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Isolation of a Moderately Halophilic and Amylolytic Bacterium from Saline Soil of a Salt Manufacturing Industry

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Screening bacteria from wet saline soil collected from a salt manufacturing industry, Chennai led to the isolation of a total of 8 moderately halophilic bacteria. One strain, designated as *MHA*, an obligate and moderate halophile, capable of hydrolyzing soluble starch and tributyrin and another strain, *MHX*, capable of hydrolyzing xylan were isolated. *MHA* showed growth in a broad pH range of 6.5-11.0; temperatures in the range of 25-55°C and NaCl from 1-25% (w/v) and failed to grow in absence of NaCl indicating its obligate halophilic nature. It produced 0.150 U/ml of amylase when grown in presence of 1% soluble starch and 10% NaCl. Activity staining of the extracellular acetone precipitated protein fraction on native polyacrylamide gel electrophoresis (PAGE) indicated the secretion of multiple amylases by the strain *MHA*.

Key words: Moderate halophile, *MHA*, Amylase, Lipase, *MHX*, Xylanase, Native PAGE.

Moderate halophiles are a group of extremophilic microorganisms that are able to grow at 1-20% NaCl concentration with an optimum at 3-15% [1]. Halophilic bacteria have developed various strategies like compatible solute accumulation, modification of proteins, and modification of membrane lipids as protective means of haloadaptation and to maintain cell structure and function at high salt concentrations. Such bacteria produce hydrolytic enzymes of industrial interest[2]. Although much progress has been made on diversity, physiology and genomics of moderate halophiles belonging to genera *Halomonas*, *Chromohalobacter*, *Salinivibrio*, and *Bacillus* [1] yet there is a lacuna in the potential applications of halophiles and halophilic enzymes in various fields of biotechnology like food, pharmaceutical industries. Moderate halophiles show degradation of a few simple sugars, amino acids and proteins. Recent studies reported the secretion of extracellular, polymer degrading enzymes that can function at low water activity from halophiles [3, 4, 5, 6, 7, 8, 9, 10]. These have potential applications in harsh industrial processes where concentrated salt solutions would inhibit enzymatic conversions [11]. Screening and isolation of moderate halophiles and characterization of industrially important enzymes are not only important in biotechnology for their novel applications but also alleviates the cost due to the genetic manipulation of enzymes to make them robust and suit for industrial applications. Therefore the objective of the present study is focused on screening of saline soils from soil

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samples collected from Maruthi Marine Industries, Poonjeri, Chennai, Tamilnadu for bacteria producing extracellular hydrolytic enzymes and isolation and characterization of aerobic moderately halophilic and amylolytic bacteria.

MATERIAL AND METHODS

Isolation of moderately halophilic bacteria

Saline soil samples were collected from a wet soil near a mud bank at a depth of 5 cm of a salt pan of Maruthi Marine Industries, Poonjeri, Chennai, Tamilnadu, India about 50 Km from IIT Madras. One gram of soil sample was mixed with 10 ml of modified casein hydrolysate medium (ATCC No 876) of the following composition (gl^{-1}): Birchwood xylan, 2.0; NaCl, 150; casein hydrolysate, 5.0; yeast extract, 5.0; $MgCl_2$. $6H_2O$, 20.0 and $CaCl_2.2H_2O$, 0.20. The pH of the medium was adjusted to 7.5 with 0.1 N NaOH prior to autoclaving and incubated for 72 h under agitation at 180 rpm at 30°C. After 3 transfers into a fresh medium, 1 ml of broth was serially diluted and was spread on the agar of similar composition as above. Single colonies obtained after 72 h were purified after repeated plating.

Determination of extracellular hydrolytic enzyme activities

Amylolytic activity

Amylolytic activity of the strains was tested on Great Salt Lake-2 (GSL-2) agar plates [12] supplemented with 1% soluble starch and 10% NaCl at 30°C. After 72 h of growth, the plates were flooded with Grams Iodine solution for the identification of amylolytic strains. The strains were also tested for starch utilization in GSL-2 broth supplemented with 1% soluble starch by measuring the total carbohydrates[13] in the culture supernatants obtained after removing the cells by centrifugation at 10000 rpm for 20 min at 4°C. Substrate utilization assay was done in duplicates. The SD values were less than 3%.

Lipolytic activity (Tributyrin hydrolysis)

The strains were screened for lipase production by growing them on GSL-2 agar plates supplemented with 1% (w/v) tributyrin and 10% NaCl. Appearance of a clearance zone surrounding the colonies indicates the lipase secretion.

Xylanolytic activity

The strains were grown on GSL-2 agar plates with 10% NaCl and 0.2% Birchwood xylan at 30°C. After 3 days of growth, the zone of hydrolysis was visualized by flooding with 1% aqueous Congo red solution for 30 min and then by washing with 0.9% NaCl solution. Also the strains were tested for xylan utilization in GSL-2 broth supplemented with 0.2% Birchwood xylan and by measuring the total carbohydrates[13] in the culture supernatants. Substrate utilization was determined in duplicates. The SD values were less than 3%.

Proteolytic activity

All the strains were grown on solid GSL-2 agar containing 1% (w/v) gelatin for 3 days at 30°C. The plates were stained with Frazier reagent (15% $HgCl_2$ in 20% Conc. HCl). Caseinolytic activity was determined by growing the strains on solid GSL-2 medium containing 10% skimmed milk powder. In both the cases formation of clearance zone around the colony indicates protease production.

Phenotypic characterization of amylolytic strain

Colony size, color, margin, elevation, surface appearance and pigmentation of the amylolytic strain were observed by growing the isolate on casein hydrolysate medium (ATCC No 876) with 1% soluble starch and 10% NaCl. Cell shape, size, motility, Gram reaction, endospore formation were tested in liquid GSL-2 [12]. The pH was adjusted to 7.5 with 0.1N NaOH prior to autoclaving. The trace metals and metal chloride solutions were autoclaved separately and added after cooling.

Scanning electron microscopy (SEM) analysis

Morphology of the amylolytic strain was determined by SEM using FMI Quanta 200 Scanning Electron Microscope.

Physiological characterization of amylolytic strain

Physiological characteristics of the amylolytic strain, *MHA*, were studied in presence of 10% NaCl and 1% soluble starch. Growth at different pH was determined by growing the cells on GSL-2 agar plates adjusted to a different initial pH (6.0-11.0). Growth at different temperatures was tested by growing the cells on agar slants of GSL-2 medium at 25, 30, 35, 40 and 50°C respectively. Salinity tolerance of the strain was tested by growing the strain on GSL-2 agar slants supplemented with 1% soluble starch and with different NaCl concentrations of 0, 1, 3.5, 10, 15,

20, 25 and 30%. In all the cases the slants were incubated for 72 h to assess the growth.

Amylase production

A 50 ml of GSL-2 broth containing 10% NaCl and soluble starch (1%) in 250 ml conical flasks was inoculated with 2% inoculum of 24 h grown seed culture of amylolytic bacterium and were incubated at 30º C at 180 rpm in a rotary shaker. Growth was monitored by measuring the absorbance of broth at 600 nm at every 6h interval. Starch utilization was monitored by determining the total carbohydrate content [13] for every 6h.

Amylase assay

Cell free supernatants were collected by centrifuging the culture broth for 30 min at 10,000 rpm at 4ºC. Amylase activity in the cell free supernatant was determined by the method of Bernfeld [14] with a slight modification as follows. One ml of reaction mixture consisting of 500 µl of 1% soluble starch (prepared in 100 mM sodium phosphate buffer, pH 7.5, and 10% NaCl), and 500 µl of culture supernatant was incubated for 60 min at 50°C. An enzyme blank with 3, 5-Dinitrosalicylic acid (DNS) added prior to the addition of enzyme at 50°C was used as control. The amount of reducing sugar released was quantified using DNS with maltose as a standard. One unit (IU) of α-amylase activity was defined as the amount of enzyme releasing 1 µmole of maltose equivalent per min from soluble starch under specified conditions. All the experiments were done in duplicates. The SD values were less than 3%.

Native PAGE or Activity staining (Zymogram analysis)

Protein in the cell free supernatant (50 ml) was separated by precipitation with 1:1 volumes of ice-cold acetone at 4ºC. After 1 h, the precipitate was centrifuged (10000, 4ºC for 30 min), dissolved in 15 ml of 100 mM Tris buffer (pH 7.5) containing 10% NaCl. Native PAGE was performed using 4.5% stacking-separating gel and 8.5% of separating gel at 4-10ºC at a constant voltage of 250 V. After electrophoresis the gel was washed and soaked in 100 mM Tris buffer (pH 7.5) containing 1% soluble salt and 10% NaCl and was incubated at 50ºC for 2 h. After 2 h the gel was washed thoroughly with 100 mM Tris buffer (pH 7.5) containing 10% NaCl and stained with 0.05% (w/v) iodine-potassium iodide (I_2-KI) solution. After 1 min of incubation at room temperature, the I_2-KI was poured out and the gel was washed with distilled water. Colorless band indicated the presence of α-amylase.

RESULTS AND DISCUSSION

Screening bacteria from saline soil collected from a salt manufacturing industry led to the initial isolation of 12 moderately halophilic bacteria out of which only 8 strains could survive on further sub-culturing. Among 8 strains, 7 were Gram positive rods and one was Gram negative coccus (Table 1).

Identification of bacteria secreting extracellular hydrolytic enzymes

Among 8 strains isolated, strain 1 showed a very good zone of hydrolysis on starch agar plate upon staining with Grams Iodine and rest of the strains did not show any zone of hydrolysis (Fig. 1a). The hydrolytic zone around the colony suggested the amylase secretion by strain 1. This strain was designated as *MHA* (Moderately Halophilic and Amylolytic bacterium). *Bacillus dipsosauri* [3] and *Halobacillus* sp. Strain MA-2 [4] were the other moderately halophilic *Bacillus* genera reported to produce amylase. Strain *MHA* showed maximum utilization of soluble starch (1% starch with 10% NaCl) when tested in submerged culture and assayed by phenol sulphuric acid (Table 1). Rest of the strains was not able to utilize soluble starch much. Therefore strain 1, *MHA*, was taken up for further studies.

Among the 8 strains tested on tributyrin agar, only strain 1 (*MHA*) showed zone of hydrolysis around the colony (Fig. 1b). Rest of the strains did not show hydrolysis. This indicated that strain 1 (*MHA*) secreted lipase when grown on tributyrin agar plate. *Salinivibrio* sp strain SA-2 is the only moderate halophile reported till to date that produce extracellular lipase [8]. Hence strain 1 (*MHA*) could be another source for the production of halophilic lipase.

Strain 7, designated as *MHX* (Moderately Halophilic and Xylanolytic bacterium), and showed the formation of a hydrolysis zone on xylan agar plate upon staining with Congo red (Fig. 1c) and rest of the strains did not show xylan hydrolysis. *MHX* showed maximum utilization of xylan (0.2% xylan with 10% NaCl) when tested in submerged culture and assayed by phenol sulphuric acid (Table 1). This indicated that strain *MHX* was able

Table 1. Colony characteristics, starch and xylan utilization by different strains

Strain	Colony morphology	Microscopic observation	Starch utilized (%)	Xylan utilized (%)
1 (*MHA*)	Smooth and glistening, initially creamy and becomes light orange colored flat, entire margin, round, 4mm	Gram positive, short rods with central endospore	80	20
2	Smooth, pink, flat, entire margin, round, 5mm	Gram positive, long rods	14	28
3	Smooth and glistening, white and central pale yellow, slightly raised, irregular margin, 8mm	Gram positive, short rods	12	21
4	Smooth pale yellow color, round, entire margin, 2mm	Gram positive, short rods	2	0
5	Smooth and slimy, yellow color, flat, entire margin, oval, 3mm	Gram negative, cocci	2	0
6	Smooth, small creamy colony, entire margin, 2mm	Gram positive, short rods	3	0
7 (*MHX*)	Smooth and glistening, round, light wheatish color, entire margin, flat, 4mm	Gram positive, short rods	9	70
8	Smooth and glistening, white slightly raised, irregular margin, 8mm	Gram positive, short rods	11	25

to produce xylanase, which caused the hydrolysis of xylan. Other strains were not able to utilize xylan much. Among halophiles the extremely halophilic archaeon, *Halorhabdus utahensis* [15], a Gram negative gamma Proteobacterium strain SX15 [12], strain CL8 [16] and *Chromohalobacter* sp. TPSV 101 [10] were reported to produce xylanase. Gelatin or casein was not hydrolyzed by any of the strains isolated in the present study indicating the absence of protease secretion.

Characterization of amylolytic and lipolytic strain (*MHA*)

Strain 1, designated as *MHA*, was studied for its morphology and biochemical characteristics due to its ability to produce two extracellular hydrolytic enzymes viz., amylase and lipase.

Morphological features of *MHA*

Cells were Gram positive, short rods; motile with a central spore (Fig. 2a). SEM analysis confirmed the rod shaped morphology of the strain *MHA* (Fig. 2b). Colonies were round, flat and the margin was entire and 4 mm in diameter. It grows as smooth and glistening colonies. They were initially cream and turned to light orange after 36 h on starch agar plates due to pigment accumulation. It was reported that the pigment accumulation by halophilic bacteria might serve as a protective measure against UV light induced damages to the cell [17].

Biochemical characteristics

Strain *MHA* was aerobic and produced respiratory enzymes like catalase and oxidase. Fructose, galactose, glucose, mannose, lactose, maltose, sucrose, trehalose, mannitol, glycerol, inulin, dextrin, glycogen and starch were fermented with acid production but without gas. Acid and gas were not produced from arabinose, *myo*-inositol, xylose, melezitose and D-salicin. It showed positive reactions for urease, lipase and negative for Voges-Proskauer, indole, nitrate reduction and H_2S production. Based on the above morphological and biochemical characteristics, strain *MHA* was identified as a strain of *Bacillus* genus according to Bergey's Manual [18].

Physiological characterization

Strain *MHA* showed good growth in a broad pH range of 6.5-11.0; temperatures in the range of 25-55°C and NaCl from 1-25% (w/v). However optimum growth was noticed between 5-10%. There was a salinity dependent amylase

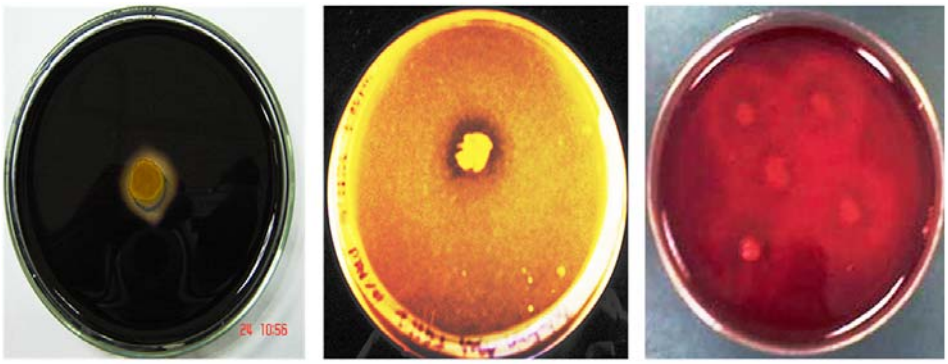

Fig. 1. Extracellular hydrolytic enzyme activities by moderately halophilic bacteria. Amylase by *MHA* (a); lipase by *MHA* (b) and Xylanase by *MHX* (c)

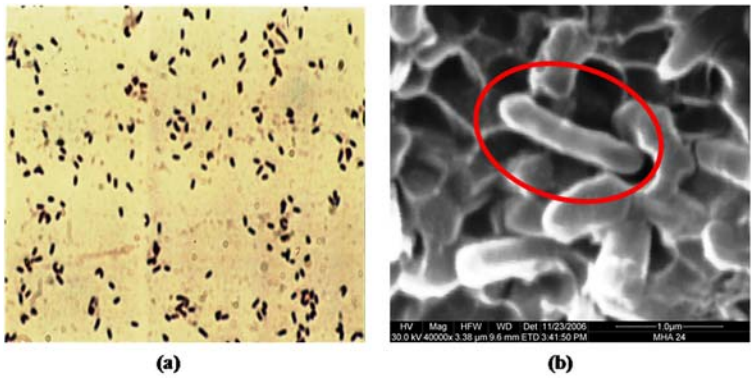

Fig. 2. Morphological characteristics of strain *MHA*. Gram staining (a) and SEM image (b)

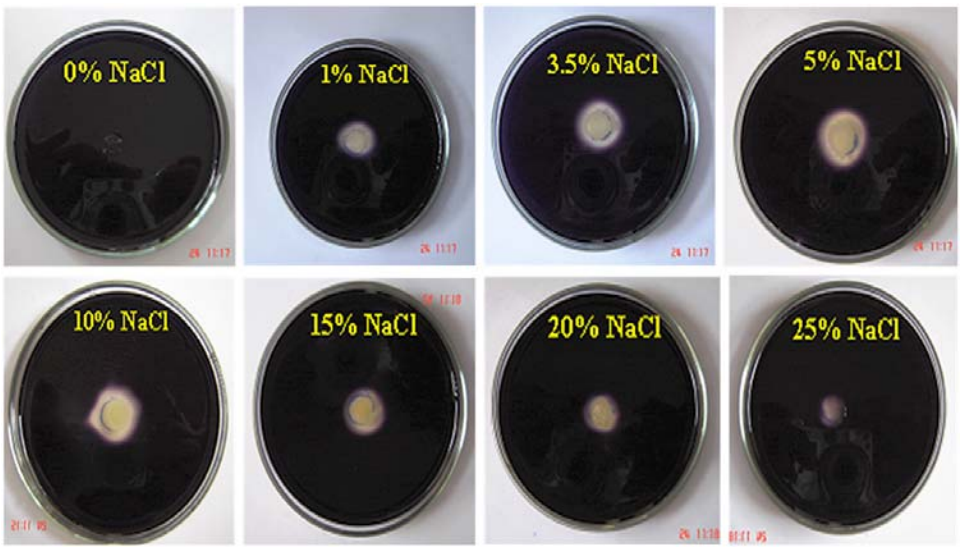

Fig. 3. Effect of NaCl concentration on the amylase secretion by strain *MHA*

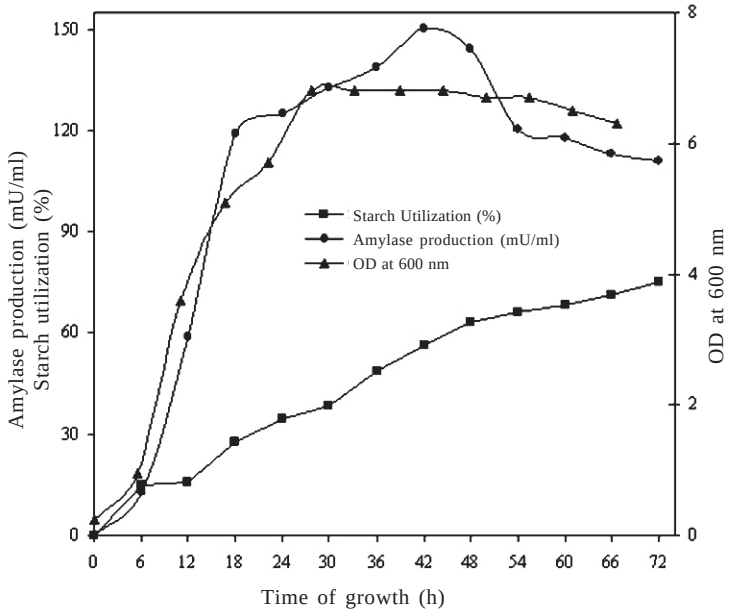

Fig. 4. Time course of amylase production by *MHA* grown in liquid GSL-2 medium supplemented with 10% NaCl and 1% soluble starch. Growth (▲); starch utilization (■) and amylase production (●)

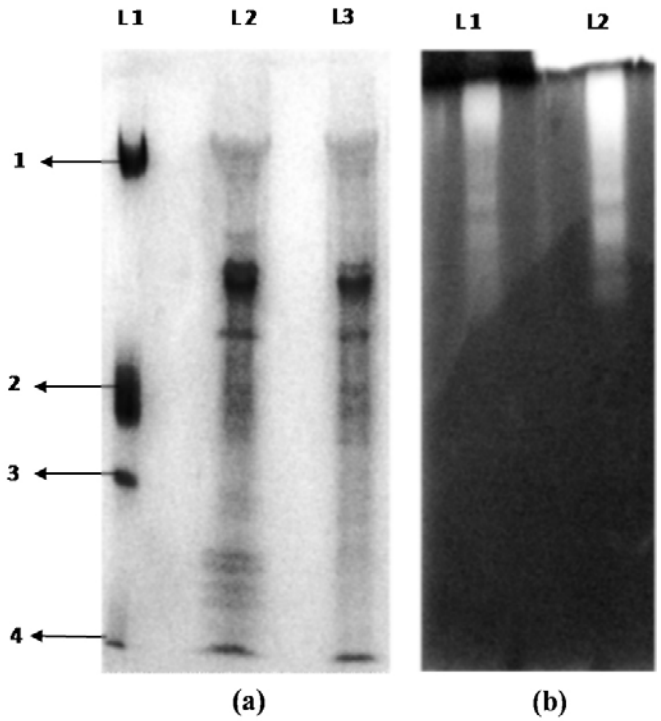

Fig. 5. Native PAGE after Coomassie blue staining (a); L1, Native page marker; 1, Catalase (240 KD); 2, Bovine serum albumin (67 KD); 3, Ovalbumin (43 KD) and 4, Tryptin soybean inhibitor (20.1KD); L2, Acetone fraction; L3, Activity staining (b); L1, Acetone fraction; L2, Cell free supernatant

production when strain *MHA* was cultured on agar plates containing 1.0% soluble starch and different concentrations of NaCl and after staining with Grams iodine. The zone of hydrolysis increased with increasing salinity up to 10% NaCl. With further increase in NaCl concentration to 25% there was a decrease in the zone of hydrolysis (Fig. 3). When *MHA* was grown in the absence of NaCl there was no growth and no starch hydrolysis. The dependency of growth and amylase production on NaCl by *MHA* confirmed it as an obligate and moderately halophilic bacterium. *MHA* utilized 70% of starch after 72 h which corresponds with maximum growth (A_{600}=6.7). Amylase production started with exponential growth, reaching its maximum at 42 h (0.150U/ml) with 1% starch (Fig. 4). This could be due to the stationary phase regulation on one side and catabolic repression by glucose released from maltose and maltotriose on the other side, as it has been thought for microorganisms as a way to save energy as was shown in *Chromohalobacter* sp. TVSP 101 [9]. The pH of the medium was dropped to 6.5 from an initial pH of 7.5 indicating the formation of acidic end products during fermentation.

Native PAGE

Activity staining of the acetone precipitated protein fraction showed presence of more than 5 amylases although the number of amylase active bands was not clear due to close alignment of bands during electrophoresis (Fig. 5a and 5b) indicating the multiple amylase secretion by *MHA*. Three reports exist on multiple amylase secretion among moderate halophiles. Amylases I and II were purified from *Acinetobacter* sp. [19]; Amy A and B were crystallized from *Halothermothrix orenii* [20, 21] and amylase I and II were purified from *Chromohalobacter* sp. TVSP 101 [9]. Among the non-halophilic bacteria, 5 amylases were purified from *Bacillus halodurans* MS-2-5 [22]. Multiple amylases could arise due to differential mRNA processing or post-secretional modification by proteolytic digestion or post-translational modification like glycosylation or by autoaggregation during non-denaturing PAGE or different alleles of same gene or expression of different genes or by the proteolytic cleavage of the mature peptide [23].

From the above results it can be concluded that strain *MHA* could be a potential source to produce halophilic enzymes like alpha amylases and lipase, which could be of valuable importance for biotechnological and industrial applications. Purification and characterization of amylases and the identification of corresponding encoding genes from the strain *MHA* is under progress.

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