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Isolation of Thermostable α-Amylase Hyperproducing *Bacillus* sp. No. 32H417 and Some Properties of the Enzyme

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耐熱性 α-Amylase 高 生產性 *Bacillus* sp. No. 32H417의 分離 및 酵素 特性

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Abstract — A bacterial strain No. 32 which produced thermostable α-amylase was isolated from soil and identified to genus of *Bacillus*. To enhance α-amylase productivity, a successive mutation of *Bacillus* sp. No. 32 was attempted with treatment of N-methyl-N'-nitro-N-nitrosoguanidine (NTG). The resulting mutant, *Bacillus* sp. No. 32H417, which is risistant to refampicin and deficient in spore formation, produced about 90-fold high level of α-amylase when compared with parental strain. The properties of the enzyme for thermostability were investigated. The optimal temperature and pH for enzyme reaction were 95℃ and pH 6.5, respectively, in the presence of 0.3 mM Ca^{2+} as an effective stabilizer.

The α-amylase (EC 3.2.1.1) with thermostable property has been focused on lately, because of its increasing application in food, adhesive, sugar production, textile and paper industries. Production of thermostable α-amylase were reported in many bacterial species; both thermophilic and mesophilic *Bacillus* sp. (1, 2), *Thermomonospora* sp. (3), *Thermoactinomyces* sp. (4) and *Streptomyces* sp. (5). But industrial applications were mainly carried out with the thermostable enzyme produced by *Bacillus* sp.. With increasing application of thermostable α-amylase, enhancement for productivity and thermostability of the enzyme has been required. Stepwise increase of enzyme productivity by a series of mutation of *B. licheniformis* with treatment of NTG was tried (6), and increase of enzyme thermostability was also reported in mutant strain of *B. licheniformis* (7). Furthermore, Bajpai *et al.* (8) isolated an alkaline thermostable α-amylase and Buonocore *et al.* (9) described about acid thermostable enzyme. In this paper, we described the isolation of thermostable α-amylase producers, the enhancement of enzyme productivity by mutation and some characteristics of the enzyme for thermostability.

Key words: Thermostable α-Amylase, *Bacillus* sp., NTG
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Materials and Methods

Media

For isolation and preservation of bacteria, BY medium consisting of 0.5% (w/v) meat extract, 1% peptone, 0.2% NaCl, 0.2% yeast extract and 2% soluble starch supplemented with 1.5% agar was used. Cell cultivation and enzyme production was carried out in ABY medium consisting of 1% meat extract, 2% peptone, 0.4% NaCl, 0.4% yeast extract and 10% soluble starch. The pH of both media were adjusted to be 7.0 with 0.01 N NaOH before autoclaving.

Isolation of thermostable α-amylase producing bacteria

Soil samples were diluted and plated on BY agar medium. After incubation for 24 hrs at 37℃, 0.01 M KI · I_2 solution was sprayed on the plates, and colonies with comparatively large clear zone were selected as α-amylase producers. Selected strains were cultivated in 30 ml ABY media in 125 ml Erlenmeyer flasks at 37℃ for 72 hrs and the culture broths were used directly for assay of α-amylase activities at 40℃ and 85℃, respectively. Strains, which showed higher α-amylase activities at 85℃ than 40℃, were selected as thermostable α-amylase producers. Isolated strains were cultured on BY slant for 30 hrs at 37℃, and preserved at 4℃.

Identification of bacterial strain No. 32

The highest thermostable α-amylase producing strain No. 32 was tested for its morphological and physiological characteristics, and identified based on "Bergey's Manual of Determinative Bacteriology" (10).

Mutagenesis

Cells of *Bacillus* sp. No. 32 were cultured in ABY medium for 16 hrs at 37℃ and harvested by centrifugation at 5000×g for 15 minutes. Harvested cells were washed with 0.05 N Tris(hydroxymethyl)aminomethane-maleate buffer (pH 7.0) and treated with NTG (50 μg/ml) for 20 minutes at 37℃ with gentle shaking. NTG-treated cells were washed 3 times with same buffer and plated on BY agar media. Strains were selected by the same method used for isolation of thermostable α-amylase producers. For isolation of antibiotics resistant mutants, NTG-treated cells were plated on BY agar media supplemented with various antibiotics as follows; ampicillin (1 μg/ml) , D-cycloserine (100 μg/ml), penicillin (2 μg/ml), rifampicin (20 μg/ml) and tunicamycin (5 μg/ml).

Cultivation of *Bacillus* sp. No. 32H417 in 30 L Jar Fermenter

The patterns of enzyme production and cell growth in ABY medium were studied in 30 l Jar Fermenter (Marubishi Model MSJ-U3). Culture medium 15 l was inoculated with precultured cells in 150 ml of ABY medium for 24 hrs at 37℃ with rotatory shaking. The cultivation temperature was 37℃ and the initial pH was adjusted to be 7.0 with 0.1 N NaOH. The aeration rate was maintained at 0.3 vvm and the agitation speed was kept at 300 rpm.

Preparation of enzyme

For investigation of the properties of α-amylase, 500 ml of culture broth was centrifuged at 5000 ×g for 20 min and solid ammonium sulfate was added to the supernatant to be the final concentration of 75%. The precipitate was dissolved in 50 ml of 0.01 M acetate buffer (pH 6.0) and dialyzed against the same buffer for about 24 hrs below 4℃. The dialyzed enzyme solution was used at once or lyophilized for further experiments.

Analytical procedures

α-Amylase activity was determined by measuring the decrease in iodine color reaction. Mixture of enzyme solution (1 ml) and 1% soluble starch solution (10 ml, pH 6.0, 0.1 M acetate buffer) was incubated at 40℃ for 10 minutes. After incubation, 0.5 ml of the mixture was added to 10 ml of 0.1 N HCl. And then 1 ml of this solution was added to 10 ml of iodine solution (0.05% iodine and 0.5% KI). The optical density of the solution was measured at 660 nm. One unit of enzyme activity was defined as the quantity of enzyme that causes 1% reduction of blue color intensity of starch-iodine solution at 40℃ per 1 min.

Cell growth was estimated by measuring the optical density at 625 nm with appropriately diluted sample. Total sugar content of the culture medium was estimated by phenol-H_2SO_4 method (11).

Results and Discussion

Identification of bacterial strain

The selected strain No. 32 was Gram positive, spore forming, motile rod and possibly identified as genus of *Bacillus* with subsequent test of characteristics as shown in Table 1 and Fig. 1.

Isolation of α-amylase hyperproducing strain *Bacillus* No. 32H417

With successive mutation of *Bacillus* sp. No. 32, a mutant strain No. 32H which produced α-amylase by 9 times as much as parental strain was isolated. Using *Bacillus* sp. No. 32H, another mutation was done and mutants resistant to minimum concentration of ampicillin, D-cycloserine, rifampicin, penicillin and tunicamycin were isolated.

From about 1800 rifampicin resistant mutants, 98 colonies appeared to show hyper-productivities of α-amylase. Among them, mutant No. 32H417, which producd α-amylase 90 times more than parental strain, was isolated and used in further experiments.

From about 2,100 D-cycloserine resistant mutants and 580 tunicamycin resistant mutants, 108 colonies and 19 colonies appeared to be α-amylase hyper-producers, respectively. But these strains did not produced α-amylase as much as strain No. 32H417. None of hyperproducer was isolated from about 1,100 penicillin resistant mutants and 720 ampicillin resistant mutants.

Five strains with the highest α-amylase activities among tested strains were listed on Table 2 and the growing colonies of the strains were illustrated

Table 1. Characteristics of strain No. 32

Characteristics studied	Results
Gram reaction	Positive
Form	Rods
Size (μm)	0.4~0.6×3.0~5.0
Spore shape and position	Ellipsoidal, central
Motility	Positive
Growth	
7% NaCl	Positive
Medium pH	pH 5~9
Maximum temperature	55℃
0.001% lysozyme	Negative
Catalase	Positive
Hydrolysis of	
starch	Positive
casein	Positive
gelatine	Positive
Degradation of tyrosine	Negative
Egg yolk lecithinase	Negative
Use of	
citrate	Positive
propionate	Positive
Reduction of nitrate to nitrite	Positive
Methyl red test	Negative
Voges-Proskauer test	Positive
Sugar fermentation	
Glucose	Acid, Gas
Mannitol	Acid

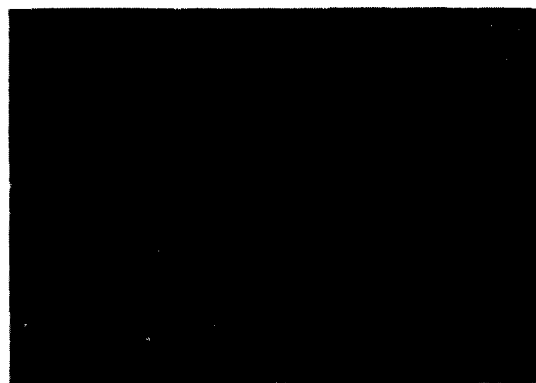

Fig. 1. The phase contrast microscope of thermostable α-amylase producing *Bacillus* sp. No. 32.
*One scale indicates 2 μm.

Table 2. α-Amylase productivities of isolated mutants

Strain No.		α-amylase activity (U/ml)	Characteristics
Bacillus sp.	No. 32	30	Parental type
	No. 32H	265	
	No. 32H106	750	D-Cycloserine resistant
	No. 32H144	1050	D-Cycloserine resistant
	No. 32H192	580	D-Cycloserine resistant
	No. 32H389	725	Rifampicin resistant
	No. 32H417	2700	Rifampicin resistant, Asporogenous

*Each strain was cultivated in ABY medium at 37℃ for appropriate time

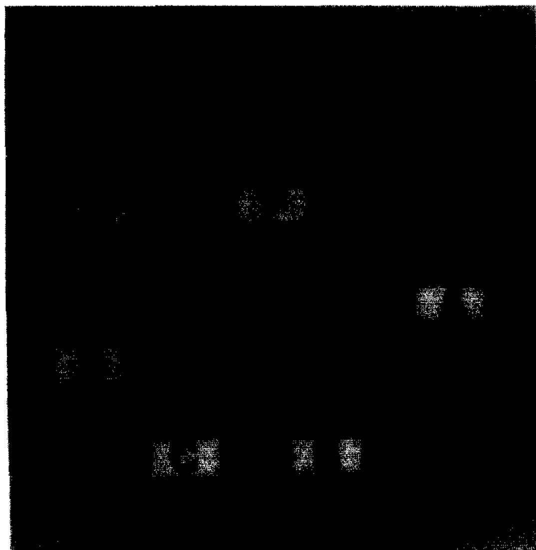

Fig. 2. Growing colonies and formation of clear zone of isolated mutants on BY agar medium containing 2% soluble strach.

*Symbols: A; *Bacillus* sp. No. 32H, B; No. 32H106, C; No. 32H144, D; No. 32H192, E; No. 32H389, F; 32H417

in Fig. 2. Although there is no direct evidence for relations between α-amylase production and antibiotics resistance except tunicamycin resistance (12), antibiotics resistance has been used as a guide mark for the isolation of α-amylase hyperproducer in *Bacillus* (6, 13). In this experiment, also, some antibiotics resistances showed efficiencies in isolating hyperproducers.

The highest α-amylase producing mutant, *Bacillus* sp. No.32H417, which was resistant to rifampicin, showed clear characteristics-no spore formation. It was reported that rifampicin resistance could be related to sporulation (14) and sporulation could affect some kinds of enzyme formation (14-16). Although it is not certain whether α-amylase productivity is related to rifampicin resistance and/or asporogenicity, the extra-hyperproductivity of the mutant No. 32H417 compared to the other mutants (Table 2) gives the possibility that this type of mutation affects on α-amylase production.

Production of enzyme in 30 *l* Jar Fermenter

Time course of α-amylase production, cell growth, variation of pH and consumption of sugar content were investigated using 30 *l* Jar Fermenter as shown in Fig. 3.

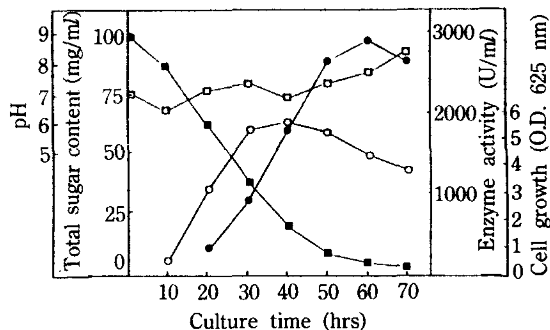

Fig. 3. Time course of α-amylase production in fermenter.

*Cell of *Bacillus* sp. No. 32H417 were cultivated in 15 *l* of ABY medium using 30 *l* Jar Fermenter (Marubishi Model MSJ-U3).

*Culture conditions were as follows; temperature 37℃, initial pH 7.0, agitation speed 300 rpm, aeration rate 0.3 vvm.

*Symbols; ●—●; α-amylase activity, ○—○; Cell growth, □—□; pH, ■—■; Total sugar content.

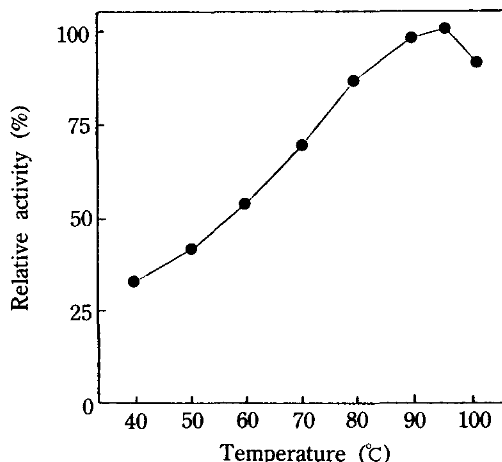

Fig. 4. Effect of temperature on enzyme activity.

*Enzyme was diluted with 0.01 M acetate buffer (pH 6.0) containing 4 mM $CaCl_2$

The production of enzyme was drastically increased after mid-exponential phase and reached at maximum level on late stationary phase. The culture time for maximum level of enzyme production was 60 hrs in fermenter, while the time was 72 hrs in shake flask. But the maximum yields of the enzyme were almost the same, 2,900 U/m*l* for fer-

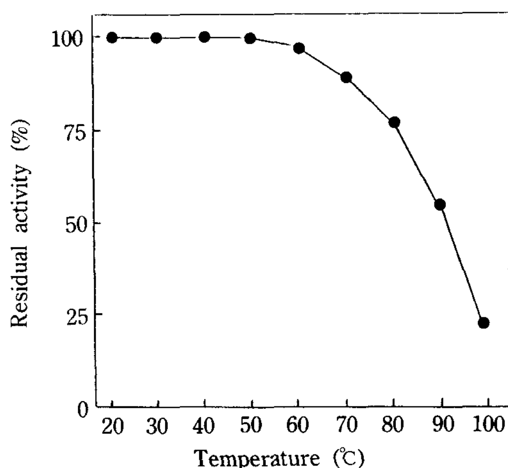

Fig. 5. Effect of temperature on enzyme stability.
*Five ml of enzyme solution (pH 6.0, 0.01 M acetate buffer) with 4 mM $CaCl_2$ was preincubated for 30 min at different temperature

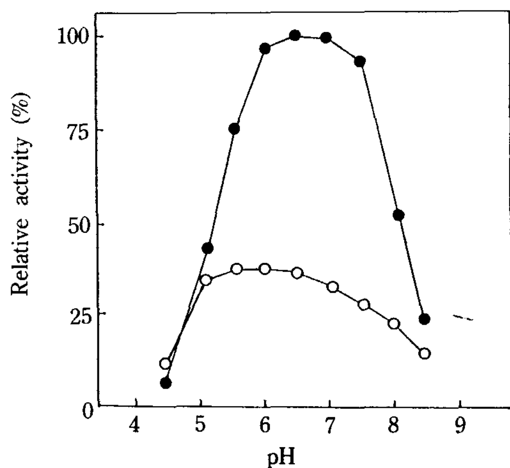

Fig. 6. Effect of pH on enzyme activity.
*Symbols: ●—●; 95℃, ○—○; 40℃
*Enzyme was diluted with buffer (pH 6.0) containing 4 mM $CaCl_2$.
*Following buffer systems were used; 0.1 M acetate buffer for pH 4~6, 0.1 M tris-maleate buffer for pH 6~8.5

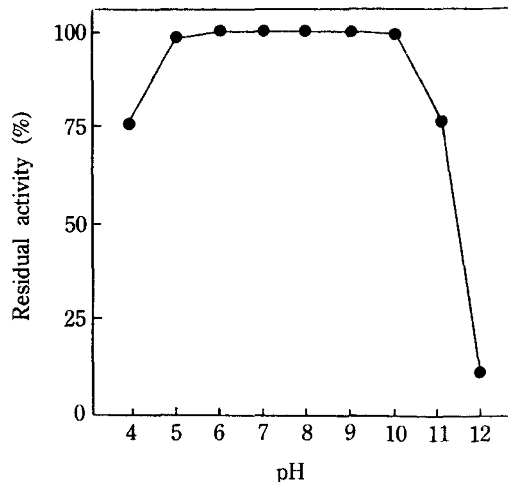

Fig. 7. Effect of pH on enzyme stability.
* Five ml of enzyme solution with 4 mM $CaCl_2$ was preincubated for 24 hrs at 25℃.
*Following buffer systems were used; 0.1 M acetate buffer for pH 4~6, 0.1 M tris-maleate buffer for pH 6~9, 0.1 M glycine-NaOH buffer for pH 9~12

Table 3. Effects of various metal ions on enzyme activities

Metal ions	Relative activity (%)
None	100
Na^+	105
Ag^+	23
Ca^{2+}	130
Mg^{2+}	92
Mn^{2+}	100
Cu^{2+}	40
Co^{2+}	75
Zn^{2+}	83
Ba^{2+}	98
Fe^{2+}	80
Pb^{2+}	24
Hg^{2+}	0

*Metal ions were added to be a final concentration of 1 mM.

menter and 2,700 U/ml for shake flask.

Effect of temperature on enzyme activity and stability

As shown in Fig. 4, α-amylase of *Bacillus* No. 32H417 has highest activity at 95℃ and retains high activity even at 100℃. The residual activity after 30 minutes incubation was 100% at 40~60℃ and decreased sharply at temperature above 60℃ (Fig. 5).

Effect of pH on enzyme activity and stability

The pH optima for enzyme activity were 6.0 at reaction temperature of 40℃ and 6.5 at 95℃, showing the optimum pH was slightly increased by

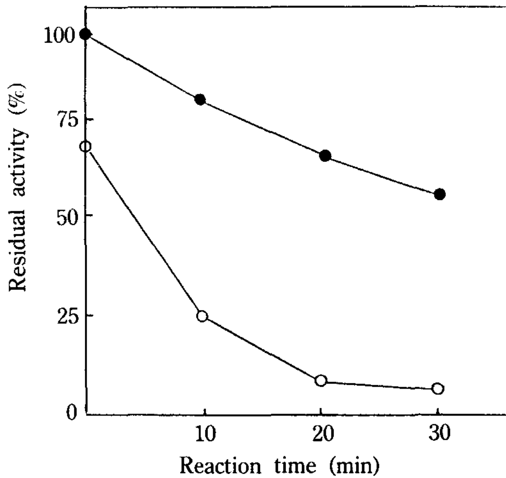

Fig. 8. Effect of Ca^{2+} on enzyme stability.
*Five ml of enzyme solution (pH 6.0, 0.01 M actate buffer) with or without 4 mM $CaCl_2$ was incubated for given time at 90℃.
*Symbols: ●—●; With 4 mM $CaCl_2$, ○—○; Without $CaCl_2$

elevating the reaction temperature (Fig. 6). The enzyme activity was stably maintained in pH ranging from pH 5.0 to pH 10.0 after incubation for 24 hrs at 25℃ (Fig. 7).

Effects of metal ions on enzyme activity and stability

The activity and stability of α-amylase could be affected by metal ions (17, 18). The effects of various metal ions on crude enzyme of *Bacillus* sp. No. 32H417 were investigated (Table 3). Among tested metal ions, Ca^{2+} stimulated α-amylase activity by about 130% while Ag^+, Pb^{2+}, Hg^{2+}, Cu^{2+} showed inhibitory effects. The minimum concentration of Ca^{2+} for optimal enzyme activity and stability was 0.3 mM. The effect of Ca^{2+} for enzyme stability was predominant at high temperature as shown in Fig. 8.

요 약

耐熱性 α-amylase를 生産하는 *Bacillus* sp. No. 32를 土壤에서 分離하였다. α-amylase의 생산성을 높이기 위하여 이 No. 32 菌株에 반복적으로 突然變異原인 NTG를 처리하고 抗生劑 耐性을 이용하여 α-amylase 高 生産性 菌株를 探索한 結果, α-amylase 生産性이 약 90배 向上된 異變株 No. 32H417을 얻었으며 이 菌株는 rifampicin 耐性 및 胞子 形成能이 缺餘된 特徵을 보였다. 變異株 No. 32H417의 耐熱性 α-amylase는 95℃의 온도 및 pH 6.5에서 가장 높은 活性을 보였으며, 0.3 mM 이상의 Ca^{2+}에 의해 높은 安定性을 나타내었다.

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