International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 7, Issue 2, 2015, pp.-616-622.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

ISOLATION AND CHARACTERIZATION OF HYDROLYTIC ENZYME PRODUCING HALOPHILIC BACTERIA *Salinicoccus roseus* FROM OKHA

RAVI R.K.[1]* AND PATEL P.[2]

[1]Department of Biotechnology, Shree M. & N. Virani Science College, Rajkot - 360 005, Gujarat, India.
[2]Department of Biotechnology, Christ College, Rajkot - 360 005, Gujarat, India.
*Corresponding Author: Email- raviranjan@vsc.edu.in

Received: January 15, 2015; Revised: May 14, 2015; Accepted: May 18, 2015

Abstract- Halophilic bacteria were isolated and purified from marine water of Okha region of Gujarat, India and further subjected to screen for extracellular hydrolytic enzyme production. Two strains (R1 & R2) showed extracellular hydrolytic enzyme activities, such as amylases, proteases, lipases and gelatinase, where as optimum activity observed with amylase. They were characterized on the basis of morphology, physiology and biochemical tests. The phylogenetic tree revealed that the strains R1 & R2 fit in to an evolutionary cluster comprising members of *Salinicoccus roseus* with 99% similarity. 16S rDNA sequence was submitted at NCBI and named as *Salinicoccus roseus* strain rvscokh1 & rsk1 respectively. The DNA G+C content rvscokh1 & rsk1 was 55.6 & 55.7 mol% respectively. The GenBank accession number of the 16S rDNA sequences of strain rvscokh1 & rsk1 is HQ190916 & HQ258884 respectively. *Salinicoccus roseus* is gram positive coccus bacteria. Strain rvscokh1 & rsk1 sustain to 10% and 6% NaCl concentration respectively while optimum pH was 7.4 for growth. Optimum amylase activity was found at temperature 37°C, pH 8 and 5% salt concentration for both the strains reveals that these strains can produce potentially industrially important enzymes.

Keywords- Phylogenesis, Halophiles, Hydrolytic enzymes, Amylase, *Salinicoccus roseus*

Citation: Ravi R.K. and Patel P. (2015) Isolation and Characterization of Hydrolytic Enzyme Producing Halophilic Bacteria *Salinicoccus roseus* from Okha. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 7, Issue 2, pp.-616-622.

Introduction

In recent years, there has been more interest in studying the microbiology of halophiles since these naturally occurring hypersaline environments are the potential source of diverse microorganisms [1]. Halophilic bacteria are found in marine, great salt lakes, natural or artificial salterns, salina etc. Those halophiles whose optimum salt concentration for growth is above approximately 5-20% (w/v) NaCl are considered moderate halophiles and 20-30% (w/v) NaCl are considered extreme halophiles which are of valuable use for research regarding hereditary, physiological and biochemical features and strain resources [2]. These extreme environments have been found to contain halophilic organisms that developed strategies or live optimally in such environments and lives occurring in such environments have found to possess an elite biotechnological potential [3]. Many halophiles and halotolerant microorganisms can grow over a wide range of salt concentrations depending on environmental and nutritional factors for the growth and tolerance [4]. Studies on halophilic bacterial extracellular enzymes are important with respect to their industrial applications mainly in fermented food, textile, pharmaceutical, leather industries, nutrient recycling and biodegradation process. Halophilic microorganisms have increased, and additional applications of industrially important enzymes are still under development. These uses have placed greater stress on increasing indigenous amylase production and search for more efficient processes [5]. The purpose of our study was to isolate and characterize pure cultures of extreme or moderate halophiles from enrichments obtained from marine water samples of coastal region of Gujarat and potentials to check extracellular hydrolytic enzyme production. Extracellular hydrolytic enzymes such as amylases, proteases, lipases etc have diverse potential usages in industrial and chemical sectors. Bacterial amylases are potentially beneficial in terms of ease to obtain, higher yield, ease to purify and thermostablity. Screening of marine microorganisms with higher amylase activity could therefore facilitate the discovery of novel amylases suitable for new biotechnological and industrial applications [6]. In present investigation, extracellular hydrolytic enzyme producing halophilic bacteria *Salinicoccus roseus* strain rvscokh1 & rsk1 was isolated and purified from marine water of Okha region of Gujarat and optimization study for amylase activity has been performed. Based on determining parts of physiological and biochemical index, 16S rDNA sequence was obtained, after which the homology was compared and phylogenetic analysis was established.

Materials and Methods

Source of Strains

Halophilic bacteria were isolated from a Marine water sample collected from Okha region (22°28'11"N 69°4'47"E) at Gujarat, India. The samples were collected using a sterile container and were

transferred to laboratory. All the samples were stored in cold condition.

Culture and Isolates

The samples were serially diluted and each diluted samples were plated on Nutrient agar (NA) and Seghal and Gibbons (SG) agar media, supplemented with 5%-15% NaCl for salinity and incubated at 37˚C for 2-4 days. After incubation, representative isolated colonies were transferred to nutrient broth medium. Isolates were routinely cultured aerobically at 37°C in Nutrient broth medium [7] containing (g/L): Peptic digest of animal tissue 5.0 g, Sodium chloride 5.0 g, Beef extract 1.5 g and Yeast extract 1.5 g, pH 7.4 supplemented with 5-15% NaCl for 72 h. Solid medium was prepared by adding 15 g/L agar. The selected colonies were purified three or four times in the solid medium before they were put into the liquid medium to culture for 3 days at 37°C at 120 rpm. Same method followed for Seghal and Gibbons media containing Yeast extract (HiMedia) 1.0 g, Casamino acids (HiMedia) 0.75 g, Na citrate 0.3 g, MgSO4· 7 H_2O 2.0 g, KCl, 0.2 g, $FeCl_2$ 0.0023 g, NaCl 25 g, distilled water 100 ml [8].

Screening of Extracellular Hydrolytic Enzymes

The isolates were screened for the production of extracellular hydrolytic enzymes such as amylase, protease, lipase and gelatinase using different plate assays method as detailed below:

Amylase Production

The presences of amylolytic activity on plates were determined using method described by Coronado [9] using HM medium supplemented with 0.5% (w/v) soluble starch. After incubation at 37°C for 3 days, the plates were flooded with 0.3% - 0.6% KI solution, a clear zone around the growth indicated hydrolysis of starch.

Protease Production

Proteolytic activity of the cultures was screened qualitatively in a saline medium containing 2% (w/v) sterile skim milk [10]. Formation of clear zone around the colonies over the next 725 h was considered as indication of protease production.

Lipolytic Production

Lipolytic activity of the isolates was detected by screening for zones of hydrolysis around colonies growing on HM plates containing 1% Tween-80, turbid around the growth indicated hydrolysis of Tween-80 [11].

Gelatin Hydrolyzing Activity

Gelatin (150 g/L) was supplemented to the saline medium and 2 milliliters were transferred to small testing tubes that were inoculated with the tested strains and incubated at 30°C. For the control, after incubation, the cultures were maintained for 10 minutes at 4°C. The liquefaction of gelatin that indicates the production of gelatinase was recorded [12].

Assay and Optimization of Amylase

Enzyme assay were performed with cell free supernatant of fermentation broth obtained by centrifugation at 15000 rpm for 10 minutes at 4˚c. Amylase activity of crude enzyme was determined by incubating a mixture of 0.5 ml 1% soluble starch dissolved in 0.1 M phosphate buffer and 0.5 ml crude enzyme at pH 7, temperature 37˚c for 15 minutes [13]. The liberated reducing sugar was estimated by addition of 3, 5 dinitrosalicylic acid and followed by boiling for 10 minutes [14]. The final volume was made up to 12 ml with distilled water and optical density was measured at 540 nm. One unit of amylase activity was defined as amount of µg of maltose equivalent liberated per minute per ml of enzyme under assay condition. The amount of maltose was determined from the maltose standard curve. Enzyme productions were further optimized using various parameters like temperature, pH and salinity in different ranges.

Characterization and Growth Condition

The isolates were examined for their colony shape, spore formation, motility and pigmentation. Gram staining was performed by using acetic acid-fixed samples as described by Dussault [15]. Growth rates at different salt concentrations were determined by using maintenance medium prepared with salt concentrations of 5-15% (w/v). Biochemical tests were performed according to Bergey's Manual of Systemic Bacteriology (1994).

The optimal conditions for growth of isolates and amylase production were tested with varying parameters. The pH growth range was pH 5-10, NaCl growth range 5-15 % and temperature range was 15°C-50°C taken for optimization. To determine antibiotic sensitivity, an active culture was inoculated onto Mueller-Hinton (Hi-media) agar plates, octadiscs impregnated with antibiotics were placed on their surface. The plates were incubated and the zones of inhibition around the antibiotic discs were recorded [16].

All the growing tests were carried out according to the following methods: 5 ml liquid medium was interfused with 0.5 ml bacterium liquid in each tube; then, the strain was cultured for 3 days at 37°C, optical density was detected at λ=600 nm, test was repeated three times.

DNA Extraction

DNA extraction was performed according to following methods: Cells were cultured to approximately the late exponential phase and then harvested by centrifugation in 15 ml portions at 10,000 rpm for 10 min. The deposit was suspended in solution [glucose 50mM (pH 8.0), Tris-HCl 25 mM (pH 8.0), and EDTA 10 mM]. The cells were lysed by adding 200 ml of 5% lysozyme, 20 ml of 1% pronase, 8 ml of mutanolysin (500 U/ml) and 4 ml of 1% RNase. DNA was extracted by adding 250 ml of extraction buffer (20 mM Tris-HCl, 100 mM EDTA, 1% sodium dodecyl sulfate, 0.01% proteinase K). The lysate was extracted two times with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Nucleic acid was precipitated with double -volume ethanol and washed with ethanol 70%. Nucleic acid was resuspended in 20 µl TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0) [17]. Then, it was quantified by spectrophotometry and kept at −20°C. The G-C content of the DNA was determined by the thermal denaturation method [18].

16S rRNA Gene Amplification and Purification

Ribosomal 16S rRNA genes were amplified using the universal bacterial primers Fd1 5'-ttccggttgatcctgcc-3' (forward); and Rd1, 5'-aaggaggtgatccagcc-3' (reverse). Bacterial 16S rDNA was amplified in a thermal reactor thermocycler (GeneAmp® PCR System 9700, Applied Biosystems) for 35 cycles (3 min denaturing step at 94°C in the first cycle; 30 sec denaturing at 94°C, 30 sec annealing at 55° C, and 2 min elongation at 72°C, with a final extension step at 72°C for 10 min). The amplification product was purified by 1.2% agarose gel using the GeNei™ Quick PCR Purification Kit (Bangalore Genei, India) to prepare it for sequencing.

Sequencing of 16S rRNA-encoding DNA

The purified DNA amplicons were lyophilized and sent for sequencing to M/s Macrogen Inc., Seoul, Korea. Sequence fragments generated from a given template were edited against electropherograms and then assembled into contigs using SeqMan (Lasergene DNASTAR, Inc, Madison, WI). The sequences were tested for chimeras by using the CHIMERA-CHECK program of the Ribosomal Database Project. The partial 16S rDNA sequences corresponding to 1-1468 positions were then subjected to BLAST search to obtain some of the best matching sequences [19]. The sequences were aligned using the Clustal W multiple-alignment program [20] and by comparison with sequences deposited in the Ribosomal Database Project [21].

The pair-wise scores of input sequences were transformed to distance matrix by kimura two-parameter model before solved by neighbor-joining algorithm. Phylogenetic relationships among the sequences were inferred using PHYLIP package, version v.3.57 [22]. Phylogenetic trees were constructed by the neighbor-joining method of Saitou & Nei [23]. The sequences reported in this paper were deposited in GenBank with accession number HQ190916 & HQ258884 for rvscokh1 & rsk1 respectively.

Results

Isolation & Screening of Extracellular Hydrolytic Enzymes

Total 36 halophilic bacteria has been isolated and subjected for screening of extracellular hydrolytic enzymes. Among various isolates ten isolates produces amylases, 8 isolates produces gelatinase, 5 showed proteolytic activity and 3 shows lipolytic activities. Finally two isolates R1 & R2 producing all four extracellular hydrolytic enzymes amylases, proteases, lipases and gelatinase has been selected. Both strains are found to be potent producer of amylase enzyme on the basis of zone of inhibition. Since both of the strains are found to potent amylase producer, further study was restricted for amylase assay and optimization of amylase enzyme in various parameters. Standardization of various parameters such as pH, temperature and salinity was carried out for the optimum growth of bacteria and selecting most commercial usage of amylase enzyme. Maximum enzymatic activity of all isolates was observed in the logarithmic phase of growth at temperature 37°C, pH 7-8 and 5% salinity. Maximum amylase activity for both strain rvscokh1 and rsk1 was observed 32 hour after inoculation in assay medium.

Physiological and Biochemical Features

Strain rvscokh1 & rsk1 is aerobic, Gram-positive, cocci, non-motile and non-spore-forming bacteria. The colony color on both media tested is orange. Growths of these two halophilic strains *Salinicoccus roseus* strain rvscokh1 & *Salinicoccus roseus* strain rsk1 were observed with NaCl concentrations of 5-15% (w/v) with optimum growth at 10% for rvscokh1 & 6% for rsk1 in both SG & NA media. They Grows at pH 6.0-9.0 (optimum growth at pH 7.4) and with temperature range of 20-45°C (Optimum 37°C). Both strains gives a positive reaction in tests for catalase and oxidase; but negative in test for Nitrate reduction, Indole production, Methyl red, H_2S production & Voges-Proskauer tests. Phenotypic characteristics of isolated Strain rvscokh1 & rsk1 were mentioned in [Table-1]. Strain rvscokh1 was found to be highly sensitive to Cephalothin, Clindamycin, Ofloxacin, Penicillin-G, Colistin and Vancomycin but resistant to Ampicillin, Ciprofloxacin, Co-timoxazole, Gentamicin, Nitrofurantoin, Streptomycin, Tetracycline and Erythromycin. Strain rvscokh1 was found to be highly sensitive to Ciprofloxacin but resistant to Cephalothin, Clindamycin, Ofloxacin, Penicillin-G, Colistin Vancomycin, Ampicillin, Co-timoxazole, Gentamicin, Nitrofurantoin, Streptomycin and Tetracycline.

Table 1- Phenotypic features of isolates studied

Characteristic	Strain	
	rvscokh1	rsk1
Colony	Flat	Flat
Motility	-	-
Colony pigmentation	Orange	Orange
NaCl range of growth (%w/v)		
Range	5-15%	5-15%
Optimum	10%	6%
pH of growth		
Range	6-9	6-9
Optimum	7.4	7.4
Temperature for growth		
Range	20-45°C	20-45°C
Optimum	37°C	37°C
Acid production from		
Maltose	-	-
Galactose	-	-
D-glucose	-	-
Fructose	-	-
Sucrose	-	-
Hydrolysis of		
Casein	+	+
Gelatin	+	+
Starch	+	+
Tween 80	+	+
Methyl Red test	-	-
Urease	-	-
Nitrate Reduction	-	-
Indole Production	-	-
H_2S production	-	-
Voges-Proskauer Tests	-	-
G+C content	55.60%	55.70%

Amylase Assay

Studies on crude enzyme revealed that optimum amylase activity was at temperature 37°C [Fig-1], pH 8 [Fig-2] and 5% salt concentration [Fig-3] for both the strain. There is a sharp decline in enzyme activity at temperature 45°C-50°C and 6-7% salt concentration. Although alpha-amylase production was evident at temperatures range of 35°C-40°C studied for fermentation, 37°C was the best among them.

Phylogenetic Analysis

An amplified fragment of about 1.5 kb was obtained when PCR primers targeted phylotypes of the bacterial domain. The DNA G+C content of strain rvscokh1 & rsk1 was 55.7% and 55.6 mol% respectively, determined by the thermal denaturation temperature (Tm) using a Cintra20 spectrophotometer (GBC Scientific Equipment). The most closely related sequences against GenBank and Ribosomal Database Project II were identified using BLAST results indicated 99% of the similarity to 16S rDNA group represented by the cluster of halophilic member of the genus *Salinicoccus* in GenBank sequences. We used ClustalW programs of the PHYLIP package to identify the similarities between 16S rDNA sequences of different strains. The DNADIST program was used to construct a

Kimura two-parameter evolutionary distance matrix [Table-2]. Phylogenetic tree was then constructed from evolutionary distances using neighbor-joining method with global rearrangements and randomized input order [Fig-4] & [Fig-5]. Bootstrap analysis (1,000 replications) was performed using the additional programs SEQBOOT and CONSENSE.

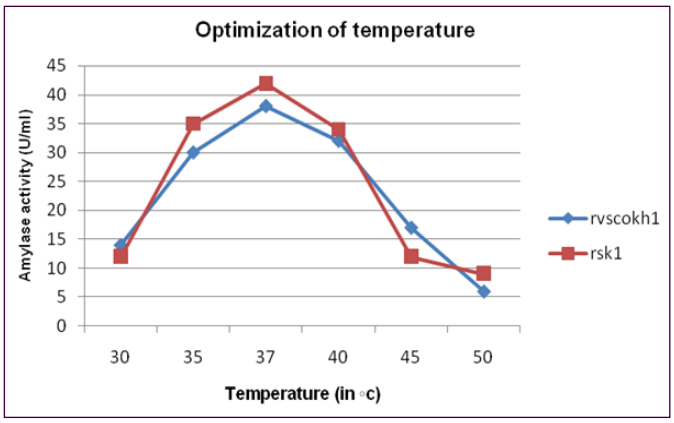

Fig. 1- Effect of temperature on enzyme activity in strain rvscokh1 & rsk1

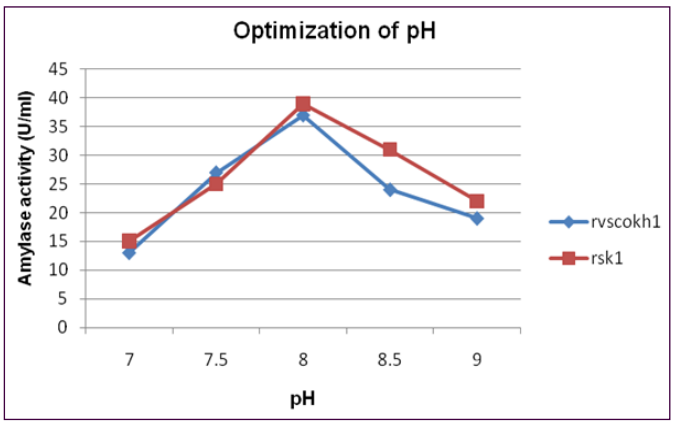

Fig. 2- Effect of pH on enzyme activity in strain rvscokh1 & rsk1

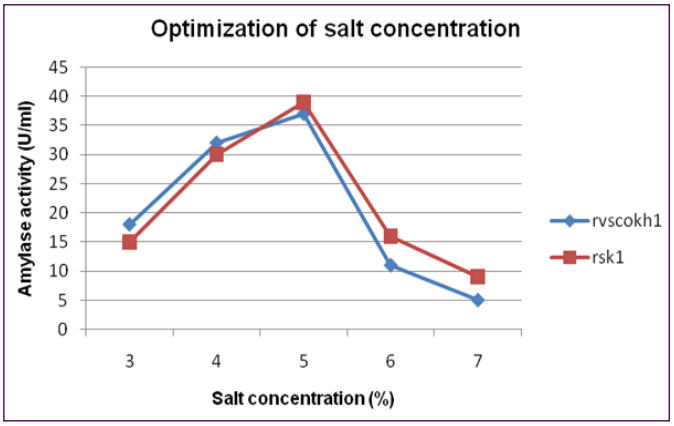

Fig. 3- Effect of salt concentration on enzyme activity in strain rvscokh1 & rsk1

Discussion

During the past decades extreme environments have been extensively explored, and the halophilic bacteria has been found in a wide range of saline environments. Gujarat have largest coastal region in India, and hence tremendous possibility are present for diverse biomolecule producing halophiles. Our study demonstrated the presence of extracellular hydrolytic enzyme producing halophilic bacteria in Okha region of Gujarat. With the advancement of molecular approaches [24], diverse bacterial genotypes have been discovered [25]. However, isolation is still a necessary approach to obtain novel microbes and physiological characteristics for understanding their physiological and environmental functions and for their application potentials [26]. *Bacillus okhensis* has been already identified in Okha region of Gujarat [27]. Various kinds of moderately/extremely halophilic bacteria and archaea have been isolated from ancient salt deposits, in which several new taxa were identified [28,29]. Isolated strains rvscokh1 & rsk1 is Gram-positive cocci, non-motile and non-spore-forming bacteria. Its optimal growth conditions are 10% NaCl for rvscokh1 & 6% for rsk1, 37°C temperature and pH 7.4 with NA & SG medium. It is considered as moderate halophile on the basis of salinity.

Table 2- Distance matrix of 16S rDNA sequence of *Salinicoccus* strains

No	Abbreviation	% Distance Matrix															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	SR	0.00															
2	SSP	0.00	0.01														
3	SSP	0.00	0.00	0.00													
4	SSP	0.00	0.01	0.00	0.00												
5	SSP	0.01	0.01	0.01	0.01	0.01											
6	Ub	0.00	0.01	0.01	0.01	0.01	0.01										
7	SSP	0.00	0.00	0.00	0.00	0.00	0.01	0.01									
8	Ub	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01								
9	Ub	0.00	0.01	0.00	0.00	0.01	0.01	0.01	0.00	0.01							
10	Ub	0.00	0.01	0.00	0.00	0.01	0.01	0.01	0.00	0.01	0.00						
11	Ub	0.00	0.01	0.00	0.00	0.01	0.01	0.01	0.00	0.00	0.01	0.01					
12	rvscokh1	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.01	0.00				
13	rsk1	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.01	0.00	0.00			
14	SSP	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00		
15	SR	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	
16	UB	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01

SR- *Salinococcuc roseus*, SSp- *Salinococcuc* Sp., Ub- Uncultured bacterium

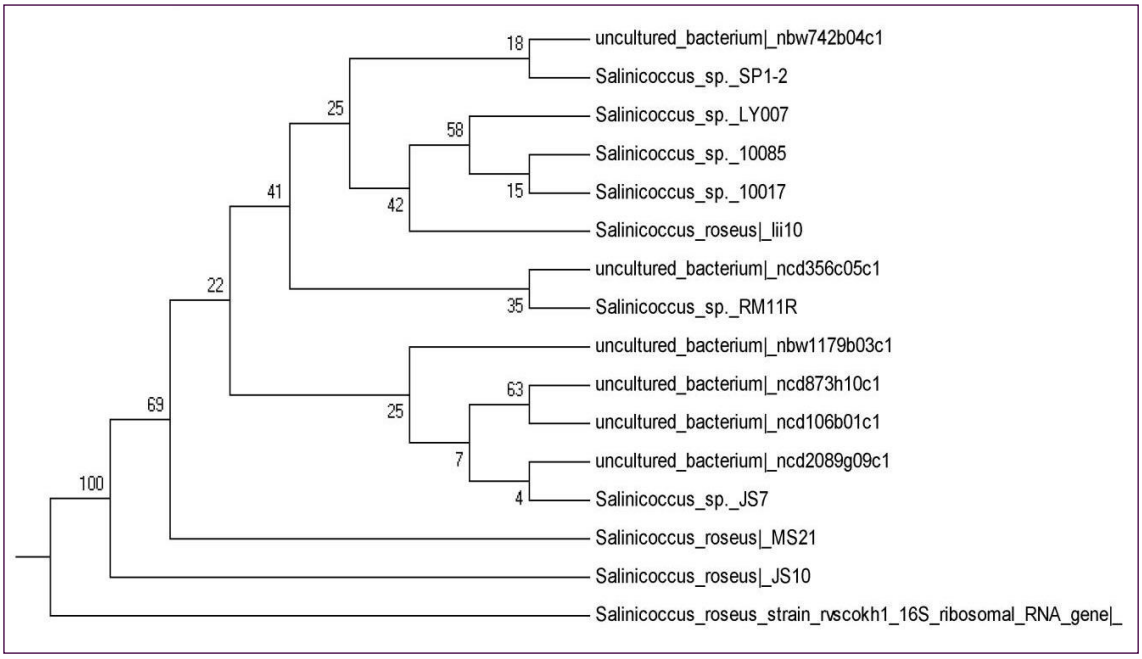

Fig. 4- Phylogenetic dendrogram based on 16S rRNA gene sequences and constructed using the neighbour-joining method showing the phylogenetic positions of *Salinicoccus roseus* strain rvscokh1 and related taxa. The phylogenetic tree was constructed by using the neighbourjoining method and Kimura two-parameter evolutionary distance matrix data obtained from unambiguous aligned nucleotides. Bootstrap values (expressed as percentage of 1000 replications) greater than 50 % are shown at the branch points. Bar 1 substitution per 100 nucleotide positions.

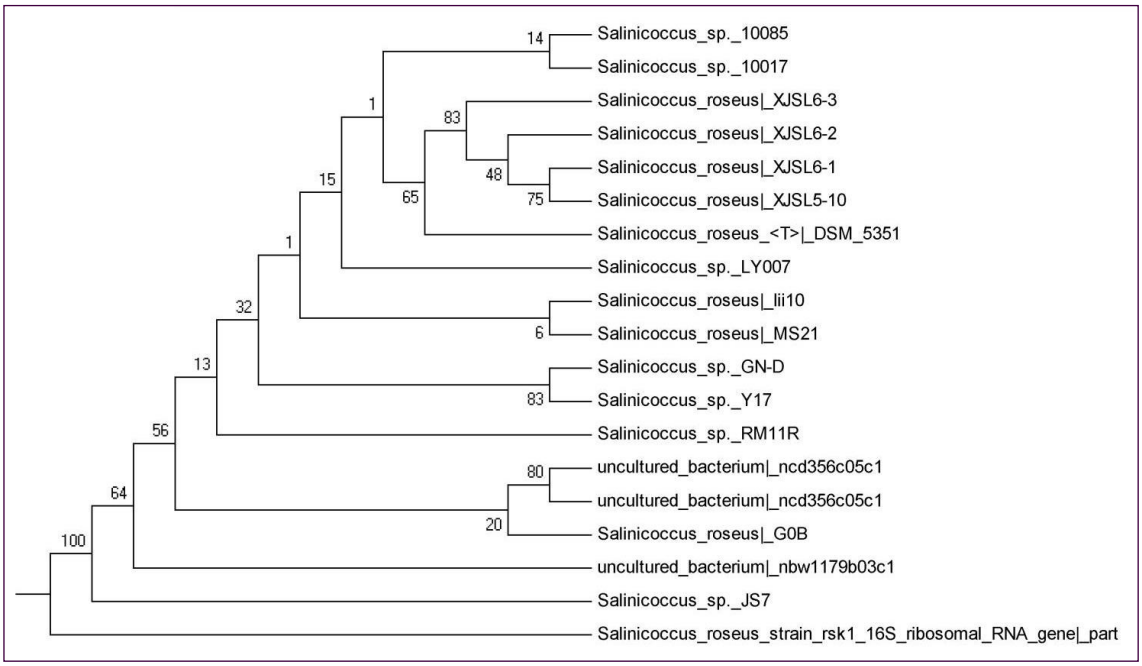

Fig. 5- Phylogenetic dendrogram based on 16S rRNA gene sequences and constructed using the neighbour-joining method showing the phylogenetic positions of *Salinicoccus roseus* strain rsk1 and related taxa. The phylogenetic tree was constructed by using the neighbourjoining method and Kimura two-parameter evolutionary distance matrix data obtained from unambiguous aligned nucleotides. Bootstrap values (expressed as percentage of 1000 replications) greater than 50 % are shown at the branch points. Bar 1 substitution per 100 nucleotide positions.

According to taxonomical classification, both of the strain belongs to domain Bacteria; phylum Firmicutes; Class Bacilli; order Bacillales; family Staphylococcaceae and genus *Salinicoccus.* The phylogenetic evolutionary distances based on 16S rDNA sequences of halophilic bacteria have better relativity with strain morphology and classified biochemistry data. Phylogenetic analysis and distance matrix value indicate that strain rvscokh1 & rsk1 clusters in the same microbiological population with other *Salinicoccus* genus. Their similarities vary from 95 to 99%. Therefore, strain rvscokh1 & rsk1 is a novel strain of the genus *Salinicoccuc roseus.*

Salinicoccuc roseus was not well reported earlier with extracellular enzyme production, but this strain shows their capability of extracellular hydrolytic enzyme production. Recently, Sulochana, et al [30] reported enzyme production capacity by *Salinicoccus* species by Arabal soil of west coast of Karnataka, India. Stability of the extremozymes, in the face of adverse condition will lead to their use in a variety of biotechnological application in which such tolerance is advantageous and beneficial. Proteins from halophilic organisms have a biased amino acid composition in order to remain stable and active at high ionic strength. Halophilic proteins typically have an excess of acidic amino acid (i.e. aspartate and glutamate) on their surface. Negative charges on the halophilic proteins bind significant amount of hydrated ions, thus reducing their surface hydrophobicity and decreasing the tendency to aggregate at high salt concentration. Although such a high proportion of acidic amino acids is not present in the amylase from the thermophilic halophile [31,32]. An enzyme from the marine source may be a unique protein molecule not found in any terrestrial organism or it may be a known enzyme from a terrestrial source but with novel properties [33].

Salinococcuc roseus strains produce extracellular Amylase, protease, lipase and gelatinase enzymes having wide applications in various industries and research. halophilic amylases highlighted due to their stability and versatility and having some hence interesting applications in starch saccharification, textile, food, brewing, distilling industries, clinical and analytical chemistries [34]. Haloproteases have been widely used in industry for a long time, especially in washing detergent, baking, brewing, cheese industry and tanning industry [35,36]. Lipases are often hampered by their low stability in the processes and loss of activity in presence of the organic solvents, hence the lipases isolated from halophilic microorganisms constitute an excellent alternative in the industrial processes. Bacterial lipolytic enzymes are valuable biocatalysts due to their broad substrate specificity and high chemo-, regio- and stereoselectivity [37,38]. Thus, these enzymes are currently used as detergent additives, in the food and paper industries, and as enantioselective biocatalysts for the production of fine chemicals [39,40]. Gelatinase is one type of diverse group protease, an extracellular metallo- endopeptidase or metalloproteinase which is able to hydrolyze gelatin and other compounds such as pheromone, collagen, casein and fibrinogen [41]. Gelatinase enzymes are widely used in chemical, medical, food and basic biological science [42]. Among all enzymes, amylase was found to be potentially produced by *Salinococcuc roseus* which has been further optimized for enhanced production. Optimization of crude hydrolytic enzyme production using various temperature, pH and salinity revealed that optimum amylase activity was at temperature 37°C, pH 8 and 5% salt concentration for both the strain. Optimum temperature report by various worker was also found to be 37°C for optimum amylase activity. Therefore, we can conclude that enzymes produced by *Salinicoccuc roseus* strains can show interesting applications in future for industrial purpose.

Conflicts of Interest: None declared.

References

[1] Ventosa A., Nieto J.J. & Oren A. (1998) *Microbiol. Mol. Biol. Rev*., 62(2), 504-544.

[2] Margesin R. & Schinner F. (2000) *Extremophiles*, 5(2), 73-83.

[3] Dave S.R. & Desai H.B. (2006) *Current Science*, 60(4), 497-500.

[4] Das S., Lyla P.S. & Khan S.A. (2006) *Current Science*, 90(10), 1325-1335.

[5] Aiyer P.V. (2005) *Afr. J. Biotechnol*., 4(13), 1525-1529.

[6] Chandrasekaran M. (1997) *Journal of Marine Biotechnology*, 5, 86-89.

[7] Saito A., Mitsui H., Hattori R., Minamisawa K. & Hattori T. (1998) *FEMS Microbiol. Ecol*., 25, 277-286.

[8] Sehgal S.N. & Gibbons N.E. (1960) *Can. J. Microbiol*., 6, 165-169.

[9] Coronado M.J., Vargas C., Hofemeister J., Ventosa A. & Nieto J. (2000) *FEMS Microbiol. Lett*., 183, 67-71.

[10] Zhang X.H. & Austin B. (2000) *J. Fish. Dis*., 23, 93-102.

[11] Hasan F., Shah A.A. & Hameed A. (2009) *Biotechnol. Adv*., 27, 782-798.

[12] Jayachandra S.Y., Parameshwar A.B., Mohan R.K. & Sulochana M.B. (2012) *World Journal of Science and Technology,* 2(2), 23-26.

[13] Bernfeld P., Colowick S.P. & Kaplan N. (1955) *Methods in Enzymology*, Academic Press Inc., New York, 149-158.

[14] Miller G.L. (1959) *Anal Chem.,* 31, 426-428.

[15] Dussault H.P. (1955) *J. Bacteriol*., 70, 484-485.

[16] Matsen J.M. & Coghlan C.R. (1972) *Antibiotic testing and susceptibility patterns of streptococci*, Streptococci and streptococcal diseases, Academic Press, New York, 189-204.

[17] Oren A. & Ventosa A. (1996) *Int. J. Syst. Bacteriol*., 46(4), 1180.

[18] Marmur J. & Doty P. (1961) *J. Mol. Biol*., 3, 585-594.

[19] Altschul S.F., Gish W., Miller W., Myers E.W. & Lipman D.J. (1990) *J. Mol. Biol*., 215, 403-410.

[20] Thompson J.D., Higgins D.G. & Gibson T.J. (1994) *Nucleic Acids Res*., 22(22), 4673-4680.

[21] Cole J.R., Chai B., Marsh T.L., Farris R.J., Wang Q., Kulam S.A., Chandra S., McGarrell D.M., Schmidt T.M., Garrity J. & Tiedje J.M. (2003) *Nucl. Acid. Res*., 31, 442-443.

[22] Felsenstein J. (1993) *PHYLIP* (*Phylogeny Inference Package*) version 3.57, Dept. Genet., Univ. Wash.

[23] Saitou N. & Nei M. (1987) *Mol. Biol. Evol*., 4, 406-425.

[24] Amann R.I., Ludwig W. & Schleifer K.H. (1995) *Microbiol. Rev*., 59, 143-169.

[25] Schloss P.D. & Handelsman J. (2004) *Microbiol. Mol. Biol. Rev.,* 68, 686-690.

[26] Vandamme P., Pot B., Gillis M., de V.P., Kersters K. & Swings J. (1996) *Microbiol. Rev*., 60, 407-438.

[27] Nowlan B., Dodi M.S., Singh S.P. & Patel B.K.C. (2006) *Int. J. Syst. Evol. Microbiol*., 56,1073-1077.

[28] Grant W.D., Gemmell R.T. & McGenity T.J. (1998) *Extremophiles*., 2, 279-287

[29] Vreeland R.H., Rosenzweig W.D. & Powers D.W. (2000) *Nature*, 407, 897-900.

[30] Jayachandra S.Y., Parameshwar A.B., Mohan R.K. & Sulochana M.B. (2012) *World Journal of Science and Technology*, 2(2), 23-26.

[31] Mijts B.N. (2001) *Genes and Enzymes of Halothermothrix orenii,* PhD thesis, Griffith University.

[32]Danson F.M., Steven M.D., Malthus T.J. & Clark J.A. (1992) *International Journal of Remote Sensing*, 13, 461-470.

[33]Debashish G., Malay S., Barindra S. & Joydeep M. (2005) *Adv. Biochem. Engin/Biotechnol*., 96, 189-218.

[34]Pandey A., Nigam P., Soccol C.R., Soccol V.T., Singh D. & Mohan R. (2000) *Biotechnol. Appl. Biochem*., 31, 135-152.

[35]Chand S. & Mishra P. (2003) *Adv. Biochem. Eng. Biotechnol*., 85, 95-124.

[36]Li A.N. & Li D.C. (2009) *J. Appl. Microbiol*., 106, 369-380.

[37]Houde A., Kademi A. & Leblanc D. (2004) *Appl. Biochem. Biotechnol*., 46, 155-170.

[38]Snellman E.A. & Colwell R.R. (2004) *J. Ind. Microbiol. Biotechnol*., 31, 391-400.

[39]Jaeger K.E. & Teggert T. (2002) *Curr. Opin. Biotechnol*., 13, 390-397.

[40]Schmid A., Dordick J.S., Hauer B., Kiener A., Wubbolts M. & Witholt B. (2001) *Nature*, 409, 258-268

[41]Makinen P. & Makinen K.K. (1994) *Biochem. Biophys. Res. Commun*., 200, 981-985.

[42]Hisano T., Abe S., Wakashiro M., Kimura A. & Murata K. (1989) *J. Fermen. Bioeng*., 68(6), 399-403.