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OPTIMIZATION OF PROTEASE PRODUCTION FROM PLANT GROWTH PROMOTING *Bacillus amyloliquefaciens* SHOWING ANTAGONISTIC ACTIVITY AGAINST PHYTOPATHOGENS

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ABSTRACT

Plant growth promoting rhizobacteria are the group of bacteria, which actively colonize plant root and exert beneficial effects on plant. They promote plant growth either by solubilizing inorganic phosphate, producing IAA or by inhibiting pathogens by producing variety of extracellular enzymes. An antagonistic bacterial strain with potent protease producing capacity was isolated from the jute rhizosphere. The isolate was identified as *Bacillus amyloliquefaciens* by means of 16S rDNA sequencing. Optimization study revealed that the optimum protease activity was 270.2 U/ml/min after 36 hours. The optimum temperature was found to be 40°C and optimum pH 8.5. Experiments with different carbon and nitrogen sources revealed fructose and yeast extract as the most favoured carbon and nitrogen. The isolate showed potent antagonistic activity against phytopathogens: *Macrophomina phaseolina* (61.5%) *Fusarium oxysporum* (82.6%) *F. Semitectum* (85.4%), *Alternaria alternata* (85.2%). Thus the isolate showed quite promising activity to be exploited as source of industrial production of protease as well as biocontrol agent.

KEY WORDS: ***Bacillus amyloliquefaciens*, 16S rDNA, Protease, phytopathogens and Antagonistic activity.**

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INTRODUCTION

Proteases, generally obtained from microbial sources are a group of very important enzymes, having wide range of applications in detergents, pharmaceuticals, leather and food and agriculture industries.[1-2] Among the microbial produces very few have been used successfully for industrial use. *Bacillus* species are the main producers of extracellular proteases.[3] Plants are always subjected to biotic and abiotic stress. Biotic stress causes severe loss in productivity every year. The infection with fungal pathogens results in huge loss of yield as well as quality of yield. The use of harmful chemicals is a common practice to control phytopathogens. But, non-judicial use of chemical fertilizers for last few decades already caused severe problem to human health and existence of normal microflora of soil. Therefore, an alternative approach, biocontrol of pathogens with plant growth promoting bacteria (PGPR) is of great interest and drawn much interest of research community. [4] Several genera have been reported as PGPR since the term was coined by Kloepper et al [5], among them *Bacillus* and *Pseudomonas* are the most prevalent. The genus *Bacillus* has been used predominantly as they can persist in the soil due to production of endospore. The mechanisms by which PGPR promote plant growth can be categorised in two types, direct and indirect. The indirect mechanisms involve production of different extracellular enzymes, which can hydrolysis fungal cell wall. Solanki et al[6] characterized mycolytic enzymes like chitinase, β-1, 3-glucanase and protease from four *Bacillus* strains, which produced these enzymes in different range with the presence of *Rhizoctonia solani* cell wall as a carbon source. Protease mediated inhibition of devastating plant pathogen *Macrophomina phaseolina* was demonstrated by Illakkiam et al.[7] Lytic enzymes produced by PGPR are capable of lysis and dissolution fungal cell wall by digesting chitin, β-1, 3-glucan and protein components present in cell wall. Bacterial isolates with capacity to produce extracellular hydrolytic enzymes have been used to inhibit deleterious plant pathogens and thereby increasing the growth of crop plants. Though production of protease by PGPR is a common phenomenon but very less work has been done on the optimization of extracellular protease, produced by plant growth promoting rhizobacteria. Hence, this study was conducted to optimize the protease production by plant growth promoting rhizobacteria *Bacillus amyloliquefaciens* Acti-6, which was isolated from jute rhizosphere.[8]

MATERIALS AND METHODS

Isolation, characterization and identification

The strain used in this study was previously isolated the rhizosphere of healthy jute plant (*Corchorus olitorius*) and sub-cultured in nutrient agar slant for their further use. For the long term use bacterial strains were maintained in 50% glycerol at -20º C.[8]

Scanning electron microscopic studies

To understand the morphology of the isolate scanning electron microscopy was performed. For this, isolates were grown in Luria Bertani broth for 48 hours and collected by centrifugation at 6000 r.p.m. for 15 minutes. The pellet was collected and washed with 0.1 M phosphate buffer saline. Then the samples were prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 6.8 followed by dehydration of the samples with different gradation of ethanol starting from 30%, 50%, 70%, 80%, 90% and 100% for 10 minutes in each. After serial dehydration samples were subjected under critical drying in CO_2 then mounted on sample stab, coated with gold palladium alloy in a mini sputter coater and examined under a JEOL JSM-6610LV Scanning Electron Microscope.

Molecular and biochemical characterization

The isolate was characterized by Gram staining, motility test, methyl red, Voges Proskauer, citrate oxidase test, catalase test, H_2S production and starch hydrolysis as per the standard methods.[9]

16 S rdna Sequencing

Extraction of genomic DNA of the isolate was done from 24-h-old culture following the method of Stafford et al.[10] For PCR amplification, DNA was amplified by mixing the template DNA (50 ng) with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase chain reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10× Taq polymerase buffer, 1 µl of 1U Taq polymerase, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 3.5 µl of 50 ng template DNA. The amplification of 16S rRNA gene of Acti-6 was carried out by PCR using the universal forward (ACT16S FP 5' AGA GTT TGA TCC TGG CTC AG 3') and reverse primer (ACT 16S RP 5' ACG CTT ACC TTG TTA CCG CTT 3'). The purified DNA was sequenced from Credora Life Sciences, Bengaluru, India, and the 16S rDNA sequence obtained from PCR product was subjected to BLAST analyses. The DNA sequence was deposited to NCBI GenBank through BankIt procedure and approved as the sequence after complete annotation and given accession numbers.

Qualitative assay

The qualitative assay for protease production was performed on sterile skim milk agar (SMA) plates (Panc. Digest of caseine 5.0, Yeast extracts 2.5, Glucose 1.0, Agar 15.0, Distilled water 1000 ml, Skim milk 7% was added as inducer). Isolates were spot inoculated and followed by incubation at 30^0 C and zone of clearance around the colony indicating the enzymatic degradation of protease.[11]

Enzyme activity assay

The protease activity was assayed by adding 5 ml of 0.65% casein as substrate (prepared in 50mM potassium phosphate buffer pH 7.5), which was incubated at 37ºC. Then 1ml crude enzyme was added and mixed thoroughly and was incubated for 10 minutes at 37ºC. After incubation, 5ml of 110 mM trichloroacetic acid (TCA) was added and mixed thoroughly to terminate the enzyme reaction and incubated for 30 minutes at 37ºC. Then the solution was centrifuged at 5000 r.p.m. for 10 minutes. Next the solution was filtered, then 2 ml of filtrate solution was taken, after that 5ml 500mM Na_2CO_3 solution and 15% Folin ciocalteau

reagent was added mixed thoroughly and incubated at 37ºC for 30 minutes. After incubation the OD was taken at 660 nm. Simultaneous controls containing enzyme, heat-killed enzyme and substrate were maintained. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmol of tyrosine min^{-1} ml^{-1}.The protein content of the culture filtrate was estimated by following the method of Bradford.[12] All enzyme assay experiments were carried out in triplicate, and the mean values were recorded. The enzyme activity was calculated by the following formula. Units/ml= (µmol tyrosine equivalent liberated X total volume of assay) / volume of enzyme used in assay X length of assay X volume used in colorimetric determination.[3]

Optimization of protease production

Optimization of extracellular protease from the isolate was done to study the effect of different parameters on enzyme production. Six parameters were tested for optimizing the protease enzyme production.

Incubation period

To study the effect of incubation period on protease production, the isolate was grown in skim milk broth at 37ºC temperature at 150 rpm. Enzyme assay was carried out at 12 hours intervals ranging from 12-96 hours.

Temperature

Effect of temperature on protease production was studied by growing the isolate in the skim milk broth for protease production at different temperature ranging from 30ºC-60ºC at 5ºC intervals. pH The effect of pH protease production in relation to initial medium pH was studied by inoculating the bacteria in skim milk broth, by adjusting the pH ranging from 5.0 to 10.

Carbon source

To investigate the influence of different carbon sources on protease production by the selected strain, the production medium was substituted with other carbon sources, including 1% (w/v) glucose, fructose, mannitol, sucrose.

Nitrogen sources

The effect of nitrogen sources on protease production of the organisms were determined by using different organic and inorganic nitrogen sources (0.5%). Nitrogen compounds are very important for growth and enzyme production. The nitrogen source of the skim milk broth is substituted with –beef extract, yeast extract, peptone, urea.

Metal ions

To study the effect of metal ions on protease production, media was substituted with different metal ions-calcium chloride, magnesium chloride, manganese chloride, zinc chloride at a concentration of 5 mM.

Antagonistic activity

Antagonistic activity of the isolate was evaluated against fungal pathogens, *Macrophomina phaseolina, Fusarium oxysporum, Fusarium semitectum, Alternaria alternata.* For this the bacterial isolates were streaked at a distance of 3.5 cm from rim of individual Petri plate containing PDA medium. 6mm mycelial disc from a 7days old PDA culture o fungal pathogens were then placed on the other side of the Petri dish and the plates were incubated ay 28^0 C for 7 days. Simultaneously, one control plate only with fungal disc was also maintained. Antagonistic activity was estimated from the inhibition of mycelial growth of fungus in the direction of actively growing bacteria. The level of inhibition was calculated by subtracting the distance (mm) of fungal growth in the direction of an antagonist from the fungal radius. The percent inhibition was calculated using the formula[13]:

PI = (R-r) × 100 / R, Where,
PI = Percent inhibition; **r** = Redial growth of the fungal colony opposite the bacterial colony and
R = Radial growth of the pathogen in control plate.

All the experiments were carried out in triplicate, and the standard deviation for each test was calculated using SPSS 1.0. The standard deviations (n = 3) are indicated as error bars. Data were analyzed using One-way analysis of variance (ANOVA) and Duncan multiple range test (DMRT).

RESULTS

Morphological and biochemical characterization

The isolate, previously isolated from jute rhizosphere was characterized morphologically, biochemically. Gram staining revealed that the isolate was a Gram positive rod shaped bacterium. The shape of the bacterium was further confirmed by scanning electron micrographs, which reveals that it was a large rod shaped bacterium with a length of 2.29 µm (Fig 1).

Identification

The protease producing strain was identified on the basis of 16S rDNA sequencing. BLAST analysis identified the isolate Acti-6 as *Bacillus amyloliquefaciens* with 100% similarity with the respective strains in NCBI GenBank database. This nucleotide sequence submitted to GenBank was provided an accession number KT192627.The isolate was characterized biochemically. Results of biochemical tests are summarized in Table 1.

Table 1
Morphological and biochemical characteristics of Bacillus amyloliquefaciens.

Tests		*Bacillus amyloliquefaciens* (Acti-6)
Gram nature		+
Endospore		+
Catalase Test		+
Oxidase		+
Gelatin liquefaction		+
Urease		-
Nitrate reduction		+
MR-VP reaction	MR	-
	VP	+
Casein hydrolysis		+
Starch hydrolysis		+
Indole production		-

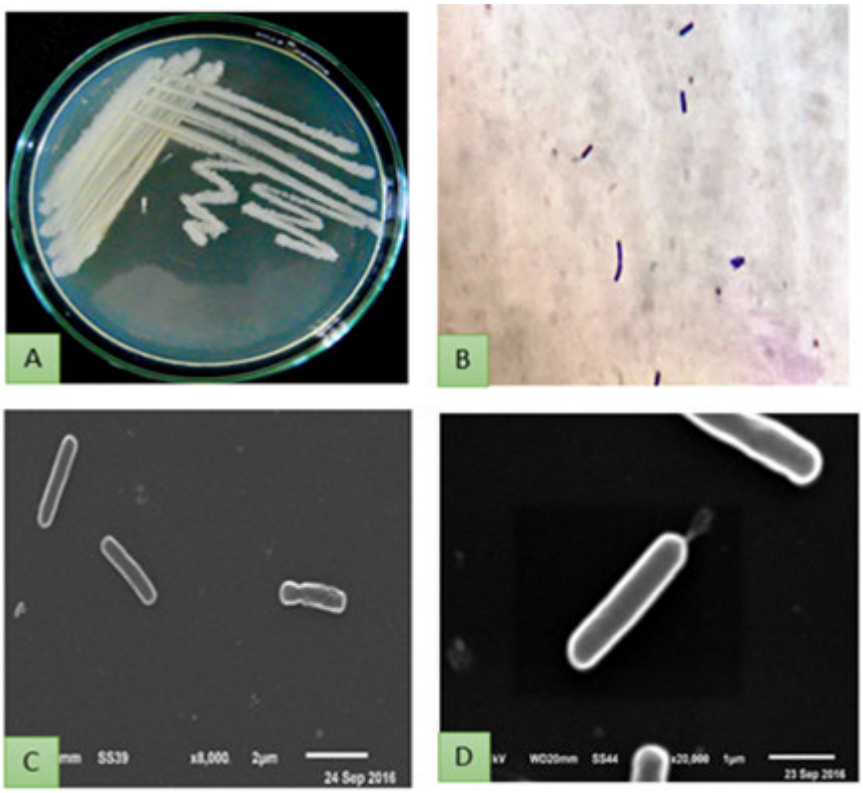

Figure 1
Light microscopic view and scanning electron micrographs of *B. amyloliquefaciens*: pure culture (A), Acti-6 under light microscope (B), Scanning electron micrographs (C & D).

The isolate was preliminary screened for its protease production on SMA for protease production. The isolate produced a clearing zone of 18 mm, which prompted for further optimization of protease production (Fig 2).

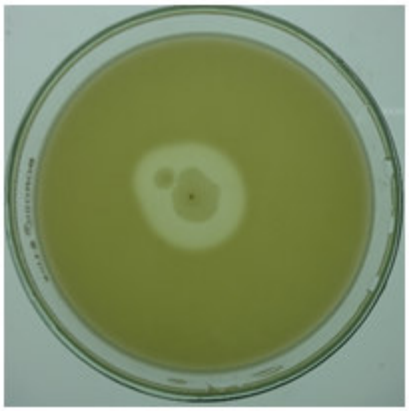

Figure 2
Protease production by *B. amyloliquefaciens* on skim milk agar plate

Optimization of protease production

Protease production by the isolate *B. amyloliquefaciens* was quantified and optimized against different parameters, such as temperature, initial pH, carbon source, nitrogen source and metal ions.

Effect of incubation period

To understand the effect of incubation period on protease production, culture filtrate was pulled off at different time intervals and assayed. The study revealed that the isolate *B. amyloliquoefaciens* showed highest protease production after 36 hours of incubation (p=0.05). After that, a gradual decrease of protease production was recorded (Fig. 3A).

Effect of incubation temperature

Effect of temperature of protease production was evaluated by growing the isolate at different temperature at a range between 30°C to 60°C, with a difference of 5°C. It was found that the isolate showed optimum protease production was at 40°C (Fig. 3B).

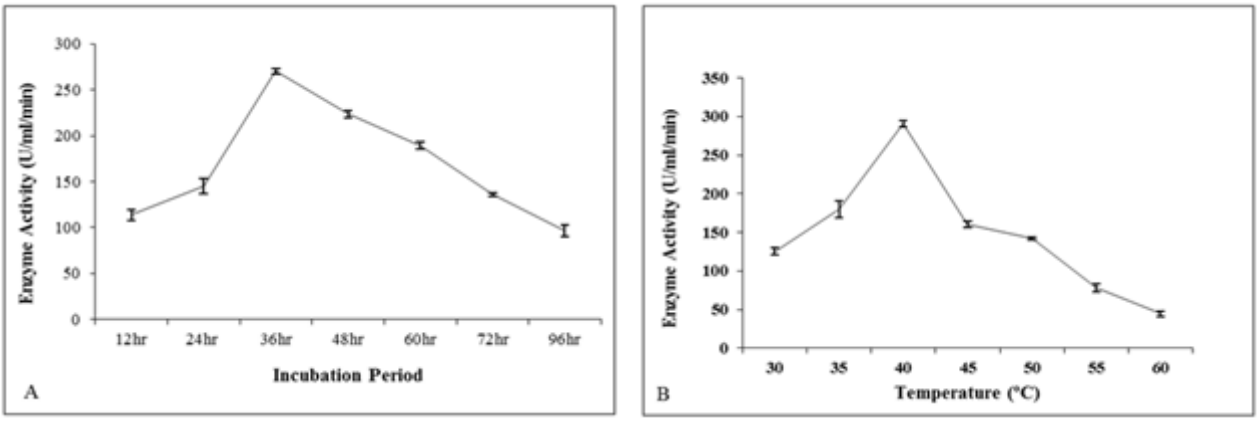

Figure 3
Effect of incubation period (A) and incubation temperature (B) on protease production by B. amyloliquefaciens
Data represent mean ± SD (n=3)

Effect of initial pH of Medium

The pH of skim milk broth was adjusted from pH 5.0 to pH 10.0, and protease activity was recorded after 36 hours. It was observed that the production of protease was maximum in the range of pH 8 to 9 (Fig. 4A). Though apparently highest protease activity was quantified at pH 8.5 but statistical analysis revealed that there is no significant difference between the enzyme activity at pH 8, 8.5 and 9.

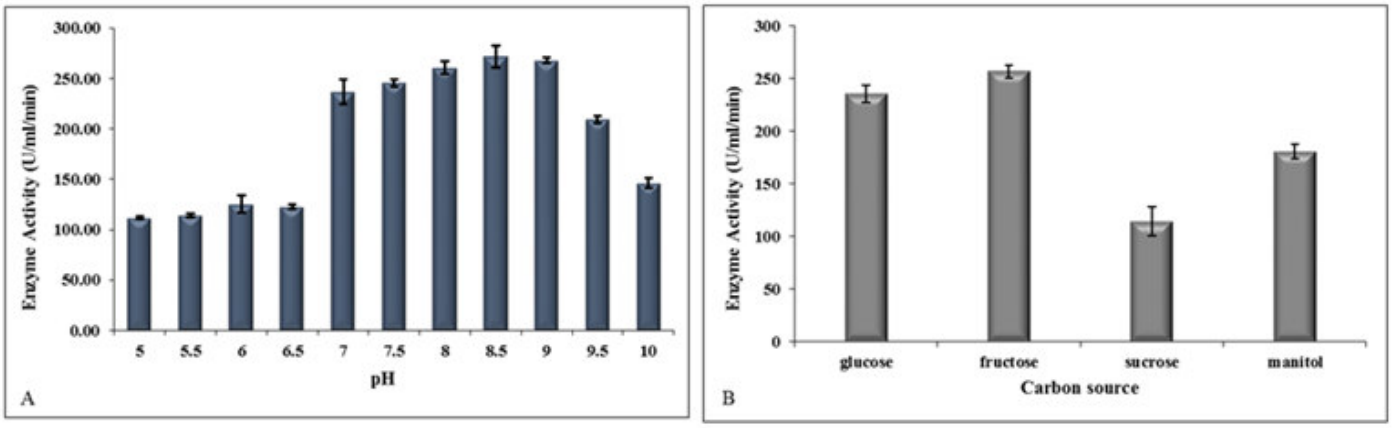

Figure 4
Effect of pH (A) and carbon source (B) on protease production by B. amyloliquefaciens. Data represent mean ± SD (n=3). Different letters above the bars indicate significant difference in DMRT (p=0.05).

Effect of carbon source

The influence of different carbon sources, including glucose fructose sucrose mannitol, on protease production was investigated. The results indicated that different carbon sources have a different impact on the production of extracellular protease by *B. amyloliquefaciens*. All tested carbon sources supported the growth of the isolate. However, among the various carbon sources, fructose was found to support maximum protease production (256.6 U/ml), whereas lowest production was observed in presence of sucrose (Fig. 4B).

Effect of nitrogen source

The influence of various nitrogen sources on protease production was evaluated using the production medium containing fructose as the sole carbon source. The results indicated that among the nitrogen sources, the protease production was recorded highest in yeast extract and the protease production is lowest in urea (Fig. 5A).

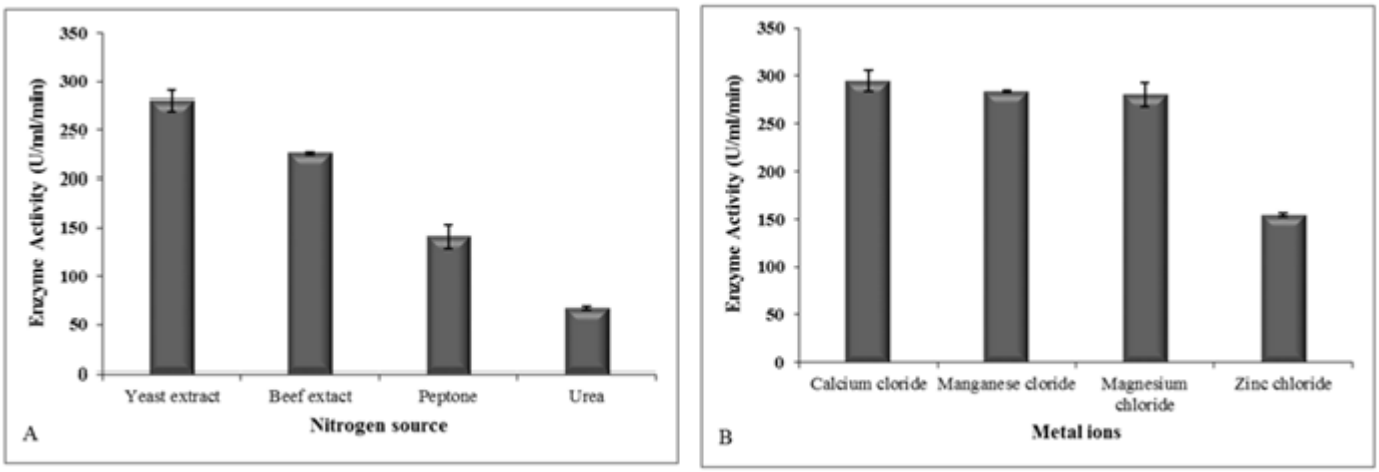

Figure 5
Effect of nitrogen source (A) and metal ions (B) on protease production by B. amyloliquefaciens. Data represent mean ± SD (n=3). Different letters above the bars indicate significant difference in DMRT (p=0.05).

Effect of metal ions

The effect of various metal ions on protease production was evaluated .The results indicated that most of the metal ions positively influenced protease production, except zinc (Fig. 5B).

Antagonistic activity

In dual culture method, after 7 days of incubation the isolate *B. amyloliquefaciens* inhibited the pathogens to varying degree. Growth reduction of *M. phaseolina*, *F. oxysporum*, *F. semitectum*, *A. alternata* was observed when dual cultured on PDA medium. The percentage of inhibition (PI) was calculated by the equation R= (R-r) x 100 /R. The results revealed that *B. amyloliquefaciens* inhibited the growth of all test pathogens used in study. The percentage of inhibition varies between 61.5-86.8% (Table 2; Fig. 6, A-F). The highest percent of inhibition was observed against *F. semitectum*, followed by *A. alternata*, *F. Oxysporum* and *M. phaseolina*.

Table 2
***In vitro* antagonistic tests of *B. amyloliquefaciens* against Phytopathogens**

Phytopathogens	Solid medium		
	Radial growth in control plate R	Radial growth in treated plate r	Percent inhibition (PI)
M. phaseolina	9.1±0.12	3.5±0.01	61.5%
F. oxysporum	8.6±0.5	1.5±0.04	82.6%
F. semitectum	8.2±0.21	1.2±0.08	85.4%
A. alternata	6.1±0.09	0.9±0.01	85.2%

Data represented as mean ±SD, n=3

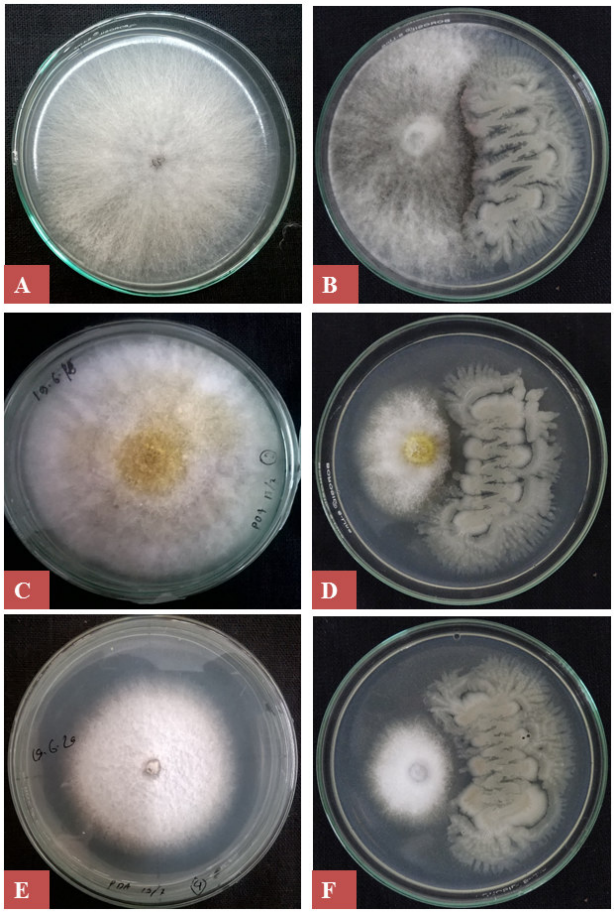

Figure 6
A-F: *In vitro* antagonistic activity of *B. amyloliquefaciens* against test pathogens *B. amyloliquefaciens* paired with *Macrophomina phaseolina* (B), *Fusarium semitectum* (D) and *F. oxysporum* (F) with A, C & E being respective control

DISCUSSION

The isolate (Acti-6) obtained from jute rhizosphere showed several PGPR activities *in vitro* and was used in this study to evaluate its capacity for protease production. The ability of this bacterium to produce protease was confirmed through the production of halo zone. Biochemical characterization and molecular studies confirmed the identity of Acti-6 as *Bacillus amyloliquefaciens*. Protease production by the members of *Bacillus* group was reported by several authors. Moreover, members of *Bacillus* group are the most common soil bacteria with plant growth promoting activities. In their study Mandal et al[14] isolated six isolates belonging to the genus *Bacillus* showed protease activity on skim milk agar plates. Protease production by *Bacillus amyloliquefaciens* was reported by Nassar et al. [15] When optimization study was carried out, the isolate *B. amyloliquefaciens* showed optimum protease production after 36 hours of incubation, after that production of protease showed continuous decrease. Similar type of decrease of protease production after 36 hours was observed by Pant et al.[3] The isolate showed optimum protease production at 40°C, after that with increase in incubation temperature protease production decreased also which indicates thermolabile nature of the enzyme. *B. amyloliquefaciens* showed capacity to grow in a wide range of pH form 5 to 10. The optimum pH of medium for protease production was found to be in the range between 8 t0 9, which indicates its alkaline nature. Haddar et al [16] showed optimum protease production by *Bacillus mojavensis* around pH 9. Other experiments conducted by previous workers showed that an incubation temperature of 37°C and pH 9.0 was the optimum requirement for protease production. [17, 18] The most suitable substrate for production of protease from *B. amyloliquefaciens* strain was Fructose as carbon source and yeast extract as nitrogen source. Sangeetha et al[19] reported similar type of observations in *Bacillus pumilus* SG 2. The optimum protease production was observed in presence of fructose as carbon and yeast extract and casein as nitrogen sources. *Bacillus cereus* strain 146 was reported to use fructose for maximum amount of alkaline protease production.[20] Fructose is easily available and cost effective. In presence of metal ions used in this study, production of protease was influenced differentially. In presence of calcium and manganese chloride the production of protease increased. Stimulatory effects of Mn^{2+} ions on microbial proteases also have been reported by other authors.[21-22] Decrease in protease activity was observed in case of Zn^{+2}.When antagonistic potentiality of the isolate was evaluated by dual culture assay, it showed quite a promising activity against all the phytopathogens used in this study. Many antagonistic bacteria secrete mycolytic enzymes among which proteases, in particular, play an important role in the cell lysis process. Proteases bind to the outer mannoprotein layer of fungal cell wall and expose inner glucan and chitin layer.[23-24]

CONCLUSION

From the above study it can be concluded that, the isolated *Bacillus amyloliquefaciens* showed capacity of protease production at pH 8.5 and after 36 hours of incubation. The isolate also showed antagonistic activity against a number of pathogens. Further study is needed to establish the exact role of protease in control of phytopathogens by *B. amyloliquefaciens*. Thus the isolate shows quite promising activity to be exploited as biocontrol agent.

CONFLICT OF INTEREST

Conflict of interest declared none.

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