JOURNAL OF BACTERIOLOGY, Feb. 1972, p. 570-574

Vol. 109, No. 2
Printed in U.S.A.

Halophilic Amylase from a Moderately Halophilic *Micrococcus*

HIROSHI ONISHI

Noda Institute for Scientific Research, Noda-shi, Chiba-ken, Japan

Received for publication 18 October 1971

A moderately halophilic *Micrococcus* sp., isolated from unrefined solar salt, produced a considerable amount of extracellular dextrinogenic amylase when cultivated aerobically in media containing 1 to 3 M NaCl. The *Micrococcus* amylase had maximal activity at *p*H 6 to 7 in 1.4 to 2 M NaCl or KCl at 50 C. Calcium ion and a high concentration of NaCl or KCl were essential for activity and stability of the amylase. The salt response of the amylase depended greatly on the *p*H and temperature of the enzyme assay.

It is known that about 20 different enzymes of extreme halophiles can function in high salt concentrations, and most of them are inactive in the absence of salts (3). Gibbons (1) reported that 9 out of 49 halophiles tested hydrolyzed starch, but the responsible enzymes of these strains were not studied in detail. Recently, Good and Hartman (2) investigated properties of the amylase from *Halobacterium halobium* and Nachum and Bartholomew examined the relationship between temperature and salt concentration on the activity and stability of the amylase from a *Halobacterium* species (R. Nachum and J. W. Bartholomew, Bacteriol. Proc., p. 137, 1969).

The author has isolated a moderately halophilic *Micrococcus* sp. from unrefined solar salt. This bacterium produces an extracellular amylase which depends on divalent cations and a high concentration of NaCl or KCl for activity and stability. This paper describes the effect of salt concentration of medium on amylase production as well as some of the characteristics of the enzyme.

MATERIALS AND METHODS

Bacterial strain. *Micrococcus* sp. 28-3, used in this study, was obtained from unrefined solar salt by aerobic enrichment culture in Sehgal and Gibbons complex medium (SGC; reference 4) followed by isolation of a colony on a plate of this medium. A detailed taxonomic study of the bacterium will be published elsewhere.

Measurement of growth. The bacterium was grown in SGC medium and nutrient broth (NB), to which designated amounts of NaCl, KCl, or glucose were added. Inocula (0.2 ml) of a 2-day-old culture grown in 2 M NaCl-SGC medium were added to 500-ml flasks containing 80 ml of medium. The flasks were shaken at 30 C on a reciprocal shaker operating at 140 rev/min with a stroke of 7.5 cm. The incubation times are given in the text and figures. Turbidity was measured in 10-mm cuvettes at 660 nm against an uninoculated blank.

Cultural conditions for amylase production. The SGC and NB media supplemented with 1% soluble starch and NaCl as indicated were used for enzyme production.

Assay of amylase activity. Dextrinogenic amylase activity was assayed by the method of Yamaguchi et al. (6); 2 ml of 0.5% soluble starch in 0.04 M phosphate buffer (*p*H 6.0) was mixed with 1 ml of enzyme solution. After appropriate incubation at 40 C, a 0.2-ml portion of the reaction mixture was added to 5 ml of 0.167 mM I_2-KI solution. The optical density at 700 nm was measured in a spectrophotometer. Hydrolysis of 0.1 mg of soluble starch in 1 min was defined as 1 unit of the enzyme activity. In some experiments, changes in reaction *p*H and temperature of enzyme assay were made. Saccharifying activity was checked by determining reducing sugar liberated according to the Cu^{2+} reduction method (5).

Preparation of cell homogenates. Bacterial cells harvested from 2- to 6-day-old cultures in 2, 3, or 4 M NaCl-SGC medium were washed three times in a solution containing 2% $MgSO_4 \cdot 7H_2O$ and the same NaCl concentration as the broth, were taken up as a dense suspension in the same solution, and were disintegrated by treatment in a Braun homogenizer for 1 min.

Chemicals. The soluble starch used in this study was a special grade of high purity for amylase assay (Wako Pure Chemical Ind. Ltd., Osaka, Japan).

RESULTS

Effects of NaCl, KCl, and glucose on growth of Micrococcus sp. 28-3. Even after incubation for 7 days, growth did not occur in SGC medium or NB without added NaCl.

Rapid growth was obtained in the two test media in the presence of 1, 2, or 3 M added NaCl; maximum population densities were reached within 2 to 4 days. The lag phase was extended by about 3 days when 4 M NaCl was added to SGC medium and NB. Similar growth patterns were obtained when the NaCl was replaced by KCl; however, no growth was observed in media containing 1, 2, or 3 M glucose but no added NaCl. Thus, *Micrococcus* sp. 28-3 is a moderate halophile.

Effect of NaCl on amylase production. Little amylase was produced when the halophile was cultivated in the growth media without starch, regardless of the NaCl concentration. Amylase production was greatly increased by the addition of soluble starch to the media. The starch could not be replaced by glucose in this experiment, and amylase production was markedly repressed when 0.5% glucose was added to the medium containing 1% starch.

A considerable amount of amylase was produced in SGC media that were 1 and 2 M in NaCl after 2 days of cultivation (Table 1); the amylase yield was fair also in the 3 M NaCl medium after 4 days of incubation. After 5 day of incubation in the 4 M NaCl medium, bacterial growth was moderately good but little amylase was produced. About twice as much amylase was produced in SGC medium as was producd in NB medium containing similar levels of added NaCl. The amylase activity was 1.5 to 2 times higher in 1.31 to 1.42 M NaCl reaction mixtures than in lower NaCl levels of 0.03 to 0.10 M (Table 1). Only 13.7% of the starch utilized was transformed to reducing sugar (calculated as glucose).

Since all Braun homogenates of the washed bacterial cells from amylase-producing cultures contained only 0 to 0.5% of total activity, the amylase of the halophile seems to be extracellular.

Effects of salts, temperature, and pH on the amylase activity. The amylase activity of the culture supernatant fluid after 2 days of growth in 2 M NaCl-SGC medium was assayed at two NaCl concentrations and a range of temperatures and *p*H values. The amylase was almost equally active in high and low levels of NaCl at 30 C (Fig. 1). In contrast, the enzyme was much more salt-dependent over the *p*H range 5.0 to 8.0 at temperatures of 40 C and higher. At *p*H 4.0, 9.0, and 10.0, little activity remained. The activity was generally low at 60 C and almost negligible at 70 C.

More detailed experiments on effects of *p*H (5.0 to 8.0) and temperature (30 to 50 C) on salt requirements of the amylase were made. At 30 C (*p*H 5.0), the amylase required 0.2 to 0.3 M NaCl or KCl for maximal activity and at 40 C (*p*H 6.0) it required 0.7 M NaCl or KCl (Fig. 2). But at both assay conditions, the amylase was inhibited by higher salt concentrations. At 50 C (*p*H 7.0), it required 1.4 to 2 M NaCl or 1.4 to 2.7 M KCl and was tolerant to 3.4 M NaCl or KCl, maintaining 80 to 90% of maximum activity. At 50 C (*p*H 8.0), the amylase required 1.4 to 3.4 M NaCl or 3.4 M KCl for maximal activity. Thus, at *p*H 8.0 and 50 C, the *Micrococcus* amylase resembles enzymes of extreme halophiles. Maximum activity was observed at *p*H 6.0 to 7.0 (50 C) in 1.4 to 2.0 M NaCl or KCl. These results showed that the salt requirement of the *Micrococcus* amylase could be greatly influenced by the temperature and *p*H of the assay.

TABLE 1. *Production of amylase by a halophilic Micrococcus sp. 28-3*

NaCl added to SGC medium[a] (M)	NaCl concn at enzyme assay (M)	Dextrinogenic amylase activity[b] (units/ml) at culture day				
		1	2	3	4	5
0	0					0
	1.31					0
1	0.03	0	17.6	18.5	16.3	
	1.35	0	30.1	30.3	30.6	
2	0.07	0	17.6	18.7	20.4	
	1.39	0	26.7	28.4	30.1	
3	0.10	0	Trace	10.9	18.1	
	1.42	0	Trace	17.0	28.7	
4	0.14				Trace	Trace
	1.45				Trace	Trace

[a] SGC medium with 1% soluble starch and NaCl as shown. When soluble starch was not added, the highest amylase activity was 3.0 units.

[b] Assayed at 40 C, *p*H 6.0.

Dialysis experiments. When NaCl was removed by extensive dialysis of the cell-free broth against distilled water, amylase activity was completely lost (Table 2). Dialysis overnight against solutions of 2 M NaCl, 2 M KCl, or 0.5 M $CaCl_2$ lowered the enzyme activity only slightly. A lower concentration of $CaCl_2$ (0.05 M) prevented enzyme inactivation, suggesting its useful application for enzyme purification. Magnesium sulfate (0.5 M), ammonium sulfate (2 M), and glucose (2 M) were not as effective as NaCl or $CaCl_2$ for the prevention of the enzyme inactivation. Dialysis against 0.2 M NaCl or against 2 M NaCl con-

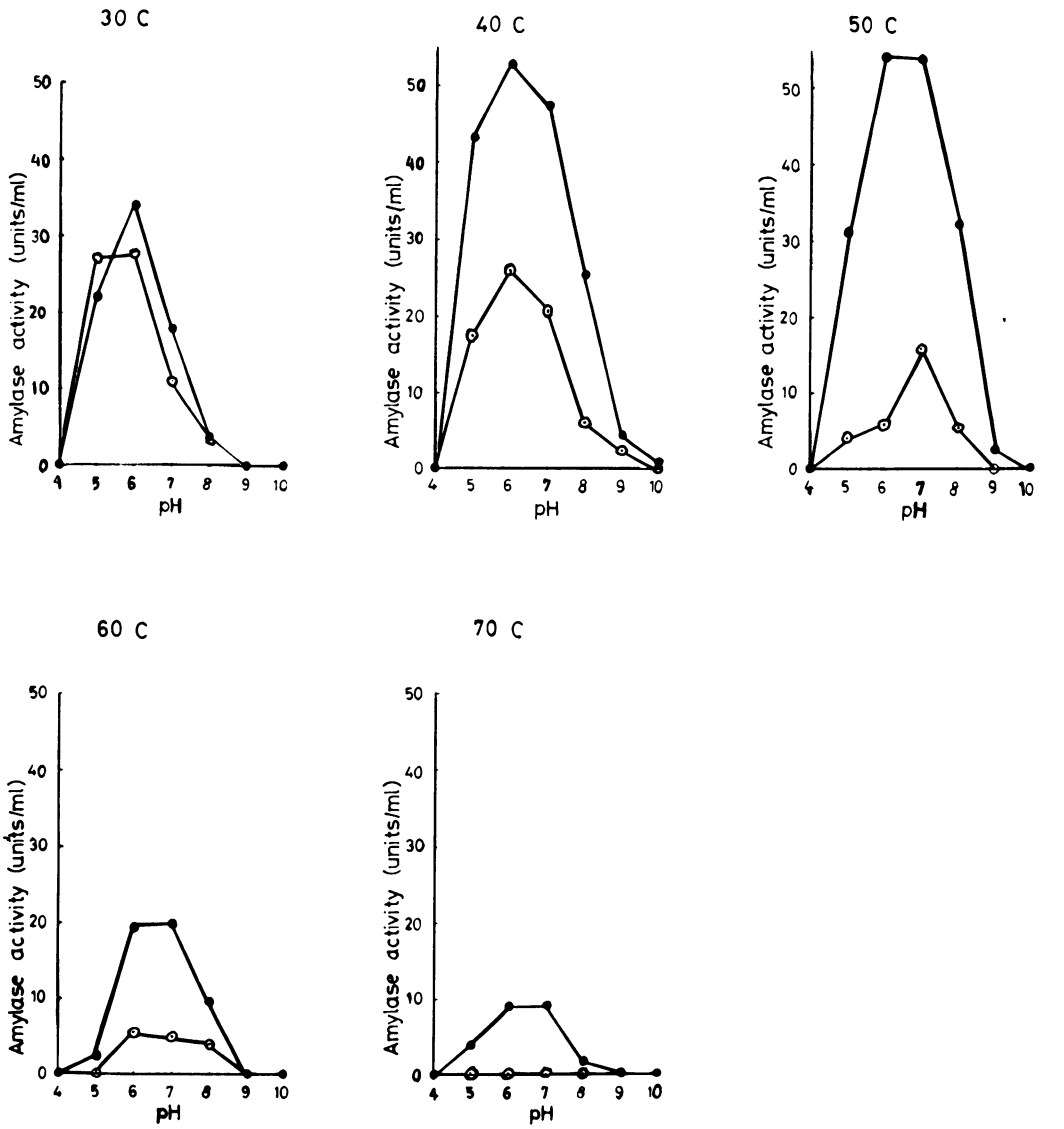

FIG. 1. *Effects of pH and temperature on the activity of Micrococcus amylase. The assay was made in the pH range of 4.0 to 10.0 at 30 to 70 C in NaCl levels of 0.1 M and 1.4 M. Open symbols, 0.1 M; closed symbols, 1.4 M. Buffers used were pH 4.0, 5.0 and 6.0, 0.071 M Veronal-acetate, pH 7.0 and 8.0, 0.1 M tris(hydroxymethyl)aminomethane, and pH 9.0 and 10.0, 0.1 M glycine.*

taining 0.01 M ethylenediaminetetraacetic acid (EDTA) at pH 6.2 caused almost as much inactivation as dialysis against water. After overnight dialysis against distilled water, attempts to obtain reactivation by dialysis against solutions containing 2 and 3 M NaCl or 2 M KCl were unsuccessful. Dialysis against a 0.5 M $CaCl_2$ solution was partly effective in reactivating the sample dialyzed overnight but not for the sample dialyzed 3 days against water.

These results indicate that the enzyme is denatured in solutions that do not contain high concentration of NaCl or KCl and is also dependent upon a divalent metal ion such as Ca^{2+} for stability or activity, or both.

DISCUSSION

The properties exhibited by the *Micrococcus* amylase differ in several respects from those described by Good and Hartman (2) for the amylase of *Halobacterium halobium:* both amylase activities were completely lost after

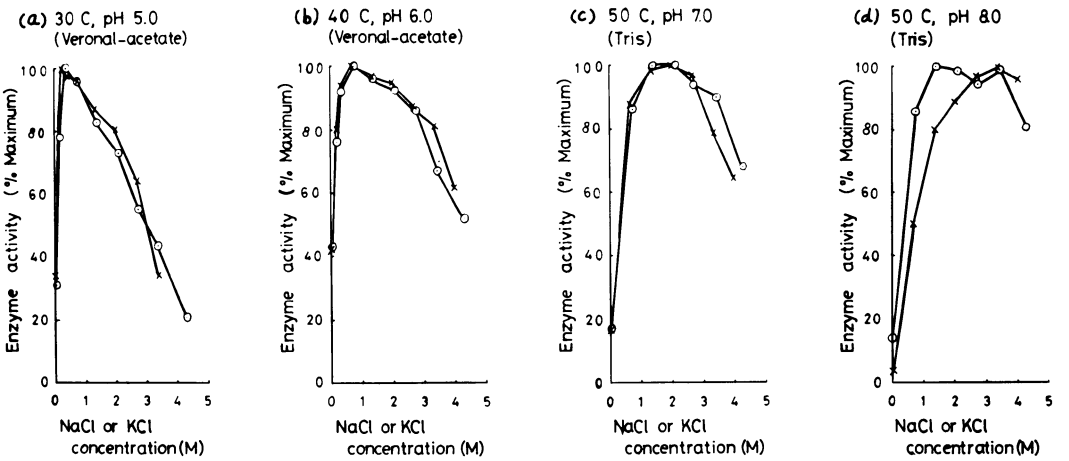

FIG. 2. *Effects of NaCl and KCl on the activity of Micrococcus amylase. The assay was carried out at 30 to 50 C in pH 5.0 to 8.0 of the same buffers used in Fig. 1 containing various concentrations of NaCl (⊙) or KCl (×). The amylase activity taken as 100% in each assay was (a) 63.8 units/ml in 0.36 M NaCl and 57.6 units/ml in 0.17 M KCl, (b) 90.4 units/ml in 0.74 M NaCl and 92.2 units/ml in 0.67 M KCl, (c) 90.5 units/ml in 1.4 M NaCl and 95.2 units/ml in 2.0 M KCl, and (d) 49.4 units/ml in 1.4 M NaCl and 91.3 units/ml in 3.4 M KCl.*

TABLE 2. *Dialysis experiments of the amylase*[a]

Solution	Time (hr)	Inactivation[b] (%)
Distilled water	3	20
Distilled water	24	89
Distilled water	70	100
0.2 M NaCl	20	81
2.0 M NaCl	20	7
2.0 M KCl	20	0
2.0 M $NaNO_3$	20	37
2.0 M $(NH_4)_2SO_4$	20	55
0.5 M $CaCl_2$	20	6
0.05 M $CaCl_2$	20	0
0.5 M $MgSO_4$	20	19
2.0 M glucose	20	34
2.0 M NaCl + 0.01 M ethylenediaminetetraacetic acid (pH 6.2)	20	62

[a] Dialysis was carried out at 5 C, and the amylase activity was assayed in 1.3 M NaCl at 40 C, pH 6.0.
[b] Initial amylase activity: 24.9 units/ml.

extensive dialysis against distilled water, but the *Halobacterium* amylase required only a very low concentration of NaCl such as 0.05 to 1.0% for maximal activity compared with the *Micrococcus* amylase which needed much more NaCl or KCl. Moreover, it was found that salt requirements of the *Micrococcus* amylase could be strikingly varied from the moderately halophilic type to the extremely halophilic type by changing the temperature and pH of the assay. My results show, unexpectedly, that the salt requirements of amylase from a moderate halophile can be much greater than those of amylase from an extreme halophile. Unlike the amylase of *H. halobium*, the inactivated enzyme of *Micrococcus* after dialysis against distilled water was not restored upon the addition of, or by dialysis against, either 2 M NaCl or KCl. Since dialysis of the *Micrococcus* amylase against 2 M NaCl solution containing 0.01 M EDTA caused much inactivation and the dialysis against only 0.05 M $CaCl_2$ prevented the loss of activity, the enzyme is thought to be dependent on Ca^{2+} ion for stability or activity, or both. A requirement for Ca^{2+} could not be demonstrated for the *Halobacterium* amylase (2).

The *Micrococcus* amylase is different from the *Halobacterium* amylase in that its pattern of salt requirement is more dependent on pH-temperature relationships. Studies were conducted on the effect of temperature and salt concentration on the activity and stability of another exocellular amylase from *Halobacterium* (R. Nachum and J. W. Bartholomew, Bacteriol. Proc., p. 137, 1969). These showed that in 5% NaCl the enzyme had maximal activity at 25 C and almost none at 40 C, whereas at 25% NaCl the maximal activity was at 55 C. As the best activity was observed at pH 5.6 in 25% NaCl at 55 C, Nachum and Bartholomew's amylase, like most enzymes from extreme halophiles, required high NaCl concentration for maximal activity.

From the viewpoint of enzyme production, the *Micrococcus* amylase which could be produced in a large scale by submerged fermenta-

tion, would be preferable to the *Halobacterium* amylase which gave much greater yield on soft agar than in tubes or shake flasks of broth (2). The amylase activity of supernatant fluids of the *Micrococcus* culture is fairly strong (90 units/ml) showing higher activity than *Bacillus natto* (18 units/ml) (6).

ACKNOWLEDGMENTS

I thank D. J. Kushner, Department of Biology, University of Ottawa, for aid in revising this manuscript.

LITERATURE CITED

1. Gibbons, N. E. 1957. The effect of salt concentration on the biochemical reactions of some halophilic bacteria. Can. J. Microbiol. **3**:249–255.
2. Good, W. A., and P. A. Hartman. 1970. Properties of the amylase from *Halobacterium halobium*. J. Bacteriol. **104**:601–603.
3. Kushner, D. J. 1968. Halophilic bacteria. Advan. Appl. Microbiol. **10**:73–99.
4. Sehgal, S. N., and N. E. Gibbons. 1960. Effect of some metal ions on the growth of *Halobacterium cutirubrum*. Can. J. Microbiol. **6**:165–169.
5. Van der Haar, A. W. 1920. Anleitung zum Nachweis zur Trennung und Bestimmung der Monosaccharide und Aldehydsäuren. Gebrüder Borntraeger, Berlin.
6. Yamaguchi, K., H. Matsuzaki, and B. Maruo. 1969. Participation of a regulator gene in the α-amylase production of *Bacillus subtilis*. J. Gen. Appl. Microbiol. **15**:97–107.