Mechanisms of Cell Wall Degrading Enzymes from *Bacillus methylotrophicus* and *Bacillus subtilis* in Suppressing Foliar Blight Pathogens

Abstract:

Bacillus strains are the potent biocontrol agents (BCAs) and identified as an effective way to control the growth of phytopathogens of wheat. Through direct inhibition mechanism which involves the production cell wall degrading enzymes (proteases, glucanase, and chitinases) and siderophore they suppress the foliar blight disease. Both the strains of *Bacillus* P10 and UP11 shows % growth inhibition *Alternaria triticina* (77.56 %, 67.83%) and *Bipolaris sorokiniana* (73.97%,62.16%) through dual culture assay. Then these strains were further studied for the production of cell wall-degrading enzymes (chitinase, protease and *β*-1,3 glucanase) and antifungal metabolites (siderophore) production. After that it was found that when the antagonist's bacteria were co- culture with fungal pathogens then maximum production of hydrolytic enzymes and siderophore achieved at 96 hrs but when both strains P10 and UP11 are alone then maximum production found at 48 hrs i.e., exponential phase. The current study determined that *Bacillus methylotrophicus* (P10) and *Bacillus subtilis* (UP11) highly effective strain for controlling foliar blight disease. Direct or Antibiosis is the main mechanism that are involved in this study.Further research on the interaction mechanisms between *Bacillus*-derived compounds and host plants is necessary.

Keywords: Cell wall degrading enzymes, chitinase; β-1,3-glucanse; protease, *Bacillus subtilis*, *Bacillus methylotrophicus*

INTRODUCTION

Fungal diseases in plants pose a major threat to global agriculture, causing significant economic losses. Key soil-borne pathogens of wheat include *Pythium, Pyrenophora triticirepentis, Fusarium, Alternaria triticina, Rhizoctonia, Bipolaris sorokiniana*. Majorly *Bipolaris sorokiniana* and *Alternaria triticina* both cause

foliar blight disease. It is most destructive wheat disease causing 50% to up to 85 % yield loss under conducive conditions (relative humidity > 70 % and an average temperature of 20 – 30°C) (Aboukhaddour *et al.,* 2020; Al-Sadi 2021).An eco-friendly alternative to chemical methods for protecting crops from phytopathogenic fungi is the use of Biological control agents (BCAs). Bacteria from the genus *Bacillus* are key microorganisms for biological control of plant diseases across various crops. They are highly effective against phytopathogens and offer advantages like easy culture, storage, and manufacture due to their ability to produce endospores (Etesami et al., 2023).

Recently, *Bacillus*-based biological pesticides have seen increased global use. *B. subtilis* and *Bacillus methylotrophicus* is one of the most utilized species in this genus effective in controlling plant diseases through direct and indirect mechanisms (Miljaković et al., 2020). It produces antimicrobial compounds, including cyclic lipopeptides (surfactins, iturins, and fengycins), which antagonize a range of pathogens like bacteria, fungi, and oomycetes. Additionally, they produce cell wall degrading enzymes (CWDEs) or pathogenesis related enzymes such as chitinases, β-1, 3-glucanase, and proteases, which degrade essential components of fungal cell walls of pathogens, limiting their growth and activity (Sahgal, M. 2022). The antifungal activity of extracellular metabolites of bacteria against several fungi has already been investigated and reported by Dahiya et al. 2006 and Gajera et al., 2012. Chitin, protease and β-1,3-glucan are key structural components found in the cell walls of many phytopathogenic fungi, characterized by their regularly arranged materials. Enzymes like chitinases, proteases and β-1,3-glucanases are crucial in various biological processes and interactions in nature, including plant defense mechanisms, fungal cell wall degradation, and the competitive interactions between organisms like Trichoderma spp. and phytopathogenic fungi (Saravanakumar et al., 2016). The several study related to *Bacillus subtilis* and *Bacillus methylotrophicus* were also found that they produce secondary metabolites like phytohormones, hydrolytic enzymes, siderophores, and antibiotics, enhancing soil fertility, suppressing phytopathogens, and serving as eco-friendly biocontrol agents (Ku et al., 2021; Guo et al., 2019; Chakraborty et al., 2022; Mulk et al., 2022). In this study, we compared the enzymatic characteristics, effects on fungal cell walls, and

antifungal activities against *Bipolaris sorokiniana* and *Alternaria triticina* at different time intervals. Therefore, understanding the induction process of these enzymes is crucial for selecting the most effective *Bacillus* strains for biocontrol purposes.

2.Material and Methods:

2.1 Biocontrol agent

The antagonistic wheat rhizobacteria strains (P10, UP11), previously isolated and stored at the Rhizosphere Biology Laboratory, Department of Microbiology, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand (Table 1) were cultured and maintained on Nutrient Agar (NA).

Table 1: Percent similarity and GenBank accession numbers of the bacterial isolates

Strain code	Description	% Identity	Accession no
P10	*Bacillus methylotrophicus*	94.04%	MN099430.1
UP11	*Bacillus subtilis*	97.43%	MN099431.1

2.1.2. Fungal pathogens

The fungal pathogens *Bipolaris sorokiniana* (ITCC 4869) and *Alternaria triticina* (ITCC 1186) were acquired from the Indian Type Culture Collection (ITCC), Indian Agricultural Research Institute, New Delhi, India. These fungal pathogens were cultured and kept on Potato Dextrose Agar (PDA) plates at 25±2°C for 5-7 days to ensure viability.

2.2 Dual culture activity of *Bacillus* strains (P10 & UP11) against foliar pathogens

The antagonistic activity of *Bacillus* strains (P10 & UP11) against phytopathogens (*Bipolaris sorokiniana and Alternaria triticina*) was examined using the dual culture plate method (Skidmore and Dickinson 1976).

Fungal discs, 5 mm in diameter, were taken from 5-7 day-old cultures and placed in the center of plates containing a medium composed of an equal mix of NA and PDA. The *Bacillus* strains were then inoculated on either side of the fungal disc, each positioned 2.0 cm away from the disc. The plates were incubated at 27±1°C for 5-7 days. Control plates, which only contained the fungal growth without Bacillus inoculation, were also prepared. The percentage inhibition of fungal growth was determined by comparing the growth in the dual culture plates to that in the control plates, using the formula:

% Inhibition = $[(C - T)/C]\ X\ 100$

(Where, C= Radius of fungal growth in control plate, T = Radius of fungal growth in dual culture plate)

2.3 Production of hydrolytic enzymes

2.3.1 Chitinase production

Preparation of colloidal chitin -

Chitin flakes obtained from HI Media were finely powdered. Five grams of this chitin powder were slowly added to 90 ml of concentrated HCl with vigorous stirring. The resulting mixture was then poured into 500 ml of ice-cold ethanol while stirring rapidly, and the solution was left overnight at 4°C. The precipitate formed was collected by centrifugation at 8000 rpm for 10 minutes at 4°C, followed by washing with distilled water until neutral pH was achieved. The resulting colloidal chitin, which contained 85-90% moisture, was subsequently air-dried and stored at 4°C for future use (Hsu and Lockwood, 1975).

Procedure

The qualitative assay for chitinase production by bacterial isolates was conducted on a minimal agar medium ($MgSO4.7H_2O$, 0.2 g; K_2HPO_4, 0.9 g; KCl, 0.2 g; NH_4NO_3, 1.0 g; $FeSO_4$_7H2O, 0.002 g; $MnSO_4$, 0.002 g; $ZnSO_4$, 0.002 g; distilled water, 1.0 l; pH 6.8); 0.5g of yeast extract, and 15g of agar. Additionally, the medium was supplemented with 0.5% colloidal chitin. The minimal agar plates were spot inoculated

with log phase bacterial cultures and plates were incubated at 30^0C for 3-4 days (Sadfi et al., 2001). The appearance of halo zone around the colony indicated positive result.

2.3.2 β-1,3 glucanase production -

β-1,3-Glucanase activity was assayed using *Laminaria digitata* laminarin as the substrate (Aktuganov *et al.,* 2007). The log phase culture was spot inoculated on minimal agar media plates supplemented with 0.25% laminarin and incubated at 30^0C for 72 hrs. Thereafter the plates were stained with 0.1% Congo red solution. The halo zone observed around bacterial colony indicated positive test.

2.3.3 Proteolytic activity

A skim milk agar medium was created by dissolving 5.15 grams of skim milk powder in 100 milliliters of agar medium. Freshly grown 48-hour-old cultures were inoculated onto these skim milk agar plates and incubated at 28±2°C for two to three days (Berg et al., 2002). The formation of a clear zone around *Bacillus* sp. colonies indicated protease enzyme production.

2.4 Siderophore production

P10 and UP11 was spot inoculated onto chrome-azurol S-agar medium (CAS) agar medium, incubated at 30^0C for 72 hrs after that appearance of zones around the colony shows positive result (Schwyn and Neilands 1987).

2.5 Quantification of hydrolytic enzymes and siderophore in presence and absence of fungal pathogens

2.5.1 Culture preparation

To quantitatively estimation of bacterial culture and co-cultures with fungal pathogens were cultivated in a 1:1 mixture of Nutrient Broth and Potato Dextrose Broth for 5 days. At various time intervals (24-120 hours), 10 ml samples were taken from the flasks and centrifuged. The supernatants from both the

antagonistic bacteria (control) and co-cultures with fungal pathogens were used for enzyme assays. The experiment was conducted in triplicate.

2.5.2 Enzymatic assay

Chitinase (EC 3.2.1.14) activity was determined using the DNS method (Miller, 1959). The reaction mixture contained 0.5 ml of 0.5% colloidal chitin in phosphate buffer (pH 5.5), 0.5 ml of crude enzyme, and 1 ml of distilled water, making a total volume of 2 ml. After thorough vortexing, the mixture was incubated in a water bath shaker at 40°C for 1 hour. The reaction was halted by adding 3 ml of DNS reagent, followed by boiling in a water bath for 5 minutes. After cooling to room temperature, enzyme activity was measured at 540 nm. One unit of chitinase activity corresponded to the amount of enzyme needed to release 1 µmol of N-acetyl-β-D-glucosamine under the specified assay conditions (Juarez-Jimenez et al., 2008).

β-1,3 glucanase (EC 3.2.1.39) activity was assessed by measuring the amount of reducing sugars released from laminarin, with glucose serving as a standard (Masih & Paul, 2002). The assay involved combining 250 µl of 0.05 M potassium acetate buffer (pH 5.0) containing 2.5 mg/ml laminarin with 250 µl of culture filtrate. This enzyme-substrate mixture was then incubated at 40°C for 2 hours. After incubation, 0.5 ml of DNS reagent was added, followed by boiling at 100°C for 5 minutes. Upon cooling, 2 ml of deionized water was added, and the absorbance was measured at 595 nm using a spectrophotometer. β-1,3-glucanase activity was quantified as the amount of enzyme required to release 1 µmol of reducing sugar equivalents (measured as glucose) under the specified assay conditions.

Protease activity (EC 3.4.21.4) was determined using casein as the substrate, following the assay method described by (Takami et al. 1989). 0.5 ml of the enzyme solution was incubated in a shaker water bath with 1.5 ml of 1.0% casein dissolved in a glycine-NaOH buffer (50 mM, pH 7) at 40°C for 30 minutes. The reaction was stopped by adding 2.5 ml of TCA solution (0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid). The mixture was then filtered, and 0.5 ml of the filtrate was combined with 2.5 ml of 0.5 M Na2CO3 and 0.5 ml of Folin-Ciocalteau reagent. After allowing the reaction mixture to stand for

30 minutes, the absorbance measured at 660 nm. One unit of protease activity was defined as the amount of enzyme required to produce 1 µg of tyrosine under the specified assay conditions.

Siderophore production was determined by antagonists and co culture were grown in an iron-free minimal medium incubated for different time intervals 24-120 hr at 25±2°C. Afterwards samples were withdrawn, centrifuged at 3000 rpm for 15 minutes. Culture supernatant (1.5 ml), CAS dye solution (1.5ml) and 30 µl of shuttling solution (sulfo-salicyclic acid) were added, mixed, kept for 20 minutes. The presence of siderophores in the culture supernatant leads to the removal of iron from the dye complex, resulting in a reduction in the intensity of the blue coloration absorbance recorded at 630 nm (Payne 1994). The minimal medium (blank) and % siderophore units were calculated by the following formula:

% Siderophore units = $[(A_r - A_s)/ A_r] \times 100$

Where A_r = absorbance of reference; A_s= absorbance of the sample

2.6 Statistical analysis

All experiments were conducted in triplicates. Statistical analysis of the data was done using Statistical Package for Social Studies (SPSS), version 21.0. software. Values $p < 0.05$ and $p< 0.01$ were considered to indicate statistical significance. All data are expressed as means ± SE.

3. Result & Discussion

3.1 Antagonistic activity of P10 & UP11 against foliar blight pathogens

The percent mycelial growth inhibition by biocontrol agents P10 against the *Alternaria triticina* (77.56%), followed by UP11 (67.83%) and *Bipolaris sorokiniana* (73.97%), followed by UP11 (62.16%) at 6 DAI (Table 2 and Fig 1). P10 shows the maximum inhibition for foliar blight pathogens as compared to UP11.

Table 2: Bacterial isolates against foliar blight pathogens

Bacterial isolates	**Percent inhibition**	**Percent inhibition**

	(*Alternaria triticina*)	(*Bipolaris sorokiniana*)
P10	$77.56^{a} \pm 2.15$	$73.97^{a} \pm 2.53$
UP11	$67.83^{b} \pm 2.24$	$62.16^{b} \pm 2.76$

Values are expressed as the means of three replications ± standard deviation. Means within each column followed by the same letter are not significantly different ($p<0.05$), according to one-way ANOVA and Duncan's multiple range test (DMRT).

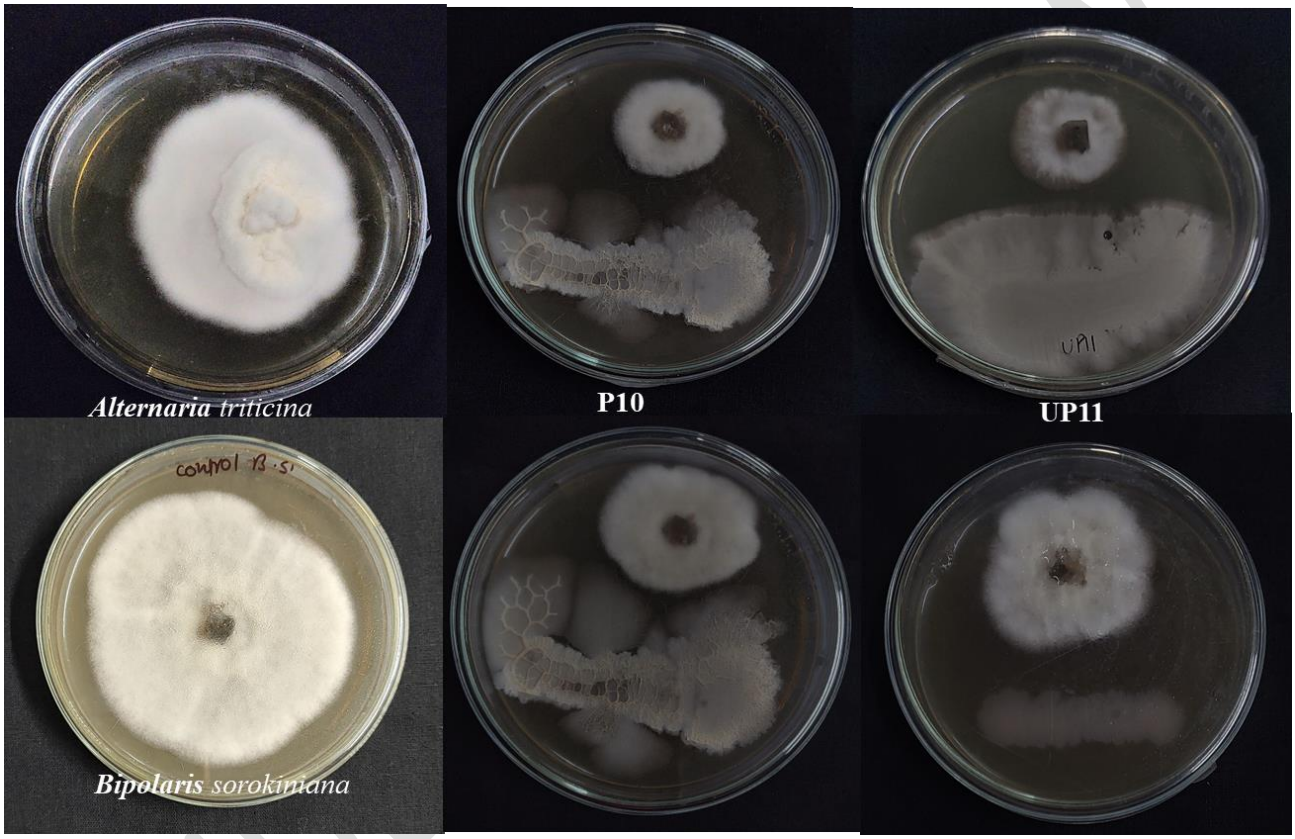


Figure 1: Antagonistic activity of P10 & UP11 against the *Alternaria triticina* and *Bipolaris sorokiniana* after 6 DAI (Day after infection).

3.2 Production of hydrolytic enzymes and siderophore:

Clear zone around the colony of *Bacillus* strains P10 & UP11 indicated positive results for chitinase, β-1,3-glucanase, protease and as well as siderophore (Fig 2). These positive results shows that both *Bacillus* strains are potent antagonists to suppress the fungal pathogen growth because they degrade the main

components of fungal cell wall. They also show siderophore positive that means they compete for nutrition or iron availability.

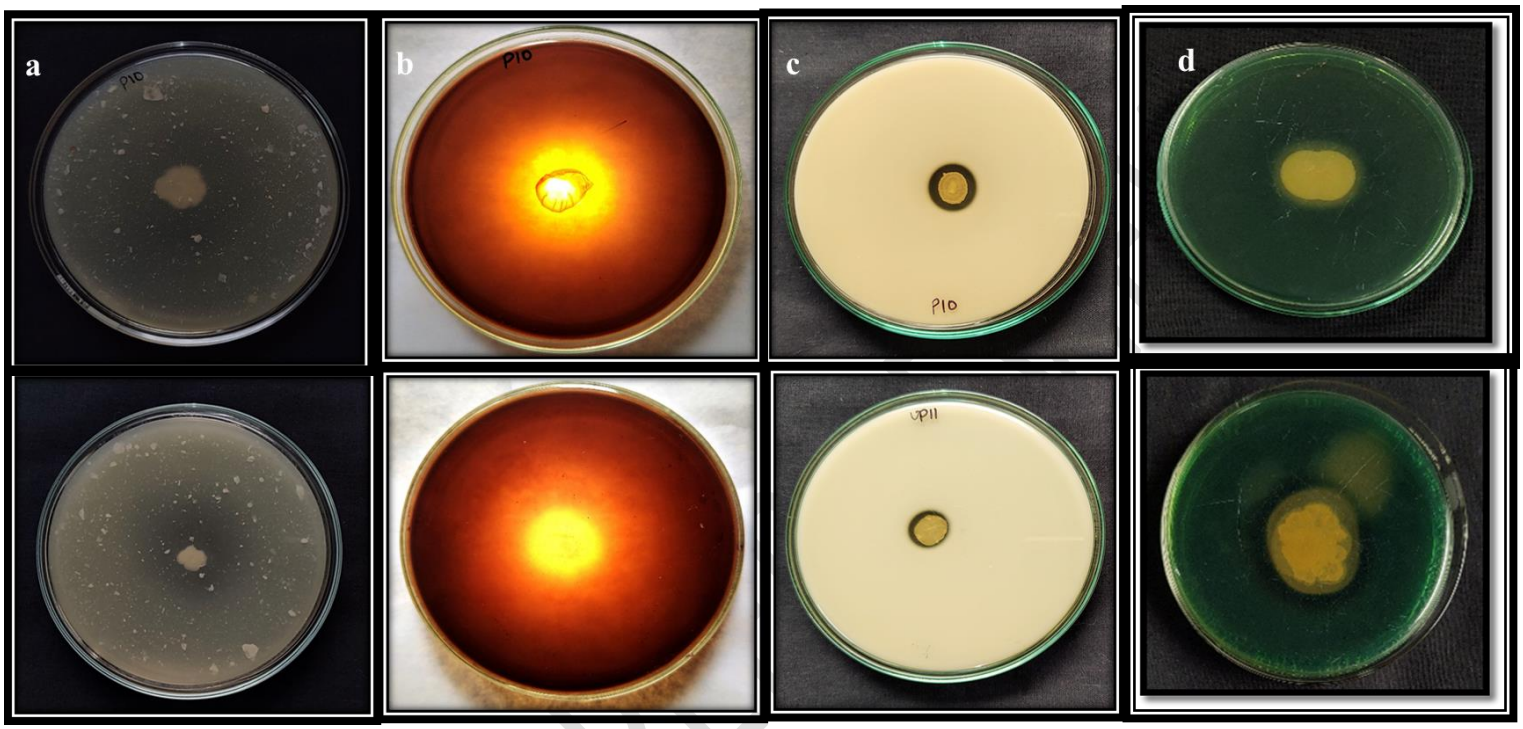


Figure 2: Production of antagonistic properties of *Bacillus* spp. (P10 & UP11) strains (a) chitinase (b) β-1,3 glucanase (c) protease (d) siderophore

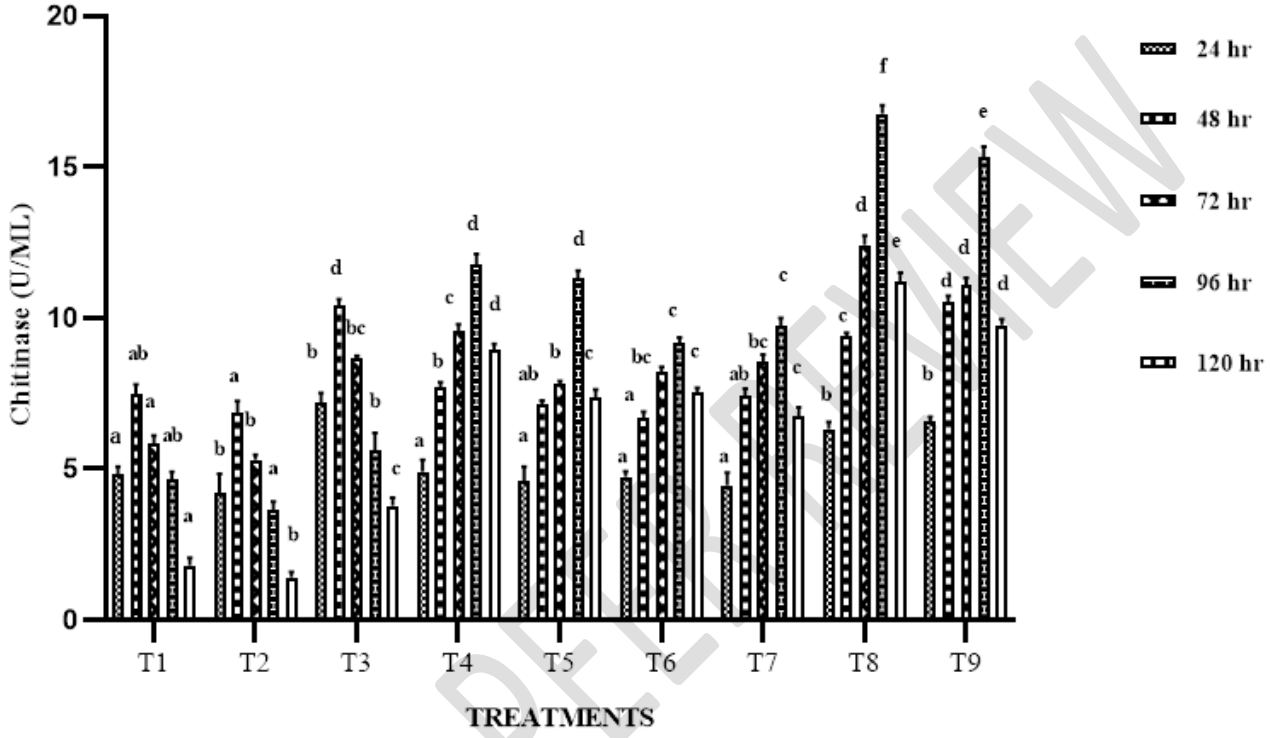
3.3 Effect of different time on total hydrolytic enzymes and siderophore of *Bacillus* strains alone and co-cultured with fungal pathogens

Bacillus methylotrophicus and *Bacillus subtilis* both the strains show the increased expression of lytic enzymes, in presence of pathogens (*Alternaria triticina* & *Bipolaris sorokiniana*) such as chitinases, β-1,3-glucanase, and protease. Chitinase activity, expressed as (U/ML), significantly found maximum at 48 hr in alone or without pathogen but in presence of pathogens the chitinase activity was found increased upto 96 hr and decreased at 120 hr so the maximum duration for *Bacillus* strains alone at

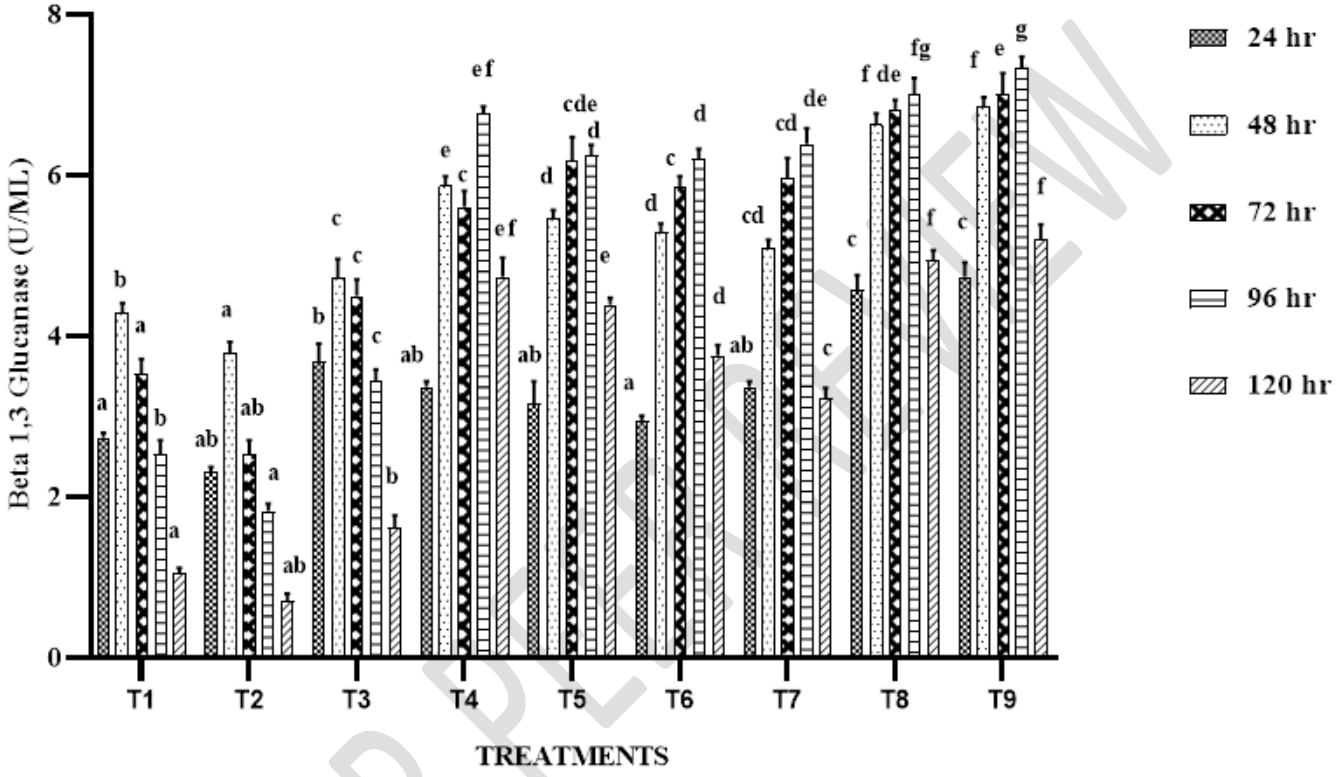
48 hr and with pathogen at 96 hr. The similar observation was also found in another cell wall degrading enzymes (β-1,3-glucanase, and protease) and siderophore. So in absence of pathogen P10 and UP11 shows maximum activity of cell wall degrading enzymes i.e., chitinase (7.45 and 6.83) β-1,3-glucanase (4.28 and 3.79), and protease (1.91 and 1.76) as well as siderophore (59.61 and 52.63) at 48 hr.

In presence of pathogens I.e., *Alternaria triticina* & *Bipolaris sorokiniana* consortium (CNS) of P10 & UP11 shows the maximum chitinase at 96 hrs T8 CNS (4.47), T9 CNS (4.28) followed by the treatments T4(4.10), T5(3.91), T6(3.85), T7(3.80), T3(1.08), T1(0.60) and T2 (0.47). β-1,3-Glucanase activity (U/ML) was also found maximum in consortium T9 CNS (7.32), T8 CNS (7.04) followed by the treatments T4(6.76), T7(6.38), T5(6.24), T6(6.20), T3(3.43), T1(2.52) and T2 (1.82). The protease activity (U/ML) also found maximum in consortium T9 CNS (4.47), T8 CNS (4.28) followed by the treatments T4(4.10), T5(3.91), T6(3.85), T7(3.80), T3(1.08), T1(0.60) and T2 (0.47). the siderophore activity was expressed as in % siderophore units T8 CNS (77.03), T9 CNS (76.37) followed by the treatments T5(58.29), T4(55.94), T3(53.55), T6(51.24), T7(48.73), T1(39.25) and T2 (30.42) (Fig. 3).

So, this study revealed that the amount of cell walls degrading enzymes and siderophore of P10 and UP11 alone was highest in early growth phase and when both the antagonist's bacteria and pathogenic fungi are growth both in same media then it was found that the highest during the stationary phase because due to the synergetic action the antagonist's strains secretes more cell wall degrading enzymes and compete for nutrition to suppress the growth of foliar blight pathogens. The similar results were also reported in the study by Alamri, S. A. (2015) and Khatri et al., (2017). Additionally, antagonists often release higher amounts of proteases to inhibit the activity of pectinase and chitinase enzymes produced by various plant pathogens (Radjacommare et al., 2010).



(a)



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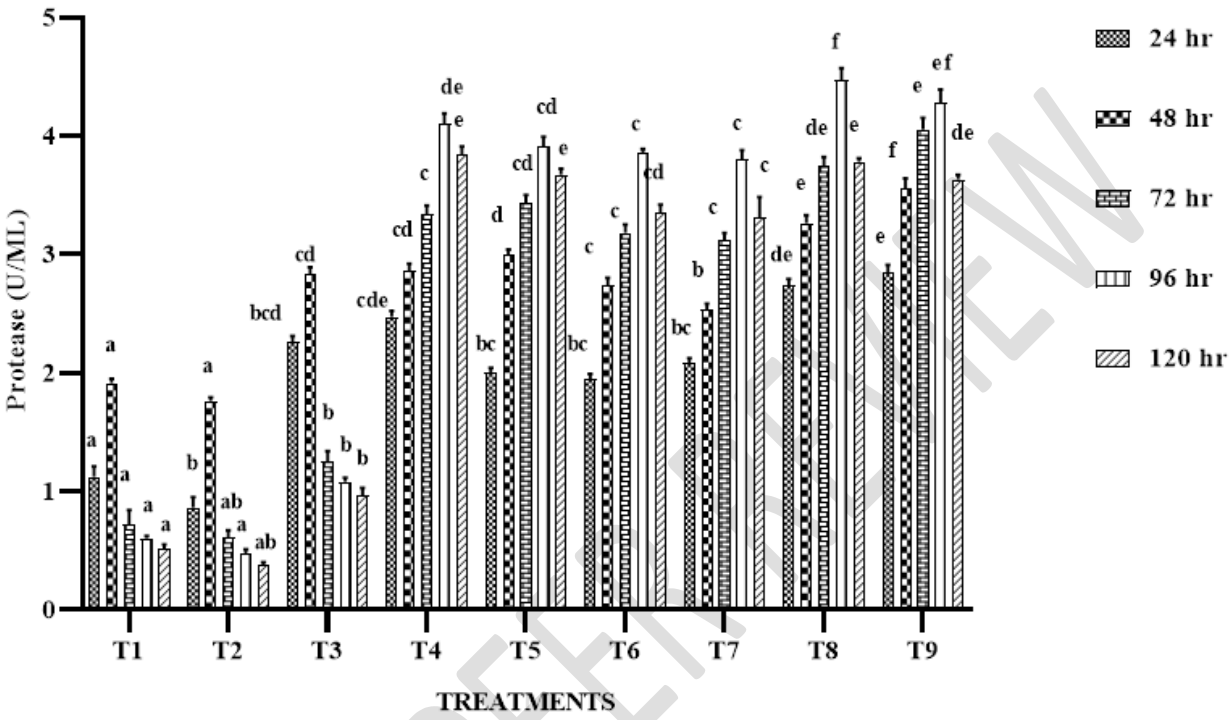

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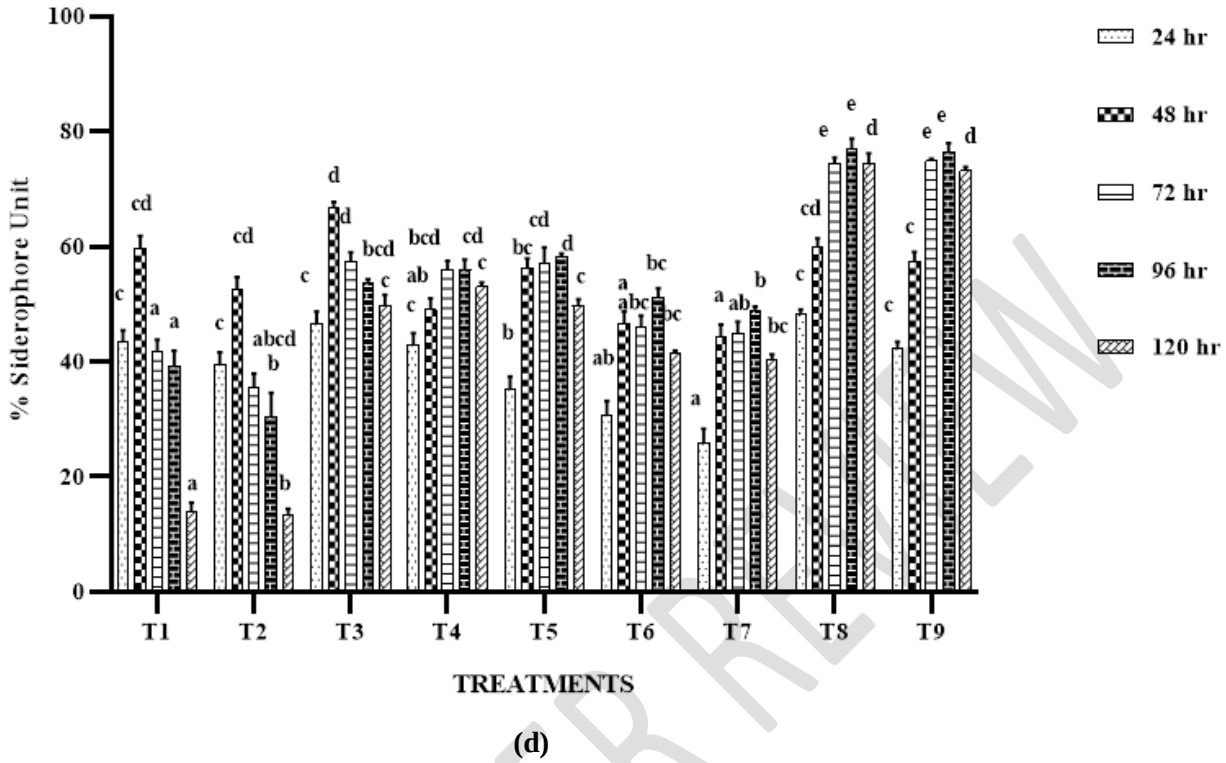

(d)

Figure 3: Production of cell wall degrading enzymes in absence and presence of fungal pathogens *Alternaria triticina* (F1) & *Bipolaris sorokiniana* (F2) (a) shows effect of different time duration on chitinase activity (b) shows effect of different time duration on β-1,3-glucanase activity (c) shows effect of different time duration on protease activity (d) Siderophore production at different time duration (T1- P10 alone, T2- UP11 alone, T3- P10+UP11 (CNS) T4- P10+F1, T5- P10+F2, T6- UP11+F1, T7- UP11+F2, T8- CNS+F1, T9- CNS+F2. Values are presented as mean ± SE (n = 3).

4. CONCLUSION

Bacillus amyloliquefaciens and *B. subtilis* were found as biocontrol antagonists against foliar wheat plant pathogens. The cell wall serves as the primary defense against pathogen attacks. Biological control agents (BCAs) generate various cell wall-degrading enzymes (CWDEs) to break down the cell wall of phytopathogenic fungi. For instance, *Bacillus subtilis* produces chitinases and glucanase, which play a crucial role in managing fungal plant pathogens (Shrestha et al., 2015). Moreover, CWDEs can trigger host

defense responses (Giovannoni et al., 2020). The quantification of cellulase, protease, and β-1,3-glucanase in P10 and UP11 indicates its role in inhibiting the growth of various fungal phytopathogens. Additionally, our findings showed that both *Bacillus* strain which demonstrated effectiveness against fungal pathogens in vitro, also produced siderophores. These cell wall degrading enzymes and siderophores production are linked to direct antagonistic mechanisms, suppress the foliar blight pathogen growth. So, from this study it was concluded that when both antagonistic strains and pathogen are in same media they act synergtically shows direct mechanism and inhibit the fungal pathogen mycelial growth. Moreover, these *Bacillus* strains are potent biocontrol agents and further used for suppression of wheat plant pathogens.

5. Future scope

Further research is needed to understand the antagonistic mechanisms of P10 and UP11 against foliar blight pathogens, along with pot and field trials to evaluate their effectiveness and compatibility with agricultural practices.

Ethical approval

There are no ethical concerns regarding the organisms and the topic of this research.

Informed consent

Informed consent was obtained from all individual participants included in the study.

REFERENCES:

Aboukhaddour, R., Fetch, T., McCallum, B. D., Harding, M. W., Beres, B. L., & Graf, R. J. (2020). Wheat diseases on the prairies: A Canadian story. *Plant Pathology*, *69*(3), 418-432.

Aktuganov GE, Galimzyanova NF, Melent'Ev AI, Kuz'mina LY (2007) Extracellular hydrolases of strain *Bacillus* sp. 739 and their involvement in the lysis of micromycete cell walls. Microbiol 76:413-420.

Alamri, S. A. (2015). Enhancing the efficiency of the bioagent Bacillus subtilis JF419701 against soil-borne phytopathogens by increasing the productivity of fungal cell wall degrading enzymes. *Archives of Phytopathology and Plant Protection*, *48*(2), 159-170.

Alamri, S. A. (2015). Enhancing the efficiency of the bioagent Bacillus subtilis JF419701 against soil-borne phytopathogens by increasing the productivity of fungal cell wall degrading enzymes. *Archives of Phytopathology and Plant Protection*, *48*(2), 159-170.

Al-Sadi, A. M. (2016). Variation in resistance to spot blotch and the aggressiveness of *Bipolaris sorokiniana* on barley and wheat cultivars. *Journal of Plant Pathology*, 97-103.

Berg G, Roskot N, Steidle A, Eberl L, Zock A, Smalla K (2002) Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different Verticillium host plants. Appl Environ Microbiol 68(7):3328-3338.

Chakraborty, K., Kizhakkekalam, V. K., Joy, M., & Chakraborty, R. D. (2022). Bacillibactin class of siderophore antibiotics from a marine symbiotic Bacillus as promising antibacterial agents. *Applied Microbiology and Biotechnology*, *106*(1), 329-340.

Dahiya, N., Tewari, R., & Hoondal, G. S. (2006). Biotechnological aspects of chitinolytic enzymes: a review. *Applied microbiology and biotechnology*, *71*, 773-782.

Etesami, H., Jeong, B. R., & Glick, B. R. (2023). Biocontrol of plant diseases by *Bacillus* spp. *Physiological and Molecular Plant Pathology*, 102048.

Fan, B., Wang, C., Song, X., Ding, X., Wu, L., Wu, H., ... & Borriss, R. (2018). Bacillus velezensis FZB42 in 2018: the gram-positive model strain for plant growth promotion and biocontrol. *Frontiers in microbiology*, *9*, 2491.

Gajera, H. P., & Vakharia, D. N. (2012). Production of lytic enzymes by Trichoderma isolates during in vitro antagonism with *Aspergillus niger*, the causal agent of collar rot of peanut. *Brazilian Journal of Microbiology*, *43*, 43-52.

Ghasemi, S., Safaie, N., Shahbazi, S., Shams-Bakhsh, M., & Askari, H. (2020). The role of cell wall degrading enzymes in antagonistic traits of Trichoderma virens against Rhizoctonia solani. *Iranian Journal of Biotechnology*, *18*(4), e2333.

Ghasemi, S., Safaie, N., Shahbazi, S., Shams-Bakhsh, M., & Askari, H. (2020). The role of cell wall degrading enzymes in antagonistic traits of Trichoderma virens against Rhizoctonia solani. *Iranian Journal of Biotechnology*, *18*(4), e2333.

Giovannoni, M., Gramegna, G., Benedetti, M., & Mattei, B. (2020). Industrial use of cell wall degrading enzymes: the fine line between production strategy and economic feasibility. *Frontiers in bioengineering and biotechnology*, *8*, 356.

Guo M, Li M, Zhang C, Zhang X, Wu Y (2020) Dietary administration of the *Bacillus subtilis* enhances immune responses and disease resistance in chickens. Front microbiol 11:1768.

Hsu, S. C., & Lockwood, J. (1975). Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. *Applied microbiology*, *29*(3), 422-426.

Juarez-Jimenez, B., Rodelas, B., Martinez-Toledo, M. V., Gonzalez-Lopez, J., Crognale, S., Gallo, A. M., ... & Fenice, M. (2008). Production of chitinolytic enzymes by a strain (BM17) of Paenibacillus pabuli isolated from crab shells samples collected in the east sector of central Tyrrhenian Sea. *International journal of biological macromolecules*, *43*(1), 27-31.

Khatri, D. K., Tiwari, D. N., & Bariya, H. S. (2017). Chitinolytic efficacy and secretion of cell wall degrading enzymes from Trichoderma spp. in response to phyto-pathological fungi. *Journal of Applied Biology and Biotechnology*, *5*(6), 1-8.

Khatri, D. K., Tiwari, D. N., & Bariya, H. S. (2017). Chitinolytic efficacy and secretion of cell wall-degrading enzymes from Trichoderma spp. in response to phytopathological fungi. *J. Appl. Biol. Biotechnol*, *5*(6), 1-8.

Ku Y, Yang N, Pu P, Mei, Cao L, Yang X, Cao C (2021) Biocontrol mechanism of *Bacillus subtilis* C3 against bulb rot disease in *Fritillaria taipaiensis* PY Li. Front Microbiol 12:756329.

Masih, E. I., & Paul, B. (2002). Secretion of β-1, 3-glucanases by the yeast Pichia membranifaciens and its possible role in the biocontrol of Botrytis cinerea causing grey mold disease of the grapevine. *Current microbiology*, *44*, 391-395.

Miljaković, D., Marinković, J., & Balešević-Tubić, S. (2020). The significance of Bacillus spp. in disease suppression and growth promotion of field and vegetable crops. *Microorganisms*, *8*(7), 1037.

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical chem 31(3):426-428.

Mulk S, Wahab A, Yasmin H, Mumtaz S, El-Serehy HA, Khan N, Hassan MN (2022) Prevalence of wheat associated *Bacillus* spp. and their bio-control efficacy against fusarium root rot. Front microbiol 12:798619.

Radjacommare, R., Venkatesan, S., & Samiyappan, R. (2010). Biological control of phytopathogenic fungi of vanilla through lytic action of Trichoderma species and Pseudomonas fluorescens. *Archives of Phytopathology and Plant Protection*, *43*(1), 1-17.

Sadfi, N., Cherif, M., Fliss, I., Boudabbous, A., & Antoun, H. (2001). Evaluation of bacterial isolates from salty soils and Bacillus thuringiensis strains for the biocontrol of *Fusarium* dry rot of potato tubers. *Journal of Plant Pathology*, 101-117.

Sahgal, M. (2022). Fungal enzymes in biocontrol of phytopathogens. In *Progress in mycology: biology and biotechnological applications* (pp. 327-356). Singapore: Springer Nature Singapore.

Saravanakumar, K., Yu, C., Dou, K., Wang, M., Li, Y., & Chen, J. (2016). Synergistic effect of Trichoderma-derived antifungal metabolites and cell wall degrading enzymes on enhanced biocontrol of Fusarium oxysporum f. sp. cucumerinum. *Biological control*, *94*, 37-46.

Shrestha, A., Sultana, R., Chae, J. C., Kim, K., & Lee, K. J. (2015). Bacillus thuringiensis C25 which is rich in cell wall degrading enzymes efficiently controls lettuce drop caused by Sclerotinia minor. *European Journal of Plant Pathology*, *142*, 577-589.

Skidmore, A. M., & Dickinson, C. H. (1976). Colony interactions and hyphal interference between Septoria nodorum and phylloplane fungi. *Transactions of the British Mycological Society*, *66*(1), 57-64.

Takami, H., Akiba, T., & Horikoshi, K. (1989). Production of extremely thermostable alkaline protease from Bacillus sp. no. AH-101. *Applied Microbiology and Biotechnology*, *30*, 120-124.