Production of a Raw Starch Saccharifying Enzyme by *Corticium rolsfii*

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Corticium rolsfii AHU 9627, which we isolated from a tomato stem, is one of the most promising producers of a raw starch saccharifying enzyme. The effects of the cultural conditions and medium components on the enzyme production were investigated. The enzyme production was improved by increasing both the concentrations of carbon sources and organic nutrients in the medium. Under the optimum cultural conditions, the enzyme activity of the culture supernatant against raw starch reached a maximum after 8-days incubation at 27°C and the activity reached 80 units per ml (when determined at 40°C and pH 4.0). The optimal pH and temperature for the enzyme reaction were 4.0 and 65°C, respectively. The saccharifying reaction was scarcely inhibited even with a high substrate concentration, and raw starch was rapidly hydrolyzed into glucose.

The enzymatic saccharification of raw starch has become the subject of much attention in recent years from the viewpoints of energy saving and effective utilization of natural resources. Starch has been gelatinized at first by cooking in the course of conventional enzymatic saccharification. In order to reduce the cost of cooking, several studies have been undertaken on the enzymatic hydrolysis of raw starch. However, it was not only technically but also economically difficult, because an inexpensive enzyme which can saccharize raw starch effectively was not available. The production of an extremely active enzyme depends on the selection of a suitable microorganism for the purpose. *Aspergillus awamori*,^{1,2)} *Rhizopus* sp.,^{3,4)} *Streptococcus bovis*,⁵⁾ *Bacillus circulans*,⁶⁾ *Chalara paradoxa*,⁷⁾ *Aspergillus* sp.,⁸⁾ and some other microorganisms have been reported to be producers of microbial amylases capable of digesting raw starch.

Recently, we found that *Corticium rolsfii* AHU 9627, a fungus isolated from a tomato stem, could be a potential source for the production of raw starch saccharifying enzyme.⁹⁾ The present study was conducted to

examine the optimum conditions for the enzyme production and some properties of the crude enzyme.

MATERIALS AND METHODS

Microorganism. The organism studied was one of our isolates, *Corticium rolsfii* AHU 9627. It was isolated from a tomato stem and maintained on potato dextrose agar slants.

Medium. The organism was cultivated in a basal medium containing, in g/liter: $(\text{NH}_4)_2\text{SO}_4$, 1.4; KH_2PO_4 , 2.0; CaCl_2 , 0.3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3; urea, 0.3; Polypepton (Daigo Nutritive Chemicals, Ltd.), 10.0; raw nonglutinous rice starch (Shimada Chemical Industry Co., Ltd.), 20.0; Tween 80, 2.0; and a mineral solution, 1.0 ml. The mineral solution contained, in g/liter: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.6; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.4; and CoCl_2 , 2.0 (Mandels and Weber¹⁰⁾). The medium without raw starch was autoclaved at 15 psi pressure for 10 min. Raw nonglutinous rice starch was sterilized with ethylene oxide and then aseptically added to the flasks containing the autoclaved medium. The concentrations of some components were occasionally changed and the effects on the enzyme production were examined. Raw nonglutinous rice starch and Polypepton in the basal medium were also replaced by some carbon sources and organic nutrients respectively, and the optimum conditions for enzyme production were determined.

Cultivation. Inocula were prepared by seeding 3 sclerotia from stock cultures onto 15 ml of potato dextrose agar medium in 9 cm petri dishes. After 4-day incubation at 27°C, the organisms on 3 plates were put into a cup together with the agar medium and homogenized in 50 ml of deionized water. For enzyme production, 1-ml each of the homogenates was inoculated into 100 ml of liquid medium in 500-ml shaking flasks. Each flask was incubated at 27°C on a reciprocating shaker.

Enzyme assays. About 3-ml portions of a culture broth were aseptically taken out at the indicated intervals and centrifuged at 3,500 rpm for 10 min at 4°C. The supernatant thus obtained was used for the enzyme assays.

(a) *Raw starch saccharifying activity.* The reaction mixture, containing 0.3 ml of a suspension of 5% raw non-glutinous rice starch in 0.1 M Na-acetate buffer (pH 4.0) and 0.3 ml of an appropriately diluted enzyme solution, was incubated with shaking at 40°C for 30 min. After centrifugation, the reducing sugar liberated into the supernatant was measured by the dinitrosalicylic acid (DNS) method.¹¹⁾

(b) *Gelatinized starch saccharifying activity.* The reaction mixture, containing 0.1 ml of a solution of 2% gelatinized nonglutinous rice starch, 0.1 ml of 0.2 M acetate buffer (pH 4.0) and 0.2 ml of a diluted enzyme solution, was incubated at 40°C for 10 min. The reducing sugar produced was assayed by the method of Somogyi-Nelson.^{12,13)}

(c) *Glucoamylase activity.* The reaction mixture, containing 0.2 ml of a solution of 1.25% cooked soluble starch, 0.25 ml of 0.1 M acetate buffer (pH 4.0) and 0.05 ml of a diluted enzyme solution, was incubated for 30 min at 40°C. The reducing sugar was assayed by the method of Somogyi-Nelson.^{12,13)}

(d) *α -Amylase activity.* Liquefying activity toward gelatinized starch was determined by the JIS K 7001 method.¹⁴⁾

(e) *α -Glucosidase activity.* The reaction mixture, containing 0.1 ml of a solution of 1% maltose in 0.1 M acetate buffer (pH 4.0) and 0.1 ml of a diluted enzyme solution, was incubated at 40°C for 30 min. After the incubation, the reaction was stopped by heating in a boiling water bath for 5 min. The amount of glucose in the solution was determined by the glucose-oxidase method (Glucose B-Test, Wako). One unit of glucoamylase activity was defined as the amount of enzyme that produced 1 mg of glucose in 1 ml of the reaction mixture under the above-mentioned conditions. One unit of α -amylase activity was expressed as the amount of enzyme, which was determined from the increase in fluidity in 15 min under the specified conditions.¹⁴⁾ One unit of the activities of the other enzymes was defined as the amount of enzyme that liberated 1 μ mol of glucose per min.

Measurement of mycelial weight. Culture broth, 100 ml, containing mycelia and starch granules was boiled with 100 ml of 2 N HCl for 15 min and then filtered through

Toyo No. 4 filter paper. The mycelia retained on the filter paper were washed several times with deionized water to remove most of the acid. The weight of the mycelia was determined after drying in an oven at 80°C for 6 hr and cooling in a desiccator.

Analysis of hydrolysis products. The reaction mixture was centrifuged and the amount of reducing sugar in the supernatant was determined as glucose by the DNS method.¹¹⁾ The sugar produced in the supernatant was then quantitatively analysed by high performance liquid chromatography (HPLC).

RESULTS AND DISCUSSION

Examination of culture conditions

Effects of organic nutrients. Polypepton in the basal medium was replaced by each of the organic nutrients shown in Table I, and the effects on the production of the raw starch saccharifying enzyme were investigated.

The microorganism grown on the basal medium (Polypepton) produced the highest level of raw starch saccharifying activity among the cultures in this experiment. When

TABLE I. EFFECTS OF ORGANIC NUTRIENTS ON ENZYME PRODUCTION

Polypepton in the basal medium was replaced by each of the organic nutrients in the table.

Organic nutrient	Maximum saccharifying activity (IU/ml)	
	Concentration	
	0.1%	1.0%
Polypepton	3.1	11.9
Yeast extract	3.6	9.8
Meat extract	2.1	8.6
Corn steep liquor	2.5	8.2
Casein	3.0	5.5
Molasses	1.6	3.4
Malt extract	2.8	3.1
Wheat bran	2.4	3.0
Distillers' soluble	1.9	2.8
Rice bran	1.8	2.5
Casamino acid	1.8	2.3
Potato extract	1.9	2.1
Glycine	1.6	2.0
L-Asparagine	1.5	1.6
L-Alanine	1.4	1.6
L-Tryptophan	1.2	1.0
None	1.6	

yeast extract, meat extract or corn steep liquor was used as an organic nutrient, the enzyme was also produced, but the levels of the enzyme activity were less than in the case of the Polypepton-grown culture.

Effects of carbon sources. The various kinds of carbohydrates shown in Table II were individually added to the basal medium with raw starch omitted, and their effects on the enzyme production were examined.

A high level of raw starch saccharifying activity were observed not only in the culture grown on raw starch, but also in the cultures grown on the monosaccharides, such as glucose and fructose, and disaccharides shown in the table.

Raw starch saccharifying activity appeared after 5 days cultivation along with marked growth, and increased thereafter, but the enzyme productivities were not proportional to the carbohydrate content of the culture media. At an early stage of cultivation, the pH fell to about 2.2 in cultures grown on sugar because of the accumulation of formic acid, and repression of the enzyme production was observed very frequently.

The effects of various kinds of starch were examined, in the cooked and uncooked states. The starch content of culture media was adjusted to 0.5%, in order to avoid an increase in viscosity on cooking. No significant difference was observed in enzyme productivity among the various kinds of starch examined, especially when cooked starches were used (Table III). When the starches were used at high concentrations, reducing sugar accumulated in most of the culture broths, but not in the culture grown on raw potato starch. The effects of various combinations of two nutrients, raw potato starch and Polypepton, are shown in Table IV. The results showed that the combination of 4% raw potato starch and 3% Polypepton gave the highest level of enzyme activity. In this case, the pH of the culture broth fell to about 2.5, but reducing sugar didn't accumulate in the broth.

Effects of inorganic nitrogen compounds. Several kinds of inorganic nitrogen com-

TABLE II. EFFECTS OF VARIOUS CARBOHYDRATES ON ENZYME PRODUCTION

Each carbon source in the table was added to the basal medium at a concentration of 0.5% in place of 2% raw starch.

Carbon source	Maximum saccharifying activity (IU/ml)
Arabinose	3.2
Xylose	8.5
Ribose	5.0
Glucose	11.4
Galactose	7.0
Mannose	14.9
Fructose	11.3
Maltose	13.1
Cellobiose	8.4
Lactose	13.4
Sucrose	12.7
Soluble starch	10.6
Amylose	10.8
Dextrin	10.7
Laminarin	9.9
Pectin	9.7
Inulin	6.9
Xylan	5.3
Avicel	2.4
Glycerol	5.9
α -Methyl glucoside	5.3
None	4.8

TABLE III. EFFECTS OF VARIOUS KINDS OF STARCH ON ENZYME PRODUCTION

Each carbon source was added to the basal medium at a concentration of 0.5% in place of 2% raw nonglutinous rice starch.

Carbon source	Maximum saccharifying activity (IU/ml)	
	Cooked starch	Raw starch
Nonglutinous rice	11.8	10.6
Glutinous rice	11.3	9.9
Waxy corn	10.8	12.0
Corn	12.3	11.3
Wheat	11.7	12.0
Sweet potato	11.6	11.5
Sago	10.5	9.9
Potato	11.5	8.8

pounds were tested in the medium containing 4% raw potato starch and 3% Polypepton. Each compound was added to a nitrogen content equivalent to that of $(\text{NH}_4)_2\text{SO}_4$. Both

TABLE IV. EFFECTS OF RAW POTATO STARCH AND POLYPEPTON ON ENZYME PRODUCTION

This examination was carried out with the basal medium, to which raw potato starch and Polypepton were added at various concentrations.

Raw potato Starch (%)	Maximum saccharifying activity (IU/ml)					
	Polypepton (%)					
	0.5	1.0	1.5	2.0	2.5	3.0
0.5	5.8	9.5	10.2	17.2	18.7	14.9
1.0	6.5	10.1	17.3	25.3	32.8	37.9
2.0	7.1	10.6	22.5	29.0	39.6	38.2
4.0	6.9	9.9	22.6	31.2	60.0	60.9
6.0	7.0	9.1	17.9	21.5	51.9	60.1
8.0	8.1	8.9	17.5	20.0	35.6	49.0
10.0	6.3	9.7	16.0	14.0	23.3	36.6

TABLE V. EFFECTS OF INORGANIC NITROGEN SOURCES ON ENZYME PRODUCTION

Inorganic nitrogen sources were added to the basal medium containing 4% raw potato starch and 3% Polypepton. Each compound was added to a nitrogen content equivalent to that of $(\text{NH}_4)_2\text{SO}_4$.

Inorganic nitrogen source	Concentration (%)	Maximum saccharifying activity (IU/ml)
$(\text{NH}_4)_2\text{SO}_4$	0.035	42.3
	0.07	43.3
	0.14	43.1
	0.28	48.7
	0.56	45.1
NH_4Cl	0.028	37.5
	0.055	39.0
	0.11	35.2
	0.22	45.3
	0.44	37.6
NaNO_3	0.045	44.0
	0.09	45.4
	0.18	46.4
	0.36	56.9
	0.72	53.8
NH_4NO_3	0.023	45.5
	0.045	46.7
	0.09	50.3
	0.18	54.3
	0.36	52.7
None	—	38.2

TABLE VI. EFFECTS OF ADDITIVES ON ENZYME PRODUCTION

Each additive was added to the basal medium containing 4% raw potato starch and 3% Polypepton.

Additive	Concentration (%)	Maximum saccharifying activity (IU/ml)
Tween 80	0.05	34.2
	0.1	49.5
	0.2	42.9
	0.4	48.8
	0.8	44.3
Triton X-100	0.05	0
	0.1	0
	0.2	0
	0.4	0
	0.8	0
Phenethyl alcohol	0.05	40.5
	0.1	31.9
	0.2	0
	0.4	0
	0.8	0
None	—	34.3

nitrate tested, sodium nitrate and ammonium nitrate, allowed effective enzyme production (Table V).

Effects of additives. The effects of organic compounds and surfactants on extracellular enzyme production has been studied to improve the productivity of the enzyme.^{10,15,16} In this experiment, Tween 80, Triton X-100 and phenethyl alcohol were separately added to the basal medium containing 4% raw potato starch and 3% Polypepton. The enzyme activity reached a maximum after cultivation for 15 days. The addition of Tween 80 resulted in excellent stimulation of the enzyme production (Table VI). Tween 80 at the level of 0.1% increased the yield of the enzyme 1.4 fold. Phenethyl alcohol added to the medium at a level of 0.05% stimulated the enzyme production. Addition of phenethyl alcohol at concentrations higher than 0.2% and Triton X-100 inhibited growth and repressed the enzyme production.

Effects of the initial pH of the medium and growth temperature. The pH of the basal medium was adjusted to different levels with

TABLE VII. OPTIMUM MEDIUM FOR RAW STARCH SACCHARIFYING ENZYME PRODUCTION

Raw (potato) starch	40.0 (g/liter)
Polypepton	30.0
NH ₄ NO ₃	1.8
MgSO ₄ ·7H ₂ O	0.3
Tween 80	1.0

Initial pH: 6.0.

2N hydrochloric acid and sodium hydroxide solution, and then the effect of the initial pH was examined. The results showed that the organism produced the raw starch saccharifying enzyme effectively and there was no significant difference in the pH range of 4.0 to 6.5. The fungus was cultivated in the temperature range of 22 to 42°C and the optimum temperature for enzyme production was examined. The enzyme activity appeared at an early stage of cultivation when the organism was grown at 32°C, however, the optimum temperature for enzyme production was found to be 27°C. The growth was poor and there was no enzyme production at 37°C (data not shown).

Optimum conditions for enzyme production. According to the above mentioned results and those of other experiments, the optimum medium shown in Table VII was decided upon. Figure 1 shows the time course of enzyme production, growth of the organism and pH change under the optimum culture conditions. In this experiment, the inoculum was pre-cultured twice in the optimum medium to stimulate the initial growth.

Saccharifying activities against raw and gelatinized starch appeared in the medium after 2 days cultivation along with the growth of the organism and increased rapidly thereafter. The activity against raw starch reached a maximum after cultivation for 8 days (80 IU/ml) and it remained at this level for another 6 days. On the other hand, the activity against gelatinized starch increased for 10 days.

The activities of glucoamylase and α -glucosidase were almost completely proportional to the raw starch saccharifying activity. α -

FIG. 1. Time Course of the Enzyme Production in the Optimum Medium.

Symbols: ●, raw starch saccharifying activity; □, gelatinized starch saccharifying activity; ■, glucoamylase activity; ○, α -amylase activity; ▲, α -glucosidase activity; △, dry weight of mycelium; ○, pH of culture supernatant.

Amylase activity was observed a little later than the increase in glucoamylase activity. The activities of α -glucosidase and α -amylase were less than that of glucoamylase throughout the cultivation period. These results strongly suggest that the raw starch digesting-amylase of *C. rolf sii* is a glucoamylase.

Properties of the crude enzyme

Effects of pH and temperature on the enzyme activity. The activity of the raw starch saccharifying enzyme was determined at different pHs (Fig. 2). A crude enzyme solution, the supernatant of the culture broth (44 IU/ml), was used in this experiment. The optimum pH for the enzyme activity was found to be 4.0. The enzyme was considered to be an acid-durable one, because the enzyme exhibited about 50% of the maximal activity even at pH 2.0.

The effect of temperature on the enzyme activity was examined at pH 4.0 (Fig. 3). The

FIG. 2. Effect of pH on the Enzyme Activity.

The reaction mixture consisted of 0.2 ml of 2% raw starch suspension, 0.2 ml of the crude enzyme solution and 0.4 ml of 0.1 M buffer solution. The buffers used were HCl-KCl buffer (pH 1.5~2.0) and McIlvaine buffer (pH 2.5~7.5). The enzyme activity was assayed after adjustment of the pH to 4.0.

FIG. 3. Effect of Temperature on the Enzyme Activity.

The reaction mixture consisted of 0.2 ml of 2% raw starch suspension, 0.2 ml of the crude enzyme solution and 0.4 ml of 0.1 M McIlvaine buffer solution (pH 4.0). The enzyme activity was assayed under the standard conditions.

activity increased with temperature up to 65°C, especially over 50°C. The maximum activity was seen at 65°C, but this temperature was considered unsuitable as the optimal temperature for the raw starch saccharifying en-

FIG. 4. Effect of pH on the Enzyme Stability.

The enzyme solution was incubated in various buffers (0.1 M) at 40°C for 60 min. The residual activity was assayed after adjustment of the pH to 4.0. The buffers used were HCl-KCl buffer (pH 1.5~2.0), McIlvaine buffer (pH 2.5~7.0) and Tris-HCl buffer (pH 7.5~8.5).

FIG. 5. Effect of Temperature on the Enzyme Stability.

The enzyme solution in 0.1 M McIlvaine buffer (pH 4.0) was incubated at different temperatures for 10 min. The residual activity was assayed after cooling to 4°C.

zyme, because gelatinization of starch would begin at this temperature.

Effects of pH and temperature on the enzyme stability. The pH stability of the enzyme was determined in the pH range of 1.5 to 8.5. The enzyme solution adjusted to different pHs was incubated at 40°C for 60 min, and then the pH

of the solution was adjusted to 4.0. The residual activity was assayed under the standard conditions. The enzyme activity against raw starch was fairly stable in the pH range of 3.0 to 7.0 (Fig. 4).

The thermal stability of this enzyme was tested at elevated temperatures of 30 to 75°C. The enzyme in McIlvaine buffer solution (pH 4.0) was incubated for 10 min at the desired temperature, and then cooled to 4°C. The residual activity was determined under the standard conditions. The enzyme was found to be quite stable up to 50°C for 10 min without a significant loss of activity. The enzyme solution exhibited about 80% of the initial activity after 10 min incubation at 60°C, but the enzyme almost completely lost its activity when the solution was incubated at 75°C for 10 min (Fig. 5).

Hydrolysis of various kinds of raw starch by the crude enzyme. Various kinds of raw starch were added to the crude enzyme solution to a final concentration of 1% (w/v), and hydrolyzed at 40°C. The pH of the reaction mixture was adjusted to 4.0. The rate of saccharification (*S*) was calculated with the following equation.

$$S = \frac{\text{Glucose produced by enzymatic hydrolysis}}{\text{Glucose produced by acid hydrolysis}} \times 100$$

As shown in Fig. 6, rice starch and waxy corn starch were almost completely hydrolyzed within 2 hr, wheat starch and corn starch within 4 hr, and tapioca starch and sweet potato starch within 8 hr. About 65% of the potato starch was hydrolyzed after 12 hr incubation in spite of its resistance to some other amylolytic enzymes. The enzymatic hydrolyzates were analyzed by HPLC. The results showed that the hydrolysis product from these starches was almost all glucose.

Enzymatic hydrolysis of raw corn starch at a high concentration. Fifty milliliters of a raw starch suspension in 0.1 M acetate buffer (pH 4.0), which contained either 10, 20 or 30 g of raw corn starch, was mixed with 50 ml of a crude enzyme solution (44 IU/ml), and the mixtures were incubated on a shaker at 40°C for 48 hr. About 0.6 ml portions of the reaction mixtures were taken out at regular intervals, and reducing sugar liberated was determined as glucose.

Figure 7 shows the time course of enzymatic

FIG. 6. Hydrolysis Curves for Various Kinds of Raw Starch with the Crude Enzyme.

The reaction mixture, consisting of 0.5 ml of 2% raw starch suspension in 0.1 M acetate buffer (pH 4.0) and 0.5 ml of the crude enzyme solution (44 IU/ml), was incubated on a shaker at 40°C.

Symbols: ○, rice; ▲, waxy corn; □, wheat; ■, corn; △, tapioca; ⊙, sweet potato; ◻, sago; ●, potato.

FIG. 7. Enzymatic Hydrolysis of Raw Corn Starch at High Concentrations.

Ten, 20 or 30 grams of raw corn starch was suspended in 0.1 M acetate buffer (pH 4.0) and then diluted to a volume of 50 ml with the same buffer. Fifty-milliliter portions of the crude enzyme solution (44 IU/ml) were added to each of the above suspensions and then the mixtures were incubated at 40°C on a shaker.

Symbols: ○, 10% raw corn starch; ◐, 20% raw corn starch; ●, 30% raw corn starch.

FIG. 8. High Performance Liquid Chromatograms of the Supernatants of Reaction Mixtures Obtained in the Experiments in Fig. 7.

G₁, G₂ and IM denote glucose, maltose and isomaltose, respectively. Column, Shodex NHpak J-411; eluent, acetonitrile–water (75:25); detector, Erma Optical Works, Ltd., model ERC-7510.

hydrolysis of raw starch. A suspension of 10% (w/v) raw corn starch was almost completely hydrolyzed within 8 hr. When the raw starch content of the mixture was increased to 20 and 30% (w/v), the saccharification started a little later than in the case of the 10% suspension, and 100 and 90% hydrolysis was attained, respectively, after 48 hr incubation. The supernatants of the reaction mixtures were analyzed

by high performance liquid chromatography after 8 and 48 hr incubations. Figure 8 shows the oligosaccharide contents of each supernatant. The major hydrolysis product was glucose and only a very small amount of oligosaccharides was detected at the early stage of the hydrolysis process (8 hr). Glucose was also the major hydrolysis product at the end of the reaction, however, a detectable

amount of isomaltose was found in the hydrolyzates after 48 hr incubation. An increase in the isomaltose content was observed in a suspension of raw corn starch when it was hydrolyzed at a higher concentration or with a prolonged incubation time. These results suggest that the production of oligosaccharides is due to the reaction of glucoamylase in the reverse direction.

These results show that *C. rolfii* is a promising source of a saccharifying enzyme that is capable of hydrolyzing raw starch to glucose rapidly, even with a high substrate concentration.

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