ISOLATION OF NEW THERMOPHILIC AEROBIC BACTERIA WHICH PRODUCE THERMOSTABLE α -AMYLASE

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Three strains of endospore forming, thermophilic and aerobic bacteria tentatively named JF₁, JF₂ and D strains were isolated from Chinese traditional koji. These strains were gram positive and belonged to the genus *Bacillus*. The optimal growth temperature was 55 to 65°C; and the optimal pH, 6 to 8. They produced extracellular thermostable α -amylase in media containing lactose, galactose, raffinose, ethanol or other carbohydrates.

It is suggested that strains JF and D are two new species of the *Bacillus* genus.

α -Amylase (EC 3.2.1.1, α -1,4-glucan-glucanhydrolase) is one of the enzymes which are produced industrially in large amounts. It hydrolyses the endo- α -1,4-linkages of starch at random by bypassing the α -1,6 linkages. The occurrence of such amylolytic enzymes in bacteria, molds and yeast is well known (2, 4). Recently, the thermostable α -amylase from thermophilic microorganisms has attracted attention (3, 11) because of the remarkable properties of these microorganisms are associated with a rapid rate of cell growth, and the possibility of economically cooling water in the enzyme production process; the thermostable α -amylase is effectively used in high-temperature industry for starch hydrolysis to reduce the viscosity of starch paste.

This paper reports some new thermophilic aerobic bacteria which produce thermostable α -amylase. They were isolated from Chinese traditional koji (Daqu in Chinese; a hard liquor from grain such as wheat is cultured commonly in the open, usually at 60–70°C) which is used to brew the Chinese traditional sake (Baiju in Chinese). Their α -amylase production was studied.

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MATERIALS AND METHODS

Media and cultivation. The medium for the microorganism isolation contained (per liter): peptone, 2 g; K_2HPO_4 , 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $FeCl_3$, 0.01 g; $MnSO_4$, 0.001 g; and soluble starch, 4 g; the pH was 7.2.

The medium used for the α -amylase production contained (per liter): peptone, 3 to 5 g; carbohydrate, 3 to 5 g; K_2HPO_4 , 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $FeCl_3$, 0.01 g; $MnSO_4 \cdot 7H_2O$, 0.001 g; pH 7.2; culture temperature, 60°C.

Isolation method. The JF and D strains were isolated from the koji of the Liaoning Sake Plant, China.

Enrichment cultures were initiated by inoculating 0.2 to 0.4 g of source materials into a flask (250 ml) containing 45 ml of soluble starch medium, and incubated at 60°C for 1 to 2 days, 0.1–0.2 ml of the culture was then transferred to 45 ml of starch medium. This operation was carried out several times.

The enrichment culture was diluted with 0.9% NaCl liquid and spread on the agar-starch plates (agar, 2.2%; soluble starch, 0.3 to 0.4%), then incubated at 60°C for 12 to 18 h. The positive colonies were obtained by flooding the plates with iodine solution. These were cultured, and the α -amylase production was examined.

Measurement of α -amylase activity. α -Amylase activity was determined by the Yoo method (10). A soluble starch (Hongxing Chemicals, China) solution was prepared by dissolving 1.33 g of starch in 100 ml of 0.01 M acetate buffer, pH 5.6. Fresh working iodine reagent was prepared by mixing 1.0 ml of stock solution (1.2 g iodine and 5 g potassium iodate/100 ml water) with 10 ml of 1 N HCl solution, and diluting to 300 ml.

A 1.5 ml solution of soluble starch was placed in a test tube and incubated at 60°C for 5 min, then 0.5 ml of enzyme solution was added. After the enzyme reacted for 10 min, 0.1 ml of the reaction mixture was added to 10 ml of the working iodine reagent, and the absorbance at 680 nm was measured.

One unit of α -amylase was defined as the amount of enzyme which reduced 10% of the absorbance shown by 1 mg of soluble starch blue color per minute.

Cell concentration. The dry cell concentration of a culture broth was measured with the method of harvesting the cells by centrifugation and drying them at 105°C to a constant weight.

Characterization of the organisms. The gram reaction, hydrogen sulfide production, catalase and oxidase production, nitrate reduced to nitrite, indole production, gelatin digestion, milk reaction and other taxonomic features of the test microorganisms were all determined by the methods described by Smibert and Krieg (8) and by the methods described in Bergey's Manual of Systematic Bacteriology, Vol. 2 (9).

The gram reaction was determined by the method of Hucker (8).

Hydrogen sulfide production was tested with lead acetate filter paper (8). The medium contained 0.2% tryptone (Difco) 0.05% L-cysteine, 0.3% maltose, 0.005% K_2HPO_4 , 0.03% $MgSO_4 \cdot 7H_2O$, 0.0001% $FeCl_3$. Three ml of the medium was

added to a test tube, and the lead acetate paper was hung on the cotton stopper. After inoculation, the samples were incubated at 60°C for 12 to 24 h to observe the blackening of the lead acetate paper.

Catalase was determined by adding 1.5 ml of 3% hydrogen peroxide to the same volume of the culture to observe continuous bubbling (8).

Oxidase was examined by adding a few drops of 1% α -naphthol in 95% ethanol and a few drops of 1% aqueous dimethyl-*p*-phenylenediamine oxalate to 3 ml of culture to observe the appearance of purplish blue (8).

Acid production from carbohydrate was determined with bromcresol purple medium (9) containing 0.5% carbohydrate.

Milk reaction was tested using litmus milk (8).

Gelatin hydrolysis was examined with a medium containing 12% gelatin (8). After inoculation and incubation at 60°C for 1 to 2 days, the cultures were chilled in a refrigerator and the solidification was tested.

Indole production was tested with a medium containing 1% tryptone (Difco) (8). After incubation at 60°C for 2 days, the Kovacs reagent was added to the culture to observe the red color.

Nitrate reduction was examined with a nitrite test reagent (8). The test broth was contained 0.5% succinate, 0.005% K_2HPO_4 , 0.1% $NaNO_3$, 0.03% $MgSO_4 \cdot 7H_2O$, 0.02% yeast extract (Difco) and 0.0001% $FeCl_3$. After incubation at 60°C for 1 to 2 days, a few drops of nitrite reagent was added to 3 ml of culture to observe the red color.

Esculin hydrolysis was tested with a medium containing 0.01% esculin and 0.05% ferric ammonium citrate. After incubation at 60°C for 1 to 2 days, the blackening of the culture was observed.

G + C content of DNA. DNA was isolated by the method of Saito and Miura (7). The DNA guanine-plus-cytosine (G + C) mol% content was estimated by the method of thermal denaturation temperature (T_m). The thermal denaturation temperature was the temperature which produced a 50% increase in hyperchromicity at 260 nm. The G + C mol% content was calculated using the equation of Marmur and Doty (6).

RESULTS AND DISCUSSION

Taxonomic features

Three strains of endospore-forming, aerobic, thermophilic and amylolytic bacteria were isolated from traditional Chinese koji. The characteristics of these microorganisms, symbolized as JF₁, JF₂ and D strains, are shown in Tables 1, 2.

In 0.3% starch broth after incubation at 60°C for (Figs. 1, 2), the rods of these strains were: JF₁ strain, 0.5 to 0.9 μm wide by 2.5 to 5.0 μm long; JF₂ strain, 0.5 to 0.9 μm wide by 1.9 to 5.0 long; D strain, 0.6 to 0.9 μm wide by 2.0 to 3.0 μm long. These strains form terminal spherical endospores (diameter: 0.9 to 1.3 μm for JF₁ and JF₂ strains, 0.9 to 1.2 μm for D strain). They are strict aerobes, do not

Table 1. Taxonomic features of the new strains.

Characteristics	JF ₁	JF ₂	D
Rods wide (μm)	0.5–0.9	0.5–0.9	0.5–0.9
long (μm)	2.5–5.0	1.9–5.0	2.0–3.0
Gram reaction	+	+	+
Acid fast	—	—	—
Growth temperature ($^{\circ}\text{C}$)	40–70	40–70	30–65
Growth pH range	5–10	5–10	5–10
H ₂ S production	+	+	+
Catalase	+	+	—
Oxidase	+	+	+
Nitrate reduced to nitrite	+	+	—
Indole production	—	—	—
Milk reaction	+	+	+
Gelatin digestion	+	+	+
Esculin hydrolysis	+	+	+
Growth in 7% NaCl	—	—	—
Growth in 0.02% sodium azide	w	w	w
G+C mol% content of DNA	57.8	53.7	49.3

Symbols: +, positive reaction; —, negative reaction; w, weak reaction.

Table 2. Acid production from carbohydrates.

	JF ₁	JF ₂	D
D-Glucose	+	+	+
L-Arabinose	+	+	+
D-Xylose	+	+	+
D-Fructose	+	+	+
D-Galactose	+	+	(—)
Maltose	+	+	+
Lactose	(—)	+	(—)
Melibiose	(—)	(—)	(—)
Sucrose	+	+	(—)
Starch	+	+	+
Cellulose	—	—	—
Ethanol	(—)	(—)	+

Symbols: +, positive reaction; —, negative reaction; (—), acid production is negative, but cell growth is good.

Batch culture at 60°C for 2 to 3 days; 0.4% carbohydrate.

grow under anaerobic condition, and are gram positive. Colonies on starch or maltose agar medium are smooth and about 2 mm in diameter after growth at 60°C for 12 to 18 h. The optimal growth temperature for JF₁ and JF₂ strains is 60 to 65°C, but growth occurs at 40 to 70°C. The optimal growth temperature of D strain is 55 to 60°C, but growth occurs at 30 to 65°C. The growth pH range for three strains is 5.0 to 10, the optimum pH is 6.0 to 8.0 for three strains. The three strains

Fig. 1. Electron microphotograph of JF₂ strain. Bar = 1.0 μ m.

Fig. 2. Electron microphotograph of D strain. Bar = 0.5 μ m.

produce oxidase. The JF₁ and JF₂ strains produce catalase, but the D strain does not. Gelatin is digested. Esculin is hydrolyzed. The JF₁ and JF₂ strains reduce nitrate to nitrite, but the D strain does not. Indole is not produced. The litmus milk reaction for the three strains is acid curd, proteolysis and reduction of litmus. Growth is weak in medium containing 0.02% sodium azide. The three strains do not grow in the medium containing 7% NaCl. The G + C mol% content is 57.8 for JF₁ strain, 53.7 for JF₂ strain, and 49.3 for D strain (Table 1).

All three strains utilize sugars such as D-glucose, L-arabinose, D-xylose, D-fructose, D-galactose, maltose, lactose, melibiose, sucrose, raffinose and ethanol; they hydrolyze starch, but do not digest cellulose. JF₁ strain produces acid from D-glucose, L-arabinose, D-xylose, D-fructose, D-galactose, maltose, sucrose and starch, but not from lactose, melibiose, raffinose and ethanol. The JF₂ strain produces acid from D-glucose, L-arabinose, D-xylose, D-fructose, D-galactose, maltose, sucrose and starch; but not from melibiose, raffinose and ethanol. The D strain produces acid from D-glucose, L-arabinose, D-xylose, D-fructose, maltose, ethanol and starch, but not from D-galactose, lactose, melibiose, sucrose and

raffinose (Table 2).

Comparing the above taxonomic features with those of the endospore-forming genus described in Table 13.1 of Bergey's Manual of Systematic Bacteriology, Vol. 2, p. 1104(9), the three strains, characteristics are the same as those of *Bacillus* genus except for the hydrogen sulfide production: the *Desulfotomaculum* genus produces hydrogen sulfide, but this genus is strictly anaerobic. Therefore, these strains are clearly members of the *Bacillus* genus.

Comparison of JF₁, JF₂ and D strains with other similar strains of Bacillus genus

Two species of the genus *Bacillus* described previously, *Bacillus stearothermophilus* (1, 9) and *Bacillus caldolyticus* (5, 9), are similar to our strains (Table 3).

B. stearothermophilus grows at 65°C; but does not grow at pH 5.7 or in 0.02% sodium azide medium, and it does not produce hydrogen sulfide. This clearly differentiates it from our strains.

B. caldolyticus grows at 72°C; but does not grow at pH 5.7 and in 0.02% sodium azide medium, and it does not produce hydrogen sulfide or catalase. This differentiates it from our strains.

Unlike *B. stearothermophilus* and *B. caldolyticus*, our new strains produce hydrogen sulfide, grow at pH 5.7 and grow weakly in the medium containing 0.02%

Fig. 3. Effects of nitrogen source on α -amylase production.

Strain, JF₁; batch culture at 60°C for 2 days in the 0.3% and 0.05% soluble starch medium; 100% enzyme, about 12,000 U/ml.

Symbols: 1, tryptone; 2, peptone; 3, yeast extract; 4, beef extract; 5, (NH₄)₂SO₄; 6, urea.

sodium azide. Compared with JF₁ and JF₂ strains, strain D grows at 30°C and does not reduce nitrate to nitrite, catalase is negative, rods are shorter, G + C mol% content is 49.3. This differentiates it from the JF₁ and JF₂ strains. Therefore, D strain is a different species from JF₁ and JF₂ strains.

For the above reasons, it appears that the JF strains and D strain are two different species of *Bacillus* genus.

Effects of nitrogen on α -amylase production

The effects of different nitrogen sources, such as tryptone, peptone, yeast extract, beef extract, (NH₄)₂SO₄ and urea, on α -amylase production were examined in the medium containing 0.3% nitrogen source, 0.3% lactose and 0.05% soluble starch (Fig. 3).

The high α -amylase production was observed in the media containing organic nitrogen, such as tryptone, peptone, yeast extract and beef extract; but the enzyme production failed in media containing (NH₄)₂SO₄ or urea. When the lactose of the medium was replaced by maltose, the effect on α -amylase production was similar to that of the lactose medium. The effects of nitrogen sources on α -amylase production by JF₂ were similar to those by the JF₁.

Carbohydrate effects on α -amylase production

The α -amylase production was closely related to the carbohydrate sources (Table 4). When the medium contained different carbohydrates, the enzyme production was markedly different. The α -amylase production of JF₁ strain was 15,300 U/ml in raffinose medium; more than 10,000 U/ml in media with maltose, lactose, melibiose and ethanol; and less than 126 U/ml in the media with D-fructose, L-arabinose and sucrose. The α -amylase production was in the order of raffinose > lactose, maltose, ethanol > D-glucose, D-galactose, D-xylose, starch > D-fructose, L-arabinose, sucrose.

The α -amylase production of JF₂ strain was 17,100 U/ml with raffinose; more than 10,000 U/ml with D-galactose, lactose, or ethanol; and less than 100 U/ml with

Table 3. Comparison of taxonomic features.

	JF ₁	JF ₂	D	<i>B. stearothermophilus</i>	<i>B. caldolyticus</i>
Growth at 65°C	+	+	(+)	+	+
30°C	—	—	+	—	—
Catalase	+	+	—	d	—
Growth at pH 5.7	+	+	+	—	—
Growth in 0.02% sodium azide	w	w	w	—	—
Nitrate reduced to nitrite	+	+	—	d	+
H ₂ S production	+	+	+	—	—
G + C mol% content of DNA	57.8	53.7	49.3	51.3(Type)	52.3

Symbols: +, positive reaction; —, negative reaction; (+), maximum; d, 11–89% strain positive; w, weak growth.

D-glucose or D-fructose. Therefore, the order of α -amylase production was raffinose > lactose, D-galactose, ethanol > starch, maltose, D-xylose, L-arabinose > sucrose > D-glucose, D-fructose.

The α -amylase production for D strain was only 5 to 15% of that of JF₁ and JF₂ strains.

The thermostability of α -amylase was examined. The optimum temperature of α -amylases from the three strains was 88 to 95°C; when incubated at 80 to 85°C

Table 4. α -Amylase production in different carbohydrate media.

	JF ₁		JF ₂	
	Enzyme activity (U/ml)	Cells (mg/ml)	Enzyme activity (U/ml)	Cells (mg/ml)
D-Glucose	7,580	6.0	n	5.4
D-Fructose	126	4.9	n	4.8
D-Galactose	6,820	5.2	10,080	5.4
L-Arabinose	n	5.7	1,110	4.8
D-Xylose	3,400	6.0	2,760	5.0
Maltose	10,800	5.2	2,460	5.2
Sucrose	n	4.0	399	3.5
Lactose	10,500	4.2	10,400	5.9
Raffinose	15,300	4.9	17,100	4.7
Starch	4,600	4.1	3,500	4.0
Ethanol	10,100	4.0	10,800	4.2
Carbohydrate free	n	0.7	n	0.8

Carbohydrate, 0.4%. Batch culture at 60°C for 24 to 48 h.

Symbol: n, α -amylase was less than 100 U/ml.

Fig. 4. α -Amylase production by JF₁ strain. Batch culture at 60°C; carbohydrate, 0.4%.

Fig. 5. α -Amylase production by JF₂ strain. Batch culture at 60°C; carbohydrate, 0.4%.

for 3 h at pH 6.5 and 0.02 M acetate buffer containing 0.005 M CaCl₂, there was hardly any enzyme activity reduction recognized; when incubated at 100°C for 10 min, the enzyme activity was remained more than 15%. So the enzymes from the three strains are thermostable.

The α -glucosidase (EC. 3.2.1.3) production was weak in the cultures of the three strains with the *p*-nitrophenyl- α -glucoside (nacalai Chemicals) as a substrate. The cultures of the raffinose medium rapidly hydrolyzed 2% amylose (Hongxing Chemicals, China), as shown by the iodine reagent, but there was hardly recognized reducing sugar after reaction for 10–30 min. Therefore, the amylase was clearly α -amylase.

The time course of enzyme production for JF₁ and JF₂ strains were examined (Figs. 4, 5). The highest α -amylase activity by JF₁ strain occurred after 45 to 50 h of culture; the enzyme production was associated with the cell growth in media with raffinose, lactose and maltose. The highest α -amylase activity by JF₂ strain occurred after 24 h culture, and this enzyme production was also associated with the cell growth.

Comparison of three strain

The enzyme productivity of the JF strains was markedly higher than that of the D strain; the enzyme production of JF₂ was more rapid than that of JF₁; in all three strains, α -amylases were thermostable.

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