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SCREENING OF EXTREMLY HALOPHILIC ARCHAEA FOR ITS BIOTECHNOLOGICAL POTENTIAL.

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ABSTRACT

Halophiles are salt loving organisms requiring about 3M- 5M NaCl concentration in their growth. Eleven halophilic archaea and nine bacteria were isolated from various samples from extremely high salt concentrated ecosystem from western parts of Maharashtra, India. The isolates were studied using morphological and biochemical studies. Most of the isolates were gram negative in nature. These organisms were tested for their ability to produce industrially important extracellular enzymes like caseinase, gelatinase, amylase, and protease. The current study indicates that, haloarchaea are of potential importance in pharmaceutical, industrial, textile and food industry.

KEYWORDS: **Haloarchaea, Halophilic bacteria, Solar salterns, Hydrolytic enzymes**

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INTRODUCTION

Microorganisms represent the highest population inhabiting all the habitats and are an excellent source of biogenic substances. Halophiles are a group of organism present in all the three domains*, Archaea, Bacteria and Eukarya*. Prior to 1977, microrganisms were only classified as prokaryotes and eukaryotes, but in 1977, Carl Woese proposed on the basis of 16SrRNA sequencing and Phylogenetic tree rooting there also exixts a group of distinct organisms that are more closely related to the bacteria than the eukaryotes known as the "ARCHAEA". Archaea are divided into two kingdoms Crenarchaeota and Euryarchaeota, the former inhabiting the extremely thermophilic region and the latter are methanogens and halophiles[1]. These organisms can only burgeon in presence of high salt concentration. All the environments have been well explored, but, hypersaline niche remains a richness of halophilic and halotolerant organisms meagerly investigated. Hypersaline environment is an enriched habitat containing about 2.5-5M of NaCl and the organisms thriving in it require this niche for their growth and survival. The halophilic microbial population is often seen to be dwelling the salt lakes, solar salterns, salt mines and soda lakes. The salterns are a kind of thalossaline man made habitat constructed on a large piece of land having rectangular demarcations and connected through common channels. Sea water is allowed in the rectangular patches and allowed to stay until evaporation occurs and salt is harvested [2]. Archaea are a group of prokaryotic organisms living in the salt marshes, hypersaline lakes, saline ponds, hot springs and solar salterns are widespread all around the globe. The survival of the archaeal microbes is by using the "salt-in" strategy where it accumulates the potassium ions in the cytoplasm of the cell to counteract the load of sodium ions in the environment, thus maintaining the osmoadaptation of the cell. It is also observed that though there is a treasure of sodium ions in the outside environment of the cell, the inner cytoplasm does not contain any amount of those ions. Haloarchaea belonging to order halobacteriales and family halobacteriaceae are the ruling and dominant microorganisms requiring hypersaline environment for their growth. This family is said to have around 130 species of organisms [3,4,5,6]. These organisms are distinguished as pigmented species attributing its coloration to the presence of ruberins and carotenoid C-50[7,8,9]. As these microorganisms grow in strict conditions, of direct exposure to UV radiation, extreme salinity, alkaline pH, high temperature, they have developed a mechanism to combat such harsh environments. Hence, these organisms have evolved to be of industrial use, viz. textile industry, food industry, pharmaceutical industry. They are also a decent source of enzymes that can tolerate stringent environmental conditions. There is a need and demand for more industrially useful products to the existing ones, so it is important to explore this new habitat which has been particularly abandoned until date [10,11,12]. Therefore, in this era researchers are turning their attention towards this less explored and useful microbial kingdom. The western belt of Maharashtra, especially the coastal areas, harbors a variety of ecosystems for such studies, some of the areas have been already excavated while some are underway. In the current investigation, we have explored the biotechnological potential of haloarchaeal community from the western belt of coastal India.

MATERIALS AND METHODS

2.1. Site of Sample Collection

Samples of extremely high salt containing sea water and soil were collected from the western belt of Maharashtra, mainly the coastal areas. These areas collect sea water which is used for protraction of sodium chloride for edible purpose. The local community residing in Mumbai is involved in the traditional process of salt making. All the samples were collected in sterile containers.

2.2. Enrichment and isolation of samples

These samples were enriched by adding one gram or ml of respective sample in Sehgal and

Gibbon's medium [13]. The broths were incubated at 40-42°C for 7-27 days for 2- 3 enrichments until intense pink coloration was observed. Thereafter, the cultures were isolated on Sehgal and Gibbon's medium [13]

2.3.Morphological and biochemical characterization

Gram staining was performed using Dussault's method [14] which is a variation of the conventional Gram staining method. Salt tolerance test was performed to check the growth of the cultures on varying salt concentrations ranging from (0% - 30%). A confirmatory test for archaea and bacteria was performed by growing the organism on medium containing 0.25 g/L sodium taurocholate and 20mg/ L Chloramphenicol. Growth on Chloramphenicol and no growth on sodium taurocholate plate confirmed that the organisms were haloarchaea[15].The oxidase activity was performed by adding a drop of oxidase reagent to the bacterial cells on Whatmann filter paper no1. The colonies turning blue in color were marked oxidase positive. Catalase activity of the cultures was performed by adding hydrogen peroxide to the cells of the culture and effervescence showed that the Archaea were catalase positive. Indole test was performed by inoculating 5ml culture in tryptone water. After 8 days add 1ml of Kovac's reagent (Himedia, Mumbai) was added to each tube and check for ring of cherry red coloration. Anaerobic growth on Arginine, DMSO and Potassium Nitrate was performed on Standard growth medium (g/L) (Yeast extract- 10, Casamino acids-7.5[9], NaCl-250, $MgSO_4$. $7H_2O$- 40, KCl- 2, Trisodium Citrate- 3, Trace solution- 10ml/L). Trace solution contains (in 100 ml, $FeCl_2.4H_2O$-2.3mg, $CaCl_2$. $7H_2O$- 7mg; $MnSO_4.H_2O$-0.3mg; $ZnSO_4$- 0.44mg; $CuSO_4.5H_2O$- 0.050mg, pH-7.2) [16]. $5gL^{-1}$ Arginine; 5 gL^{-1} DMSO and 30mM of KNO_3 is added to the medium in individual oakridge tubes. After laying the medium with 5ml of the culture, the tubes were tightly fastened and incubated in the dark for 8 days. Growth was checked by measuring the O.D at 600nm [17,18]. The utilization of various sugars like Sucrose, Glucose, Maltose and Lactose by the isolates was checked by using 0.05 % concentration of sugar in sugar utilization medium (Himedia, Mumbai) [4].

2.4. Screening for extracellular hydrolytic enzymes

Sehgal and Gibbons medium with 1% Starch was used to screen amylase production. The culture was spot inoculated on the plate. The plates were incubated the plates until the colonies appear, and then overlayed with 1% Iodine solution. The zone of hydrolysis is indicative of amylase production. Sehgal and Gibbon's with 1% Tween 80 was prepared and spot inoculated. The plates were incubated at 40°C and checked for the zone of hydrolysis. Sehgal and Gibbons medium with 1.5% Gelatin was used to check the production of gelatinase. After a period of incubation, overlay the plate with 0.5M Mercuric Chloride and observe the zone of hydrolysis. Sehgal and Gibbon's with 1% Casein was prepared and checked for the zone of clear hydrolysis on the plate indicative of caseinase production[10,11,19,20,21].

2.5. Test for optimum growth in $MgSO_4$ and $MnCl_2$ (0mM-500mM)

15% SG medium containing varying $MgSO_4.7H_2O$ and $MnCl_2$ concentration was prepared and the cultures were spot inoculated with it. The incubation was carried out for 7 days at 40^0C to check the optimum growth and coloration.

RESULTS AND DISCUSSION

The temperature for each sample was noted between 32°C – 37°C and alkaline pH was observed. Some beds had salt already formed while some were still budding.

3.1. Isolation and Characterization

Sea water and soil samples were collected from the coastal areas of Western Maharashtra (Fig. 1).

Figure 1
The site for traditional process of salt production from sea.

The temperature recorded of the samples at the time of sampling was in the range of 32□C- 36□C and pH was in the range of 7.5-9. In total nine Halobacteria and eleven Haloarchaea were isolated from the samples by incubating them in different medium and incubation durations. The samples were rich in sodium and chloride ions.During enrichment, most of the enrichment broths showed intense red coloration (Fig. 2a) and upon isolation, typical red colored colonies was observed.

a) b)

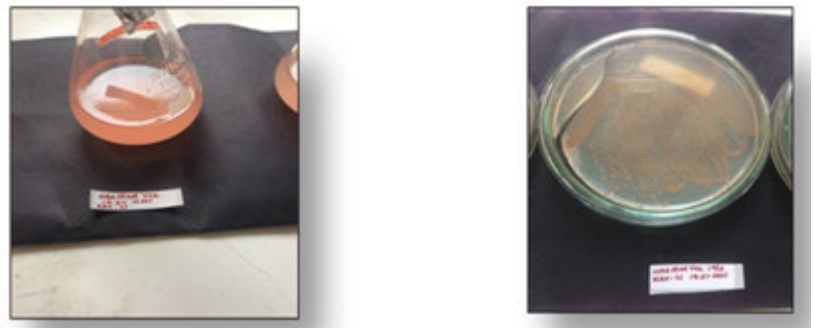

Figure 2 a
Enrichment of sea water sample in Sehgal and Gibbon's medium ,
b. Sehgal and Gibbon's plate containing 15% NaCl.

3.2. Morphological Characterization

The isolated organisms were mostly gram negative in nature except for a few, were gram positive and coccoid in morphology (Table 1).

Table1
Morphological characterization and growth pattern of the Archaea

Isolate no.	Sample type	Gram nature	Cell morphology	Color	Incubation days
1	Sea Water	-ve	Coccobacilli	Pink	7
2	Sea Water	-ve	Cocci	Orange	7
3	Sea Water	+ve	Cocci	Red	7
4	Sea Water	-ve	Cocci	Pale Red	7
5	Sea Water	-ve	Cocci	Orange	7
6	Salt bed soil	+ve	Cocci	Pinkish Red	7
7	Sea Water	-ve	Cocci	Dark orange	15
8	Sea Water	-ve	Cocci	Reddish orange	15
9	Concentrated Sea Water	-ve	Cocci	Pale pink	15
10	Concentrated Sea Water	-ve	Cocci	Pink	27
11	Concentrated Sea Water	+ve	Cocci	Pink	27

Some of the colonies were pinpoint while others were 2-5mm large. The organisms were irregular, mucoid in shape and had entire margin with transparent opacity and convex elevation. They were seen in varied in the shades of pink, red and orange that depicted the presence of characteristic pigments in them (Fig. 3).

Figure 3
Single isolated orange color colony of Isolate No. 5

3.3. *Biochemical Characterization*

The biochemical tests like oxidase, catalase, indole, anaerobic growth for these organisms were performed as described in Bergey's manual of Systematic Bacteriology and Aharon Oren [2,4,7,24,25].

3.4. *Salt tolerance and confirmatory test for Archaea*

Most of the organisms are extreme halophiles and require 25% and above for their lavish growth, while a few can grow in presence of moderate salt concentration.

Table 2
Salt tolerance and confirmatory test for Archaea

Isolate no.	0%	2%	5%	8%	10%	15%	25%	30%	Chloramphenicol	Sodium Taurocholate
1	-	-	-	+	+	-	+	+	+	-
2	-	-	+	-	-	-	-	-	+	-
3	-	-	-	-	-	-	+	+	+	-
4	-	-	-	-	-	+	+	+	+	-
5	-	-	-	-	-	-	-	+	+	-
6	-	-	+	+	-	-	-	-	+	-
7	-	-	-	+	+	-	+	+	+	-
8	-	-	+	+	+	+	+	+	+	-
9	-	-	-	-	-	+	+	+	+	-
10	-	-	+	+	+	+	+	+	+	-
11	-	-	+	+	+	+	+	+	+	-

3.5. *$MnCl_2$ Utilisation*

Most of the organisms did not grow in presence of $MnCl_2$, except KN6 which showed poor growth at 500mM. Further work to check for production of any extracellular metabolite is underway.

Table 3
Growth of organisms on varying concentration of $MnCl_2.4H_2O$

Isolate no.	0mM	5mM	10mM	50mM	100mM	200mM	300mM	400mM	500mM
1	-	+	+	-	-	-	-	-	-
2	-	-	+	+	+	-	-	-	-
3	-	-	-	-	-	-	-	+	-
4	-	-	-	-	-	+	+	+	+
5	-	+	-	-	-	-	-	-	-
6	-	-	+	-	+	-	-	-	-
7	-	-	+	-	+	-	-	+	-
8	-	-	-	+	+	-	+	+	-
9	+	+	+	-	+	-	-	+	-
10	-	-	-	-	-	-	-	-	-
11	-	-	+	-	-	-	-	+	-

Key: '+' - growth, '-' – no growth

3.6. *$MgSO_4$ Utilisation*

All the organisms showed luxurious growth and coloration in the presence of high concentration of $MgSO_4$. Hence, it can be concluded from this data that $MgSO_4$ is one of the essential chemical required for the growth of Archaea.

Table 4
Growth of organisms on varying concentration of $MgSO_4.6H_2O$

Isolate no.	0mM	5mM	10mM	50mM	100mM	200mM	300mM	400mM	500mM
1	-	+	+	+	+	+	+	+	+
2	-	+	+	+	+	+	+	+	+
3	-	-	-	-	-	-	+	-	+
4	-	-	-	-	-	-	+	+	-
5	-	+	+	+	-	+	+	+	+
6	-	-	+	+	-	-	-	-	-
7	-	+	+	+	+	+	+	+	+
8	-	+	+	+	+	+	+	-	-
9	+	+	+	+	+	+	+	+	+
10	-	-	+	+	+	+	+	+	+
11	-	+	+	+	+	+	+	+	+

Key : '+' - growth , '-' - no growth

3.7. Sugar utilization and Hydrolysis of Starch Tween 80, Gelatin and Casein

It can be concluded from the table below that all the sugars were utilized but not fermented as it did not show the formation of air bubble in the Durham's tube. Sucrose is the only exception to sugars which was not utilized by any of the organisms except one i.e. Isolate no. 9. These Archaea isolated did not show the presence of gelatinase and caseinase but some produced amylase and lipase enzyme.

Table 5
Sugar utilization and production of extracellular enzymes

Isolate no.	Glucose	Sucrose	Maltose	Lactose	Amylase	Caseinase	Gelatinase	Protease
1	+	-	+	-	-	+	-	-
2	-	-	-	-	-	-	-	-
3	+	-	+	-	-	-	-	-
4	+	-	-	+	-	-	-	-
5	+	-	-	-	-	+	-	-
6	+	-	+	-	-	-	-	-
7	+	-	+	-	+	+	-	-
8	+	-	+	+	+	+	-	-
9	+	+	+	+	+	+	-	-
10	+	-	+	-	-	+	-	-
11	-	-	-	+	+	+	-	-

Key : '+' - growth , '-' – no growth

3.8. Anaerobic growth

These organisms are aerobes but can also survive anaerobically by utilizing Arginine, DMSO and potassium nitrate as electron donors.

Table 6
Anaerobic growth in presence of Arginine, DMSO and potassium nitrate

Isolate no.	Arginine Utilisation	KNO_3 Utilisation	DMSO Utilisation
1	0.418	0.788	0.574
2	0.565	1.150	0.454
3	0.438	0.941	0.535
4	0.585	1.150	0.454
5	0.450	1.038	0.468
6	0.587	0.685	0.464
7	0.470	0.633	0.574
8	0.180	0.259	0.191
9	0.521	0.809	0.508
10	0.486	0.901	0.456
11	0.536	0.856	0.635

Biochemical tests: All of these organisms are catalase negative, while some showed oxidase activity and were positive for indole test.

Table 7
Biochemical tests.

Isolate no.	Oxidase	Catalase	Indole test
1	-	-	+
2	-	-	+
3	-	-	+
4	-	-	-
5	+	-	+
6	+	-	-
7	+	-	+
8	-	-	-
9	+	-	+
10	+	-	+
11	-	-	+

Key : '+' - growth , '-' – no growth

CONCLUSION

On the basis of this study and biochemical tests it can be concluded that halophilic Archaea isolated from coastal areas of Western Maharashtra may belong to Haloferax species however, this needs to be confirmed using 16SrRNA sequencing. They are of potential use in various industries as these organisms are capable to hold a treasure box of bio-active molecules required for various industrial, pharmaceutical, textile and food processing industry.

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