*NBU Journal of Plant Sciences*, Vol.2 : 30-38, 2008

# Searching for phosphate solubilizing fungal isolates from soil

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**Abstract**

A total of 354 fungal isolates were obtained from soil samples collected from forests, river basins and agricultural fields of North Bengal using serial dilution, direct soil plating, serial root washing and root maceration techniques. Cultural characteristics of the isolated fungi were studied and microscopic observations were made for identification of these isolates. All the isolates were screened for their phosphate solubilizing activities in vitro. A total of 70 fungal isolates showed phosphate solubilizing activities as detected in Pikovskaya's agar medium. Quantitative evaluation of phosphate solubilization in liquid medium supplemented with two phosphate sources (tricalcium phosphate and rock phosphate) was carried out for all the isolates showing phosphate solubilizing activity. Maximum phosphate solubilizing capacity was shown by three isolates of A. niger while A. clavatus showed minimum activity. Genomic DNA was extracted from sixteen isolates showing high activity and PCR amplification of DNA from nine isolates was done.

## Introduction

Microorganisms form a vital component of all known ecosystems of earth. Soil bacteria and fungi play pivotal roles in various biochemical cycles (BGC) (Molin and Molin, 1997) and are responsible for the cycling of organic compounds. Soil microorganisms also influence above-ground ecosystems by contributing to plant nutrition (George *et al.*, 1995), plant health (Smith and Goodman, 1999), soil structure (Wright and Upadhyaya, 1998) and soil fertility (Yao *et al.*,2000). An estimated 1,500,000 species of fungi exist in the world (Giller *et al.*, 1997). In a study conducted on the dicersity of filamentous fungi on decomposing leaf and woody litter of mangrove forest of south west coast of India, it was found that the frequency of occurrence of *Aspergillus, Cirrenalia, Penicillium* and *Trichocladium* was more than 10% and that woody litter collected during summer season showed highest fungal diversity than in the monsoon (Ananda and Sridhar, 2004). The role of rhizospheric organisms in mineral phosphate solubilization was known as early as 1903. Since then, there have been extensive studies on mineral phosphate solubilization by naturally abundant rhizospheric microorganisms. Important genera of mineral phosphate solubilizers include *Bacillus* and *Pseudomonas* (Illmer and Schinner, 1992), while *Aspergillus* and *Penicillium* form the important fungal genera. The high proportion of PSM is concentrated in the rhizospheres and is known to be more metabolically active than those isolated from sources other than the rhizosphere. Conversely, the salt-, pH- and temperature-tolerant phosphate-solubilizing bacteria have been reported to be maximum in the rhizoplane followed by the rhizosphere and root-free soil in alkaline soils (Johri *et al.*, 1999). Among PSMs, fungi perform better in acidic soil conditions (Ahmad and Jha, 1968). Species of *Aspergillus, Penicillium* and yeast have been widely reported solubilizing various forms of inorganic phosphates (Asea *et al.*, 1988; Whitelaw, 2000). Fungi have been reported to possess greater ability to solubilize insoluble phosphate than bacteria.

In this context, the present work has been envisaged for studying the microbial diversity of the north Bengal region which encompasses diverse habitats including high altitude regions, forests at different altitudes, rivers, cultivated lands as well as plantations. Since these fall under the Biodiversity Hotspots, microbial diversity is also expected to be high. Most of these regions have not yet been previously worked out.

## Material and Methods

### *Isolation and Identification of Microorganisms*

Soil samples were collected from three districts (Darjeeling, Jalpaiguri and Cooch Behar) of North Bengal. Source of soil samples were forests ( Sukhna, Lohagarh, Cinchona, Mongpong, Gorumara ) riverine soil from river basin ( Balasan, Mahananda, Dhorola, Torsa, Raidak) agricultural land ( paddy, bamboo ) rhizosphere of tea, rubber, mandarin (plantation crops) and *Cryptomeria.* Fungi from these soil samples were isolated following Warcup's soil plate method (1950) for isolating fungi from the rhizosphere with a few modifications.

### *Screening for phosphate solubilizing activity*

Screening for primary phosphate solublizing activity of the isolates was carried out by allowing the fungi to grow in selective media, i.e., Pikovskaya's agar (Pikovskaya, 1948) for 7 to 10 days at 25 °C. The appearance of a transparent halo zone around the fungal colony indicated the phosphate solublizing activity of the fungus.

### *Estimation of phosphorus*

Phosphorus solubilizing ability of fungal strains was tested in two different types of Pikovskaya (PVK) liquid medium (Pikovskaya, 1948) amended with with 0.5% tricalcium phosphate ('P' = 997 µg/ml) and 0.25% (w/v) Rock phosphate (RP-140) having $P_2O_5$ ("P" 18.8 %) pH of the medium was adjusted to 7 before autoclaving. Dissolved phosphate concentration in the culture filtrate was determined by ammonium molybdate-ascorbic acid method (Kundsen and Beegle, 1988). Difference between the amount of tricalcium phosphate and Rock phosphate added and their remainder after incubation gave the exact phosphate solubilizing potential of the isolates.

### *Isolation of fungal genomic DNA*

Isolation of fungal genomic DNA was done as outlined by Boreman (1996) with modifications.

### *PCR amplification*

PCR amplification of fungal isolates with ITS specific primer HCHITSF-1 5' GCGGAAGGATCATTACTGAG 3' and HCHITSF-2 5' GGGTATCCCTACCTGATCCG 3' was carried out in a PCR-Thermocycler Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, 2.5 mM dNTP mix, primers and Taq polymerase (1U). Polymerase Chain Reaction was performed in a total volume of 100 µl,.containing. PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 40 s and extension at 70 °C for 90 s and the final extension at 72 °C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl), and then loaded in 0.8% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

**Results and Discussion**

A total of 354 fungal isolates were obtained from soil samples collected from Darjeeling, Jalpaiguri and Cooch Behar districts of North Bengal. Source of soil samples from forest, riverine and agricultural land (rhizosphere of plantation and agricultural crops) yielded 117, 52 and 185 fungal isolates respectively (Tables 1, 2 & 3). Cultural characteristics of the isolated fungi were studied and microscopic observations were made for identification of these isolates (Fig 1). A total of 70 fungal isolates obtained from different sources showed phosphate solubilizing activities as detected in Pikovskaya's agar medium (Table 4) showing halo zones after 4-5 days of incubation (Fig 2) In a study carried out by Pradhan and Sukla (2005). The phosphate solubilization potential of *Aspergillus* isolates was determined in three different liquid medium where PVK medium showed maximum phosphate solubilization. In the present study isolates were further taken up for evaluation of phosphate solubilization potential in liquid medium, isolate RHS/P 51 and FS/ L04 showed maximum solubilization when medium was amended with tricalcium phosphate, whereas isolate RHS/R 12, FS/L 13 and FS/L 17 showed maximum amount of phosphate solubilization when the medium was amended with rock phosphate, with an average drop in the pH from 7 to 3.5. Acid production and drop in the pH of the medium have been reported in the earlier studies (Abd Alla, 1994; Whitelaw, 2000), however no significant relationship could be established in terms of phosphate solubilization and drop in the pH of the liquid medium. Some researchers have suggested that of the medium increasing P concentration in the phosphate solubilizing fungus containing medium was releated to the organic acid- types metabolities, which should correlate with the pH (Illmer and Schinner, 1992). Many studies have showed the ineffectiveness of rock phosphate use due to low solubility of its P content. Phosphate solubilizing fungus (PSF) have demonstrated the utilization of these poorly soluble phosphate source and PSFs were used as bioactivators of poorly soluble Rock phosphate (Didiek *et al.*2000).

Total genomic DNA of selected isolates were obtained efficiently following the method as outlined (Fig 2 A). ITS-PCR finger prints obtained with the primer HCHITSF-1 and HCHITSF-2 of *Aspergillus niger*, *A. melleus* and *A.clavatus* yielded single molecular weight product of 168 bp (Fig 2 B). Ribosomal DNA (rDNA) is suited for phylogenetic studies and developing molecular markers because the degree of conservation differs from one component of rDNA to another (Hibbett 1992).Internal transcribed spacer regions (ITS I and ITS II ) are found to be more variable than the three ribosomal gene subunits in an organism (Molina *et al.* 1994). ITS I region targeted in the present study to utilize ITS region to develop molecular markers for *Aspergillus* group of phosphate solubilizing fungus gave a clue that ITS specific universal primers can be used for further investigation and analysis. It has been reported that universal primer do not work consistently for some fungi (Li *et al.* 1992), the PCR products could be obtained in case of all the DNA samples taken for amplification.

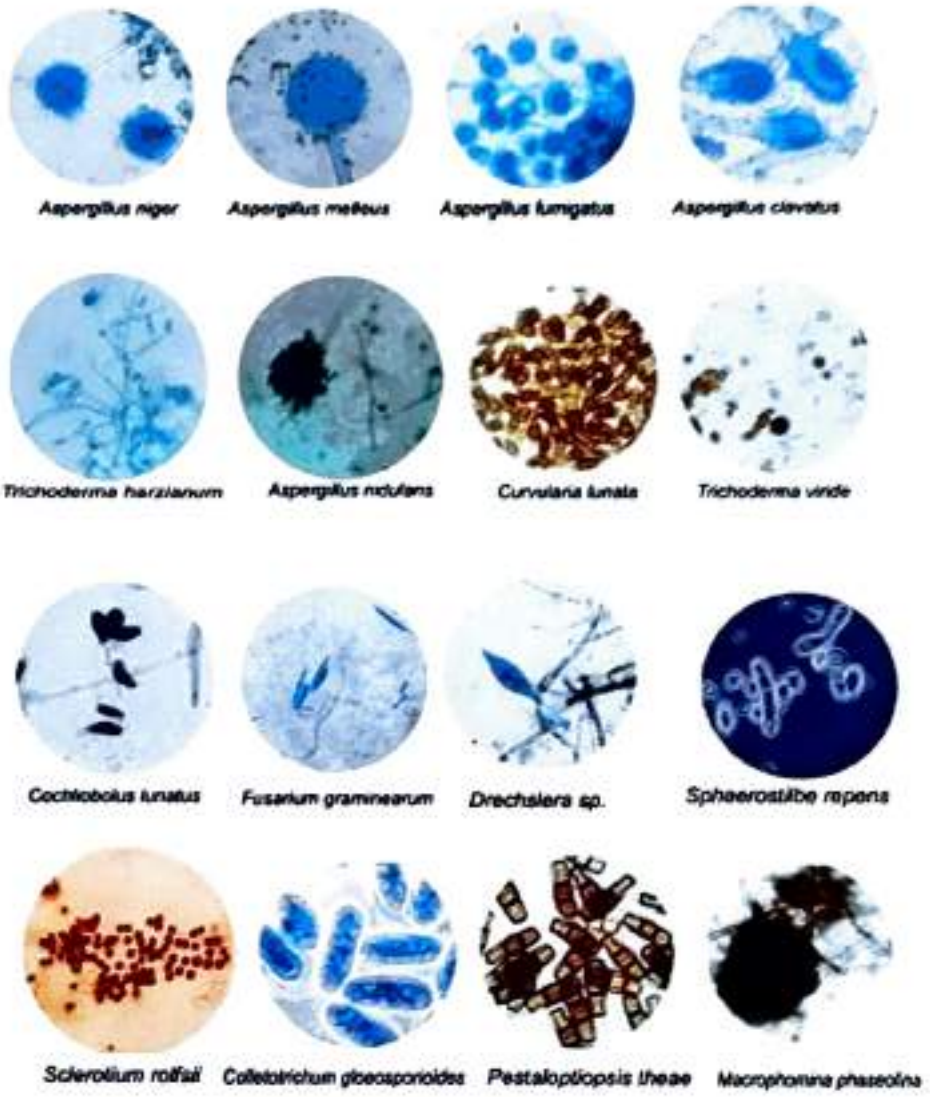


**Fig 1. Microscopic view of selected fungal isolates**

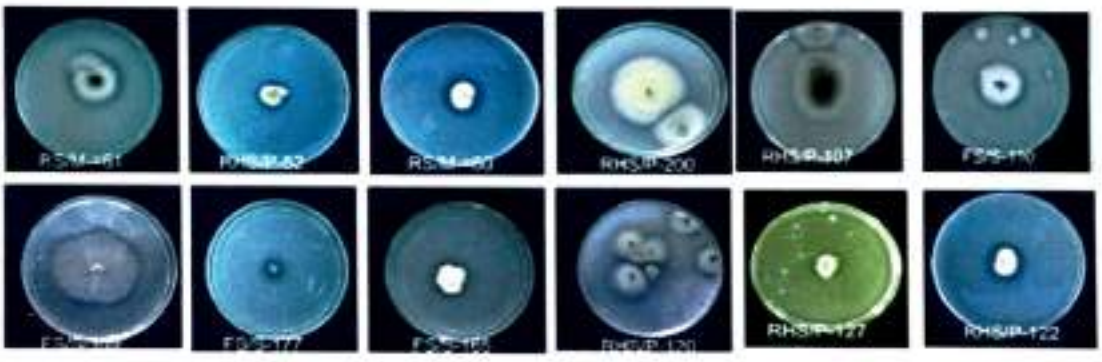

**Fig 2. Screening of fungal isolates on Pikovskaya's agar medium showing of halo zones after 4-5 days of incubation indicating the phosphate solubilization activity of the isolates**

**Table 1. Fungal isolates obtained from forest soil**

| Name of the forest | Geographical location | Isolated fungi | Total |
|---|---|---|---|
| Sukna Forest | Darjeeling | FS/S-63, FS/S-64, FS/S-108, FS/S-109, FS/S-110, FS/S-185, FS/S-186, FS/S-187, FS/S-188, FS/S-189, FS/S-278, FS/S-279, FS/S-280, FS/S-281, FS/S-282, FS/S-311, FS/S-312, FS/S-313, FS/S-314, FS/S-315, FS/S-316, FS/S-352, FS/S-353, FS/S-354, FS/S-355, FS/S-356, FS/S-357, FS/S-358, FS/S-359, FS/S -165, FS/S -166, FS/S -167, FS/S -168, FS/S -169, FS/S -170, FS/S -171, FS/S -172, FS/S -173, FS/S -174, FS/S -175, FS/S -176, FS/S -177, FS/S -178, FS/S -179 | 44 |
| Lohagarh Forest | Darjeeling | FS/L-4, FS/L-11, FS/L-13, FS/L-16, FS/L-17, FS/L-18, FS/L-19, FS/L-20, FS/L-24, FS/L-39, FS/L-40, FS/L-41, FS/L-42, FS/L-55, FS/L-56 | 15 |
| Cinchona Forest | Darjeeling | FS/C-140, FS/C-141, FS/C-142, FS/C-143, FS/C-144, FS/C-145, FS/C-146, FS/C-147, FS/C-148, FS/C-149, FS/C-150, FS/C-151, FS/C-152, FS/C-153, FS/C-154, FS/C-155, FS/C-156, FS/C-157 FS/C-160 | 19 |
| Mongpong Forest | Darjeeling | FS/M-111, FS/M-112, FS/M-113, FS/M-255, FS/M-256, FS/M-257, FS/M-258, FS/M-259, FS/M-260, FS/M-261, FS/M-262, FS/M-263, FS/M-264, FS/M-265, FS/M-266. | 15 |
| Gorumara Forest | Jalpaiguri | FS/G-226, FS/G-228, FS/G-317, FS/G-318, FS/G-319, FS/G-320, FS/G-321, FS/G-322, FS/G-323, FS/G-324, FS/G-325, FS/G-326, FS/G-327, FS/G-328, FS/G-329, FS/G-330, FS/G-360, FS/G-361, FS/G-362, FS/G-363, FS/G-364, FS/G-365, FS/G-366, FS/G-367. | 24 |

**Table 2. Fungal isolates obtained from riverine soil**

| Name of the river | Geographical location | Isolated fungi | Total |
|---|---|---|---|
| Panighata | Darjeeling | RS/P-01, RS/P-02, RS/P-03, RS/P-05, RS/P-14, RS/P-15 | 6 |
| Mahananda | Darjeeling | RS/M-60, RS/M-61, RS/M-62, RS/M-161, RS/M-162, RS/M-163, RS/M-164 | 7 |
| Dhorola | Jalpaiguri | RS/D-274, RS/D-275, RS/D-276, RS/D-277, RS/D-283, RS/D-284, RS/D-285, RS/D-286, RS/D-287, RS/D-289, RS/D-374, RS/D-375, RS/D-376, RS/D-377, RS/D-378, RS/D-379, RS/D-380 | 17 |
| Torsha | Cooch Behar | RS/T-30, RS/T-31, RS/T-32, RS/T-33, RS/T-34, RS/T-57, RS/T-58, RS/T-59, RS/T-74, RS/T-75, RS/T-76, RS/T-85, RS/T-86, RS/T-182, RS/T-183, RS/T-184 | 16 |
| Raidak | Cooch Behar | RS/R-88, RS/T-89, RS/T-115, RS/T-116, RS/T-118, RS/T-119 | 6 |

**Table 3. Fungal isolates obtained from agricultural soil**

| Name of the Plant | Geographical location | Isolated fungi | Total |
|---|---|---|---|
| Tea | Darjeeling | RHS/T-267, RHS/T-268, RHS/T-269, RHS/T-270, RHS/T-272, RHS/T-273 | 06 |
| Tea | Jalpaiguri | RHS/T-70, RHS/T-71, RHS/T-72, RHS/T-73, RHS/T-90, RHS/T-99, RHS/T-91, RHS/T-99, RHS/T-100, RHS/T-225, RHS/T-227 | 11 |
| Rubber | Darjeeling | RHS/R-06, RHS/R-07, RHS/R-08, RHS/R-09, RHS/R-10, RHS/R-10, RHS/R-12 | 07 |
| Mandarin | Darjeeling | RHS/M-01, RHS/M-02, RHS/M-03, RHS/M-04, RHS/M-05, RHS/M-06, RHS/M-07, RHS/M-08, RHS/M-09, RHS/M-10, RHS/M-11, RHS/M-12, RHS/M-13, RHS/M-14, RHS/M-15, RHS/M-16, | 16 |
| Large cardamom | Darjeeling | RHS/LC-21, RHS/LC-22, RHS/LC-23, RHS/LC-25, RHS/LC-26, RHS/LC-27, RHS/LC-28 | 07 |
| Paddy | Darjeeling | RHS/P-43, RHS/P-44, RHS/P-45, RHS/P-46, RHS/P-47, RHS/P-48, RHS/P-49, RHS/P-50, RHS/P-51, RHS/P-52, RHS/P-53, RHS/P-54, RHS/P-65, RHS/P-66, RHS/P-67, RHS/P-68, RHS/P-69, RHS/P-120, RHS/P-121, RHS/P-122, RHS/P-123, RHS/P-124, RHS/P-125, RHS/P-12, RHS/P-127, RHS/P-128, RHS/P-129, RHS/P-130, RHS/P-131, RHS/P-132, RHS/P-133, RHS/P-134, RHS/P-135, RHS/P-136, RHS/P-137, RHS/P-138, RHS/P-139, RHS/P-221, RHS/P-222, RHS/P-223 | 42 |
| Paddy | Cooch Behar | RHS/P-35, RHS/P-36, RHS/P-37, RHS/P-38, RHS/P-105, RHS/P-106, RHS/P-107, RHS/P-114, RHS/P-117, RHS/P-180, RHS/P-181, RHS/P-196, RHS/P-197, RHS/P-198, RHS/P-199, RHS/P-200, RHS/P-201, RHS/P-202, RHS/P-203, RHS/P-204, RHS/P-205, RHS/P-206, RHS/P-207, RHS/P-208, RHS/P-209, RHS/P-210, RHS/P-211 | 31 |
| Bamboo | Cooch Behar | RHS/B-218, RHS/B-219, RHS/B-220 | 03 |
| Bamboo | Darjeeling | RHS/B-241, RHS/B-242, RHS/B-243, RHS/B-244, RHS/B-245, RHS/B-246, RHS/B-247, RHS/B-290, RHS/B-291, RHS/B-292, RHS/B-293, RHS/B-294, RHS/B-295, RHS/B-296, RHS/B-297, RHS/B-298, RHS/B-299, RHS/B-300, RHS/B-301, RHS/B-302, RHS/B-303, RHS/B-304, RHS/B-305, RHS/B-306, RHS/B-307, RHS/B-331, RHS/B-332, RHS/B-343, RHS/B-344, RHS/B-345, RHS/B-346, RHS/B-347, RHS/B-348, RHS/B-350, RHS/B-351 | 35 |
| Bamboo | Jalpaiguri | RHS/B-247, RHS/B-248, RHS/B-249, RHS/B-250, RHS/B-251, RHS/B-252, RHS/B-253, RHS/B-254, RHS/B-368, RHS/B-369, RHS/B-370, RHS/B-371, RHS/B-372, RHS/B-373 | 14 |
| Cryptomeria | Darjeeling | RHS/C-308, RHS/C-309, RHS/C-310, RHS/C-333, RHS/C-334, RHS/C-335, RHS/C-336, RHS/C-337, RHS/C-338, RHS/C-339, RHS/C-340, RHS/C-341, RHS/C-342 | 13 |

**Table 4.Fungal isolates showing phosphate solubilizing activity**

| Soil type | Isolate code | Total no. of isolates |
|---|---|---|
| Forest soil | FS/L04, FS/L-13, , FS/L-17,FS/L-18,FS/L-24, FS/L-40, FS/L-41, FS/L-42, FS/S-63, FS/S-64, FS/S-108, FS/S-109, FS/S110, FS/S-112, FS/S-113, FS/C-140, FS/C143, FS/C-160, FS/S-165, FS/S-173, FS/S-177, FS/S-262, FS/S-278, FS/G-226, | 23 |
| Rhizosphere soil | RHS/R-12, RHS/P-43, RHS/P-45, RHS/P-46, RHS/P-47, RHS/P-48, RHS/P-49, RHS/P-50, RHS/P-50, RHS/P-51, RHS/P-52, RHS/P-54, RHS/P-65, RHS/P-82, RHS/P-120, RHS/P-112, RHS/P-125, RHS/P-127, RHS/P-130,RHS/T-99, RHS/T-190, RHS/T-191, RHS/P-37, RHS/P-38, RHS/P-105, RHS/P-106, RHS/P-107, RHS/P-114, RHS/P-117, RHS/P-198, RHS/P-200, RHS/P-201, RHS/P-202, RHS/P-205, RHS/P-209, RHS/B-220 | 37 |
| Riverine soil | RS/P05, RS/P/14 RS/M-60, RS/M-61, RS/D-288, RS/T-57, RS/T-58, RS/T-59, RS/R-115, RS/T-182, RS/T-183, | 10 |

**Table 5.Evaluation of fungal isolates for phosphate solubilization potential in liquid medium amended with 0.5% tricalcium phosphate (TCP) and 0.25% (w/v) Rock phosphate (RP-140)**

| Isolates | TCP | RP | Isolates | TCP | RP | Isolates | TCP | RP |
|---|---|---|---|---|---|---|---|---|
| RHS/R-12 | 810 | 385 | RHS/P-200 | 838 | 345 | FS/S110 | 842 | 367 |
| RHS/P-37 | 807 | 345 | RHS/P-201 | 836 | 342 | FS/S-112 | 842 | 354 |
| RHS/P-38 | 799 | 288 | RHS/P-202 | 829 | 350 | FS/S-113 | 848 | 360 |
| RHS/P-43 | 812 | 350 | RHS/P-205 | 842 | 340 | FS/C-140 | 824 | 344 |
| RHS/P-45 | 842 | 287 | RHS/P-209 | 827 | 331 | FS/C143 | 821 | 345 |
| RHS/P-48 | 841 | 342 | RHS/P-114 | 838 | 335 | FS/C-160 | 824 | 346 |
| RHS/P-46 | 829 | 360 | RHS/B-220 | 837 | 344 | FS/S-165 | 830 | 352 |
| RHS/P-47 | 811 | 348 | RHS/P-105 | 807 | 349 | FS/S-173 | 802 | 343 |
| RHS/P-49 | 849 | 350 | RHS/P-106 | 813 | 344 | FS/S-177 | 843 | 341 |
| RHS/P -50 | 830 | 342 | RHS/P-107 | 807 | 355 | FS/G-226 | 847 | 352 |
| RHS/P-51 | 849 | 374 | RHS/P-117 | 837 | 360 | FS/S-262 | 795 | 360 |
| RHS/P -52 | 830 | 351 | FS/L04 | 856 | 366 | FS/S-278 | 829 | 339 |
| RHS/P -54 | 839 | 350 | FS/L-13 | 817 | 381 | RS/P05 | 854 | 370 |
| RHS/P-65 | 851 | 340 | FS/L-17 | 820 | 379 | RS/P/14 | 852 | 360 |
| RHS/P-82 | 838 | 350 | FS/L-18 | 821 | 376 | RS/T-57 | 809 | 352 |
| RHS/T-99 | 832 | 341 | FS/S-24 | 810 | 338 | RS/T-58 | 802 | 354 |
| RHS/P-112 | 797 | 355 | FS/L-40 | 847 | 370 | RS/T-59 | 830 | 350 |
| RHS/P-120 | 808 | 355 | FS/L-41 | 843 | 214 | RS/P -60 | 840 | 340 |
| RHS/P-125 | 819 | 342 | FS/L-42 | 830 | 360 | RS/P -61 | 847 | 343 |
| RHS/P-127 | 820 | 345 | FS/S -63 | 839 | 332 | RS/R-115 | 836 | 338 |
| RHS/P-130 | 839 | 332 | FS/S-64 | 842 | 211 | RS/T-182 | 810 | 309 |
| RHS/T-190 | 825 | 350 | FS/S-108 | 808 | 350 | RS/T-183 | 850 | 317 |
| RHS/T-191 | 827 | 351 | FS/S-109 | 802 | 355 | RS/D-288 | 830 | 350 |
| RHS/P-198 | 841 | 346 | | | | | | |

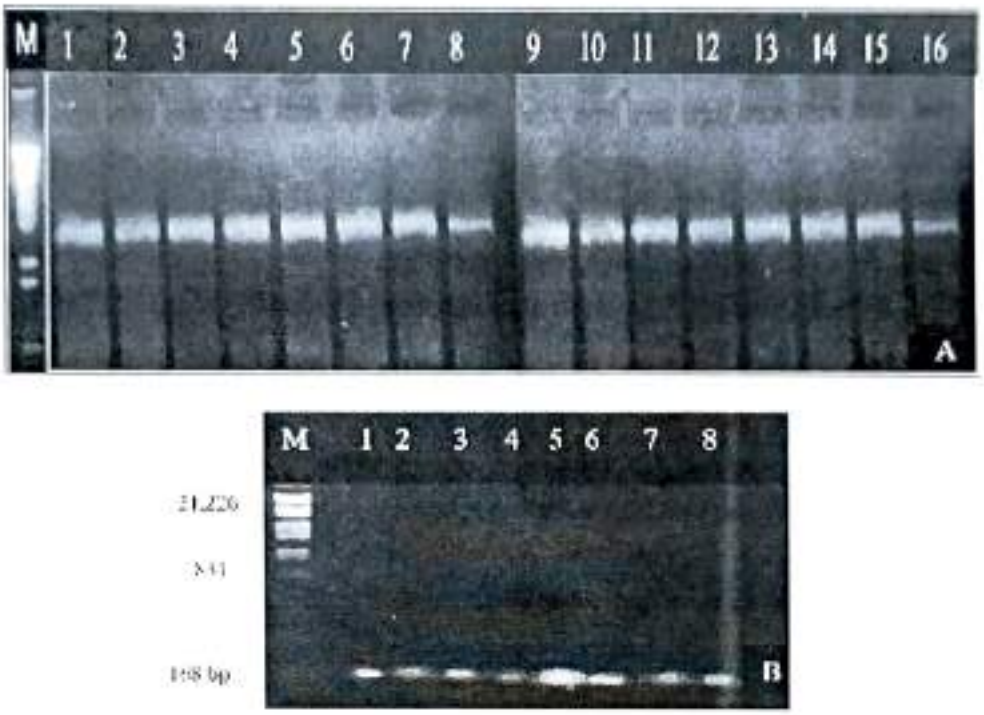


**Fig 3. (A) Agarose gel electrophoresis of genomic DNA of 1-RHS/P-82, 2-FS/S-108, 3-FS/S-109, 4-FS/S110, 5-FS/S-112, 6-FS/S-113, 7-RHS/P-120, 8-RHS/P-112,9-RHS/P-125, 10-RHS/P-127, 11-RHS/P-130, 12-FS/C-140, 13-FS/C143, 14-FS/C-160, 16-FS/S-165. (B) ITS-PCR finger prints obtained with the primer HCHITSF-1 and HCHITSF-2 of *A. niger* (lanes 1-3), *A. melleus* (lanes 4-7) and *A.clavatus* ( lane 8). M - DNA Ladder Eco R1 HindII double digest**

**Acknowledgement**

Financial support from ICAR, as a part of network project entitled Application of Microorganisms in Agriculture and Allied Sectors (AMAAS) of National Bureau of Agriculturally Important Microorganisms (NBAIM), is gratefully acknowledged.

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