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Full Length Research Paper

Evaluation of extracellular lytic enzymes from indigenous *Bacillus* isolates

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The aim of the investigation was to study the hydrolytic enzymes viz., chitinase, protease, , β-1, 3 glucanase and cellulase from the isolates of *Bacillus* sp. (twenty eight) which isolated from tomato rhizospheric soil in IIVR farm (DPNSB-1 to 7), IIHR farm (DPNSB-8 to 15), IARI farm (DPNSB-16 to 20) and farm of APHU (DPNSB-21 to 28). Among the strains, IARI isolate of DPNSB-18 exhibited the highest chitinase activity (4.65 IU/ml), IIHR isolate of DPNSB-15 produce highest protease activity (0.79 IU/ml), maximum , β-1, 3 glucanase production was noted in *Bacillus* strains viz., DPNSB-14 (IIHR isolate), DPNSB-2 (IIVR isolate) and DPNSB-20 (IARI isolate), range from 0.24 IU/ml to 0.39 IU/ml, cellulase production was made by isolates of IIVR, DPNSB-3 (0.75 IU/ml) and DPNSB-1 (0.60 IU.ml) respectively.

Keywords: *Bacillus* spp., Chitinase, Protease, β-1, 3 glucanase, Cellulase

INTRODUCTION

Developing predictive models for biocontrol is undoubtedly a complex endeavor because of the multi component nature of the system. Biological control is a disease management strategy employed to protect agricultural areas would not only be economical but also durable by sustaining the reduction of inoculums potential and amount of disease produced by pathogens. Since biological control is a result of many different types of interactions among microorganisms, scientists have concentrated on characterization of mechanisms occurring in different experimental situations (De Meyer and Hofte, 1997; Elad and Baker, 1985; Bar-Shimon et al., 2004; Compant et al., 2005; Cota et al., 2007). Indeed a number of rhizobacteria, in particular *Bacillus* spp., have already been reported to inhibit the growth of fungal pathogen (Deepa et al., 2010; Kumar et al., 2011; Gopalakrishnan et al., 2011; Gajbhiye et al., 2010)

Rhizosphere bacteria are excellent agents to control soil-borne plant pathogens. Bacterial species like *Bacillus* have been proved in controlling the fungal diseases. Earlier reports showed that they capable of lysing chitin, which is a major constituent of the fungal cell wall, play an important role in biological control of fungal pathogens (Mitchell and Alexander, 1962). Purified forms of such metabolites and lytic enzymes inhibit the mycelial growth of certain fungi (Basha and Ulaganathan, 2002; Yoshida et al., 2001; Yu et al., 2002). The mechanisms for the suppression of pathogens by *Bacillus* has primarily known to be caused by three following mechanisms include mycoparasitism, competition for space and resources and antibiosis. The extracellular cell wall degrading enzymes excreted by many strains of *Bacillus* are traditionally included in the concept of mycoparasitism, due to their integral role in direct physical interactions (Yu et al., 2002; Zhang and

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Fernando, 2004; Abdullah et al., 2008).

The objective of the present study was to test the possible role on *in vitro* production of lytic enzymes viz., chitinase, β-1, 3-glucanase, protease and cellulase from rhizospheric *Bacillus* spp.

MATERIALS AND METHODS

Strains used in the study

Twenty eight *Bacillus* strains were isolated from various tomato rhizospheric soil of India viz., 1).IIVR farm (DPNSB-1 to 7), 2). IIHR farm (DPNSB-8 to 15), 3). IARI farm (DPNSB-16 to 20) and 4).farm of APHU (DPNSB-21 to 28) were used in this study. The current investigation focuses on the exact mechanism involved in pathogenic mycelia inhibition via cell wall degrading enzyme production (qualitatively).

Estimation of cell wall degrading enzymes

Estimation of chitinase

Strains were cultured at 28 °C for 96 h on a rotary shaker in 250 ml conical flasks containing 50 ml of chitin–peptone medium as described by Lim et al., 1991. The cultures were centrifuged at 12,000g for 20 min at 4 °C and the supernatant was used as enzyme source. Colloidal chitin was prepared from crab shell chitin according to Berger and Reynolds (1958). The reaction mixture contained 0.25 ml of enzyme solution, 0.3 ml of 1M sodium acetate buffer (pH 5.3) and 0.5 ml of colloidal chitin (0.1%). The reaction mixture was incubated at 50°C for 4 h in a water bath. Chitinase activity was determined by measuring the release of reducing sugars by the method of Nelson (1944). One unit of chitinase was determined as 1 nmol of GlcNAc released per minute per mg of protein.

Estimation of β-1,3 glucanase

For determination of β-1,3 glucanase activity, bacterial strains were grown in synthetic medium which contains (per liter) 10 g maltose, 0.68 g KH_2PO_4, 0.87 g K_2HPO_4, 0.2 g KCl, 1 g NH_4NO_3, 0.2 g $CaCl_2$, 0.2 g $MgSO_4$, 0.002 g $FeSO_4$, 0.002 g $MnSO_4$, (pH 6). After two days of incubation at 28 ± 2°C, supernatant was separated by centrifugation at 10,000rpm for 5min, followed by estimation of β- glucanase using Azo-Barley Glucan method ((McCleary and Shameer 1985). One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1µM glucose in 1min at 50°C.

Estimation of protease

Alkaline protease activity was determined by applying a modified form of the method given by Takami et al. 1989. According to this procedure 0.25 ml of glycine:NaCl:NaOH (50 mM, pH 7.0) buffer was incubated with 2.5 ml of 0.6% casein (Merck) dissolved in the same buffer at 30◦C until equilibrium was achieved. An aliquot of 0.25 ml of the enzyme solution was added to this mixture and incubated for 20 min. The reaction was stopped by adding 2.5 ml TCA solution (0.11M trichloroacetic acid, 0.22M sodium acetate, and 0.33 M acetic acid). After 10 min the entire mixture was centrifuged at 5000×g for 15 min. The supernatant in the amount of 0.5 ml was mixed with 2.5 ml of 0.5M Na_2CO_3 and 0.5 ml of Folin-Ciocalteu's phenol solution and kept for 30 min at room temperature. The optical densities of the solutions were determined with respect to the sample blanks at 660 nm using spectrophotometer. For these studies, one protease unit was defined as the enzyme amount that could produce 1 g of tyrosine in 1 min under the defined assay conditions.

Estimation of Cellulase

One ml spore suspension ($6x10^6$ spores ml^{-1}) of each strain (DPNSB1-DPNSB28) was inoculated individually into 100 ml Erlenmeyer flasks containing low viscosity carboxy methyl cellulase (CMC) medium [(g 1000 ml^{-1}): CMC, 10.00; KH2PO4, 1.00; NaNO3,2.00; MgSO4 _ 7H2O, 0.01; yeast extract, 10.00 and the pH adjusted to 6.0]. The cellulase activity (mg of reducing sugar produced 24 h^{-1} ml^{-1}) was estimated by the procedure described by Mahadevan and Sridhar (1998).

Statistical analysis

All the values of enzyme activity are the mean values of at least three replicates. Data obtained from all the experiments were analyzed by analysis of variance ANOVA using SPSS statistical Package. Least significance difference (LSD) at 5 % level of significance (P = 0.05) was used to compare the mean values of different treatments in the experiment.

RESULTS

Estimation of cell wall degrading enzyme production

The production of different types viz., chitinolytic, proteolytic, glucanolytic and cellulolytic enzyme activities by the *Bacillus* isolates were quantified using standard methods and assayed (Figure 1 – 4). Results were

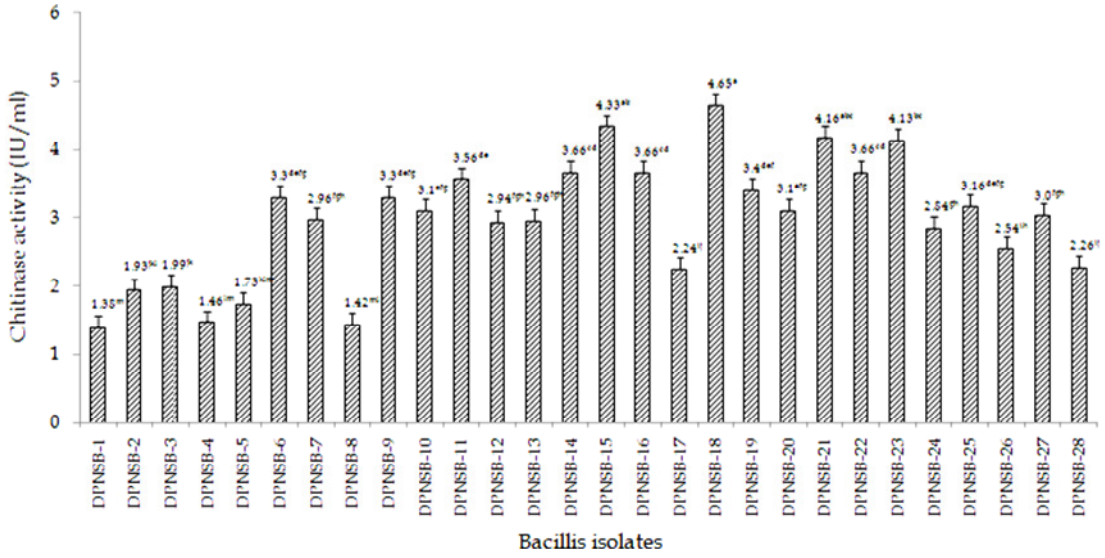

Figure 1. Production of Chitinase from Bacillus isolates

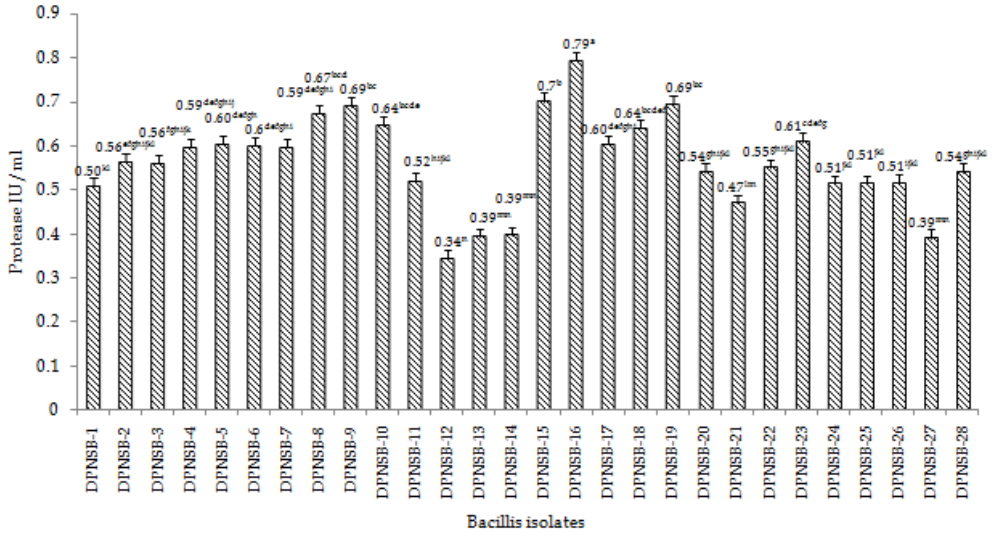

Figure 2. Production of Protease from Bacillus isolates

recorded according to the IU/ml produced in the enzyme assays. Chitinase activity was analyzed using a chitin amended medium. Among the 28 strains of *Bacillus* tested for production of chitinase, IARI isolated of DPNSB-18 recorded the highest chitinase activity up to 4.65 IU/ml followed by DPNSB-15 (4.33 IU/ml) from IIHR farm and DPNSB-21 (4.16 IU/ml) from APHU farm respectively. The inefficient strain DPNSB-1, DPNSB-4 and DPNSB-8 which produced more or less similar to 1.42 IU/ml (Figure 1). Protease activity was analyzed using casein as a whole protein source. It is evident from the data in Figure 2 that the *Bacillus* strain DPNSB-15 (0.79 IU/ml), isolate of IIHR recorded the highest protease activity followed by one isolates from IARI (DPNSB-19), isolate from IIHR (DPNSB-9) produce similarly (0.69 IU/ml) and IIHR isolate DPNSB-8 produce up to 0.67 IU/ml (Figure 2). The lowest production (0.34 IU/ml) was produced by IIHR isolate i.e DPNSB-12 respectively.

β- 1, 3 glucanase was estimated by a kit method. The maximum glucanase production was recorded in *Bacillus* strain DPNSB-14 (IIHR isolate), DPNSB-2(IIVR isolate), and DPNSB-20 (IARI isolate), range from 0.24 IU/ml to 0.39 IU/ml. Where isolate of IIVR (DPNSB-7) produce

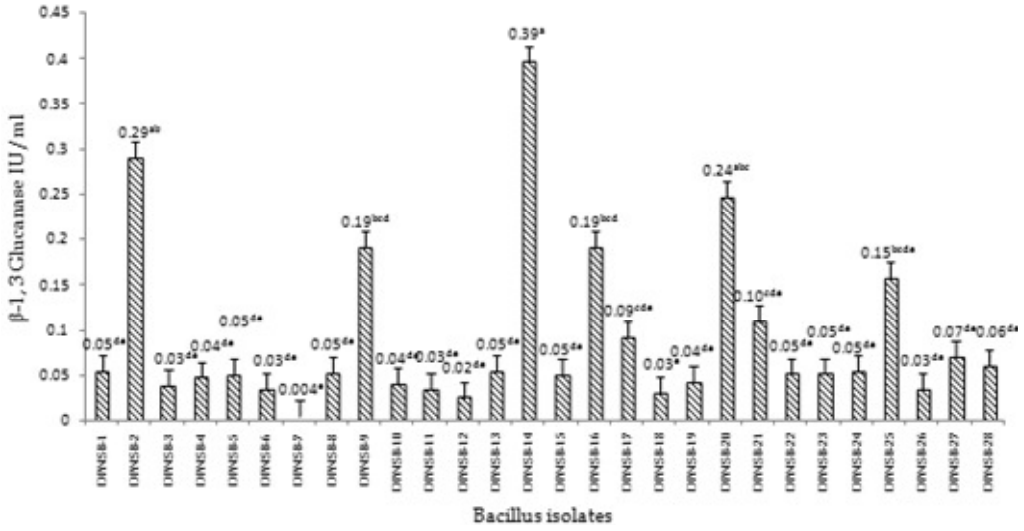

Figure 3. Production of β-1, 3 glucanase from Bacillus isolates

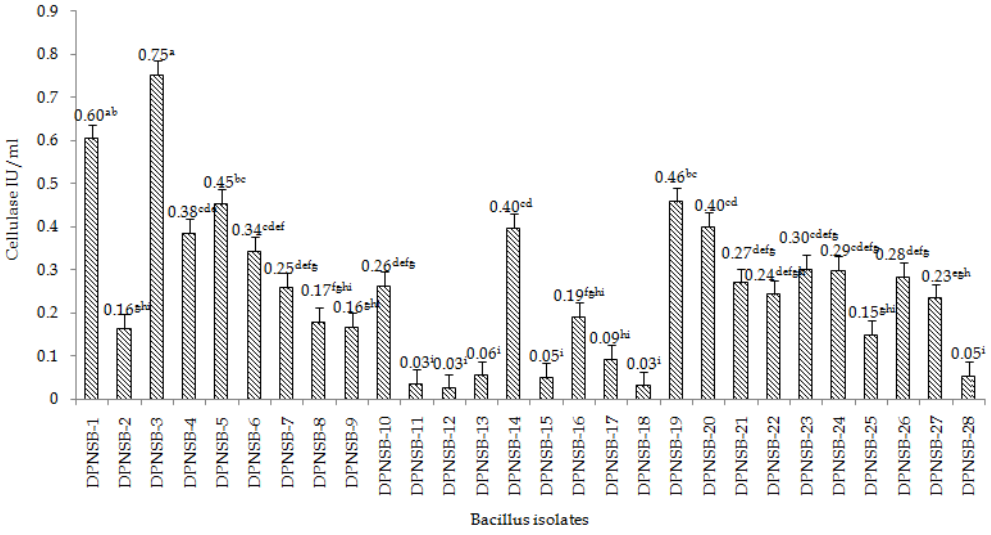

Figure 4. Production of Cellulase from Bacillus isolates

least production (0.004 IU/ml) among the isolates (Figure 3). Cellulase activity was determined by using CMC as a substrate. The data in Figure 4 showed that among the isolates, two isolates of IIVR, DPNSB-3 (0.75 IU/ml) and DPNSB-1 (0.60 IU.ml) showed maximum cellulase production. Similar level of minimal production 0.03 IU/ml has observed in two isolates of IIHR (DPNSB-11, DPNSB-12) and one isolate of IARI (DPNSB-18) (Figure 4). The results of all were showed no significant relationship between the antagonistic potential of strains.

DISCUSSION

Although evidence for suppression of soil borne plant pathogens by both antibiotic and lytic enzyme producing *Bacillus* strains have recently been described (Praveen et al., 2010; Podile and Prakash, 1996; Abdullah et al., 2008). In the present study *Bacillus* sp. were isolated from the rhizosphere of tomato and studied for cell wall degrading enzymes (CWDE) viz., chitinase, protease, β-1,3 glucanase and cellulase was done. The role of microbial CWDE's against phytopathogenic fungi (Praveen et al., 2011; Neuhans, 1999; Aziz et al., 2008; Shanmugam and Kanoujia et al., 2011). Chitinase production in the tested isolates were ranged from 1.38 to 4.65 IU/ml and maximum activity was observed in DPNSB-18 (4.65 IU/ml). Production of β-1, 3-glucanase also investigated and results shows that few of the strains (DPNSB-14, DPNSB-2 and DPNSB-20) were efficient

producers; overall range for all strains was 0.004 to 0.39 IU/ml respectively. Several cell wall degrading enzymes such as chitinase and β-1, 3-glucanase are involved in this study. Biocontrol agents such as *Serratia marcescens* (Ordentlich et al., 1988; Lee et al., 1992), *P. cepacia* (Fridlender et al., 1993), *P. stutzeri* (Lim et al., 1991), *P. fluorescens* (Velazhahan et al., 1999; Meena et al., 2001) and *Stenotrophomonas maltophilia* (Zhang and Yuen, 2000) secrete chitinase and β-1,3 glucanase capable of degrading chitin and β-1,3-glucan, respectively, which are the major components of fungal cell walls. As examples, isolates related to *Bacillus* sp. (Hoster et al., 2005) produce chitin-degrading enzymes while *Bacillus subtilis* AF1 displays some fungitoxicity through the secretion of N-acetyl glucosaminidase and glucanase (Manjula and Podile, 2005).

Members of the *Bacillus* genus are generally found in soil and most of these bacteria have the ability to disintegrate proteins, namely proteolytic activity. Protease enzymes not only have important industrial uses, but the proteases of these microorganisms play an important role in the nitrogen cycle, which contributes to the fertility of the soil (Heineken et al., 1972). Among the strains synthesis of protease level was quite good as compare to other enzymes, range is from 0.34 to 0.79 IU/ml. Márcia P. Lisboa et al., 2006 proves that *Bacillus* strain produces an antimicrobial substance which related to proteases that inhibits *B. cereus* and also involved in biocontrol traits (Kamensky et al., 2003). Cellulase production from bacteria can be an advantage as the enzyme production rate is normally higher due to the higher bacterial growth rate as compared to fungi (Ariffin et al., 2006). Out of the 28 strains 30% produce consequential cellulase production, range from 0.40 to 0.75 IU/ml. Reports on strains belonging to species such as *Bacillus sphaericus* and *Bacillus subtilis* express high cellulase degradation activities (Singh et al., 2004, Mawadza et al., 1996). The results of the study indicated that there was no significant relationship between the assayed enzymes.

Knowledge of the mechanisms involved in the *Bacillus* sp. is important for genetic enhancement (Baker, 1990). Mode of action of *Bacillus* sp. differs depending upon the strains. Recently, we demonstrated that highest chitinase activity found in IARI isolate (DPNSB-18); protease activity in IIHR isolates (DPNSB-15); maximum glucanase production was recorded in isolates of IIHR (DPNSB-14), IIVR isolate (DPNSB-2) and IARI isolate (DPNSB-20); cellulase production was made by isolates of IIVR (DPNSB-3 and DPNSB-1) respectively. This study provides additional information about the unique profiling of cell wall degrading enzymes from rhizospheric Bacilli sp. has a potential used as to be bioresource of bacterial species for the benefit of the agriculture industry.

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