Screening for Raw Starch Digesting Bacteria

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Screening for raw starch digesting bacteria revealed that bacteria producing amylase, which hydrolyzes raw starch, are widely distributed in nature. From among 155 raw starch digesting bacterial isolates, 20 strains were selected and examined as to both the characteristics of their enzymes and some bacteriological properties. According to the action patterns of their enzymes on starch, these strains could be divided into two groups, i.e., exolytic and endolytic amylase producers. The phenotypic properties of a representative bacterial isolate belonging to each group were examined. One of them (isolate TB1012) was assigned to *Bacillus polymyxa* (Prazmowski) Mace, which produced an exolytic β -amylase. The other (isolate B1018) was identified as *Bacillus* sp., which produced an endolytic amylase giving a single activity band corresponding to an approximate molecular weight of 75,000 on SDS-polyacrylamide gel electrophoresis.

In the course of conventional enzymatic saccharification, starch has to be gelatinized at first by cooking at a rather high temperature and liquefied with α -amylase at the same time. In recent years, the importance of enzymatic saccharification of raw starch without heating has become well recognized, mainly from the viewpoints of energy saving and effective utilization of the biomass. Although effort has been made to achieve more efficient breakdown of raw starch, it is still worthwhile to search for a very active enzyme and to develop an efficient production system with such an enzyme.

It is more difficult for amylases to act on raw starch granules than on gelatinized starch.¹⁾ Previous studies indicated that the saccharification of raw starch by amylolytic enzymes might be related to the extent of adsorption of an enzyme to the starch granules. Pancreatic α -amylase and fungal glucoamylase adsorbed well to starch granules, which resulted in effective digestion of raw starch.^{2,3)} Although mi-

croorganisms reported to be good producers of amylase capable of digesting raw starch have mostly been fungi, such as *Aspergillus* sp.³⁾ *Rhizopus* sp.^{4,5)} and *Corticium rolfsii*⁶⁾, there have been reports of the possibility of raw starch degradation by bacterial α - and β -amylases.^{7~9)}

Our primary research aim here was to find novel bacterial amylases which efficiently hydrolyze raw starch, for practical use in the starch industry. By cloning the genes coding for such amylases and analyzing their structures, we may expect the very efficient production of raw starch digesting amylases through expression of the genes or genetically engineered genes in a host-vector system such as the *Bacillus brevis* system.

Materials and Methods

Microorganisms. All the bacteria were isolated from soil and compost from various places.

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Media. The media used in the screening were as follows. Medium A contained (g/l): raw corn starch, 5; polypepton, 0.5; and yeast extract, 0.1. Medium B contained (g/l): raw corn starch, 5; $(\text{NH}_4)_2\text{SO}_4$, 0.5; urea, 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; K_2HPO_4 , 0.5; and yeast extract, 0.2. Raw starches were separately sterilized with ethylene oxide. Solid media contained 1.5% agar. T_{21} medium, used as the enzyme production medium, contained (g/l): polypepton, 10; meat extract, 5; yeast extract, 2; and raw corn starch, 5.

Screening method. Each soil sample was suspended at the approximate ratio of 1 g of moist sample to 5 ml of sterile water and then streaked onto the plates of the two screening media described above, of which the pH was adjusted to 4, 7 or 10. The plates were incubated at 30°C or 45°C for 72 hr. Bacteria that formed clear zones were purified by streaking on plates and single colonies were obtained. Each of the selected isolates was spotted onto screening agar plates containing 6 kinds of starch, which were kindly supplied by Dr. K. Kainuma of the National Institute of Food Science. They were corn, tapioca, potato, rice, wheat and soluble potato starches. After cultivation of the bacteria, the widths of the clear zones were determined. The isolates that formed rather large clear zones on starch agar plates were selected.

Enzyme Production. Each isolate was grown aerobically at 37°C or 45°C for 36 hr in 30 ml of T_{21} medium. After removal of cells by centrifugation (5000 rpm for 15 min at 4°C), the supernatant was used to assay amylase activity.

Assaying of amylase activity. A reaction mixture, consisting of 1 ml of a 0.5% (w/v) raw corn starch suspension in 20 mM phosphate buffer, pH 7.0, and 1 ml of the enzyme solution, was incubated with gentle shaking at 37°C or 45°C for 4 hr. After removal of residual starch granules by centrifugation, the reducing sugar liberated was determined by the dinitrosalicylic acid method with glucose as the standard.¹⁰ One unit of saccharifying activity was defined as the amount of enzyme which liberated 1 mg of reducing sugar in 1 hr under the assay conditions.

The soluble starch digesting activity was determined at 40°C by measurement of the dextrinizing power according to Fuwa.¹¹ The hydrolysis of 1 mg of soluble starch in 1 hr was defined as 1 unit of enzyme activity.

Action patterns on substrates. Multiple-ascend paper chromatography was performed for analysis of the products of enzymic hydrolysis as described by Kainuma and French.¹² The substrates used were soluble and raw corn starch, α -cyclodextrin and β -cyclodextrin, at a concentration of 0.5%. One part of the enzyme solution was added to four parts of a substrate solution, except for raw corn starch, where the enzyme solution to substrate solution ratio was 1:1. The reactions were carried out at pH 7.0 and 45°C. Aliquots were withdrawn at various times

and then boiled for 10 min to stop the reaction. When raw corn starch was used as the substrate, the reaction mixtures were centrifuged to remove residual starch granules before boiling. Twenty μ l of each sample was spotted on Whatman No. 1 paper. After three to five ascents at room temperature with the solvent system of 1-propanol-water (7:3, v/v), the chromatograms were developed according to a silver nitrate dip procedure following glucoamylase treatment of the paper.¹²

Other methods. Extracellular protease activity was assayed qualitatively. Bacteria were grown on the nutrient agar medium containing 0.5% of skim milk at 37°C or 45°C for 2 days and then 5% TCA was poured onto the plates. Apparent protease activity was indicated by a clear zone around a colony. Sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis was performed as described by Laemmli.¹³ The amylase band in the gel after electrophoresis was detected *in situ* as described by Lacks and Springhorn.¹⁴

Identification of the bacterial isolates. Cultural, morphological, physiological and biochemical characterization of the bacteria isolated was performed according to the methods recommended by Gordon *et al.*¹⁵ and Komagata.¹⁶ *Bacillus polymyxa* ATCC 842 (type strain) and *B. circulans* ATCC 4513 (type strain) were used as references. The results were analyzed using "Bergey's Manual of Determinative Bacteriology."¹⁷

Results

Screening for raw starch hydrolysing bacteria

From one sample under one set of screening conditions, about 20~400 microorganisms grew on a plate, but only 1~5 bacteria pro-

Table I. DISTRIBUTION OF RAW CORN STARCH DIGESTING BACTERIA

Screening medium	Temperature (°C)	Initial pH of the medium	Number of bacteria isolated	Percent of total isolates
A	30	4	31	20
	30	7	12	8
	30	10	9	6
B	30	4	19	12
	30	7	17	11
	30	10	14	9
A	45	4	—	—
	45	7	8	5
	45	10	11	7
B	45	4	—	—
	45	7	19	12
	45	10	15	10

Table II. CHARACTERISTICS OF SOME RAW STARCH DIGESTING BACTERIA

Strain number	Cultivation temperature	Clear zone on plate ^a	Raw starch saccharifying activity ^b ($\times 10^2$)	Soluble starch liquefying activity ^b	Probable amylase type ^c	Cell morphology		Gram stain	Spore formation	Catalase	Extracellular protease
						Shape	Size (μ)				
A 406	37°C	++	3.8	69	exo	Rod	$0.9 \times 1 \sim 2.5$	+	—	+	+
A 407	37°C	+	—	56		Rod	$0.6 \times 1 \sim 2$	—	—	+	—
A 1007	37°C	++	4.8	64	exo	Short rod	$0.3 \sim 0.5 \times 1$	+	—	+	—
A 1020	37°C	+	1.0	612	endo	Rod	$0.3 \times 1 \sim 1.2$	+	+	+	—
B 410	37°C	+	—	510	endo	Rod	$0.5 \sim 0.7 \times 1.5 \sim 2.5$	—	—	—	+
B 715	37°C	++	17	794	exo	Rod	$0.7 \sim 1 \times 2 \sim 3$	—	—	—	+
B 720	37°C	+	1.8	456	endo	Short rod	$0.3 \sim 0.5 \times 0.7 \sim 1.2$	+	+	+	+
B 1006	37°C	++	2.5	54	exo	Rod	$0.5 \times 1.5 \sim 4$	—	—	—	—
B 1007	37°C	+	2.2	482	endo	Short rod	$0.3 \sim 0.5 \times 1$	+	+	+	—
B 1012	37°C	+	—	12		Rod	0.5×1.5	+	—	+	+
TB 720	37°C	+	—	372	endo	Short rod	$0.5 \times 0.7 \sim 1.2$	+	+	+	+
TB 1012*	37°C	+	13	792	exo	Rod	$0.7 \sim 1 \times 2 \sim 5$	+	+	+	+
TB 1020	37°C	+	1.0	548	endo	Rod	$0.3 \times 1 \sim 1.2$	+	+	+	—
A 723	45°C	+++	5.5	34	exo	Rod	$0.5 \times 3 \sim 5$	+	—	+	+
B 724	45°C	+	15	728		Rod	$0.6 \times 1.5 \sim 3$	+	+	+	—
B 1009	45°C	+	10	756		Rod	$0.4 \times 2 \sim 4$	+	+	+	—
B 1017	45°C	+	—	778	endo	Rod	$0.5 \sim 0.7 \times 1 \sim 4$	+	+	+	+
B 1018*	45°C	++	13	2640	endo	Rod	$0.5 \sim 0.7 \times 2 \sim 5$	—	+	+	+
B 1019	45°C	++	4.2	360	exo	Rod	$0.6 \times 3 \sim 4$	—	+	—	—
B 1020	45°C	+	2.0	728	endo	Rod	$0.6 \times 1 \sim 3$	+	+	+	—

Raw Starch Digesting Bacteria

* Representative raw starch digesting bacteria studied in more detail.

^a Width of clear zones formed by the bacteria on raw corn starch containing plates. +, 1~2 mm; ++, 3~4 mm; +++, >5 mm.

^b The activity is expressed in units per ml of culture supernatant.

^c The type was determined on the basis of the starch digesting activities. An amylase showing higher saccharifying activity relative to the liquefying activity was regarded as being of the exo-type and one showing the opposite was assumed to be of the endo-type.

duced clear zones around their colonies on raw starch plates. Although the isolates were screened under different growth conditions, *i.e.*, with varying medium components, pH and temperature, probable duplicates, *i.e.*, bacteria isolated from the same sample and possessing the same colony morphology, were omitted. A total of 155 isolates were obtained from 25 soil and compost samples. Table I shows the numbers of raw starch hydrolysing bacteria isolated under the different conditions.

All the 155 isolates were then spotted onto screening agar plates containing six kinds of starches. Twenty isolates were selected which exhibited the ability to hydrolyse almost every kind of starch. After cultivation of these 20 isolates in liquid medium T₂₁, both soluble starch digesting and raw corn starch saccharifying activities were determined as described under **Materials and Methods** (Table II). Some of the isolates formed clear zones on the agar plates but did not show any raw starch saccharifying activity. This might be because the cultivation conditions were not yet optimal. The bacteria produced enzymes

which showed soluble starch liquefying activity and raw starch saccharifying activity of various levels. Some of the microbiological properties are also shown in Table II. Many good amylase producers were gram-positive rods and catalase positive. Some of them produced proteases. Two isolates, A1007 and TB1012, which produced amylases in higher amounts than other isolates grown at 30°C, and three strains, B724, B1009 and B1018, which were apparently good amylase producers among those isolated at 45°C, were selected for further studies.

Properties of amylases produced by the selected bacteria

The action patterns of amylases produced by strains B724, B1009 and B1018, which were isolated at 45°C, on soluble or raw corn starch were similar. The pattern of B1018 is shown in Fig. 1A. At early stages of the reaction, higher molecular-weight oligosaccharides were the major products. As the reaction proceeded, the quantities of G₁–G₄ increased. The enzyme could liberate a series of low molecular-weight saccharides when α - or β - cyclodextrin was

Fig. 1. Paper Chromatograms of the Products formed from Raw Corn Starch (A) and α -Cyclodextrin (B) by the B1018 Amylase.

Chromatography was performed as described under **Materials and Methods**. The culture supernatant of B1018 was concentrated 8 times by 20–80% ammonium sulfate precipitation and then used to digest raw corn starch. 2 to 7, aliquots of the reaction mixture withdrawn at 0, 10, 20, 30, 60 and 120 min incubation, respectively; M, standard oligosaccharides; 1, maltose.

used as the substrate. The product derived from α -cyclodextrin is shown in Fig. 1B. These observations suggest that the enzymes are of the endolytic type. In the case of strains A1007 and TB1012, which were isolated at 30°C, the action patterns on starch were different from those of the amylases of the bacteria (B724, B1009 and B1018) isolated at 45°C. The amylase of strain A1007 could be considered, from its action pattern, to be of the endolytic

type, but it digested neither α - nor β -cyclodextrin, even on prolonged incubation (data not shown). When the amylase of strain TB1012 was used, the main product at various stages of hydrolysis of soluble starch or raw corn starch was apparently maltose (Fig. 2). This enzyme liberated some low molecular weight oligosaccharides, mainly maltose, when β -cyclodextrin was used as the substrate, but did not liberate any when the substrate was α -cyclodextrin. Such different action patterns cause much difficulty when determining whether an enzyme is of the endolytic or exolytic type, and might suggest that A1007 and TB1012 produce more than one type of amylase.

Figure 3 shows that the activities toward various raw starches of two representative isolates, TB1012 and B1018, varied considerably, and they were weak in digesting raw potato starch, which is known to be often resistant to amylases of microbial, plant and animal origin.

The number of amylase activity bands as well as the extracellular protein composition were determined by SDS-polyacrylamide gel electrophoresis. The crude enzymes from strains B1018 and B724 each gave one activity band corresponding to an approximate molecular weight 75,000. The crude enzyme of strain TB1012 gave at least four activity bands with molecular weights ranging from 40,000 to 100,000 (Fig. 4B). Apparently, these bacteria

Fig. 2. Paper Chromatogram of the Products formed Raw Corn Starch by the TB1012 Amylase.

Chromatography was performed as described under **Materials and Methods**. The reaction mixture contained the culture supernatant of TB1012 and the raw corn starch suspension in the ratio of 1:1. 1, maltose; 2, standard oligosaccharides; 3 to 9, aliquots of the reaction mixture withdrawn at 0, 10, 20, 30, 60, 120 and 240 min, respectively.

Fig. 3. Comparison of the Digestibilities of Various Raw Starches.

The saccharifying activities of the amylases produced by TB1012 (A) and B1018 (B) toward each raw starch were analyzed as described under **Materials and Methods**. The relative activities are shown on the ordinate.

Fig. 4. SDS-Polyacrylamide Gel Electrophoresis of the Culture Supernatants of Raw Starch Digesting Bacteria.

Electrophoresis was performed as described under **Materials and Methods**. Panel A: Protein staining with Coomassie blue. Lane 1, marker proteins; lane 2, A1007; lane 3, TB1012; lane 4, B1018; and lane 5, B724. Panel B: in situ detection of amylase activity. Lane 1, TB1012; lane 2, B1018; and lane 3, B724. The arrow indicates the protein bands of B1018 and B724 which migrated the same distance as the amylase activity band.

produced various kinds of extracellular proteins (Fig. 4A).

Figure 5 shows the effect of temperature on the soluble starch digesting activity of the enzymes. A similar effect was seen on the raw starch saccharifying activity. The enzyme of strain B1018 showed relatively high thermal stability, nearly 100% amylase activity being retained even after incubation for 30 min at 55°C. Strain TB1012 produced amylases with a lower thermal stability, the enzyme being stable up to about 45°C.

Characterization of the two raw starch digesting bacteria

The characteristics of strains TB1012 and B1018 are shown in Table III. Chemotaxonomical studies and analysis of the DNA base composition, which were not performed in the present work, are necessary for definitive identification of these bacteria. Their phenotypic properties, see Table III, however, indicate that they both belong to the genus *Bacillus*. Isolate TB1012 is assigned to *Bacillus polymyxa* (Prazmowski) Mace, although

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

The culture supernatant was made to 10 mM CaCl_2 , 50 mM Tris-HCl, pH 7.5, by adding the respective concentrated solutions, followed by incubation at different temperatures for 30 min. The residual activity was assayed after cooling to room temperature. ○, B1018; △, B724; ×, TB1012.

it has some characteristics that are different from those of *B. polymyxa*; i.e., it did not reduce nitrate and did not form gas from D-xylose. On the other hand, isolate B1018 was similar to *B. circulans* in some respects, but could not be identified as a definite species. Although *B. circulans* could not grow above 50°C, B1018 grew even at 55°C. B1018 did not produce acid from fructose, trehalose or

Table III. PHENOTYPIC CHARACTERIZATION OF STRAINS TB1012 AND B1018

	TB1012	B1018
Cultural characteristics		
Nutrient agar colonies:	Moderate growth, smooth, round, yellowish-grey, flat	Good growth, smooth, rhizoid, pale yellow-brown, flat
Nutrient agar slant:	Moderate growth, smooth or slightly rough, yellowish-grey	Moderate growth, smooth or slightly rough, yellowish-grey
Nutrient broth:	Slight turbidity, formation of a pellicle and a tiny amount of precipitate	Slight turbidity, no pellicle formation, a small amount of precipitate
Gelatin stab:	Growth on the surface, some liquefaction	Growth on the surface, some liquefaction
Litmus milk:	No reduction, liquefaction	No change
Morphological characteristics		
	Rods, $0.7 \sim 0.9 \times 3 \sim 4 \mu\text{m}$	Rods, $0.5 \sim 0.7 \times 2 \sim 5 \mu\text{m}$
	Non-pleomorphic	Non-pleomorphic
	motile, peritrichous flagella	motile, peritrichous flagella
	endospore forming, heat resistant	endospore forming, heat resistant
	Spores: spherical, $1 \sim 1.2 \mu\text{m}$	Spores: spherical, $0.8 \sim 1 \mu\text{m}$, central or terminal location, swollen sporangium
	central location, swollen sporangium	gram negative and non-acid fast
	gram positive and non-acid fast	
Physiological and biochemical characteristics		
Nitrate reduced to nitrite:	Negative	Negative
Denitrification:	Negative	Negative
V-P test:	Positive	Negative
pH in V-P broth	< 6	< 6
MR test:	Negative	Positive
Catalase:	Positive	Positive
Oxidase:	Negative	Negative
Urease:	Negative	Negative
Formation of		
Indole	Negative	Negative
Hydrogen sulfide	Positive	Positive
Utilization of		
Citrate	Negative	Negative
Nitrate or ammonia	Positive	Positive
O-F test:	Fermentation	No decomposition
Oxygen relation:	Facultative anaerobic	Aerobic
Growth range:	pH 6~8 up to 43°C	pH 6~8.5 up to 55°C
pH and temperature for good growth:	pH 6, 30~40°C	pH 6, 35~45°C
Growth in NaCl	No growth in about 2%	Grow in 2%, no growth in about 5% NaCl
Water soluble pigment:	Negative	Negative
Hydrolysis of casein:	Positive	Positive
Hydrolysis of starch:	Positive	Positive
Hydrolysis of DNA:	Negative	Negative
Acid or gas production from sugars:		
	Both acid and gas from D-glucose, L-arabinose, D-mannose, D-galactose, maltose, sucrose, lactose, trehalose, D-mannitol, glycerol, starch, D-fructose. Acid but no gas from D-xylose, D-sorbitol. No acid and no gas from inositol.	Acid but no gas from D-glucose, L-arabinose, D-mannose, D-galactose, maltose, sucrose, lactose, starch, D-xylose. No acid and no gas from D-fructose, trehalose, D-sorbitol, D-mannitol, inositol, glycerol,

mannitol, whereas *B. circulans* ATCC 4513 did. Tentatively, we named it *Bacillus* sp. strain B1018.

Discussion

On screening, it became clear that raw starch digesting bacteria are widely distributed in nature and are able to grow under various growth conditions, such as different temperatures and pHs (Table I). However, when a medium with an acidic initial pH was used, such bacteria were isolated from among those grown at 30°C, but not those grown at 45°C. During the course of screening, it was observed that many fungi and streptomyces-like organisms formed large clear zones on raw starch plates. Since the next aim of our research was to analyze the gene for a raw starch digesting enzyme, we only selected bacteria which produce a raw starch digesting amylase, the gene for which may be easy to analyze.

The starch liquefying and saccharifying activities of the amylases produced by the selected bacteria suggested that there are mainly two groups of bacteria, one of which produces an exolytic amylase and the other an endolytic amylase (Table II). Strain TB1012 was identified as *B. polymyxa* and produced an enzyme which exclusively formed maltose from starch (Fig. 2). *Bacillus polymyxa* is known to produce β -amylase, which splits starch into maltose in a stepwise fashion from its non-reducing end, and to produce multiple β -amylases with different molecular weights.¹⁸⁾ Therefore, TB1012 is most likely a β -amylase producing bacterium.

On the other hand, strain B1018 produced an amylase which appeared to split starch endolytically (Fig. 1) and gave one activity band upon SDS-polyacrylamide gel electrophoresis, as shown in Fig. 3B. Although there have been a few reports on raw starch digesting amylases produced by bacteria,^{7,9,19)} they seem to differ from that produced by *Bacillus* sp. B1018 in some respects, such as the action patterns on starches and molecular weight. Although the B1018 amylase can digest raw

potato starch only poorly, the latter is well degraded by the amylase of *B. circulans*.⁸⁾ The microbiological properties of B1018 are also different from those of raw starch digesting *B. circulans*⁸⁾ and *B. subtilis*¹⁹⁾ (Table III). A detailed study on the B1018 enzyme will be described in a subsequent report.

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