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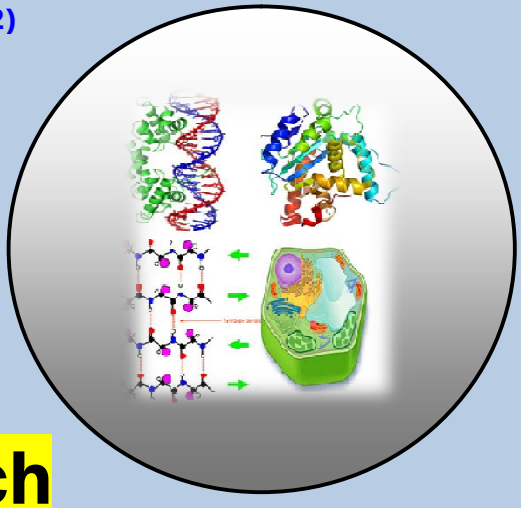

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RESEARCH PAPER
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Isolation and Characterization of Phosphate Solubilising and Growth Promoting Bacteria Isolated from Soil of Bihar

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ABSTRACT

In the present investigation five sampling sites namely Nayka Tola (Maner Block, Patna), Motirampur (Bihia Block, Bhojpur), Wetland of Parbatti (Bhagalpur), Phulbari Sharif Block Pond (Patna) and Mahane River East (Mokama, Patna) were selected for isolation of microbial strains and total 93 microbial strains were isolated. Out of total 93 isolated microbial strains, 15 strains were selected for their assessment as phosphate solubliser (phosphatase activity), starch (amylase) hydrolysis, catalase activity, casein (caseinase) hydrolysis and gelatinase activity. Out of 15 tested isolates, isolate C(43)K showed positive result on pikovskays's medium, starch (amylase) hydrolysis, catalase activity, casein (caseinase) hydrolysis and gelatinase activity and isolate D(43)A gave positive result on catalase activity, casein (caseinase) hydrolysis, gelatinase activity and oxidase positive. Based on positive test of enzymatic activities, microbial strains were further selected for their morphological, physiological and biochemical characterization. Subsequently, the characterized and identified isolates of test culture were catalogued and database prepared by the institute IMTECH, Chandigarh for future references. This study reveals that, the identified bacterial strains may be useful in agricultural field for better growth of crop plants.
Keyword: Phosphate Solubliser, Enzyme Producer, Bacteria and Agricultural Importance.

INTRODUCTION

Soil is a dynamic, living matrix that is an essential part of the terrestrial ecosystem. It is a critical resource not only for agricultural production and food security but also towards maintenance of most life processes. The functions of soil biota are central to decomposition processes and nutrient cycling.

Soil is considered a store house of microbial activity, though the space occupied by living microorganisms is estimated to be less than 5% of the total space. Therefore, major microbial activity is confined to the 'hot-spot', i.e. aggregates with accumulated organic matter, rhizosphere (RS) (Lynch, 1990; Pinton, et al., 2001).

Microorganisms involved in phosphorus acquisition include mycorrhizal fungi and PSMs (Fankem, et al., 2006). Among the soil bacterial communities, ectorhizospheric strains from *Pseudomonas* and *Bacilli*, and endosymbiotic rhizobia have been described as effective phosphate solubilizers (Igual, et al., 2001).

Evidence of naturally occurring rhizospheric phosphorus solubilizing microorganism (PSM) dates back to 1903 (Khan, et al., 2007). Bacteria are more effective in phosphorus solubilization than fungi (Alam, et al., 2002). Among the whole microbial population in soil, PSB constitute 1 to 50 %, while phosphorus solubilizing fungi (PSF) are only 0.1 to 0.5 % in P solubilization potential (Chen, et al., 2006). In particular, soil microorganisms are effective in releasing P from inorganic P through solubilization (Subba, 1982a; Subba, 1982b; Goldstein, 1986; Tandon, 1987; Kucey, et al., 1989; Richardson, 1994; Narula, et al., 2000) and from organic pools of total soil P by mineralization (Greaves and Webley, 1965; Raghu and Mac, 1966; Abd-Alla, 1994; Bishop, et al., 1994). The microbial biomass in soil also contains a significant quantity of immobilized P that is potentially available to plants (Hedley and Steward, 1982; Brookes, et al., 1984; Oberson, et al., 2001).

Strains from bacterial genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi are the most powerful P solubilizers (Whitelaw, 2000). *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa, B. sircalmous, Pseudomonas striata*, and *Enterobacter* could be referred as the most important strains (Subbarao, 1988; Kucey, et al., 1989). A nematofungus *Arthrobotrysoligospora* also has the ability to solubilize the phosphate rocks (Duponnois, et al., 2006). High proportion of PSM is concentrated in the rhizosphere, and they are metabolically more active than from other sources (Vazquez, et al., 2000). Therefore, studies have been focused on the inoculation of P-solubilizing bacteria (PSB) into the soil so as to increase the availability of native fixed P and applied phosphates as well as nutrients such as Fe and Zn through production of plant growth promoting substances and to reduce the use of fertilizers (Adesemoye and Kloepper, 2009).

MATERIAL AND METHODS

For isolation of microbes from soil samples, 0.1 gram of each soil sample was dissolved in 10 ml of normal saline. The soil suspension was diluted serially to obtain 10^{-1}, 10^{-2} and 10^{-3} dilution. After dilution, one ml of soil suspension was dispensed into 40 ml of sterilized nutrient agar medium (45^0C.) and mixed thoroughly. The medium was poured in sterilized petri plates and allowed to solidify. The plates were incubated at 37 ± 1^0C. for 3 to 7 days separately. However, the plates were examined every day with respect to their growth, morphology and number of colonies per plate. Colour of the colonies and pigment secretion of the isolates, if any, on the media and even the colony size were also examined. Number of viable colonies per gram of soil was calculated by conventional method and strains isolated from different sampling sites were recorded. Only mesophilic isolates were considered for their further investigation i.e., morphological, physiological and biochemical characterization.

Pure culture of isolate consisting of single type of species was picked up with the help of sterile needle. Purity of isolates was maintained by subsequent microscopic and physiological studies as the microbial studies involve use of pure culture. Streak plate method was used for obtaining pure culture.

RESULTS AND DISCUSSION

It is well known that a considerable number of bacterial species, mostly those associated with the plant rhizosphere, are able to exert a beneficial effect upon plant growth. Therefore, their use as biofertilizers or control agents for agriculture improvement has been a focus of numerous researchers for a number of years (Suslov, 1982; Davinson, 1988; Lemanceau, 1992; Kloepper, 1994; Glick, 1995a)

Among the bacterial genera with this capacity are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium* and *Erwinia.* There are considerable populations of phosphate-solubilizing bacteria in soil and in plant rhizospheres (Sperberg, 1958; Katznelson, et al., 1962; Raghu and Mac, 1966; Alexander, 1977). These include both aerobic and anaerobic strains, with a prevalence of aerobic strains in submerged soils (Raghu and Mac, 1966).

This group of bacteria has been termed 'plant growth promoting rhizobacteria' (PGPR) (Kloepper and Schroth, 1978) and among them are strains from genera such as *Pseudomonas, Azospirillum, Burkholderia, Bacillus, Enterobacter, Rhizobium, Erwinia, Serratia, Alcaligenes, Arthrobacter, Acinetobacter* and *Flavobacterium.* A considerably higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere in comparison with nonrhizosphere soil (Katznelson, et al., 1962; Raghu and Mac, 1966).

The concentration of soluble P in soil is usually very low, normally at levels of 1 ppm or less (10 $MH_2PO_4^-$) (Goldstein, 1994). The cell might take up several P forms but the greatest part is absorbed in the forms of HPO_4^{2-} or $H_2PO_4^-$ (Beever and Burns, 1980). Several reports have examined the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Goldstein, 1986).

For the purpose of isolation and screening of microbes, sample collection was done at the site which was highly fertile mainly due to the revaluates of Ganges and wetland areas which carry a lot of growth promoting biotic and abiotic components for common crops. Hence, the soil samples were collected from sampling sites namely Maner (village Nayka Tola), Bhagalpur (wetland of Parbatti), Patna (Phulbari Sharif block pond), Bhojpur (village Motirampur, Bihia block) and Mokama (sediments of Mahane River east) for isolation of microbial strains. Total ninety three (93) different microbial strains were isolated from all selected sampling sites. Due care was taken during sample collection. Samples were collected from varying depth i.e., from 3 to 6 inches. Soil samples were collected in separate sterilized sampling bottles. Each sample bottle was labelled properly, once they were filled with sample. The Sampling details and number of microbial strains isolated from every individual sampling station are shown in Table 1.1.

Table 1.1 Season-wise Isolation details of Microbial Strains.

Sl. No.	Sampling Site	Sampling Station	Sampling Season	No. of Microbial Strain Isolated
01	Patna	S_1-Phulbari Sharif Block Pond	June Sept. Dec. March	01 02 02 03
02	Maner	S_2-Nayka Tola (Maner Block)	June Sept. Dec. March	00 01 01 02
03	Bhagalpur	S_3-Wetlands of Parbatti (Bhagalpur)	June Sept. Dec. March	07 08 07 00
04	Bhojpur	S_4-Motirampur (Bihia Block)	June Sept. Dec. March	09 15 06 17
05	Mokama	S_5-Mahane River East	June Sept. Dec. March	04 03 05 00
Total				**93**

The soil samples were collected into separate sampling bottles and labelled properly. The sampling bottles were sterilized by proper detergent treatment followed by autoclaving (in case of reusable sample containers). Due care was taken to open the sampling bottles only at the time of sample collection and next time in laboratory under aseptic condition at the time of use. Attempts were also made to take out the soil and sediments samples by digging the soil at various depths varying from 3 to 6 inches. During the phase of isolation of microbial strains, seasonal variations were also considered rather the samples were collected in every quarter of the year i.e. pre monsoon season (June), post monsoon season (September), winter season (December) and spring season (March). For the isolation of microbes from soil samples collected, 0.1 gram of each soil sample was dissolved in 10 ml of normal saline. The soil suspension was diluted serially to obtain 10^{-1}, 10^{-2} and 10^{-3} dilution. After dilution, one ml of soil suspension was dispensed into 40 ml of sterilized nutrient agar medium (45^0C.) and mixed thoroughly. The medium was poured in sterilized petri plates and allowed to solidify. The plates were incubated at 37 ± 1^0C. for 3 to 7 days separately. However, the plates were examined every day with respect to their growth, morphology and number of colonies per plate. Colour of the colonies and pigment secretion of the isolates on the media and even the colony size were also examined. The microscopic examination revealed different types of microbial isolates on plates from different sources of soil samples.

The microbial colonies were picked up by sterilized inoculation needle and streaked on nutrient agar plates. The plates were further incubated for 3 to 7 days. The types of microbial isolates were found to be maximum in humus rich soil of Motirampur village (Bihia Block), Bhojpur.

In the present investigation, total 93 microbial strains were isolated in the laboratory. Most of the isolates were found to be mesophilic in nature growing luxuriantly at a temperature ranging from 27^0C. to 37^0C. Few of them were also found thermophilic or thermotolerant which grew and proliferated well at 45^0C. and above were isolated from soil rich in humus. Only mesophilic isolates were considered for further investigation.

Out of total 93 microbial isolates isolated from different sampling sites, fifteen (15) isolates were selected for the test for identification of phosphate solublizing potential and other enzymatic tests supporting the growth of the common crops. Initially out of fifteen (15) selected test isolates, one isolate shown halo zone on plates containing Pikovskays's medium and various positive results of enzymatic tests and another isolate gave the positive results of various enzymatic tests.

After confirmation of positive results by selected test isolates on Pikovskays's medium (phosphate solubilizing activity) and various enzymatic tests, screening and purification of test isolates were done. After screening and purification, both test isolates were preserved in the laboratory as a pure culture in slanted test tubes (containing NA media) for their further investigation i.e., characterization, identification and cataloguing. The phosphate solublizing microbes (PSM) are commonly used as inoculants for improving the growth and yield of agricultural crops.

Cultural characteristics of strains were observed on various media in order to have an idea of the nature of isolates. Various isolates of microbial strains showed a notable array of macroscopic features like diversity in spore colour, colony morphology and presence of diffusible exo-pigment by some. Characteristic pigment produced by strain may be used as a distinguishing taxonomic character. Colour of the colonies and pigment secretion of the mycelium if any revealed differences with variation in temperature of incubation, pH and composition of medium of growth. In the present study, one isolate i.e., D(43)A with visible yellow colour was observed and another isolate i.e., C(43)K didn't show any pigment colour. The heterogeneous morphology of isolates revealed high degree of phenotypic diversity among microbes in the soil of wetland and rainfed area.

Both isolates i.e., C(43)K and D(43)A grew luxuriantly on Nutrient Agar (NA) medium. Cultural characteristics of all selected 15 isolates including isolates C(43)K and D(43)A were investigated on starch casein agar (SCA), czapek-dox agar (CzDA), yeast extract malt extract agar (YEMA), starch agar (SA) besides nutrient agar (NA) media.

On yeast extract malt extract agar media, both isolates i.e., C(43)K and D(43)A showed very less growth. Poor and moderate growth of both isolates was observed on SCA medium. Moderate growth was observed by both isolates on starch agar medium. Both Isolates showed luxuriant growth on nutrient agar medium. The result of present investigation showed that nutrient agar medium was suitable for growth and sporulation of the isolates.

This suggests that the pattern of growth and pigment production as well as spore production was found to be characteristically different on different medium. Nutrient agar and starch agar were the most preferred suitable media for both strains. An overview of the result reveals that nutrient rich medium favours rapid growth and sporulation of microbial isolates.

Morphological Characterization

The external appearance of colonies of microbial strains of test isolate C(43)K and D(43)A varies. Colonies have smooth, raised, round, entire, flat or wrinkled (IMTECH Workshop Manual, 2004). Morphology of colonies on plate was the characteristic feature of bacteria and was quite useful in preliminary identification procedure. Purified colonies were allowed to grow on petri plate on nutrient agar medium (NA). Morphological features like configuration, margin, elevation, surface, pigment, opacity, Gram's reaction, spore and motility were observed. Many of these features were observable with naked eye but were confirmed by looking under a microscope. Above mentioned characteristics were noted down from the freshly grown cultures of the isolates i.e., C(43)K and D(43)A. For isolates C(43)K and D(43)A, morphological characteristic i.e., configuration, margin, elevation, surface, pigment, opacity, gram's reaction, cell shape, size (μm), arrangement, spore (s), shape, position, sporangia bulging and motility are shown in Table1.2

Table 1.2 Morphological Characteristics of the Selected Isolates

Test	C(43)K	D(43)A
Colony Morphology		
Configuration	Circular	Circular
Margin	Entire	Entire
Elevation	Flat	Convex
Surface	Round	Smooth
Pigment	-	Yellow
Opacity	Opaque	Opaque
Gram's reaction	+ve	+ve
Cell shape	Rods	Cocci
Size (um)	Length:-2-3μ Width:- 1μ	Diameter: <2μ
Arrangement	Mostly singles & pairs	Pairs & tetrads
Spore (s)	+	-
Shape	Cylindrical	-
Position	Terminal/Subturminal	-
Sporangia Bulging	-	-
Motility	-	-

+ = Present, - = Absent

Physiological Characterization

Both test isolates i.e., C(43)K and D(43)A grown at temperature between 15^0C. to 42^0C. and are thus regarded as mesophilic. Very good growth of both test isolates was observed at pH 7, 8 and 9. Isolate D (43)A was shown weak growth at pH 6.0. Present investigation also

suggests that both selected isolates were failed to proliferate or have negative activity below pH 6.0. Salinity of the medium also affects the growth and sporulation pattern of the selected isolates. Both test isolates could tolerate up to 9% NaCl concentration in the growth medium. Isolate D(43)A shown the positive growth at 10% NaCl concentration while the isolate C(43)K didn't shown the positive growth at the same concentration. The detailed physiological characteristics of the selected test isolates are shown in Table 1.3

Table 1.3 Physiological Characteristics of the Selected Isolates.

Test	C(43)K	D(43)A
Growth at Different Temperature		
4^0C	-	-
15^0C	+	+
20^0C	+	+
25^0C	+	+
37^0C	+	+
42^0C	+	+
52^0C	-	-
Growth on NaCl (%)		
2.0	+	+
5.0	+	+
7.0	+	+
10.0	-	**+**
Growth at pH		
pH 4.0	-	-
pH 5.0	-	-
PH 6.0	-	W
pH 7.0	+	+
pH 8.0	+	+
pH 9.0	+	+
Growth under anaerobic condition	+	-

+ = Present, - = Absent, W= Weak

Biochemical Characterization

During the study, isolate C(43)K had luxuriant growth on starch and pikovskays's medium while isolate D(43)A had negative growth on starch and pikovskays's medium. The isolate C(43)K produce acid from lactose while isolate D(43)A hadn't produce acid from the lactose. Isolate C(43)K had shown the hydrolysis of tween 40 and 60 and hadn't hydrolyse the tween 80 while isolate D(43)A hadn't hydrolyse the tween 40, 60 and 80.

Ammonium nitrate, amino acids, peptones and a number of proteins are utilized as nitrogen sources. Isolate C(43)K showed luxuriant growth in presence of Arginine decarboxylase, Ornithine decarboxylase and Lysine decarboxylase while isolate D(43)A had shown the negative growth on the same test. The carbohydrate utilization spectrum of selected test isolates showed that lactose and starch was being utilized by the isolate C(43)K while isolate D(43)A was not utilised by the lactose and starch.

Isolate C (43) K degrade starch, gelatine, esculin and casein by producing appropriate enzymes while isolate D(43)A degrade the casein and gelatin only. Both isolates could utilize citrate indicating that enzyme citrate permease was produced. Isolate C(43)K and D(43)A were able to reduce nitrate to nitrite. Isolate C(43)K was found to be MR +ve while D(43)A was found to be MR –ve. Both isolates was found to be VP -ve. Among both isolates no one could liberate H_2S on triple sugar iron agar. Both test isolates showed –ve test on Mc conkey agar and indole test respectively. The detailed biochemical characteristics of the selected test isolates are shown in Table 1.4

Table 1.4 Biochemical Characteristics of the Selected Isolates.

Test	**C(43)K**	**D(43)A**
Growth on Mc. Conkey agar	-	-
Indole test	-	-
Methyl red test	+	-
Voges Proskauer test	-	-
Citrate Utilisation	+	+
H_2S production	-	-
Casein hydrolysis	+	+
Esculin hydrolysis	+	-
Gelatin hydrolysis	+	+
Starch hydrolysis	+	-
Urea hydrolysis	-	-
Catalese test	+	+
Oxidase test	+	+
Nitrate reduction	+	+
Arginine dihydrolase	+	-
Ornithine decarboxylase	+	-
Lysine decarboxylase	+	-
Tween 20 hydrolysis	NG	-
Tween 40 hydrolysis	+	-
Tween 60 hydrolysis	+	-
Tween 80 hydrolysis	-	-
Acid from glucose	-	-
Acid from lactose	+	-
Gas from glucose	-	-
Phosphatase	+	-
Tyrosine	+	+
O/F test	-	-
ONPG	-	-
DNAase	-	-
Hippurate hydrolysis	-	-

+ = Present, - = Negative NG = No Growth

Cataloguing of Microbes

The microbial test isolates C(43)K and D(43)A after its characterization and identification from laboratory were sent to the IMPECH, Chandigarh for their final characterization, identification, cataloguing and preservation for future reference. The IMTECH, Chandigarh catalogued the selected test isolates with MTCC number i.e., C(43)K - *Bacillus cereus* - MTCC 8754 and D(43)A - *Micrococcus luteus* - MTCC 8923.

CONCLUSION

On the basis of overall findings (cultural, morphological, physiological and biochemical characterization) using Bergey's Manual of Systematic Bacteriology (Bergey's, 1986) of the present investigation, it may be concluded that both selected test isolates were species of genus *Bacillus* and *Micrococcus* i.e. isolate C(43)K - *Bacillus cereus*, D(43)A - *Micrococcus luteus*. Both characterized and identified test isolates from laboratory were sent to IMTECH, Chandigarh for final characterization, identification, preservation and cataloguing. The IMTECH, Chandigarh catalogued the selected test isolates with MTCC number i.e., C(43)K- *Bacillus cereus* - MTCC 8754 and D(43)A-*Micrococcus luteus* - MTCC 8923. Based on the positive enzymatic test i.e., phosphatase activity, starch (amylase) hydrolysis, catalase activity, casein (caseinase) hydrolysis and gelatinase activity and final identification of microbial strains from IMTECH, Chandigarh, this study further reveals that both bacterial isolates i.e., *Bacillus cereus and Micrococcus luteus* either individually or in consortia may be useful as agriculturally important bacteria for better growth of crop plants.

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