Kor. J. Appl. Microbiol. Biotechnol.
Vol. 19, No. 5, 464-469 (1991)

Isolation of α-Amylase Hyperproducing Strain HG4 from *Bacillus* sp. and Some Properties of the Enzyme

Kim, Moo-Sung and Pyong-Su O*
Fermentation Technology Laboratory, R & D Center, Pacific Chemical Co., Ltd., Ansan 425-120, Korea

α-Amylase 생산성이 높은 *Bacillus* sp. HG4의 분리 및 효소 특성

김무성 · 오평수*
태평양화학(주) 기술연구소 발효공학연구실

Abstract — An α-amylase producing bacterium, strain 2B, was isolated from soil and identified to genus *Bacillus*. To enhance α-amylase productivity, strain 2B was mutagenized successively with nitrosoguanidine. For an efficient selection of α-amylase hyperproducers, mutants which produced α-amylase in the presence of glucose were isolated. The resultant mutant HG4, which was classified as constitutive and catabolite derepressed hyperproducer of α-amylase, produced about 30 folds more α-amylase than parental strain in medium containing lactose as carbon source. The strain HG4 grew rapidly and produced enzyme in parallel with cell growth. Moreover, its cell lysis did not occur until time of maximal yield of enzyme, which was considered to be a favorable characteristic for the production and purificiation of enzyme in industrial scale. The enzymatic properties of parental strain 2B and mutant strain HG4 were almost the same. The optimal temperature and pH for enzyme reaction was 70℃ and pH 6.0, respectively, in the presence of 0.6 mM Ca^{2+} as an effective stabilizer.

α-Amylase (EC 3.2.1.1) from bacteria is one of the most widely used commerical enzymes, but high productivity of the enzyme is strongly required for the production of industrial scale. Many efforts have been made to isolate amylase hyperproducing strains and to elucidate the control mechanisms of the amylase synthesis (1-3). However, there are diverse reports and some disagreement about patterns of enzyme synthesis even in the same species of bacteria. α-Amylase synthesis may be constitutive, constitutive but catabolite repressible, or inducible but catabolite repressible according to individual strain of bacteria (3-5). The secretion patterns of the enzyme are also variable for the strains and environmental factors (3, 6, 7). From this point of view, the enzyme productivity of a bacterium could be enhanced effectively by controlling or changing the patterns of enzyme production in the bacterial strain.

In present paper, we described the isolation of α-amylase producing bacterium, the enhancement of the enzyme productivity through mutation and effective selection method, and some properties of the enzyme.

Key word: α-Amylase, *Bacillus* sp., catabolite repression
***Corresponding author**

Materials and Methods

Isolation of α-amylase producing bacteria

Soil samples were plated on BY agar media (8) containing 2% starch, and α-amylase producers were selected by clear zone formation with iodine solution on the plates as described in previous paper (8). Selected strains were cultivated in 500 ml of sakaguchi flasks containing 50 ml of ABY media (8) with 5% starch at 37℃ for 48-96 hrs and the culture broths were used for assay of α-amylase

at 40℃.

Identification of bacterial strain 2B

The highest α-amylase producing strain 2B was tested for its morphological and physiological characteristics, and identified based on "Bergey's Manual of Determinative Bacteriology" (9).

Mutation and selection method

Cells of *Bacillus* sp. 2B in logarithmic growth phase were treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG, 50 μg/m*l*) by the same method described in previous paper (8). For isolation of mutants producing α-amylases in the presence of glucose, mutagenized cells were plated on BY agar media containing 0.4% glucose and 1.6% starch. After incubation for 12 hrs at 37℃, iodine solution was sprayed on the plates and colonies forming clear zone were selected. For further isolation of mutants not repressed α-amylase production in the presence of higher concentration of glucose, NTG-treated cells of strain G17 were plated on BY media containing 1% glucose and 1% strach. Selected strains were investigated their patterns of enzyme production and enzyme productivities in BY media and ABY media, respectively.

Cultivation in 30 *l* jar fermenter

Effect of lactose concentration and time course of α-amylase production were investigated in 30 *l* jar fermenter (Marubishi Model MSJ-U3) containing 18 *l* of ABY medium. The cultivation temperature was 37℃ and initial pH was adjusted to be 7.0 with 0.1 N NaOH. The aeration rate was maintained at 0.5 vvm and the agitation speed was kept at 300 rpm.

Preparation of enzyme

Crude enzyme solution was prepared to investigate the properties of α-amylase by the same method described in previous paper (8).

Analytical procedures

α-Amylase was assayed by starch-iodine color reaction as described in previous paper (8). One unit of enzyme activity was defined as the quantities of enzyme that cause 1% reduction of blue color intensity of starch-iodine solution at 40℃ per 1 min. Glucose concentration was enzymatically determined by hexokinase-glucose-6-phosphate dehydrogenase method (10). Total sugar content was measured by phenol-H_2SO_4 method (11) and cell growth was estimated by measuring optical density at 625 nm.

Results and Discussion

Identification of bacterial strain 2B

The characteristics of isolated strain 2B were shown in Table 1 and Fig. 1. The isolate was Gram positive, spore forming, motile and rod shaped bacterium that belonged to genus *Bacillus*.

Table 1. Characteristics of strain 2B

Characteristics Studied	Results
Gram Reaction	Positive
Form	Rods
Size (μm)	0.5~0.7×2.5~5.5
Spore Shape and Position	Ellipsoidal, Central
Motility	Positive
Growth in	
7% NaCl	Positive
pH of Medium	pH 5.0~8.5
Maximum Temperature	45℃
0.001% Lysozyme	Negative
Anaerobic Growth	Negative
Catalase	Positive
Hydrolysis of	
Starch	Positive
Casein	Positive
Gelatine	Positive
Degradation of Tyrosine	Negative
Egg Yolk Lecithinase	Negative
Use of Citrate	Postive
Use of Propionate	Positive
Reduction of Nitrate to Nitrite	Positive
Methyl Red Test	Negative
Voges-Proskauer Test	Positive
Sugar Fermentation of	
D-Glucose	Acid, Gas
D-Mannitol	Acid
L-Arabinose	Acid
D-Xylose	Acid

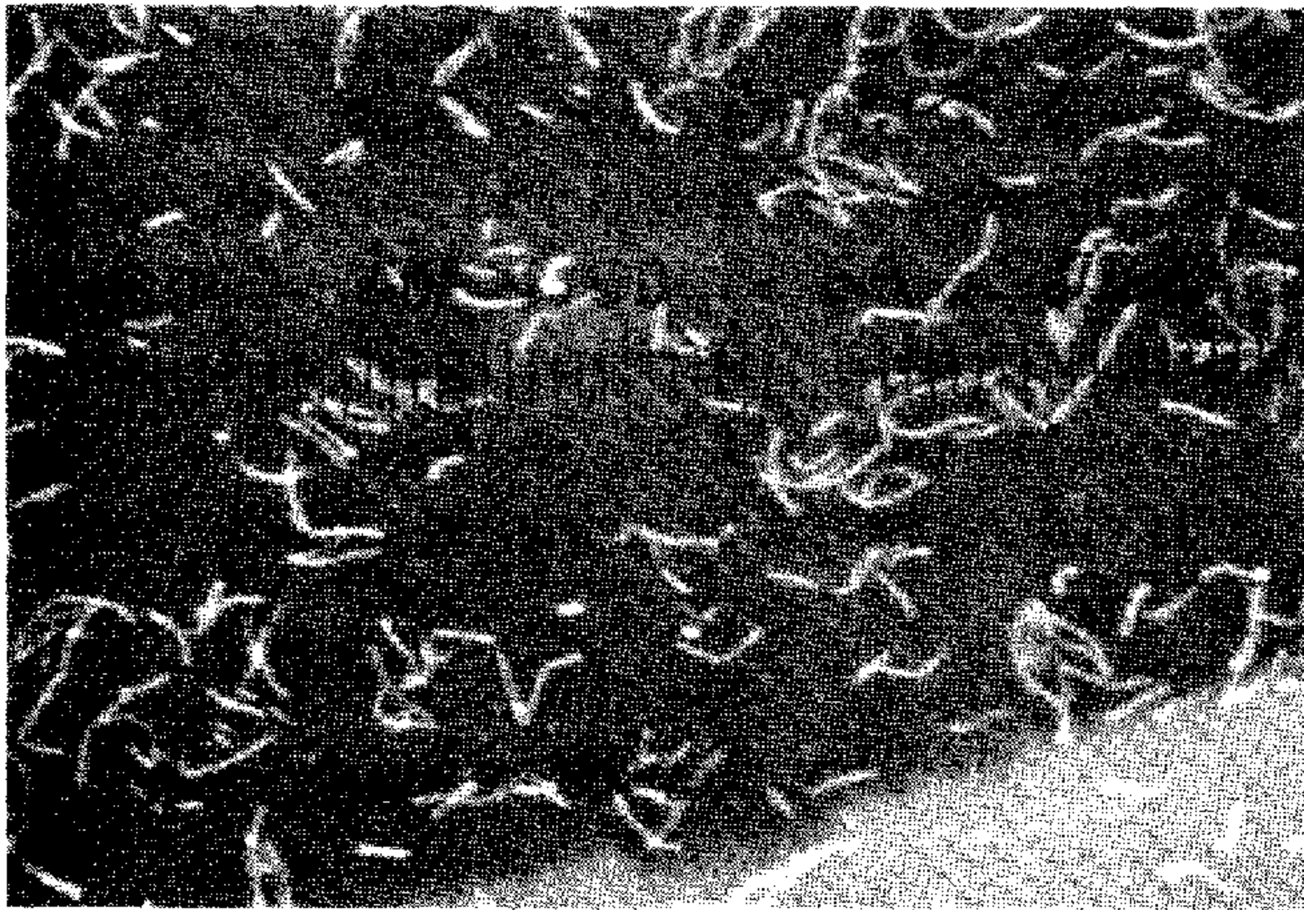

Fig. 1. Phase contrast microphotograph of *Bacillus* sp. 2B.
One scale indicates 2 μm.

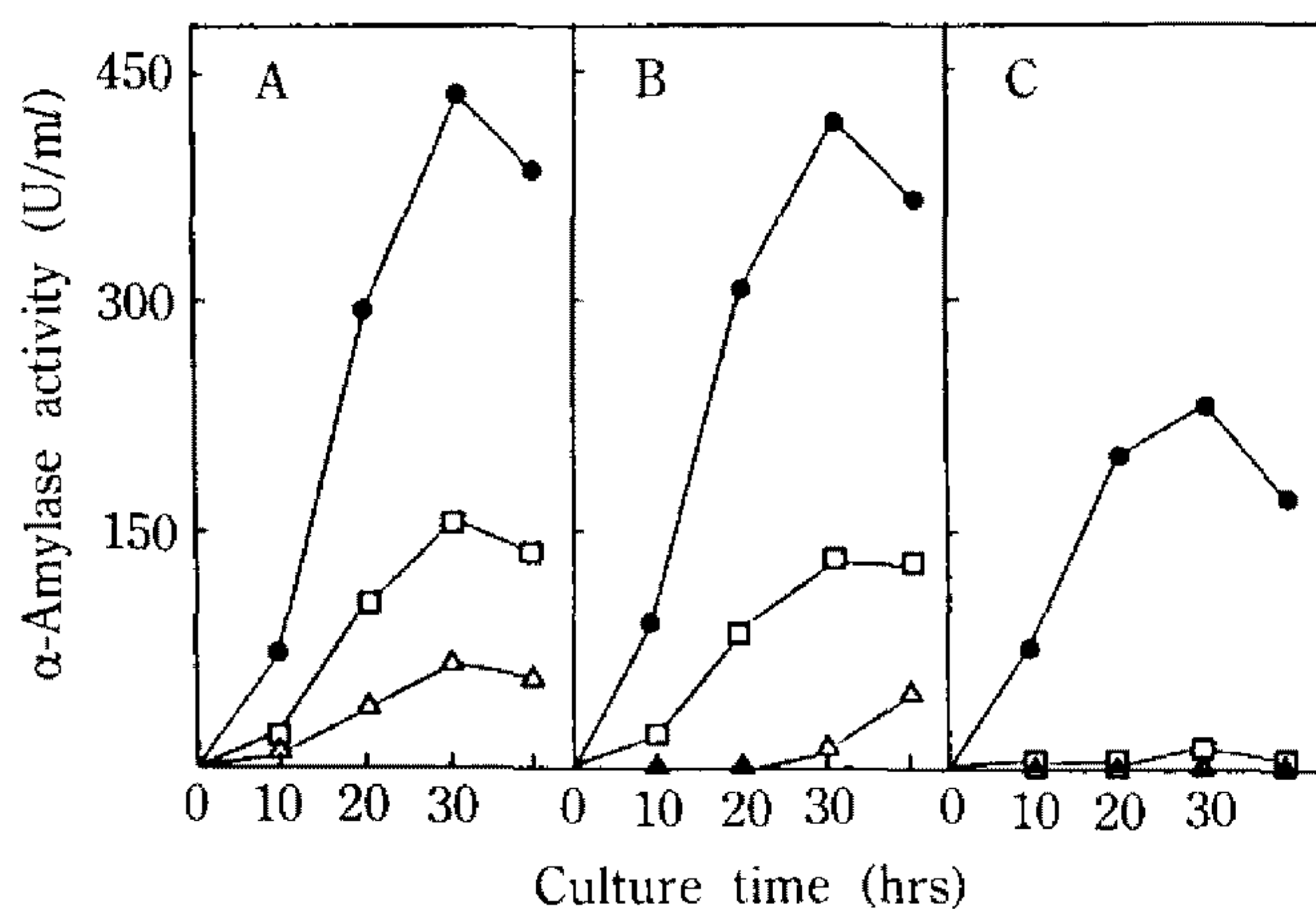

Fig. 2. α-Amylase production of parental and mutant strains according to various carbon sources.
A: BY media containing 2% strach
B: BY media containing 0.4% glucose and 1.6% starch
C: BY media containing 2% glucose
Symbols: △−△ Strain 2B, □−□ Strain G17, ●−● Starin HG4.

Isolation of α-amylase hyperproducing strains

In general, syntheses of amylases are repressed by low molecular weight substrates, such as glucose, and many attempts have been made to overcome the repression for increase of enzyme productivities (12, 13). The α-amylase of *Bacillus* sp. 2B was produced from log phase and reached at maximum on stationary phase in medium containing 2% starch as sole carbon source, but the production was much retarded in medium containing 0.4% glucose and 1.6% starch (Fig. 2). Furthermore, no α-amylase was measured in medium containing glucose alone as carbon source. These meant that the α-amylase synthesis of strain 2B was not constitutive, but might be inducible and catabolite repressible. To obtain mutants relieved from glucose repression, strain 2B was mutagenized with NTG and colonies which produced α-amylase on media containing 0.4% glucose and 1.6% starch were selected. Among 19 isolated mutants, strain G17 produced 4 folds more α-amylase than parental strain 2B in ABY-5% strach medium (Table 2). Although α-amylase synthesis of strain G17 was not repressed by 0.4% glucose, high cencentration of glucose above 0.8% still repressed the enzyme synthesis. For further increase of enzyme productivity, strain G17 was mutagenized again and mutants which produced α-amylase on media containing 1% glucose and 1% starch were isolated. As results, 5 strains were selected and the highest α-amylase producing strain HG4 which produced 3 times as much α-amylase as G17 in ABY-5% starch medium was isolated (Table 2).

Table 2. α-Amylase productivities and characteristics of isolated strains

Strain	α-Amylase productivity (U/ml)	Pattern of α-amylase production
2B	145	Inducible, catabolite repressed
G17	594	Inducible, catabolite derepressed
HG4	1885	Partially constitutive, catabolite derepressed

Characterization of α-amylase hyperproducing strains

The course of α-amylase production of strain G17 in glucose-strach medium was similar to that in starch medium, but the enzyme was not produced in glucose medium (Fig. 2). These meant that the α-amylase synthesis of strain G17 was inducible but catabolite derepressed. The level of glucose consumption in strain G117 was low compared to that in parental strain 2B, while cell growth was similar in both strains (Table 3). Therefore it might be suggested that the high productivity of α-amylase in G17 be due to the low cytoplasmic concentration of glucose, which made the cell relieved from catabolite repression.

Table 3. Comparison of some features of parental and mutant strains after cultivation

Strain	Cell growth (625 nm)	α-Amylase activity (U/ml)	Residual glucose concentration (mg/ml)
2B	3.4	45	0.30
G17	3.3	148	1.20
HG4	4.4	440	0.38

Each strain was cultivated in BY medium containing 0.4% glucose and 1.6% starch.

Like strain G17, the course of α-amylse production of strain HG4 in glucose-starch medium was similar to that in starch medium. But strain HG4 also produced the enzyme in glucose medium, although the amounts of the enzyme was about half of that in starch medium (Fig. 2). These results meant that the α-amylase synthesis of HG4 might be partially constitutive and catabolite derepressed. The glucose consumption of strain HG4 was similar to that of parental strain 2B, but cell growth of HG4 was greater than that of 2B or G17 after cultivation (Table 3). These results indicated that the high enzyme productivity of strain HG4 was not related to glucose transport into the cell, but another regulational change in α-amylase production and some metabolic alternation might occur in this strain.

Effects of carbon sources of α-amylase production

As shown in Table 4, the parental and mutant strains could produce α-amylase not only on starch or maltose but also on other carbon sources which did not have α-1,4-glucosidic linkage. Unlike other bacilli, lactose was the most effective carbon source for α-amylase production in parental and mutant strains. These meant that starch or starch-breakdown product was not the specific inducer of α-amylase in this *Bacillus* sp., but another intracellular turn-on mechanisms of α-amylase synthesis might be exist in this bacterial species.

Effect of lactose concentration on production of α-amylase in fermentor

Effect of lactose concentration on enzyme produ-

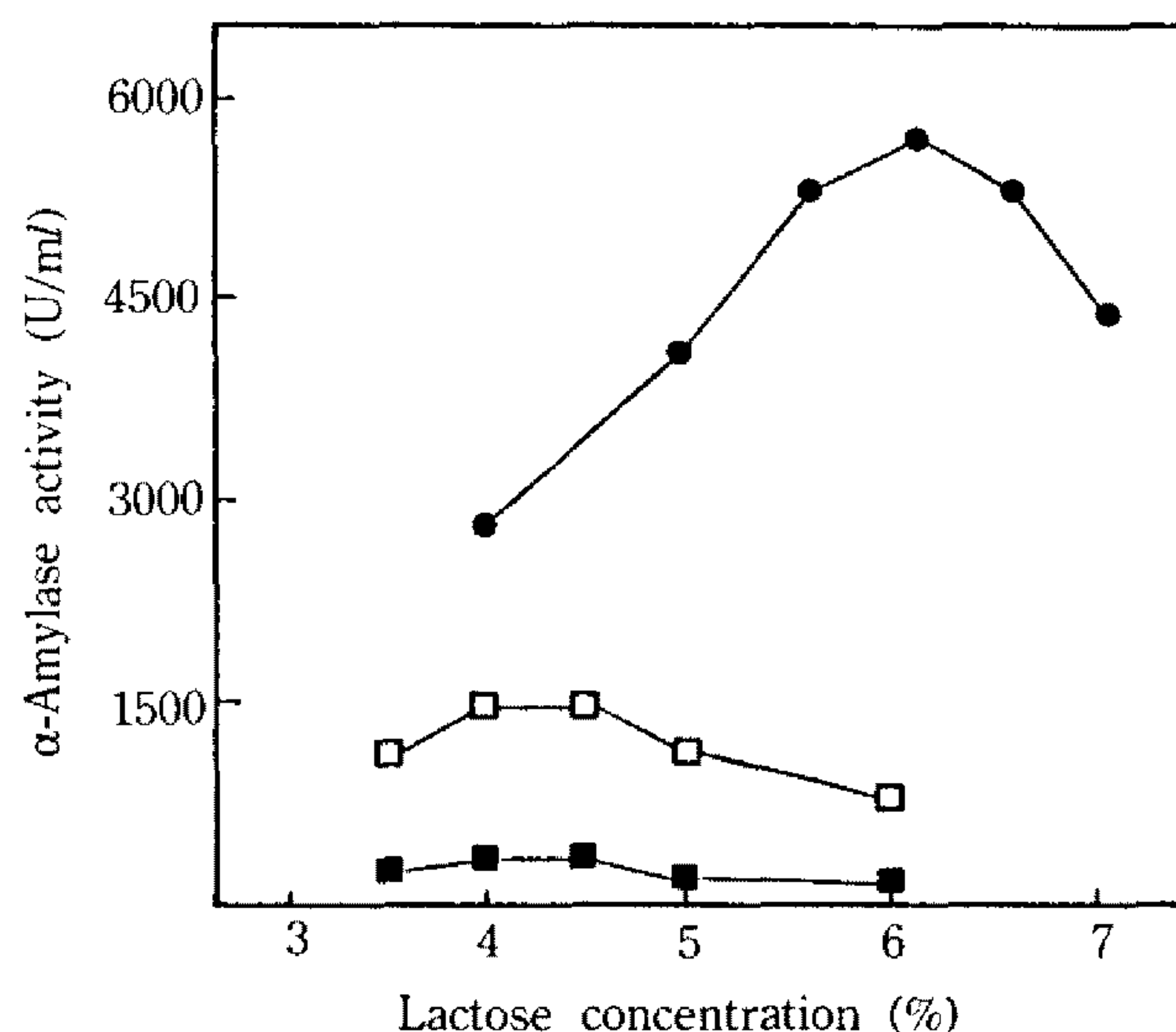

Fig. 3. Effect of lactose concentration on α-amylase production.

Each strains were cultivated in 18 *l* of ABY medium containing lactose as carbon source using 30 *l* jar fermentor.

Symbols: ■−■ Strain 2B, □−□ Strain G17, ●−● Strain HG4

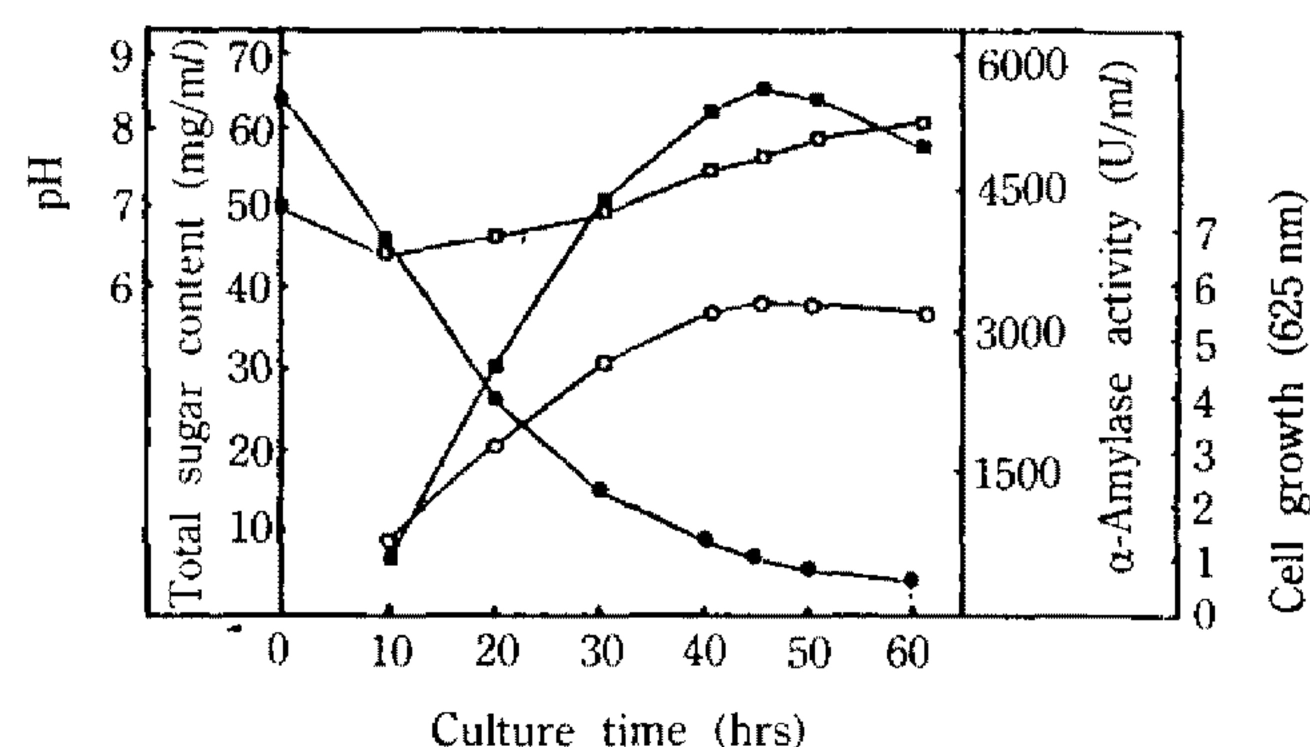

Fig. 4. Coures of α-amylase production in fermentor.

Cells of *Bacillus* sp. HG4 were cultivated in 18 *l* of ABY medium containing 6% lactose using 30 *l* jar fermentor.

Culture conditions were as follows: temperature 37℃, initial pH 7.0, agitation speed 300 rpm, aeration rate 0.5 vvm.

Symbols; ■−■ α-Amylase activity, □−□ pH, ○−○ Cell growth, ●−● Total sugar content.

ction of parental and mutant strains were investigated using 30 *l* jar fermenter. The optimal concentration of lactose was 6% for strain HG5, while it was 4% for both strain G17 and 2B (Fig. 3). The maximal yield of α-amylase in strain HG4 was 5850 U/m*l* at 45 hrs cultivation while it was 1470 U/m*l* at 55 hrs cultivation in strain G17 and 200 U/m*l*

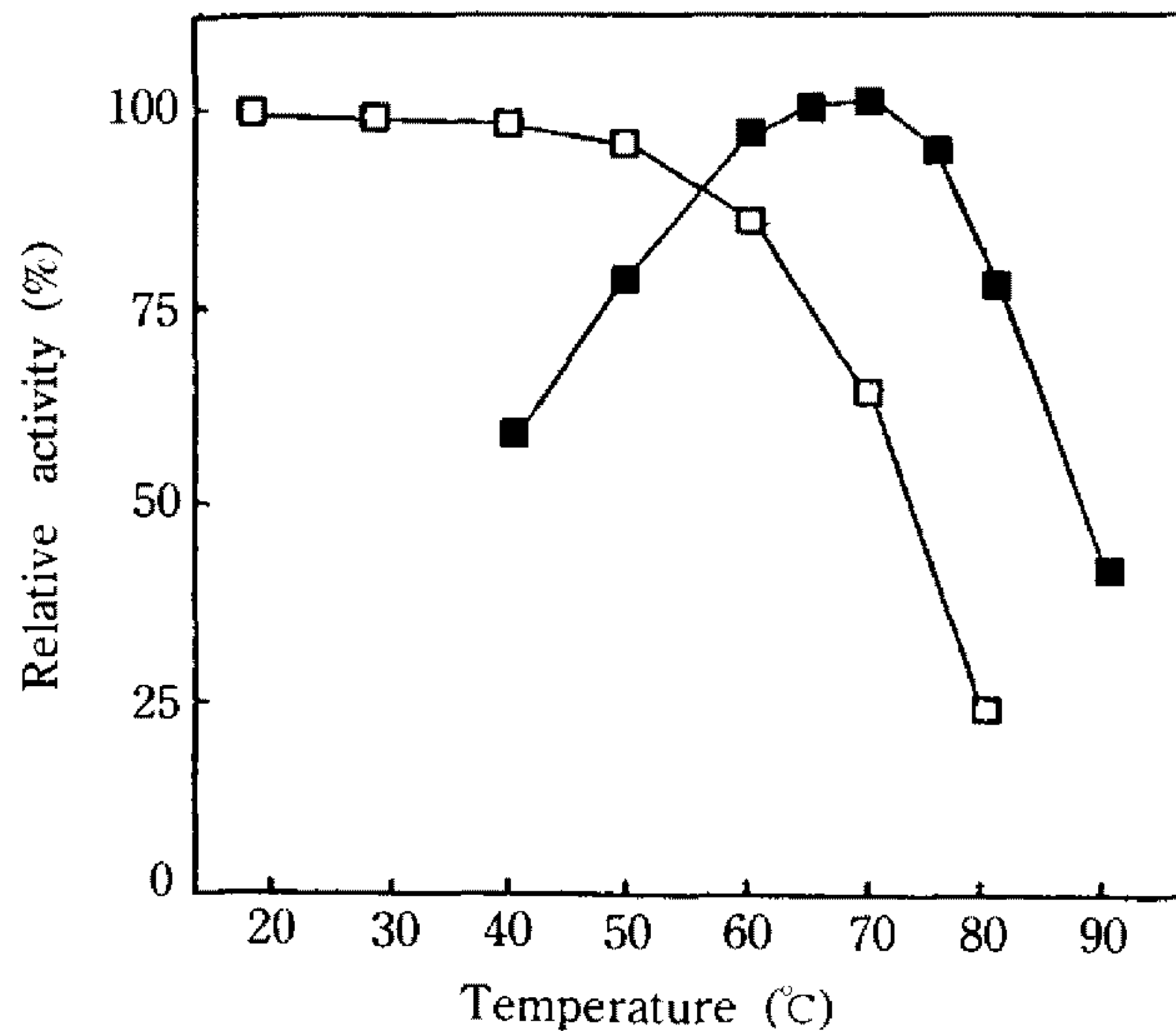

Fig. 5. Effect of temperature on enzyme activity and stability.
Enzyme was diluted with 0.01 M acetate buffer solution (pH 6.0) containing 7 mM $CaCl_2$.
For the experiment of enzyme stability, Five ml of enzyme solution was preincubated for 60 min at different temperature.
Symbols: ■—■ Optimal temperature, □—□ Temperature stability.

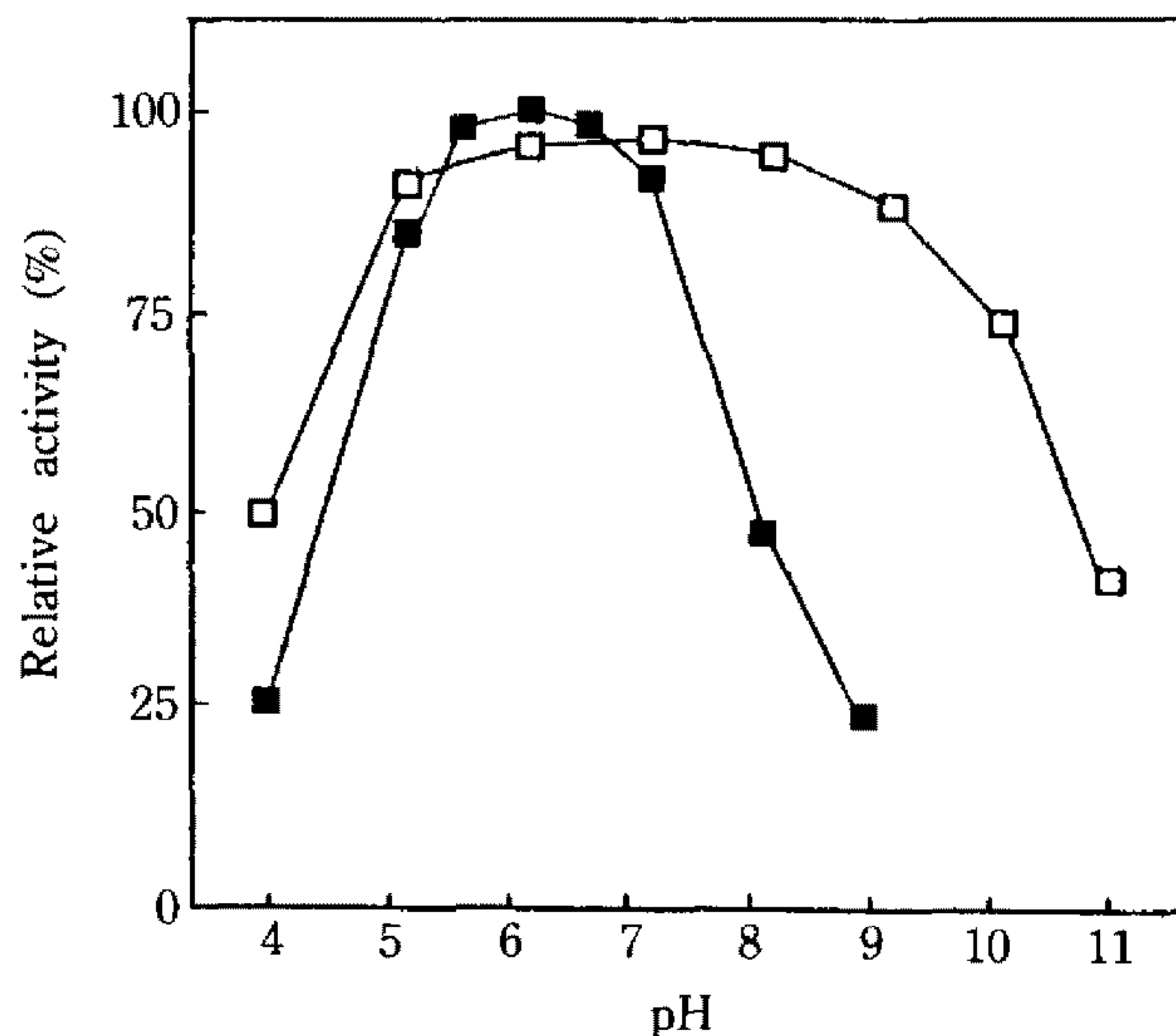

Fig. 6. Effect of pH on enzyme activity and stability.
Enzyme was diluted with buffer solution containing 7 mM $CaCl_2$.
For the experiment of enzyme stability, Five ml of enzyme solution was preincubated for 24 hrs at 25℃. Following buffer systems were used; 0.1 M acetate buffer for pH 4~6, 0.1 M Tris-maleate buffer for pH 6~9, 0.1 M glycine-NaOH buffer for pH 9~11.
Symbols: ■—■ Optimal pH, □—□ pH stability

at 60 hrs cultivation in strain 2B. The α-amylase production of HG4 was about 30 folds high compa-

Table 4. Effects of carbon sources on α-amylase production.

Carbon Sources	α-Amylase activity (U/ml)		
	Strain 2B	Strain G17	Strain HG4
Citrate	70	184	0*
Glucose	0	5	988
Fructose	0	0	225
Galactose	19	55	680
Maltose	105	455	1700
Lactose	145	728	2925
Cellobiose	52	202	1085
Sucrose	0	0	289
Soluble Starch	145	594	1885

Each strain was cultivated in ABY medium containing 5% of carbon source.
*Cell growth was not observed.

Table 5. Effects of various metal ions and reagent on α-amylase activities

Metal ions or reagent	Concentration (mM)	Relative activites (%)
None		100
Na^+	10	105
Ag^+	1	31
Ca^{2+}	1	127
Mg^{2+}	1	95
Mn^{2+}	1	105
Cu^{2+}	1	38
Co^{2+}	1	82
Zn^{2+}	1	94
Ba^{2+}	1	88
Fe^{2+}	1	80
Pb^{2+}	1	42
EDTA	5	90

EDTA: Ethylenediaminetetraacetic acid

red to that of parental strain 2B. As shown in Fig. 4, strain HG4 grew rapidly and produced enzyme in parallel with cell growth, moreover, its cell lysis did not occur until the time of maximal yield of enzyme. These could be considered to be favorable characteristics for the production and purification of enzyme in industrial scale.

Properties of the enzyme

As shown in Fig. 5, the α-amylase exhibited ma-

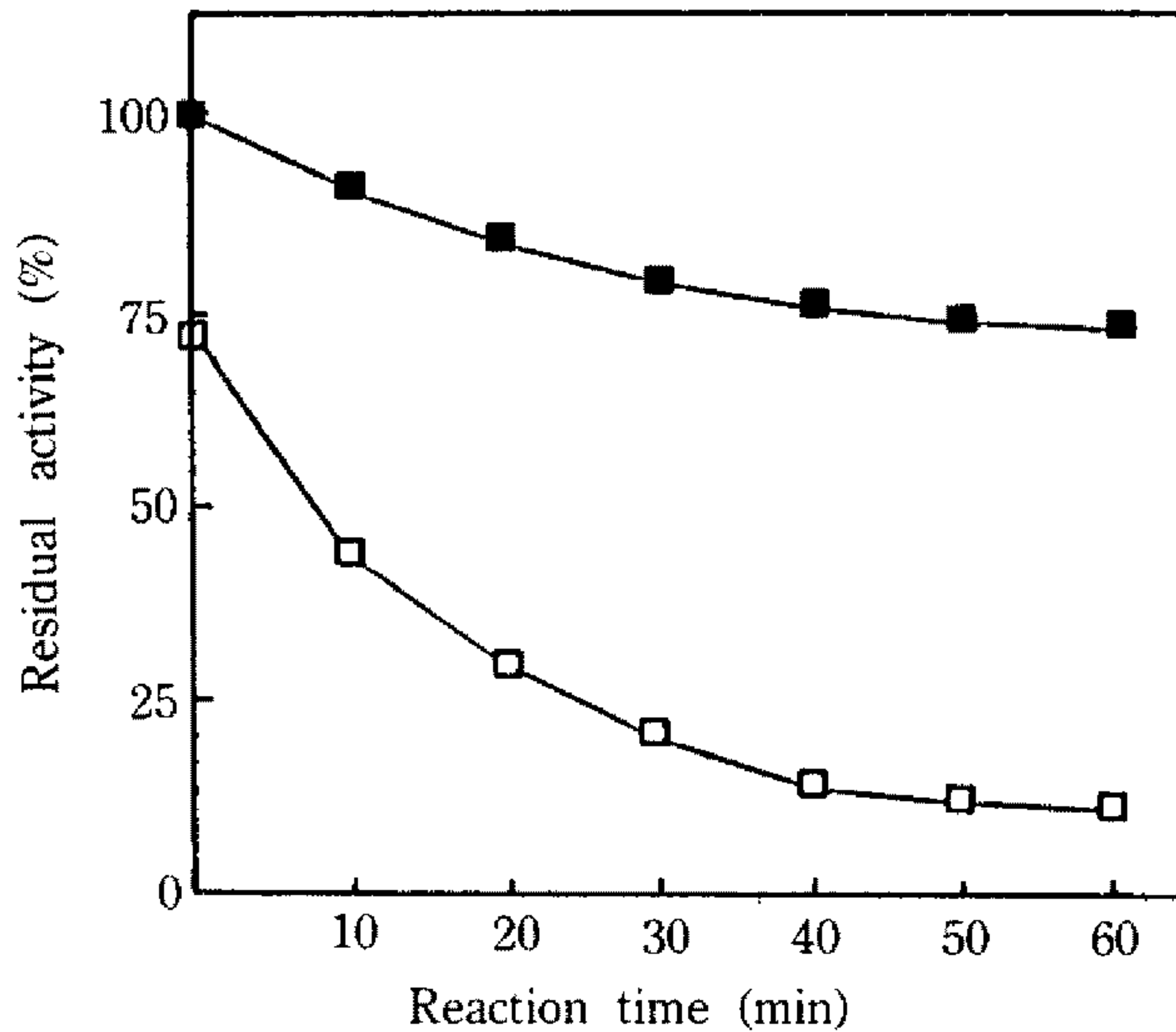

Fig. 7. Effect of Ca^{2+} on enzyme stability
Five milliliters of enzyme solution (pH 6.9, 0.01 M acetate buffer) with or without $CaCl_2$ was princubated at 70℃ for given time.
Symbols; ■−■ with 7 mM $CaCl_2$, □−□ without $CaCl_2$

ximum activity at 70℃, but its stability decreased sharply at temperature above 50℃ after being incubated for 60 min. The optimal pH for the enzyme reaction was 6.0, but the enzyme stability was maintained between pH 5.0~9.5 at 25℃ for 24 hrs (Fig. 6). The thermal and pH stabilites of the enzymes in parental strain 2B and mutant strain HG4 were almost the same (data not shown). Table 4 summerized the effects of various metal ions on enzyme activity. Pb^{2+}, Ag^{+}, Cu^{2+} exhibited inhibitory effects while Ca^{2+} activated the enzyme. EDTA inhibited the enzyme reaction slightly, however, the enzyme dialyzed against 10 mM EDTA for 24 hrs retained 38% of its original activity. After washing EDTA, the enzyme recovered its activity by addition of Ca^{2+}. These indicate that the α-amylase require Ca^{2+} for its structual stability and full activity. The minimum concentration of Ca^{2+} for enzyme stability was 0.6 mM. Fig. 7 showed the marked effect of Ca^{2+} for enzyme stability at high temperature.

요 약

α-Amylase를 생산하는 *Bacillus* sp. 2B를 토양에서 분리하였으며 이 균주에 반복적으로 돌연변이원인 NTG를 처리하여 효소생산성이 증대된 변이주를 유도하였다. α-Amylase 고 생산성 균주의 효율적인 획득방법으로 glucose에 의한 α-amylase의 생성억제를 받지않는 변이주를 분리한 결과, 효소생산성이 약 30배 향상된 변이주 *Bacillus* sp. HG4를 획득하였다. 이 균주는 lactose를 탄소원으로 하여 최대 효소생성능을 나타내었으며 빠른 균체성장 및 최대 효소생성시기에 균체 lysis가 적은 점 등 산업적으로 사용하기에 유리한 특성을 가진 것으로 판단된다. 변이주 HG4의 α-amylase는 70℃의 온도 및 pH 6.0에서 가장 높은 활성을 보였으며 0.6 mM 이상의 Ca^{2+}에 의해 높은 안정성을 나타내었다.

References

1. Saito, N. and K. Yamamoto: *J. Bacteriol.*. **121**, 848 (1975)
2. Rothstein, D.M., P.E. Devlin and R.L. Cate: *J. Bacteriol.*, **168**, 839 (1986)
3. Yoo, Y.J., T.W. Cadman, J. Hong and R.T. Hatch: *Biotechnol. Bioeng.*, **31**, 357 (1988)
4. Welker, N.E. and L.L. Campbell: *J. Bacteriol.*, **86**, 681 (1963)
5. Thirunavukkarasu, M. and F.G. Priest: *FEMS Microbiol. Lett.*, **7**, 315 (1980)
6. Sasaki, T., M. Yamasaki, B. Maruo, Y. Yoneda, K. Yamane, A. Takatsuki and G. Tamura: *Biochem. Biophy. Res. Commun.*, **70**, 125 (1976)
7. Yoneda, Y., K. Yamane and B. Maruo: *Biochem. Biophy. Res. Commun.*, **50**, 3 (1973)
8. Kim, M.S. and P.S.O.: *Kor. J. Appl. Microbiol. Biotechnol.*, **19**, 122 (1991)
9. Breed, R.S., E.G.D. Murray and N.R. Smith: Bergey's Manual of Determinative Bacteriology, Vol. 2, Williams and Wilkins, Baltimore (1986)
10. Bergmeyer, H.: Method of Enzymatic Analysis, Academic Press. Inc., New York (1965)
11. Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith: *Anal. Chem.*, **28**, 350 (1956)
12. Yoshigi, N. and M. Kamimura: *Agric. Biol. Chem.*, **52**, 2365 (1988)
13. Hyun, H.H. and J.G. Zeikus: *J. Bacteriol.*, **164**, 1162 (1985)
14. Sata, H., H. Taniguchi and Y. Maruyama: *Agric. Biol. Chem.*, **51**, 1521 (1987)

(Received Angust 26, 1991)