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Optimization of chitinase produced by a biocontrol strain of *Bacillus subtilis* using Plackett-Burman design

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ABSTRACT

A novel Bacillus subtilis (JN032305), strain was isolated from Chilli rhizosphere soil. It exhibited broad-spectrum antifungal activity against nine potent chilli pathogens. The Strain was detected for chitinase production on colloidal chitin plates.. The strain was able to produce Chitinase (1 U/mL) on Nutrient broth (NB) amended with colloidal chitin (10g/L). The optimization of fermentation medium for the production of chitinase by this strain was done using Plackett- Burman design. The procedure limited the number of actual experiments performed while allowing for possible interactions between the components. The Design revealed that for chitinase production, CMC (10g/L), incubation period of 72 h, and temperature (30ºC) were very significant. The Chitinase activity with unoptimised medium was 1 U/mL and that was enhanced ten folds to 11.5 U/mL under optimized conditions using Plackett- Burman design.

Key words: Chitinase, *Bacillus subtilis*, production, Plackett-Burman

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INTRODUCTION

Chitin, an insoluble linear β1,4-linked polymer of N-acetylglucosamine, is a common constituent of fungal cell walls and of the exoskeletons of arthropods [1]. Chitinases are the enzymes responsible for biological hydrolysis of chitin to its monomer N-acetyl glucosamine [2] and have been found to be produced by a number of microorganisms. Chitin degradation is a key step in cycling of nutrients in the environment. These enzymes are known to perform several biological functions. Bacteria produce chitinase to digest chitin and utilize it as carbon and energy sources [3]. In addition, some chitinases of chitinolytic bacteria, such as the *chiA* gene produced from *Serratia marcescens* and *Enterobacter agglomerans* are potential agents for the biological control of plant diseases caused by various phytopathogenic fungi [4, 5]. The latter enzymes inhibit fungal growth by hydrolyzing the chitin present in the fungal cell wall. Various species of *Bacillus* have been shown to secrete chitinase, including *B. Circulans* [6], *B. Licheniformis* [7,8] and some others [9]. The ability of *B. subtilis* to suppress a wide range of plant pathogens and also its reputation as a safe and friendly microorganism is attractive. Many isolates of *B. subtilis* display antagonistic activity against a number of pre-harvest and post-harvest pathogens on several different crops [10-12]. Chilli (*Capsicum annuum* L.) is an important vegetable and spice crop worldwide, produced and consumed as fresh or processed. The popularity and demand for chilli are providing a boost to the chilli industry, but production is increasingly constrained by chilli plant diseases. The chilli wilt has been found as the most frequently encountered disease problem [13]. Several microorganisms may be involved in causing wilt diseases in chilli. The four diseases that lead to wilting in chilli are *Phytophthora* root rot, *Verticillium* wilt, *Rhizoctonia* root rot, and *Fusarium* wilt.

Studies on medium optimization for chitinase production is a worthwhile technique for multifactor experiments because it is less time consuming and capable of detecting the true optimum level of the factor. In addition, medium

compositions greatly influence the microbial production of extracellular chitinase and their interaction play an important role in the synthesis of this enzyme [14]. Optimizing the parameters by statistical method reduces the time and expense. Several statistical and non statistical methods are available for optimization of medium constituents [14]. Plackett-Burman and response surface methodology are the most widely used statistical approaches [15].

The objective of the present study was to optimise the medium and growth conditions for the production of highly active chitinase by a biocontrol strain of *Bacillus subtilis* (JN032305)
using statistical design of Plackett-Burman .

MATERIALS AND METHODS

Organism and culture conditions

Bacillus sp. capable of producing chitinase was isolated from the Chilli rhizosphere. It was identified as *Bacillus subtilis* by morphological, biochemical and 16S rDNA sequence analysis. The Gene-Bank accession no. for the nucleotide sequence is JN032305.

Dual plate assay

The fungal growth inhibition capacity of the strain was determined [16]. A few modifications were made to suit the need. One 5 mm disk of a pure culture of the pathogen was placed at the centre of a Petri dish containing PDA. The *Bacillus subtilis* was inoculated at 2 opposing corners. Plates were incubated for 72 h, at 28^{o}C, and growth diameter of the pathogen was measured and compared to control growth, where the bacterial suspension was replaced by sterile distilled water. Each experiment using a single pathogen isolate was run in triplicate. Results were expressed as the means of the percentage of inhibition of growth of the corresponding pathogen in the presence of the strain. Percent inhibition was calculated using the following formula: % inhibition = [1 - (Fungal growth / Control growth)] x 100

Enzyme assay

The culture was grown at 30°C with 120 rpm for 24 h. 200 µL of the culture inoculum was transferred to 250 mL Erlenmeyer flask containing 100mL of broth medium with (g/L): Peptone, 5; Beef extract, 3; NaCl, 5 and Colloidal chitin, 10. The pH of the medium was adjusted to 7.0 using 1N NaOH before autoclaving. All the experiments were performed in duplicate. The culture broth after 72 h of incubation was centrifuged at 10,000 g for 10 min at 4°C to separate the cells. The cell-free supernatant was analyzed for enzyme activity.

Chitinase assay

Chitinase activity was measured with colloidal chitin as a substrate. The cell free supernatant obtained was added to 1.0 ml of substrate solution, which was made by suspending 1% of colloidal chitin in Phosphate buffer (pH 7.0). The mixture was incubated at 50°C for 30 minutes. 1ml of DNS was added and incubated at 100^{o}C in boiling water bath. The amount of reducing sugar produced in the supernatant was determined by Dinitrosalicylic acid Method (DNS) [17]. One unit of chitinase activity was defined as the amount of enzyme that produced 1 µmol of reducing sugars per min [18].

Screening of the Most Significant Medium Components by Plackett-Burman Design

Optimization of medium components for chitinase production by *B. subtilis* isolate JN032305 was performed in two stages. At the first stage, the components that have significant effect on enzyme production were identified. At the second stage, the optimum values of these components for chitinase production were determined.

Initial screening of the most important components affecting chitinase production by *B. subtilis* was performed by Plackett-Burman design. A total number of eleven components in twelve different combinations were selected for this study, with each being represented at two levels, high (+1) and low (-1) as shown in Table 1. All trials were performed in triplicate and the averages of degradation observation results were treated as the responses. The main effect of each variable was determined with the following equation:
$Exi\_(SMi\_\_SMi\_)/N$

where *Exi* is the concentration effect of the tested variable, *Mi_* and *Mi_* are chitinase activity in trials where the independent variable (*Xi*) was present in high and low concentrations, respectively, and *N* is the number of trials divided by 2. When the sign is positive, the influence of the variable upon chitinase activity is greater at a high concentration, and when negative the influence of the variable is greater at a low concentration. Using Microsoft Excel, statistical *t*-values for unequal paired samples were calculated for determination of variable significance. Because three trials of the design could not be measured, the main effects of the variables were calculated taking this fact in consideration.

Enzyme assay with optimised conditions

The culture was grown at 30°C with 120 rpm for 24 h. 200µL of the culture inoculum was transferred to 250mL Erlenmeyer flask containing 100mL of broth medium with alterations made after statistical optimization was employed, (g/L): CMC, 10; Peptone, 5; Beef extract, 3; NaCl, 5. The pH of the medium was adjusted to 7.0 using 1N NaOH before autoclaving. All the experiments were performed in duplicate. The culture broth after 72 h of incubation was centrifuged at 10,000 g for 10 min at 4°C to separate the cells. The cell-free supernatant was analyzed for enzyme activity [19,20].

RESULTS AND DISCUSSION

Members of the genus *Bacillus* are well known for their potential to secrete a number of degradative enzymes such as chitinase [21]. Detection of chitin-degrading bacteria from natural sources such as rhizosphere soil is useful in the isolation of bacteria that produce antifungal or other novel compounds. A high correlation between chitinolysis and production of bioactive compounds has been reported [22-24]. Many members of the family Bacillaceae are generally soil inhabiting bacteria or exist as epiphytes and endophytes in the spermosphere [25] and rhizosphere [26-28]. For this reason, *Bacillus* species are ideal candidates for use as biocontrol agents in seed treatment programs against soil-borne pathogens [25].

The present organism under study, *B.subtilis* (JN032305) exhibits a broad spectrum inhibition of potent chilli pathogens by the production of chitinases. The *B.subtilis* was subjected to dual plate assay with nine different chilli fungal pathogens. It showed broad spectrum antagonism against *Alternaria* (3) spp. (55%), *Colletotrichum gloeosporioides* (57%), *Phytophthora capsici* (55%), *Rhizoctonia solani* (42%), *Fusarium solani* (42%), *Fusarium oxysporum* (40%) and *Verticillium theobromae* (36%), the range of percentage inhibition varied from 40-62 [29].

Evaluation of effect of environmental factors on Chitinase production using Plackett-Burman experimental design

The production of extracellular enzymes by microorganisms is greatly influenced by media components and other environmental factors. The 11 different independent variables examined with their levels, main effect, *t*-value and Significance has been represented in Table 1. The main effect of each variable on the enzyme activity has been analysed and depicted in the figure 1.

For the production of the enzyme chitinase, different variables exhibited positive and negative effects. The factors which had a positive effect were CMC, KNO_3, CSL, TX100, incubation period, temperature and pH, however, glucose, YCW, $CaCl_2$ and Chitin had a negative effect (Figure 1). Statistical analyses of the data (*t*-test) showed that variations of YCW, CMC, incubation time and temperature affects Chitinase very significantly (Table 1).

The comparison of chitinase activity between unoptimised and optimised media showed a ten fold increase from 1 U/mL to 11.5U/mL respectively. The *t*-value was 18.999 and was found to be statistically very significant ($P < 0.05$). The Plackett Burman design has helped in screening of the essential components for chitinase production and also has identified the factors which are very significant and not significant for production. The findings are concurrent with the earlier research papers [30].

Table 1: Degree of positive or negative effect of independent variables on B.subtilis chitinase according to the levels in the Plackett-Burman experimental design

Variable	Symbol	-Level	+ Level	Main effect	*t*-value	Significance
Glucose (g/L)	G	0	5	-2.16	*-6.70*	S
YCW (g/L)	Y	0	10	-2.56	*-11.61*	VS
CMC (g/L)	CM	0	10	2.26	**11.01**	VS
KNO_3 (g/L)	K	0	0.1	0.73	**2.96**	NS
Corn SL(g/L)	CSL	0	0.1	0.33	**1.80**	NS
TX100 (mM)	T X 100	0	5	1.33	**2.30**	NS
$CaCl_2$ (mM)	C	0	50	-0.36	*-1.35*	NS
Time(Hrs)	T	24	72	2.16	**27.57**	VS
Ph	P	5	7	1.2	**5.96**	S
Chitin(g/L)	Ch	0	10	-2.0	*-8.66*	S
Temp(°C)	Tm	24	72	2.0	**97.45**	VS

Bold type indicates positive effect, italic type indicates negative effect. Very Significant (VS); Significant (S); Non-Significant (N.S) (t < 0.05).
(Key: YCW-Yeast Cell Wall extract, CMC- Carboxy Methyl Cellulose, Corn SL-Corn Steep Liquor, TX100- Triton X 100)

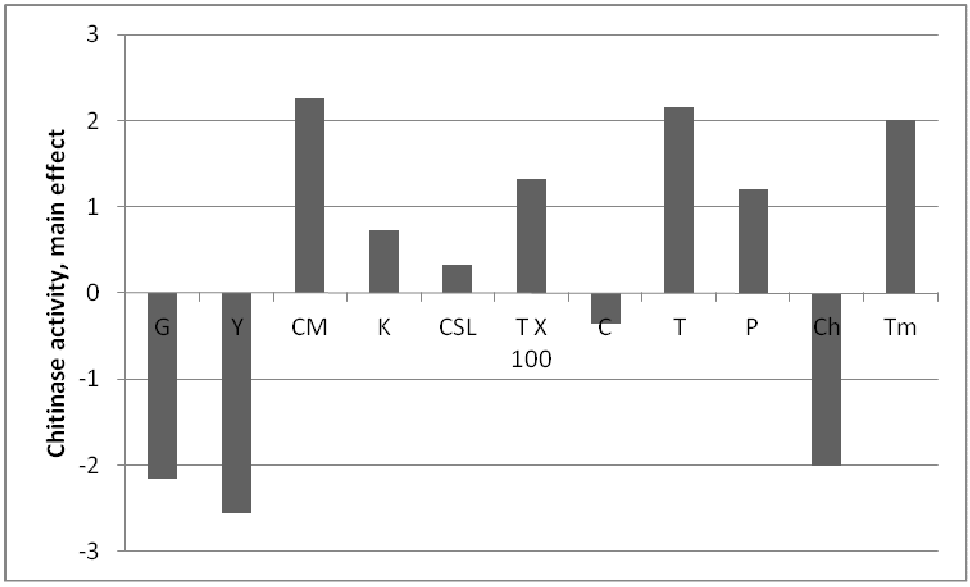

Figure 1: Elucidation of factors affecting chitinase activity

CONCLUSION

The role of chitinase in the biological control of various fungal pathogens has already been established [31-35]. Several chitinolytic organisms such as *Pseudomonas sp*. and *Streptomyces sp*. isolated from the rhizosphere have been shown to be potential biocontrol agents [36-38]. Since the chitinase producing *B. subtilis* (JN032305) was originally isolated from the Chilli rhizosphere, this bacterium could be an ideal candidate for biological control of a broad range of Chilli fungal pathogens.

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