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Isolation and characterization of extreme halophilic bacterium *Salinicoccus sp.* JAS4 producing extracellular hydrolytic enzymes

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Abstract
A extreme halophilic bacterium, strain JAS4 was isolated from the Arabal soil of west coast of Karnataka, India. The isolate is Gram positive, strictly aerobic, ferments several carbohydrates and has motile, coccoid shaped cells and non sporing, catalase- and oxidase- positive, that grew in presence of 2-25% (w/v) NaCl and at pH 6.5-11, with optimum growth at 10%(w/v) NaCl, with an optimum growth temperature of 34^{0}C, has potential to produce the extracellular enzymes such as Amylase, Protease, Inulinase and Gelatinase, but production of lipase was found to be negative. The phenotypic studies and genotypic analysis by 16S rRNA analysis showed that the bacterium belonged to the genera *Salinicoccus* of 98% BLAST sequence similarity and it is named as Salinicoccus sp. JAS4 and phylogenetic study was carried out using Mega5 software.

Keywords: *Salinicoccus sp.*, Extracellular enzymes, 16S rRNA, Phylogenetic analysis

INTRODUCTION

The majority of important industrial processes are conducted under specific parameters, pH, ionic strength, temperature etc., but some of these enzymes cannot exhibit all the times their optimal activities (Arnold, 2001). In respect of these requirements, extremozymes (Eichler, 2001), the extracellular enzymes produced by extremophiles, microorganisms that are able to flourish in extreme environments, could help to develop new biotechnology.

Halophilic bacteria are found in different environments such as salt lakes, saline soils and salted food. The majority of halophilic microorganisms studied so far produce compounds with great potential in industrial process and they have physiological properties which facilitate its use with commercial aims. Enzymes produced by halophilic microorganisms have developed particular features which confer them stability and solubility at salt concentrations, thus, low water concentrations. However, inspite of a growing interest in the use of halophilc enzymes for biotechnological applications, there are relatively few reports in the literature about their production and characterization (Bhatnagar *et al*, 2005).

Most of the industrial processes are carried out under specific physicochemical conditions which may not be definitely adjusted to the optimal points required for the activity of the available enzymes; thus, it would be of great importance to have enzymes that exhibit optimal activities at various ranges of salt concentration, pH and temperature (Rohban, R., et al., 2008). Halophiles are an excellent source of such enzymes which are not only salt tolerant, but also may be active at high temperature and pH values (Gomez J and Steiner W, 2004). The isolation of moderate halophiles able to produce hydrolases will provide the possibility to have optimal activities at different salt concentrations that could be useful in some industrial processes (Gomez J *et al*, 2004 and Sanchez- Porro *et al*., 2002).

India consists of various saline environments including hypersaline lakes, ocean and salt pans, in which the microbial diversity has not been characterized; thus the potential of producing different hydrolytic enzymes and antibiotic resistant profile among them remains unknown. The present study reveals the importance of *Salinicoccus sp.* JAS4 in producing extracellular hydrolytic enzymes.

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MATERIALS AND METHODS

Screening of Strain for extracellular hydrolytic activities

Isolated strain was subjected to screen for the production of extracellular hydrolytic enzymes such as protease, amylase, lipases, inulinases and gelatinase, different plate assays were performed as detailed below.

Extracellular protease production

Proteolytic activity of the isolate was screened in skim milk agar containing 10% (w/v) skim milk, 2% (w/v) agar, supplemented with 20% (w/v) total salt for determining the hydrolytic activity of extremely halophilic *Salinicoccus sp*. Clear zones around the growth after 7 days was taken as evidence of proteolytic activity (Rohban, R., *et al*., 2008)

Extracellular amylase production

The presence of amylolytic activity on plate was determined qualitatively following the method described by (Amoozegar *et al*.2003), using starch agar medium containing 20% (w/v) total salts. After incubation at 34- 37^0 C for 7 days, the plate was flooded with 0.3% I_2- 0.6% KI solution, a clear zone around the growth indicated hydrolysis of starch.

Extracellular lipolytic activity

Lipolytic activity of the isolate was detected by screening for zone of hydrolysis around colonies growing on saline medium plates containing 1% Tween-80, after incubation for 48 h. A spectrophotometric assay permitted the determination of lipolytic activity in the culture supernatant with p-nitrophenol esters as substrates. Cleavage of p-nitrophenol esters (Sigma) was measured at 37^0C using 0.1 M phosphate- buffer (pH 7.5) according to the Winkler and Struckmann (1979). One unit was defined as the amount of enzyme which caused the release of 1 µmol of p-nitro phenol per minute under test conditions.

Extracellular gelatinase activity

Gelatin (150 g l^{-1}) was supplemented to the saline medium and 2 mililiters were transferred to small testing tubes that were inoculated with the tested strains and incubated at 30^0C. For the control, after incubation, the cultures were maintained for 10 mins at 4^0C. The liquefaction of gelatin that indicates the production of gelatinase was recorded.

Determination of extracellular inulinase activity

The production of halophilic isolates was detected by preparing media containing inulin 2g l^{-1}, $(NH_4)_2$ SO_4 0.5 g l^{-1}, $MgSO_4$. $7H_20$ 0.2 g l^{-1}, KH_2PO_4 3 g l^{-1}, agar 20 g l^{-1}, supplemented with the appropriate concentration (20%) of salts for extreme halophile. Inulin was used as the sole source of carbon in this medium; thus, bacterial growth after 48 h of incubation at 37^0C, indicated the presence of inulinase activity (Allais J.J., 1986).

16S rRNA sequencing

The genomic DNA was isolated as described by Ausubel *et al.,* (1987). The PCR assay was performed using Applied Biosystems, model 9800 with 1.5µl of DNA extract in a total volume of 25µl. The PCR master mixture contained 2.5µl of 10X PCR reaction buffer (with 1.5 M $MgCl_2$), 2.5µl of 2 mM dNTPs, 1.25µl of 10pm/µl of each oligonucleotide primer 16S_8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S_1391R (5'-GACGGGCGGTGTGTRCA -3'), 0.2µl of 3 U/µl Taq DNA polymerase and 15.76µl of glass-distilled PCR water.

Initially denaturation accomplished at 94°C for 3 min. Thirty-two cycles of amplification consisted of denaturation at 94°C for 30sec, annealing at 55°C for 30sec and extension at 72°C for 1.30 min. A final extension phase at 72°C for 10 min was performed. The PCR product was purified by PEG-NaCl method. The sample was mixed with 0.6 times volume of PEG-NaCl, 20% [PEG (MW 6000) and 2.5 M NaCl] and incubated for 20 min at 37°C. The precipitate was collected by centrifugation at 3,800 rpm for 20 min. The pellet was washed with 70% ethanol, air dried and dissolved in 12 µl sterile distilled water.

The sample was sequenced using a 96-well Applied Biosystems sequencing plate as per the manufacturer's instructions. The thermocycling for the sequencing reactions began with an initial denaturation at 94°C for 2 min, followed by 35 cycles of PCR consisting of denaturation at 94°C for 10 s, annealing at 50°C for 10 s, and extension at 60°C for 4 min using primers 704F (5'-GTAGCGGTGAAATGCGTAGA- 3') and 907R (5'-CCGTCAATTCMTTTGAGTTT-3'). The samples were purified using standard protocols described by Applied Biosystems, Foster City, USA. To this, 10 µl of Hi-Di formamide was added and vortexed briefly. The DNA was denatured by incubating at 95°C for 3 min, kept on ice for 5-10 min, and was sequenced in a 3730 DNA analyzer (Applied Biosystems) following the manufacturer's instructions. The obtained sequences were analysed using Sequence Scanner (Applied Biosystems) software. The rDNA sequence contigs were generated using Chromas Pro and then analysed using online databases viz. NCBI-BLAST to find the closest match of the contig sequence.

Phylogenetic analysis was performed using the software packages PHYLIP (Felsenstein J., 1993) and MEGA version 4 (Kumar S., *et al., 2004*) after obtaining multiple alignment data available from databases by CLUSTAL_X (Thomson *et al*., 1997). Pairwise evolutionary distances were computed using the correction method (Jukes T.H and Cantor C.R., 1969) and clustering was performed using the neighbor-joining method (Saitou N and Nei M., 1987). Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 1,000 resampling (Felsenstein J.,1985). Sequence was deposited in the NCBI GenBank under the accession number HQ426914.

RESULTS AND DISCUSSION

Both the molecular and microbiological studies have revealed the presence of extremely halotolerant microorganisms, especially bacteria in a wide range of these saline environments.

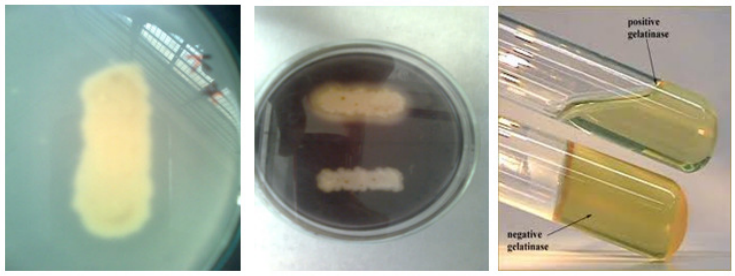
Phenotypic analysis

After several dilutions and repeated sub culturing process, isolation of extreme halophilic bacteria was carried out by using the halobacterium medium. Luxuriant growth was observed at pH 7 and temperature of 30^0C for 48 hours.

The phenotypic characteristics of all isolates studied were determined and compared to phenotypic data of known organisms described in the Bergey's manual of systemic bacteriology (Claus and Barkeley*et al*., 1986). The isolate is Gram positive, strictly aerobic, ferments several carbohydrates and has motile, coccoid shaped cells and non sporing, catalase and oxidase positive, that grew in presence of 2-25% (w/v) NaCl and at pH 6.5-11, with optimum growth at 10%(w/v) NaCl, with an optimum growth temperature of 34^0C. Isolated bacteria showed close resemblance to the genera *Salinicoccus* in regards to physiological, biochemical and genotypic characteristics, with pink pigmentation, three species are known for the genus, *S. roseus* (Ventosa et al., 1990), *S. hispanicus* (Ventosa A. *et al*.,1992) and *Salinicoccus alkaliphilus* (Zhang W *et al*., 2002).

Extracellular enzyme production, genotypic and phylogenetic analysis

The isolated strain JAS4 has got potential produce many extracellular enzymes such as amylase, protease, gelatinase and Inulinase, it was confirmed by the repeated plate assay methods. Further, standardization of various parameters such as pH, temperature and salinity is to be carried out for the bacteria selecting most commercial usage of enzymes.



1 2 3

Fig1,2,3. Showing the hydrolysis of casein, starch and gelatin by the halophilic bacterium *Salinicoccus sp.* strain JAS4

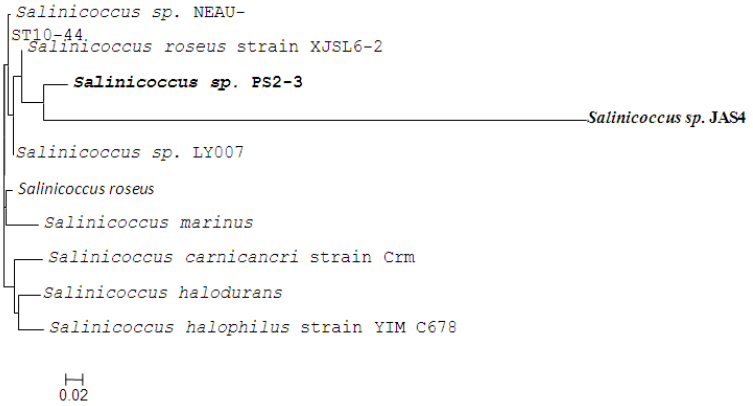


Fig 4. Showing the phylogenetic relationship among the Salinicoccus sp., tree is constructed by using MEGA5 software

Table1. Showing the characteristic features of *Salinicoccus sp.* JAS4

Sl. No	Characteristics features	Results
1	Colony pigmentation	Pinkish
2	Gram's stain	+ve, cocci
3	Motility	Motile
4	Spore stain	Non-sporing
5	Catalase	+
6	Oxidase	+
7	Citrate utilization	-
8	Esculin hydrolysis	-
9	D-arabinose	-
10	Malonate utilization	+

The 16SrRNAgene sequence was determined by direct sequencing of PCR product. Thus obtained 16S rRNA gene sequence was applied for the BLASTN analysis that almost confirms that the bacteria belong to the genera *Salinicoccus* with the maximum identity of just 98%.

Phylogenetic analysis was performed using the software packages PHYLIP (Felsenstein J, 1993) and MEGA version 4 (Kumar S., *et al., 2004*) after obtaining multiple alignment data available from databases by CLUSTAL_X (Thomson *et al*., 1997). Pairwise evolutionary distances were computed using the correction method (Jukes T.H and Cantor C.R., 1969) and clustering was performed using the neighbor-joining method (Saitou N and Nei M., 1987). Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 1,000 resampling (Felsenstein J, 1985).

Lastly the isolation and screening of the Salinicoccus sp.JAS4 reveals the significant impact in producing the extracellular enzymes such as protease, amylase, inulinase and gelatinase in broad range of salt concentration which could have wide range of industrial application and the isolate showing just 98% sequence similarity with respect to the NCBI BLAST sequence suggests that the strain may be belonging to the novel taxon, further it needs to be proved by performing polyphasic analysis.

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