

Protease Formation by a Moderately Halophilic *Bacillus* Strain

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A moderately halophilic strain of *Bacillus*, isolated from unrefined solar salt, was capable of growth in the presence of 4 M NaCl. Maximal growth was obtained in a medium containing 1 to 2 M NaCl. The organism produced protease when cultivated aerobically in media containing 0 to 3 M NaCl or 0 to 2 M KCl. The protease activity was optimal at 0.5 M NaCl and 0.75 M KCl.

We reported previously that halophilic α -amylases (8) and nucleases were produced by moderately halophilic strains of *Micrococcus*. (A preliminary report on this topic was presented at the annual meeting of the Society of Fermentation Technology, Japan, November 1972.) During the course of an investigation on halophilic and halotolerant bacteria, we found that a spore-forming bacterial strain produced protease in the presence of a high concentration of NaCl. The microorganism (strain no. 21-1) used in this study was isolated from unrefined solar salt by aerobic enrichment culture in Sehgal and Gibbons complex medium (10) containing 25% NaCl. The organism was a gram-positive, catalase-positive, aerobic, sporogenous rod. On the basis of these characteristics, the organism was placed in the genus *Bacillus*. The detailed taxonomic study of this bacterium will appear in another paper.

Little is known about halophilic and halotolerant bacilli. Matsumoto et al. (5-7), Ishimaru (1-3), and Sakaguchi (9) described microbial properties, including salt tolerance of *Bacillus* spp. isolated from soy sauce mash. Turner et al. (12) isolated many strains of *Bacillus* from salt marshes and found that all of the nonpigmented strains failed to grow in media containing 20% NaCl. Recently, Thomson et al. (11) isolated a halophilic *Bacillus* strain from crude hides from South Africa. This culture grew in the salt range of 0.85 to 20%, producing extracellular collagenase in the presence of 2.3% NaCl but not 7% NaCl.

Growth of *Bacillus* sp. no. 21-1 in nutrient broth containing various concentrations of NaCl is shown in Fig. 1. Inocula (0.05 ml) from a 2-day-old culture grown in 1 M NaCl medium were added to side-arm test tubes containing 10 ml of media. The tubes were shaken at 30 C on a reciprocal shaker operating at 350 rpm with a

stroke of 2 cm. Turbidity was measured with a Klett-Summerson colorimeter (no. 66 filter) against an uninoculated blank. Rapid growth was obtained in the presence of 1 or 2 M NaCl. Addition of 4 M NaCl resulted in a lag phase of 5 days. When NaCl was replaced by KCl, the bacterium grew well at a concentration of 2 M, but failed to grow in the presence of 3 M KCl (Fig. 2). At 40 C, 3 M KCl was not inhibitory to growth. Thus, *Bacillus* sp. no. 21-1 is a moderately halophilic bacterium.

After cultivation of *Bacillus* sp. no. 21-1 in media of various salt concentrations, culture filtrates were dialyzed against 400-fold-distilled water with two changes of water, and the protease activities were measured. To 1 ml of 1% casein solution in 0.1 M tris(hydroxymethyl)aminomethane-chloride buffer (pH 8.0)

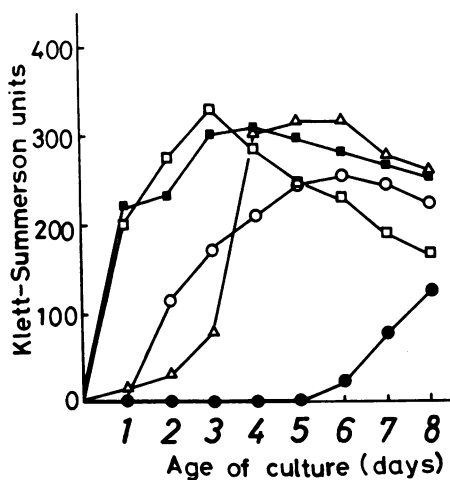


FIG. 1. Growth curves of *Bacillus* sp. no. 21-1 in media containing various concentrations of NaCl. Symbols: Δ , 0 M NaCl; \square , 1 M NaCl; \blacksquare , 2 M NaCl; \circ , 3 M NaCl; \bullet , 4 M NaCl.

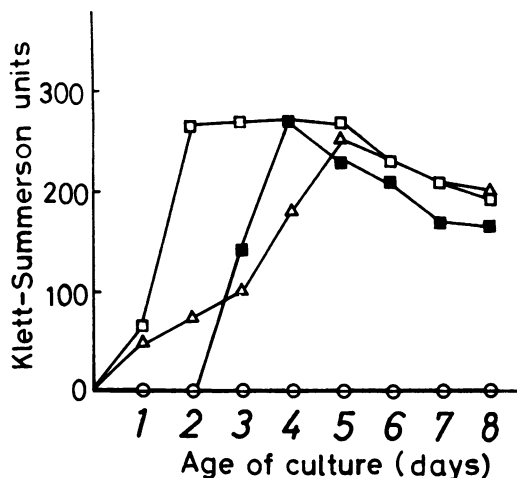


Fig. 2. Growth curves of *Bacillus* sp. no. 21-1 in media containing various concentrations of KCl. Symbols: Δ , 0 M KCl; \square , 1 M KCl; \blacksquare , 2 M KCl; \circ , 3 M KCl.

was added 1 ml of the dialyzed enzyme solution. After incubation for 1 h at 40 C, the reaction was stopped by the addition of 3 ml of 5% trichloroacetic acid, followed by 1 h of incubation at 40 C. The absorbance of the filtrate was measured at 280 nm against a reaction blank for each sample. One unit of the protease activity was defined as the quantity of enzyme which caused an increase of 0.1 in the absorbance at 280 nm under these conditions. Figure 3 shows that 1 M NaCl was optimal for the production of the enzyme. Protease formation was depressed markedly by the addition of 2 M KCl. Addition of casein to the nutrient broth had no effect on the production of the enzyme.

The halophilic *Bacillus* sp. isolated from crude hide (11) resembles in many respects *Bacillus* sp. no. 21-1 that we isolated, but differs in the mode of enzyme production. *Bacillus* sp. no. 21-1, capable of growth at 23% NaCl, produced the protease even in the presence of 17% NaCl. A marine psychrophilic bacterium, *Pseudomonas* sp. no. 548 isolated by Kato et al. (4), secreted protease in a medium containing full-strength sea water, but growth and enzyme production at higher salt concentrations were not described.

The activity of the enzyme produced at 1 M NaCl was assayed as described above except that NaCl or KCl was added to the reaction mixture as indicated. Maximal activity was obtained at 0.5 M NaCl and 0.75 M KCl, almost no activity being observed at 3 M NaCl. KCl

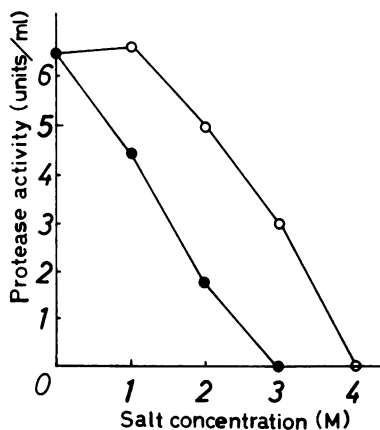


Fig. 3. Production of the enzyme in medium of various concentrations of NaCl (O) and KCl (●). Culture filtrates were dialyzed against distilled water, and protease activities were measured as described in the text.

was not as inhibitory as sodium chloride. Two optimum pH values (8 and 12) were observed when the pH activity profile was determined in the absence of NaCl. Purification and general properties of the enzyme will be published elsewhere.

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