Alkaline Amylases of Alkalophilic Bacteria†

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In the previous paper of this series,¹⁾ it was reported that alkalophilic bacteria, *Bacillus* No. A-40-2 produced an alkaline amylase, and some properties of this amylase were discussed.

Recently, it has been found that alkalophilic bacteria isolated from soil in Japan produces amylases, whose pH-activity curves can be classified into four types. These findings are discussed in this note.

An isolation medium (II-medium) and isolation method of alkalophilic bacteria were reported previously.¹⁾ Eight strains, A-59, 27-1, 124-1, 135, 169, 38-2, 13 and 17-1 were selected from about 300 strains isolated. These bacteria were aerobic, sporeforming, motile and rod-shaped bacteria. It is clear that the all strains should belong to the genus *Bacillus*. The characteristic point of the bacteria was that the growth was especially good in alkaline media, the optimum pH for growth being about 10. Giant colonies of *Bacillus* No. 38-2 were motile colonies rotating from point of inoculation. Table I summarizes the morphological and cultural characteristics of the isolated strains.

The isolated strains were grown aerobically at 37°C in II-medium. After 3 days' incubation with shaking, cells were removed by centrifugation at $6,000 \times g$ for 10 min. Amylase activities of the supernatant fluid were measured at various pH's. A modified blue

value method¹⁾ was applied to assay amylase activity. The following buffer systems were used: pH 2.5~6, 0.2 M acetate buffer; pH 5~8, 0.2 M Tris-maleate buffer; pH 7~9, 0.2 M Tris-HCl buffer; pH 8.5~13, 0.2 M glycine-NaCl-NaOH buffer. As shown in Fig. 1, four types of pH-activity curve were observed: type 1, optimum pH is about 10; type 2, optimum pH are 4 and 10; type 3, optimum pH are 4 and 9; type 4, optimum pH is 4 and shoulder at pH 10. Type 2, 3 and 4 may be a mixture of two or more amylases. *Bacillus* No. 38-2 amylase which has type 3 pH-activity curve was purified about 50 fold, but still has same pH-activity curve. However, hydrolysis rate at pH 4.7 and 8.5 were remarkably different. Further details will be reported elsewhere. It is noteworthy that those four types of pH-activity curve were also observed under the tested culture conditions as shown in Table II.

The culture broth was mixed with 0.1 M Borax-NaOH buffer (pH 10.0) in the presence or absence of 10 mM CaCl_2 , incubated at 55°C or 60°C for 15 min, and the residual activity was measured at pH 10.0. As shown in Table III, all of the amylases tested were stabilized by Ca^{2+} . Amylases which have type 1 pH-activity curve were thermolabile and competely inactivated by heating at 55°C for 15 min. Amylases of type 2 and 4 were fairly heat stable, and about 70~95% of activity was remained after heating at 55°C for 15 min. *Bacillus* No. 38-2 amylase which

† Production of Alkaline Enzyme by Alkalophilic Microorganisms. Part IV.

TABLE I. MORPHOLOGICAL, CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF ISOLATED STRAINS

Strain No.	A-59	27-1	124-1	135	169	38-2	13	17-1
Morphological characteristics								
From	rod	rod	rod	rod	rod	rod	rod	rod
Size	0.4~0.5	0.5~0.8	0.5~0.6	0.6~0.8	0.5~0.6	0.5~0.6	0.8~0.9	0.7~0.8
(μ)	$\times$	$\times$	$\times$	$\times$	$\times$	$\times$	$\times$	$\times$
Motility	2.5~4.0	2.0~3.0	2.0~3.0	2.5~4.0	2.0~3.0	2.0~3.0	2.0~3.0	2.0~3.0
Gram stain	motile	motile	motile	motile	motile	motile	motile	motile
Sporangia	variable	positive	positive	variable	positive	positive	positive	positive
	bulged	slightly swollen	slightly swollen	slightly swollen	slightly swollen	swollen	slightly swollen	bulged
Spore	0.8~1.0	0.9~1.2	0.9~1.2	0.9~1.2	1.0~1.2	0.9~1.0	1.0~1.2	1.3~1.4
(μ)	$\times$	$\times$	$\times$	$\times$	$\times$	$\times$	$\times$	$\times$
Cultural characteristics								
	1.3~1.4	1.2~1.5	1.2~1.5	1.5~1.8	1.3~1.7	1.2~1.5	1.2~1.5	1.5~1.6
Nutrient broth								
pH	7 $\pm$	—	—	$\pm$	$\pm$	—	—	—
pH	10 +	+	+	++	+	$\pm$	++	++
Glucose nutrient broth								
pH	7 +	—	—	$\pm$	$\pm$	—	—	$\pm$
pH	10 ++	++	++	++	++	++	++	++
I-medium								
pH	7 $\pm$	—	—	+	—	+	—	—
pH	10 ++	++	++	++	++	++	++	++
Glucose nitrate								
agar slant	pH 10 +	$\pm$	$\pm$	$\pm$	$\pm$	+	$\pm$	++
Glucose asparagine								
slant	pH 10 +	++	++	++	++	+	++	+
Anaerobic growth								
in glucose								
broth	pH 10 ++	—	+	+	+	++	—	—
I-medium +								
5% NaCl	pH 10 +	—	—	—	—	—	+	+
Utilization of								
citrate	pH 10 $\pm$	++	++	—	—	$\pm$	+	++

Biochemical characteristics									
Hydrolysis of casein	+	+	+	+	+	+	+	+	+
Hydrolysis of starch	+	+	+	+	+	+	+	+	+
Reduction of nitrate	—	—	+	+	+	+	+	+	+
Voges-Proskauer reaction	±	±	+	+	+	+	+	+	±
Max. temp. for growth in I-medium (°C)	45	45	42	45	45	45	42	42	42
pH 10	+	+	+	+	+	+	+	+	+
pH 10	+	+	+	+	+	+	+	+	+
pH 10	±	±	+	+	+	+	+	+	±

TABLE II. AMYLASE ACTIVITIES AT VARIOUS pH'S UNDER THE DIFFERENT CULTURE CONDITIONS

Exp. No.	1		2		3		Amylase activities						5		6		7				
	4	7	10	4	7	10	4	7	10	4	7	10	4	7	10	4	7	10			
pH of Assay																					
A-59	0	0	0	0	3.3	10.5	0	4	12	0	1.5	4.5	0	2	6	0	3	10	0	3	9.5
27-1	0	0	0	0.5	3	10	0	3.5	11	0	1	3.1	0	1.6	5.1	0	3	9.8	0	4	13
124-1	0	0	0	0	1.5	6	0	2	7	0	0.5	1.5	0	1.4	4	0	2.5	10	0	2.2	8
Strain No.	135	1.7	0.7	1		14	6.5	7								10	5.3	6.1			
169	0	0	0	22	8	10	14	5.4	7	13	5	6.5	21	8	10	30	8.5	12	27	8.2	11
38-2	4.5	3	3	10	7.5	7.4	9	6	6	21	14	13	10	7.4	7.3	22	15	14	23	16	14
13-1	0	0	0			12	5	4								6	2.5	2			
17-1	1.6	0.7	0.6	22	10	8	30	14	12	20	9	8	28	12	8	32	15	13	12	5	4

Exp. 1, the culture conditions are as follows: basal medium (starch, 2%; polypeptone, 1%; yeast extract, 0.5%; K_2HPO_4 , 0.1%; $MgSO_4 \cdot 7H_2O$, pH 7.2); 37°C; 3 days.

Exp. 2, basal medium + 0.5% Na_2CO_3 ; 37°C; 3 days; pH 9.7.

Exp. 3, basal medium + 1.0% Na_2CO_3 ; 37°C; 3 days; pH 10.2.

Exp. 4, basal medium + 1.5% Na_2CO_3 ; 37°C; 3 days; pH 10.5.

Exp. 5, basal medium + 0.5% NaHCO₃; 37°C; 3 days; pH 8.9.

Exp. 6, basal medium + 1.0% NaHCO_3 ; 37°C; 3 days; pH 9.0.

Exp. 7, basal medium + 2.0% NaHCO_3 ; 37°C; 3 days; pH 9.3.

Amylase activity is expressed as unit $\times 10^{-2}$ /ml of culture broth.

TABLE III. THERMAL STABILITIES OF AMYLASES

Enzymes were dissolved in pH 10 Borax buffer and incubated for 15 min at indicated temperatures. Residual activity was measured at pH 10.

Strain No.	Type	Residual activity (%)			
		55°C		60°C	
		CaCl ₂ 10 mM		CaCl ₂ 10 mM	
		—	+	—	+
A-40-2†	1	4.6	55	0	5
A-59	1	7	67	0	5
27-1	1	8	42	0	0
124-1	1	6	22	0	0
135	2	66	95	4	20
169	2	57	95	7	25
38-2	3	94	100	35	85
17-1	4	87	97	5	35
13	4	48	73	0	0

† See ref. 1.

TABLE IV. PROPERTIES OF AMYLASES OF ALKALOPHILIC BACTERIA

Type	Strain No.	Opt. pH ^{a)}	act. 10 ^{b)} Max. act	pH stability ^{c)}	Protection by Ca ²⁺	DEAE ^{d)}
1	A-40-2	10.5	1.0	7~9.5	+	+
	A-59	10.5	1.0	7~9.5	+	+
	27-1	10.5	1.0	7~9	+	+
	124-1	10.5	1.0	7~9	+	+
2	135	4~4.5,10	0.8	7~9	+	+
	169	4~4.5,10	0.7	7~9	+	—
3	38-2	4.5, 9.0	0.6	5.0~10.5	+	—
4	13	4.5	0.3	6.5~10	+	—
	17-1	4.5	0.35	6.5~10	+	—
Commercial <i>B. subtilis</i> ^{e)} amylase		6	0.05			

^{a)} optimum pH, ^{b)} maximum activity/activity at pH 10.0, ^{c)} 50% of the activity was remained, ^{d)} adsorption by DEAE-cellulose equilibrated with 0.1 M Na₂CO₃ (pH 9.0); +, adsorped; —, not adsorped, ^{e)} obtained from Nagase Sangyo Co.

FIG. 1. Effect of pH on Enzyme Activity. See the text for the experimental details.

has type 3 pH-activity curve was heat stable and about 50% of activities which measured at pH 4.5 and 10 were remained after the incubation at 65°C for 40 min. Effects of pH on stability of the enzymes were investigated in the buffers described above containing 10 mM CaCl_2 . The mixture was incubated at 50°C for 15 min and the residual activity was assayed at pH 10.0. Table IV shows pH range, under which more than 70% of the activity was remained. When the culture filtrate was loaded on a column of DEAE-cellulose equilibrated with 0.1 M Na_2CO_3 (pH was adjusted to 9.5 with HCl), type 1 was completely adsorbed by DEAE-cellulose. These results are summarized in Table IV.

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REFERENCES

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