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Production of fungal cell wall degrading enzymes by a biocontrol strain of *Bacillus subtilis* AF 1

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Fungal cell wall degrading chitinases and glucanases attained significance in agriculture, medicine, and environment management. The present study was conducted to describe the optimum conditions required for the production of β-1,4-*N*-acetyl glucosaminidase (NAGase) and β-1,3-glucanase by a biocontrol strain of *Bacillus subtilis* AF 1. *B. subtilis* AF 1 was grown in minimal medium with colloidal chitin (3.0%) and yeast extract (0.3% YE) and incubated at *p*H 7.0 and 30°C on constant shaker at 180 rpm for 6 days produced highest amounts of NAGase. Presence of 0.5 m*M* of phenyl methyl sulfonyl fluoride (PMSF) and 0.04% of Tween 20 further improved the enzyme production. *B. subtilis* AF 1 grown in minimal medium with laminarin (1%) and yeast extract (0.3%) for 3 days produced maximum amount of β-1,3-glucanase. These conditions can be further scaled-up for large-scale production of NAGase and β-1,3-glucanase by *B. subtilis* AF 1.

Keywords: *Bacillus subtilis*, Biocontrol, Fungal cell wall, Wall degrading enzyme

Chitin, a linear β-(1,4)-linked N-acetyl glucosamine polysaccharide, is the second most abundant natural polysaccharide after cellulose in nature[1]. Chitin is a major structural component of marine invertebrates, insects, fungi and algae. The content of chitin varies from 22-44% in fungal cell walls, 3-5% in green algae, and 25-50% in the cuticles of arthropods and mollusks. Due to the important biological role of chitin as a structural component, its synthesis and degradation has been the subject of extensive research. Most of these studies have focussed on biofungicides, bioinsecticides, fungal morphogenesis and commercial utilization of chitinous wastes for single cell protein production. Important application of chitinases/chitinolytic microorganisms is as biofungicides and bioinsecticides. Commercial applications of chitin degrading enzymes are in agriculture, medicine, ecological and environmental fields[2,3].

β-1,3-Glucanase is another important enzyme in biological control of phytopathogenic fungi, which helps in degradation of glucan content of fungal cell walls. Chitinases and glucanases are inducible and secreted by many microorganisms in cultures containing chitin or its oligomers and glucan compounds as sole carbon source. The two enzymes are of special interest in control of fungal diseases of plants because of their ability to degrade the cell walls of phytopathogenic fungi. *Bacillus subtilis* was known to produce high levels of both chitinase and glucanase enzymes. *Bacillus* spp. produce large amounts of extracellular enzymes and are among the most extensively studied organisms for industrial enzyme production[4].

A biocontrol strain of *Bacillus subtilis* AF 1 has shown broad-spectrum antifungal activity against phytopathogenic fungi[5]. *B. subtilis* AF 1 produced β-1,4-N-acetyl glucosaminidase (NAGase) and β-1,3-glucanase in presence of colloidal chitin and laminarin as sole carbon source[6]. The chitinolytic ability of *B. subtilis* AF 1 has been utilized to develop improved formulations with chitin supplements for effective fungal disease control[7]. Partially purified NAGase of *B. subtilis* AF 1 has been used for control of groundnut rust and soft rot of lemons[8]. In the present study, we have reported the optimal conditions for production of NAGase and β-1,3-glucanase by *B. subtilis* AF 1.

Materials and Methods

B. subtilis AF 1 was isolated from wilt non-conducive soils of pigeonpea rhizosphere[5]. All the chemicals and media components used in the study

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were from Himedia, SD Fine chemicals, India and chitin was procured from Sigma Chemical Co., USA.

β-1,4-N-Acetyl glucosaminidase (NAGase) assay—Cell free culture filtrate of AF 1 was used as enzyme source and chitinase activity was measured as described by Boller and Mauch[9] with minor modifications, using colloidal chitin prepared according to Berger and Reynolds[10], in 10 m*M*, sodium phosphate buffer (*p*H 7.0) as substrate. NAGase activity unit was expressed as amount of enzyme required to release 1 μ*M* of N-acetyl glucosamine (NAG) per ml per hr. Protein content in culture filtrate was estimated according to Bradford[11] and specific activity of enzyme expressed in terms of units per mg protein.

β-1,3-Glucanase assay—The reaction mixture consisted of 50 μl each of enzyme source, laminarin (4%) and 20 m*M* of sodium acetate buffer (*p*H 5.2) and incubated at 37°C for 60 min. β-1,3-glucanase was assayed colorimetrically by measuring the reducing sugars released from enzyme substrate reaction with dinitro salicylic acid (DNS) reagent using glucose as standard[12]. *β*-1,3-Glucanase activity unit was expressed as amount of enzyme required to release 1 μ*M* of glucose per ml per hr. Specific activity was represented as units of enzyme per mg protein.

Optimum conditions for production of NAGase and β-1,3-glucanase—*B. subtilis* was grown in minimal medium (MM; contained 0.05 g of yeast extract; 0.30 g, magnesium sulfate; 1.36 g, potassium phosphate; 1.0 g, ammonium sulfate; and 5 g of chitin in one litre of double distilled water), *p*H adjusted to 8.5 with 1N NaOH. In all the experiments, 5 ml of broth in 25 ml flask was inoculated with 50 μl of log phase *B. subtilis* AF 1 culture and incubated at 180 rpm and 30°C.

Production of NAGase by *B. subtilis* AF 1 was studied under different conditions including fermentation time, nature and concentration of carbon source (colloidal chitin, N-acetyl glucosamine, N,N'-diacetyl chitobiose and *Aspergillus niger* mycelium), *p*H of medium (*p*H 4-10 with an increment of 1), concentration of yeast extract (YE; 0.1-0.5% with an increment of 0.05%), phenyl methyl sulfonyl fluoride (PMSF; 0.1-1.0 m*M* with an increment of 0.1 m*M*) and Tween 20 (0.01-0.1% with an increment of 0.01%).

The production of extracellular β-1,3-glucanase by *B. subtilis* AF 1 was studied under different conditions like fermentation time (12hr-3 days at 12 hr interval), *p*H of the medium (4-10 with an increment of 1), laminarin (0.2-2% with an increment of 0.2%), YE (0.05-0.5% with an increment of 0.05%), PMSF (0.1-1.0 m*M* with an increment of 0.1 m*M*) and Tween 20 (0.01-0.1% with an increment of 0.01%).

All the experiments for optimization of NAGase and β-1,3-glucanase production by *B. subtilis* AF 1 were repeated thrice (with three replications each time) and presented mean values.

Results and Discussion

B. subtilis AF 1, when grown in MM containing chitin (0.5%), NAGase activity of the culture filtrates increased steadily up to six days and decreased thereafter (Fig. 1A). Similarly, β-1,3-glucanase activity of the culture filtrates increased steadily up to 48 hr from 1 to 2.8 units/mg protein and decreased thereafter. Colloidal chitin was the most suitable substrate to induce production of NAGase by *B. subtilis* AF 1 (Fig. 1B) followed by chitobiose, *A. niger* mycelium, NAG and chitosan and the enzyme levels were maximum when 3.0% colloidal chitin was used as the inducing substrate (Fig. 1C). For β-1,3-glucanase, laminarin (1.0%) supported maximum production (3.8 units/mg protein), when a range from 0.2 to 2.0% was used. Further increase in laminarin concentration did not affect the production of β-1,3-glucanase.

NAGase production was high at neutral *p*H and declined both in alkaline and acidic *p*H (Fig. 1D). Similarly, at *p*H 7.0 and 8.0, β-1,3-glucanase production was higher, where it reached 4.0-4.4 specific activity units compared to other *p*H conditions tested.

AF 1 produced maximum NAGase in presence of yeast extract (0.3%), which served as a nitrogen source (Fig. 1E). Further increase in YE concentration negatively influenced the specific activity of the enzyme. For β-1,3-glucanase production also, 0.3% YE supported maximum activity of 7.1±0.21 units, and further increase in the concentration had no effect on enzyme production.

In presence of PMSF (0.5 m*M*), the NAGase activity was high (Fig. 1F). β-1,3-glucanase production by *B. subtilis* AF 1 was not affected by addition of PMSF from 0.1 to 1.0 m*M* (data not shown). *B. subtilis* AF 1 was grown in MM supplemented with different concentrations of Tween 20 (0.01 to 0.1%) with all other optimum conditions described above.

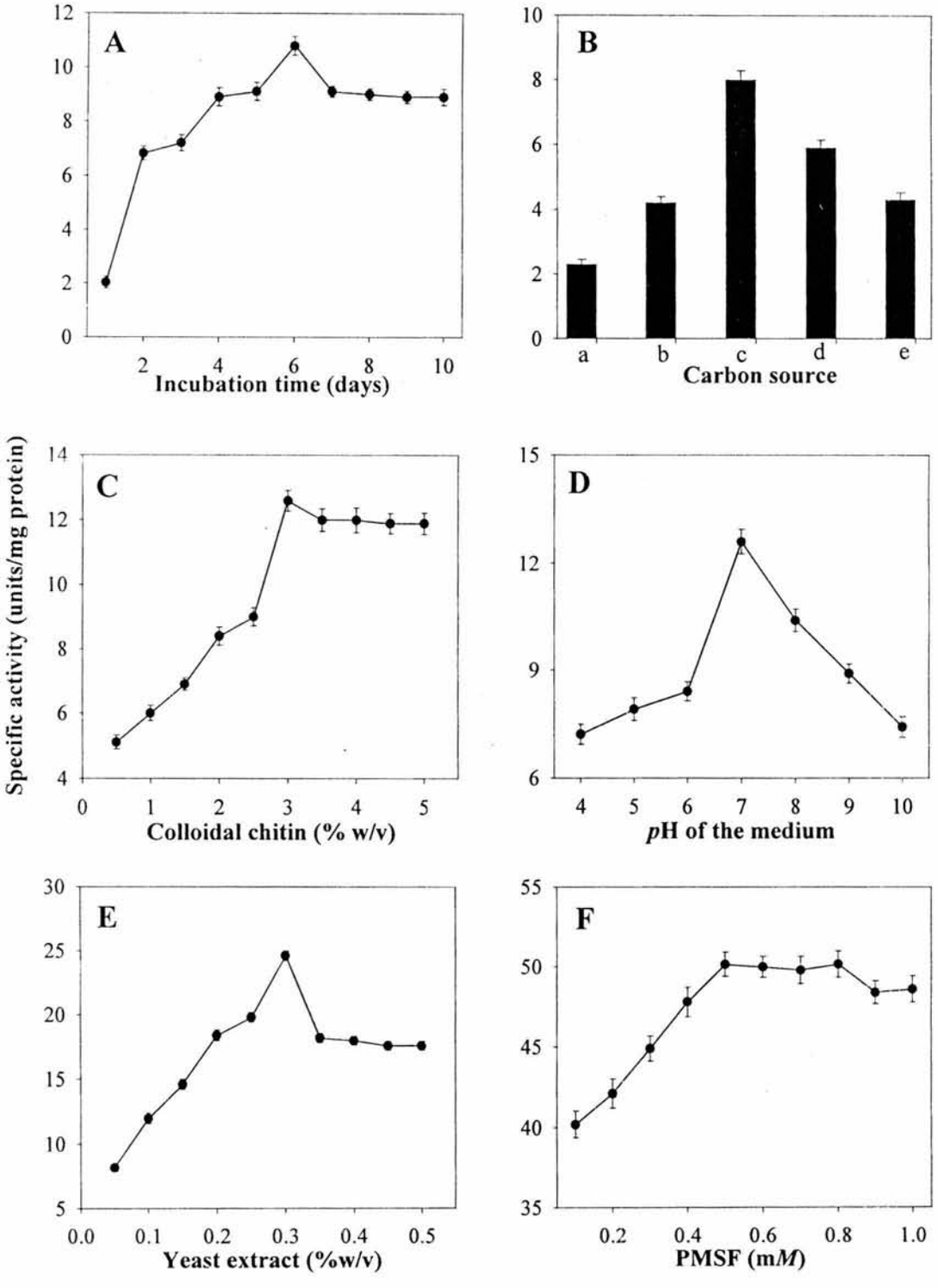

Fig. 1—Effect of different factors on the production of β-1,4-*N*-acetyl glucosaminidase by *B. subtilis* AF 1 grown in submerged culture (180 rpm) with different supplements in MM at 30°C. The activity of NAGase was expressed in terms of specific activity—(A) Incubation period (MM with 0.5% CC), (B) Carbon sources (a–chitosan, b–N-acetyl glucosamine, c–colloidal chitin, d–chitobiose, e – *A. niger* mycelium); (C) Concentration of colloidal chitin, (D) *p*H of the medium (MM with chitin), (E) Concentration of yeast extract and (F) Concentration of PMSF. [Each data point represents mean of three determinations and the vertical bars represent standard deviation].

Increase in NAGase levels were observed up to 0.04% of Tween 20 where the increase was from 32 to 40 units/mg protein approximately. Addition of Tween 20 at concentrations of 0.01 to 0.1% did not alter β-1,3-glucanase production by *B. subtilis* AF 1 (data not shown).

Extra-cellular β-1,3-glucanase and chitinases have been shown to be important in dissolution of hyphal walls in most cases of the antagonistic interaction between biocontrol bacteria and plant fungal pathogens. In the genus *Bacillus*, chitin degrading activity is very common[13]. *B. cereus* produces a chitobiosidase and is antagonistic to root rot pathogen *Rhizoctonia solani*[14] in cotton seedlings. *B. circulans* IAM1165 produces three major extracellular β-1,3-glucanases, of which, 42 kDa enzyme lyses the fungal cell wall effectively[15]. Similarly, *B. circulans* WL-12, when grown in liquid medium with yeast cell walls or yeast glucan produces five β-glucanases[16]. Selective enzymolysis of cell walls of *Pyricularia oryzae* by single and combined actions of β-1,3- and 1,6-glucanases and chitinase produced by *B. circulans* WL-12 has been reported by Tanaka and Watanabe[17].

B. subtilis AF 1 inoculated into medium containing *A. niger* suppressed >90% fungal growth in terms of dry weight. In dual cultures the fungal growth was not accompanied by formation of spores. The mycelial preparation of *A. niger* as principal carbon source supported the growth of *B. subtilis* AF 1 as much as chitin[18]. Crude and partially purified NAGase from culture filtrate of *B. subtilis* AF 1 inhibited the growth of *A. niger* and uredinospore germination of groundnut rust pathogen *Puccinia arachidis*. The enzyme was also effective against post harvest soft rot in lemons[8].

Production of NAGase and β-1,3-glucanase could be increased by 5- and 3-folds, respectively compared to initial levels by altering cultural and nutrient conditions. The NAGase activity increased from 2 to 30 units/mg protein after optimizing growth and culture conditions. Similarly, units of β-1,3-glucanase activity increased from 1 to 10 unit after optimizing growth conditions. Presence of endochitinase activity in the culture supernatant was ruled out since further incubation of enzyme substrate reaction mixture with cytohelicase did not produce additional N-acetylated sugars. Increase in the concentration of chitin and yeast extract in the growth medium of *Serratia marcescens*, *Enterobacter liquefaciens* and *Aspergillus fumigatus* resulted in increased chitinase production[19]. Oligomers and monomers of chitin from different sources have been reported as inducers of chitinolytic enzymes. The suitability of mono/oligomers like N-acetyl glucosamine, diacetyl chitobiose, colloidal chitin and *A. niger* mycelium was tested and it was found that colloidal chitin at a higher concentration of 3.0% served as better inducer of NAGase in the present study. In the present study, yeast extract might have contributed to better proliferation of bacterial cells in the medium, whereas Tween 20 facilitated better release of enzyme into the external medium. Enhanced NAGase activity in presence of PMSF could be due to inhibition of proteases that degrade NAGase produced into the extracellular medium. The present study indicated the possibility of increased production of NAGase and glucanase of *B. subtilis* AF 1 with alterations in the culture conditions, which can be used to enhance the biocontrol activity.

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References

1 Gooday G W, Chitinases: Enzymes in Biomass Conversion, in *American Chemical Society Symposium*, No. 460, edited by G F Letham and M E Himmel (American Chemical Society, Washington DC) 1991, 478.

2 Li Q, Dunn E T, Grandmaison E W & Goosen M F A, Applications and properties of chitosan, in *Applications of chitin and chitosan*, edited by Goosen M F A (Technomic Publishing Company, Inc. Pennsylvania, USA) 1997, 346.

3 Hirano S, Applications of chitin and chitosan in the ecological and environmental fields, in *Applications of chitin and chitosan*, edited by M F A Goosen (Technomic Publishing Company, Inc. Pennsylvania, USA) 1997, 31.

4 Schallmey M, Singh A & Ward O P, Developments in the use of *Bacillus* species for industrial production, *Can J Microbiol*, 50 (2004) 1.

5 Podile A R, Prasad G S & Dube H C, *Bacillus subtilis* AF 1 as an antagonist of vascular wilt fungi, *Curr Sci*, 54 (1985) 864.

6 Manjula K, *Characterization of chitinolytic ability of Bacillus subtilis AF1 and its use in development of improved formulations for plant growth promotion and disease control*. Ph.D. Thesis submitted to University of Hyderabad, India, 1999.

7 Manjula K & Podile A R, Chitin-supplemented formulations improve biocontrol and plant growth promoting efficiency of *Bacillus subtilis* AF 1, *Can J Microbiol*, 47 (2001) 618.

8 Manjula K, Kishore G K & Podile A R, Whole cells of *Bacillus subtilis* AF 1 proved effective than cell free and chitinase-based formulations in biological control of citrus fruit rot and groundnut rust, *Can J Microbiol*, 50 (2004) 737.

9 Boller T & Mauch F, Colorimetric assay for chitinase activity, *Methods Enzymol,* 161 (1988) 430.

10 Berger L R & Reynolds D M, Colloidal chitin preparation, *Methods Enzymol,* 161 (1988) 140.

11 Bradford M M, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal Biochem,* 72 (1976) 248.

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

13 Kuroshima K I, Satane T, Takata R & Yokota A, *Bacillus ehimensis* sp. nov. and *Bacillus chitinolyticus* sp. nov., New chitinolytic members of the genus *Bacillus*, *Int J Syst Bacteriol*, 46 (1996) 76.

14 Pleban S, Chernin L & Chet I, Chitinolytic activity of an endophytic strain of *Bacillus cereus*, *Lett Appl Microbiol*, 25 (1997) 284.

15 Hamamura M, Yamamoto M & Asano T, Isolation of extracellular 28- and 42-kilodalton beta 1,3-glucanases and composition of three beta 1,3-glucanases produced by *Bacillus circulans* IAM 1165, *Appl Environ Microbiol* , 61 (1995) 122.

16 Rombouts F M & Phaff H J, Lysis of yeast cell walls: Lytic β 1,3-glucanases from *Bacillus circulans* WL-12, *Eur J Biochem*, 63 (1976) 121.

17 Tanaka H & Watanabe T, Glucanases and chitinases of *Bacillus circulans* WL-12, *J Indus Microbiol*, 14 (1995) 478.

18 Podile A R & Prakash A P, Lysis and biological control of *Aspergillus niger* by *Bacillus subtilis* AF 1, *Can J Microbiol*, 42 (1996) 533.

19 Monreal J & Reese E T, The chitinase of *Serratia marcescens*, *Can J Microbiol*, 15 (1969) 689.