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A study of moderate and extreme halophilic bacteria for the presence of bromoperoxidase

Swapna Deepak Mayya
San Jose State University

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**A STUDY OF MODERATE AND EXTREME HALOPHILIC BACTERIA
FOR THE
PRESENCE OF BROMOPEROXIDASE**

A thesis

Presented to

**The Faculty of the Department of Biology
San Jose State University**

In Partial fulfillment

of the Requirements for the Degree

Master of Science

by

Swapna Deepak Mayya

May 2000

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APPROVED FOR THE DEPARTMENT OF BIOLOGY

Patricia L. Grilione
Dr. Patricia L. Grilione, Prof. of Microbiology

Ruth Ann Kibler
Dr. Ruth Ann Kibler, Associate Prof. of Microbiology

Sabine Rech
Dr. Sabine Rech, Assistant Prof. of Microbiology

APPROVED FOR THE UNIVERSITY

William Fisher

ABSTRACT

A STUDY OF MODERATE AND EXTREME HALOPHILIC BACTERIA FOR THE PRESENCE OF BROMOPEROXIDASE

by Swapna Deepak Mayya

Bromoperoxidases have been reported from a variety of bacteria, lichens, marine algae and invertebrates. This enzyme has potential for use as a catalyst in bromination of organic compounds and might reduce the use of bromine as a brominating agent due to its toxicity and special disposal needs.

Halophilic bacteria live in a saline environment which has abundant elemental halogens. Although many marine algae possess bromoperoxidase, bromoperoxidases have not been reported as occurring in any of the halophilic eubacteria or extreme halophilic archaeobacteria. The focus of this research was to enrich and to isolate moderate or extreme halophilic bacteria which produce bromoperoxidases. Sixty four halophilic bacterial isolates were obtained. These were grouped into 23 groups based on preliminary morphological and biochemical characterization. An isolate was randomly selected from each group and screened for bromoperoxidase production using the phenol red assay. Two isolates, E3S-2 and E4S-5, were found to possess bromoperoxidase activity in crude protein extracts.

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LITERATURE REVIEW

Introduction.

Marine organisms have become an important focus of study in the search for novel microbial products (6). Many marine organisms have been reported to produce secondary metabolites which have biomedical applications. Ocean water contains 0.5 M chloride, 1 mM bromide and 1 μ M iodide (1). Given its high halogen content, it is not surprising that marine organisms have developed means to incorporate halogens into their metabolites. Many of these halogenated compounds are of pharmacological interest due to their biological activities which include, anti-fungal, anti-bacterial, anti-viral and anti-inflammatory activities (1).

Halogenating enzymes capable of very specific halogenation reactions are attracting increasing attention. Since the production of halogenated compounds by chemical halogenation is not very specific and usually also results in the formation of unwanted halogenated by-products, the use of highly specific halogenating enzymes is an attractive alternative (21).

Bromination is an important process in the manufacture of a range of chemicals, including flame retardants, disinfectants, anti-bacterial and anti-viral drugs (2, 11, 14). Conventional bromination methods typically use elemental bromine, a pollutant and safety hazard.

Based on the current interest in 'green chemistry', *i.e.*, environmentally friendly chemical technology, other methods are being explored to replace existing toxic chemical processes. Bromoperoxidases can be used to carry out safer and more specific bromination reactions. The presence of bromoperoxidases has been reported in a variety of bacteria, lichens, marine algae and invertebrates (10, 11).

Haloperoxidases.

Haloperoxidases are enzymes that catalyze the oxidation of halide ions (where X^- is chloride, bromide or iodide) in the presence of hydrogen peroxide. The resulting electrophilic halide (X^+) can then react with organic substrates (11).

Haloperoxidases are divided into three classes : heme type, eukaryotic non-heme type and bacterial non-heme type (1, 13). The heme-enzyme has protoporphyrin IX as its prosthetic group (4, 12, 16). Haloperoxidases of the eukaryotic non-heme type contain vanadium instead of heme, whereas the bacterial non-heme type do not require metal ions or other cofactors (17, 20).

The nomenclature for haloperoxidases has traditionally been based on the most electro-negative halide which can be oxidized by hydrogen peroxide (1). Thus, chloroperoxidase catalyzes the oxidation of chloride, bromide and iodide by hydrogen peroxide; bromoperoxidase catalyzes the oxidation of bromide and iodide by hydrogen peroxide and iodoperoxidase catalyzes the oxidation of only iodide by hydrogen peroxide (1, 11).

Halophilic bacteria.

Halophiles are organisms that have specific requirements for the sodium ion (9). Halophilic bacteria can be found in a wide range of habitats such as saline lakes, saltern ponds, deserts, hypersaline soils and salted foods (18). Kushner (7) defines moderate halophilic bacteria as those requiring 0.5 - 2.5 M NaCl for growth while extreme halophiles require 2.5 - 5.2 M NaCl. Many extreme halophiles are archaeobacteria and cannot grow in the absence of high salt concentrations in their environment.

Among bacteria, bromoperoxidases have been found in many soil bacteria belonging to the genera *Streptomyces* and *Pseudomonas* (5, 14, 16). Although ocean water and other halophile habitats have high halogen content and bromoperoxidases have been found in numerous marine algae (10), they have not been reported as occurring in any of the halophilic eubacteria or extreme halophilic archaeobacteria. Study and screening of halophilic bacteria isolated from halophile habitats for the presence of bromoperoxidase is essential.

Detection of bromoperoxidase.

Methods commonly employed to detect bromoperoxidase include the phenol red assay, the monochlorodimedone (MCD) assay and activity staining of protein bands on polyacrylamide gels with the phenol red assay reagent (15, 19).

The MCD assay is commonly used to determine the unit of activity of bromoperoxidase using MCD as the substrate. Bromoperoxidase activity is measured by monitoring the decrease in absorbance due to bromination of MCD at 290 nm using a spectrophotometer. This assay requires clarified enzyme fractions, since turbid samples tend to give faulty readings. It also requires much standardization and therefore is not practical for rapid screening of numerous experimental bacterial samples.

Activity staining of protein bands on polyacrylamide gels is an excellent technique but again, for the purpose of large scale screening is impractical. The protein samples are run on a denaturing polyacrylamide gel and then stained with phenol red assay solution. Protein bands containing bromoperoxidase brominate phenol red to bromophenol blue and form blue bands. The preparation and running of gels is laborious and expensive.

The phenol red assay is a very simple, quick and inexpensive assay for rapid screening of a large number of samples. It requires only clean sterile test tubes and phenol red assay reagent. Bromoperoxidase brominates phenol red (sodium salt) to yield bromophenol blue in the presence of bromide (X^-) and hydrogen peroxide (8) as shown in Fig. 1.

FIG. 1. Phenol red assay for bromoperoxidase.

Bromoperoxidase activity is detected colorimetrically by change in color of phenol red from an initial yellow to blue due to the bromination of phenol red. Incorporation of sodium azide in the phenol red assay reagent helps to distinguish between heme and non-heme bromoperoxidases since azide inhibits the activity of heme bromoperoxidases and heme catalases but not that of non-heme bromoperoxidases (19).

OBJECTIVES

The objective of this research is to isolate moderate and extreme halophilic bacteria which produce bromoperoxidases. Soil and water samples taken from salt ponds will be subjected to enrichment technique. All isolates obtained from the enrichment will be characterized on the basis of selected morphological and biochemical tests. The isolates will be grouped based on the similarities in morphological and biochemical characteristics. Members from each group will be randomly selected and screened for bromoperoxidase activity. Crude protein extracts and cell pellet debris of each selected isolate will be screened for the presence of bromoperoxidase using the phenol red assay.

MATERIALS AND METHODS

Media.

Gibbon's medium (3) was used for enrichment and isolation of the halophilic bacteria. It was modified by the addition of 5 g proteose peptone and 500 mg sodium bromide per liter of medium. The Modified Gibbon's medium (MG) was supplemented with 1.5, 2, 3, 4 or 5 M NaCl.

A complex medium (19) was employed to grow the seed cultures of *Streptomyces aureofaciens* ATCC 10762, while a mineral salt (19) medium was used to grow and harvest the *Streptomyces* for screening purposes.

Enzyme assay reagents.

Bromoperoxidase activity was detected by the phenol red assay reagent (1). This reagent was used with and without 10mM sodium azide. To detect chloroperoxidase activity, sodium chloride was substituted at an identical concentration for sodium bromide in the phenol red assay reagent.

Source of moderate and extreme halophile soil and water samples.

Samples of soil and water for the enrichment of moderate halophiles were obtained from salt ponds located near the eastern end of the Dumbarton Bridge, Newark, California. At the time of sampling the ponds, their pH was 8.5, their temperature 17°C and their salinity approximately 1 M NaCl on the salinometer scale. Soil samples taken adjacent to the salt ponds had a pH of 8.0 and a temperature of 18°C. Salinity could not be determined. The extreme halophile samples were obtained from crystallization ponds and levee soils on the property of Cargill Salt Co., Newark, CA. Liquid portions from the crystallization ponds had a pH of 7.0, temperature of 17° C and salinity approximately equivalent to 5.2 M NaCl. The levee soil sample had a pH of 6.0 and a temperature of 16°. All samples were maintained on ice and brought immediately to the lab for enrichment.

Enrichment technique.

All liquid and soil samples were serially diluted using 2 M or 5 M sterile saline water for the moderate and extreme halophiles, respectively. One tenth ml of each dilution was spread onto MG agar in glass petri dishes containing 1.5 M, 2 M, 3 M, 4 M or 5.2 M NaCl. The plates were incubated at room temperature in partially closed metal cans. After two weeks of incubation, the 4 and 5.2 M NaCl agar plates were exposed to ambient light to facilitate possible carotenoid pigment production. These plates also required a longer incubation period, with some needing up to 10 weeks to exhibit growth.

To obtain pure cultures, colonies from the primary enrichment media were streaked for isolation onto MG agar having the corresponding salinity. MG agar slants of the appropriate NaCl concentration were prepared for each isolate and used to inoculate MG broth containing 15 % glycerol and the appropriate NaCl concentration (3). These broths were stored at - 70° C for future use.

Each isolate was given a strain designation as follows: E for Extreme halophile, M for Moderate halophile, S for Soil, W for Water (salt water), 1.5, 2, 3, 4 & 5.2 for molar concentrations of NaCl, *e.g.*, 'E4S-5' stands for the fifth isolate obtained from an extreme halophile enrichment having a 4 M sodium chloride concentration.

Preliminary morphological and biochemical characterization of the isolates.

All reagents for wet mounts, Gram stain and media were modified by the addition of NaCl to meet the osmotic requirements of either moderate or extreme halophiles. The morphological characteristics of each isolate were determined using wet mounts, phase microscopy and the Gram stain. Colony and slant growth descriptions were done macroscopically. Biochemical characterization included a fermentation test using Russell's double sugar (Difco) and an oxidase test (Difco). The isolates were grouped according to similarities of morphological and biochemical characteristics. One member from each group was randomly selected for bromoperoxidase screening. These isolates were also

challenged for their ability to grow on each of the five different molar concentrations of NaCl.

Growth, harvest and partial purification of bromoperoxidase from *Streptomyces aureofaciens* ATCC 10762.

Streptomyces aureofaciens ATCC # 10762 (19), a known bromoperoxidase producer, served as the control for the phenol red assay. Seed cultures of *Streptomyces* (100 ml) were used to inoculate 1000 ml mineral salt broth in 2 liter flasks. The flasks were incubated on a rotary shaker (220 rpm) for 4 days at room temperature. Cells were harvested by pelleting and sonicated (Branson sonifier 350, eight 30 second periods, 40 W, 50 % duty cycle, 4 ° C) to obtain a crude protein extract (19). The cell debris was removed by centrifugation and discarded (Sorval RC2-B, 22100 g, 30 min, 4°C). The supernatant fluid from the crude protein extract was partially purified as shown in Fig. 2.

The partially purified fractions were assayed for brominating activity using the phenol red assay reagent. Bromoperoxidase activity was observed in the 0.5 M NaCl fraction. This fraction was collected in sterile tubes and stored at - 20°C for future use. The enzyme was very stable at - 20°C for up to four months. The enzyme fraction was not quantified for its protein content prior to use as the control in the phenol red assay.

FIG. 2. Partial purification of bromoperoxidase from *Streptomyces aureofaciens* ATCC# 10762 used as control enzyme.

Growth and harvest of the halophilic bacteria for screening.

Frozen stocks of each isolate were used to inoculate 100 ml MG broth for seed cultures. The seed cultures were then inoculated into 1000 ml MG broth in 2 liter flasks. All broth media contained the appropriate NaCl concentration for a given isolate. The flasks were incubated as described for *Streptomyces* on a rotary shaker for 4 days at room temperature with the exceptions of strains E4W - 2 and E5.2W - 3 which required 7 days at 37° C to reach maximum cell growth. Cells of the halophilic isolates were harvested by pelleting, sonicated and centrifuged as described for *Streptomyces*. The cell pellet debris and the supernatant fluid of each isolate were collected for screening.

Screening Protocol.

Crude protein extract from each isolate in the form of supernatant and cell pellet debris was tested for brominating activity by the phenol red assay. Both the supernatant and the cell pellet debris were tested since bromoperoxidase may be membrane-bound and not occur in the supernatant. The protocol for screening the isolates for bromoperoxidase activity is shown in Fig. 3. For each isolate, 100 µl of supernatant and a loop full of cell debris were placed separately into 1 ml of phenol red assay reagent in sterile 5 ml glass tubes. The tubes were incubated at room temperature and observed periodically over a period of 5 days for a color change which would indicate the presence of bromoperoxidase activity. Only those samples exhibiting a change in color from yellow (negative) to blue

(positive) within the 12 hour time period taken by the *Streptomyces* bromoperoxidase sample to give a positive result, were considered to have bromoperoxidase activity.

FIG. 3. Screening protocol to detect bromoperoxidase activity in the isolates.

RESULTS

Sixty four isolates were obtained from the moderate and extreme halophile enrichments. Nineteen of these 64 isolates were from the 1.5 M NaCl enrichment, 21 from the 2 M, 10 from the 3 M, 9 from the 4 M and 5 from the 5.2 M NaCl enrichment.

On the basis of slant and colony growth morphology, Gram stain, sugar fermentation and oxidase production, the 64 isolates were placed into one of twenty three groups. One isolate from each group was randomly selected for bromoperoxidase screening. The morphological and preliminary biochemical characteristics of the isolates which were selected for screening are summarized in Table 1.

Fifty of the 64 isolates characterized were gram-negative rods, eight were gram-positive cocci and the remaining six could not be gram stained. Among the gram-negative rods, 10 of the 37 non-fermenters were oxidase positive, 12 of the 13 fermenters were oxidase positive and the rest were oxidase negative. Among the eight gram-positive cocci, seven non-fermenters were oxidase positive and only one isolate was a fermenter and oxidase negative.

Isolates selected for screening were challenged for their ability to grow on MG agar slants having different molar concentrations of NaCl (Table 2). The growth of isolates at different salt concentrations were scored on a scale of +1 to +3 based on density of growth observed on the slants. Some isolates, when challenged, failed to grow on any salt concentration. As expected, the M1.5S and M1.5W isolates grew equally well on 1.5 M and 2 M NaCl but

not at higher salinities. The majority of M2S and M2W isolates grew on 1.5 M NaCl but exhibited better growth on 2 M and 3 M NaCl. This was also the case with the E3S and E3W isolates except for E3S - 2, which grew best on slants having 3 M NaCl. E4S - 5 grew best in 4 M NaCl, while the remaining E4 isolates grew better in 3 M NaCl or failed to grow.

Bromoperoxidase activity was detected in two of the 23 isolates screened. It was detected in the supernatant portion of E3S - 2 and E4S - 5 within four hours of initiation of the assay (Table 3). These samples exhibited bromoperoxidase activity six hours before the *Streptomyces* bromoperoxidase positive control sample. E3S - 2 was a gram-negative non-motile rod shaped bacterium which was oxidase negative and did not utilise glucose or lactose. E4S - 5 was a motile gram -negative rod which was oxidase positive and utilised glucose but not lactose.

Neither of the two isolates which exhibited bromoperoxidase activity showed chlorinating activity when the chloroperoxidase assay was performed using phenol red assay reagent in which sodium chloride was substituted for sodium bromide. Both isolates were tested for catalase activity and were found to possess it.

Table 1. Morphological and biochemical characterization of halophilic isolates

	STRAINS									
	M1.5S - 1	M1.5S - 4	M1.5S - 6	M1.5S - 12	M1.5S - 13	M1.5S - 14	M1.5W - 2	M2S - 1		
Slant growth	Filiform	Filiform to echinulate	Filiform to echinulate	Filiform to echinulate	Filiform	Filiform	Filiform	Filiform		
Slant color	Offwhite to tan	Creamy	Yellowish to creamy	Tan to creamy	Light tan to creamy	Creamy	Offwhite	Creamy to tan		
Slant surface	Glossy	Glossy	Glossy	Glossy	Glossy	Glossy	Glossy	Glossy		
Colony shape	Irregular	Circular	Circular	Circular	Irregular	Circular	Circular	Circular		
Colony edge	Lobate	Entire	Lobate	Undulate	Lobate	Entire	Entire	Mild undulate		
Colony Elevation	Umbonate	Umbonate	Umbonate	Umbonate	Umbonate	Umbonate	Umbonate	Mild umbonate		
Colony color	Offwhite to tan	Creamy	Yellowish to creamy	Tan to creamy	Light tan	Creamy	Offwhite	Creamy to tan		
Surface	Glossy	Glossy	Glossy	Glossy	Glossy	Glossy	Glossy	Glossy		
Optical nature	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Translucent		
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod		
Motility	Motile	Motile	Motile	Motile	Motile	Motile	Motile	Motile		
Gram stain	gram - negative	gram - negative	gram - negative	gram - negative	gram - negative	gram - negative	gram - negative	gram - negative		
Oxidase	Positive	Negative	Negative	Positive	Negative	Positive	Positive	Positive		
RDS slants										
Glucose	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Positive		
Lactose	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative		

Table 1. continued

STRAINS										
	M2S - 2	M2S- 5	M2S - 6	M2S - 7	M2W - 2	M2W - 5	M2W - 10	E3S - 1		
Slant growth	Filiform	Filiform	Filiform	Filiform	Filiform	Filiform	Filiform	Filiform		
Color	Tan	Tan to peach	Tan	Tan	Creamy	Creamy to light peach	Creamy	Creamy		
Surface	Glossy	Glossy	Glossy	Glossy	Glossy	Glossy	Glossy	Glossy		
Colony shape	Circular	Circular	Circular	Circular	Circular	NA*	NA	Circular		
Edge	Entire	Irregular	Entire	Entire	Entire	NA	NA	Entire		
Elevation	Flat	Umbonate	Convex to umbonate	Convex	Convex	NA	NA	Convex		
Color	Tan	Tan to peach	Tan	Tan	Creamy	NA	NA	Creamy		
Surface	Glossy	Glossy	Glossy	Glossy	Glossy	NA	NA	Glossy		
Optical nature	Opaque	Opaque	Translucent	Opaque	Opaque	NA	NA	Opaque		
Cell shape	Rod	Rod	Rod	Rod	Coccus	Rod	Coccus	Rod		
Motility	Non - motile	Non - motile	Non - motile	Non - motile	Non - motile	Non - motile	Non - motile	Non - motile		
Gram stain	gram - negative	gram - negative	gram - negative	gram - negative	gram - positive	gram - negative	gram - positive	gram - negative		
Oxidase	Positive	Positive	Positive	Negative	Negative	Positive	Negative	Negative		
RDS slants										
Glucose	Negative	Positive	Negative	Negative	Negative	Positive	Positive	Negative		
Lactose	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative		

* Isolates did not grow on agar plates.

Table 1. continued

STRAINS								
	E3S - 2	E3S - 8	E4S - 1	E4S - 5	E4S - 17	E4W - 2	E5.2W - 3	
Slant growth	Filliform	Filliform	Filliform	Filliform	Filliform	Beaded, slimy	Beaded, slimy	
Color	Creamy	Creamy	Creamy	Creamy	Beige	Pinkish to orange	Pinkish to orange	
Surface	Glossy	Glossy	Glossy	Glossy	Glossy	Glossy	Glossy	
Colony shape	Circular	NA	Confluent colonies, few punctiform	Confluent colonies, few punctiform	NA*	NA	NA	
Edge	Entire	NA	Irregular to entire	Irregular to entire	NA	NA	NA	
Elevation	Convex	NA	Flat	Flat	NA	NA	NA	
Color	Creamy	NA	Creamy	Creamy	NA	NA	NA	
Surface	Glossy	NA	Glossy	Glossy	NA	NA	NA	
Optical nature	Opaque	NA	Opaque	Opaque	NA	NA	NA	
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	
Motility	Non - motile	Non - motile	Motile	Motile	Motile	Motile	Motile	
Gram stain	gram - negative	gram - negative	gram - negative	gram - negative	gram - negative	NA	NA	
Oxidase	Negative	Negative	Positive	Positive		NA	NA	
RDS slants								
Glucose	Negative	Negative	Negative	Positive	Negative	NA	NA	
Lactose	Negative	Negative	Negative	Negative	Negative	NA	NA	

* Isolates did not grow on agar plates.

Table 2. Growth of isolates at different NaCl concentrations

Strains	Molar concentrations of NaCl				
	1.5	2	3	4	5.2
M1.5S - 1	- ^a	-	-	-	-
M1.5S - 4	-	-	-	-	-
M1.5S - 6	-	-	-	-	-
M1.5S - 12	+3 ^b	+3	-	-	-
M1.5S - 13	-	-	-	-	-
M1.5S - 14	+3	+3	-	-	-
M1.5W - 2	+1 1/2	+3	-	-	-
M2S - 1	+3	+3	-	-	-
M2S - 2	+3	+2	+2	-	-
M2S - 5	+2	+3	+3	-	-
M2S - 6	+2	+3	+3	-	-
M2S - 7	+2	+3	+3	-	-
M2W - 1	+1	+2	-	-	-
M2W - 5	+3	+3	+2	-	-
M2S - 10	-	-	-	-	-
E3S - 1	+3	+3	+3	-	-
E3S - 2	+2	+2	+3	+1	-
E3S - 8	+3	+3	+3	-	-
E4S - 1	NA ^c	NA	+3	-	-
E4S - 5	NA	NA	+2 1/2	+3	-
E4S - 17	NA	NA	+2	1 colony	-
E4W - 2	NA	NA	-	-	-
E5.2W - 3	NA	NA	-	-	-

^a No Growth

^b Growth

^c Not available, isolates challenged only on 3M, 4M and 5.2 M NaCl.

Table 3. Screening Results

Strain	Sample	Reaction	Time in hours
Negative Control	PhR ^a (no H ₂ O ₂)	No change	120
Negative Control	PhR + H ₂ O ₂	Violet	48
Positive control (Streptomyces)	0.5 M NaCl fraction + Azide	Blue	10 – 12

M1.5S - 1	Pellet debris + / - Azide	Blue	48
	Supernatant + Azide	Blue	84
	Supernatant - Azide	Blue	120
M1.5S - 4	Pellet debris + Azide	Blue	48
	Pellet debris - Azide	No change	120
	Supernatant + / - Azide	No change	144
M1.5S - 6	Pellet debris + / - Azide	Blue	48
	Supernatant + Azide	Blue	144
	Supernatant - Azide	No change	120
M1.5S - 12	Pellet debris + Azide	Blue	48
	Pellet debris - Azide	Blue	72
	Supernatant + / - Azide	No change	120
M1.5 - 13	Pellet debris + Azide	Blue	72
	Pellet debris - Azide	No change	120
	Supernatant + / - Azide	No change	120
M1.5S - 14	Pellet debris + / - Azide	Blue	72
	Supernatant + / - Azide	No change	120
M1.5W - 2	Pellet debris + / - Azide	Blue	96
	Supernatant + / - Azide	No change	120
M2S - 1	Pellet debris + / - Azide	Blue	36
	Supernatant + / - Azide	No change	120
M2S - 2	Pellet debris + / - Azide	Blue	48
	Supernatant + / - Azide	No change	120
M2S - 5	Pellet debris + / - Azide	Blue	48
	Supernatant + / - Azide	No change	120
M2S - 6	Pellet debris + Azide	Blue	96
	Supernatant + Azide	Yellow blue	120
	Pellet debris - Azide	Brown	120
	Supernatant - Azide	No change	120

^a Phenol red reagent

Table 3. continued

Strain	Sample	Reaction	Time in hours
M2S - 7	Pellet debris + Azide	Pale Violet	96
	Supernatant +/- Azide	No change	120
	Pellet debris - Azide	No change	120
M2W - 2	Pellet debris + Azide	Blue	48
	Supernatant + Azide	Magenta	72
	Pellet debris - Azide	No change	120
	Supernatant - Azide	Magenta	120
M2W - 5	Pellet debris + / - Azide	Blue	48
	Supernatant + Azide	Blue	60
	Supernatant - Azide	No change	120
M2W - 10	Pellet debris + / - Azide	Blue	60
	Supernatant + Azide	Blue	60
	Supernatant - Azide	No change	120
E3S - 1	Pellet debris + / - Azide	Violet	48
	Supernatant + / - Azide	Blue - grey	120
E3S - 2	Pellet debris + Azide	Blue	16
	Supernatant + Azide	Blue	4
	Pellet debris & Super. - Azide	No change	120
E3S - 8	Pellet debris + Azide	Blue	36
	Supernatant + / - Azide	No Change	120
	Pellet debris - Azide	Blue	72
	Pellet debris & Super. - Azide	No Change	120
E4S - 1	Pellet debris + / - Azide	Blue	60
	Supernatant + / - Azide	Blue	72
E4S - 5	Pellet debris & Super. + Azide	Blue	4
	Pellet debris & Super. - Azide	No change	120
E4S - 17	Pellet debris + / - Azide	Blue	60
	Supernatant + Azide	Blue	84
	Supernatant - Azide	No change	120
E4W - 2	Pellet debris + / - Azide	Blue	96
	Supernatant + / - Azide	No change	120
E5.2W - 3	Pellet debris + / - Azide	Blue	60
	Supernatant + / - Azide	No change	120

DISCUSSION

The marine environment has a huge diversity of organisms and also a high halogen content. Halogenated natural products have been reported from marine algae, fungi, invertebrates and bacteria (6). Haloperoxidases have been reported in many marine algae and marine invertebrates (10, 11). Current research involving screening for haloperoxidases has focused on soil bacteria such as *Streptomyces* and *Pseudomonas* genera. Some of their species are known to produce chlorinated antibiotics. It is presumed that halogenating enzymes are involved in the halogenation and biosynthesis of these antibiotics. Considering the fact that thalassohaline salt ponds have a high halogen content and a diverse population of moderate and extreme halophilic bacteria (7, 18), these habitats are ideal environments to screen for bromoperoxidase producing halophilic bacteria.

Sixty four bacterial isolates were obtained from halophile enrichments of soil and water samples collected from salt ponds. Based on preliminary morphological and biochemical characterization, these isolates were grouped into 23 groups and one member from each group was screened for bromoperoxidase. Bromoperoxidase activity was detected in two isolates, E3S - 2 and E4S - 5. These organisms originated from the 3 M and 4 M NaCl containing enrichment agars inoculated with soil samples collected near a salt pond having approximately 5.2 M NaCl.

The phenol red assay was used to screen halophilic isolates for the presence of bromoperoxidase. Bromoperoxidase of *Streptomyces aureofaciens* ATCC# 10762 was partially purified in order to have a stable stock of enzyme for use as a control in the assay. In the negative controls of the phenol red assay, it was observed that the addition of hydrogen peroxide (15.6 mM) to the phenol red assay reagent (in the absence or presence of cell sample) slowly altered the color of the phenol red reagent from yellow to violet-blue in 2 days. The bromoperoxidase of *Streptomyces aureofaciens* ATCC# 10762 required only 10 -12 hours to show positive enzyme activity. Therefore, only isolates which showed bromoperoxidase activity within 12 hours of the initiation of assay were considered as positive for bromoperoxidase activity.

For the bromoperoxidase-producing isolates E3S - 2 and E4S - 5, activity was observed only when sodium azide was present in the phenol red assay reagent. Sodium azide inhibits heme containing enzymes including catalases and heme bromoperoxidases (15, 17). Catalases, when present in the enzyme extract, can compete with bromoperoxidase for hydrogen peroxide and make it difficult to detect bromoperoxidase activity (16). Both isolates showed catalase activity and this may be responsible for inhibiting bromoperoxidase activity in the absence of azide. Since the enzyme activity was seen in the presence of azide, these isolates are likely producing non-heme bromoperoxidases.

Cell pellet debris of E3S - 2 showed very little activity, suggesting the enzyme may not be membrane-bound. The faint activity observed in the pellet upon prolonged incubation of the assay sample is most likely due to the presence of traces of supernatant enzyme as a contaminant. Bromoperoxidase activity in E4S - 5 was observed in both the pellet debris and the supernatant. The enzyme of this isolate is possibly membrane-bound but since sonication disrupts the entire cell, the enzyme could have become distributed uniformly in both the pellet debris and the supernatant, accounting for the observed activity.

Generally a chloroperoxidase can catalyze the oxidation of chloride, bromide and iodide in the presence of hydrogen peroxide, while a bromoperoxidase catalyzes the oxidation of only bromide and iodide. To confirm that the brominating activity observed in E3S - 2 and E4S- 5 was not due to a chloroperoxidase, the phenol red assay was done in which NaCl was substituted for NaBr. The enzyme did not chlorinate phenol red.

Although not mentioned under “Materials and Methods” and “Results”, a MCD assay (19) was attempted to quantify the activity of the bromoperoxidases of both isolates. This attempt failed, most likely due to inadequate purification of the enzymes.

Most of the 23 isolates screened for bromoperoxidase activity grew best at salt concentrations equivalent to that of the enrichment media from which they were isolated. E3S - 2 and E4S - 5 grew better at NaCl concentrations corresponding to that present in the enrichment plates from which they were isolated. Some of the M1.5S and M2S isolates did not grow in any of the NaCl concentrations. This was also the case with M2W - 10,

E4SW - 2 and all the E5.2W isolates. The cell suspensions used were likely too dilute to initiate growth, as some strains do not grow well unless they are heavily inoculated.

Based on the habitat from which the two bromoperoxidase producers were enriched and the fact that they were consistently growing at high salt concentrations, it is assumed that they are moderate and / or extreme halophiles. If future taxonomic investigation of the isolates confirm this, it will be the first report of the occurrence of bromoperoxidase in halophilic bacteria. Further studies need to be done to classify these two isolates and also to purify the enzymes in order to determine their molecular weight, unit of activity, spectral properties, substrate specificity and kinetics.

These bromoperoxidase-producing isolates are very easy to grow in a short period of time compared to marine algal bromoperoxidase producers. The bacterial enzymes therefore have tremendous potential as brominating agents in 'green chemistry' to replace current hazardous chemical bromination processes.

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