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

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Article

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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 soluilizing 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^{0}-00^{1} and 15^{0}-00^{1} and longitudes 79^{0}-35^{1} and 80^{0}-00^{1}. The chemical properties of Nellore Mica were listed in Table-1.

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Table 1: Chemical composition of Mica mines

Composition	Nellore Mica (%)
Al_2O_3	36.77
Fe_2O_3	0.21
FeO	1.64
CaO	1.28
MgO	0.72
SiO_2	46.42
F	trace
K_2O	0.94
Na_2O	0.72
Water combined	3.24
Total	99.94

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 alkaliphilic *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-defiency 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^{0}C. Later this suspension was placed on to trypticase soy agar with a pH of 10 and incubated at 37^{0}C for 4 days.

Colony Selection

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^{0}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, H_2S 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 GGC 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.

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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^{0}C, 40^{0}C, 50^{0}C and 60^{0}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 (Na_2HPO_4) 2.0g / l; Magnesium Sulphate ($MgSO_4.7H_2O$) 0.5g / l; Ferric Chloride ($FeCl_3$) 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 ingradients were dissolved in 1000ml distilled water .Then Mica powder was added. The flasks were plugged with cotton and sterilized at 1200C 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% H_3PO_4 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 phylogenic 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).

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Table 2: Physiological and Biochemical Characteristics of M9 isolate

Name of the Test	Response
Grams Staining	Gram Positive
Shape of the Cell	Rods
Arrangement of the cells	Chains
Pigment production	-
Motility	Motile
O / F test	Oxidative
Catalase	+
Indole Production	-
Methyl Red	+
Voges Proskauer reaction	+
Utilization of	
Citrate	-
Glucose	+
Lactose	+
Sucrose	+
Nitrate reduction	+
Hydrolysis of	
Starch	+
Gelatin	+
Urea	+

Table 3: Showing alignment view using combination of genbank and RDP database

Alignment view using combination of NCBI GenBank and RDP database:

Alignment View	ID	Alignment results	Sequence Description
	M9	1.00	Studied Sample
	JN086147	0.99	*Bacillus amyloliquefaciens St.Rx-35*
	JN086146	0.99	*Bacillus amyloliquefaciens St.Rx-34*
	JF899287	0.99	*Bacillus methylotrophicus St.Ht10-2*
	JN086143	0.99	*Bacillus amyloliquefaciens St.Dx-18*
	HM055608	0.99	*Bacillus amyloliquefaciens St.JS*
	GU323369	0.99	*Bacillus amyloliquefaciens St.HS8*
	JF460743	0.99	*Bacillus methylotrophicus St.Ks8-18*
	HQ831404	0.99	*Bacillus methylotrophicus St.Ns1-29*
	HQ433576	0.99	*Bacillus sp. 19(2010)*
	HQ662601	0.99	*Bacillus methylotrophicus St.Mo-Bm-16*

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 grew 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 with stand the osmotic stress at 15% (w/v) sucrose.Table-4 summarizes the growth properties of the strain under different experimental conditions

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Figure 1: Growth of the M9 isolate at different Hydrogen ion(pH) concentration

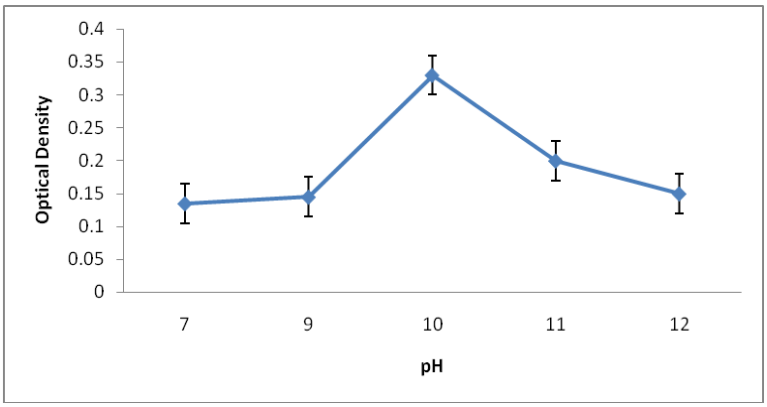

Table 4: Effect of Salinity, Osmotic pressure and Temperature on growth of M9 Mica isolate

Property	Isolate M9
Growth in the presence of NaCl (%)	
0.5	+++
5	++
10	+
Growth in the presence of sucrose (%)	
0.5	+
15	+++
60	++
Growth at different temperatures (°C)	
0	-
10	+
25	++
37	++
44	+++
55	++

Potassium Dissolution Assay

The potassium dissolution assay was performed with the identified alkalophilic Bacillus sp., NMM9 (GenBank accession JQ 9221141) 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

S.No	Parameter	Control	Culture
1.	% 'K' Release	-	40
2.	pH	7.0	4.8
3.	Total Acidity (%)	0.05%	0.4
4.	Organic Acid		
	a) Gluconic acid	-	313 ng/l
	b) Citric acid	-	108 ng/l
	c) Acetic acid	-	64 ng/l
	d) Malic acid	-	89 ng/l

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 *Bacllus sp*.19 (GenBank Accession No.HQ662601). Although Bacillus.Sp19and 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 grew 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, chitinases 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.

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