Isolation and Characterization of Haloarchaeal Strain from Puthalam Salt Pan located in the Southern Peninsular Coast of India

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Abstract

Halophiles are salt loving organisms that inhabit hypersaline environments. They possess the potential to provide significant opportunities for pharmacology. Moreover, by the concentration of seawater in arid environments, hypersaline environments may easily be created. These facts, along with the presence of novel and stable biomolecules in halophilic bacteria and Archaea, suggest that these microorganisms will prove even more treasured in coming. In the present investigation, water samples were collected from three different sites of Puthalam salt pan. The samples were aseptically transported to the laboratory and subjected to serial dilution using sea water. Among that $10^{-6}$ dilution was taken for the study. 3 different coloured colonies were observed on Zobell marine agar plates, incubated at $37^\circ$C for 12 days whereas red coloured colony was taken for further study such as biochemical characterization, pH and temperature optima, halophilicity, growth in the presence of various carbon, $N_2$ and inorganic sources and various organic solvents. The observed results indicated that the strain is a red coloured, motile, gram negative rod with evenly spreaded colonies. It shows positive results with catalase, oxidase, gelatin liquefaction, starch hydrolysis, casein production, glucose, sucrose, dextrose and mannitol tests. The isolate shows wide range of carbon, $N_2$ and inorganic sources as well as organic solvents for its growth. The isolate was identified as *Halomonas utahensis* by 16S rRNA sequencing. The nucleotide sequence was submitted to Gen Bank and assigned the accession number KY986725.

Keywords: Zobell marine agar; Hypersaline; Halophilicity; Halophiles

Introduction

An extremophile is an organism that thrives in physiologically or geochemically extreme conditions that are detrimental to most life on Earth [1]. Most known halophiles are relatively easy to grow, and genera such as *Halobacterium*, *Halofex*, and *Haloarcula* have become popular models for studies of the archael domain as they are much simpler to handle than methanogenic and hyperthermophilic Archaea. **Haloarchaea** belongs to order halobacteriales and family halobacteriaceae are dominant microorganisms requiring hypersaline environment for their growth. A wide variety of media are used for the cultivation of halophiles and halotolerant bacteria. The media for extreme halophiles have elevated levels of magnesium and the source of water used for preparing hypersaline media varies [2]. Defined media for the extreme halophile, *Halosimplex*, has broad application [3]. Filtered sea water or salt plains brine were effective bases for growing halotolerant *Cyanobacteria* and algae [4]. Many halophilic and halotolerant microbes isolated to date are neutrophils, growing best in media with pH ranging from 6.8 to 7.5 [5]. Alkaliphilic organisms are grown at the pH range of 8-10 [6]. There is a need and demand for more industrially useful products to the existing ones, so it is important to explore this new habitat which has been particularly abandoned until date [7]. Therefore, in this era researchers are turning their attention towards this less explored and useful kingdom. The southern coast of Kanyakumari harbors a variety of ecosystems for such studies; some of the areas have been already excavated while some are underway. Hence the present investigation deals the isolation, optimization and characterization of haloarchaeal strain from water samples of Puthalam salt pan.

Materials and Methods

Collection of samples

Water samples were collected from Puthalam saltpan at 3 different sites. The collected samples were transferred to sterile polythene bags to prevent direct contact with air and are transported to the laboratory in an ice box for further examination. Physico-chemical characterization of water samples were analyzed according to Abbasi, [8].

Isolation and screening of Haloarchaeae

The water samples were serially diluted by taking 1 mL from each sample individually and a total of ten dilutions ($10^{-1}$ to $10^{-10}$) were made by sea water. The $10^{-4}$ dilution is used for plating on Zobell marine agar plates by spread plate method. The plates were incubated at $37^\circ$C for 12 days [9].

Biochemical characterization

The culture characterization was identified according to Kamekura et al. [10].

Growth characteristics of isolated strain

In order to find out growth potential, the broth culture was supplemented with NaCl of different concentrations (g/dL) ranging from 25% to 30%, different temperatures of $37^\circ$C to $43^\circ$C and various pH ranges from 8.0 to 9.2. The broth cultures were incubated at $37^\circ$C for 12 days. Growth rate was assessed by recording O.D values at 540 nm. From the O.D values average mean and S.D were calculated.

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Determination of growth curve and generation time

The generation time of isolated strain was determined by inoculating the isolate in marine broth at 0 to 12 days at 37°C and kept in rotary shaker at 120 rpm. The growth rate was assessed by recording OD values at 540 nm.

Growth in various organic and inorganic sources

To find out the efficacy of organic and inorganic sources, the isolated strain was inoculated on Zobell marine agar plates supplemented with various carbon sources such as glucose, fructose, lactose, starch, maltose, galactose and sucrose and N\textsubscript{2} sources such as tryptophan, tyrosine, arginine, cysteine, methionine, histidine, glycine and proline in a concentration of 0.1 gm/dL (w/v) as well as inorganic sources such as BaCl\textsubscript{2}, Lead acetate, MnCl\textsubscript{2}, Mercuric chloride, Nickel sulphate, CaCl\textsubscript{2}, FeSO\textsubscript{4}, ZnSO\textsubscript{4}, Potassium thiosulphate, CuSO\textsubscript{4}, Ferrous ammonium sulphate, Sodium sulphate, Lithium carbonate, MgSO\textsubscript{4} and KCl at a concentration of 0.5 gm (w/v) were used. The plates were incubated at 37°C for 12 days. Broth cultures were also prepared with the above sources (except inorganic sources) in different concentrations (glucose: 1-10%, fructose: 1-6%, lactose: 1-5%, starch: 1-11%, maltose: 1-5%, galactose: 1-7% and sucrose: 1-6%, tryptophan: 0.5-2.0%, tyrosine: 0.5-2%, arginine: 0.2-1.0%, cysteine: 0.5-2.0%, histidine: 0.2-1.2% and glycine: 0.2-1.0%) along with Zobell marine broth. The growth rate of the strain was obtained by recording OD values at 540 nm. Moreover average mean and S.D were calculated.

Growth in various organic solvents

In order to determine the efficacy of organic solvent, the isolated strain was inoculated on Zobell marine agar plates and broth supplemented with different concentrations of organic solvents such as, methanol (1-5% (v/v)), ethanol (1-7% (v/v)), chloroform (1-6% (v/v)) and diethyl ether (1-7% (v/v)). The results were recorded by calculating average mean and S.D from OD values at 540 nm.

Antimicrobial analysis

The supernatant liquid collected from the broth was subjected to antimicrobial activity against E. coli, Staphylococcus aureus, Klebsiella sp., Pseudomonas aeruginosa, Enterobacter sp., and Proteus vulgaris by standard paper disc assay method [11].

### 16S rRNA sequencing

Identification of the isolate was confirmed by 16S rRNA sequencing. Genomic DNA of isolate was extracted directly from the colonies grown on solid medium and was used as template for PCR. A single isolated colony was suspended in 10 μL of distilled water and heated in a boiling water bath for 2-3 min. The content was centrifuged at 10,000 xg for 3 min and the supernatant was used as DNA template for PCR system. 16S rRNA gene of isolate was amplified using archael primers. Forward primer was designed in the laboratory according to Gupta et al. [12] and reverse primer was referred from Xuet et al. [13]. Phylogenetic tree was constructed from evolutionary distances with the help of neighbour-joining method of MEGA 6 program package [14]. The 16S rRNA sequences of the isolated bacterium was submitted to NCBI Gene Bank Database.

### Results and Discussion

**Physico-chemical analysis**

The physico-chemical characteristics of water samples are given in Table 1. The average temperature of the sampling sites was 27°C at morning and 32°C at noon. Colour of the water collected from three sites were observed as reddish pink in site 1, Light pink in site 2 and white in site 3. Dissolved oxygen content was found to be decreased (2 mg/L) in water collected from site 3 and was more (9.02 mg/mL) in site 1. Free CO\textsubscript{2} content was found to be 278 ppm at site 1 and 220 ppm at site 2 respectively. The water sample collected nearby the saltpan had a low free CO\textsubscript{2} of about 120 ppm. Carbonate content of the water sample from site 3 was found to be 10 ppm. The bicarbonate content was found to be 60 ppm, 56 ppm and 54 ppm in site 1, site 2 and site 3 respectively. The present study describes that chloride content of the water sample collected from site 3 was high (24.82 mg/L), compared to site 1 and 2. When compared to other water sampling sites, the salinity of the water sample present along seawater (site 1) was found to be 40.78 ppm, which was higher than the other samples. The water sample in site 1 had hardness of about 220 mg/L and it is due to the presence of above said minerals. The conductivity of the water sample nearby the collected salt was found to be 0.08 million/mho. Magnesium content was found to be more (1.5 g/Kg) in site 1 comparing to other sites. Nitrogen content of water samples was maximum (32 ppm) at site 1, whereas iron content was more at site 2 and site 3 (4.6 and 5 ppm). Copper content was more.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Sample site 1</th>
<th>Sample site 2</th>
<th>Sample site 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>Reddish pink</td>
<td>Light pink</td>
<td>White</td>
</tr>
<tr>
<td>Dissolved oxygen (mg/mL)</td>
<td>9.02</td>
<td>3.54</td>
<td>2</td>
</tr>
<tr>
<td>Free CO\textsubscript{2} (ppm)</td>
<td>278</td>
<td>220</td>
<td>120</td>
</tr>
<tr>
<td>Carbonate (ppm)</td>
<td>10</td>
<td>24</td>
<td>10</td>
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<tr>
<td>Bicarbonate (ppm)</td>
<td>60</td>
<td>56</td>
<td>54</td>
</tr>
<tr>
<td>Chloride (mg/L)</td>
<td>24.82</td>
<td>20.28</td>
<td>14.50</td>
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<tr>
<td>Salinity (ppm)</td>
<td>40.78</td>
<td>36.63</td>
<td>26.22</td>
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<tr>
<td>Hardness (mg/L)</td>
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<td>100</td>
<td>800</td>
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<tr>
<td>Electrical conductivity (ohm)</td>
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<td>0.03</td>
<td>0.04</td>
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<tr>
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<td>8</td>
<td>8.3</td>
</tr>
<tr>
<td>Magnesium (g/L)</td>
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<td>1.05</td>
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<tr>
<td>Nitrogen (ppm)</td>
<td>32</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>Potassium (ppm)</td>
<td>125</td>
<td>118</td>
<td>110</td>
</tr>
</tbody>
</table>

**Table 1:** Physicochemical characteristics of solar saltern located at Puthalam near Kanyakumari coast of India.
at site 1 (1.0 ppm) and zinc was found to be more (0.924 ppm) at site 3. Calcium content was high at site 2 (4.3 ppm) compared to other sites.

**Isolation and screening**

Different shades of red, yellow and white coloured colonies were seen (Figure 1). The observed colonies were sub cultured individually on Zobell marine agar plates. In the present inquest red coloured colonies were used for further analysis. The study based on extremely halophilic Archaea are capable of producing red pigments known as carotenoids which gives red, yellow and orange colour to the organism and also to compact the high salt and intense UV radiation [15]. It was shown that these colored pigments have strong antioxidant, immune boosting activities and protecting premature ageing [16]. It was shown that these colored pigments have strong antioxidant, immune boosting activities and protecting premature ageing [16]. Since carotenoids of extremely halophilic Archaea are widely applied in pharmaceutical and medical fields such as antitumour and heart disease prevention agents [17] (Figure 1).

**Biochemical characterization of isolate**

The isolated pure culture was subjected to various biochemical characterizations and the results were presented in Table 2 [10]. The results indicate that the strain is red coloured, Gram negative rod with evenly spreaded colonies. It shows motility and positive reaction in catalase, oxidase, gelatin liquefaction, starch hydrolysate, casein production, glucose, sucrose, dextrose, arabinose and mannitol tests. Positive catalase test indicated that some bacteria contain flavoprotein that reduce O₂, resulting in the production of H₂O₂ or superoxide. These are extremely toxic because they are powerful oxidizing agents and destroy cellular constituents very rapidly. A bacterium must be able to protect itself against such O₂ products or it will be killed. Many bacteria possess enzymes that afford protection against toxic O₂ products. Add few drops of H₂O₂ on bacterial colony bubbles of O₂ represent a positive catalase test. Methyl red test showed positive by isolated strain which indicates the bacterium undergoes carbohydrate metabolism leading to the formation of acidic end products like organic acid [18].

**Growth characteristics of isolated strain**

The results clearly indicated that the strain exhibited significant growth at 29% (w/v) (0.95 ± 0.48), whereas delayed growth response was observed above 29% NaCl, as represented in Figure 2. This is because halophiles show more or less wide ranges of growth at different salinities, but experience optimum growth at salinity significantly higher than sea water [19]. Studies on halophilic microorganisms isolated from ponds of China and Karak region showed their optimum growth at the temperature of 35°C to 40°C [20]. Generally haloarchaea grow best at 37°C and others grow below 35°C or higher at 40°C [21] and optimum growth temperature of 50°C have also been reported [22]. In the present investigation the isolated strain shows optimum growth at 42°C, over that growth was found to be decreased drastically (Figure 3). This might be due to denaturation of proteins responsible for growth at higher temperature.

The strain attained well established growth at pH 8.8 (0.70 ± 0.18),
beyond that the growth rate was found to be decreased substantially as shown in Figure 4. Alkaliphilic organisms are grown well at pH of 8-10 using Na₂CO₃ to maintain the pH [23]. Mohsinet et al. [24] reported that Halophilic bacterial species grow best at pH 7-8. A novel extreme alkaliphilic isolate named as SAGMI was grown in medium containing optimum pH of 10 [25].

**Growth curve and generation time**

During the growth curve determination, the lag phase occurred in between 1st and 2nd day of inoculation. The onset of log phase was occurred between 3rd and 6th day of inoculation, whereas onset of stationary phase occurred on 6th day up to 9th day and the growth was declined after 9th day as depicted in Figure 5.

**Growth in various carbon sources**

The obtained results indicated that high growth rate was occurred in medium containing 10% starch (0.98 ± 0.27), followed by 5% sucrose (0.79 ± 0.17), 9% glucose (0.78 ± 0.24), 6% galactose (0.76 ± 0.13), 4% lactose (0.74 ± 0.13), 5% fructose (0.74 ± 0.18) and 4% maltose (0.68 ± 0.14) respectively. The biomass production increased enormously in medium containing starch and sucrose compared to other sources and control (0.32 ± 0.14), as represented in Figure 6.

It was earlier thought that all extremely haloarchaea required a number of amino acids and no carbohydrates for their growth [19]. Halotolerant bacteria have wider metabolic activities so they use many carbon sources for growth [26]. Caton et al. [5] suggested that glucose is the most common carbohydrate added to complex hypersaline media for the growth of haloarchaeal species. More over the strain of present work explains that optimum growth of the isolate was reached on 6th day of inoculation beyond that the growth was found to be decreased drastically. This is because halobacteria were considered organisms of very slow growth able to utilize only a stricted range of organic compounds mostly amino acids as energy sources some groups...
described more recently utilize a wide range of substrates including sugars, and grow more rapidly. Retardation in growth rate may be due to drop in pH of culture medium because of accumulation of alcohol formed during carbohydrate fermentation. From the results it can be concluded that starch is the best carbon source for the growth of isolated strain. Moreover the obtained results revealed that the strain showed better growth at 2% tryptophan (0.85 ± 0.13), 1.5% tyrosine (0.73 ± 0.10), 0.8% arginine (0.76 ± 0.14), 0.8% glycine (0.74 ± 0.13), 1% histidine (0.75 ± 0.11) and at 1.5% cysteine (0.71 ± 0.09) respectively as shown in Figure 7. Comparative analysis indicated that in all the aminoacids, the growth was found to be increased when concentration of aminoaids was increased.

In addition to these, prominent growth was observed in the presence of MnCl₂, CaCl₂, FeSO₄, Potassium thiosulphate, CuSO₄, Sodium sulphate, Lithium carbonate, MgSO₄ and KCl as presented in Table 3. According to these results it can be concluded that these metal ions are appeared to be essential for the growth of isolated strain. Similar study based on culture media supplemented with metal cations such as Ca²⁺, Mg²⁺ and Mn²⁺ substantially improved protease production and bacterial biomass of Halobacterium sp. Halobacterium halobium, Pseudomonas salinarum, Pseudomonas cutirubra and Sacinalittoralis failed to grow in the absence of K⁺. KCl upto 2% mimic concentrated sea water [27]. Halotolerant and halophilic organisms grow well in medium supplemented with high concentration of MgSO₄ [28].

**Effect of organic solvents**

Growth of isolate increased gradually when increasing the concentration of solvents as depicted in Figure 8. Moreover optimum biomass production was found at 4% methanol (0.54 ± 0.17), 6% diethyl ether (0.70 ± 0.22), 6% ethanol (0.60 ± 0.20) and 5% chloroform (0.63 ± 0.10) respectively. Research on solvent tolerance has been detected in many microbes [29] and the mechanism of tolerance has been investigated [30]. Most of the strains were able to grow in hexylether (log Pow=5.1), but none of them grow in the presence of n-octane (log Pow=4.9) except Halogemetricum borinquense J CM 10706T and Halorubrum sacchrovorum J CM 8865T. On the other hand, two strains Haloarcula Spp. OHF-1 and 2 exhibited strong tolerance when grown in isooctane (log Pow=4.8). Growth of some strains was retarded in the presence of n-octane, but the final densities were greatly repressed by the presence of solvents [31].

Thus, organic solvent tolerance of halophilic archaea seems to be weaker than that of gram negative bacteria [29]. The present analysis explains that cell densities were found to be greater in the presence of diethylether, compared to other solvents. This suggests that the chromosomes of isolated strain contain genes responsible for organic solvent tolerance.

**Antimicrobial assay**

Results were compared with commercially available antibiotic Choremphenicol and are given in Table 4. The isolate shows significant activity against Enterobacter sp., Klebsiella sp., E.coli and Proteus vulgaris.
Figure 7: Effect of N2 sources on growth.

Figure 8: Growth of isolate in different organic solvents.

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Characteristics</th>
<th>Reaction</th>
</tr>
</thead>
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<tr>
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<td>Source</td>
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</tr>
<tr>
<td>2</td>
<td>Colony Morphology on Zobell Marine Agar (ZMA)</td>
<td>Red colour</td>
</tr>
<tr>
<td>3</td>
<td>Gram reaction</td>
<td>-ve</td>
</tr>
<tr>
<td>4</td>
<td>Shape</td>
<td>Rod</td>
</tr>
<tr>
<td>5</td>
<td>Arrangement</td>
<td>Evenly spread</td>
</tr>
<tr>
<td>6</td>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Oxidase</td>
<td>+</td>
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<tr>
<td>8</td>
<td>Motility</td>
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</tr>
<tr>
<td>9</td>
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<tr>
<td>10</td>
<td>Methyl red</td>
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</tr>
<tr>
<td>11</td>
<td>Vogesprossauer test</td>
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</tr>
<tr>
<td>12</td>
<td>Citrate utilization</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>TSI</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>Urease production</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>Gelatin liquefaction</td>
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</tr>
<tr>
<td>16</td>
<td>Starch hydrolysate</td>
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</tr>
<tr>
<td>17</td>
<td>Casein production</td>
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</tr>
<tr>
<td>18</td>
<td>Glucose</td>
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<tr>
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<td>Sucrose</td>
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<td>21</td>
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</tr>
<tr>
<td>22</td>
<td>Arabinose</td>
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</tr>
<tr>
<td>23</td>
<td>Mannitol</td>
<td>+</td>
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</tbody>
</table>

Table 2: Biochemical characterization of isolated strain.
and the zone of diameter of inhibition was about 24 mm, 21 mm, 20 mm and 20 mm respectively. Report on antibacterial activity among marine bacteria is much familiar and has been studied in a number of research works [32,33]. Similar studies on extract of *Pseudomonas putida* (B151) demonstrated extreme inhibitory effects on *S.aureus* (26 mm), *M. phenylpyruvica* (27 mm), *M.luteus* (25 mm) and *Rhodovulum species* (28 mm) [34]. *Pseudomonas* strains are one of the most important groups can able to produce antibiotics. In this investigation, the isolated strain shows significant antimicrobial activity against *E.coli* (81.8%), *Klebsiella sp.* (250%), *Enterobacter sp.* (140%) and *Proteus vulgaris* (52.8%). This may be due to the inhibition of cell wall synthesis, accumulation of lysozymes or inhibition of cell multiplication [35]. Based on these observations, it was confirmed that the test organism of the present study was found to produce secondary metabolites against *E.coli, Klebsiella sp., Enterobacter sp.* and *Proteus vulgaris* depicted in Figure 9. Extremophiles are a source of active biomolecules and secondary metabolites [14].

16SrRNA sequence analysis

On the basis of NCBI/BLAST database the 16S rRNA gene
sequence of the isolate showed 99.8% similarity with Halomonas utahensis SM1. From the results obtained, the test strain belongs to the genus Halomonas. The nucleotide sequence 16S rRNA gene of the strain Halomonas utahensis SM1 was submitted to GenBank and assigned the accession number KY986725 and the constructed phylogenetic tree is shown in Figure 10.

Conclusion

The strain isolated in the present investigation was identified as Halomonas utahensis SM1 by 16S rRNA sequencing. It exhibited well established growth at pH 8.8 and temperature 42°C. Based on the growth characteristics, the isolated strain can be considered as an “Alkaliphilic moderate thermophile”. It utilizes maximum number of carbon as well as nitrogen sources used in this study for its growth. Prominent growth was found to be appeared in plates supplemented with MnCl₂, CaCl₂, FeSO₄, Potassium thiosulphate, CuSO₄, Sodium carbonate as well as nitrogen sources used in this study for its growth. “Alkaliphilic moderate thermophile” is related to the extreme conditions of a terrestrial, hypersaline environment. It uses maximum number of growth characteristics, the isolated strain can be considered as an alkaliphilic moderate thermophile.

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References


