Potential of plant growth promoting microorganisms as biofertilizers and biopesticides and it’s exploitation in sustainable agriculture

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ABSTRACT

Thirty three isolates were isolated from rhizospheric soil of soybean and mungbean and screened for its plant growth promoting and biocontrol activity in vitro against phytopathogenic fungi. Among them two bacteria and one fungi were selected and were screened for plant growth promoting activity based on their ability to produce IAA and phosphate solubilization activity. Their biocontrol potential was checked by siderophore, ammonia, HCN, chitinase, β-1, 3 glucanase, cellulase production against pathogenic fungi. Two bacterial isolates were denoted as MJ-1, MJ-2 and third was fungi denoted as MJ-3. MJ-1 & MJ-2 were identified as Pseudomonas spp and MJ-3 was identified as Trichoderma spp. All isolates were studied for their antifungal activities against two fungal pathogens viz. Aspergillus niger and Fusarium oxysporum by using dual culture technique. Isolate MJ-2 showed maximum 79.58% inhibition of Aspergillus niger and 53.25% inhibition of Fusarium oxysporum by dual culture method. MJ-2 also showed chitinase (72.62 µg/ml), β-1, 3 glucanase (59.36 µg/ml) after 48 hours of incubation. Highest cellulase production was observed by MJ-3 that was 90.32 µg/ml after 5 days of incubation. The results observed make all three organisms appropriate for future application as potential biocontrol agents.

Keywords: Lytic enzymes, PGPM, Biofertilizers, Biopesticides

INTRODUCTION

The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of soil are influenced by the root. This zone is about 1 mm wide, but has no distinct edge. Rather, it is an area of intense biological and chemical activity influenced by compounds exuded by the root, and by microorganisms feeding on the compounds. Rhizosphere microorganisms produce vitamins, antibiotics, plant hormones and communication molecules that all encourage plant growth. Plant growth-promoting rhizobacteria (PGPR) were first defined to describe soil bacteria that colonize the roots of plants following inoculation onto seed and that enhance plant growth [1]. PGPR enhance plant growth by direct and indirect means, but the specific mechanisms involved have not all been well-characterized. PGPR that indirectly enhance plant growth via suppression of phytopathogens do so by a variety of mechanisms. These include the ability to produce siderophores that chelate iron, making it unavailable to pathogens; the ability to synthesize anti-fungal metabolites such as antibiotics, fungal cell wall-lysing enzymes, or hydrogen cyanide, which suppress the growth of fungal pathogens; the ability to successfully compete with pathogens for nutrients or specific niches on the root; and the ability to induce systemic resistance [2].
Biological control of plant disease is defined as the involvement of the use of beneficial microorganisms, such as specialized fungi or yeast or bacteria to attack and control the plant pathogens and the diseases they are causing. Biological control of plant disease can occur through different mechanisms. A few strains of *Pseudomonas* and *Trichoderma* are also known to produce antifungal antibiotics, elicit induced systemic resistance in the host plant or interfere specifically with fungal pathogenicity factors [3]. Mostly *Pseudomonas spp.* and *Trichoderma spp.* are proved as potent biocontrol agents. The direct plant growth promoting effect of biocontrol agents, such as *Pseudomonas spp.*, in pathogen-free environments, is often associated with the following mechanisms: solubilization of insoluble P sources [4] and/or regulation of the concentration of plant growth regulators either through their production or their degradation [5,6]. Most likely, the growth promoting ability of bacteria from the genus *Pseudomonas* results from the synergistic effect of more than one mode of action. With regard to the added secondary benefits of *Trichoderma spp.* on plant growth, two main mechanisms of action have been revealed in the past decade: (i) increased plant nutrition through solubilization and/or enhanced uptake of macro- and micronutrient sand (ii) production of plant growth factors. These mechanisms are responsible for the so-called ‘biofertilizer effect’ of *Trichoderma*. Plant growth promoting *Pseudomonas* sp. generally employ an array of mechanisms like production of antibiotics, HCN, chitinase, siderophore, ammonia, fluorescent pigment and an anti fungal volatiles to antagonize pathogens [7].

The objective of the present study was to assess the plant growth promoting as well as biocontrol potential of new bacterial and fungal isolates.

**MATERIALS AND METHODS**

**Culture collection and isolation**

Thirty three isolates were isolated from the fertile soil collected from the rhizosphere of soybean and mungbean. Three isolates were selected on the basis of PGPR and biocontrol characteristics and identified and authenticated on the basis of Bergey’s manual of systematic bacteriology and Handbook of soil fungi [8]. Two known fungal phytopathogens were procured from corresponding author’s culture collection, Dept. of Microbiology, Gujarat University, Ahmedabad, India and maintained on PDA plates and they were *Aspergillus niger* and *Fusarium oxysporum*. Plates were preserved at 4°C.

**Characterization of rhizobacteria for PGP traits**

**Phosphate solubilization**

For qualitative estimation of phosphate solubilization was tested on Pikovskaya’s medium agar medium [9]. The culture were placed on Pikovskaya’s agar plate (containing insoluble tricalcium phosphate 2.5 gm, glucose 13 gm, \((\text{NH}_4)\text{SO}_4\) 0.5 gm, NaCl 0.2 gm, MgSO\(_4\).7H\(_2\)O 0.1 gm, KCl 0.2 gm, yeast extract 0.5 gm, MnSO\(_4\) trace, FeSO\(_4\).7H\(_2\)O trace, Agar 15 gm, pH adjusted to 7.2 and dissolved in 1000 ml distilled water) and the plates with bacterial cultures were incubated at 37° C for 24-48 h where as plate with fungal culture were incubated at 28±2° C for 3 to 6 days. The halo zone formation was observed after the incubation period on each plate. The colonies which forming a clear zone around them, indicating P solubilization, were counted and further used to determine the P-solubilization index (SI) was measured using following formula [10].

\[
\text{SI} = \frac{\text{Colony diameter} + \text{Halozone diameter}}{\text{Colony diameter}}
\]

Fungal and bacterial cultures were inoculated in Pikovskaya broth in 250 ml Erlenmeyer flask and incubated at 30° C for 3 days and 37° C respectively on a shaker at 120 rpm. Cultures were harvested at 24 hr intervals, centrifuged at 12,000 rpm for 20 min and the dissolved phosphate concentration in the culture filtrate was determined by vanado-molybdate method [11]. Initial pH and change in pH was noted at each 24 hr interval after 3 days of inoculation in case of fungal culture and after each 24 hr interval in case of bacterial culture by digital pH meter.

**IAA Production**

IAA was determined in vitro by the method of [12]. All the test strains were screened for IAA production. Briefly, test bacterial culture was inoculated in the nutrient broth with tryptophan (0.1%) or without tryptophan and incubated at 37° C and fungal culture was inoculated in PD broth with tryptophan (0.1%) or without tryptophan incubated at 30°C. Cultures were centrifuged at 3000 rpm for 30 min. 2 ml of the supernatant was mixed with 2
drops of orthophosphoric acid and 4 ml of Salkowski (50 ml, 35% perchloric acid; 1 ml 0.5 FeCl₃) reagent. Allowed at R.T. for 10 min and O.D. was measured at 540 nm.

Identification of IAA
Bacterial cells were separated from the supernatant by centrifugation at 10,000 rpm for 30 min. The supernatant was acidified to pH 2.5 to 3.0 with 1 N HCl and extracted twice with ethyl acetate at the double volume of the supernatant. Extracted ethyl acetate fraction was evaporated to dryness in a rotary evaporator at 40°C. Ethyl acetate fraction (10-20 ml) were plated on TLC plate and developed in solvent system (chloroform:methanol:water in the ratio of 90:20:6). Sample spots identical to authentic IAA were identified under U.V. light by spraying the TLC sheet with salkowsky reagent [13].

Siderophore Production
Cultures were grown for 72 hr at 25°C with shaking (200 rpm) in 500 ml Erlenmeyer flasks containing 125 ml Succinate medium [14]. To remove traces of iron, glassware was cleaned with 6M HCl and with double distilled water. After 72hrs withdraw the sample and centrifuge it, take 50 µl supernatant and mix 50 µl CAS dye solution and the change in colour of the sample was observed from blue to orange that shows the presence of siderophore.

Characterization of rhizobacteria for biocontrol traits
In vitro fungal inhibition on solid media
Biocontrol of phytopathogenic fungi by bacterial cultures using sterile cup borer the ditch was made in the centre of the each PDA plate in which fungal pathogens were placed and streak one to four bacterial cultures around the edge of 10 mm diameter of the plates around the ditch with bacterial cultures. The plates were incubated at 30°C for 5 days. Inhibition of the fungal growth was assayed by measuring the distance between fungal and bacterial growth as compared to control that contain only the test fungus [15]. Biocontrol of phytopathogenic fungi by fungal culture: Using sterile cup borer the ditch was made in the centre of the each PDA plate in which fungal pathogens were placed and around these ditch near the edge of the Petri plate four ditches were made in which fungal culture was placed. Inhibition of the fungal growth was assayed by measuring the distance between test fungal and fungal isolate colonies as compared to control that contain only the test fungus.

In vitro fungal inhibition assay on liquid media
In the flask containing PD broth, 10 ml bacterial culture was added and two disks of the different phytopathogenic fungal isolates were added. In case of fugal strain, 2 disk of fungal culture and two disk phytopathogenic fungi were added. Flasks were incubated on shaker at 30° C. After 7 days the percent biocontrol was measured using the control flask containing only phytopathogenic fungi [15].

HCN production
Production of HCN was determined on slants [16]. Each isolates were cultured in nutrient broth and streaked on nutrient agar supplemented with 4.4 gm glycine/L. filter paper soaked in 0.5% picric acid in 2% sodium carbonate were attached to the upper side of the test tube and the tube sealed. HCN production was estimated after 2 days of incubation at 37˚C by observing a color change in the filter paper from yellow to light brown or reddish brown.

Ammonia production
Qualitative test for ammonia was performed by using picric acid [16]. Pure cultures were strike on Nutrient agar slant. Litmus paper dipped into picric acid and NaOH containing solution was kept at the age of slant tube. Than incubated for 24 hr, after incubation it showed that pink color of litmus paper change to blue color that indicate the ammonia production. Quantitative test of ammonia was done using Nessler’s reagent in that peptone water and culture was added and incubated for 48 hr after that centrifuged it and supernatant was collected. 1ml supernatant and 1ml Nessler’s reagent were mixed with each other. Quantitative test for ammonia was done using Nessler’s reagent and O.D. taken at 640 nm [17].

Chitinase production
Chitinase production was done by using chitin as a substrate [18]. Pseudomonas sp. was grown in 100 ml of liquid medium in an Erlenmeyer flask (250ml) containing 1% chitin, 0.1% K₂HPO₄, and 0.05% MgSO₄.7H₂O (pH 7). Two ml of culture was transferred into 100ml of the same medium and grown in an orbital shaking incubator for one day at 30°C and pH 7. After incubation, the culture broth was centrifuged (4°C and 10000 rpm for 20 min), and the supernatant was collected for enzyme assay. Fungal culture was grown on media containing (g/L): MgSO₄.7H₂O
0.2gm, K₂HPO₄ 0.9gm, KCl 0.2gm NH₄NO₃ 1.0gm, FeSO₄. 7H₂O 0.002gm, MnSO4 0.002gm and ZnSO₄ 0.002gm. The appropriate carbon source (0.5 % chitin) was supplied and the pH was adjusted to 5.5 with 50 mM acetate buffer. Flasks were inoculated with either 1 ml of fungal spore suspension. Flasks were incubated in a shaker incubator (125 rpm and 30 °C) for 7 days. At the end of the incubation period, cultures were separated by filtration and centrifuged at 6000 rpm for 10 min in a cooling centrifuge at 4 °C and the clear supernatant was used as crude enzyme preparations. The effects of chitin concentration (0.1–1.5 %), initial pH (3-8) and temperature (20–45˚C) on the production of enzymes were tested. 0.5 ml substrate (1% chitin in sodium phosphate buffer, pH 8), 0.5ml enzyme and 1ml of buffer added and incubated for 10 min at 55˚C. After that 1ml DNSA solution added and further incubated for 10 min at boiling water bath and O.D. were taken at 540 nm. The amount of reducing sugar are released was calculated from standard curves for N-acetylglucosamine.

β-1,3 Glucanase production

The bacterial culture were grown in the 100 ml Broth containing K₂HPO₄ 0.1 %, MgSO₄.7H₂O .05 %, Laminarin 1 %, pH adjusted to 7.0 and kept on shaker at 150 rpm for 1-5 days at 370C. The fungal culture was grown in 100 ml broth containing(g/l) MgSO₄.7H₂O 0.2 gm, K₂HPO₄ 0.9 gm, KCl 0.2 gm, NH₄NO₃ 1.0 gm, FeSO₄.7H₂O 0.002 gm, MnSO₄ 0.002 gm, ZnSO₄ 0.002 gm, Laminarin 1%, pH adjusted to 4.8 with 50 mM acetate buffer. And kept on shaker at 125 rpm for 7 days at 30° C [15]. β-1,3 glucanase was assayed based on the release of reducing sugar from laminarin as substrate. Cultures were centrifuged at 6000 rpm for 10 min. The activity was routinely determined by incubating 0.5ml 1% substrate in 50mM acetate buffer (pH 4.8) with 0.2 ml of supernant for 30 min at 45° C and the reducing sugar produced were determined by the method by Miller using DNSA reagent.

Cellulase production

Cellulose agar plates were prepared using the CMC as a carbon source was stricked on the solidified agar and allowed to incubate for 48 hr to express cellulose depolymerization through cellulase production into its surrounding medium. The plate was stained with 0.1% Congo red and counterstaining with 1M NaCl for 15-20 min. the zone of cellulose hydrolysis was appear as a clear area against the Congo red stain background. The cultures were grown under optimam condition for cellulase production. The Pseudomonas strain grown in basal medium containing 1% CMC and incubated for 24h on a rotary shaker at 37°C. The cultures were centrifuged aseptically and supernatant were used for enzyme assay [19]. The activity was estimated using 1% solution of CMC in 0.05M citrate buffer (pH 5) as substrate. The reaction mixture contained 1ml citrate buffer 0.5ml of substrate solution and 0.5 ml enzyme solution. The reaction was carried out at 40°C for 30 min. the amount of reducing sugar released in the hydrolysis was measured using DNSA.

Statistical Analysis

Statistical analysis was done using a two way analysis of variance (ANOVA) for individual parameters. All hypotheses were tested at the 1% confidence level and ANOVA statistics were calculated using SPSS statistical software.

RESULTS AND DISCUSSION

Isolation and Identification

Two Bacterial and one fungal isolates were selected on the basis of their biocontrol potential from the thirty three isolates isolated from rhizosphere soil and sub cultured and studied (table 1). All the cultural and microscopic characteristics of colonies grown on nutrient agar plates were noted.

Table 1 Morphological characteristic of bacterial isolates on nutrient agar

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Isolate 1</th>
<th>Isolate 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>Small</td>
<td>Big</td>
</tr>
<tr>
<td>Shape</td>
<td>Round</td>
<td>Irregular</td>
</tr>
<tr>
<td>Margin</td>
<td>Entire</td>
<td>Irregular</td>
</tr>
<tr>
<td>Elevation</td>
<td>Convex</td>
<td>Convex</td>
</tr>
<tr>
<td>Texture</td>
<td>Smooth</td>
<td>Smooth</td>
</tr>
<tr>
<td>Opacity</td>
<td>Semi transparent</td>
<td>Opaque</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>Light green</td>
<td>Blush green</td>
</tr>
</tbody>
</table>

Available online at www.scholarsresearchlibrary.com
From the cultural, morphological and biochemical characteristics two isolates were identified as *Pseudomonas spp.* And one was identified as *Trichoderma spp.* [8]. They were denoted as MJ1, MJ2 and MJ3 respectively. *Trichoderma spp.* (MJ-3) colonies fast growing (5-9cm) conidiation forming compact tufts or more effuse, glaucous to dark bluish-green. Reverse typically uncolored, less often pale yellowish. Odour usually distinctly aromatic, as of coconut. Conidiophores usually not extensively branched and having a relatively loose arrangement, branches most often paired, or single or 3-verticillate, often appearing flexous. Phialides frequently paired, or arising singly or 3-verticillate, narrowly logeniform, 8-14µm X 2.4-3.0µm. Conidal globose to ellipsoidal, usually conspicuously warty, bluish-green to dark green, 4.0-4.8 X 3.5-4.0µm. Known phytopathogenic fungi was taken and they were *Aspergillus niger* and *Fusarium oxysporum.* were denoted as JPA and JPB.

**Phosphate solubilization**

In vitro solubilization of phosphate was determined on Pikovskaya agar media. Clear zone was recorded on Pikovaskaya agar media after 24 hr in case of MJ-1 and MJ-2 while after 3 days in case of MJ-3 (figure 1). Higher solubilization index was recorded of MJ-2 then of MJ-1 and the least SI index was recorded was of MJ-3. Similarly results of solubilization of phosphate in broth culture were recorded, higher Phosphate concentration was found in MJ-2 (330 µg/ml after 48 hr) then in MJ-1 (252.16 µg/ml after 48 h) and the least in MJ-3 (130.4 µg/ml after 5 days). In each culture broth pH was decreased at each 24 h interval. The results of phosphate solubilization are depicted in table 2. Evidence for phosphate solubilization by the strains of fluorescent *pseudomonads* has been reported by others [20,21]. Similar results have been reported that the solubilization of tricalcium phosphate varied from 111.5 to 404.07 µg/ml in the culture filtrates of various *Trichoderma* cultures [22].

![Figure 1 Phosphate solubilization](image)

<table>
<thead>
<tr>
<th>Name of organisms</th>
<th>Solubilization Index (SI)</th>
<th>Solubilization efficiency</th>
<th>pH in broth</th>
<th>P-solubilization (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJ-1</td>
<td>2.9</td>
<td>195.75%</td>
<td>4.9</td>
<td>252.16</td>
</tr>
<tr>
<td>MJ-2</td>
<td>3.0</td>
<td>200%</td>
<td>5.5</td>
<td>330</td>
</tr>
<tr>
<td>MJ-3</td>
<td>2.27</td>
<td>151.33%</td>
<td>6.2</td>
<td>130.4</td>
</tr>
</tbody>
</table>

**Siderophore Production**

Siderophore production was determined by the universal method method [14]. 72 hr grown culture supernatant turned to orange which the production of siderophore. Siderophore production was observed in MJ-1 and MJ-2.

It was observed that the ability *P. aeruginosa* to produce the pyoverdine type of siderophores that has good antifungal activity against plant deleterious fungi namely *A. niger* and *A. flavus* [23].
IAA Production
The IAA concentration was observed maximum in presence of tryptophan by MJ-2 after 48 hr of incubation (78.77 µg/ml), then by MJ-1 (24.52 µg/ml) and the least by MJ-3 (15.56 µg/ml) after 5 days of incubation. The results of IAA production in presence as well as in absence of tryptophan are shown in the table 3. P. fluorescens and P. chlororaphis, two known biocontrol agents with growth promoting ability, were also shown to synthesize IAA [24]. It was also reported 60% isolates of Trichoderma spp. produced IAA from total 101 isolates from Colombia [25].

<table>
<thead>
<tr>
<th>Name of organisms</th>
<th>With 0.1 % tryptophan (µg/ml)</th>
<th>Without tryptophan (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>MJ-1</td>
<td>24.52 ±2.4</td>
<td>11.32 ±2.1</td>
</tr>
<tr>
<td>MJ-2</td>
<td>78.77 ±1.8</td>
<td>47.64 ±1.5</td>
</tr>
<tr>
<td>MJ-3</td>
<td>15.56 ±1.3</td>
<td>2.83 ±1.8</td>
</tr>
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</table>

Biocontrol on solid media
The biocontrol activity of all three isolates against all three phytopathogenic fungi was observed on the PDA plates after 7 days incubation. The positive results for biocontrol activity against fungal pathogens were observed with all three isolates. The highest zone of inhibition was observed with MJ-3 against JP-B. In this case the zone of inhibition was 1.9 cm that shows the highest biocontrol of MJ-3 against fungal pathogen JP-B. Results of biocontrol on solid media are shown in table 4.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>JP-A</th>
<th>JP-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJ-1</td>
<td>1.15 cm</td>
<td>1.05 cm</td>
</tr>
<tr>
<td>MJ-2</td>
<td>1.05 cm</td>
<td>0.53 cm</td>
</tr>
<tr>
<td>MJ-3</td>
<td>1.8 cm</td>
<td>1.9 cm</td>
</tr>
</tbody>
</table>

In vitro fungal inhibition assay on liquid media
Fungal growth inhibition ability of all three isolates was tested in liquid media. All three isolates were inoculated with three different fungal pathogens in three different flasks. The maximum biocontrol was showed by MJ-3 against JP-A which was 90.49% while MJ-2 showed the maximum biocontrol against JP-B and JP-C and was about 79.58% and 64.94% respectively. The results of biocontrol in liquid media are depicted in table 5. Enhanced plant growth in addition to disease control has been achieved with Pseudomonas and Bacillus [26, 27]. It was also reported that 66%, 40.42%, 63.82%, 48.94% and 27.65% of the strains showed antagonistic effects against R. solani, M. phaseolina, Pythium sp., P. nicotianae var. parasitica and Fusarium sp., respectively by fluorescent pseudomonads under in vitro conditions [28]. Also the isolate MJ-3 showed maximum inhibition of Aspergillus spp. i.e. 90.49% by dual culture method. Similar results also shown that Trichoderma viridae strain Tr 8 showed 70%, 68.2%, 70%, 73.3%, 69.3% and 70.1% growth inhibition against R. solani, S. rolfsii, M. phaseolina, A. alternata, F. solani and C. capsici respectively [29].

<table>
<thead>
<tr>
<th>Isolates</th>
<th>JP-A</th>
<th>JP-B</th>
</tr>
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<tbody>
<tr>
<td>MJ-1</td>
<td>74.41% ±1.6</td>
<td>43.95% ±1.1</td>
</tr>
<tr>
<td>MJ-2</td>
<td>79.58% ±2.2</td>
<td>53.25% ±0.09</td>
</tr>
<tr>
<td>MJ-3</td>
<td>78.49% ±2.5</td>
<td>51.62% ±1.5</td>
</tr>
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</table>

HCN production
HCN production was observed after 24 hr of incubation at 37°C by using picrate assay method (tube method). After 48 hr MJ1 and MJ2 both shows the positive result. MJ1 showed orange brown color and MJ2 showed brown colour (table 6). The broad spectrum antagonistic activity of pseudomonads is executed by the secretion of number of metabolites including antibiotics, [30], volatile HCN [21] and siderophores [20].

Fluorescent Pigment Production
Fluorescent Pigment Production was observed under U.V. light and bluish green pigment was observed in MJ1 and MJ2.
Ammonia production

**Qualitative test:**
Pure cultures were strike on N agar slant. Litmus paper dipped into picric acid and NaOH containing solution was kept at the age of slant tube. Than incubated for 24 h, after incubation it showed that pink color of litmus paper change to blue color. That indicate ammonia production was there in MJ1 and MJ2. Quantitative ammonia production was done by the method of [17]. After 48 hr of incubation by addition of Nessler’s reagent in peptone water indicate the presence of ammonia by forming brown color. For the measurement of quantity of ammonia produced by MJ1 and MJ2, O.D was taken at 640 nm. MJ1 produce 320µmol/ml and MJ2 produce 280µmol/ml ammonia (table 7) Accumulation of ammonia in soil may increase in pH creating alkaline condition of soil at pH 9–9.5. It suppresses the growth of certain fungi and nitrobacteria due to it potent inhibition effect. Ammonia production in 95% of isolates of *Bacillus* followed by *Pseudomonas* (94.2%), *Rhizobium* (74.2%) and *Azotobacter* (45%) [31].

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Concentration in µmol/ml</th>
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<tbody>
<tr>
<td>MJ1</td>
<td>320±2.1</td>
</tr>
<tr>
<td>MJ2</td>
<td>280±1.7</td>
</tr>
</tbody>
</table>

**Chitinase production**

All the three isolates were checked for the production of chitinase in vitro using chitin as substrate gave hydrolysis zone in chitin agar plate. Subsequently, all isolates were evaluated in liquid medium using chitin as substrate. Maximum amount of chitinase production found by MJ2 after 48 hr incubation 72.62 µg/ml. MJ1 give the production of chitinase after 48 hr of incubation 61.32 µg/ml and 63.41µg/ml chitinase was produced by MJ-3 after 5 days of incubation (figure 2). Chitinolysis plays an important role in antagonism and extracellular antifungal metabolite production [5,32]. Cell wall lysis occurred by concentrated action of chitinases and β 1, 3 glucanases. The role of chitinases and its importance in biological control as well as in plant defense mechanism has been reported by [33]. *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, β1,3 glucanases and proteases [34,35,36].

**β-1, 3 Glucanase production**
The β-1, 3 glucanase production was in praise worthy amount in case of all three isolates which was determined by finding the enzyme activity of this enzyme. The maximum enzyme activity was found in case of MJ-2 (59.36 µg/ml after 48 h of incubation) while in case of MJ-3 (52.98 µg/ml after 5 days) and in case of MJ-1 (42.63 µg/ml after 72 hours). Similar observations wherein the activity of lytic enzymes (β1,3 glucanases, chitinases and proteases) was responsible for lysis of *R. solani* hyphae through digestion of major cell wall components [37,38].

**Cellulase production**
Cellulase production was checked in liquid media [19]. CMC was used as a substrate and maximum enzyme activity is found at 48 h in MJ1 and MJ2 which is 78.14 µg/ml and 65.12 µg/ml respectively and after 5 days in MJ3 which is 90.32 µg/ml which is shown in graph. It was also found similar results while *T. reesei* Rut-C30 grown on Rice bran and corn straw ratio of 5:5 gave the maximum production of Cellulase on the 6th day (18.5 IU/ml) [39]. Fagade and Bamigboye observed the 0.44mg/ml cellulase production by *Bacillus subtilis* on day 4 at 40°C [40].
CONCLUSION

On basis of results obtained it can be concluded that two species of *Pseudomonads* and one *Trichoderma* spp. have proved to be potential biocontrol agents against fungal pathogens in addition to their plant growth promoting attributes under in vitro conditions. Among these isolates, *Pseudomonas spp* (MJ-2) showed the best biocontrol towards the all fungal pathogens by maximum fungal cell wall lysing enzymes and as well as PGPR characteristics. Further studies are, however, needed to investigate the mode of action of these strains in terms of inducing systemic resistance and enhancing their antibiosis activity against fungal pathogens, as well as confirming the antagonistic ability of these strains in field trials.

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