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Screening for the Unexplored Potential of Haloalkaliphilic Bacteria, *Oceanobacillusoncorhynchi*

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Abstract

Recent studies have clearly shown that halotolerant and alkalophilic species are good sources of rare biomolecules of industrial interest. A haloalkaliphilic bacterium was isolated from the solar saltpans of the Thoothukudi district located in Tamil Nadu, India. The isolate is a gram-positive, rod-shaped, aerobic bacterium and ferments several carbohydrates. The physiological characterization (optimum pH, temperature and salt) was determined. On the basis of 16S rRNA gene sequence similarity, the isolate was shown to belong to the genus *Oceanobacillus* and species *oncorhynchi*. This is the first article indexing the unknown properties of this unexplored bacterium. To better understand the role of this isolate, several experiments were carried out in this study to investigate its metabolic peculiarities, particularly regarding the production of exopolysaccharides or industrially important enzymes. Overall, the results determined that this isolate could be established for its specific metabolic products that are of biotechnological interest.

Keywords

Oceanobacillusoncorhynchi, Haloalkaliphile, Saltpans, Thoothukudi District

1. Introduction

Several reviews have been focused on various aspects of halophilic and alkalophilic microorganisms. These organisms, because of their adaptability, have always been of biotechnological interest for their vast commercial applications. To our knowledge, few studies have been published on the microbial diversity of Thoothukudi saltpans and certainly, none of them reported on the biotechnological relevance of haloalkaliphiles from these locations. The organism isolated from the salt pans of the Thoothukudi district was identified as *Oceanobacillusoncorhynchi*. The genus *Oceanobacillus* was found by Lu et al. [1] to designate some haloalkaliphilic isolates. Other species include *O. limi* [2], *O. picturae* [3], *O. profundus* [4] etc. In this study, a rare member of the genus *Oceanobacillus* was screened to identify its ability to produce exopolysaccharides, biosurfactants and enzymes of industrial interest. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the genus *Oceanobacillus* was most closely related to *Oceanobacillusoncorhynchi*subsp. incaldanensis. During our study, we spotted the isolate *O. oncorhynchi*served as a good reservoir of biomolecules, that can be utilized in environmental cleanup procedures. The isolate was found to degrade petroleum compounds and synthetic dyes, emulsify hydrocarbons and carry out biosorption of metals. No such studies have been conducted on this haloalkalophilcbacteria.

2. Materials and Methods

2.1. Isolation of Halophilic Bacteria from the Saline Soil Sample

In this study, saline soil samples were collected from solar salt pans of Thoothukudi district, Tamil Nadu,

India. The collected soil sample was placed in a sterile container and immediately transported to the laboratory for further analysis. The sample was serially diluted in the range of 10^{-1} to 10^{-7} in a series of test tube. The samples were spread plated in the Mineral Salt (MM63) medium with 6g/100 ml concentration of NaCl. The pH of the medium was adjusted to 8–9. The plates were incubated at 30°C and the colonial appearance was examined after incubation for 3-7 days.

2.2. Gram Staining

Gram staining was performed by using air-dried slides which were fixed and simultaneously desalted in 2% acetic acid for 5 minutes, dried before staining by standard procedures [5]. Thin smear of bacterial colony was prepared on a clean slide. Slide was fixed by passing through flame. The smear was covered with crystal violet for 30 sec, then rinsed with water and drained. Next, the smear was covered with iodine for 30 sec, then with rinsed water. Decolorized with ethanol 95% and washed with water, then it was counter stained about 30 sec with safranin. Blot slide was dried and examined under oil immersion.

2.3. Physiological Characterization

2.3.1. Temperature Optimum

All the isolates were tested for the temperature tolerance by subjecting them to different temperature (30°C, 37°C, 40°C, 45°C, 50°C, 55°C & 60°C) in nutrient broth and subsequently measuring their optical density [6]. Salt concentration was maintained at 15% and pH at 8.0.

2.3.2. pH Optimum

Nutrient broth was prepared at different pH range (1, 3, 5, 7, 10 & 14) to detect the tolerance level of the strains and subsequently measuring their optical density at each pH [6]. Salt concentration was maintained at 15% and temperature at 37°C.

2.3.3. Salt Concentration Optimum

The isolates were screened for their salt tolerance level by growing them on to nutrient broth tubes with concentrations of salt (NaCl) ranging from 0.5%, 1%, 2%, 3%, 5% & 10%, and subsequently measuring their optical density [6]. pH was maintained at 8.0 and temperature at 37°C.

2.4. Biochemical Testing

The following biochemical tests were carried out in the laboratory using standard procedures. For the indole test, the isolate was grown in medium containing peptone water. Starch (5%), casein (5%), lipid (5%), and gelatine (12%) hydrolysis and oxidase, urease, and catalase activities [7] were tested in minimal (MM63) medium. Anaerobic growth in the presence of DMSO was determined by inoculating the isolate onto agar plates in the presence of DMSO (10 g/L) and incubating the plates for 1–2 weeks. Cultures were incubated under anaerobic conditions at 37°C. Growth on single carbon sources was tested in a liquid medium containing the following: D-glucose, D-fructose, D-xylose and sucrose. For nitrate reduction, minimal (MM63) medium plus 0.1% KNO_3 was employed. After incubation, alpha-naphthylamine and sulphanilic acid were added, and the change in colour in the tubes was observed. Results were verified by adding zinc powder. A positive result was indicated by the appearance of a reddish brown colour in the test tubes. Extraction of intracellular solutes and their quantification was performed by Motta et al. [8].

2.5. Molecular Identification

2.5.1. 16S rRNA Gene Sequencing

The haloalkaliphilic isolate was identified based on 16S rRNA gene sequencing. Briefly, the gDNA was extracted that served as a template for the 16S rRNA gene amplification using universal forward primer (27F) (5'-AGAGTTTGATCMTGGCTCAGTAC-3') and reverse primer (1492R) (5'- GGYTACCTTGTTACGACTT-3') (Biozone (India) Pvt. Ltd.) via standard polymerase chain reaction (PCR) protocol. The amplified gene product (1 Kb) was checked on a 1.5% agarose gel against a 1 Kb DNA ladder. The BLASTn search program was employed to find nucleotide sequence homology.

2.5.2. Molecular Phylogenetic Analysis by Maximum Likelihood Method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [9]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 [10].

2.6. Biodegradation of Crude Oil

The overnight culture at the log phase of growth were transferred to 250 ml flasks, each containing 100 ml of sterile defined mineral salt (MM63) medium with [1% (v/v) and 2.5% (v/v)] two concentrations of crude oil. The flasks were incubated in a shaker at 200 rpm at 30°C for 7 days. The total hydrocarbons in the treatments were determined spectrophotometrically at 420 nm wavelength. Degradation was estimated as the difference between the initial and the final concentrations of total hydrocarbons [11].

2.7. Screening for Biosurfactant Production

2.7.1. Haemolytic Activity

Haemolysis was carried out on blood agar plates (blood agar medium supplemented with 5% human blood). The isolate was streaked in blood agar plates and were incubated at 37°C for 24 hours. Plates were examined for clear zone around the colonies [12].

2.7.2. Drop Collapsing Test

Biosurfactant production was determined using the qualitative drop-collapse test [13]. Mineral oil was added to

96-well microtiter plates and halophilic culture supernatant was added to the surface of the oil in the well and plates were incubated for 1 hr at 37°C. The shape of drop on the oil surface was observed after 1 min. The culture supernatant makes the drop collapsed, it indicates as a positive result and if the drops remain intact, it indicates as a negative result. Distilled water was used as control treatment.

2.7.3. Oil Displacement Assay

10 µl of crude oil is added to the surface of 40 ml of distilled water in a Petri dish to form a thin oil layer. Then, 10 µl of culture or culture supernatant are gently placed on the centre of the oil layer. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed [14].

2.8. Dye Degradation

The commonly used dyes Congo red, crystal violet and safranin were chosen to screen for dye degradation by the halophilic isolate. All the chemicals used were of the highest purity and of analytical grade. In screening, decolorization (%) was measured as a decrease in optical density from day 0 (initial absorbance) today 15 (final absorbance) after the inoculation of the isolate using the spectrophotometer (UV-1800, Shimadzu, Japan). Decolorization was calculated by the following formula [15].

$$\text{Decolorization (\%)} = [(A0 - A1) / A0] \times 100$$

where,

A0: Initial absorbance;

A1: Final absorbance

2.9. Hydrocarbon Degradation Assay

The halophilic isolate was applied to hydrocarbon degradation assays using two classes of hydrocarbons, namely naphthalene and diesel fuel using 2,6-dichlorophenol indophenol (DCPIP) as the indicator [16]. The isolates were grown in fermentation broth containing minimal saltmedium supplemented with glucose (10 g/L) and yeast extract (1.0 g/L) and incubated at 30°C for 72 h with shaking at 120 rpm. After incubation, the culture was centrifuged at 10,000 g for 10 min. The pellet was re-suspended in phosphate buffer and again centrifuged to remove all culture medium residues. The pellet was finally re-suspended in phosphate buffer and the OD adjusted to McFarland 0.5. The assay was carried out by using DCPIP as an indicator in sterile tubes. Each well contained 20 µL of the isolate, 168 µL of minimal salt medium, 12 µL of DCPIP and 1 µL of hydrocarbon (naphthalene and diesel) in turn. The tubes were incubated at 30°C and the readings were taken at 600 OD in a photometer after 24, 48 and 72 hours of incubation, respectively and the percentage of significant reduction was obtained using the following equation:

$$\text{DCPIP reduction (\%)} = (\text{Initial O.D} - \text{Final O.D}) \div \text{Initial O.D} \times 100\%$$

2.10. Heavy Metal Tolerance

Heavy metal resistances of the isolate were determined by agar dilution method [17]. Plates containing 20 ml of one-half strength Mineral Salt medium containing 0.5 g concentration of metal were poured on day of experiment. Metals to be tested were as follows $NiCl_2.6H_2O$, $Pb(CH_3COO)_2.3H_2O$, $CuSO_4.5H_2O$, $CaCl_2$, and $HgCl_2$. Plates were dried at 37°C for 30 min and inoculated with 0.1 ml from exponentially grown cultures. Plates were incubated at 37°C for 2 days and examined for colony growth. Plates containing media with no added metal were inoculated in the same manner to serve as controls.

3. Results and Discussions

3.1. Isolation and Characterization of Haloalkaliphilic Bacteria

The saline soil sample collected was serially diluted and plated on mineral salt (MM63) medium. After incubation for 7 days, colonial growth was observed from all the dilutions 10^{-1}–10^{-7}. Colonies were circular and creamy white. Cells were gram positive, motile and non-sporulating rods. The mesophilic bacterium showed optimum growth at 37 °C but was able to grow at 30–40°C and able to survive at pH 6–9 exhibiting optimum growth at pH 8. Growth occurs at 3%–12% (w/v) NaCl, with an optimum appearing at 10%. Anaerobic growth in the presence of DMSO was observed after 7 days of incubation. The isolate was positive for oxidase, catalase, reduction of nitrate to nitrite, starch, lipase and urease and negative for gelatine, casein and indole production. The isolate could grow on D-glucose, D-fructose, D-xylose and sucrose as carbon sources. It accumulated an osmoprotectantintracellularly called glycine betaine during osmotic stress conditions. A similar study was reported by Yumoto et al. [18], in which a halophilic bacterium was isolated from the rainbow trout, *Oncorhynchusmykiss*.

3.2. 16S rRNA Gene Sequencing and Phylpgenetic Analysis

16S rRNA gene sequence analysis revealed that the isolated haloalkaliphile is a member of the genus *Oceanobacillus*. The closest phylogenetic relative of the isolate was *Oceanobacillusoncorhynchi*, with a sequence similarity of 92%. The phylogenetic tree with the highest log likelihood (–1519.30) based on the 16S rRNA gene is shown (Figure 1). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. There were 515 positions in total in the final dataset. The tree also shows the relationship between the isolate *O. oncorhynchi* and the type strains *O. oncorhynchi* subsp. *incaldanensis*and *O. aidingensis*. Phylogenetic analysis of the 16S rRNA gene sequence obtained from the

isolate of *O. oncorhychi* showed a close affiliation with the obligate aerobe haloalkaliphilic *Oceanobacillusoncorhychi* subsp. *oncorhynchi isolated* from an algal mat collected from a sulphurous spring in Italy of which the bioremediation abilities have not been demonstrated.

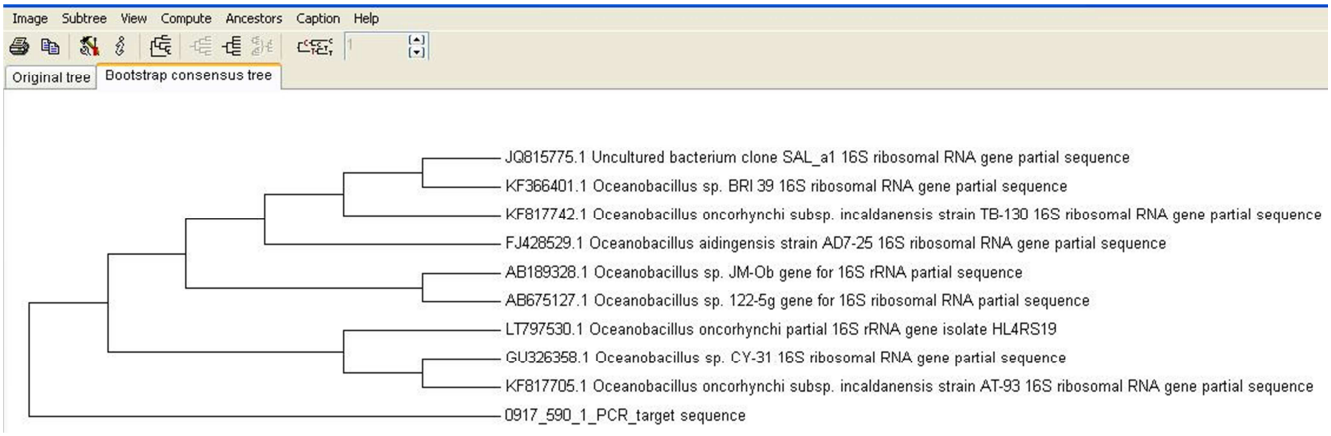

Figure 1. *Molecular Phylogenetic Analysis by Maximum Likelihood Method.*

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-1519.30) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 515 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

3.3. Biodegradation of Crude Oil

In this study, the isolate was tested for the efficiency of crude oil degradation at different oil concentrations on mineral salt (MM63) medium. The results showed that at a 1% (v/v) crude oil concentration, the isolate could exhibit a maximum of 64% degradation after incubation for 7 days, whereas at a 2.5% (v/v) crude oil concentration, the isolate showed 52% degradation after 7 days' incubation.

3.4. Screening for Biosurfactant Production

Biosurfactants are non-toxic, biodegradable compounds produced by bacterial strains that can degrade petroleum hydrocarbon. The haloalkaliphilic isolate was screened for biosurfactant production. Positive results of haemolytic activity (clear zone around the colonies) (Figure 2), the drop collapsing test and the oil displacement test (Figure 3) demonstrated that the isolate was a biosurfactant producer that can use petroleum oil as a carbon and energy sources.

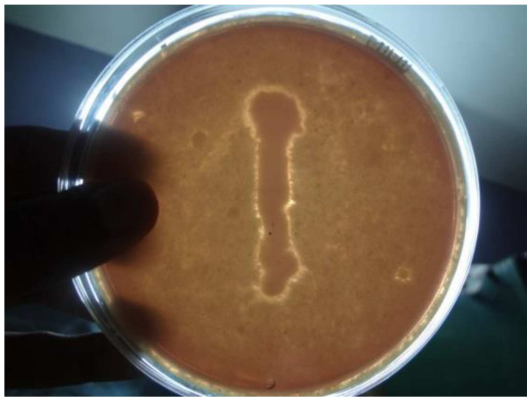

Figure 2. *Haemolytic activity.*

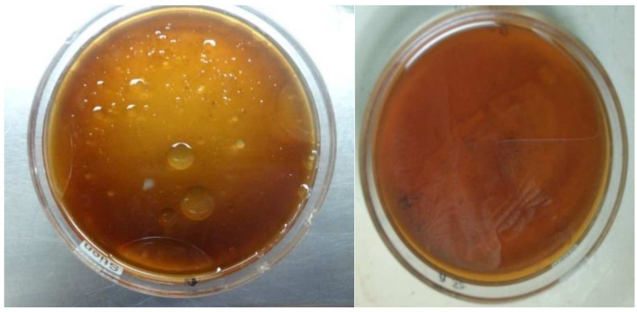

Figure 3. *Oil displacement test.*

3.5. Dye Degradation

The isolate was analysed for its ability to degrade dyes (Congo red, crystal violet and safranin) by decolorization. The decolorization percentage was calculated by measuring the decrease in optical density using the spectrophotometer. The isolate was able to degrade crystal violet (18%) more effectively than Congo red (17%) and safranin (11%) (Figure 4). Biological methods being cheap and simple to use are resorted to as the proposed solution. The ability of microorganisms to carry out dye decolorization has received

much attention and is seen as a cost-effective method for removing these pollutants from the environment.

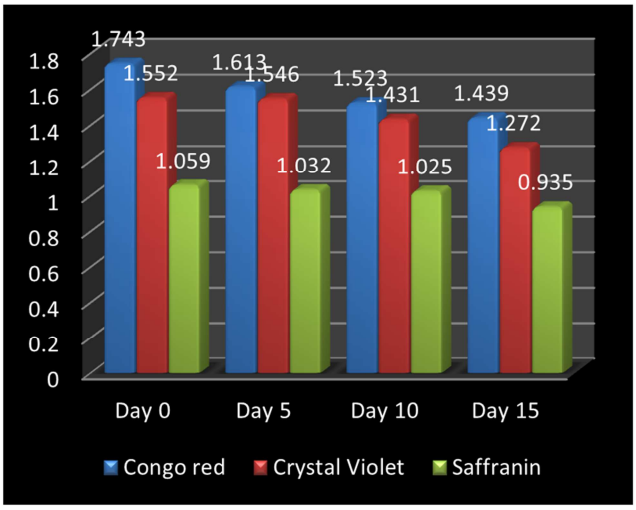

Figure 4. *Degradation of dye by O. oncorhynchi.*

3.6. Hydrocarbon Degradation Assay

The potential of the isolate to degrade hydrocarbons (diesel fuel and naphthalene) was profiled in this study using DCPIP as the indicator. This DCPIP is said to be an electron acceptor that becomes reduced (decolorized) when redox reactions occur during microbial degradation of hydrocarbons (Figure 5 & 6). This significant reduction reaction is due to the conversion of NADH to NAD^+ during microbial metabolism of poly aromatic hydrocarbons under hypersaline conditions. The isolate showed a higher hydrocarbon degrading activity toward naphthalene (74% DCPIP reduction) compared with diesel fuel (60% DCPIP reduction). Bio-based remediation approaches using metal-resistant microbes, however, have recently received global acceptance as alternative, cost-effective and eco-friendly remediation processes.

Figure 5. *Hydrocabon Degradation using DCPIP indicator.*

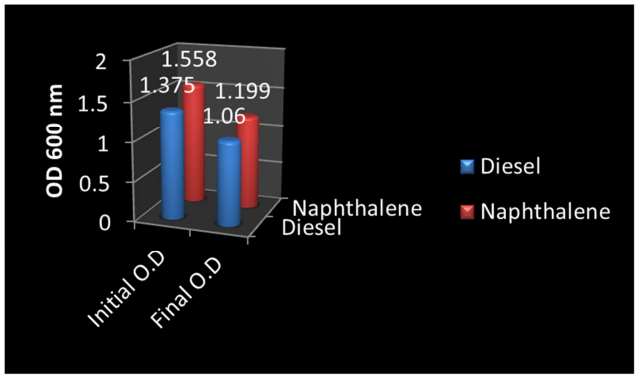

Figure 6. *Hydrocarbon Degradation by O. oncorhynchi in Spectrophotometer analysis.*

3.7. Heavy Metal Tolerance

The growth of the isolate in medium inoculated with metals showed increased resistance towards the particular heavy metal ions. The isolate was able to tolerate and grow in calcium chloride, copper sulphate, nickel sulphate and lead acetate but not in mercuric chloride (Table 1). The heavy metal resistivity was due to the biosorption of metal ions inside the cells of the halophilic isolate.

Table 1. *Determination of Heavy Metal tolerance of O. oncorhynchi.*

Heavy Metals	Resistance	Sensitive
$NiCl_2.6H_2O$	+	-
$Pb(CH_3COO)_2.3H_2O$	+	-
$CuSO_4.5H_2O$	+	-
$CaCl_2$	+	-
$HgCl_2$	-	+

4. Conclusion

The present study shows moderate salt-tolerance, multiple heavy metal resistivity and antibiotic sensitivity of the halobacterial isolate *Oceanobacillus* sp. However, a long-term analysis on this aspect is required to unravel the potential of this haloalkaliphilic isolate as an agent of bioremediation and to understand the detailed mechanism of heavy metal biotransformation. Finally, this study introduces an excellent bacterial strain with promising roles in oil pollution remediation, metal recycling and dye degradation.

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