

Characteristics of Halophilic Bacteria in Solar Salts

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ABSTRACT

In this paper, we have studied a number of halophilic bacteria in solar salts produced in various places throughout the world. It was found that the halophilic bacteria in solar salt crystals after the surface had been thoroughly washed were 10^1 – 10^4 /g as measured by the plate cultivation method using a 25% NaCl–SGC medium, and that the viable cells increased with increasing amounts of impurities in the solar salts. We isolated various bacteria from the colonies which appeared on an agar plate for the purpose of the counting of viable cells. From the test of tolerance for NaCl, these isolates appeared to be extremely halophilic bacteria which were red in color and moderately halophilic or halotolerant bacteria which were not red. Further, the group of red colored bacteria and that of non-red color bacteria had characteristic components of proteins, amino acids and lipids. In particular, the lipids of all the red color bacteria isolated were composed of the ether type, and it was confirmed that they were extremely halophilic bacteria.

INTRODUCTION

In the manufacturing process of solar salts and of processed foods using raw or crushed solar salts, there are many problems with micro-organisms. In many cases, coloring phenomena occur through an unusual propagation of red color bacteria. (Masui, 1979). In order to investigate this phenomenon, we studied the biological aspects of halophilic micro-organisms, especially extremely halophilic bacteria present in solar salts.

First, we determined the number of halophilic bacteria in solar salts produced in various places throughout the world, and found differences in the distribution of halophilic bacteria depending on where the solar salts were produced. On the basis of measurements of viable cells, we discuss the relationship between the viable cells and the main constituents of impurities contained in solar salts.

We isolated bacteria from the colonies which appeared on agar plates by counting the viable cells and furthermore, investigated their bacteriological properties and tolerance for NaCl. We attempted to classify chemically these halophilic bacteria based on the components of proteins and amino acids. (Baxter, 1959; Kersters, 1980).

Furthermore, using thin layer chromatography, we confirmed the presence of an ether bond, which is a significant characteristic, in the typical structure of lipids of extremely halophilic bacteria. In addition,

we investigated these isolates in respect to differences in the thin-layer chromatograms of lipids. (Ross et al., 1981; Grant and Larsen, 1989).

MATERIALS AND METHODS

Samples of solar salts

Thirty-seven samples of solar salts from nine producing districts were used in this test; the details are shown in Table 1.

TABLE 1
The solar salt samples

Origin	Date of arrival	No. of samples
Mexico	Jul.–Aug. 1990	5
Australia		
L. Macleod	Jun.–Jul. 1990	5
Dampier	Jul. 1990	5
Shark Bay	Mar.–Jul. 1990	5
Port Hedland	Apr.–Jun. 1990	5
Thevenard	Dec. 1989–Jul. 1990	4
Port Alma	Apr. 1990	1
China	Nov. 1989–Jun. 1990	5
India	Dec. 1989–Mar. 1990	2
Total		37

Measurements of viable cells

The total number of viable cells in solar salts was determined by the following procedure:

Ten grams of solar salt were dissolved in 60 ml of tap water, and 1 ml of the solution was put into a test tube, which contained 9 ml of Sehgal & Gibbons complex (SGC) medium (25% NaCl), and mixed well. This dilution procedure was repeated five or six times. The samples at each dilution step were cultivated at 200 rpm on a reciprocal shaker for a month at 35°C.

The test tubes were then visually checked at the last dilution step for growth. In addition, 0.2 ml of the solutions were then smeared on the surface of petri dishes containing SGC medium with 1.5% agar. The samples were incubated at 35°C for over 3 weeks and the colonies appearing on the plate were counted.

To determine the viable cells in solar salts, the surface of 20 g of solar salts was thoroughly washed with NaCl saturated solution and NaCl saturated ethanol, and then dissolved in 120 ml of tap water. The dissolved sample solutions were filtered through a cellulose ester membrane filter of 0.45 μm pore size. The filter was then placed on an agar plate of the above medium. The plates were incubated under the same conditions as described above, and the colonies appearing on the plate were counted.

Analyses of major components

Solar salts were analyzed by the procedure described in "Methods for Salt Analysis" (Japan Tobacco Inc., 1989).

Characteristics of the isolates

Colonies differing in shape or color were isolated from the agar plate in order to count the viable cells, and the following properties of the isolates were investigated.

Tolerance for NaCl

A loop of each isolate was inoculated in tubes containing SGC medium with 1.5, 2.5, 4.0 and 5.2 M NaCl, respectively. These tubes were incubated at 37°C at 200 rpm for 2 weeks on a reciprocal shaker, and were checked for tolerance at each concentration of NaCl.

Morphology

Morphological properties of the isolated bacteria cultivated in the best growth medium in the tolerance for NaCl test were observed under a microscope.

Protein analysis

Each isolate grown at 37°C in the best growth medium was harvested by centrifuge and washed. The washed bacterial cells were then homogenized with a glass homogenizer and centrifuged. The cell-free extract of the isolates obtained was analyzed by SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of 10% acrylamide gel content. The densities of the bands on the gel were measured by the chromatoscanner, SHIMAZU CS-930.

Amino acid analysis

Bacterial cells were grown and treated with the same procedure as for the protein analysis and then were freeze-dried. After the addition of 6 N HCl, the dry cells were hydrolyzed at 110°C for 22–24 h, evaporated to dryness on a water bath and dissolved in 0.02 N HCl. These samples were analyzed by amino acid analyzer, HITACHI L-8500.

Lipid analysis

Twenty mg of freeze-dried cells produced as described above were mixed with methanol, toluene and concentrated H_2SO_4 and heated at 50°C for 15–18 h for methanolysis. The long-chain constituents were extracted from this mixture by adding hexane. The hexane extracts were chromatographed on silica gel thin-layer plates (TLC) using the method of Ross et al. (1981). The hexane extract of each isolate was investigated, regardless of whether or not glycerol diether moieties (GDEM) and fatty acid methyl esters (FAME) had been ascertained as being present from Rf values. Further, the GDEM were chromatographed in the same direction by development using two different developing solvents (two-step method).

RESULTS AND DISCUSSION

Bacterial population size

The viable cells determined by the dilution end-point method were 10^4 – 10^6 /g in crude solar salts and 10^1 – 10^5 /g in the crystals of solar salt of which the surface had been thoroughly washed. The viable cells in these salts determined by the plate dilution method were 10^2 – 10^5 /g and 10^1 – 10^4 /g, respectively.

The viable cells differed greatly according to the source of the solar salts, and many bacteria also existed in the crystals.

From these results, we believe that it is inadvisable to use solar salts in food manufacturing without confirming their safety.

Relationship between major components and viable cells in the crystals (Figs. 1-3)

The viable cells increased with increasing concentration of Ca^{2+} , Mg^{2+} , K^+ or SO_4^{2-} in the solar salts, and reached a maximum at a certain concentration of these ions. Furthermore, a similar tendency was observed in the relationship between the viable cells and the drying loss of mass or the insoluble matter in solar salts.

From this, we conclude that these bacteria require these ions for growth (Kamekura and Onishi, 1976). It is also clear that the number of viable cells increased with increasing amounts of impurities in the solar salt.

Characteristics of isolates

Tolerance for NaCl concentration

Many red isolates could grow in the SGC medium at concentrations of 4.0 M NaCl and above, but could not grow at concentrations under 2.5 M NaCl. On the other hand, while non-red isolates grew best in the SGC medium of 2.5 M NaCl, they could also grow in 1.5 M NaCl. Therefore, red color isolates were classified as extremely halophilic bacteria, and many non-red color isolates did not grow well because an SGC medium for extremely halophilic bacteria was used.

Morphology

In these experiments, a great many red colonies appeared but some other bacteria colonies were light ochre or ivory in color. Almost all red color isolates were triangular, square or another shape mixed with rods in the liquid culture.

Components of protein

From the pattern of densitometric tracing shown in Fig. 4, it can be seen that big peaks of molecular weight of about 30,000, 45,000, and 66,000 exist in almost all isolates. Moreover, a comparison of the red color and non-red color isolate in Fig. 4 shows that proteins of molecular weights greater than 66,000 were detected in non-red color isolates, but that they were absent from red color isolates.

Components of amino acid

In this study, the amino acid components of each whole cell were analyzed. It can be seen from Fig. 5 that all isolates contained large amounts of acidic amino acids. However, it can also be seen that the acidic amino acid content of red color isolates is higher than that of the non-red color isolates. In connection with this fact, it has been reported (Lanyi, 1974) that enzymes of extremely halophilic

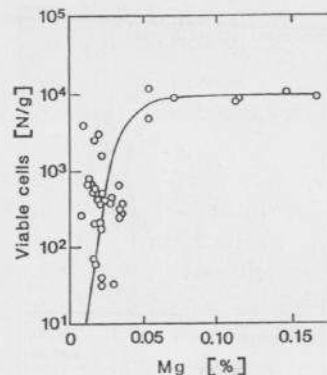


Fig. 1. Relationship between viable cells and Mg^{2+} content in the solar salts.

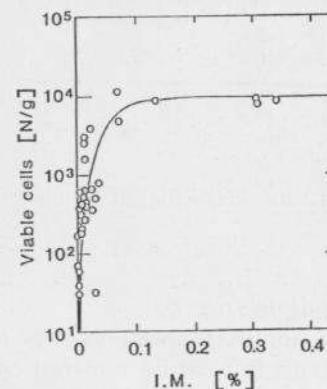


Fig. 2. Relationship between viable cells in the insoluble matter content in the solar salts.

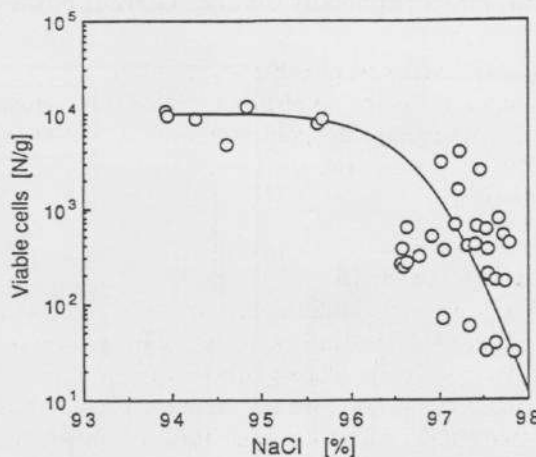


Fig. 3. Relationship between viable cells and NaCl in the solar salts.

bacteria contain large amounts of acidic amino acids. In this report, we propose that the protective effect of salt was due to the shielding of the negative charges on these structures.

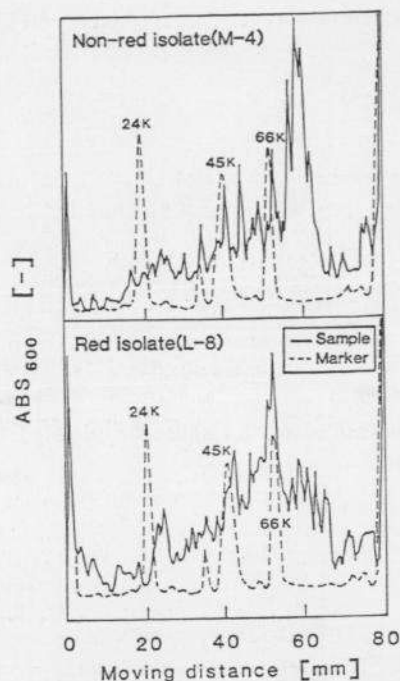


Fig. 4. The densitometric tracings of the electrophoretic protein patterns.

Components of lipid (Fig. 6)

All red color isolates showed only one spot with an Rf value of about 0.2, while non-red color isolates contained only FAME with an Rf value above 0.6. These results agree with the reported characteristics of extremely halophilic bacteria (Ross et al., 1981).

The majority of red color isolates contained only one chromatographically distinct GDEM, but a few

strains produced two distinct moieties when using a two-step method. They were isolated from Chinese and Indian solar salts. These results show that species of extremely halophilic bacteria are different according to the source of the solar salt.

After assembling the results of all these characteristics, it was noticed that red color isolates were different from non-red color isolates in respect of their protein, amino acid and lipid components. In particular, the results of lipid analysis show that all isolated red isolates could be classified as extremely halophilic bacteria, i.e. the structures of the lipid were all ether type. On the other hand, it has been confirmed that non-red color isolates do not contain a lipid of ether type and cannot be classified as extremely halophilic bacteria.

Moreover, from the results of the morphological and physiological studies, we confirmed that most of the red color isolates belonged to the genus *Halobacterium*.

CONCLUSIONS

We have studied the biological aspects of halophilic micro-organisms in solar salts and the results were obtained as follows:

(1) We determined the number of halophilic bacteria in the solar salts produced in various places throughout the world.

(2) It has become apparent that the number of viable cells increased with increasing the amount of impurities in solar salt and the Mg content a limiting factor for the cell growth.

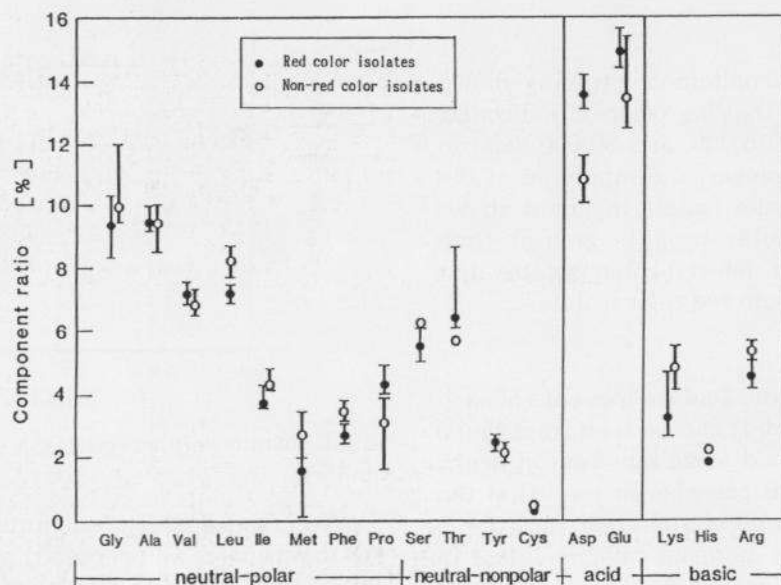


Fig. 5. Comparison of the amino acid components between the red and the non-red isolates.

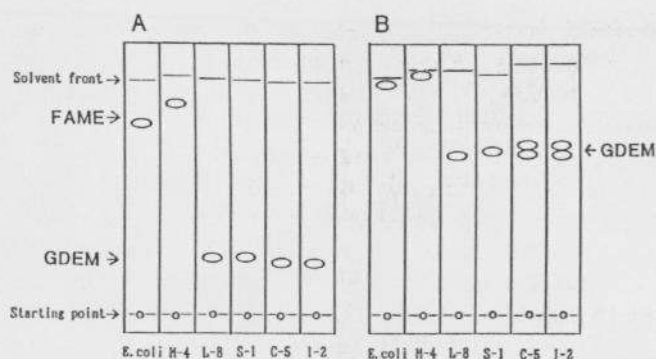


Fig. 6. Thin-layer chromatograms of the lipids in isolates. A. Developer, petroleum ether : diethyl ether = 85 : 15. B. First developer, petroleum ether : acetone = 95 : 5; second developer, toluene : acetone = 97 : 3. Color reagent, 10% dodecamolybdo-phosphoric acid in absolute ethanol.

(3) We isolated various bacteria from the colonies that appeared on the agar plates and then investigated their morphological properties and the tolerance for NaCl concentration. Further, we analyzed the proteins, the amino acids and the lipids in these isolates.

(4) We confirmed that most of the red color iso-

lates were *Halobacterium* that were extremely halophilic bacteria.

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