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# Exploring Microbes from Bhavnagar and Okhamadhi Marine Salterns for Production of Industrially Important Enzymes

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**ABSTRACT:** Marine solar salterns, is an extreme hypersaline environment which is used for commercial production of salt and other marine chemicals from sea-water. The hypersaline water is predominantly inhabited by halophilic and halotolerant microorganisms. With the rise of green chemistry to protect the environment, the use of biocatalysts in industry has increased enormously over the past two decades. The aim of this research is to isolate, identify halotolerant microorganisms of two marine salterns of Gujarat (Bhavnagar and Okhamadhi), and to examine their salt tolerance/requirement and enzyme profile. Four bacterial (BVUC\_01, BVUC\_02, BVUCh\_01, BVUCh\_02) culture isolated from marine salterns of Bhavnagar. One bacterial (MOVUCh\_01) and one fungal (MOVUP\_01) culture were isolated from Okhamadhi water samples. MOVUP\_01 and MOVUCh\_01 isolates showed 98.75% and 100% similarity with *Aspergillus versicolor* and *Staphylococcus epidermidis*, respectively by NCMR – NCCS, Pune, India through nucleotide BLAST analysis based on ITS and 16S rRNA gene sequencing. BVUC\_02 and BVUCh\_01 were identified as *Bacillus megaterium* and *Bacillus licheniformis* with 93% and 97% probability, respectively by Supratech Micropath Laboratories, Ahmedabad, India through VITEK2 system. Isolates from Bhavnagar marine salterns produced all of the extracellular enzymes tested, except pectinase in screening media without NaCl. MOVUCh\_01 showed only protease activity, while MOVUP\_01 produced all enzymes in media without NaCl. BVUC\_01, BVUC\_02, BVUCh\_01 and BVUCh\_02 produced amylase and protease in screening media containing 10% NaCl. MOVUCh\_01 showed protease activity in media containing 20% NaCl. This study reveals the diversity of microorganisms present and their hydrolyase production.

**KEYWORDS:** Marine salterns, Bhavnagar, Okhamadhi, Hydrolytic enzymes, *Aspergillus versicolor*

## I. INTRODUCTION

Marine ecosystem is extremely diverse because of high salt content and other physical factors which make it different from other ecosystems. Marine microorganisms are key components of such an extreme environment and are diverse as their ecosystem. Marine salterns are extreme hypersaline environment which commercially use for salt production. They are predominantly inhabited by halophilic and halotolerant microorganisms (*Archaea*, *Bacteria*, *Eukarya*). Some of the marine salterns in India that have been studied for the microbial diversity are located in: Gujarat [1-5], Karnataka [6], West Bengal [7], Maharashtra [8] and Orissa [7].

India is the third largest Salt producing country in the World after China and USA. In India, major marine salterns are located along the coastal regions of Gujarat, Tamil Nadu, Maharashtra, Karnataka, Orissa, West Bengal and Goa (tnsalt.com). Gujarat alone accounts for 76% of total salt production in India, it become largest salt producer state in India and third largest in the world. Bhavnagar, Jamnagar, Porbandar, and Rann of Kutch are the major salt producing districts of Gujarat [9].

Microorganisms that grow in the presence of salt (NaCl) are categorized into following groups: (i) slight halophiles that grow optimally at 0.2-0.5 M, (ii) moderate halophiles at 0.5-2.5 M, (iii) extreme halophiles at 2.5-5.2 M salt concentration. (iv) Halotolerant can grow in both low and high salt concentration. In some cases, they can tolerate 5.5M salt concentration [10].

Adaptation to hypersaline conditions microorganisms, require specialized cellular as well as enzymatic adaptations to preserve osmotic balance with the environment and to avoid excessive water loss. There are two mechanisms known

that help microorganisms to survive in presence of high salinity: (i) High salt-in strategy in which osmotic balance is achieved by accumulating high concentrations of inorganic salts intracellularly [11]; and (ii) Compatible solute or low salt-in strategy. Compatible solutes also called as osmolytes, allow all essential cell processes to function effectively in the presence of high salt concentration [12]. This strategy requires accumulation of osmolytes inside the cell. Their accumulation helps to maintain turgor pressure, cell volume, and concentration of electrolytes - all important elements for cell proliferation [44]. Concentration of these substances within the cell are regulated according to the salt concentration outside the cell [13]. Apart from it, osmolytes protects microbial protein from denaturation in water of low salt concentration while enhancing the microbes tolerance to dramatic fluctuations in the external saline environment [14]. Osmolytes present in halophiles and halotolerant organisms are categorized into following groups [44]: (i) Zwitterionic solutes like, betaine, ectoine [15], hydroxyectoine [16], beta-glutamine [17]. (ii) Noncharged solutes like, alpha-glucosyl glycerol [18], trehalose [19], sucrose [20], alpha-mannosyl glyceramide [21]. (iii) Anionic solutes like, L-alpha-glutamate [22], beta-glutamate [22], hydroxyl butyrate [23], alpha-glucosyl glycerate [21], alpha-mannosyl glycerate [21].

With the rise of green chemistry to protect the environment, the use of biocatalysts in industry has increased enormously over the past two decades [25]. The global enzyme market was about 3.9, 6.1, 8.18 and expected to reach 13.79 US billion dollars in 2011, 2016, 2018 and 2026 respectively [24]. Most of industrial processes carried out under harsh operational conditions (various range of temperature, pH and salt concentrations) at which normal enzyme denature, aggregate and precipitate. For that reason, the finding of novel enzymes that exhibit optimal activity at these extreme conditions is continuous to be a desirable objective in enzyme research and its demand is high [26, 27]. Halophilic enzymes extremozymes, which are capable to function under hypersaline conditions. Some of them are also capable to function under high temperature and pH ranges [27, 28]. So, halozymes becomes promising for industrial applications. Growing demand for halozymes has focused attention on halophilic microorganisms, as its enzyme are furnishing with unique structural features and catalytic power to sustain metabolic and physiological processes under extreme conditions. As a result, halophiles emerge as an ideal, promising, unexplored and potentially valuable resource of halozymes [1, 29, 30]. The aim of this research is to isolate, identify halotolerant microorganisms from two marine salterns of Gujarat (Bhavnagar and Okhamadhi), and to examine their salt requirement / tolerance and screen for their extracellular enzyme profile.

## II. MATERIALS AND METHODS

### Sampling site and sample collection

Saline soil and water sample collected from Okhamadhi and Bhavnagar (along the NH47) marine salterns in sterile containers and stored in refrigerator until used for isolation of microorganisms.

### Enrichment and Isolation

Samples were enriched by salt (100.0/200.0 g/L, NaCl; for Bhavnagar and Okhamadhi respectively) and substrate (starch, carboxymethylcellulose, chitin, pectin, gelatin, tributyrin) enrichment [29]. Diluted samples were added into enrichment medium and incubated at 37<sup>o</sup>C, 120 rpm for 5-7 days. After enrichment, sample streak on Nutrient agar (for bacteria) and Potato dextrose agar (for fungi) with 10 & 20% NaCl, plates were incubated at 37<sup>o</sup>C and 29<sup>o</sup>C respectively for 5-7 days. Resultant colonies were purified by repeated streaking on respective isolation media. Pure cultures are preserved on slants at 4-10<sup>o</sup>C, in a refrigerator.

### Morphological studies

Morphology of pure cultures were studied microscopically using 10X, 45X (for fungi) and 100X (for bacteria) objectives with microscope.

### Screening for production of extracellular hydrolytic enzymes

Qualitative detection of extracellular enzyme production by isolates done by using, different enzymatic agar plate assays [35]. The pH of all media was adjusted on 7.2-7.4 (for bacteria) 6.5-7.0 (for fungi). All enzyme assay carried out in media with (10% and 20%) and without NaCl. The different assay media used are described below.

Amylase activity:



Amylase activity of the cultures was screened on starch nutrient agar plate, as earlier described by [31]. After incubation the plates were flooded with 0.3% I<sub>2</sub>-0.6% KI solution; a clear zone around the growth indicated the hydrolysis of starch.

Cellulase (CMCase) activity:

CMCase activity of cultures was screened in media containing (g/L): Carboxymethyl cellulose 5, NaNO<sub>3</sub> 1, K<sub>2</sub>HPO<sub>4</sub> 2, KCl 1, MgSO<sub>4</sub> 0.5, yeast extract 0.5, glucose 1, agar 17. After incubation, the plates were flooded with 0.1% Congo red solution. The clear zone around the colony indicated cellulase activity [33].

Chitinase activity:

Screening of the isolates for chitinase activity was performed by using Chitin agar plates containing (g/L): colloidal chitin 5, Na<sub>2</sub>HPO<sub>4</sub> 0.65, KH<sub>2</sub>PO<sub>4</sub> 1.5, NaCl 0.25, NH<sub>4</sub>Cl 0.5, MgSO<sub>4</sub> 0.12, CaCl<sub>2</sub> 0.005 and agar 18. After incubation the plates were flooded with 0.3% I<sub>2</sub>-0.6% KI solution; a clear zone around the growth indicated chitinase activity [36].

Lipase activity:

Cultures were screened for lipolytic activity on Tributyrin agar plates containing: (g/L) tributyrin 10, yeast extract 3, peptone 5, calcium carbonate 10, Nile blue sulphate 0.01, agar 20. Clear zone of tributyrin hydrolysis surrounding growth indicate lipase activity [29].

Pectinase activity:

Pectinase activity of isolates was determined by using media containing (g/L): pectin 10, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 1.4, K<sub>2</sub>HPO<sub>4</sub> 2, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2, nutrient solution 1 (FeSO<sub>4</sub>·7H<sub>2</sub>O 5 mg/L; MnSO<sub>4</sub>·H<sub>2</sub>O 1.6 mg/L; ZnSO<sub>4</sub>·7H<sub>2</sub>O 1.4 mg/L; CaCl<sub>2</sub> 2 mg/L), agar 20. After incubation the plates were flooded with 0.3% I<sub>2</sub>-0.6% KI solution. A clear zone around the growth showed pectinolytic activity [34].

Protease activity:

The isolates screened for protease activity on gelatin nutrient agar plates containing gelatin (1.0% w/v). The gelatinase activity was observed as a clear zone around the growth after adding Frazier's reagent for 5 min. [32].

#### Identification of isolates

Selected fungal (MOVUP\_01) and bacterial (MOVUCh\_01) isolates genetically identified by National Centre for Microbial Resource – NCCS, Pune, India through nucleotide BLAST analysis based on ITS and 16S rRNA gene sequencing respectively. Other two bacterial isolates (BVUC\_02, BVUCh\_01) identified by Supratech Micropath Laboratories, Ahmedabad, India through VITEK2 system.

### III. RESULTS AND DISCUSSION

#### Isolation and characterization of microorganisms from sample

Four Gram positive bacterial (BVUC\_01, BVUC\_02, BVUCh\_01, BVUCh\_02) cultures were isolated from marine salterns of Bhavnagar. One bacterial (MOVUCh\_01) and one fungal (MOVUP\_01) cultures were isolated from Okhamadhi marine salterns. Isolates from Bhavnagar and Okhamadhi salterns were able to grow in medium containing up to 10.0 and 20.0 (% w/v) salt concentration. All these isolates also grew in the isolation medium without salt (NaCl). Morphology and gram's character of isolates are given in Table 1.

Table 1		
Designation	Morphology	Gram's Reaction
BVUC_01	Short rod	+
BVUC_02	Big Rod	+
BVUCh_01	Rod	+
BVUCh_02	Rod	+
MOVUCh_01	Cocci	+

**Table 1. Characteristics of the bacterial isolates.**

### Screening for hydrolytic enzyme production

All isolates were screened for extracellular enzymes (amylase, cellulase, chitinase, lipase, pectinase, protease) production in respective media, with NaCl (10.0 and 20.0 % w/v), and without NaCl. Results showed a variety of enzyme production by these halotolerant isolates.

Isolates from Bhavnagar marine saltern (BVUC\_01, BVUC\_02, BVUCh\_01, BVUCh\_02) produced all enzymes (amylase, cellulase, chitinase, lipase, protease) except pectinase in screening media without NaCl, as shown in Table 2. The isolate MOVUCh\_01 showed only protease activity, while MOVUP\_01 produced all enzymes in media without NaCl. The isolates BVUC\_01, BVUC\_02, BVUCh\_01 and BVUCh\_02 produced amylase and protease in screening media containing 10.0 (% w/v) NaCl. MOVUCh\_01 showed protease activity in media containing 20.0 (% w/v) NaCl (Table 3).

Table 2							
No.	Designation	Zone of Hydrolysis					
		Amylase	Cellulase	Chitinase	Lipase	Pectinase	Protease
1.	BVUC_01	++	+++	+++	++	-	++
2.	BVUC_02	+	+	+	++	-	+++
3.	BVUCh_01	++	+++	+++	+	-	+++
4.	BVUCh_02	+++	+++	+++	+	-	++++
5.	MOVUCh_01	-	-	-	-	-	++
6.	MOVUP_01	++	+++	+++	+	++	+++

+ Degree of hydrolysis

**Table 2. Screening of isolates for hydrolytic enzyme production in media without NaCl.**

Table 3			
No.	Designation	Zone of Hydrolysis	
		Amylase	Protease
1.	BVUC_01	+++	+++
2.	BVUC_02	++	+++
3.	BVUCh_01	+++	++++
4.	BVUCh_02	+++	++++
5.	MOVUCh_01	-	++
6.	MOVUP_01	-	-

+ Degree of hydrolysis

**Table 3. Screening of isolates for hydrolytic enzyme production in media containing NaCl.**

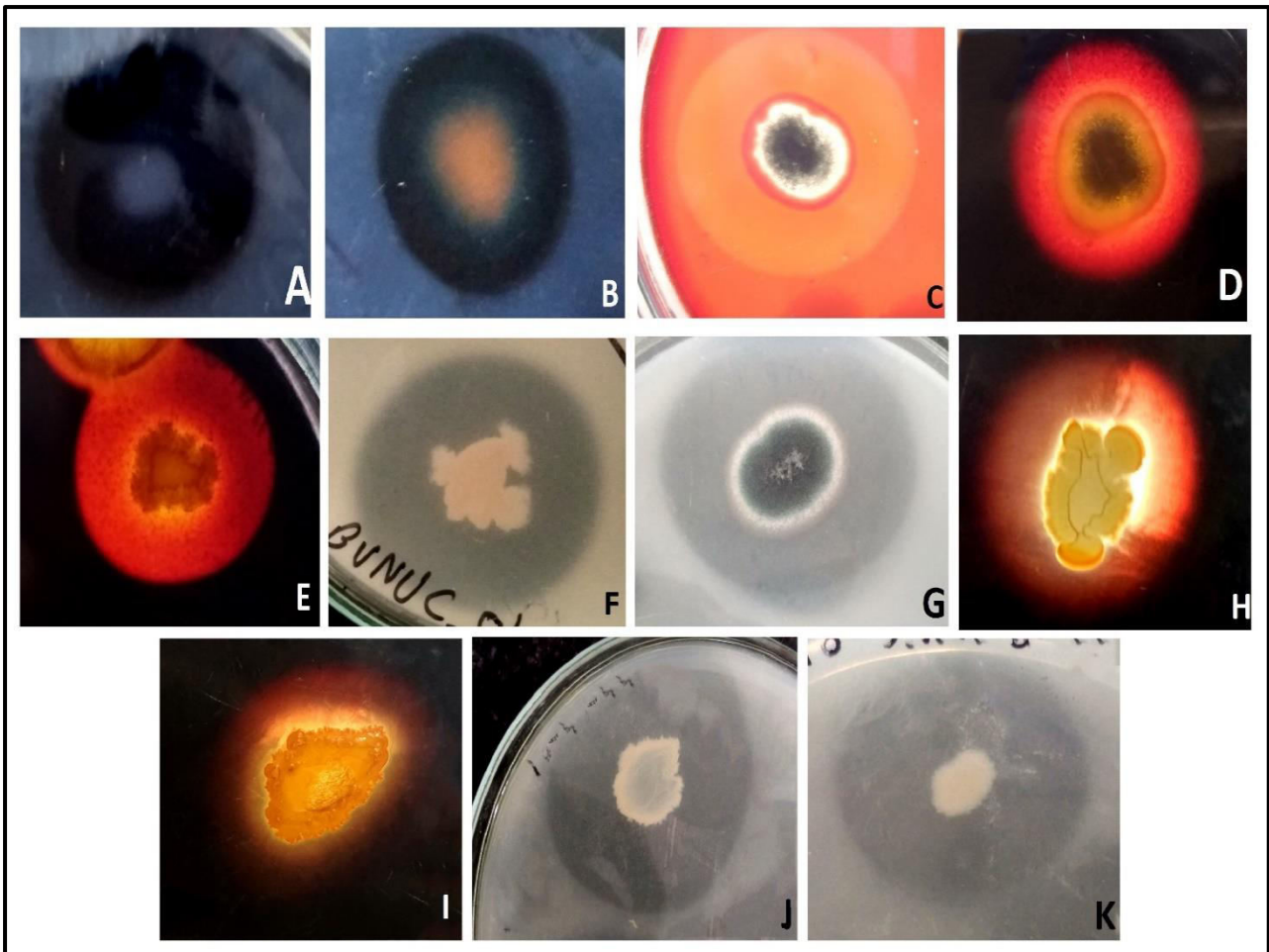


Fig. 2 Photographs of screening of isolates for Amylase (A-B), Cellulase (C), Chitinase (D-E) and Protease (F-G) enzyme production, in respective media without NaCl. (H-I); and (J-K) Screening of isolates for Amylase and Protease enzyme production respectively, in respective media containing NaCl (10.0 & 20.0 % w/v).

**Identification of isolates**

MOVUP\_01 and MOVUCh\_01 isolates showed 98.75% and 100% similarity with *Aspergillus versicolor* (Strain: ATCC 9577) and *Staphylococcus epidermidis* (Strain: NCTC 11047(T)) with Genbank accession number NR\_131277.1

and UHDF01000003 respectively; according to National Centre for Microbial Resource – NCCS, Pune, India based on nucleotide BLAST analysis based of ITS and 16S rRNA gene sequencing, respectively.

BVUC\_02 and BVUCh\_01 were identified as *Bacillus megaterium* (Bionumber: 0252510565456220) and *Bacillus licheniformis* (Bionumber: 1376271757577271) with 93.0% and 97.0% probability respectively, by Supratech Micropath Laboratories, Ahmedabad, India based on VITEK2 system. The fungal isolate MOVUP\_01 has been deposited at National Centre for Microbial Resource, Pune, Maharashtra, India with Accession number: MW376894.

There are very few reports on microbial diversity of Okhamadhi marine salterns [4, 5]. To the author's best knowledge this is the first report concerning the screening and characterization of hydrolytic enzyme producing halotolerant bacteria and fungi from Okhamadhi marine salterns. Microbial diversity of Bhavnagar salterns has been studied by some researcher, but there are very few report which shows halotolerant diversity of this area [1]. We report here for first time the presence of *Aspergillus versicolor* ATCC 9577 and *Staphylococcus epidermidis* NCTC 11047(T) in marine salterns of Gujarat.

Various strains of *A. versicolor* have been reported from saline sites of Goa [37], Little Rann of Kutch [38], Lake Magadi, Kenya [39]. *Staphylococcus epidermidis* previously been isolated from Valparaíso and viña del Mar – Chile [40]. Various strains of *Bacillus licheniformis* have been isolated and described as halotolerant from saline sites at Tutikorin [41] and Bhavnagar [1].

Hydrolytic enzymes such as amylase, cellulase, chitinase, lipase, pectinase and protease have been commercialized in various industries including food, baking, feed, chemical and pharmaceutical, paper and pulp, detergent, leather industries, fish sauce and soy sauce preparations, saline waste water, and oilfield waste treatment [30, 42, 43].

#### IV. CONCLUSION

Different halotolerant isolates have been isolated from marine salterns at Bhavnagar and Okhamadhi. These cultures were screened for production of important enzymes such as amylase, cellulase, chitinase, lipase, pectinase and protease; in media with and without NaCl. Results shows variety of enzyme production by isolates. The isolates MOVUP\_01 and MOVUCh\_01 isolates showed 98.75% and 100% similarity with *Aspergillus versicolor* (Strain: ATCC 9577) and *Staphylococcus epidermidis* (Strain: NCTC 11047(T)), according to NCMR – NCCS, Pune, India. The bacterial isolates BVUC\_02 and BVUCh\_01 were identified as *Bacillus megaterium* and *Bacillus licheniformis* with 93.0% and 97.0% similarity, respectively based on VITEK2 system. Some of the isolates showed very good extracellular hydrolyase production for commercial exploitation.

#### V. ACKNOWLEDGEMENT

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#### VI. CONFLICT OF INTEREST

Authors not have conflict of interest.

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