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Molecular characterization and screening of halophiles for the production of biopolymers

[1] Shifa A.G Jamadar, [2] Zeba A.S Shaikh, *[3] Vinod P.S, [4] M.B. Sulochana

[1, 2, 3] Walchand Centre for Biotechnology, Solapur, Maharashtra, India.

[4] Dept. of Biotechnology, Gulbarga University, Kalaburagi, Karnataka, India

Abstract
Biopolymers are polymers, generated from renewable natural sources, are often biodegradable and not toxic to produce. They can be produced by microorganisms, plants and animals. Halophiles are organisms that prefer to live in habitats "cum grano salis" (with a grain of salt), like saline and hypersaline environments with sodium chloride concentrations upto saturation. The aim of the present work includes isolation of halophiles from saline soil and water samples obtained from coastal regions of Karnataka like Karwar, using halophilic medium with 10% NaCl. Out of 10 strains isolated, strains VSZ1 and VSZ2 were screened for the production of biopolymers like biosurfactants and extracellular hydrolytic enzymes. The 16S rRNA gene sequencing, molecular characterization and identification of strains was carried out and their evolutionary relationship was studied by phylogenetics. These biopolymers from halophiles are gaining tremendous importance because of their high stability in extreme salt conditions, with wide industrial and pharmacological applications.

Keywords: Biopolymer, Halophiles, Biosurfactants, Extracellular hydrolytic enzymes, 16S rRNA

1. Introduction

Halophiles are salt-loving organisms that inhabit hypersaline environments. They include mainly prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts. Halophiles may be classified according to the degree of their salt requirement: slight halophiles grow optimally at 0.2–0.85M (1–5%) sodium chloride (NaCl); moderate halophiles grow optimally at 0.85–3.4M (5–20%) NaCl; and extreme halophiles grow optimally at 3.4–5.1M (20–30%) NaCl. In contrast, non- halophiles grow optimally in less than 0.2M NaCl concentrations [1, 2]. Halophiles produce a large variety of stable and unique biopolymers which have diversified biological activities and hence are emerging with greater and novel industrial and therapeutic applications. Their novel characteristics and capacity for large scale culturing make halophiles potentially valuable for biotechnology [3]. It should be pointed out that the salt requirement and tolerance of many species vary according to growth conditions such as temperature and medium composition [4]. Halophiles have been used in several common fermentation processes [5]. They have also been utilized in some new industrial processes like the production of bioactive compounds such as beta-carotene and ectoine [6, 7]. Similarly they have been reported for the production of biorhodopsin for optical computing, biosurfactants for enhanced biodegradability, exopolysaccharides for efficient oil recovery, food additives and compatible solutes working as stress protectants [8, 9].The halophiles have emerged as a vast repository of novel enzymes in recent years. Enzymes derived from halophiles are endowed with unique structural features and catalytic power to sustain the metabolic and physiological processes under high salt conditions. Some of these enzymes have been reported to be active and stable under more than one extreme condition [10, 11, 12]. Research using halophiles in the area of biosurfactants has expanded in recent years due to its potential use in different areas, such as the food industry, agriculture, pharmaceuticals, oil industry, petro chemistry, and paper and pulp industry [13].

2. Materials and Methods

2.1 Isolation and culturing of halophiles

The halophiles were isolated from soil and water samples collected from coastal area of Karnataka i.e. Karwar. The isolated halophilic strains VSZ1 and VSZ2 were used in the present investigation. The isolated strains were revived and sub-cultured on halophilic medium containing 10% sodium chloride, pH 7.0±0.2. The inoculated plates were incubated at 37±2 °C for 24 to 48 hours.

2.2 Screening of strains for the production of Biopolymers

Screening of Biosurfactants

The halophilic strains VSZ1 and VSZ2 were grown aerobically in Erlenmeyer flask with 50ml of mineral salt medium containing (g/L) 1.0g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 0.05g $FeSO_4.7H_2O$, 0.1g $CaCl_2.2H_2O$, 0.001g $Na_2MoO_4.2H_2O$, 30g NaCl and crude oil (1.0%, w/v). Flasks containing sterilized mineral salt medium were inoculated with a loopful of bacterial culture. The culture flasks were incubated for 7 days at 37 ºC [14]. The potential biosurfactant producer was screened by oil displacement method which was the most convenient to perform. In oil displacement method 30ml of distilled water was taken in a two separate petridishes. 1 ml of used crude oil was added to the centre of each dish containing distilled water. 20µl of culture supernatant of each strain was added at the centre of their respective plates and observed for oil displacement [15].

Screening of Strains for Extracellular Hydrolytic Enzymes

The two isolated strains VSZ1 and VSZ2 were screened for

Their capacity to secrete extracellular hydrolytic enzymes as follows.

Extracellular Gelatinase Activity

The ingredients of Fraizer's gelatin agar medium is weighed and dissolved in 100ml of distilled water in 250ml of conical flask by shaking and swirling. pH is adjusted to 7.2 and autoclaved at 121 ºC for 15mins. The inoculated plates were incubated at 37 ºC for 2 days. The plates were then flooded with solution of mercuric chloride ($HgCl_2$) to observe the clear zone of hydrolysis.

Extracellular Protease Activity

Proteolytic activity of the isolates was screened in skim milk agar containing 10% (w/v) skim milk, 2% (w/v) agar, supplemented with 10% and 20% (w/v) NaCl for determining the hydrolytic activity of moderate halophiles. Clear zone of hydrolysis was observed after 7 days of incubation indicating positive for the proteolytic activity [16].

Extracellular Amylase Activity

The presence of amylolytic activity on plates was determined qualitatively following the method described by Amoozegar *et al.* [17], using starch agar medium containing 10% or 20% (w/v) NaCl. After incubation at 30 °C for 7 days, the plates were flooded with 0.3% I2 or 0.6% KI solution, a clear zone was formed around the colonies indicated the hydrolysis of starch.

Extracellular DNase Activity

The DNase test agar medium containing 10% (w/v) total salt and 2g DNA powder was used for the isolation of strains with DNase activity. After incubation for 2 days, the plates were flooded with 1 N HCl solution and clear halos around the colonies showed the presence of DNase activity [18].

2.3 Molecular characterization of strains

16S rRNA gene sequencing

Genomic DNA of VSZ1 and VSZ2 strains was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel). The quality of the DNA isolated was checked using agarose gel electrophoresis. PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer, 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward and reverse primers as shown in Table. 1 and FTA disc as template.

Table 1: Showing details of primers for 16SrRNA sequencing

Target	Primer Name	Direction	Sequence (5' → 3')
16S rRNA	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The PCR products were checked in 1.2% agarose gels prepared in 0. 5X TBE buffer containing ethidium bromide. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37 ºC for 15 minutes followed by enzyme inactivation at 80 ºC for 15 minutes. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystemss, USA) [19]. The cleaned up air dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6 [20].

Characterization of strains

The characterization and identification of two strains was carried out by using the 16S rRNA sequences. The individual sequences were subjected to similarity search and comparison in NCBI database using the similarity search tool BLASTn. The identification of strains was based on the maximum similarity hits obtained from BLAST.

Submission of sequences to NCBI

The 16S rRNA sequences of halophilic strains VSZ1 and VSZ2 were submitted to GenBank nucleotide database of NCBI. Both the sequences were accepted and the accession numbers for each sequence was obtained separately.

Phylogenetic analysis of halophilic strains

The phylogenetic analysis evolutionary relationship of both the strains with the other major halophilic strains was determined by using bioinformatics tool Omega, with respect to their 16S rRNA sequences. The relatedness between the species was calculated by evolutionary distance and represented in the form of phylogenetic tree.

3. Results and Discussion

Hypersaline environments, with salinity ranges at or near saturation are extreme environments; yet, they often maintain remarkably high microbial cell densities and are biologically very productive ecosystems [21]. Bacteria have developed various strategies to maintain cell structure and function to adapt themselves for saline condition. Studies of such bacteria are of great importance, as these produce compounds of industrial interest, such as extracellular, hydrolytic enzymes that have diverse potential usage in biomedical science and chemical industries [9, 22, 21].

3.1 Isolation and culturing of halophiles

The two isolated halophilic strains VSZ1 and VSZ2 were sub cultured and revived on the halophilic agar medium containing 10% of NaCl. After 24 to 48 hours of incubation distinct colonies appeared as shown in Fig. 1 and 2, differing in colony characters and pigmentation [16].

Fig 1: Agar plate showing Colonies of VSZ1 On Halophilic medium containing 10% NaCl

Fig 2: Agar plate Showing Colonies ofVSZ2 On Halophilic medium containing 10% NaCl

3.2 Screening of strains for the production of Biopolymers
Screening of Biosurfactants

Isolated strains VSZ1 and VSZ2 were grown aerobically in Erlenmeyer flask with 50 ml of mineral salt medium which shows turbidity after 24-48 hrs of incubation and observed for all the 7 days as shown in Fig. 3. The oil displacement by biosurfactant from both the isolated strains was screened for all the 7 days. By using oil displacement method, none of these bacterial strains displaced the crude oil and clear zone was not observed.

Fig 3: Mineral salt medium

Screening of strains for extracellular hydrolytic enzymes

The two isolated strains VSZ1 and VSZ2 were screened for extracellular hydrolytic enzymes, such as Gelatinase, Protease, Amylase and DNase. There was no formation of zone of hydrolysis for the enzymes protease, amylase and DNase by both the strains named VSZ1 and VSZ2. The strains showed clear zone of Gelatinase Hydrolysis as shown in Fig. 4. The diameter of the zones of Gelatinase was measured as shown in Table. 2.

Fig 4: Plate showing zone of hydrolysis for Gelatinase

Table 2: Zone diameter of Gelatinase

Strains	Zone Diameter(cm)
VSZ1	2.2
VSZ2	3.8

3.3 Molecular characterization and identification of strains
16S rRNA gene sequencing

The quality of the genomic DNA of both the strains isolated, was checked using agarose gel electrophoresis Fig. 5. The PCR products were checked in 1.2% agarose gels prepared in 0. 5X TBE buffer containing ethidium bromide. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad) Fig. 6. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit [19]. The cleaned up air dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems). The 16S rRNA gene sequences of VSZ1 and VSZ2 strains have respective sequence lengths which are shown in Table. 3.

Table 3: Showing sequence lengths of VSZ1 and VSZ2

SI. No	Organism	Sequence length
1	VSZ1	1272 bp
2	VSZ2	1281 bp

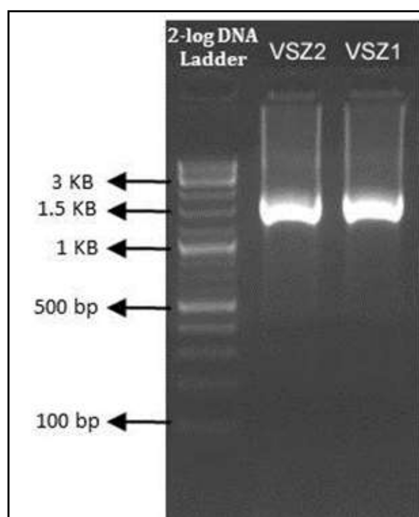

Fig 5: Genomic DNA **Fig 6:** PCR amplicon

Characterization of strains

The molecular characterization and identification of strains was performed by sequence similarity search using NCBI BLASTn tool. The BLAST 16S rRNA sequence analysis for both the strains suggested that strain VSZ1 and VSZ2 shared maximum sequence similarity with *Virgibacillus sp.* (100% similarity). Hence it can be interpreted that the strains VSZ1 and VSZ2 belong to *Virgibacillus sp.* and named as *Virgibacillus* VSZ1 and *Virgibacillus* VSZ2 sp. respectively.

Submission of sequences to NCBI

The 16S rRNA sequences of VSZ1 and VSZ2 strains were deposited in NCBI - GenBank nucleotide database with respective accession numbers as mentioned in Table. 4.

Table 4: GenBank Accession Numbers of VSZ1 and VSZ2

SI. No.	Organism	Code Names	GenBank Accession Number
1	Virgibacillus sp.	VSZ 1	KU248096
2	Virgibacillus sp.	VSZ 2	KU248097

Phylogenetic analysis of halophilic strains

The phylogenetic analysis evolutionary relationship of both the strains with the other major halophilic strains was determined by using bioinformatics tool Omega, with respect to their 16S rRNA sequences. The relatedness between the species was calculated by evolutionary distance and represented in the form of phylogenetic tree as shown in Fig.7.

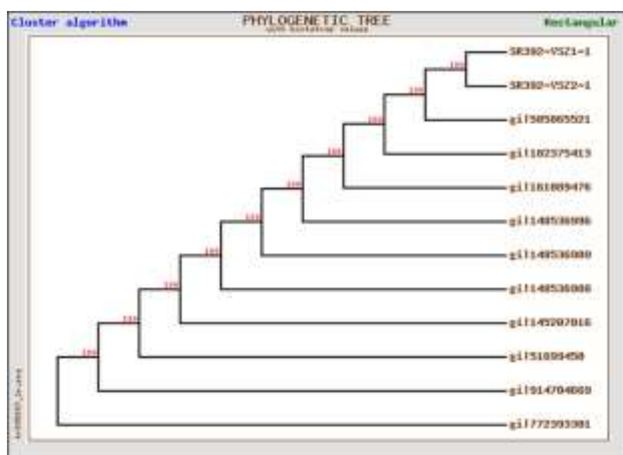

Fig 7: Phylogenetic Tree of VSZ1 and VSZ2

4. Conclusion

Halophiles are salt-loving organisms that inhabit hypersaline environments. They include mainly prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts. The halophilic strains were isolated from coastal regions of southern India like Karwar, Karnataka. Total ten strains were isolated from the saline soil and water samples. Two strains coded as VSZ1 and VSZ2 were used in the present studies. The two halophilic strains VSZ1 and VSZ2 were revived and pure cultured using salt and halophilic medium with optimum physico-chemical parameters. The two halophilic strains VSZ1 and VSZ2 were screened for the production of biosurfactants for seven days using oil displacement method, in which none of the two strain proved potent for biosurfactant production.VSZ1 and VSZ2 strains were also screened for the production of extracellular hydrolytic enzymes using standard protocols for respective enzymes, both the strains proved to be potent for the production of gelatinase enzyme as compared to others. The 16S rRNA sequencing, molecular characterization and identification of two among ten strains were carried out and their evolutionary relationship was studied by phylogenetics. Halophiles produce a large variety of stable and unique biopolymers which have diversified biological activities and hence are emerging with greater and novel industrial and therapeutic applications.

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