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CHARACTERIZATION AND POTENTIAL APPLICATIONS OF *HALOVIBRIO VARIABILIS* MLA3 (A HETEROTROPHIC BACTERIUM) ISOLATED FROM A SALINE-ALKALINE LAKE OF RAJASTHAN, INDIA

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ABSTRACT

Halophiles are salt loving microorganisms. These have ability to produce industrially important enzymes showing activity at high salt concentration and alkaline condition. This study describes the isolation, physiological and biochemical characterization, phylogenetic analysis of a moderately halophilic bacterial strain MLA3 and screening of the strain for industrially important enzymes production. The strain MLA3 was isolated from Sambhar Lake, Rajasthan, India. It is a gram-negative, motile, rod shaped with an optimal growth observed at 10% (w/v) NaCl concentration, pH 8.0 and 30 °C. On the basis of 16S rRNA gene sequence analysis, the strain MLA3 was phylogenetically identified as members of the genus Halovibrio and clustered in clade with type species Halovibrio variabilis DSM 3050. The occurrence of the studied strains is an addition of the genus Halovibrio in microbial diversity of Sambhar Lake. The MLA3 was found to be capable to hydrolyse skim milk, Tween80 and starch. It showed maximum hydrolysis zone for starch and skim milk. The amylase and protease enzyme production were estimated to be 7.3 ± 0.05 U/ml and 1.7 ± 0.03 U/ml, respectively. The present study indicates that Sambhar Lake is a treasure of still many unexplored microorganisms which are capable of producing industrially significant enzymes.

KEY WORDS

Sambhar Lake, Halophile, 16S rRNA gene, Phylogenetic study, Halovibrio, Halophilic enzymes

1. INTRODUCTION

Hypersaline environments such as salt lakes, salt pan and marine water are habitats for survival of microbial community of halophiles in the entire world [1-4]. Soda lakes such as Howz Soltan Lake, Iran [5]; Wadi Natrum, Egypt [6, 7]; Great salt lake, Utah [8]; Mono Lake, California [9] and Lonar lake, India [10] were more emphasized for exploration of culture dependent diversity of halophilic bacteria. In this context, Sambhar Lake attracted researchers more due to its dual nature i.e., salinity and alkalinity [11]. On the basis of salinity and alkalinity, many earlier studies focused on the phylogenetic diversity of halophiles and haloalkaliphiles of the lake and characterized different genera such as *Natronobacterium* [12, 13], *Halobacterium* [14], *Gracilibacillus* [15], *Geomicrobium* [16], *Alkalibacillus* [17], *Hallobacillus* [18], *Halobiforma* [19], *Oceanobacillus* [20] and *Lentibacillus* [21]. Due to unique characteristics of halophiles, many studies focused not only on phylogenetic diversity but also their industrial and biotechnological applicability [15, 22-25]. It was reported that subgroups members of genus *Halovibrio* and *Halomonas* were phylogenetically distinct [26]. A number of strains belonging to *Halomonas* have been reported from Sambhar lake and also examined for industrial applications [27, 28].

Considering the novelty and significance of halophilic heterotophic bacteria and paucity of work on microbial diversity of Sambhar lake, the present study was undertaken to characterize and phylogenetic analysis of a strain MLA3 isolated from this soda lake. The strain MLA3 was also evaluated for the qualitative production of extracellular hydrolytic enzymes such as protease, lipase and amylase as well as quantitative production of amylase and protease.

2. MATERIALS AND METHODS

Chemicals

All chemicals used in the present study were procured from HiMedia Laboratories, India

Sample collection

The water sample was collected in summer season from opposite site of sludge gate, Guda Jhapog dam area (26°58'N 75°5'E) of Sambhar lake (a saline-alkaline lake), Rajasthan, India. Sample was stored in sterile bottles and transported to laboratory, at Central University of Rajasthan, Ajmer for further analyses.

Isolation and purification of strain MLA3

Water sample was initially inoculated for enrichment in the modified MGM broth medium [29] containing (g L^{-1}) NaCl, 220; KCl, 5.4; $MgSO_4.7H_2O$, 26.9; $MgCl_2.6H_2O$, 23.1; $CaCl_2.2H_2O$, 0.8; peptone, 10.0, 0.8; sodium lactate, 2.5; and yeast extract, 2. The pH 8.0 of the medium was adjusted with NaOH and incubated at 37 °C on an orbital shaker incubator (Waiometra, India) at 120 rpm for 7 days. Further, the colony of the strain MLA3 was purified by successive streaking on MGM medium agar plates. Isolated strain was stored at 4 °C in refrigerator for further investigations.

Determination of optimum temperature, salinity and pH for growth

The growth studies were carried out in MGM modified broth medium. The different ranges of temperature (25-55 °C), pH (6-12) and salinity (0-30%) were employed to find out optimal growth of the isolated strain [30]. The turbidity was measured at OD_{600} nm by using spectrophotometer (HALO DB-30 UV-Visible double beam, Dynamica, Singapore). All experiments were performed in triplicates.

Morphological studies of colony and cell

The appearance of colony such as shape, colour, opacity, margin and elevation was observed by magnifying lens (10X) on agar plate containing MGM modified agar medium. Gram staining was performed using earlier protocol [31]. The other morphological features of bacterial strain were observed at 1000X using Axio Lab. A1 compound microscope (Carl Zeiss, Germany).

Biochemical characterization

A series of biochemical tests such as indole production, methyl red, Voges-Proskauer, catalase activity, nitrate reduction [32], citrate utilization and oxidase activity [33] were performed in triplicates by placing the culture at 30 °C and pH 8.0 for 7 days.

Extracellular lipolytic activity

Lipolytic activity of the strain was determined using 1% Tween 80 in MGM modified broth medium containing (g L^{-1}) NaCl, 100; KCl, 5.4; $MgSO_4.7H_2O$, 26.9; $MgCl_2.6H_2O$, 23.1; $CaCl_2.2H_2O$, 0.8; peptone, 10.0, 0.8; sodium lactate, 2.5 yeast extract, 2 and pH 8.0. The strain MLA3 was incubated on solided plates at 30 °C for 7 days. The formation of conspicuous halos/zone around the growing isolate indicated the lipolytic activity [34].

Extracellular protease activity

The proteolytic activity of the strain was examined in MGM modified broth medium containing (g L^{-1}) NaCl, 100; KCl, 5.4; $MgSO_4.7H_2O$, 26.9; $MgCl_2.6H_2O$, 23.1; $CaCl_2.2H_2O$, 0.8; peptone, 10.0, 0.8; sodium lactate, 2.5; skim milk, 10; yeast extract, 2 and pH 8.0. For protease activity, the strain MLA3 was inoculated on skim milk agar plates and incubated at 30 °C for 7 days. The hydrolysis around the culture gave indication of proteolytic activity [35].

Quantitative assay of protease enzyme production

Quantitative protease production was performed in 50 ml of MGM modified broth medium containing (g L^{-1}) gelatin, 10; casein enzymatic hydrolysate, 10; NaCl, 100; KCl, 5.4; $MgSO_4.7H_2O$, 26.9; $MgCl_2.6H_2O$, 23.1; $CaCl_2.2H_2O$, 0.8; peptone, 10.0, 0.8; sodium lactate, 2.5 yeast extract, 2 and pH 8.0 in 250 ml Erlenmeyer flasks. The MLA3 culture was incubated on orbital incubator shaker (Waiometra, India) at 30 °C with 150 rpm for 7-10 days. Samples were withdrawn at 1-day interval and centrifuged at 10,000 rpm for 10 min at 4 °C. The cell free extract was used as crude enzyme to measure protease activity [36]. The enzyme substrate reaction mixture containing 0.5 ml enzyme was added with 3.0 ml casein (0.6% in 20mM Tris-HCl buffer, pH 8.0) and reaction mixture incubated at 37 °C for 10 min. The reaction was terminated by adding 3.2 ml of TCA mixture (0.11 M trichloro acetic acid, 0.22 M sodium

acetate, 0.33 M acetic acid) and incubated for 30 min at room temperature. The reaction mixture was centrifuged at 14000 g for 10 min at 4 °C and supernatant absorbance was observed at 280 nm by spectrophotometer (HALO DB-30 UV-Visible double beam, Dynamica). One unit of alkaline protease activity was defined as the amount of enzyme liberating 1 µg of tyrosine/min under assay conditions. Enzyme units were measured using tyrosine (0–100 µg) as standard [37].

Extracellular amylase activity

The amylase activity was determined in MGM modified broth medium containing (g L^{-1}) starch, 10; NaCl, 100; KCl, 5.4; $MgSO_4.7H_2O$, 26.9; $MgCl_2.6H_2O$, 23.1; $CaCl_2.2H_2O$, 0.8; peptone, 10.0, 0.8; sodium lactate, 2.5 yeast extract, 2 and pH 8.0. Isolate MLA3 was inoculated on solidified plates and incubated at 30 °C for 7 days. After incubation, plates were flooded with Gram's iodine solution (3% (w/v), KI and 0.3% (w/v) iodine). A clear zone around the growth indicated hydrolysis of starch [38].

Quantitative assay of amylase enzyme production

Quantitative amylase production was performed in 50 ml of MGM modified broth medium containing (g L^{-1}) starch, 10; NaCl, 100; KCl, 5.4; $MgSO_4.7H_2O$, 26.9; $MgCl_2.6H_2O$, 23.1; $CaCl_2.2H_2O$, 0.8; peptone, 10.0, 0.8; sodium lactate, 2.5 yeast extract, 2 and pH 8.0 in 250 ml Erlenmeyer flasks. The strain MLA3 was inoculated on orbital shaker (Waiometra, India) at 30 °C with 150 rpm for 7-10 days. Samples were withdrawn at 1-day intervals and centrifuged at 12000 g for 20 min at 4 °C. The cell-free supernatants were used for the amylase assay. The amylase activity was measured by using DNS method and maltose as the standard. One unit of amylase is defined as the amount of enzyme that liberates one l µmol reducing sugar (maltose) ml^{-1}, min^{-1} at 30 °C [39-41].

Genomic DNA extraction

Genomic DNA of halophilic bacterial strain MLA3 was extracted using protocol [42] with some modifications. Briefly, bacterial culture was centrifuged at 9000 g for 5 minutes followed by addition of lysis solution (500 µl) and incubated at 65 °C for 5 minutes in water bath. Cell suspension was extracted three times with organic solvent using equal amount of Chloroform: Isoamyl alcohol (24:1) and gently emulsified by inversion 3-5 times and centrifuged at 9000 g for 5 minutes. Aqueous phase was separated into sterile centrifuge tube and extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) at 9000 g for 5 minutes. Obtained aqueous phase were transferred into sterile centrifuge tube and extracted with equal amount of chloroform: isoamyl alcohol (24:1) at 9000 g for 5 minutes. Upper aqueous phase containing DNA was transferred to a new tube and equal amount of Propan-2-OL (iso-propyl alcohol) was added and mixed gently by inversion at room temperature (37°C) followed by centrifugation at 9000 g for 3 minutes. supernatant was removed completely, and DNA pellet was dissolved in 70 µl of NaCl solution. Thereafter, 300 µl of cold absolute ethanol was added, the DNA precipitated out in 7 minutes at -20 °C which was centrifuged at 9000 g for 5 minutes. Ethanol was removed, and the was pellet washed once with 70% cold ethanol at 9000 g for 5 minutes. Later, the pellet was dissolved in 50 µl deionzed water by gentle vortexing and kept at -20 for further use.

16S rRNA gene sequencing

The 16S rRNA gene was amplified using PCR master mix (Genei™ Company) using universal primers 27F and 1495R [43]. Amplified genomic DNA was purified with GeneJET PCR purification kit (Thermo Fisher, India) as per the manufacturer's protocol. Purified product was sequenced commercially (Eurofins Genomics India, Bangalore) to get partial sequences of 16S rRNA gene.

Phylogenetic analysis

A clean 1466 bp sequence was generated by commercial sequencing of amplified product. The similarity search was done using the 'Identify' option of the EzTaxon database http://www.eztaxon.org [44] to find out the closest strain for molecular identification. Other appropriate 16S rRNA gene sequences of related strains particularly from strains originated from Sambhar Lake were also retrieved for the phylogenetic analysis. Multiple sequence alignment was performed using MUSCLE program [45] inbuilt available MEGA 7 [46]. The alignment was manually edited to obtain unambiguous data. A total of 1262 positions in the final dataset was used for phylogenetic analysis. All the phylogenetic analyses were carried out using the software package MEGA version 7. The suitable model of phylogeny with the lowest Bayesian Information Criterion (BIC) score was selected using the MEGA 7 program which led to the selection of the TN93+G model having the lowest BIC score. The phylogenetic tree was constructed using the maximum likelihood (ML) method. Bootstrapping of

1000 replications was calculated to find out confidence values for the edges of the maximum likelihood tree. *Planococcus citreus* (X62172) was chosen as the outgroup.

Nucleotide sequence accession numbers

The nucleotide sequence has been deposited in the GenBank (NCBI database) under accession number MK156419.

3. RESULTS AND DISCUSSION

Colony and cell morphology

The present study revealed that colonies of the strain MLA3 were circular with entire margin, smooth surface, sticky nature, orange colored, raised and opaque on MGM modified medium agar plate, as shown in Table 1. The morphological characters rather than colony color are similar to *Halovibrio variabilis* and *Pseudomonas halophila* [47]. Cell morphology result showed that cells were gram negative and rod shaped (fig.1b and Table 1). These results correspond to earlier reported strains *Halovibrio variabilis*, *Pseudomonas halophila* and *Halovibrio denitrificans* strain HGD 3 [47, 48].

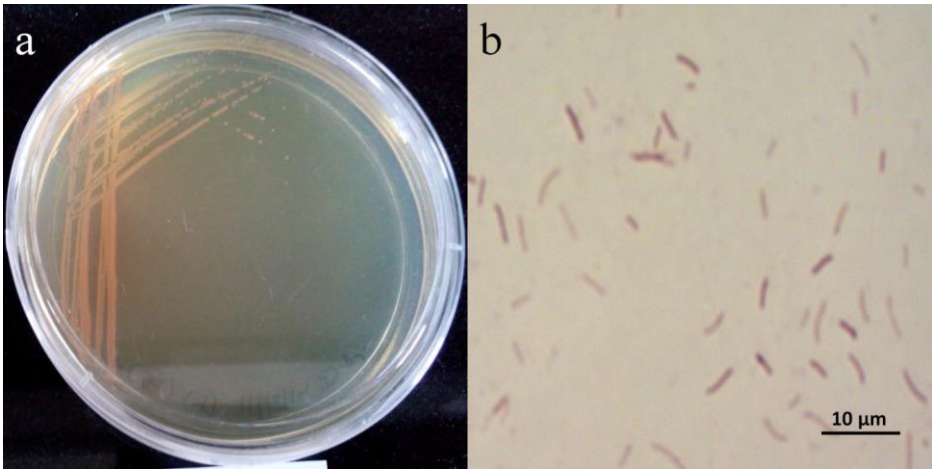

Fig. 1. (a) Colonies of the *Halovibrio* sp. strain MLA3 on MGM modified medium. (b) Bright field microscopy showing the negative rod cell

Table 1: The colony and cell morphology similarity features of *Halovibrio* sp. strain MLA3 with type strain *Halovibrio variabilis* and *Pseudomonas halophila*

Characters	*Halovibrio* sp. MLA3	*Halovibrio variabilis* DSM 3050	*Pseudomonas halophila* DSM 3051
Isolation source	Sambhar lake, India	Great Salt Lake, Utah, USA	Great Salt Lake, Utah, USA
Shape	Circular	Circular	Circular
Colour	Orange	Light brown	Reddish-brown
Margin	Entire	Entire	Entire
Surface	Smooth	Smooth	Smooth
Elevation	Raised	Raised	Raised
Gram reaction	Negative	Negative	Negative
Cell shape	Curved rod	Curved rod	Rod
Cell size (µm) (width and length)	0.3-0.5 x 1.0- 3.8	0.5-0.8 x 1.0-3.0	0.8-1.0 X 1.5-5.0
Motility	Motile	Motile	Motile

Physiological and biochemical characterization

The results of physiological parameters for the growth showed that the strain MLA3 was able to grow optimally at 30 °C, 10% (w/v) NaCl concentration and at pH 8.0 (Fig. 2a, 2b, 2c and Table 2). Whereas, previously reported halophilic strain of *Halovibrio variabilis* DSM 3050 from Great Salt Lake, Utah, USA showed optimal growth at 33 °C, 9.35 % (w/v) NaCl concentration and at

pH 7.5. Another strain of *Pseudomonas halophila* was able to grow optimally at 28°C, 4.56% (w/v) NaCl concentration and at pH 7.0 [47]. Likewise, *Halovibrio denitrificans* HGD 3 from hypersaline lake sediment from Central Asia showed optimal growth at 28 °C, 11.6 % (w/v) NaCl concentration and at pH range 6.7-8.5 [48]. Moreover, *Halomonas* sp. strain SL121 from Sambhar lake could grow optimally at 11% (w/v) NaCl concentration and pH 8.9 [28].

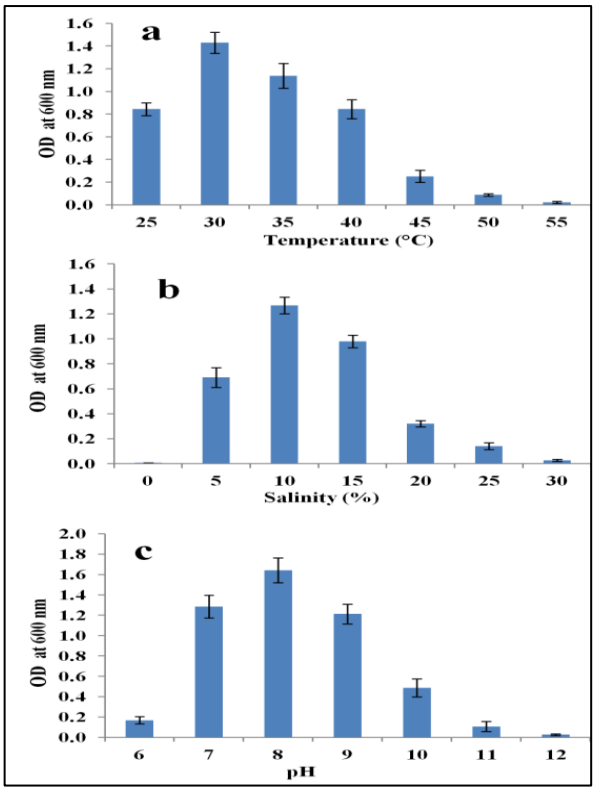

Fig. 2. Growth pattern of the *Halovibrio* sp. strain MLA3 (a) Temperature 25-55 °C. (b) 0-30 % NaCl concentration in MGM modified medium. (c) pH ranges 5 -12. Each value is a mean of three replicates data with ± standard deviation.

The strain MLA3 showed negative result for indole formation, methyl red and voges-proskauer, citrate utilization, and urease activity (Table 2). It showed positive result for nitrate reduction, oxidase and catalase activity. Similarly, *Halovibrio variabilis* DSM 3050 showed positive result for oxidase and catalase activity [47].

Characters	*Halovibrio* sp. MLA3	*Halovibrio variabilis* DSM 3050	*Pseudomonas halophila* DSM 3051
NaCl optimum range (% w/v)	10	9.35	4.56
pH optimum	8.0	7.5	7.0
Temperature optimum (°C)	30	33	28
Indole	-	-	-
Methyl Red	-	-	-
Voges-Proskauer	-	-	-
Nitrate	-	-	-
Citrate	-	ND	ND
Catalase	+	+	+
Oxidase	+	+	+

Table 2: Differential features of physiological and biochemical characterization of *Halovibrio* sp. strain MLA3 with type strain *Halovibrio variabilis* and *Pseudomonas halophila*

Phylogenetic analysis

The strain MLA3 showed 99.97% identity with coverage of 100% to *Pseudomonas halophila* DSM 3050(T) in search 1466 bp of 16S rRNA gene using EzTaxon database. During the course of studies on halophilic, Gram-negative bacteria, a comparison of two strains of halophilic bacteria*, Pseudomonas halophila* DSM 3050 and *Halomonas variabilis* DSM 3051 (formerly *Halovibrio variabilis*) demonstrated that the characteristics of strain DSM 3050 corresponded to the original description of *Halovibrio variabilis* and those of DSM 3051 to *P. halophila*, both of which had been isolated from the Great Salt Lake in Utah [47, 49]. It was concluded that type species *Halovibrio variabilis* should be associated with type strain DSM 3050 [49]. Thus, MLA3 is identified as the strain of *Halovibrio variabilis* on the basis of percentage identity of 16S rRNA gene. The phylogenetic tree constructed to determine the affiliation of the strain MLA3 are shown in Fig.3. The investigated strain MLA3 falls in the cluster in which type strain of *Halovibrio variabilis and H. denitrificans* HGD3 is included with support of significant bootstraps. The occurrence of the studied strains is an addition of the genus *Halovibrio* in microbial diversity of Sambhar Lake. This cluster also comprised strains related to *Pseudomonas halophila,* those may be affiliated to genus *Halovibrio*. Moreover, phylogenetically close species of the genus *Halomonas* have been reported from Sambhar Lake [28].

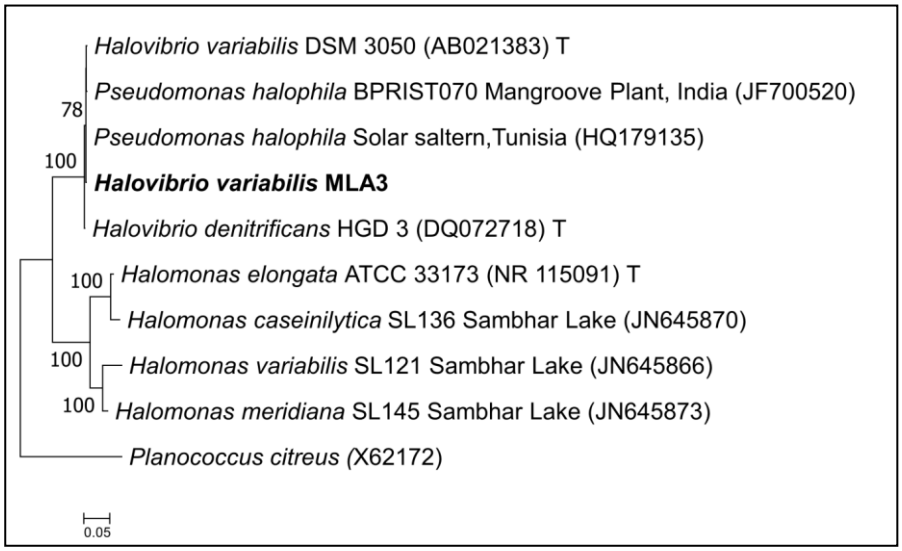

Fig 3. Phylogenetic tree showing the relationships between the strain MLA3 and closely related bacterial species based on 16S rRNA gene sequences

Production of extracellular hydrolytic enzymes

Halovibrio variabilis MLA3 was able to produce the protease, amylase and lipase (fig. 4, fig. 5 and fig. 6). Whereas, type strain *Halovibrio variabilis* DSM 3050 showed production of protease alone confirmed by hydrolysis of casein [47, 50]. In the present study, quantitative assay was further performed for amylase and protease enzyme production on the basis of maximum hydrolyse zone. The maximum protease production 1.7 ± 0.02 U/ml was observed after 7 days of incubation (fig.7). Our result corresponds to earlier findings showing that halophilic bacteria are well known for the protease enzyme production [19, 22, 51]. However, *Halomonas variabilis* SL121 isolated from Sambhar Lake showed only amylase enzyme production and protease production was not reported by this strain. The present study describes the production of protease enzyme by the strain MLA3 at qualitative as well quantitative level. The amylase production started after 1 day and maximum production was recorded on 7^{th} day (7.3 ± 0.05 U/ml) and later on production of enzyme decreased (fig. 8). Some other studies also showed amylase production by halophilic bacteria such as *Halomonas meridian* [52], *Bacillus* sp. Strain TSCVKK [53], *Bacillus cereus* [54]. Further, study will be focused on the optimization of carbon sources, nitrogen sources (organic and inorganic), effect of different factors such as agitation, inoculum size, inoculum age, metal ions, surfactant, additives, inhibitors and chelating agents on amylase production and its activity.

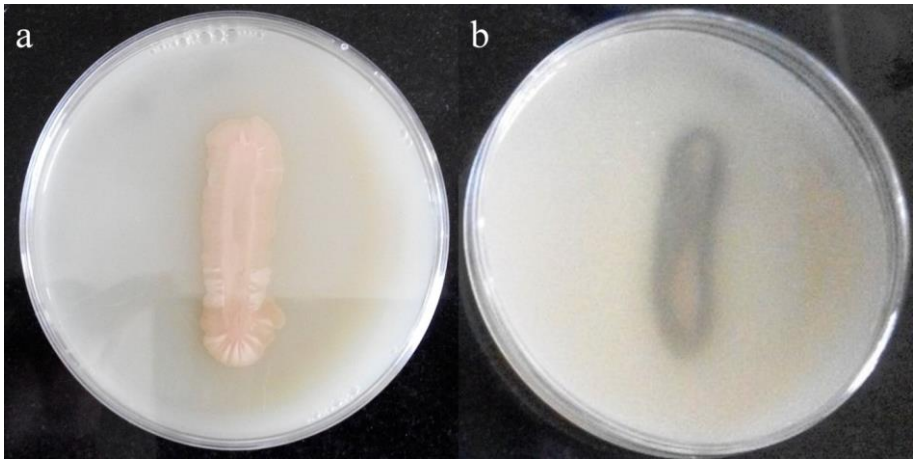

Fig 4. Skim milk hydrolysis (a) control (b) *Halovibrio* sp. strain MLA3

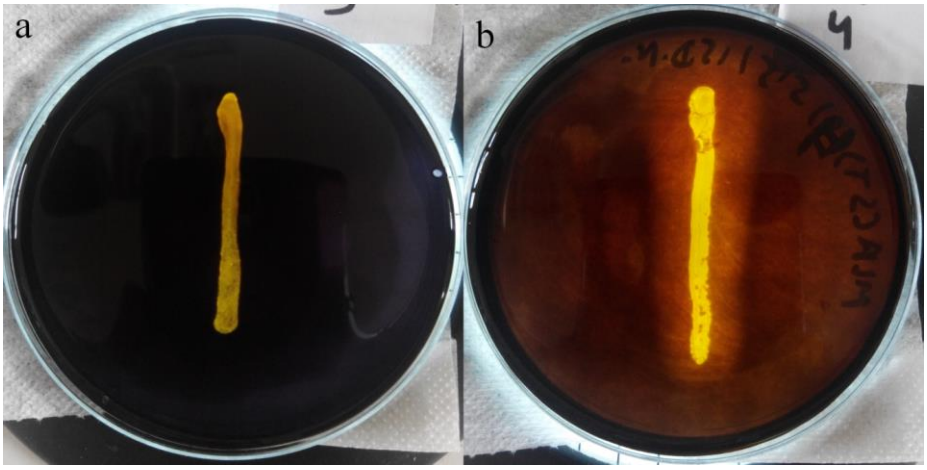

Fig 5. Starch hydrolysis (a) control (b) *Halovibrio* sp. strain MLA3

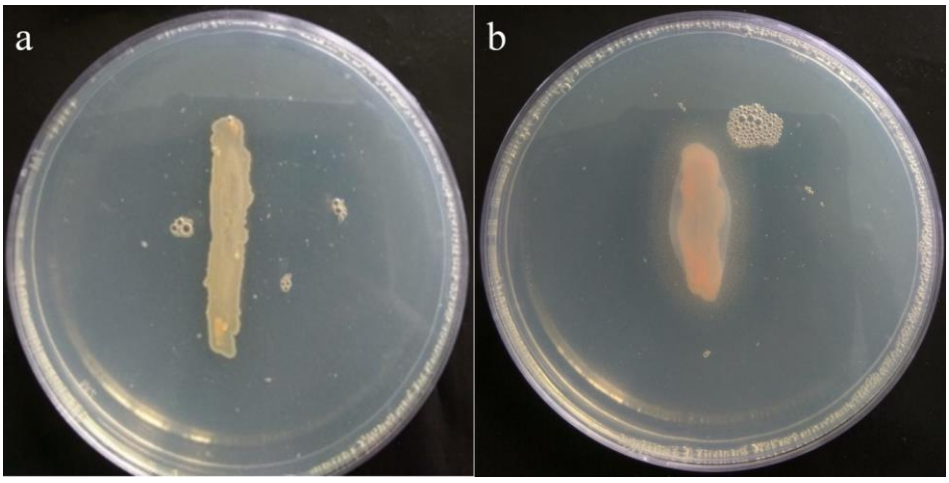

Fig. 6. Tween 80 hydrolysis (a) control (b) *Halovibrio* sp. strain MLA3

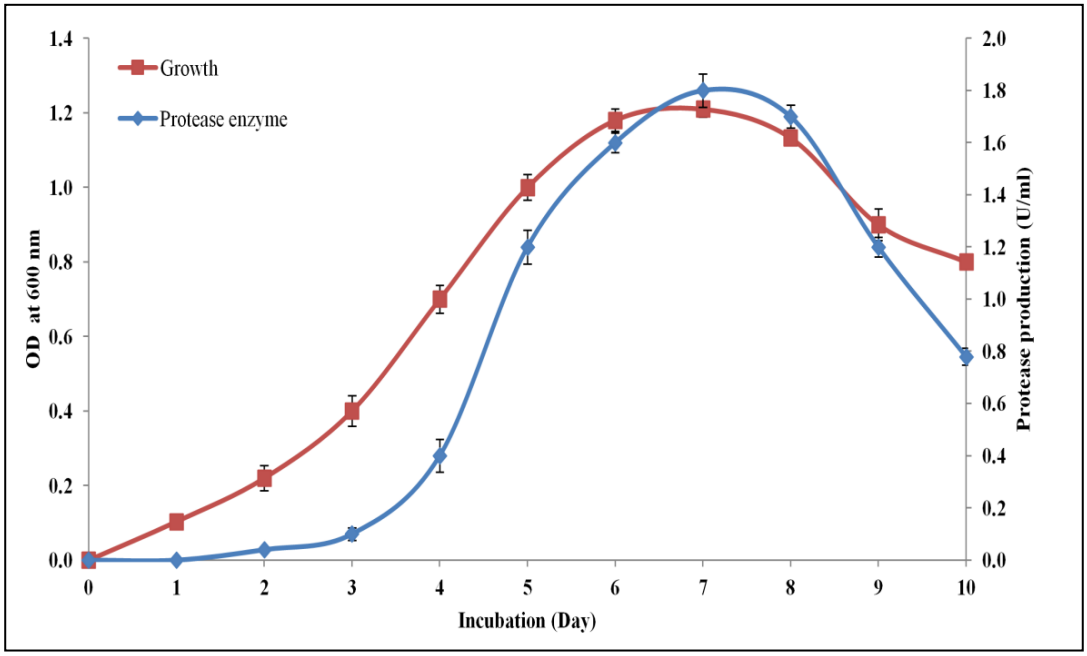

Fig. 7. Protease production during growth of *Halovibrio* sp. strain MLA3. Each value is a mean of three replicates data with ± standard deviation.

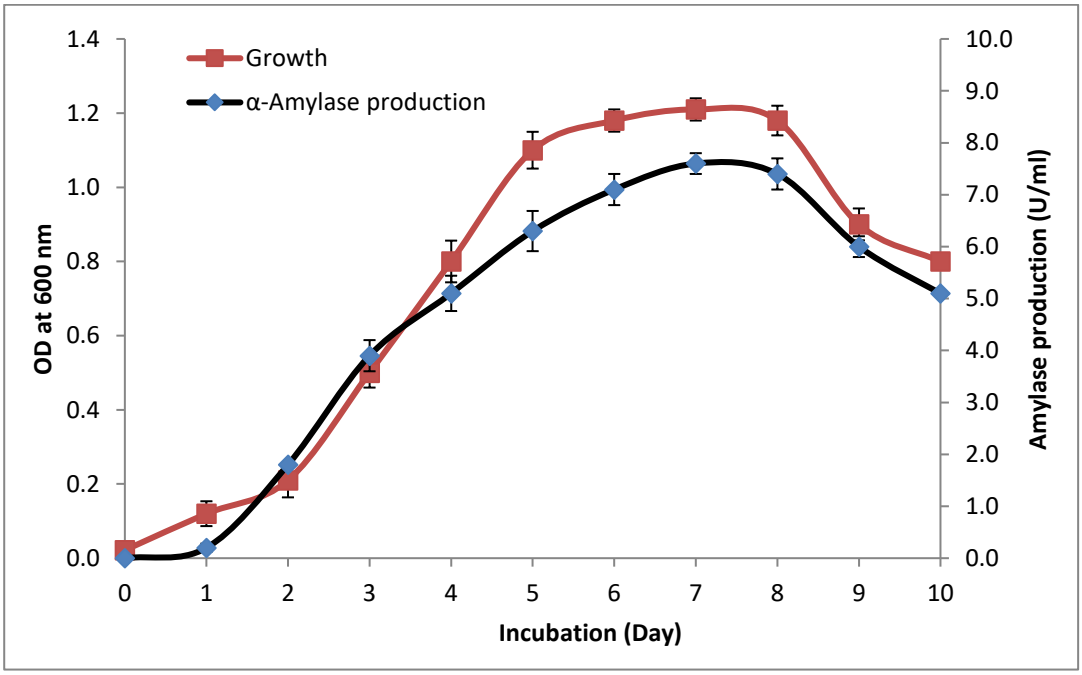

Fig. 8. Amylase production during growth of *Halovibrio* sp. strain MLA3. Each value is a mean of three replicates data with ± standard deviation.

4. CONCLUSIONS

The present study exhibited the occurrence of bacteria of genus *Halovibrio* in Sambhar Lake, India. Moreover, the strain MLA3 found ability to produce industrially important enzymes such as amylase, protease and lipase which could meet the industrial needs. Thus, the present study shows that Sambhar lake is a treasure of many unexplored microorganisms. It should be explored extensively for evaluation of microorganisms for phylogenetic studies and also for their biotechnological applications.

5. ACKNOWLEDGEMENTS

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