

ISOLATION AND CHARACTERIZATION OF HALOPHILIC BACTERIA PRODUCING AMYLASE AND PROTEASE ENZYME FROM MARAKKANAM SALT PAN

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ABSTRACT

Halophilic bacteria are organisms which inhabit the salt-rich environment and are capable of producing wide variety of bioactive compounds. Sediment samples were collected from four different sites of Marakkanam salt pan, Tamil Nadu. Totally, 23 halophilic bacteria were isolated and their colony characteristics were recorded. Based on the colony morphology and gram staining results, ten isolates were selected and screened for their ability to produce commercially important hydrolytic enzymes such as amylase and protease. The results showed that the isolates MSP1 and MSP2 produce highest amylase and protease enzyme activity. Further, the isolates MSP1 and MSP2 were identified as Bacillus sp. and Pseudomonas sp. respectively by 16SrRNA method.

KEYWORDS: Halophiles, Marakkanam, Salt Pan, Amylase, Protease, Bacillus sp & Pseudomonas sp

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INTRODUCTION

Solar salterns or salt pans are artificial, shallow open ponds used to evaporate brine for the salt production. The high salt concentrations at these environment represent a unique group of organisms that survive at high salinities, high temperatures and tolerate severe solar radiations (Jamadar *et al.*, 2016). The microorganisms that live under these extreme salt conditions are said to be halophilic and halotolerant microorganisms. These halophilic microorganisms are also adapted to high pressure of the environment resulting from high salinity. Halophiles include all the three domains, namely Archaea, Bacteria and Eucarya and contain representatives of many different physiological types adapted to a wide range of salt concentrations as high as salt saturation (Aljohny, 2015).

In general, halophiles adapted to two special defensive mechanisms to cope with the osmotic pressure induced by the high NaCl concentration of the environment in which they live: Some extremely halophilic bacteria accumulate inorganic ions in the cytoplasm (K^+ , Na^+ and Cl^-) in order to balance the osmotic pressure of the medium. In addition to that, they have also developed specific proteins that are stable and active in the presence of salt. In contrast, moderate halophiles accumulate large amounts of specific organic osmolytes, which function as

osmoprotectants, providing osmotic balance without affecting with the normal cellular metabolism (Santos and Costa, 2002; Fraser *et al.*, 2004; Moreno *et al.*, 2013; Aljohny, 2015).

Since the halophilic bacteria can tolerate high salt concentration and low nutritional requirement, that confer them a significant potential in harsh industrial processes. Biotechnological applications of halophiles include the production of compatible solutes, biopolymers and carotenoids; they have also studied for various environmental bioremediation processes. As they are stable and active at high salt concentrations, halophilic enzymes can be used in food processing, environmental bioremediation and biosynthetic processes. Accordingly, discovering novel enzymes, showing optimal activities at various ranges of salt concentrations, temperatures and pH values are commercially important.

Halophilic microbes produce several industrially important enzymes, in which amylase and protease are known to be one of the commercially important enzymes playing vital role in the field of biotechnology (Margesin and Schinner, 2001). Amylase is used as a pharmaceutical aid for the treatment of digestive disorders (Moreno *et al.*, 2013). It is also used in various food, textile, detergent, paper and chemical industries. Proteases are used in medical, detergent and food processing industries. Amylases and proteases are derived from several microorganisms like fungi, yeast, bacteria and actinomycetes but the genus *Bacillus* is found to possess maximum amylase activity. As the isolation, identification and maintenance of these halophilic microbes are difficult; very few studies have been reported regarding their applications (Das Sarma and Arora, 1997).

With this background, the present investigation was made on isolation, characterization of halophilic bacteria from Marakkanam salt pans and screening for amylase and protease activity.

MATERIALS AND METHODS

Collection of Samples

Sediment samples were collected from four different sites of Marakkanam salt pan in a sterile plastic covers during the month of January 2014 and brought immediately to the laboratory for processing.

Isolation and Enumeration of Halophilic Bacteria

Halophilic bacteria were isolated from the sediment samples of salt pan from various sites using selective Halophilic agar medium. The sediment samples were serially diluted in a 5% NaCl solution, an aliquot of 0.1 ml of each dilution from 10^{-2} to 10^{-4} was taken and spread on the surface of halophilic agar plates. The plates were incubated at 37° C for 2 weeks. After incubation, morphologically different colonies were selected and purified for further investigation.

Morphological and Biochemical Characterization

The isolated bacterial were subjected to morphological, physiological and biochemical confirmations (Sawale *et al.*, 2013).

Screening for Amylase Producing Isolates

All the isolated bacteria were tested for amylase production by starch hydrolysis method (Kumar *et al.*, 2012). The 24hrs old cultures of isolated halophilic bacterial forms was streaked into the sterile starch agar medium and incubated at 37°C for 5 days. After incubation, the plates were flooded with iodine solution (iodine – 0.2%, KI – 0.4%, water 100 ml), the presence of clear zone was recorded and the selected bacterium species was noted for future investigations.

Screening for Protease Producing Isolates

Similarly all the isolates were tested for protease enzyme production in skimmed milk medium. The 24hrs old culture of isolated halophilic bacterial strains was streaked into the skimmed milk agar and incubated at 37°C for 48hrs. After incubation the plates were observed for the presence of clear zone and the selected bacterium species was stored for further investigations (Sánchez-Porro *et al.*, 2003).

Molecular Characterization

Genomic DNA Extraction

Extractions of genomic DNA from isolated strains were carried out using the commercially available DNA extraction kit. 1ml of bacterial cells from well grown culture in LB medium (OD = 1.2 – 1.4) at a salt concentration of 1M NaCl and pH 8 were transferred into 1.5 ml Eppendorf tube and harvested by centrifugation in a bench top centrifuge at 6000 rpm for 2 minutes at room temperature. After following all the steps the isolated genomic DNA was kept in 1.5 ml Eppendorf tube and stored at - 20°C (Asad *et al.*, 2007).

Polymerase Chain Reaction (PCR) Amplification of 16S rRNA Gene

Following the extraction of genomic DNA, polymerase chain reaction (PCR) was carried out in order to amplify 16S rRNA gene. The reaction was performed with forward and reverse primers respectively. The sequences for the forward primers were 5' – AGRGTTTGATCCTGGCTCAG- 3' (20) and the sequences for the reverse primers were 5' –CGGCTACCTTGTTACGACTT -3' (20) for the bacteria. Amplification was carried out in a thermocycler (BioRad Laboratories, Inc., USA). This began with PCR cycling steps which consisted of a 3 minutes initial pre-incubation step at 94°C followed by 30 cycles of a denaturation step at 94°C for 1minute, 1 minute annealing step at 50°C, and a 1 minute elongation period at 72°C followed by a final extension step at 72°C for 5 minutes.

DNA Sequencing and Phylogenetic Analysis

Finally the PCR products were purified and sent for DNA sequencing. The sequence was then compared to other sequences using the NCBI Blast function <http://www.ncbi.nih.gov/BLAST/>.

RESULTS AND DISCUSSIONS

Halophilic microorganisms are economically important because it produces several bioactive compounds which are useful for many industrial applications. Among the halophilic microbes, the halophilic bacterial forms are typically known for its secondary metabolites such as proteins, amino acids, pigments and enzymes. The present study was undertaken to explore the various halophilic bacteria from Marakkanam salt pan and to screen for its potential applications.

Isolation and Enumeration of Halophilic Bacteria

In this investigation, four samples were analysed from which totally 23 halophilic bacteria were isolated from Marakkanam salt pans (MSP) (Figure 1.) and it was named as MSP1 to MSP23. The isolates were maintained by sub-culturing at regular intervals of 20 days and stored at 4°C until further use. The enumeration of halophilic bacteria showed nearly equal population of bacteria in all the sampling sites (Table 1). Similar study carried out by Mayavu *et al.*, 2011 demonstrated the enumeration and isolation of halophilic bacteria from salt pans of Parangipettai.

Colony Morphology

The colony of most of the isolates was pale pink, lemon yellow, pale yellow, creamy white, pure white and bright orange in colour. Some of the colonies were very small, large, circular and irregular in shape. Smooth, mucoid and dry colonies were also recorded (Table 2). Furthermore, the results of the present study were supported by the results of Delgado-García *et al.*, 2012 who has selected the isolates based on their colony morphology.

Gram Staining

All the 23 isolates were subjected to Gram staining; their results showed that most of the isolates were Gram positive rod shaped bacilli (Table 2). Interestingly, MSP7 and MSP12 are positive and irregular in shape (pleomorphic). The study by Sawale *et al.*, 2013 had also revealed the presence of several gram positive rod and cocci and gram negative varying size rod shaped bacteria. Among all the isolates, only MSP17 and MSP23 were found to be Gram positive cocci. From the present investigation, only 10 morphologically diverse isolates were subjected to further works.

Biochemical Tests

The biochemical tests showed that most of the isolates showed negative for Indole, Methyl red, Voges Proskauer and catalase, oxidase tests (Table 3). Antón *et al.*, 2002 demonstrated the novel halophilic bacteria, *Salinibacter* sp. isolated from solar salterns of Spain. Further, they confirmed the isolates by biochemical and molecular characterization. Similarly, Saju *et al.*, 2011 isolated and characterized the halophilic bacteria such as *Vibrio fischeri*, *Halobacillus salinus*, *Halobacterium salinarum*, *Bacillus subtilis* and *Staphylococcus citreus* from salt pans of Kovalam, Kanyakumari district.

Screening for Amylase and Protease Enzyme

The ten selected isolates were screened for their ability towards amylase enzyme production by starch hydrolysis method. The present study showed that the isolates bearing code MSP1, MSP2, MSP5, MSP12, MSP13 and MSP17 were found to have clear zone in starch agar medium which indicates their amylase production. At the same time, isolates MSP3, MSP4 and MSP14 did not show any zone in the same medium. Out of the ten isolates, MSP2 (10.5 mm) showed maximum zone followed by MSP1 (Figure 2.)

The isolates were also screened for protease activity in skimmed milk agar medium. After incubation, the isolates such as MSP1 and MSP2 showed protease activity in the medium by a zone formation (Figure 3.). Halophilic and halotolerant bacteria produce several commercially important enzymes like amylase, protease and lipases. In the present study, all the selected ten isolates were screened for their ability towards amylase and protease production. Interestingly, the two isolates MSP1 and MSP2 were found to produce amylase as well as protease enzyme.

Likewise, Kumar *et al.*, 2012 demonstrated screening and isolation of halophilic bacteria producing industrially important hydrolytic enzymes including amylase and protease. Saju *et al.*, 2011 also reported the amylase producing halophilic bacteria from salt pans of Kovalam.

Molecular Identification of the Isolates

The introduction of 16SrRNA sequence analysis is considered to be a useful tool for identifying bacterial species. In order to identify the halophilic bacteria, 16SrRNA was amplified using specific primers. A PCR product of around 750bp was detected in the isolates MSP1 and MSP2 (Figure 4.). The amplicons of each bacterial isolate was sequenced and they were subjected to BLAST analysis for sequence similarity. The phylogenetic and evolutionary analysis showed that

the isolates MSP1 and MSP2 belongs to *Bacillus* sp. and *Pseudomonas* sp. respectively (Figure 5.). Previous studies also reported the morphological, biochemical and 16S rRNA analysis of halophilic bacteria like *Oceanobacillus*, *Bacillus*, *Halomonas* and *Staphylococcus* genera isolated from salt pans (Kumar *et al.*, 2012).

CONCLUSIONS

Halophiles have been perceived as an ideal source of many pharmaceutically and industrially important products. Recent studies on extreme environments including hypersaline ecosystems by molecular and microbiological aspects have revealed the presence of moderately to extremely halophilic microorganisms in a wide range of these saline environments. The present study investigated the isolation, enumeration, characterization of halophilic bacteria from Marakkanam salt pan. The study also revealed the potential of halophilic isolates towards amylase and protease production. Thus, the current study revealed that the isolates MSP1, MSP2 might be a good candidate for the industrial application regarding its enzyme. As some of the isolates are pigment producers, it can also be explored for its attractive coloured pigments.

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APPENDICES

Table 1: Enumeration of halophilic bacteria

Sampling Site	Dilution Rate	Number of Colonies
Salt pan 1	10^{-2}	101
	10^{-3}	75
	10^{-4}	65
Salt pan 2	10^{-2}	127
	10^{-3}	83
	10^{-4}	61
Salt pan 3	10^{-2}	119
	10^{-3}	87
	10^{-4}	56
Salt pan 4	10^{-2}	108
	10^{-3}	93
	10^{-4}	67

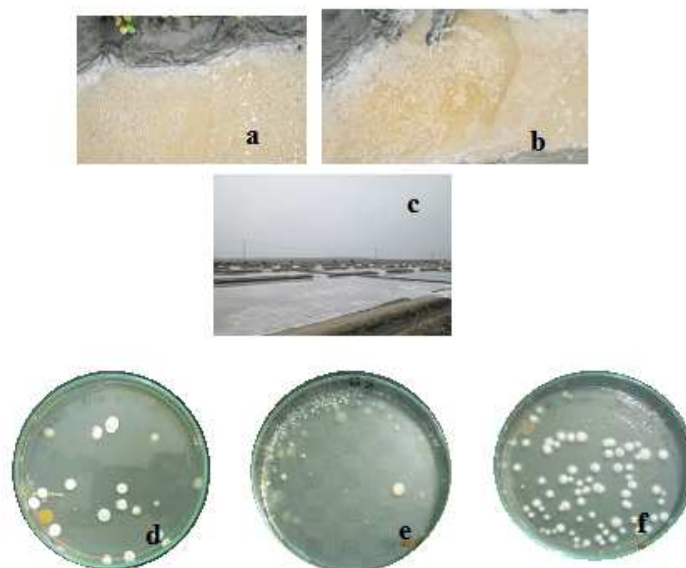


Figure 1: a & b: brine Solution, c: Marakkanam Salt Pan, d-f: Morphologically different Colonies

Table 2: Colony Morphology and Gram Staining

Culture Code	Colony Colour	Form	Surface	Gram Staining
MSP1	Creamy	Circular/Large	Smooth	Positive rods
MSP2	Light green	Circular/Elevated	Mucoid	Negative rods
MSP3	Pale yellow	Irregular/Elevated	Mucoid	Negative rods
MSP4	Lemon yellow	Circular/Elevated	Mucoid	Positive rods
MSP5	Pale yellow	Irregular/Elevated	Mucoid	Positive rods

Table 2: Contd.,

MSP6	Pale yellow	Circular/Elevated	Mucoid	Positive rods
MSP7	Orange	Circular/Medium	Smooth	Positive /Pleomorphic
MSP8	Creamy	Circular/Large	Mucoid	Positive rods
MSP9	White	Irregular/Elevated	Mucoid	Positive rods
MSP10	Pale yellow	Circular/Large	Dry	Positive rods
MSP11	Light brown	Irregular/Elevated	Mucoid	Negative rods
MSP12	Light brown	Circular/Elevated	Mucoid	Positive /Pleomorphic
MSP13	Creamy	Circular/Elevated	Smooth	Negative rods
MSP14	White	Circular/Flat	Smooth	Positive rods
MSP15	Palepink	Circular/Flat	Smooth	Positive rods
MSP16	White	Circular/Flat	Smooth	Negative rods
MSP17	Orange	Circular/Elevated	Mucoid	Positive cocci
MSP18	White	Circular/Flat	Mucoid	Positive rods
MSP19	Lemon yellow	Circular/Flat	Mucoid	Negative rods
MSP20	Creamy	Circular/Elevated	Mucoid	Negative rods
MSP21	White	Circular/Elevated	Smooth	Positive rods
MSP22	White	Circular/Elevated	Smooth	Negative rod
MSP23	White	Circular/Elevated	Smooth	Positive cocci

Table 3: Biochemical Results for Halophilic Bacterial Isolates

Culture code	Indole	Methyl Red	Voges Proskauer	Citrate	Urease	Catalase	Oxidase
MSP1	Negative	Negative	Negative	Positive	Positive	Positive	Negative
MSP2	Negative	Negative	Negative	Positive	Positive	Positive	Positive
MSP3	Negative	Negative	Negative	Positive	Positive	Negative	Positive
MSP4	Negative	Negative	Negative	Positive	Positive	Negative	Positive
MSP5	Negative	Negative	Negative	Positive	Positive	Negative	Positive
MSP6	Negative	Negative	Negative	Positive	Negative	Negative	Positive
MSP12	Negative	Negative	Negative	Negative	Positive	Negative	Positive
MSP13	Negative	Negative	Negative	Negative	Negative	Negative	Positive
MSP14	Negative	Negative	Negative	Negative	Negative	Negative	Positive
MSP17	Negative	Negative	Negative	Negative	Negative	Negative	Positive



Figure 2: a- MSP1, b- MSP2 (Showing Clear Zone), C- MSP4, d-MSP3 (No Zone)

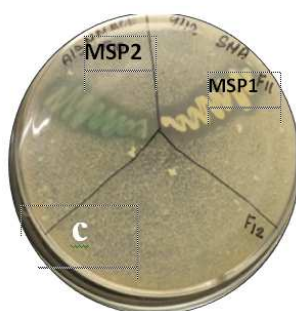


Figure 3: MSP1 and MSP2 Showing Protease Activity



Marker MSP1 MSP2

Figure 4: PCR Product of MSP1 and MSP2

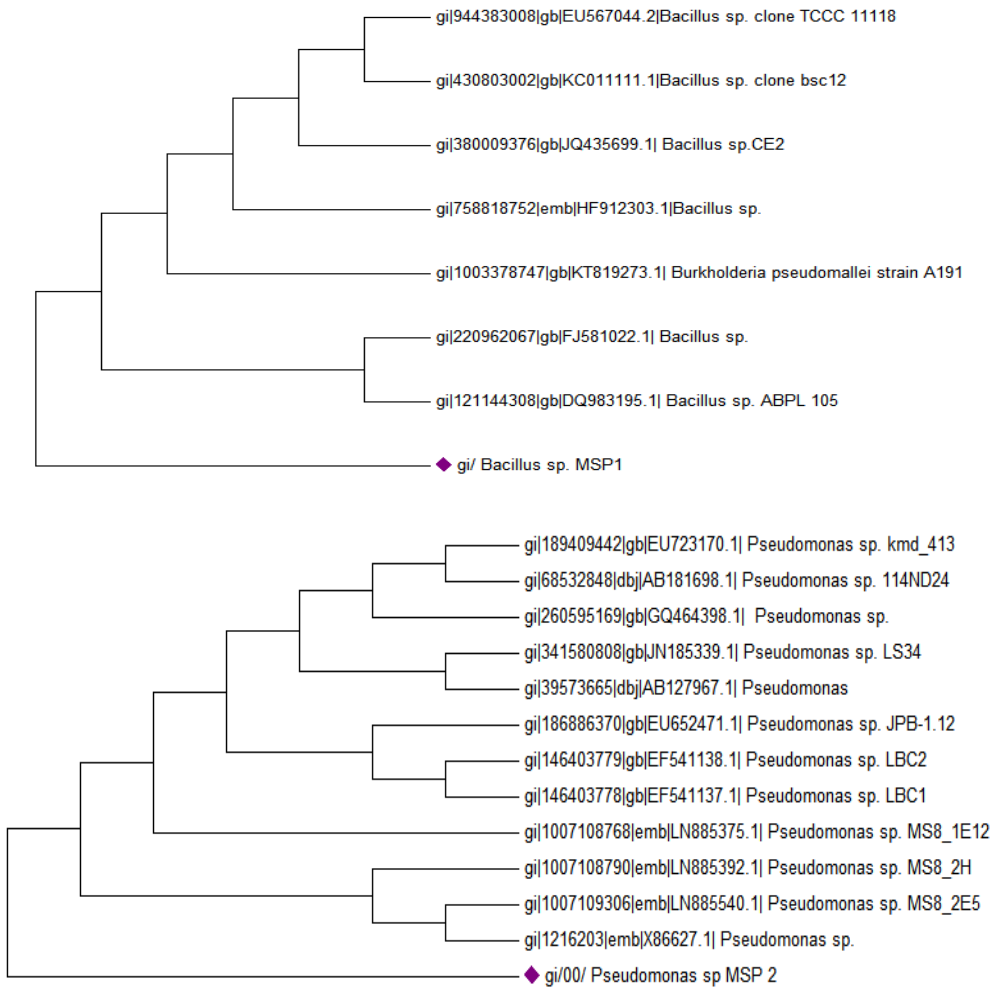


Figure 5: Phylogenetic Tree Showing the Evolutionary Relationship of *Bacillus* sp. MSP1 and *Pseudomonas* sp. MSP2