Summary

Studies on biocontrol of Fusarium wilt of Cajanus cajan by Paenibacillus sp. D1 and Streptomyces sp. A6
Out of 140 chitinolytic bacteria isolated from different sites in Uttar Pradesh 40 isolates were primarily selected based on CZ/CS ratio on chitin agar plates. These 40 isolates along with 18 other isolates showing actinomycete like morphology but having less CZ/CS ratio were further screened by chitinase assay. Isolates Cl, C2, C7, D1, D2 and SP7 exhibited high chitinase activity

- (30.6-36.42 units/ml) while, among actinomycetes, isolates A6, All and A18 had high chitinase activity.

- All 58 isolates were also screened for production of two other important mycolytic enzymes, protease and glucanase. Isolates A1, A6, A7, All, A17 and A18 exhibited high protease activity (20.11-26.14 units/ml) while isolates A4, A6, A7, All, A18, and D1 had high glucanase activity (15.04-16.80 units/ml).

- Based on mycolytic enzyme production isolates D1, A6, All and A18 were selected for further studies. Isolate D1 was a high chitinase producer with considerable amounts of protease and glucanase while, isolates A 6, All and Al 8 were high protease and glucanase producers.

- The selected isolates were also checked for antifungal compound production. Isolates A6, All and A18 produced antifungal metabolite besides mycolytic enzymes, with maximum antifungal compound being produced by the isolate A6. Hence, the isolates D1 (high chitinase producer) and A6 (high protease and antifungal compound producer) were selected for all the further studies.

- 16S rDNA sequence analysis revealed close homology of isolate D1 with Paenibacillus sp. (95% similarity) and isolate A6 with Streptomyces sp (96% similarity).

- Maximum chitinase production by Paenibacillus sp. D1 was 36.36 ± 1.42 units/ml while by Streptomyces sp. A6 was 7.16 ± 0.50 units/ml. Endochitinase and chitobiase produced by Paenibacillus sp. D1 were 22.64 ± 1.39 and 15.37 ± 0.66 and by Streptomyces sp. A6 were 4.95 ± 0.5 and 3.76 ± 0.41 units/ml, respectively. The isolates produced acidic, neutral and alkaline proteases with maximum production of neutral protease. Maximum neutral protease activity by Paenibacillus sp. D1 was observed to be 8.47 ± 0.79 units/ml while by Streptomyces sp. A6 was 26.49 ± 0.96 units/ml. Maximum glucanase production by Paenibacillus sp. D1 was 15.09 ± 1.28 units/ml while by Streptomyces sp. A6 was 16.36 ± 1.95 units/ml. Except for glucanase in Streptomyces sp. A6 which was high at 96 hours, all the other major mycolytic enzymes of both isolates showed highest production at 72 hours of growth.

- Other biocontrol and plant growth promoting activities were also determined for isolates. Paenibacillus sp. D1 produced HCN and ammonia while, Streptomyces sp. A6 did not. Both the isolates produced indole acetic acid (IAA). Maximum IAA production by Paenibacillus sp. D1 and Streptomyces sp. A6 was found to be 33.96 ± 0.77 pg/ml and 81.88 ± 6.09 pg/ml, respectively in tryptophan supplemented minimal medium which was 4.4 folds and 4.85 folds high compared to unsupplemented medium. Both the isolates produced catacholate type of siderophore as major Fe chelator with 42.36 ± 1.l1pg/ml produced by Paenibacillus sp. D1 at 144 h and 35.03 ± 1.1 pg/ml by Streptomyces sp. A6 at 96 h of growth. Slight phosphate solubilising ability was observed in Streptomyces sp. A6 while it was not detectable in Paenibacillus sp. D1.

- Both the cultures were found to be compatible with each other and antifungal agent produced by one did not inhibit the growth of other.
The efficacy of Paenibacillus sp. D1 and Streptomyces sp. A6 to protect seeds of Cajanus cajan from Fusarium infection, both individually and in combination was determined. The protection offered by Paenibacillus sp. D1 and Streptomyces sp. A6 individually was comparable to that provided by commercial biofungicide T-2 Trichoderma Monitor WP. Combined treatment with both the isolates was better compared to their individual treatments which may be due to synergism between different antifungal agents produced by both the cultures. The protection offered by combined treatment with Paenibacillus sp. D1 and Streptomyces sp. A6 together was comparable to that provided by chemical fungicide Bavistin.

Treatment of seeds with cultures also increased the growth of seedlings as evident from increased radicle and plumule length and total weight. Treatment of seeds with Streptomyces sp. A6 resulted in considerable increase in radicle length. The effect was enhanced when seeds were treated with combination of Paenibacillus sp D1 and Streptomyces sp. A6.

Treatment of Cajanus cajan seeds with Paenibacillus sp. D1, Streptomyces sp. A6 and combination of both the cultures reduced the wilt incidence in Cajanus cajan by 45.1±2.83, 51.0 ± 3.55 and 54.9 ± 6.58%, respectively as evident from field trials. While reduction in disease incidence was observed after treatment with commercial biocontrol agent T-2 Trichoderma Monitor WP and chemical fungicide Bavistin was 41.2 ± 2.6 and 58.8 ± 2.0 %, respectively.

Rhizospheric soil from plants treated with Paenibacillus sp. D1 and Streptomyces sp. A6 had high chitinase, protease and glucanase activity compared to control. Soil treated with Paenibacillus sp. D1 showed high chitinase and glucanase activities at 60 days post sowing (DPS), while protease activity at 30 DPS. Whereas, all the three enzyme activities were high at 60 DPS in soil treated with Streptomyces sp. A6.

The combined treatment with both the cultures also increased the height, number of branches and dry weight of the plants, in turn, leading to increase in average number of pods per plant and yield.

Influence of various medium components on chitinase production by Paenibacillus sp. D1 and protease and antifungal compound production by Streptomyces sp. A6 was studied using statistical approach. Plackett-Burman method was used to identify die essential medium components while, response surface methodology was applied for further optimization of screened components using central composite design. Yeast extract, chitin, K2HPO4 and urea were identified as essential medium components having significant influence on chitinase production with a confidence level of 98%, 93%, 93% and 90%, respectively. Although the confidence level of urea was less, preliminary observation had suggested urea to be the best nitrogen source for chitinase production by Paenibacillus sp. D1. The optimized medium for chitinase production contained (g/1): Yeast extract, 0.65; chitin, 3.75; K2HP04, 1.17 and urea, 0.33. Protease production by Streptomyces sp. A6 was influenced by initial pH of the medium and addition of ZnS04, FeCl3, shrimp waste. These components had confidence levels 99.45, 97.35, 96.64 and 93.18%, respectively. Composition of optimized medium for protease production was found to be (g/1): shrimp waste, 14; FeCl3, 0.035; ZnS04, 0.065 and initial pH, 8.0. Shrimp waste, glycerol, KH2PO4 and FeCl3 were identified as essential medium constituents influencing antifungal compound production by Streptomyces sp. A6 with confidence levels 99.99,
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99.99, 95.04 and 98.41%, respectively. The optimal concentration of medium components for antifungal compound production was found to be (g/l): shrimp waste, 25; glycerol, 1.75; KH2PO4, 0.35 and FeCl3, 0.035.

- Statistical optimization of the medium constituents resulted in 2.56 fold increase in chitinase production by *Paenibacillus* sp. D1; 4.96 fold increase in protease and 3.4 fold increase in antifungal compound production by *Streptomyces* sp. A6 compared to respective basal mediums. sfc As shrimp waste was found to be an important medium constituent for protease production by *Streptomyces* sp. A6. The ability of *Streptomyces* sp. A6 protease to deproteinize the shrimp waste was explored. 85.12 ± 4.7 % deproteinization of shrimp waste was obtained after 24 h of incubation by the action of protease from *Streptomyces* sp. A6 with recovery of 5.93 g of chitin (70.58 ± 1.33 %) reflecting its potential as economical and eco-friendly alternative to conventional method for reclamation of food processing waste such as shrimp waste.

- Both the selected isolates and major mycolytic enzymes produced by them i.e chitinase of *Paenibacillus* sp. D1 and protease of *Streptomyces* sp. A6, were explored for their potential use with pesticides in integrated pest management (IPM) strategy. The most important parameter for IPM is the compatibility of the integrating components. Therefore, the effect of commonly used pesticides on growth and enzyme production by both the isolates was investigated. Both the isolates were tolerant to most of the fungicides tested. No inhibitory effect was observed on growth and enzyme production in presence of fungicides belonging to M, MBC and phosphonate group. Sulphur increased growth and chitinase production by *Paenibacillus* sp. D1 while, growth and protease production by *Streptomyces* sp. A6 was enhanced in presence of sulphur, mancozeb, carbendazim, fosteayl aluminium, and triadimefon. Among the DMI group of fungicides triadimefon inhibited growth and chitinase production from *Paenibacillus* sp. D1 by 24%. However, *Streptomyces* sp. A6 exhibited strong tolerance towards all the DMI fungicides. No significant inhibition in growth and enzyme production by both the isolates was observed in presence of organophosphate and carbamate group of insecticides. Acephate increased growth of *Paenibacillus* sp. D1 by 23% with no effect on chitinase production while, growth and protease production by *Streptomyces* sp. A6 was enhanced in presence of both acephate and methyl parathion. Growth of both cultures was drastically inhibited in presence of cypermethrin, a pyrethroid insecticide while, termiticide, endosulfan, inhibited growth only at higher concentrations.

- Effect of pesticides on activity and stability of chitinase and protease was further investigated. No inhibitory effect on activity and stability of chitinase and protease was observed with test fungicides at recommended field concentration (RFC) and higher concentrations (1000 jg/ml) except for propioconazole which inhibited chitinase and protease activity by almost 40 and 30% respectively, at 100 pg/ml concentration. Both the enzymes were highly active and stable in presence of most of the insecticides and termiticide at RFC. These results suggested that the isolates *Paenibacillus* sp. D1 and *Streptomyces* sp. A6; and their enzymes chitinase and protease were highly compatible with the pesticides tested.

- The 50% effective concentration (EC50) dose of test fungicides, the isolates *Paenibacillus* sp. D1 and *Streptomyces* sp. A6 and their respective enzymes, chitinase and protease, for inhibition of fungal spore germination and fungal biomass reduction was determined.
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EC50 dose of test fungicides, mancozeb, carbendazim, fosteyl aluminum and sulphur, was in the range 16.78-47.15 pg/ml for inhibition of fungal spore germination and 18.34-50.49 pg/ml for fungal biomass reduction, respectively, with carbendazim having the lowest EC50 dose. EC50 doses of mancozeb and carbendazim (fungicides used in seed dressing) for protection of *Cajanus cajan* seed from fusarium infection were determined as 40 and 30 pg/ml, respectively. The EC50 dose of *Paenibacillus* sp. D1 and its chitinase was found to be 3.18 x 106 cells/ml and 25.03 units/ml for inhibition of fungal spore germination and 2.36 x 106 cells/ml and 31.4 units/ml for fungal biomass reduction, respectively. The cell density of 105 cells/seed served as EC50 dose of *Paenibacillus* sp. D1 for inhibition of fungal infection of *Cajanus cajan* seeds. The EC50 dose of *Streptomyces* sp. A6 and protease was found to be 1.14 x 106 spores/ml and 37.7 units/ml for fungal spore germination inhibition and 1.44 x 106 spores/ml and 40.94 units/ml for fungal biomass reduction, respectively. The spore count of 106 spores/ml served as EC50 dose of *Streptomyces* sp. A6 for prevention of fusarium infection of *Cajanus cajan* seeds.

- Interaction between the bacterial cultures, their mycolytic enzymes and test fungicides was further determined. Both the isolates, *Paenibacillus* sp. D1 and *Streptomyces* sp. A6, showed highly synergistic interaction (synergy factor, SF>1) with the test fungicides for inhibition of fungal spore germination and fungal biomass reduction. *Paenibacillus* sp. D1 exhibited maximum synergism with carbendazim for both inhibition of fungal spore germination and fungal biomass reduction while, *Streptomyces* sp. A6 exhibited maximum synergism with carbendazim for inhibition of fungal spore germination and with mancozeb for reduction of fungal biomass. Chitinase and protease from isolates D1 and A6, respectively also showed highly synergistic interaction with the test fungicides exhibiting maximum synergism with carbendazim for inhibition of fungal spore germination as well as fungal biomass reduction. Furthermore, seed protection assays and field trials revealed strong synergistic interaction between bacterial cultures (*Paenibacillus* sp. D1 and *Streptomyces* sp. A6) and fungicides (carbendazim and mancozeb) used in seed dressing, for controlling fusarium infection and wilt incidence in *Cajanus cajan* suggesting the potential of these isolates for use in integrated pest management strategy to reduce the dose of toxic pesticides in agriculture.

- Chitinase from *Paenibacillus* sp. D1; and protease and antifungal compound from *Streptomyces* sp. A6 were purified and characterized. 4’ Chitinase form *Paenibacillus* sp. D1 was purified to 52.3 folds by ion exchange chromatography using SP Sepharose with a final yield of 10.17% and specific activity 492.4 U/mg protein. A single band of molecular mass 56.56 kDa was obtained on SDS PAGE. The identity of band as chitinase was confirmed by activity staining.

- The purified chitinase was identified by MALDI-LC-MS/MS analysis. The peptide sequences obtained after tryptic digestion, R.QWDDVAK.A and R.TAFANSALQYIR.A, showed ion score of 60 in Mascot search revealing homology (p-value < 0.05) of *Paenibacillus* sp. D1 chitinase with chitinase Chi55 of *Paenibacillus ehimensis*.

- Optimum pH and temperature for chitinase activity were found to be pH 5.0 and 50°C, respectively. The enzyme was highly active at pH 4-8 and 40-60°C suggesting broad range of pH and temperature for activity.

- Catalytic constants $K_m$ and $V_{max}$ for chitin hydrolysis at 50°C and pH 5.0 were 4.97 mg/ml
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- The thermodynamic parameters enthalpy ($\Delta H^*$), Gibbs free energy ($\Delta G^*$), and entropy ($\Delta S^*$) of activation for chitin hydrolysis were 16.45 KJ/mol, 67.47 KJ/mol and -157.89 J/mol, respectively. The free energies for activation of substrate binding ($\Delta G^*$-e-s) and formation of activated (transition) complex ($\Delta G^*$-t) were 4.31 KJ/mol, -7.58 KJ/mol. These parameters showed that the enzyme was in its active conformation and had high affinity for chitin.

- The enzyme was found to be highly stable at temperatures prevailing under field conditions (40-50°C) in Uttar Pradesh region of India with $tm$ values 370.68 and 255.15 min, respectively. The enzyme was stable up to 80°C with $tm$ of 22.61 min.

- Thermal stability of chitinase in presence of fungicides mancozeb, captan and carbendazim was investigated. All the test fungicides enhanced the thermal stability of enzyme. The stability of chitinase in presence of the three fungicides was in the order: captan > carbendazim > mancozeb > control. The $tm$ values of chitinase in presence of captan at 40° and 50°C (temperatures prevailing in fields) were 542.95 min and 469.41, respectively. The thermodynamic parameters activation energy of deactivation ($E$), change in enthalpy ($AH^*$), entropy ($AS^*$) and free energy ($AG^*$) for chitinase deactivation in presence of fungicides was further investigated. These parameters were also effective in the order: captan > carbendazim > mancozeb > control.

- Extracellular protease from Streptomyces sp. A6 was purified by gel permeation chromatography using Sephacryl S-100 column. The protease was purified to 20.8 fold with a final yield of 4.96% and specific activity 107.28 units/mg protein. A single band of molecular mass 20 kDa was obtained on SDS PAGE. Identity of the band as protease was confirmed by activity staining.

- The purified protease inhibited germination of Fusarium udum spores in dose dependent manner as revealed from phase contrast microscopy. The enzyme also inhibited the advancing edge of growth of the fungus which was studied by well diffusion method. These results indicated the antifungal activity of Streptomyces sp. A6 protease.

- The protease exhibited maximum activity at pH 7-9 and 55°C. The enzyme was highly active in the temperature range 40-70°C.

- The protease activity was completely inhibited by PMSF (a serine protease inhibitor) indicating the enzyme to be a serine protease. Amongst the metal ions tested, maximum activity was retained in presence of CaCl2 (104.20 ± 2.42%) and MgSC>4 (91.705 ± 6.39%) after incubation for 30 min. Most of the detergents tested enhanced the activity of protease. Highest enzyme activity was observed in presence of 1% triton- X 100 which was 4.12 fold higher the untreated control. Urea exhibited positive effect on enzyme activity. Increase in enzyme activity was observed with increasing concentration of urea from 2 to 8 M (109.9-119% activity).

- Among the various synthetic substrates tested, highest enzyme activity (304.29 ± 13.23 units/ml) was observed with N-Suc-Ala-Ala-Val-Ala-pNA (a chymotripsin specific substrate). The protease had a higher affinity for chymotrypsin specific substrate and hydrolysed it more efficiently as revealed by low $km$, 0.201 pM and high $F_{max}$ (344,83 units/ml), compared to casein ($k_m$= 4.26 µM, $V_{max}$ = 285.71 units/ml). The turnover
number ($k_m$) and second order rate constant ($k_{cat}k_m$) for the enzyme was also high in presence of chymotrypsin specific substrate compared to casein.

- The protease was highly stable at 40°C as apparent from the $tm$ value, 106.75 min. The enzyme was stable up to 70°C with $\Delta h$ value of 16.07 min. The activation energy for protease deactivation was calculated as 50.014 KJ/mole. Change in enthalpy ($\Delta H^*$), entropy ($\Delta S^*$) and free energy ($\Delta G^*$) for deactivation of protease at 40°C were 47.41 KJ/mole, -126.95 J/mole and 87.17 KJ/mole, respectively. The low value of enthalpy and negative value of entropy and high free energy value signified the stability of protease.

- The purified protease was identified by MALDI-LC-MS/MS analysis. The peptide sequences obtained after tryptic digestion, -IAGGEAIYAAAGGGR.C, R.CSLGFNVR.S, R. AGTSFPGNYGLIR.H, R.HSNAAADGR.V, R.VLYNGSYR.D, R.DITGAGNYVGQTVQR.S., revealed 67% similarity of Streptomyces sp. A6 protease with SFase 2 of Streptomyces fradiae. The calculated molecular mass of enzyme was 19.219 kDa with pi of 6.99.

- Antifungal compound from Streptomyces sp. A6 was purified and characterized. Streptomyces sp. A6 exhibited antifungal activity against different fungal phytopathogens, Fusarium udum, Rhizoctonia solani and Aspergillus parasiticus as revealed from dual culture technique by agar diffusion method.

- The culture supernatant of Streptomyces sp. A6 containing antifungal metabolite retained 91.2 ± 4.14, 89.57 ± 3.2, 88.55 ± 5.99 and 81.43 ± 3.23% activity after treatment with proteinase K, TCA precipitation, boiling and autoclaving, respectively revealing its non-proteinaceous nature.

- Best extraction of the compound was achieved with benzene (Snyder Polarity Index, SPI=3.0) followed by n-butanol (SPI=3.9) > chloroform (SPI=4.1) > ethyl acetate (SPI=4.3).

- The compound was purified by thin layer chromatography. The three spots obtained on TLC showed similar Rf values as that of spots obtained from Streptomyces fradiae (MTCC321) extract used as reference. The spot with Rf value of 0.46 possessed antifungal activity.

- The TLC purified antifungal metabolite was subjected to FTIR and GC-MS analysis. FTIR spectra of the antifungal metabolite produced by Streptomyces sp. A6 was similar to that of antifungal metabolite from S. fradiae. The absorption peaks obtained from IR spectra suggested presence of alkyl groups, C-H, aldehyde/ketone group, C=O, nitro group, N-O and alcohol group, -OH. GC analysis of crude and purified antifungal compound from Streptomyces sp. A6 revealed a common peak at 45.89 min. MS analysis of this peak suggested the molecular weight of compound to be approximately 440.