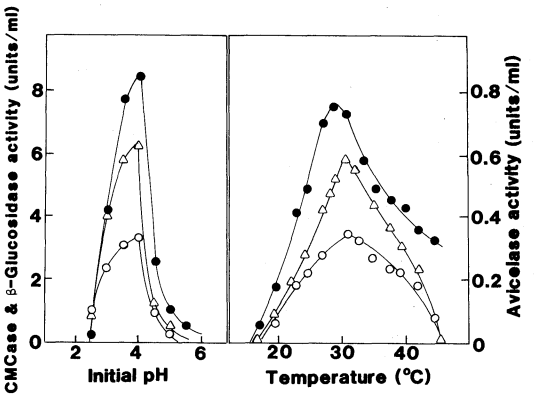

FIG. 3. Effects of Cultivation Temperature and Initial pH on Cellulolytic Enzyme Production by Strain Y-94.

In order to investigate the effect of pH, various initial pHs of the medium were obtained, within the range of 2.5 to 8.0, by adding 1 N HCl or NaOH. To elucidate the effect of temperature, cultivations were carried out using a temperature gradient cultivation system at temperatures between 15° and 50°C and at 40 oscillations per minute for 7 days. ●, CMCase; △, avicelase; ○, β-glucosidase.

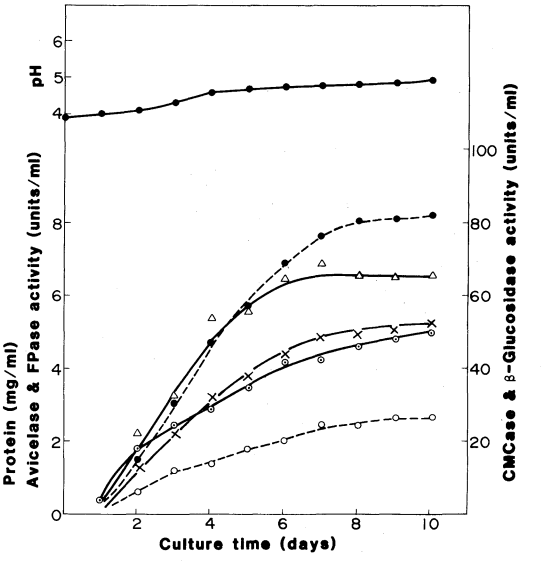

FIG. 4. Time Course of Cellulolytic Enzyme Production in the Optimum Medium by Strain Y-94.

The cultivation was carried out in a 200-ml flask containing 20 ml of the optimum medium with shaking at 30°C for 10 days at a rate of 240 rotations per minute. ●, CMCase; △, avicelase; ×, FPase; ○, β-glucosidase; ◉, extracellular protein.

the enzyme production. When a medium containing 10 mg of each of $MnSO_4 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$ and $CuSO_4 \cdot 7H_2O$ per liter was used for the cultivation of strain Y-94, the synthetic cellulase activity generated through the synergistic effects of avicelase, CMCase and β-glucosidase was about 2.5 times that in the broth on culturing without these metal salts.

The effects of several kinds of surfactants on the production of avicelase, CMCase and β-glucosidase were also examined. Among the surfactants tested, Tween 80 stimulated the production of these cellulase components by strain Y-94 most effectively when it was added to the medium at the start of the cultivation or to the culture at the early stage of cultivation.

4. *Effects of initial pH and temperature.* The production of avicelase, CMCase and β-glucosidase at various pHs and cultivation temperatures is shown in Fig. 3. The maximum yields of these cellulase components were obtained when the initial pH of the medium was adjusted to pH 4.0. The temperature for the optimum production of these cellulase components and the growth of the mycelium was 30~33°C. A temperature higher than 45°C caused a marked decrease in both the productivity of these cellulase components and mycelial growth. On the basis of the data described above, the medium shown in Table IV is proposed to be the optimum semi-synthetic medium for the production of the enzymes.

5. *Time course of enzyme production.* The production of the enzymes and the pH va-

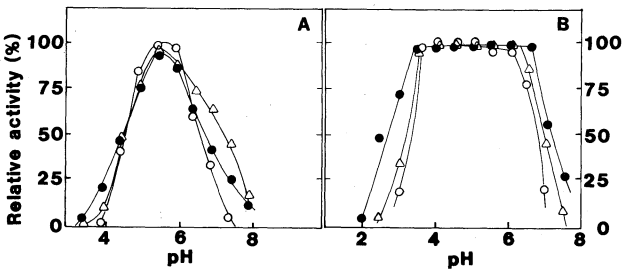

FIG. 5. Optimum pH and pH Stability of the Cellulolytic Enzyme Components Produced by Strain Y-94.

After incubation of each enzyme component solution at 50°C for 10 min with the following kinds of buffer solution, the optimum pH was examined. Acetate buffer (0.1 M) was used over the pH range of 2.5 to 7.0. Phosphate buffer (0.1 M) was used above pH 6.5. The pH stability of each enzyme component was examined by measuring its remaining activity after storage for 20 hr at 10°C in 0.1 M buffer solution, within a pH range of 2.0 to 8.0. The symbols are the same as in Fig. 3. A, optimum pH; B, pH stability.

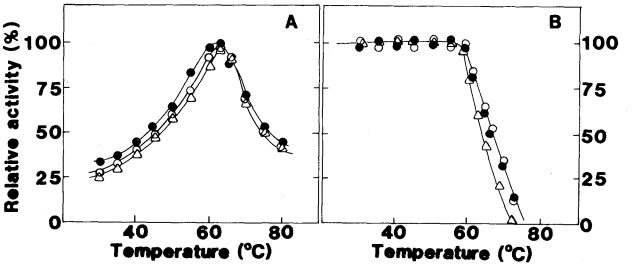

FIG. 6. Optimum Temperature and Thermostability of the Cellulolytic Enzyme Components Produced by Strain Y-94.

After incubation of each enzyme component solution at the indicated temperature for 10 min, the optimum temperature was examined. Aliquots of each enzyme components solution in 0.1 M acetate buffer (pH 5.0) were incubated at the indicated temperature for 10 min, cooled immediately to 0°C and then assayed for the remaining activities. The symbols are the same as in Fig. 3. A, optimum temperature; B, thermostability.

riation with elapsed cultivation time were examined. Figure 4 shows a typical time course for the cellulolytic enzyme production under the optimum conditions. After a one-day lag period, log-phase growth occurred. The activities of both CMCase and β-glucosidase in the broth became maximum after cultivation for 10 days. However, the hydrolytic activity toward crystalline cellulose reached a maximum on the 6th day. The increase in cellulolytic enzyme activity paralleled the mycelial growth, the pH of the culture broth increasing from 4.0 to 5.2. Whole activities of the cellulolytic enzyme components produced by strain Y-94 were present in the supernatant of the cultured broth.

Some properties of crude enzyme preparation

Figure 5 shows that the optimum pH of each component of the cellulolytic enzyme was around 4.5, and indicates that they were stable in the pH range of from 3.5 to 6.0. As shown in Fig. 6, their optimum temperatures were all about 60°C, their activities being stable even after treatment at 60°C for 10 minutes. After heat treatment at 80°C for 10 minutes, however, their activities could no longer be detected.

Comparison of the saccharification activity of cellulolytic enzymes of strain Y-94 with that of Trichoderma reesei QM 9414

Solka Floc BW-200 was added to the culture filtrate of strain Y-94, grown in medium containing 4% cellulose powder, to a final concentration of 15%, and then saccharification was carried out for 24 and 48 hr at 50°C and pH 4.5. As shown in Fig. 7, after 48 hr incubation the extent of cellulose saccharification by the culture filtrate of strain Y-94 was appreciably greater than that in the case of the cellulase system of *Trichoderma reesei* QM 9414.[24] This seems to indicate that the cellulolytic enzyme system of strain Y-94 is moderately more thermostable than that of *Trichoderma reesei*, indicating that its β-glucosidase functions effectively as a promoter for saccharification of cellulosic substances.

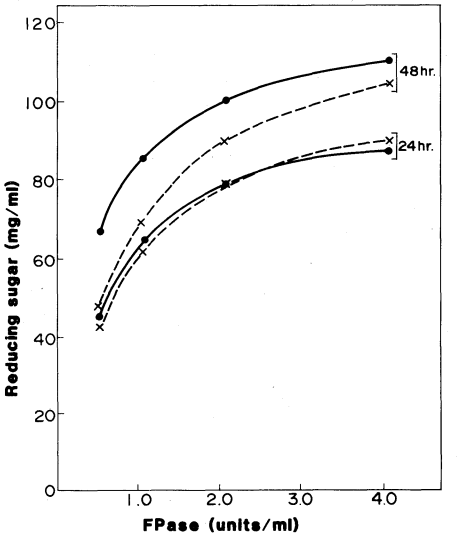

FIG. 7. Comparison of the Saccharification Activity of the Cellulolytic Enzyme System Produced by Strain Y-94 with that in the Case of *Trichoderma reesei* QM 9414.

A suspension of 15% Solka Floc BW-200 was incubated at pH 4.5 and 50°C for 24 and 48 hr with the culture filtrate of strain Y-94. —●—, strain Y-94; ···×···, *Trichoderma reesei* QM 9414.

Saccharification of microcrysalline cellulose

Figure 8 shows the amounts of reducing sugar, soluble sugar and glucose in the hy-

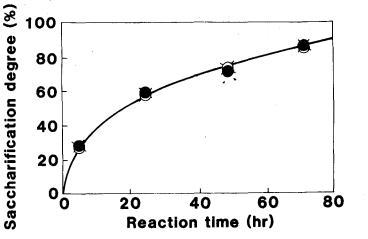

FIG. 8. Saccharification of Microcrystalline Cellulose (Avicel) with the Cellulolytic Enzyme System Produced by Strain Y-94.

An amount of avicelase corresponding to 40 units per g of Avicel was reacted with a slurry of 5% Avicel in 0.05 M acetate buffer (pH 4.5). An aliquot of the reaction mixture was withdrawn at 4, 24, 48 and 72 hr, respectively. The amount of reducing sugar was measured by the Somogyi–Nelson method with glucose as a standard, the amount of soluble sugar by the phenol–sulfuric acid method with glucose as a standard, and the amount of glucose by the glucose oxidase method, respectively. ○, soluble sugar; ●, reducing sugar; ×, glucose.

TABLE V. COMPARISON OF THE ENZYME PRODUCTIVITY OF STRAIN Y-94 WITH THAT OF *Trichoderma reesei* QM 9414

Organism	Carbon source	Enzyme activity (units/ml of c.f.[a])				Protein (mg/ml of c.f.)
		Avicelase	β-Glucosidase	CMCase	FPase[b]	
Y-94	4% Cellulose powder	6.5	25.0	82.0	5.0	5.0
QM 9414[24]	6% Solka Floc BW-200	—	0.3	89.0	9.0	9.0

[a] Culture filtrate.
[b] Filter Paper Units (F.P.U.).
The present strain was grown under its optimum culture conditions using the medium shown in Table IV.

drolysate of avicel with the culture filtrate of strain Y-94. The results indicate that the soluble sugar in the hydrolysate was almost exclusively glucose from the beginning of the reaction and no accumulation of cellobiose or other oligosaccharides was detected.

Enzyme productivities of strain Y-94 and Trichoderma reesei QM 9414

As summarized in Table V, the productivity of the cellulase system produced by strain Y-94 was enhanced by improvement of the medium composition, that is, an 8-fold increase in avicelase activity, a 9-fold increase in CMCase activity and a 13-fold increase in β-glucosidase activity, in comparison with those initially obtained in the basal medium. Table V also compares the typical contents of these cellulase components produced by strain Y-94 with those of the cellulase components produced by *Trichoderma reesei* QM 9414.[24] As can be seen in Table V, the composition ratio of FPase : CMCase : β-glucosidase of the cellulolytic enzyme system produced by strain Y-94 was 1 : 16 : 5, while in the case of the enzyme system produced by *Trichoderma reesei* QM 9414, the aforementioned ratio was 1 : 18 : 0.06. These results indicate that the enzyme system produced by strain Y-94 has a notably high β-glucosidase content.

Hydrolysis of various glycans by the culture filtrate

As shown in Table VI, the enzyme system produced by strain Y-94 was capable of hydrolyzing various kinds of glycans. These results indicate that various kinds of enzymes that hydrolyze glycans were produced by strain Y-94 in the culture filtrate.

TABLE VI. HYDROLYSIS OF VARIOUS SUBSTRATES BY THE CULTURE FILTRATE OF STRAIN Y-94

Substrate	Linkage	Hydrolysis (%)[d]
Soluble starch	α-1,4·1,6 G[b]	77.2
Dextran	α-1,6 G	46.1
Xyloglucan[a]	β-1,4 G·α-1,6 X[c]	29.4
Xylan	β-1,4 X	42.8
Schizophyllan	β-1,3·1,6 G	97.9
Curdlan	β-1,3 G	100.0
Laminarin	β-1,3 G	14.3
Avicel	β-1,4 G	52.0
CMC	β-1,4 G	47.7
Solka floc BW-200	β-1,4 G (X)	56.2
Cellobiose	β-1,4 G	100.0
Gentiobiose	β-1,6 G	100.0

Each reaction mixture, consisting of 10 μl of enzyme solution (culture filtrate containing 0.062 units avicelase, 0.609 units CMCase, 0.233 units β-glucosidase and 46 μg protein), 0.5 ml of 0.5% substrate, 0.4 ml of 0.1 M acetate buffer (pH 4.6) and 0.1 ml of toluene was incubated for 15 hr at 50°C.
[a] From tamarind kernels.
[b] Glucose.
[c] Xylose.
[d] [Reducing sugar (glucose)/Total sugar] × 100.

DISCUSSION

A fungus was isolated from soil as a cellulolytic enzyme-producing microorganism by a non-selective method. This strain was a fungus which had not previously been reported to be a potent cellulase-producing microorganism. The cellulase system produced by this

fungal strain, Y-94, exhibits notably higher β-glucosidase activity, which is very important for the enzymatic conversion of cellulose into glucose, as well as appreciable levels of avicelase and CMCase, than in the case of the conventionally well-known cellulase systems produced by several strains of the genus *Trichoderma, etc.*[9,25,26] No strain exhibiting a higher β-glucosidase activity than that of strain Y-94 has been found in the genus *Trichoderma,* although the ability to produce a cellulase system containing high levels of avicelase and CMCase is marked in several strains belonging to this genus. On the other hand, β-glucosidase-producing activity is known to be distributed among several strains belonging to the genus *Aspergillus*,[27,28] but the amounts of this enzyme produced by them are not yet satisfactory for the saccharification of cellulosic substances, even allowing for the addition of *Aspergillus* β-glucosidase[24,29] for reduction of the cellobiose levels and increasing the glucose content in the hydrolysate. It appears that the cellulolytic enzyme system produced by strain Y-94 shows superior functions which combine the solubilizing activity of the *Trichoderma* enzyme system and the saccharifying activity of the *Aspergillus* enzyme system toward cellulosic substances. Consequently, the culture broth of strain Y-94 exhibited high hydrolysis limitations on crystalline cellulose such as Avicel cellulose, in comparison with the low limitations reported for other strains.[18] In practice, almost all the enzymatic hydrolysates of pure cellulose were glucose (Fig. 8).

Furthermore, since the cellulolytic enzyme system produced by strain Y-94 is comparatively thermostable, it permits the saccharification of cellulose at temperatures 5 to 10°C higher than those at which the saccharification has been performed using *Trichoderma* cellulase,[30,31] thus possibly facilitating the protection of the saccharification reaction mixture against potential contamination by infectants.[32]

The enzyme productivity was remarkably increased by using the improved medium prepared for the enzyme production, compared with the productivity obtained in the screening medium. In addition, strain Y-94 also produced xylanase, β-1,3-glucanase and so on. We therefore considered, from these experimental data, that strain Y-94 might be useful for the production of glucose from various cellulosic substances and become an advantageous source of various enzymes required for use on the vegetable biomass.

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