


sp. lycopersici on tomato by *Brevibacillus brevis*. **J. Phytopathol.,** 158: 470–478.


xxx


Original Research Article

**In Vitro** phosphate solubilization abilities of three indigenous bacteria isolated from Muscovite mine

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**ABSTRACT**

The main objective of this study was to assess the ‘P’ solubilization abilities of three bacterial isolates indigenous to Muscovite mine. The results revealed that the isolate SVUNM17 was a potential rock phosphate solubilizer among the three tested bacteria. After 28DAI the isolate SVUNM17 showed two fold increase in release of ‘Phosphate’ from rock phosphate mineral. The rock phosphate mineral Muscovite was treated with three isolates for 14 days and 28 days. At 14\(^{th}\) day of Incubation the isolate SVUNM16 released highest content of ‘P’ (74mg/l)from muscovite followed by SVUNM9(66mg/l).However at 28\(^{th}\) day of incubation the isolate SVUNM17 was able to release highest content of ‘P’ from Muscovite. The isolate SVUNM17 showed two fold increase for longer incubation periods. This suggests that SVUNM17 is slow ‘P’ solubilizer and SVUNM16 is a fast ‘P’ solubilizer. Further ,the SEM analysis indicated that these isolates also secretes Exopolysaccharides (EPS) and by producing biofilms the bacteria will bind strongly to the Muscovite.

**Introduction**

In soil, phosphorus is sequestered by adsorption to the surface of soil particles and through precipitation reactions with soil cations, particularly iron, aluminum and calcium which becomes unavailable to plants (Harris et al., 2006). For this reason, a large amount of soluble ‘P’ fertilizer is commonly applied to agricultural soils in order to increase plant growth, which is likely to adversely affect both the environment and economy.

In many countries, there has been a steady increase in the use of ‘P’ fertilizer (Syers et al., 2008), which is considered as a major source of heavy metal contamination in agricultural soils (McLaughlin et al., 1996; Bolan et al., 2003). In addition, excess amounts of “P” fertilizer often leach from soil and cause eutrophication of surface and groundwater sources (He et al., 2003; Sharpley et al., 2003). Therefore, there has been increasing interest in the use of slow
release phosphate fertilizers, such as rock phosphate (Rajan et al., 1996; Chen et al., 2006). Insoluble phosphate compounds can be solubilized by organic acids and phosphatase enzymes produced by plants and microorganisms (Kucey, 1983; Duponnois et al., 2005). For example, phosphate solubilizing bacteria (PSB) have been shown to enhance the solubilization of insoluble 'P' compounds through the release of low molecular weight organic acids (Sahu and Jana, 2000). Phosphorous is essential for growth and productivity of plants. It plays an important role in many physiological activities such as cell division, photosynthesis, and development of good root system and utilization of carbohydrates. Phosphorous deficiency results in the leaves turning brown accompanied by small leaves, weak stem and slow development. In ancient times the use of animal manures to provide phosphorous for plant growth was common agricultural practice. Organically bound phosphorous enters in soil during the decay of natural vegetation, dead animals and from animal excretions (He et al., 2003; Sharpley et al., 2003). To achieve optimum crop yields, soluble phosphate fertilizers have to be applied at a high rates which cause unmanageable excess of phosphate application and environmental and economic problems. Direct application of rock ‘P’ materials may be more useful and environmentally more feasible than soluble ‘P’ (Ranawat et al., 2006). Rock ‘P’ materials are cheaper sources of ‘P’. However, most of them are not readily available to the plants because the minerals are released slowly.

The transformation of insoluble phosphate into soluble form is carried out by a number of microbes present in the soil. A large fraction of soil microbes can dissolve insoluble inorganic phosphates present in the soil and make them available to the plants. Microorganisms play an important role for transformation of phosphorous in water and sediments and the phosphate ions are reported to be strongly adsorbed by sediments with a high content of silt and clay (Seshadri et al., 2002). Bacteria are the predominant microorganisms that can solubilize phosphate compared to the fungi and actinomycetes (Yin, 1988). There are some species of bacteria which have potential to mineralize and solubilize organic and inorganic phosphorus (Khiari and Parent, 2005). Strains from the genera Pseudomonas, Bacillus and Rhizobium are among the most powerful phosphate solubilizers (Rodriguez and Fraga, 1999). In this context, the aim of this study was to evaluate the bio-solubilization of the muscovite a rock ‘P’ mineral for Phosphate release from the muscovite mine.

Materials and Methods

Isolation of Bacterial isolates

Muscovite ore samples were collected from Muscovite mining sites situated in Gudur division, Nellore dist. Muscovite ore was collected by employing cable tool drilling from underground mines. The collected samples were pooled and stored at 4°C for further processing. The isolation of native microorganisms present in the samples were done by dilution plate technique using nutrient agar medium. Three morphologically distinct colonies were selected and grown on nutrient agar slants for pure culture preparation. Nutrient agar plates were incubated at 37°C for 24hr. Pure culture isolates were named as SVUNM₀, SVUNM₁₆, SVUNM₁₇.

Morphological and Biochemical Characteristics of the isolates

Identification of pure cultures were made by using morphological and biochemical characteristics (Sneath Peter, 1994). The
colony characteristics were studied basing on their shape, size, elevation, margin, surface, colour and structural characteristics and Gram` +ve or Gram ` -ve nature. Various biochemical tests were also carried out to characterize the isolated bacterial strains. The biochemical tests include O/F test, Catalase, Indole production, methyl red, Voges proskauer reaction, Citrate utilization, Urea activity, Starch hydrolysis, Gelatin liquefaction, carbohydrate utilization, nitrate reduction and antibiotic resistance test.

**In Vitro Phosphate solubilization assay**

**Bacterial Cultures**

The bacterial strains SVUNM9, SVUNM16, SVUNM17 strains which were indigenous to ‘muscovite’ a rock phosphate mineral mine used for the In vitro solubilization assay. These three isolates were inoculated into 100 ml Nutrient sterile medium and incubated on a rotary shaker at 150 rpm/min and 28°C for 24 hours.

**Mineral Processing**

The rock phosphate Muscovite mineral was crushed and sieved to collect grains as large as 20-40 mesh, washed with de-ionized water and dried at 50°C to constant weight. The mineral sample was cut into pieces measuring 1 cm x 1 cm x 1 mm in size and burnished with 5um abrasive.

**Experimental settings for phosphate Mineral degradation**

A batch of experiments were made to explore different aspects.

**Release of Phosphorus from phosphate Minerals**

In phosphate minerals phosphorus is locked up in the phosphate lattice. During interaction with phosphate minerals the bacteria breaks the lattice and release phosphorus. Therefore, in this study phosphorus solubilization was quantified. A loop of 48 hrs old grown bacterial cultures were individually inoculated into 25 ml modified mineral salt medium (PSM) (NH₄)₂SO₄ : 0.10 gms / ltr; MgSO₄.7H₂O : 0.125 gms / ltr; MgCl₂.6H₂O : 2.5 gms / ltr; KCl : 0.10 gms / ltr containing 0.50% Muscovite. Mineral salt medium containing muscovite was sterilized at 121°C for 20 minutes followed by inoculating 2 ml culture broth of the bacterial strains individually and incubated on a rotary shaker at 150 rpm/min and at 28°C. The experimental setting has two batches incubated for 14 days and 28 days respectively. Control experiments were carried out in parallel to the experimental runs, whose experimental conditions were identical to those of the treatment experiments, but with autoclaved medium without bacterial culture. After, every 14 and 28 days of incubation, Phosphorus (PO₄³-⁻) content was measured using Induction Coupled Plasma (ICP-OES Optima 4300 DV, Perkin Elmer, Waltham, MA,USA).

**SEM analysis of Muscovite for structural changes**

To observe the structural changes in the phosphate minerals if any as affected by phosphate dissolving bacterium another set of treatments were selected. For this part, the Muscovite samples were collected during harvesting and washed three times to remove culture medium with sterile distilled water. These mineral samples were air dried at room temperature. This dried sample was fixed by applying 15 ml of fixing solution (2.5% Glutaraldehyde in 0.0075 M phosphate buffer) on to the ore inside Greiner tube. Mine samples were washed
three times for 15 min each with 0.0375M phosphate (Na$_3$(PO$_4$)$_3$) buffer. The samples were then dehydrated at different alcohol concentrations (50, 70, 95, 100%) at 10 min each. The dehydrated samples were repeatedly soaked in 100% alcohol twice. This stage was followed by drying of the samples that were later sputter coated in a Polaron equipment limited SEM coating unit E5200 with gold prior to observation under the scanning electron microscope (SEM). They were then assembled for observation under the microscope at 5 KV on a JEOL 5800 LV Scanning electron microscope (Tokyo, Japan).

Results and Discussion

From the isolated colonies in the petriplates three different strains were selected on the basis of district morphological characteristics such as colony shape, size, margin, colour and elevation as given in table-1. These selected strains were sub cultured repeatedly several times to obtain the pure culture. There strains were pure cultured and the microscopic and biochemical characteristics were studied. The Gram staining was carried out for six bacterial strains to differentiate between two principle groups of bacteria, such as bacillus or cocci etc. Further various biochemical tests related to the characterization of bacteria were carried out and presented in Table-2.

The isolate M9 was found to be small in size, white in colour, with irregular form, entire margin having raised elevation. This isolate was found to be Gram positive rod, showing motility. From the bio chemical test it was found that the isolate showed positive results for catalase production, methylred, Voges proskauer reaction, citrate utilization, urease activity, starch hydrolysis, zelatin liquefication, nitrate reduction and resistant to streptomycin, chloramphenicol, and tetracyclin. The isolate also showed positive results for oxidative test. The isolate produced acid from D-glucose and sucrose and gas from lactose . However, the isolate is unable produce Indole. The isolate was resistant to

The isolate M16 was found to be small, circular, brown colour with white margin, having flat elevation. This isolate was found to be Gram `-`ve cocci from the biochemical tests it was found that the bacteria was motile fermentative. The isolate also showed positive results for the production of catalase, Voges proskauer reaction, citrate utilization, urease activity, starch hydrolysis, gelatine liquifcation, nitrate reduction and able to produce acid from D-glucose, gas from lactose. However neither gas nor acid were produced from sucrose. The isolate showed resistance to streptomycin, chloramphenicol and tetracycline.

The isolate M17 was found to be pinhead in size, with circular form, entire margin, raised elevation and purple colour. This isolate was found to be Gram positive cocci with appendages. The biochemical tests were positive for fermentative, Voges proskauer, citrate utilization, urease activity, starch hydrolysis, gelatine liquifcation, nitrate reduction, resistance to streptomycin, chloramphenicol and tetracycline negative for catalase, indole production and methyl red.

In vitro phosphate Solubilizing assay

The Muscovite, rock phosphatse was subjected to phosphate solubilization assay. The amount of P released from Muscovite in a broth by the isolates was studied at 14 and 28 days of Incubation (DAI). The results indicated that the amount of P released from Mica by three strains
increased with increased incubation and was maximum at 28 DAI. The P release from mica by all the three strain at 14 DAI ranged from 44.8 – 66.50mg/l. Similarly the amount of ‘P’ released from Mica by three isolates after 28 DAI ranged from 74-96.10mg/l. Among the three isolates SVUNM16 released maximum amount of (74.3mg/l) from Mica followed by SVUNM9 (66.50mg/l). The isolate SVUNM 17 released only 44.8mg/l ‘P’ only after 14days of Incubation.

In case of 28 DAI the amount of ‘P’ release increased slightly for SVUNM9,SVUNM16 isolates as 74.0mg/l and 84.30mg/l respectively. In case of M17 longer incubation times decreased the ‘P’ release (96.10mg/l). This may be due to the biosorption of P by bacterial isolate. The ‘P’ release from potash mineral by three isolates after 14days ranged from 52-721.50 for 2/4dAI and 20-103.14mg/l after 28 days. The isolate SVUNM17 released good amount of P after 14days. However the isolate SVUNM16 released good amount of P (184.4mg/l) after -28days.

**SEM analysis:** The SEM analysis of residue of Muscovite mineral left after the 28 DAI showed noticeable structural changes. The SEM image of SVUNM9 treated Muscovite showed visible forms of biofilm formation and SVUNM16 treated Muscovite showed copious amounts of biofilm and strong attachments to the surface of mica. The isolate SVUNM17 treated Muscovite SEM image represents the high possibility of EPS, secretion that binds the mica ore strongly together. All the Images obtained were at the end of 28DAI of the Experiment. (Plate-1a,1b,1c and 1d)

Soil microorganisms play a key role in soil ‘P’ dynamics. Release of ‘P’ by PSB from insoluble and fixed/adsorbed forms is an important aspect regarding ‘P’ availability in soils.

<table>
<thead>
<tr>
<th>Name of the isolate</th>
<th>Size</th>
<th>Pigmentation</th>
<th>Form</th>
<th>Margin</th>
<th>Elevation</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVUNM9</td>
<td>Small</td>
<td>-</td>
<td>Irregular</td>
<td>Entire</td>
<td>Raised</td>
<td>White</td>
</tr>
<tr>
<td>SVUNM16</td>
<td>Small</td>
<td>-</td>
<td>Circular</td>
<td>Brown with white margin</td>
<td>Flat</td>
<td>Brown</td>
</tr>
<tr>
<td>SVUNM17</td>
<td>pinhead</td>
<td>-</td>
<td>Circular</td>
<td>Entire</td>
<td>Raised</td>
<td>purple</td>
</tr>
</tbody>
</table>

**Table 1** Morphological characteristics of the isolates

**Fig.1 Release of P from Muscovite using three bacterial strains after 14and 28 days**
Table 2: Biochemical Characteristics of the isolates

<table>
<thead>
<tr>
<th>Name of the test</th>
<th>SVUNM9</th>
<th>SVUNM16</th>
<th>SVUNM17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>‘+’ve, rods</td>
<td>‘-’ve, rod</td>
<td>‘+’ve, cocci with appendages.</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
<td>Non motile</td>
<td>Motile</td>
</tr>
<tr>
<td>O/F test</td>
<td>Oxidative</td>
<td>Fermentative</td>
<td>Fermentative</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Indole production</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methyl red</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vogesproskauer reaction</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Urease activity</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin liquification</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Glucose-acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Glucose-Gas</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Lactose-acid</td>
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<td>Lactose-Gas</td>
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<td>+</td>
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<tr>
<td>Sucrose-Acid</td>
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<td>-</td>
</tr>
<tr>
<td>Sucrose-Gas</td>
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<td>-</td>
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<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Antibiotic resistance test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Sreptomycin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. Choloramphenicol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C. Tetracyclin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3: Content of P from Mica using three bacterial strains after 14 and 28 days

<table>
<thead>
<tr>
<th>Name of the Culture</th>
<th>Content of ‘P’ in Control</th>
<th>Content of ‘P’ after 14 days</th>
<th>Content of ‘P’ after 28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>M9</td>
<td>131.80</td>
<td>198.30</td>
<td>205.80</td>
</tr>
<tr>
<td>M16</td>
<td>131.80</td>
<td>206.00</td>
<td>216.10</td>
</tr>
<tr>
<td>M17</td>
<td>131.80</td>
<td>176.60</td>
<td>227.9</td>
</tr>
</tbody>
</table>

Table 4: Release of P from Mica using three bacterial strains after 14 and 28 days

<table>
<thead>
<tr>
<th>Name of the Culture</th>
<th>Content of ‘P’ in Control</th>
<th>‘P’ release after 14 days</th>
<th>‘P’ release after 28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>M9</td>
<td>131.80</td>
<td>+66.50</td>
<td>+74.0</td>
</tr>
<tr>
<td>M16</td>
<td>131.80</td>
<td>+74.2</td>
<td>+84.30</td>
</tr>
<tr>
<td>M17</td>
<td>131.80</td>
<td>+44.8</td>
<td>+96.10</td>
</tr>
</tbody>
</table>
Plate I Scanning Electron Microscope (SEM) images showing the action of microbes on Mica sheets

Ia: SEM image represents the mica control.
Ib: SEM image represents the isolate SVUNM9 with visible forms biofilm formation.
Ic: SEM image represents the isolate SVUNM16 with copious amounts of biofilm and strong attachments to the surface of mica.
Id: SEM image represents the isolate with visible SVUNMM17 with high possibility of EPS secretions that binds the mica ore strongly together

There are strong evidence that soil bacteria are capable of transforming soil ‘P’ to the form available to plants. Rock phosphate minerals are too insoluble. However some bacterial species have mineralization and solubilization potential for organic and inorganic phosphorous respectively. Application of insoluble rock phosphate alone in the soil caused limited increase in the ‘P’ uptake, this increase in ‘P’ content could be due to the role of indigenous microorganisms in the soil. In the present study microorganisms indigenous to muscovite mine were isolated and evaluated for their rock phosphate solubilization. This solubilization may be due to the production of strong acids (Schilling et al., 1998). In our study also three indigenous microorganisms were able to increase ‘P’ release from rock phosphate by about 0.5-2.0 fold in comparison to the control. Similarly, Amer et al., (2010) reported the B. subtilis and P. fluorescense also increases ‘P’ release. PSB have been used to improve rock ‘P’ value because they convert insoluble rock ‘P’ into soluble forms. This conversion is through acidification, Chelation and exchange reaction and produce strong organic acids in the periplasm. Acid phosphatase also play a major role in the mineralization of rock phosphate minerals. In our study three indigenous bacterial isolates were able to solubilize ‘P’ from...
muscovite significantly. Similarly, Styriakova et al., (2003) reported that the activity of potassium dissolving bacteria played a pronounced role in the release of ‘K’ from Feldspar, a rock phosphate mineral. Similarly in our study also these three bacterial isolates are good candidates for Phosphate solubilization. In conclusion due to their phosphate solubilizing capacity, they can also be used as biofertilizers.

References


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Original Research Article

Isolation and Identification of Sulfate Reducing Bacterial Strains Indigenous to Sulphur Rich Barite Mines

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Abstract

The present study was aimed at isolating Sulfate Reducing Bacteria (SRB) by enriched method using Iron-Lyngby medium from barium sulphate mines situated in Mangampeta, Kadapa district of Andhra Pradesh. Isolation of bacterial strains was done by dilution plate technique continued by three-tier identification process such as morphological, microscopic studies and biochemical characterization. Identification was done using biochemical tests such as, ONPG, Lysine utilization, Ornithine utilization, Urease production, Phenylalanine Deamination, Nitrate reduction, H₂S production, Citrate Utilization, Voges Proskauer’s reaction, Methyl red, Indole production, Malonate utilization, and Esculin hydrolysis. Carbohydrate utilization potentials were also tested using Arabinose, Xylose, Adonitol, Rhamnose, Cellobiose, Melibiose, Saccharose, Raffinose, Trehalose, Glucose and Lactose. In total five species of bacterial strains viz., four species were belongs to the Enterobacter species and one species of Bacillus were identified from the barite mine sample. Physiological responses were also studied for these strains in different pH, NaCl concentration and temperatures. These strains have potential use in bioremediation of sulphur contaminated environments.

Introduction

Microbial diversity is rapidly gaining interest among the scientific community with emphasis to understand their eco physiological role and function in various ecosystem. The role of microorganisms in an ecosystem is influenced by ecosystem structure, composition, nature and location. The Sulfate reducing bacteria (SRB) are a heterogeneous group of microbes which use sulfate as terminal electron acceptor (Hansen, 1994). They use simple inorganic and organic compounds like hydrogen, ethanol, methanol, acetate, lactate, propionate, and pyruvate as electron donor (Liamleam and Annacchatre, 2007). The sulphate reducing bacteria (SRB) are unique physiological group of prokaryotes because they have capability of using sulphate as the final electron acceptor in respiration. A hallmark characteristic that distinguish SRB is the manner in which sulphate is metabolized. The sulphate reducing bacteria
are one of the wide technological interest not only for their ability to reduce metal sulphide but also for formation of insoluble metal sulfide thus removing toxic metals from waste water (Hoa et al., 2007; Biswas et al., 2008).

Barite (BaSO$_4$) is a sulphur rich deposit generally arising from mixing of soluble Barium containing fluids with sulphate rich fluids. Abiotic oxidation of sulphide to sulphate leads to barite deposition (Plummer, 1971). Alternatively barium leads brines may mix barite formation (Kasiser, 1987; Williams Jones et al., 1992). Basicallly barium is an alkaline earth element which occurs as a trace metal in igneous and sedimentary rocks. In nature it occurs principally in combined states as Barite (BaSO$_4$) and Witherite (BaCO$_3$). Barium precipitates as the mineral barite (barite). Even though recent investigations have shown that Barium (Ba) may serve as an indicator of palio oceanographic and modern conditions, a better understanding of its biogeochemistry is required before confident interpretation of its distribution and reaction ways are possible. Barium has a low solubility from Barite but it has been observed that laboratory cultures of sulphate bacteria have been indicated as good candidates for barium solubilisation from barite. The present study intends to isolate SRB which will help to mitigate the toxic effects of Ba in Eukaryotic and Prokaryotic cells.

Materials and Methods

Study area

The bedded barite deposits are located at Mangampeta (Lat. 14°0.01 N; long 70° 19'E) in Kadapa district, Andhra Pradesh (Fig-1). It is included in the survey of India Topo sheet No.57 N/8. This is the single largest deposit of its kind in the world. The mining activity is generally conducted as opencast and underground mining. Sulfur Content is high in this area.

Collection of sample

Barite mine samples have been collected from different operating opencast mines. The samples collected were from old ore deposits using randomised block design. The collected samples were placed in sterile poly bags and brought to the laboratory and stored at 4°C until further processing.

Isolation and pure culture preparation

Isolation of native microorganisms present in the mine samples were done by selective isolation and enrichment method using Iron Lyngby Medium (Lorentzen et al., 2003). This medium is generally used for the isolation of sulphur oxidizing bacteria and the ingredients of the medium are Peptone - 20g/l; Yeast Extract - 3g/l; Ferric Citrate - 0.3g/l; Sodium Thiosulphate - 0.3g/l; NaCl-5g/l at pH 7.5. Isolation of native microorganisms present in the mine samples were done by dilution plate technique. From the cultured plates the morphologically different strains were isolated and grown on the same medium for pure culture preparation. The plates were incubated at 37°C for 24hr. Pure cultures were preserved in Glycerol medium and stored at -20°C.

Morphological and Microscopic studies

The colony characteristics were studied basing on their shape, size, elevation, margin, surface, colour and structure of the colony. Microscopic studies of the isolated strains were done by Gram staining for identification of structural characteristic and Gram ‘+’ve or Gram ‘-’ve nature.

Biochemical studies

Various biochemical tests were carried out
to characterize the isolated bacterial strains. The tests include ONPG, Lysine utilization, Ornithine utilization, Urease production, phenylalanine deamination, nitrate reduction, H\textsubscript{2}S production. Citrate utilization Voges Proskauer’s reaction, Methyl red, Indole production, Malonate utilization, Esculine hydrolysis, Oxidase production, Arabinose, Xylose, Adonitol, Rhamnose, Cellobiose, Melibiose, Saccharose, Raffinose, Trehalose and Glucose and unable to utilize Arabinose, Xylose, Adonitol, Rhamnose and Lactose.

Results and Discussion

From the distinct isolated colonies in the petri plates five different strains were selected on the basis of distinct morphological characteristics such as colony shape, size, margin, colour and elevation as given Table-1. The selected strains were sub cultured repeatedly several times to obtain the pure culture and named as CSRB-1, CSRB-2, CSRB-3, CSRB-4, CSRB-5. These pure strains were subjected to Microscopic, biochemical and growth characteristics. The Gram staining was carried out for five bacterial strains to differentiate between two principal groups of bacteria i.e., Gram positive and Gram negative nature and their shape such as Bacilli or Cocci etc., were studied. Further, various biochemical tests related to the characterization of bacteria were carried out to identify them (Table-2). The carbohydrate utilization profiles were also studied. The isolate CSRB-1 was found to be circular in shape, cream in colour and the margin was lobate having crenate elevation. The colony size after 24 hours incubation was measured as 0.1 - 1.2mm. This isolate was found to be Gram negative, rod shaped bacteria. From the biochemical tests it was found that the bacteria is facultative anaerobe. The isolate also showed positive for Urease production, Ornithine Utilization, Methyl red test and nitrate reduction and negative for ONPG, Lysine utilization, Phenylalanine deamination, H\textsubscript{2}S production, Citrate utilization, Voges Proskauer’s reaction, Indole production, Malonate utilization, and Esculin Hydrolysis. The isolate was able to utilize Cellobiose, Melibiose, Saccharose, Raffinose, Trehalose and Glucose and unable to utilize Arabinose, Xylose, Adonitol, Rhamnose and Lactose.

Due to these results the isolate was identified as *Enterobacter* sp.. The isolate CSRB-2 was found to be circular in shape, yellow in colour and margin was entire having flat elevation. The colony size after complete incubation was measured as 0.1 – 1.5 mm. The isolate was found to be Gram negative rod shaped bacteria. Biochemical tests showed positive for Nitrate reduction, Methyl red and Escculin hydrolysis and negative for ONPG, Lysine utilization, Ornithine utilization, Phenylalanine deamination, H\textsubscript{2}S production, Citrate utilization, Voges Proskauer’s reaction, Indole production, and Malonate utilization. The carbohydrates Saccharose, Trehalose, and Glucose are utilized by the isolate. But, Arabinose, Xylose, Adonitol, Rhamnose, Cellobiose, Melibiose, Raffinose and Lactose were not utilized by the isolates. Due to the presence/absence of the above results the isolate was identified as *Bacillus* sp. The isolate CSRB-3 was found to be circular in shape, white in colour and the margin was lobate having umbonate elevation. The isolate was positive for Nitrate reduction, H\textsubscript{2}S production, Methyl red, Escculin hydrolysis and negative for ONPG, Lysine utilization, Urease, phenylalanine deamination, Citrate utilization, Voges Proskauer’s reaction, Indole, Malonate utilization and unable to utilize Arabinose, Xylose, Adonitol, Rhamnose, Cellobiose, Melibiose, Raffinose, and Lactose. So isolate was identified as *Enterobacter* sp.
Table 1 Biochemical Characteristics of Sulfate reducing Bacteria

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name of the Test</th>
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<th>CSRB-2</th>
<th>CSRB-3</th>
<th>CSRB-4</th>
<th>CSRB-5</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Gram Staining</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>ONPG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Lysine Utilization</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Orthinine Utilization</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Urease</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>6</td>
<td>Phenylalanine Deamination</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Nitrate Reduction</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>H2S Utilization</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Citrate Utilization</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Voges Proskauer's</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Methyl Red</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Indole</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Malonite Utilization</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>Esclun Hydrolysis</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2 Carbohydrate Utilization Potentials of sulfate Reducing Bacteria

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name of the Carbohydrate</th>
<th>CSRB-1</th>
<th>CSRB-2</th>
<th>CSRB-3</th>
<th>CSRB-4</th>
<th>CSRB-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arabinose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Xylose</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>3</td>
<td>Adonitol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Rhamnose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Cellobiose</td>
<td>+/-</td>
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<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>6</td>
<td>Melibiose</td>
<td>+/-</td>
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<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Saccharose</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Raffinose</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>11</td>
<td>Lactose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Oxidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The isolate CSRB-4 was found to be circular in shape, cream in colour and the margin was lobate having flat elevation. The colony size after complete incubation was measured as 0.1 – 1.5µm. This isolate was found to be Gram negative. The result of biochemical tests positive for Nitrate reduction, H2S production, Esclun hydrolysis and able to utilize Arabinose, Cellobiose, Saccharose, Raffinose, Trehalose, Glucose, and Lactose, so it comes under the genera Enterobacter sp.

The isolate CSRB-5 was found to be circular in shape, yellow in colour and the margin was entire having flat elevation. The colony size was after complete incubation was measured as Gram
negative rods. Due to yellow colour pigment production it comes under Enterobacteriaceae family. The biochemical tests include H2S production, methyl red, Esculin hydrolysis and able to utilize Cellobiose, Saccharose, Trehalose, Glucose. Therefore the isolate was identified as Enterobacter sp.

Morphological, cultural, physiological and biochemical characteristics of bacterial isolates from different environments have been studied by various workers (Gahan et al., 2005, Dhal and Thatoi, 2007, Elizabeth et al., 2008). Venugopal et al., 2000 suggested that application of indigenously available microbes has immense potential as bioremediating agents. The organisms isolated from barite mines also would have immense potential applications in bioremediation of sulphur or desulphurization process. Lakshman raj et al., (2012) isolated the sulfur specific microorganisms from Indian refinery plant sites and used in desulfurization process. In his studies he identified Enterobacter sp., and Pseudomonas sp., Similarly, Bacillus species were also isolated from oil contaminated soil and used in Biodiesulfurization (Kalyani et al.,2012).

Philip et al., (1998) found that Bacillus species were also involved in heavy metal reductions. Bacterial reduction of heavy metal by Enterobacter has also been reported (Ehrlich,1996; Shen,1950). The sulphur oxidizing bacteria are major potential applications in biodesulfurization, biodenitrogenation, biodemetallization , and biotransformation of heavy crude oils into lighter crude oils sulphur contaminated and heavy metal contaminated soils.

References


A New Facultative Alkaliphilic, Potassium Solubilizing, Bacillus sp. SVUNM9 Isolated from Mica Cores of Nellore District, Andhra Pradesh, India.

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1Department of Microbiology, Sri Venkateswara University, Tirupati – 517 502. Andhra Pradesh, India 2Department of Botany, Sri Venkateswara University, Tirupati – 517 502. Andhra Pradesh, India

ABSTRACT

A new facultative alkaliphilic Bacillus species isolated from mica mines of Nellore district of Andhra Pradesh, India. This strain is gram positive, rod shaped, motile bacteria capable of growth in aerobic conditions up to pH 12.0 exhibiting optimum growth at pH 10.0. The strain is oxidative, catalase positive, urease positive, able to hydrolyse starch, gelatine, able to utilize lactose, D-glucose and sucrose and produce acids. It also converts nitrate into nitrite. Further, exhibited positive results for methyl red and Voges Proskauer reaction. The strain showed optimum growth at 0.5% NaCl and able to thrive even at higher concentration of sucrose. The 16S rDNA sequence showed 99% similarity with Bacillus sp19 (HQ433576) and closest relative is Bacillus amyloliquifaciens (JN086147). The efficiency of this strain for potassium dissolution from insoluble mica was evaluated in vitro. Modified Alexandrov’s medium supplemented with 0.1% mica powder was used to investigate the potassium solubilising activity of the Bacillus spSVUNM9 strain. Final pH, total acidity, soluble K content and released organic acids were determined in culture media after 21days of incubation. The K content was increased to 2.5times greater than the control. Mineral potassium solubilisation was directly related to the pH drop by the strain. The analysis of the culture medium by high pressure liquid chromatography identified gluconic acid as the main organic acid released by Bacillus sp.SVUNM9. This study is the first report on the isolation and characterization of native potassium solubilising bacteria from mica ore.

INTRODUCTION

Alkaliphiles are a class of extremophilic microorganisms that exhibit the ability to grow at pH of 9.0 and above. The alkaliphiles have yielded a rich array of products, suitable for industrial scale[1]. The products of alkaliphiles which have industrial importance have been commercialized in the area of detergent and food industries. It is not worthy that industrial production of products from alkaliphiles is so far insufficient to meet the demands. True alkaliphiles by and large grow at and above pH of 9.0 and show optimal growth at pH of 10.0. Though considerable diversity exists among alkaliphiles but many more remain to be tapped from unexplored regions. Bioprospection for novel alkaliphiles from unexplored habitats for specific products have suitability for broad technological plant forms which can provide environment friendly and cost-effective solutions.

The present study aims to isolate and characterize alkaliphilic bacterium with Potassium solubilizing property from Mica cores. Mica is a complex mineral classified as an Alumino-silicate but occurring in combination with one or more elements like potassium, sodium, magnesium, lithium, vanadium and iron. In Andhra Pradesh rich deposit of mica are available in the Nellore District. The mica belt lying between latitudes 14°-00' and 15°-00' and longitudes 79°-35' and 80°-00'. The chemical properties of Nellore Mica were listed in Table-1.
Table 1: Chemical composition of Mica mines

<table>
<thead>
<tr>
<th>Composition</th>
<th>Nellore Mica (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al₂O₃</td>
<td>36.77</td>
</tr>
<tr>
<td>Fe₂O₃</td>
<td>0.21</td>
</tr>
<tr>
<td>FeO</td>
<td>1.64</td>
</tr>
<tr>
<td>CaO</td>
<td>1.28</td>
</tr>
<tr>
<td>MgO</td>
<td>0.72</td>
</tr>
<tr>
<td>SiO₂</td>
<td>46.42</td>
</tr>
<tr>
<td>F</td>
<td>trace</td>
</tr>
<tr>
<td>K₂O</td>
<td>0.94</td>
</tr>
<tr>
<td>Na₂O</td>
<td>0.72</td>
</tr>
<tr>
<td>Water combined</td>
<td>3.24</td>
</tr>
<tr>
<td>Total</td>
<td>99.94</td>
</tr>
</tbody>
</table>

Source: Bureau of Mines, INDIA.

Though the mica's possess acidic environment with a pH of 5.6, it is a good source for alkaliphiles about 41% among total diversity (data not presented). On the other hand so far there is no report of these microorganisms in the mica cores. This is the first report about the alkaliophilic Bacillus species from Mica Mines.

Mica is one of the major K-bearing mineral. Further Potassium is one of the three essential elements in NPK required for the growth and reproduction of the plants. However the biggest portions of ‘K’ in soil are insoluble rocks, minerals and other forms. Microbes can enhance mineral dissolution rate by producing and excreting metabolic by products that interact with the mineral surface [2]. Studies have documented the ‘K’ release by degradation of silicate minerals by bacteria[3, 4]. Due to rapid development of agriculture K-deficiency became predominant in Indian soils[5]. The Fertilizer Association of India in 2007 reported that India ranks 4th in consumption of potassium fertilizer. The whole consumption of K-fertilizers is imported in the form of potash and sulphate of potash which leads to a huge foreign exchange. In this context identification of alternative indigenous K source is essential. Therefore the present study aims to isolate and characterize indigenous culturable bacteria with potassium solubilizing potential inhabiting in mica core.

MATERIALS AND METHODS

Isolation of Microorganism

Microorganisms were isolated from mica cores obtained from Utukur mandal, Gudur division, Nellore Dt. Andhra Pradesh, India by the following method. Mica cores were splitted into pieces using sterile splitters and powdered using sterile mortar and pestle. This fine powder was dispensed into 0.85% NaCl solution and incubated for 2 hours in orbital shaker at 28°C. Later this suspension was placed on to trypticase soy agar with a pH of 10 and incubated at 37°C for 4 days.

Colonies were selected on the basis of cultural characteristics like form, elevation, margin and texture. For the present study isolate M9 was selected and streaked several times on Trypticase soy Agar and purified. For further study the purified colony was preserved in 20% glycerol stock and stored at -20°C.

Morphological and Phenotypic Characterization of the Isolate

The morphology of vegetative cells, sporangia and the shape and position of spores were observed under microscope (Olympus) using submerged 100X magnification. In addition to that the following phenotypic tests were performed. They are motility, catalase, Voges–Proskauer test, methyl red test, gas and acid production from lactose, glucose and sucrose and nitrate reduction, indole formation, H₂S production, Citrate utilization [6] and Gelatin liquification. Degradation of urea, starch and cellulose were tested according to the methods reported previously [7,8].

DNA Extraction and Sequence Determination

Genomic DNA was isolated from pure bacterial colonies using the method Ausubel et al., (1995)[9]: The 16S rRNA gene was amplified using universal primers. (Forward 5’CAG CAG CCG CGG TAA TAC -3’and reverse 5’− ACG GCC GGT GTG TAC -3’). The amplified product was subjected to 1.5% agarose gel electrophoresis. The 1.4 Kb product was extracted from the gel using silica gel columns and sequenced. The resulting DNA sequences were submitted to the non-redundant nucleotide database at Genbank using the basic local alignment search tool (BLAST) program to determine its identify.
Sequence Analysis

Multiple alignments of the M9 isolate sequence and closest relatives were performed using the Bio edit sequence alignment editor [10]. The percentage of sequence similarity was calculated between the isolate and other close relatives using the Clustal W programme[11]. Phylogenetic trees were constructed using the neighbour joining method using the phylip ver 3.67 suite of programs[12].

Growth Studies

Growth assays were performed using trypticase soy broth. To analyze pH tolerance 100 m mol/−1 sodium acetate, sodium phosphate and calcium carbonate were used to adjust pH to 7, 9, 10, 11 and 12 respectively. Tolerance to salt was determined adding different amounts of NaCl (0.5%, 5% and 10% (w/v)) to trypticase soy broth at pH 10. The effect of temperature on microbial growth was studied at 30°C, 40°C, 50°C and 60°C in Trypticase soy broth at pH 10.

To analyse osmotic stress (tolerance) sucrose at varying concentrations (0.5% 15% and 60% (w/v)) was incorporated in the medium at pH 10.

Potassium Dissolution Assay

The Bacillus strain was inoculated into modified Alexandrove’s medium [13] consisting 5.0g/l Sucrose; Sodium Hydrogen phosphate (Na2HPO4) 2.0g / l; Magnesium Sulphate (MgSO4.7H2O) 0.5g / l; Ferric Chloride (FeCl3) 0.005g / l; Calcium Carbonate(CaCo3)0.1g/l; Mica powder (Insoluble potassium source )1.0g / l; Distilled water 1000ml; pH of the medium was adjusted to 7.0 using dilute acid and/or alkali. Except mica all the ingredients were dissolved in 1000ml distilled water .Then Mica powder was added. The flasks were plugged with cotton and sterilized at 1200°C and 0.1MPa for 20 min in an autoclave. To this sterilized medium 1ml Bacillus SVUNM9 culture was added and incubated for 21days on a shaker at 180 rpm. After 21 days of incubation, the cultures were filtered through 0.2µm membrane filter and pH was directly measured by pH meter. The total acidity of the culture media was determined according to the method described by Helrich (1990)[14]. The K content in the digested solutions was measured using the induction coupled plasma Optimal Emission Spectroscopy (ICP-OES Optima 4300 DV, Perkin Elmer).

Analysis of Organic Acids

The organic acids in culture filtrate fluid were analyzed by high performance liquid chromatography HPLC (Hewell Packard 1050) with ODS columns(200mm,4.6mm,50m). The operating conditions consisting of 0.1% H3PO4 as the mobile phase detector ,VMD(210nm) and a constant flow rate of 1.0ml/m-1 the pH was adjusted to 2 by phosphatic acid and 50ul of organic acids extract was injected .The organic acids were quantitatively determined by comparing the retention times and peak areas of chromatograms with those standards. The organic acid standard included gluconic acid, acetic acid, citric acid and malic acid.

RESULTS

Microbial Isolation

The cell suspension was placed on the alkaline medium. After incubation among the various isolates the isolate M9 was selected for the present study. According to the morphological and physiological characteristics the strain belonged to the Bacillus genus. Microscopic observations showed that microorganisms were spore-forming, gram positive rods . They showed positive results for catalase, Methyl red, Voges Proskauer reaction, Nitrate reduction, Utilization of Glucose, Lactose, Sucrose able to hydrolysis of starch ,gelatin ,urea and negative for Indole production and unable to metabolize citrate (Table-2).

16S rDNA Sequence Analysis

Gene sequences were aligned and compared between them with the sequences of 10 related taxa from public databases to determine its phylogenetic position. Taxonomically M9 strain was clustered in the rRNA group-I of the phylogenetic group defined as Bacillus genus and occupy a phylogenetic position closely related to Bacillus sp 19(Gen bank accession No. HQ662601). The 16S rDNA sequences similarity value of the mica isolate M9 to Bacillus sp.19 was 99% (Table-3).
### Table 2: Physiological and Biochemical Characteristics of M9 isolate

<table>
<thead>
<tr>
<th>Name of the Test</th>
<th>Response</th>
</tr>
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<tbody>
<tr>
<td>Grams Staining</td>
<td>Gram Positive</td>
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<tr>
<td>Shape of the Cell</td>
<td>Rods</td>
</tr>
<tr>
<td>Arrangement of the cells</td>
<td>Chains</td>
</tr>
<tr>
<td>Pigment production</td>
<td>-</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile</td>
</tr>
<tr>
<td>O / F test</td>
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</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Indole Production</td>
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<tr>
<td>Methyl Red</td>
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</tr>
<tr>
<td>Voges Proskauer reaction</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of Citrate</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of Starch</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
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<tr>
<td>Urea</td>
<td>+</td>
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### Table 3: Showing alignment view using combination of genbank and RDP database

<table>
<thead>
<tr>
<th>Alignment View</th>
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<th>Alignment results</th>
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<td></td>
<td>M9</td>
<td>1.00</td>
<td>Studied Sample</td>
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<tr>
<td></td>
<td>JN086147</td>
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<td><em>Bacillus amyloliquefaciens</em> St.Rx-35</td>
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<tr>
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<td><em>Bacillus amyloliquefaciens</em> St.Rx-34</td>
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<td>0.99</td>
<td><em>Bacillus methylotrophicus</em> St.H5-12</td>
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<tr>
<td></td>
<td>JN086143</td>
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<td><em>Bacillus amyloliquefaciens</em> St.Dx-18</td>
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<td>JF460043</td>
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<td><em>Bacillus methylotrophicus</em> St.K3-18</td>
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<td>0.99</td>
<td><em>Bacillus methylotrophicus</em> St.Nx-1-20</td>
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<tr>
<td></td>
<td>HQ433576</td>
<td>0.99</td>
<td>*Bacillus sp. 19(2010)</td>
</tr>
<tr>
<td></td>
<td>HQ682601</td>
<td>0.99</td>
<td><em>Bacillus methylotrophicus</em> St.Mo-Bw-16</td>
</tr>
</tbody>
</table>

**Growth Studies**

In order to determine the effect of pH on microbial growth, isolates were incubated at pH 7, 9, 10, 11 and 12. Although the strain is able to grow at pH 7, optimal growth was observed at pH 10 and at extreme alkaline conditions like pH 12 growth was decreased (Fig -1).

The isolate M9 was able to grow from 10°C to 55°C being the optimum 44°C. Microorganism grew in the presence of NaCl concentration up to 10% (w/v) and the strain is able to withstand the osmotic stress at 15% (w/v) sucrose. Table-4 summarizes the growth properties of the strain under different experimental conditions.
Figure 1: Growth of the M9 isolate at different Hydrogen ion (pH) concentration

Table 4: Effect of Salinity, Osmotic pressure and Temperature on growth of M9 Mica isolate

<table>
<thead>
<tr>
<th>Property</th>
<th>Isolate M9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth in the presence of NaCl (%)</td>
<td>+++</td>
</tr>
<tr>
<td>0.5</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Growth in the presence of sucrose (%)</td>
<td>++</td>
</tr>
<tr>
<td>0.5</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>+++</td>
</tr>
<tr>
<td>60</td>
<td>++</td>
</tr>
<tr>
<td>Growth at different temperatures (°C)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>25</td>
<td>++</td>
</tr>
<tr>
<td>37</td>
<td>++</td>
</tr>
<tr>
<td>44</td>
<td>+++</td>
</tr>
<tr>
<td>55</td>
<td>++</td>
</tr>
</tbody>
</table>

Potassium Dissolution Assay

The potassium dissolution assay was performed with the identified alkalophilic Bacillus sp., NMM9 (GenBank accession JQ9221141) from insoluble mica mineral. Generally compared to the control the pH of the inoculated media supplemented with insoluble mica was decreased to pH4.8 and in turn increased the total acidity in culture media. The % K release is 40% in culture media. Among the organic acids produced by the Bacillus the gluconic acid content (313ng/l) is high compared to the other acids. This is everywhere observed in case of silicate solubilizing property (Table-5).

Table 5: Showing results of Potassium dissolution assay

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameter</th>
<th>Control</th>
<th>Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>% ‘K’ Release</td>
<td>–</td>
<td>40</td>
</tr>
<tr>
<td>2.</td>
<td>pH</td>
<td>7.0</td>
<td>4.8</td>
</tr>
<tr>
<td>3.</td>
<td>Total Acidity (%)</td>
<td>0.05%</td>
<td>0.4</td>
</tr>
<tr>
<td>4.</td>
<td>Organic Acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a) Gluconic acid</td>
<td>–</td>
<td>313 ng/l</td>
</tr>
<tr>
<td></td>
<td>b) Citric acid</td>
<td>–</td>
<td>108 ng/l</td>
</tr>
<tr>
<td></td>
<td>c) Acetic acid</td>
<td>–</td>
<td>64 ng/l</td>
</tr>
<tr>
<td></td>
<td>d) Malic acid</td>
<td>–</td>
<td>89 ng/l</td>
</tr>
</tbody>
</table>
**DISCUSSION**

*Bacillus species* are the major workhorse industrial microorganisms with important roles which date back in time more than a thousand years [15]. The ability of different species of *Bacillus* to ferment at different pH and temperatures has led the scientists to the development of a variety of commercial enzymes, products with the desired temperatures has led scientist to the development of a variety of commercial enzyme products with the desired temperature, pH activity and stability properties to address a vast variety of specific applications. In this study the isolate M9 was isolated from Mica cores. This strain was identified as *Bacillus* species with the typical phenotypic characteristics such as rod shape and the ability to sporulate[16]. The 16 S rDNA sequence analysis revealed that the isolate is a close relative of *Bacillus sp.*19 (GenBank Accession No.HQ662601). Although Bacillus.Sp19 and M9 isolates 16s rDNA similarity values indicate that it is 99% identical to the type strain. However there is some difference in physiological characteristics.

The mica mine *Bacillus sp.* produced acid from the fermentation of several carbohydrates. The property to modify the pH is an important characteristic. Though mica’s posses acidic environments this strain is able to grow under alkaline conditions and modified pH value close to neutrality. Similarly several authors reported the isolation of alkaliphiles from acidic environments[17].

*Bacillus.sp* synthesize commercially important enzymes like amylases, Ureases lipases, chinases and Proteases .Further the ability of enzymes to be active in alkaline pH may be advantageous in biotechnological applications. Several enzymes exhibited optimum activity at pH 9 or stable under alkaline conditions[18, 19, 20, 21]. It has been reported that when cells of facultative alkaliphiles grown at neutral pH are exposed to alkaline pH an amidase is activated and the cell wall is hydrolysed[22]. However, when facultative alkaliphiles grow at alkaline pH, the cell wall gets thicker and shows an increase in the negative charge duly protecting the cell from the alkaline environment [23]. The internal pH was maintained at around 8, despite a high external pH of 8 to 11, therefore one of the key features in alkalinity is associated with the cell surface which discriminates and maintains the intracellular neural environment separate from the extracellular alkaline environment[24]. In this study alkaliphile is isolated from acidic environment this may be because of the alkaliphilic pockets in the underground mica mine deposits.

In general Bacillus strains showed variable degrees of metabolic effectiveness in silicate mineral solubilities,Our results also indicated that ‘K’ release from mica was significantly enhanced by the isolated strain Bacillus.sp.,NMM9. The mineralogy and chemical composition of the minerals may determine their susceptibility to microbial potassium and silicon mobilization[25,26].The decrease in pH resulted in increase in total acidity was not the only reason for the release of the soluble K. solubilization of the minerals is achieved through the production of metabolites that contain organic acids as the active ingredients[27, 28, 29]. The process occurs through direct oxidation pathway where gram Negative bacteria are mostly involved .The organic acids produced in the periplasm could easily diffusible into adjacent environment and subsequently dissolves insoluble forms of minerals such as calcium phosphate[30] ability of the isolates to reduce the pH of the growth medium was taken as an indication of medium acidification [31].While those that dissolve water insoluble K were assume to have the capability of producing organic acids in high quantity particularly gluconic acid. For the first time this study provides information about Bacillus inhabitants in Mica ore.

**CONCLUSION**

This results provides information on indigenous alkalophilic inhabitants of Mica with potential potassium solubilizing ability which will have potential applications in biogeocycling, biohydrometallurgy, biodegradation of xenobiotics, Chemolithotrophy and as a biomineralizer in agriculture.

**ACKNOWLEDGEMENT**

This research work was supported by the grant from University Grant Commission (UGC), New Delhi.

**REFERENCES**


