Properties of Two Halophilic Bacteria from a Salt Pan

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Abstract

An extreme halophilic bacterium and a moderate halophilic bacterium were isolated from water from a salt pan near Bloemfontein containing 28,5% NaCl. The moderate halophile grew in medium containing between 0 and 25% NaCl. The optimum NaCl concentration for growth was 5%. The extreme halophile grew best when the medium contained between 20 and 25% NaCl, but also grew at 15% and 35% NaCl. The optimum temperature for growth of the extreme halophile was between 38° and 40°C while the moderate halophile grew best between 33° and 35°C. The growth rate of the extreme halophile decreased more rapidly below 21°C than the moderate halophile. The effect of temperature on the yield of the halophiles was less marked.

The moderate halophile had properties similar to *Pseudomonas* sp. except that flagella were absent, while the extreme halophile was *Halobacterium halobium*. The ecology of these organisms is discussed.

Introduction

Halophilic bacteria are commonly found in natural environments containing significant amounts of NaCl such as inland salt lakes and evaporated sea-shore pools, as well as environments such as curing brines, salted food products and saline soils. These bacteria often cause a reddening of brine and the salt produced from such environments may be spoilt to a limited extent by the colour. Halophilic bacteria have also been implicated in the spoilage of bacon, fish and hides preserved in solar salt prepared from sea water (Kushner, 1968).

Halophilic bacteria have been divided into groups based on their salt requirements. Flannery (1956) defined obligate halophiles as only growing in media containing more than 2% NaCl and facultative halophiles as growing in media containing less than 2% NaCl but growing best above this salt level. Halophiles have been further classified as moderate when growing best in the range of 3 to 25% NaCl or as extreme growing in the range of 17% to saturated NaCl (Kushner, 1968). Halotolerant bacteria apparently grow best in media containing less than 2% salt but may grow in a NaCl concentration of 10% or more (Flannery, 1956).

Salt lakes or "pans" occur commonly in parts of the Orange Free State and Northern Cape. The purpose of this study was to investigate the presence of halophilic bacteria in one such pan as their presence in salt pans has not previously been reported in South Africa. In this report, an extreme and a moderate halophile isolated from a pan near Bloemfontein and their growth responses to NaCl concentration and temperature are described.

Materials and Methods

Source of the isolates

Water samples were collected in sterile bottles in September, 1977 from a salt pan at Soutpan, O.F.S. being used for the preparation of solar salt, and taken to the laboratory for analysis.

Chemical analysis of the water

Sodium and potassium concentrations in the water were determined by flame photometry (Anon, 1976). Calcium, magnesium, manganese and iron were determined by atomic absorption using a Perkin-Elmer (Model 603) atomic absorption spectrophotometer (Anon, 1976). Nitrate and nitrite were determined by the method of Vogel (1961). Phosphate was determined by the molybdenum-blue method (Fogg and Wilkinson, 1958). All analyses were done in duplicate.

Experimental medium

The halophilic medium had the following composition: Casamino acids, 10 g; Yeast extract, 10 g; Proteose peptone, 5 g; Sodium citrate, 3 g; KCl, 2 g; $MgSO_4.7H_2O$, 25 g and distilled water, 1 *l*. The NaCl concentration was adjusted as necessary. The medium was solidified with 20 g agar per litre medium and the pH adjusted to 7 before sterilization.

Isolation of halophiles

Water samples were filtered through a Millipore filter (0,45 μm pore size) and the filter was placed onto a plate of halophilic medium containing 25% (m/v) NaCl as this concentration was similar to the NaCl concentration of the pan. The plates were incubated at 30°C until colonies appeared on the filter. Representative colonies were purified by repeated streaking on 25% NaCl — halophilic medium.

Growth experiments

The growth rate of the organisms was determined in 500 ml Erlenmeyer flasks with a side-arm to facilitate turbidity measurements of the culture using a Klett-Summerson colorimeter at 640 nm. The flasks were incubated at 37°C in a reciprocal-shaking waterbath. Moderate halophiles were grown at NaCl concentrations between 0 and 25% (m/v) while extreme halophiles were grown at NaCl concentrations between 15 and 35% (m/v). Growth rate determinations at each NaCl concentration were done in triplicate.

Temperature studies

The growth rates and yields of the halophiles grown in a temperature gradient incubator set between 15° and 43°C (Model TN 3, Scientific Industries Inc., Bohemia, N.Y.) were determined turbidimetrically with a Klett-Summerson colorimeter at 640 nm. The temperature at the point opposite the growth tube was measured daily and the mean calculated. The NaCl concentration of the medium was 15% (m/v) for experiments with moderate halophiles and 25% (m/v) for the extreme halophiles. Yields were determined as the maximum optical density reached at each temperature when the culture was in stationary phase. The activation energy was calculated as described by Pirt (1975).

Classification

The oxygen relationships of the organisms were determined in halophilic medium containing 1% glucose, 1,2% agar and 0,04% Bromocresol purple. Liquefaction of gelatin was determined in a medium containing Liebig's meat extract, 7,5 g; peptone, 25 g; gelatin, 120 g; 10% $FeCl_2.4H_2O$, 5 ml and water, 1 *l*. Presence of arginine dihydrolase was determined after growth in halophilic medium supplemented with 2% glucose, 0,3% arginine and the addition of Nessler's reagent. Production of urease by the moderate halophile was determined in Christensen's medium. Indole production was determined by growth in halophilic medium containing 1% casein and the addition of Ehrlich's reagent. Production of 2,3 butylene glycol was determined by growth in halophilic medium containing 1% glucose and testing for the Voges-Proskauer reaction. The NaCl concentration of the medium was adjusted to 25% for growth of the

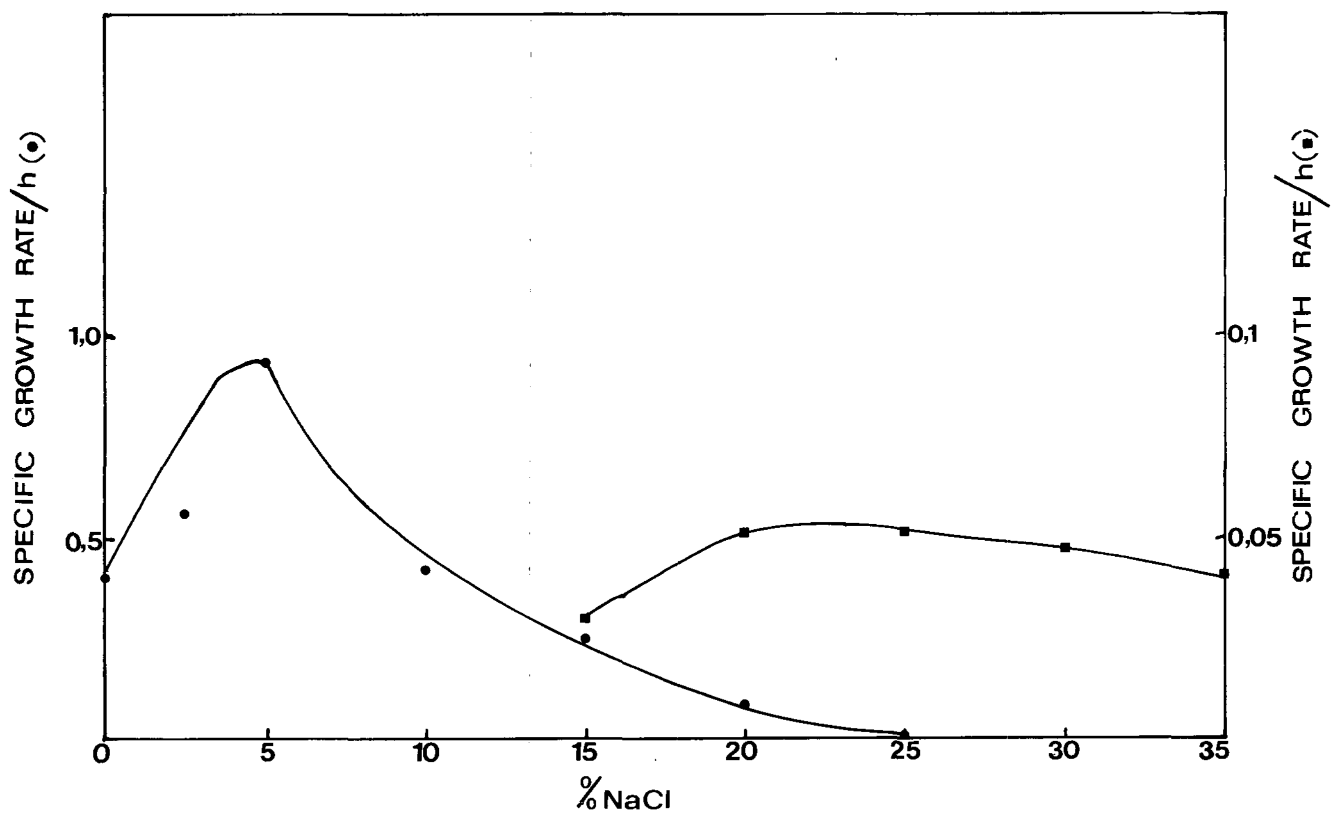

Figure 1
Growth response of a moderate halophilic bacterium (●) and an extreme halophilic bacterium (■)

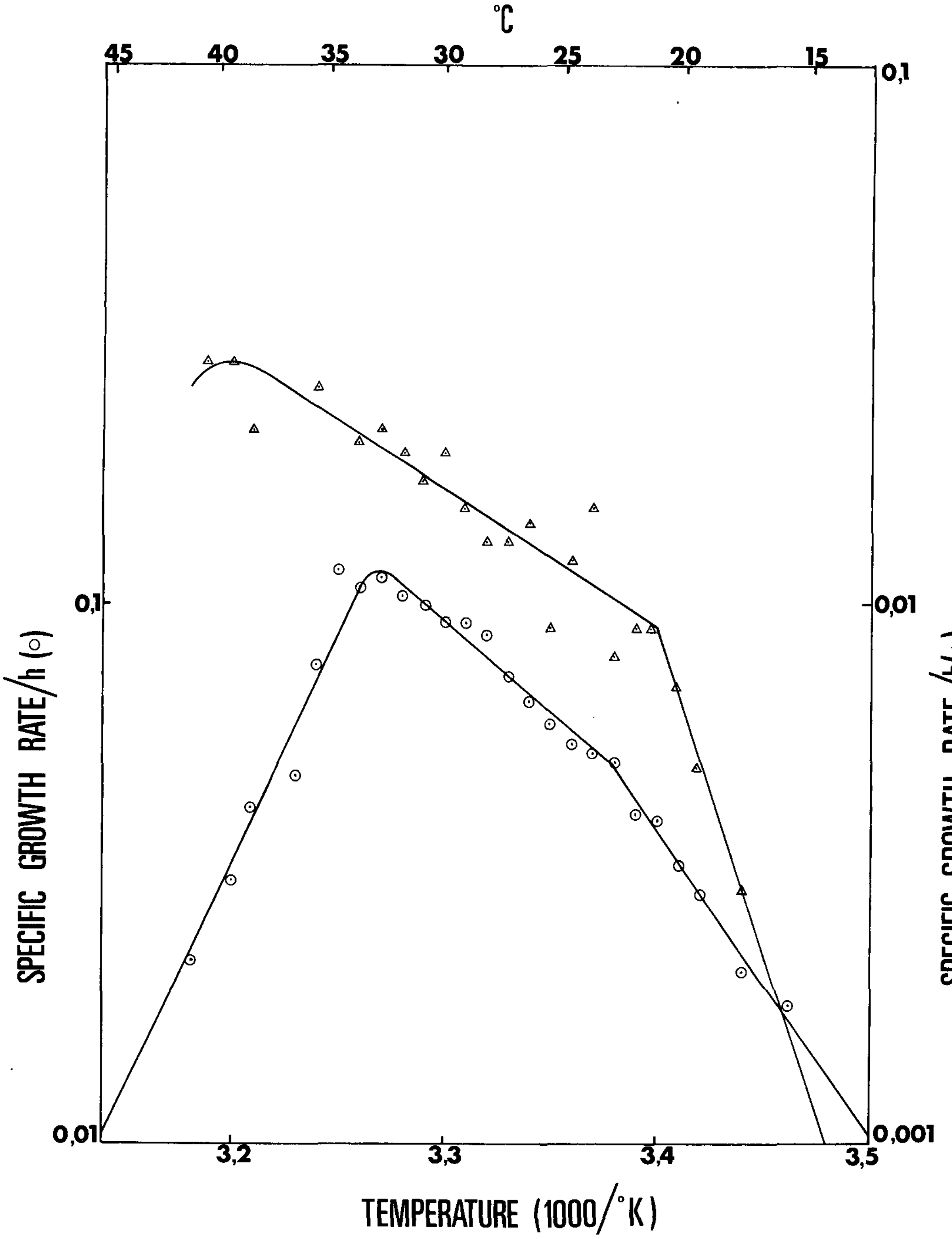

Figure 2
Arrhenius plots of the effects of temperature on the specific growth rate of a moderate halophilic bacterium (⊙) and an extreme halophilic bacterium (△). The lines were fitted by regression analysis (Snedecor and Cochran, 1967)

extreme halophile and 5% for the moderate halophile. Except for the oxygen relationship test, all tests were done under strictly aerobic conditions.

The isolates were negative-stained by the method of Horne (1965) before examination by electron microscopy.

Results and Discussion

Natural habitat and isolation

Analysis of the water from the salt pan showed a NaCl concentration of 28,5% (Table 1). Other substances present in significant amounts were potassium, magnesium, calcium, nitrate and nitrite while phosphate and iron were absent. Phosphate in the presence of high concentrations of calcium forms an insoluble precipitate (Bolt and Bruggenwert, 1976) and may explain its absence.

The high NaCl concentration present in the pan would tend to select for the extreme halophiles. Screening of the NaCl tolerance of the isolates showed that two groups of the organisms were present: red-pigmented extreme halophiles and white-coloured moderate halophiles. Rods and cocci were present in both groups. One representative of each group was selected for further study.

Growth response of the isolates to NaCl concentration

The moderate halophile grew between 0 and 25% NaCl concentration with an optimum being observed at 5% (Fig. 1). The ability to grow in the absence of NaCl is unusual for moderate halophiles. However, this may reflect the conditions of cultivation. Novitsky and Kushner (1975) observed that a moderately halophilic micrococcus was able to grow in the absence of NaCl by the lowering of growth temperature. The high $MgSO_4$ concentration in the medium may have also served as a substitute for NaCl thus enabling the organism to grow. The extreme halophile grew at 15 and 35% NaCl concentration (Fig. 1) with an optimum between 20 and 25% and these results are similar to values reported previously for extreme halophilic bacteria (Brown, 1976).

The moderate halophile grew more rapidly than the extreme halophile at their optimum NaCl concentration and while the moderate halophile reached stationary phase within 16 h, approximately 30 to 50 h was required before completion of the exponential phase by the extreme halophile. The specific growth rate of the moderate halophile was much higher than the extreme halophile at optimum NaCl concentration (Fig. 1). Low growth rates by extreme halophiles have also been reported by Kushner (1968).

TABLE 1
ANALYSIS OF WATER FROM A SALT PAN

Component	Concentration (μg/ml)
Sodium	112000
Potassium	1400
Calcium	121
Magnesium	228
Manganese	<2
Iron	0
NO_2	0,42
NO_3	68
PO_4	0
NaCl	28,5%
pH	6,4

Effect of temperature on growth rate and yield

The extreme halophile showed an optimum temperature between 38° and 40°C (Fig. 2). An Arrhenius plot of the inverse of absolute temperature against the log of specific growth rate showed two linear portions with an inflection at 21,3°C. The activation energy between 21° and 36°C was 2 700 joules while at the lower temperature range between 21° and 18°C, it was

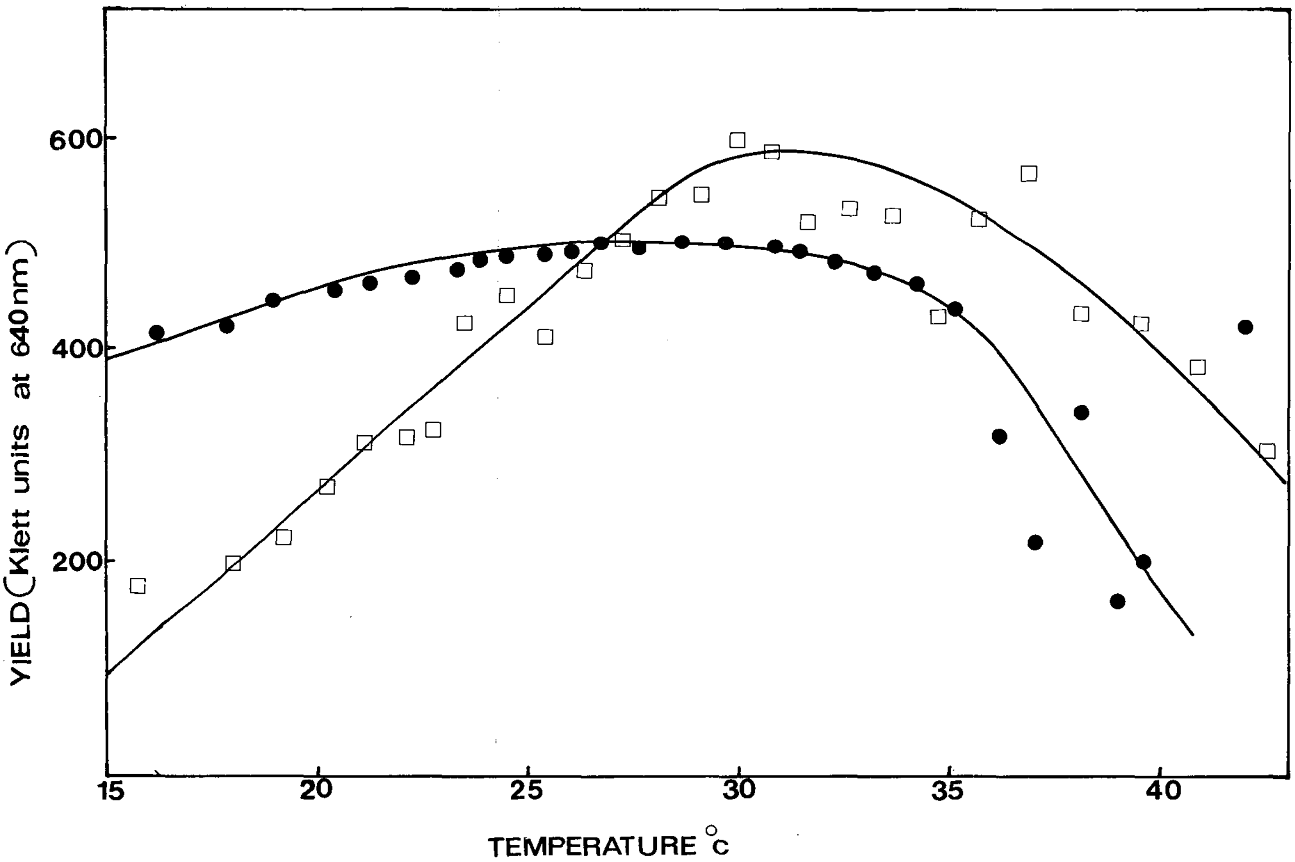

Figure 3
Effect of temperature on the yield of a moderate halophilic bacterium (•) and an extreme halophilic bacterium (□)

13 000 joules. No growth was observed at 15,8°C. The optimum temperature reported here agrees closely with values reported by other workers (Mullakhanbhai and Larsen, 1975; Kushner, 1968). Activation energies for the growth of extreme halophiles has not been previously reported. However, the activation energy between 21° and 36°C (2 700 joules) compares closely to values reported by Pirt (1975) for *Escherichia coli* (3 100—3 900), a psychrophilic pseudomonad (3 000) and *Klebsiella aerogenes* (3 400) over similar temperature ranges. However, the value at the lower temperature range (13 000 joules) is much higher than reported for *E. coli* (6 800 joules) and a psychrophilic pseudomonad (5 700 joules). This suggests that the extreme halophile is sensitive to temperatures below 21°C and this may be of ecological significance in competition with other microorganisms.

The moderate halophile had an optimum temperature between 33° and 35°C (Fig. 2) and is similar to that reported for other moderate halophiles (Breed *et al.*, 1957). Poor growth was observed above 40°C. Between 23° and 32°C, an activation energy of 3 800 joules was observed while between 16° and 23°C, the value was 6 300 joules. The moderate halophile was affected less by lower temperatures than the extreme halophile. This is borne out by observations in the field. The red colouring of the salt pans caused by pigmented halophiles is usually observed only in the summer and not in the spring when the water temperature may still be reasonably low (De Beer, 1977).

The effect of temperature on the yield of the extreme and moderate halophiles is less marked (Fig. 3). The extreme halophile showed highest yield between 28° and 36°C which is significantly lower than the optimum temperature for growth rate. The moderate halophile showed highest yield between 22° and 33°C.

Classification

The moderate halophile was a Gram-negative, non-sporing rod. In some instances, the rod was bent in the form of a vibroid. No flagella were observed in negative-stained cells observed by electron microscopy. The organism was strictly aerobic on glucose, hydrolyzed gelatin and arginine, was catalase- and oxidase-positive and was unable to break down urease or form indole. Except for the absence of flagella, the characteristics of this organism are similar to the genus *Pseudomonas*. No halophilic species of *Pseudomonas* are recognized by the 7th edition of Bergey's Manual (Buchanan and Gibbons, 1974) although halophilic pseudomonads were described in the previous edition (Breed *et al.*, 1957). Evidently it is felt that insufficient information is available to place the halophilic pseudomonads into species. Other Gram-negative rods with similar responses to salt concentrations are in the genera *Achromobacter (Alcaligenes)* and *Vibrio* (Larsen, 1962). However, the properties of the moderate halophile described here do not agree with either of these genera. Thus no genus name is ascribed to the moderate halophile isolated here.

The extreme halophile was a Gram-negative, non-sporing rod with a tuft of polar flagella. The organism was strictly aerobic, catalase- and oxidase-positive, produced 2,3 butylene glycol, indole and arginine dihydrolase. Gas vacuoles were observed and the organism liquefied gelatin. Its salt and temperature requirements and other characteristics agreed with the description of *Halobacterium halobium* (Buchanan and Gibbons, 1974), although the other species, *Halobacterium salinarium* does not appear to be well differentiated from *H. halobium*.

Conclusions

The isolation of halophilic bacteria from salt pans in South Africa has not been previously reported. However, these organisms are ubiquitous and have been reported to occur in similar environments in other countries (Kushner, 1968).

The growth criteria determined in this study show that conditions of high NaCl concentration (>25%) and high temperatures (>33°C) would tend to favour the growth of the extreme halophile. However, analyses in the laboratory (unpublished data) have shown brine samples from the salt pan with a 28% NaCl concentration, contain significant numbers of moderate halophiles and evidently these organisms are able to survive the high NaCl concentration. *H. halobium* grew slowly even under optimum conditions and Kushner (1968) has also reported slow growth rates by extreme halophiles. The moderate halophile grew much faster than the extreme halophile under laboratory conditions and these results suggest that only at high NaCl concentrations can the extreme halophiles be expected to predominate over the moderate halophile. Lower temperature of the water (<21°C) would also favour the moderate halophile. The ecological interaction of the moderate and extreme halophilic bacteria during increasing NaCl concentration in the salt pan should be investigated further in order to confirm results obtained in the laboratory.

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