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Antiphytopathogenic potential of chitosanase obtained from *Bacillus amyloliquefaciens* KJ782424

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Abstract

Plants have acquired a variety of defense mechanisms in order to protect themselves from potential pathogens. Chitosan and chitooligosaccharides can induce defense reactions in plants including the induction of chitosanase and 1,3-glucanase isoforms. Chitosanases have the ability to digest chitosan that constitutes the cell wall of many of the phytopathogens such as fungi. The present study aims to investigate the antifungal effect of chitosanase obtained from an isolated *Bacillus amyloliquefaciens*. Chitosanase of *B. amyloliquefaciens* was tested for antifungal activity against *Fusarium oxysporum*, *Mucor* sp., *Aspergillus niger* and *Aspergillus fumigatus*. The crude enzyme showed maximum inhibitory activity against *A. niger* (82%). However, different degree of inhibition (*A. fumigatus* 76%, *Mucor* sp. 75% and *F. oxysporum* 78%) was observed with other test organisms. The effect of chitosanase produced by *B. amyloliquefaciens* on the germination of *F. oxysporum* infested *Cicer arietinum* (gram seeds) was also tested and it was observed that the chitosanase treated seeds grew better in comparison to non-treated or infected seeds.

Key words: Chitosan, chitosanase, chitooligosaccharides, *B. amyloliquefaciens*, *Cicer arietinum*, antifungal, phytopathogens

1. Introduction

Chitosanase (EC 3.2.1.132) is an enzyme, able to specifically cleave β-1,4-glycosidic bond linkages in chitosan to produce a chitooligomer product. Chitosan and N-acetylated analogue of chitin are the most abundant glycans in nature (Pantaleone *et al.*, 1992) and they are substrates of chitosanases. The antimicrobial activity of chitosan and its oligosaccharide derivative has been recognized and is considered to be one of the most important properties, corresponding directly to their possible biological applications (Wei and Xia., 2003; Zhao and Xia., 2006). Recently, chitosanase and its chitooligosaccharides are attracting a wide attention because they possess versatile functional properties (Aam *et al*., 2010). Chitosan and its derivatives showed a broad spectrum of antimicrobial activity to filamentous fungi, yeasts and bacteria, being more active against gram-positive than gram-negative bacteria (Muzzarelli *et al*., 1990; Rhoades and Roller., 2000; Jeon *et al*., 2001; No *et al*., 2002). A novel *B. amyloliquefaciens* KJ 782424 has been isolated which shows a highly chitosanolytic index on LB medium at 30^0C after 22 h of incubation (Sharma and Azmi., 2014). Chitosanases are employed for the preparation of fungal protoplasts especially for Zygomycetes and used as a biocontrol agent of phytopathogens (Hsu *et al.*, 2012) and for developing transgenic plants. Biological control using microorganisms or its component to express plant disease, offers an alternative to chemical fungicide and also it is an ecofriendly approach for controlling agriculture pathogens. Several research groups reported *in vitro* antifungal activity of chitosanases and, thus, they can be used to improve the resistance of plants against different phytopathogenic fungi. The success in using chitosanase for diverse applications depends on the production of highly active enzyme at a reasonable cost. In this context, thermostable chitosanases offer several advantages in industrial applications because a high temperature can increase the solubility of raw materials, reduces the viscosity of the reaction mixture, triggers the enzymatic reactions and helps in protecting the reaction from microbial contamination (Zhou *et al*., 2015). Therefore, the present research was focused on applying chitosanase from microorganisms as antiphytopathogenic agent for better germination of seeds which could subsequently enhance the crop production.

2. Materials and Methods

2.1 Chemicals

All the chemicals used in this study were of analytical grade and procured from Merck and Hi-Media, India.

2.2 Microorganisms used

Bacillus amyloliquefaciens KJ 782424 used in present study was isolated from the forest soils of Uttrakhand, India (Sharma and Azmi., 2014). The culture was maintained on the agar plates (pH 7.0) containing (%, w/v) Luria broth 2.0, chitosan 0.7 and agar 2.0.

2.2.1 Test microorganisms

The antifungal activity of chitosanase obtained from *B. amyloliquefaciens* (Family : Bacillaceae) was observed against the four fungal strains; *Aspergillus niger, Fusarium oxysporum*, *Mucor* sp. and *Aspergillus fumigatus* which were procured from the authenticated stock culture of Department of Biotechnology, HPU, Shimla, India.

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2.3 Preparation of crude fungicide solution

B. amyloliquefaciens was grown in 100 mL of liquid medium in an Erlenmyer flask (250 mL), containing (%, w/v) yeast extract 1.0, chitosan 0.7, casein 1.0 and NaCl 0.5. to 5.0 mL of the 6 h old seed culture, was transferred into 100 mL of the same production medium and grown in an orbital shaking incubator for 22 h. The culture broth was then centrifuged at 15000 rpm for 20 min and the supernatant was used as crude chitosanase.

2.4 Assay of chitosanase activity

The chitosanase activity of *B. amyloliquefaciens* was found to be extracellular in nature and, hence, the cell supernatant was used as enzyme. Chitosanase activity was determined spectrophotometrically by measuring the reducing sugar released from glucosamine (Miller, 1959). The absorbance of colour developed was measured at 540 nm in a spectrophotometer (LABINDIA). One unit of chitosanase activity was defined as the amount of enzyme which produces one μmol of glucosamine ml^{-1} min^{-1} under standard assay conditions.

2.5 Assays for antifungal activity

The antifungal activity of the crude enzyme was tested using agar well diffusion method as well as in potato dextrose broth. In well diffusion method, spreading of the fungal cultures (50 μL) was done on the potato dextrose agar (PDA) plate with the help of spreader and different concentrations (0.514 to 2.57 IU) of the syringe filtered enzyme, was placed into different wells. The petriplates were incubated at 30^0C for 24-48 h. The zone of inhibition around each of the well (10 mm) was recorded including the well diameter (Ueno *et al*., 1997).

2.6 Effect of *B. amyloliquefaciens* chitosanase on plant-pathogenic fungi

F. oxysporum, *Mucor* sp., *A. niger* and *A. fumigatus* were grown separately in 50 mL of potato dextrose broth (PDB) in 250 mL flasks at 30^0C with an agitation speed of 150 rpm. Different amounts (5.74 IU to 63.14 IU) of filter-sterilized *B. amyloliquefaciens* chitosanase was added to each culture and incubated for 48-72 h at 30^0C. A set of control was also taken in which chitosanase was not added to the test culture. Each of the reaction was carried out in triplicate and the error bars were added by calculating standard deviation. The dry weights of the each test organism were measured by filtering the broth through Whatmann No.1 filter paper and subsequent washing with distilled water. The percentage inhibition was calculated in each case.

2.7 Effect of chitosanase on germination of *Cicer arietinum* (gram seeds) infected with fungus

The chitosanase of *B. amyloliquefaciens* was used to treat the gram seeds to investigate their effect on plant growth. Gram seeds were screened by shape, color, appearance and weight to eliminate bad ones. The selected seeds were planted in organic culturing soil in plastic tray with different segments. Seeds were sown at a suitable depth in each well and the plastic tray was placed in green house for germination for 7 days. This experiment was planned as per scheme given below (Figure 1). The plant growth was measured in each case above and below the soil.

2.8 Statistical analaysis

The data were subjected to statistical analysis for calculating the mean and the standard error.

3. Results and Discussion

3.1 Growth inhibition of different fungal strains by *B. amyloliquefaciens* chitosanase

Chitaosanase enzyme of *B. amyloliquefaciens* showed antifungal activity on PDA medium (Figure 2) as this enzyme has ability to break the chitosan present in their wall. These fungi were separately grown in potato dextrose broth and different amount of *B. amyloliquefaciens* chitosanase was added except control. The biomass of test fungi was significantly reduced with coincubation with filter sterilized culture supernatant of *B. amyloliquefaciens* chitosanase after 48 h when compared with the fungi, grown without chitosanase.

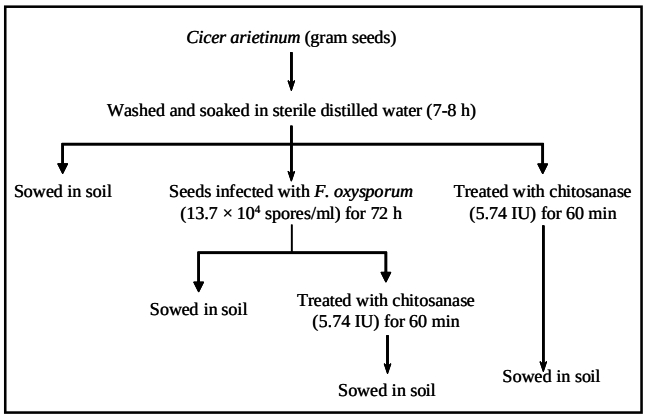

Figure 1: Experimental plan to establish the role of chitosanase on germination of fungal infected seeds

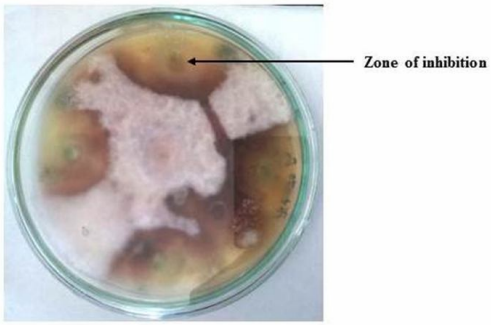

Figure 2: Zone of inhibition of *F. oxysporum*

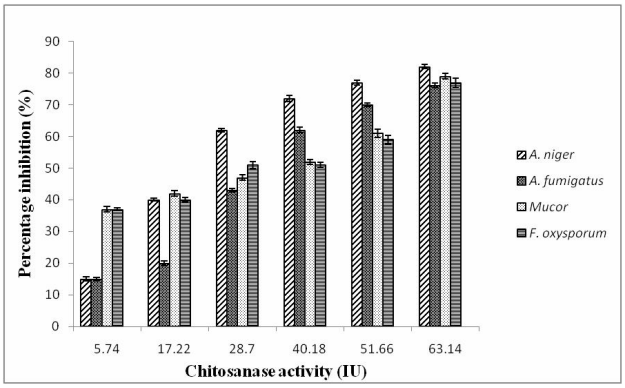

Figure 3: Inhibitory activity of chitosanase against different fungal strains

The antifungal activity of crude chitosanase of *B. amyloliquefaciens* showed marked inhibitory activity against *A. niger, A. fumigatus, Mucor* sp. and *F. oxysporum*. The crude chitosanase showed maximum inhibitory activity against *A. niger* (82%). However, *A. fumigatus*, *Mucor* sp. and *F. oxysporum* exhibited 76, 75 and 78% inhibition, respectively. Generally, if the inhibition ratio exceeded 20%, the tested fungus growth was considered inhibited (Chang *et al.*, 2007). Our results showed that antifungal activity of chitosanase against *A. niger* were higher than other fungal strain. The data indicated that inhibitory effect of chitosanase increased with increase of enzyme activity (Figure 3). This is because chitosanase hydrolyzed chitosan in cell wall of microorganism and inhibit the growth of microorganism. Antimicrobial activities of chitooligosaccharides produced by chitosanase from *Pseudomonas* CUY8 depend on their concentrations, degrees of deacetylation and polymerization. This study suggested that chitooligosaccharides and chitosanase from *Pseudomonas* CUY8 have the potential application to food and aquatic preservation (Wang *et al.*, 2007). According to Abdel-Aziz *et al.* (2012), it was reported that when the crude supernatant of the strain *Bacillus alvei* NRC-14 was examined against *Fusarium oxysporum* and *Scloratium rolfsii*, it possesses a potential antifungal activity as indicated by zone formation around the wells and by the release of reducing sugars due to fungal mycelium degradation as the strain was grown with fungal chitin which contains the dried mycelium of *A. niger*. Kilani-Feki *et al.* (2013) reported that when the hyphae of *R. oryzae* and *R. nigricans* were cultured for 24 h in a PDB medium, a significant antifungal activity was monitored as it showed a general degradation of *Bacillus subtilis* chitosanase culture compared to the control without protein. The microscopic observations showed that in the presence of *Bacillus subtilis* chitosanase, both *Rhizopus* species were strongly affected with an alteration of the mycelium walls and the appearance of swellings. This alteration was due to degradation of chitosan present in the cell walls, inducing a change in the membrane structure and mycelium permeability. The effect is more pronounced on *R. nigricans* with formation of protoplasts.

3.2 Effect of chitosanase on the germination of fungal infected gram seeds

The effect of *B. amyloliquefaciens* chitosanase on the germination of gram seeds infested with phytopathogenic fungi, *F. oxysporum* was tested and the results are presented in Figure 4.

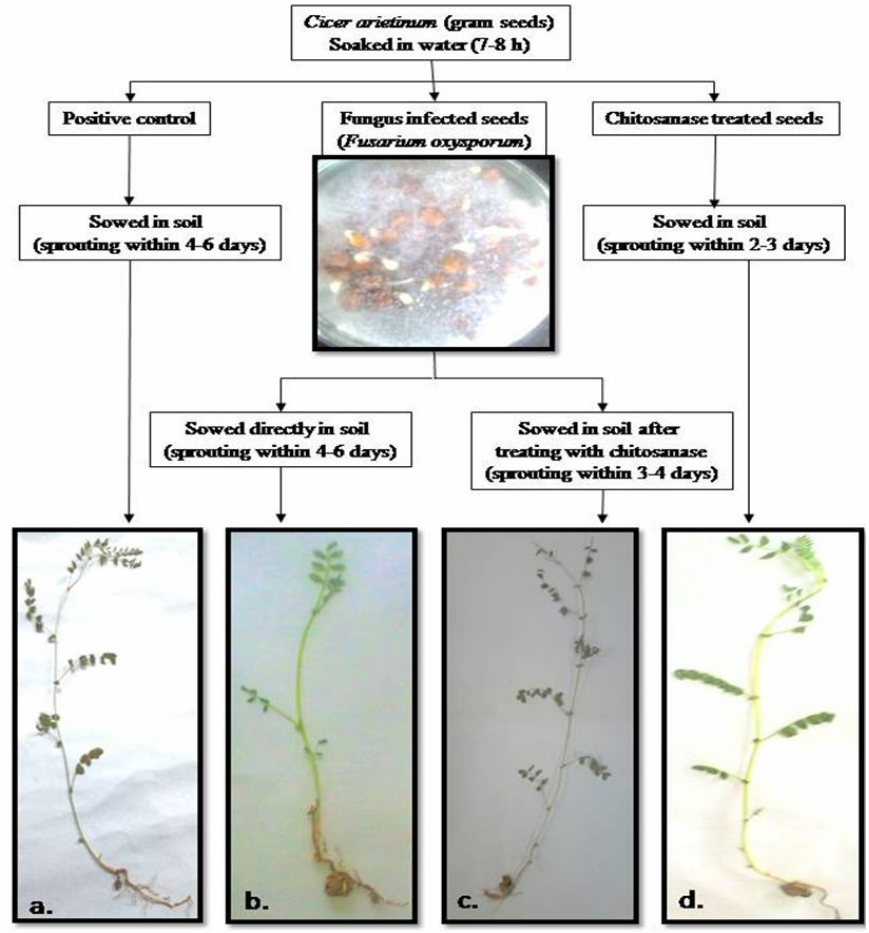

Figure 4: Germination of *Cicer arietinum* (gram seeds); a) Normal seed (Positive control), b) Fungus infected seed (Negative control), c) Fungal infected seeds subsequently treated with chitosanase and d) Chitosanase treated seeds without fungal infection

The growth of the plant pathogenic fungi, *Fusarium oxysporum* was considerably affected by *B. amyloliquefaciens* chitosanase. The fungi reduced the germination of infested seeds and seeds treated with *B. amyloliquefaciens* chitosanase showed a very good response for germination. Besides antifungal activity, *B. amyloliquefaciens* enhanced the growth of *Cicer arietinum* (Table 1). *Bacillus cereus* QQ308 produced antifungal hydrolytic enzymes, comprising chitinase, chitosanase and protease, when grown in a medium containing shrimp and crab shell powder produced from marine waste. The growth of the plant pathogenic fungi, *F. oxysporum, F. solani* and *Pythium ultimum* were considerably affected by the presence of the *B. cereus* culture supernatant. The supernatant inhibited spore germination and germ tube elongation of *F. oxysporum, F. solani* and *P. ultimum.* Beside antifungal activity, *B. cereus* QQ308, chitosanase was also reported to enhance the growth of Chinese cabbage. These characteristics were unique among known strains of *B. cereus* (Chang *et al*., 2007).

Table 1: Growth of the plant

S.No.	Experiments	Plant dry weight (mg)	Growth (in cm)	
			Shoot	Root
1.	Control (normal seed)	120 ± 1.52	18 ± 0.70	4 ± 0.13
2.	*F. oxysporum* infected seeds	70 ± 1.52	9 ± 0.25	2 ± 0.12
3.	*F. oxysporum* infected and chitosanase treated seeds	160 ± 2.08	20 ± 0.76	3 ± 0.09
4.	Normal seeds treated with chitosanase	230 ± 3.21	23 ± 0.50	5 ± 0.14

4. Conclusion

The protection of plants from the diseases, caused by the phytopathogenic fungi is one of the most important challenges in the field of agriculture. The present study reveals that the chitosanase produced by the *B. amyloliquefaciens* exhibited notable antifungal effects against various phytopathogenic fungi tested. Besides this, the *B. amyloliquefaciens* chitosanase was found to help in better germination of fungal infected *Cicer arietinum* seeds as well as in the enhancement of the plant growth. Furthermore, chitosanase obtained from a novel strain of *B. amyloliquefaciens* may also have important implications on agriculture as a biocontrol agent for insect pests.

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Conflict of interest

We declare that we have no conflict of interest.

References

Aam, B.B.; Heggest, E.B.; Norberg, A.L.; Sorlie, M.; Varum, K.M. and Eijsink, V.G. H. (2010). Production of chitooligosac-charides and their potential applications in medicine. Mar. Drugs, **8:**1482-1517.

Abdel-Aziz S. M.; Moharam M. E.; Hamed H. A. and Mouafi F. E. (2012). Extracellular metabolites produced by a novel strain, *Bacillus alvei* NRC-14. Some properties of the chitinolytic system. New York Sci. J. **5:**53-62.

Chang, W.T.; Chen, Y.C. and Jao, C. L. (2007). Antifungal activity and enhancement of plant growth by *Bacillus cereus* grown on shellfish chitin wastes. Biores.Technol., **98:**1224-1230.

Hsu, S.K.; Chung, Y. C.; Chang, C. T. and Sung, H. Y. (2012). Purification and characterization of two chitosanase isoforms from the sheaths of bamboo shoots. J. Agric. Food Chem., **60:**649-657.

Jeon, Y.J.; Park, P.J. and Kim, S.K. (2001). Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydr. Polym., **44:**71-76.

Kilani-Feki, O.; Frikha F.; Zouari, I. and Jaoua, S. (2013). Heterologous expression and secretion of an antifungal *Bacillus subtilis* chitosanase (CSNV26) in *Escherichia coli.* Bioprocess. Biosyst. Eng., **36:**985-992.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent of determination of reducing sugar. Anal. Chem., **31:**426-428.

Muzzarelli, R.; Tarsi, R.; Filippini, O.; Giovanetti, E.; Biagini, G. and Varaldo, P.E. (1990). Antimicrobial properties of *N*-carboxybutyl chitosan. Antimicrob. Agents Chemother., **34:**2019-2023.

No, H.K.; Park, N.Y.; Lee, S.H. and Meyers, S.P. (2002). Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. Int. J. Food Microbiol., **74:**65-72.

Pantaleone, D.; Yalpani, M. and Scollar, M. (1992). Unusual susceptibility of chitosan to enzymatic hydrolysis. Carbohydr. Res., **237:**325-332.

Rhoades, J. and Roller, S. (2000). Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. Appl. Environ. Microbiol., **66:**80-86.

Sharma, M. and Azmi, W. (2014). Isolation of a novel *Bacillus amyloliquefaciens* KJ 782424 with chitosanase activity. J. Adv. Microbiol., **1:**322-329.

Ueno, K.; Yamaguchi, T. and Sakairi, N. (1997). Antimicrobial activity by fractionated chitosan oligomers. Advan. in Chitin Sci., **2:**156-161.

Wang, Y.; Zhou, P.; Yu, J.; Pan, X.; Wang, P.; Lan, W. and Tao, S. (2007). Antimicrobial effect of chitooligosaccharides produced by chitosanse from *Pseudomonas* CUY8. Asia pac. J. Clin. Nutr., **16:**174-177.

Wei, X. L. and Xia, W. S. (2003). Research development of chitooligosaccharides physiological activities. Chin. Pharm. Bull., **19:**614-617.

Zhao, X.R. and Xia, W. S. (2006). Antimicrobial activities of chitosan and application in food preservation. Chin. Food Research and Dev., **27:**157-160.

Zhou, Z.; Zhao, S.; Wang, S.; Li, X.; Su, L.; Ma, Y.; Li, J. and Song, J. (2015). Extracellular overexpression of chitosanase from *Bacillus* sp. TS in *Escherichia coli*. Appl. Biochem. Biotechnol., **175:**3271-3286.