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ANTIFUNGAL PROPERTIES OF PARTIALLY PURIFIED CHITINASE ENZYME FROM AN ENDOPHYTIC BACTERIUM *Bacillus cereus* CE3 ISOLATED FROM ITS HOST *Coleus amboinicus* L.

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ABSTRACT

Chitin is the second-largest polymer in world after cellulose, which can be broken down by chitinase enzymes. Microbes have great promise and sustainability in chitinase production at industrial level. The present study was aimed to produce and partially purify the chitinase from an endophytic bacterium *Bacillus cereus* CE3. The *B. cereus* CE3 was isolated from its host *Coleus amboinicus.* followed by improving the production medium by using one factor at a time, Plackett-Burman and response surface methodologies. Further, enzyme was purified by using various extraction and chromatographic techniques. The purified enzyme was analysed for its antifungal activities. The study revealed that *B. cereus* CE3 yielded three-fold chitinase when the variables like yeast extract, fermentation time, and orange peel powder were standardized during statistical optimization of medium, as suggested by Plackett-Burman and Response surface methodologies. In optimized medium the overall yield of chitinase enzyme was 44.87%, and the enzyme was purified three-fold by using precipitation, dialysis and column chromatography. The molecular weight of partially purified chitinase was ~43 kDa as per SDS-PAGE analysis. The stability and efficiency of enzyme and its activity revealed that the enzyme was alkaline and mesophilic in nature. It showed antifungal activity by inhibiting the growth of *Fusarium* sp., and *Aspergillus* sp. The partially purified endophytic chitinase can be used as an active ingredient in cosmetics, pharmaceuticals, and agricultural products as a biocontrol agent.

Keywords: *Bacillus cereus*, biocontrol activity, chitinase, endophyte, partial purification, yield optimization

INTRODUCTION

Endophytes are the microorganisms that thrive in symbiotic relationships with their host plants without inflicting any harm to them. The extracellular enzyme produced by them greatly aid the symbiotic relationships (Khan *et al*., 2017). The ability of enzymes to resist biotic stress and suppress a variety of plant diseases has been reported (Gopalakrishnan and Shanmugam, 2021). Therefore, the ability of endophytes to coexist with their hosts and their joint synthesis of these enzymes (Ek-Ramos *et al*., 2019) helps in mitigating the new problems. Due to their great environmental adaptability, these extracellular enzymes can be used in a variety of large-scale fermentation processes. Significant enzyme activities have been shown by a few bacterial endophytes from *Acanthaceae*, *Rhizophoraceae*, and several mangrove plants (Gopalakrishnan and Shanmugam, 2021).

Chitins are polymers of N-acetylglucosamine connected by β-1,4-glycosidic linkages. It is a common constituent of fungal cell walls, insect exoskeletons and crustacean shells. The improperly used chitins from sea foods are discarded, thus leading to huge waste accumulation. The mismanagement of chitin leads to the pollution as chitin are very slowly degraded under natural conditions and the process consumes enough oxygen from the environment thereby causing an imbalance in oxygen demand in ecosystems. Also, the chitin accumulation in soil may lead to the leaching of nutrients and even pollute the groundwater. Chitin accumulation provides a favourable environment for the growth of harmful microorganisms that adversely affect terrestrial and aquatic environments (Unuofin *et al*., 2024). Hence, to overcome these problems various waste management practices for easy and quick removal of chitin from the environment are being explored. One such biotechnological approach is chitin degradation by chitinase enzyme, prevalently found in microbes. These microbes utilize chitin as nitrogen and carbon sources for their growth and development. Their lytic enzyme actions break chitin into chitin oligosaccharides which chitobiases metabolize into N-acetyl-D-glucosamine units (Rathore and Gupta, 2015). Chitin byproducts have a variety of biological properties, including antioxidant and antibacterial, anti-tumour activities (Poria *et al*., 2021a).

Many chitinolytic microbes like *Bacillus* sp., *Streptomyces* sp., etc. have been identified; however, to enhance their chitinase production several growth factors need to be optimized. Due to higher costs and time taken in one factor at a time approach, statistical optimization is used which, in turn, not only reduces the number of experiments, but also enable faster screening of enormous factors in less time. The interaction between various parameters is achieved by statistical optimization (Meriem and Mahmoud, 2017; Dukariya and Kumar, 2021). On the selection of parameters most researchers has reported that the variables of chitin, yeast extract, temperature, incubation period, etc. play major role in chitinase production (Nawani and Kapadnis, 2005; Dukariya and Kumar, 2021).

Biotic stress by weeds, insects, and diseases cause 35-40% crop yield loss. Also, the reliance on pesticide- and fungicide-use results in the buildup of hazardous chemicals in food components as well as development of resistance in the organisms that cause infections and a variety of health problems. Using biological approach involving microbes to overcome plant pathogens is a viable alternative to chemical approach. Most research has focussed on hydrolytic enzymes, including chitinases, that weaken and degrade chitin in cell walls of many pathogens through antibacterial, antifungal, insecticidal, or nematocidal activities (Swiontek Brzezinska *et al*., 2014; Veliz *et al*., 2017). This paper describes the production and purification of chitinase enzyme from an endophytic bacterium *Bacillus cereus* CE3 isolated from *Coleus amboinicus* plant and assesses its antifungal properties.

MATERIALS AND METHODS

The leaves of *Coleus amboinicus* (Karpooravalli) were collected from the garden of SRM Arts and Science College, Kattankulathur, Tamil Nadu (India) and the present research work was conducted at the College in the year 2023.

Colloidal chitin preparation

Chitin powder (HiMedia) [10 g] was added to 120 mL conc. HCl in a 250 mL flask and incubated at 37°C on a rotary shaker (180 rpm) for overnight. The mixture was transferred to 50% ethanol and thoroughly mixed to obtain a homogenous suspension. The suspension was filtered and washed with distilled water until the colloidal chitin reached pH 7. The colloidal chitin was centrifuged and the pellets stored at 4°C until use.

Surface sterilization

The samples were surface sterilized by continuous washing with running tap water for 1 min and submerged in 70% ethanol for 1 min. The samples were then soaked in 0.1% sodium hypochlorite having 1% tween 20 for 4 min and finally rinsed three times in sterilized distilled water, with last rinsed water taken as positive control to verify the sterilization (Costa *et al*., 2012; Suhandono *et al*., 2016).

Isolation of bacteria

Serial dilution and impregnation techniques were employed to isolate the surface-sterilized samples (Costa *et al*., 2012; Marchut-Mikołajczyk *et al*., 2023). Using a sterile scalpel, the samples were equally divided. The inner epidermal region was impregnated with nutrient agar medium after half of the sample was broken into smaller pieces. The remaining half was ground in 5 mL sterilized water. The solution was serially diluted and added to the medium. Then each plate was cultured for 7 days at 37°C. The size, shape, and colour were taken into consideration for choosing the colonies that developed on the plates, and they were pure-cultured for further screening.

Screening of chitinase-producing bacteria

Colloidal chitin plate assay (Gonfa *et al*., 2023) was carried out to screen the chitinase-producing bacteria. The experiment was carried out by preparing 1% colloidal chitin-containing nutrient agar. The isolated bacterium was streaked in the centre of plates and incubated at 37°C for 36 h. The plates were flooded with Gram's iodine dye to visualize the zone of inhibition. The colonies showing high inhibition zone were selected for further for estimating chitinase enzyme production.

Chitinase assay

Chitinolytic activity was estimated by standard dinitrosalicylic acid (DNS) method (Farag *et al*., 2016; Gonfa *et al*., 2023) using colloidal chitin as substrate. The reaction mixture comprised of 0.5 mL 0.5% colloidal chitin suspension in 0.1 M sodium acetate buffer (pH 5) and 0.5 mL enzyme solution. The mixture was incubated at 50°C for 30 min and the reaction was stopped by adding 2 mL DNS and incubated for 10 min. The released N-acetyl glucosamine (NAGA) was determined colorimeterically at 530 nm. The enzyme activity was calculated from the standard curve of NAGA. The IU of chitinase activity is equal to the amount of enzyme liberating 1 µmol NAGA under the assay conditions.

Strain identification and molecular characterization

The high chitinase-producing bacteria were identified using morpho-biochemical and molecular characterization. The bacterial genomic DNA was obtained by standard DNA isolation method. Universal primers were used for PCR amplification of the chosen strain's 16S rDNA sequencing. Using upstream primer 27F and downstream primer 1492R, the PCR product was purified to have a base pair of 1482, which was then sequenced. An ABI PRISM® BigDyeTM terminator cycle kit (Applied Biosystems) was used for PCR analysis. Using an ABI3730XL automated DNA sequencing instrument (Applied Biosystems), the final product was electrophoresed. Then, using NCBI's BLAST tool, a homology search was done on the acquired nucleotides (Gopalakrishnan and Shanmugam, 2021).

Factors affecting the chitinase production in fermentation

Submerged fermentation method was used for the synthesis of chitinase, owing to its advantages like consistent enzyme properties, simple downstream approach, and processing conditions. The work concentrated on developing and scaling up the chitinase purification process under ideal physical and nutritional conditions (Tsuchiya *et al*., 1991; Patil and Kurhekar, 2020). The variables of each factor influencing chitinase synthesis in bacteria were selected based on one-factor-at-a-time approach (OFAT). Physical parameters like RPM (static and aerated), incubation period (24, 48,72, 96, and 120 h), and temperature (4, 25, 37, 45, and 60°C) were studied. Similarly, nutritional factors like carbon sources *viz*., dextrose, fructose, sucrose, lactose and mannitol @ 1% (w/v) of each, organic nitrogen source *viz*., urea, cornmeal, soybean, yeast extract, and casein @ 1% (w/v) of each, inorganic nitrogen source *viz*., ammonium sulphate, ammonium chloride, sodium carbonate, sodium di-hydrogen phosphate, and sodium chloride @ 0.5% (w/v), and agro-waste like orange peel powder (OPP) and spent coffee ground (SCG) @ 1% (w/v) were optimized. All the optimization studies were carried out in triplicates. The mean value was taken for the estimation of enzyme activity by chitinase assay.

Significant variable identification using Plackett-Burman (PB) approach

Screening of variables affecting the chitinase production was performed by PB approach. In this study, two levels of 7 independent variables *viz*., sucrose, yeast extract, NaCl, OPP, pH (8) and temperature

(37°C) selected for the analysis using Plackett- Burman design were:

Factors	Units	Coded levels	
		Low	High
Sucrose (A)	% (w/v)	0.5	1.0
Yeast extract (B)	% (w/v)	0.1	1.0
NaCl (C)	% (w/v)	0.25	0.5
Orange peel powder (OPP) (D)	% (w/v)	0.5	1.0
pH (E)	-	4.0	9.0
Fermentation period (F)	H	72.0	96.0
Temperature (G)	°C	25.0	37.0

The selected variables were classified on twelve-run PB analysis and the effect of individual variable was evaluated using the first-order polynomial model:

$$Y = \beta_0 + \Sigma \beta_i X_i \qquad (1)$$

where β_0 is the model intercept, βi is the linear coefficient, Xi is the level of independent variable, and Y is the response activity (total enzyme activity in U mL^{-1}). The model assesses the important elements that affect the response, even though it does not explain how the factors interact. Each design experiment was run in triplicate, and the response (Y) was determined by averaging the enzyme activity. All the calculation of statistical analysis was performed using Analysis of Variance (ANOVA) and the significant factors ($p < 0.05$) for the chitinase production were optimized using response surface methodology (RSM). All the experiments were carried out using statistical software, Design-Expert 10.0.1, Stat-Ease Inc. Minneapolis, USA (Mishra *et al*., 2012).

Statistical analysis of variable effect using RSM: Central composite design (CCD)

The three significant effect depicting variables (fermentation period, yeast extract, OPP) were studied at five coded level (s (-α, -1, 0, +1, +α) in a set of 20 different runs performed in triplicates as under:

Factors	Units	Coded levels				
		−α	−1	0	+1	+α
Yeast extract (A)	% (w/v)	-0.20681	0.1	0.55	1	1.3068
pH (B)	-	2.29552	4.0	6.5	9	10.7045
Fermentation period (C)	H	63.81850	72.0	84	96	104.182

The experimental data were evaluated as a second order polynomial regression equation comprising individual and interactive effects of the variables.

$$Y = a_0 + \sum_{i=1}^{3} a_i C_i + \sum_{i=1}^{3} a_{ii} C_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} a_{ij} C_{ij} \qquad (2)$$

where Y represents the experimental response, a_0 is the offset term, a_i, a_{ii} and a_{ij} are the coefficients of linear terms, square terms, and the interactive terms, respectively and C_i's were A, B, and C, C_{ij}'s are AB, BC, and CA. All the experiments were performed in triplicates and their average chitinolytic activity denoted as response (Y).

To estimate the model value and its significance ANOVA was performed. The "Prob > F" (< 0.05) indicates the design model and interactive components are significant for the study. To understand the interaction and response between the variables at each factor level was fitted in the polynomial equation and represented as 3D surface contour plots. The combinatorial effect of each optimized parameter that gave maximum enzyme activity (Y) was tested experimentally to confirm the model's validity (Kumar *et al*., 2018).

Purification and characterization of chitinase

The bacterial culture was mass-produced in fermentation broth based on the optimized parameters. After fermentation, the broth culture was centrifuged at 10,000 rpm for 10 min to obtain a cell-free supernatant. Enzyme purification was done sequentially by the methods: ammonium sulphate precipitation, dialysis, followed by DEAE anion exchange chromatography, and size exclusion chromatography (Gopalakrishnan and Shanmugam, 2021). The cell-free supernatant was subjected to ammonium sulphate precipitation with 60% saturation. To 500 mL cell-free supernatant, ammonium sulphate (60%) was added to precipitate the enzyme. The mixture was left overnight at 4°C and then the precipitated enzyme was separated by centrifugation at 10,000 rpm for 10 min. The pellet collected was re-dissolved in sodium acetate buffer (pH 6), and desalted overnight using dialysis membrane bag with cut off 14 kDa using same buffer at 4°C. The collected dialysis fraction was loaded on a pre-equilibrated DEAE-Sephadex 50 column. The dialysed samples were eluted by sequential extraction with sodium acetate buffer (pH 6) containing NaCl whose concentration was gradually increased (0 to 1M). The fraction that showed high enzyme activity and protein concentration were pooled together. The pooled samples were dialyzed again with sodium acetate buffer, lyophilized and stored at -20°C for further characterization (Akeed *et al*., 2020).

SDS-PAGE analysis

Partially purified samples were analysed to determine the molecular weight of enzyme using SDS-PAGE (12% [w/v]) (Jankiewicz *et al*., 2020). The partial purified enzyme was loaded onto gel after being heated with SDS sample buffer. Molecular weight was compared and confirmed using broad range protein markers (Myosin, rabbit muscle 205 kDa, phosphorylase B 97 kDa, bovine serum albumin 66 kDa, ovalbumin 43 kDa, carbonic anhydrase 29 kDa, soybean trypsin inhibitor 20 kDa, lysozyme 14 kDa, aprotinin 6.5 kDa, insulin 3.5 kDa) (Genei, Bangalore). The protein bands were visualized by performing the silver stain method (Narayanan *et al*., 2013).

Biochemical properties of partially purified chitinase

The enzyme stability at optimal temperature and pH was characterized by incubating the enzyme at different temperatures (4, 24, 37, 50 and 60°C) and pH (3 to 12) using sodium acetate buffer. The enzyme activity was evaluated under standard procedure of 30 min of incubation and catalytic activity was stopped by DNS method using colloidal chitin as substrate. Also, the effect of metal ions on enzyme activity was determined by incubating enzyme in presence of metals Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+}. The metal ion concentration used were 50 mM in 100 mL sodium acetate buffer (Doan *et al*., 2021; Morales *et al*., 2011).

Antifungal property of chitinase

The partially purified chitinase enzyme was assessed for its inhibition activity against two fungi *viz*., *Fusarium* sp., and *Aspergillus* sp. The fungal strains were isolated from soil samples. The spore suspensions of test fungi were prepared and 1 mL spore culture inoculated in PDA medium. To the inoculated plates 200 µL partially purified chitinase from *Bacillus cereus* CE3 was added to 6 mm wells of PDA. The plates were incubated for 24 to 48 h at 37°C. The zone of inhibition on PDA was measured. All the experiments were performed in triplicates using statistical software, Design-Expert 10.0.1, Stat-Ease Inc. Minneapolis, USA (Mishra *et al*., 2012).

RESULTS AND DISCUSSION

Isolation and identification of endophytic bacterium

The leaves of *Coleus amboinicus* is a niche for both beneficial and harmless endophytic bacteria. Around seven bacterial isolates were isolated and coded as CE1, CE2, CE3, CE4, CE5, CE6 and CE7 (Fig. 1a). All the isolates showed good chitin degrading activity by using the chitin plate agar. Among them, isolate CE3 showed maximum zone of inhibition in chitin plate agar assay (Fig. 1b).

The morphological characterization of endophytic isolate CE3 revealed it to be Gram positive rod. The bacterium was positive for catalase


(a) (b)

Fig. 1: Chitinase producing bacterium (a) endophytic bacterial growth in impregnation method; (b) zone of degradation in chitin plate agar flooded with Gram's iodine

Fig. 2: Gram-positive rod-shaped *Bacillus cereus* strain CE3

and Voges Proskauer tests but negative for indole, methyl red and urease. Further, 16s rDNA sequencing of isolate CE3 revealed it to be closely related to *Bacillus cereus* so was identified as *Bacillus cereus* CE3. The detailed evolutionary relationship between the strain CE3 and *B. cereus* closely related families are shown in Fig. 3.

Optimization of key parameters using OFAT

The composition of culture medium and physical factors affected chitinase production. To enhance and optimize chitinase synthesis, the improvisation of medium components has vital role in the growth and metabolism of the microbes involved. The classical approach of 'one factor at a time' (OFAT) depends on changing the variables of one factor, while keeping the other factors constant (Gomaa, 2021). The influence of various physical and nutritional parameters on chitinase production is depicted in Fig. 4. A differential response on chitinase activity was observed when various C and N sources were varied. Among the C sources (1%) tested, sucrose (84 U mL^{-1}) proved better over fructose, sucrose, lactose, and mannitol (Fig. 4a). Reports suggest that chitin, cellulose, and maltose yield higher chitinase (Poria *et al*., 2021) and in present study the test organism produced highest chitinase enzyme in presence of sucrose. Similarly, among various N sources, yeast extract (159.6 U mL^{-1}) and urea (158.6 U mL^{-1}) [Fig. 4b] induced more chitinase production in comparison to cornmeal, soybean and casein. Among the inorganic N sources, sodium chloride affected chitinase production by CE3 strain (Fig. 4d). The results were in agreement with previous reports wherein chitinase production was higher when yeast extract, urea, casein and inorganic nitrogen were used as N-source (Cheba *et al*., 2018; Kumar *et al*., 2017). Similarly, the effect of metal ions in chitinase production was high in presence of Mg^{2+} ions (159 U mL^{-1}) as compared to other metal ions (Fig. 4c). The study on the impact of other substrate like agro-waste products revealed that the bacterial strain CE3 produced maximum chitinase in presence of orange peel powder (OPP) (Fig. 4e). Though nutritional factors play a major role in bacterial growth and metabolism, physical parameters define their cultural environment and adaptation indulge

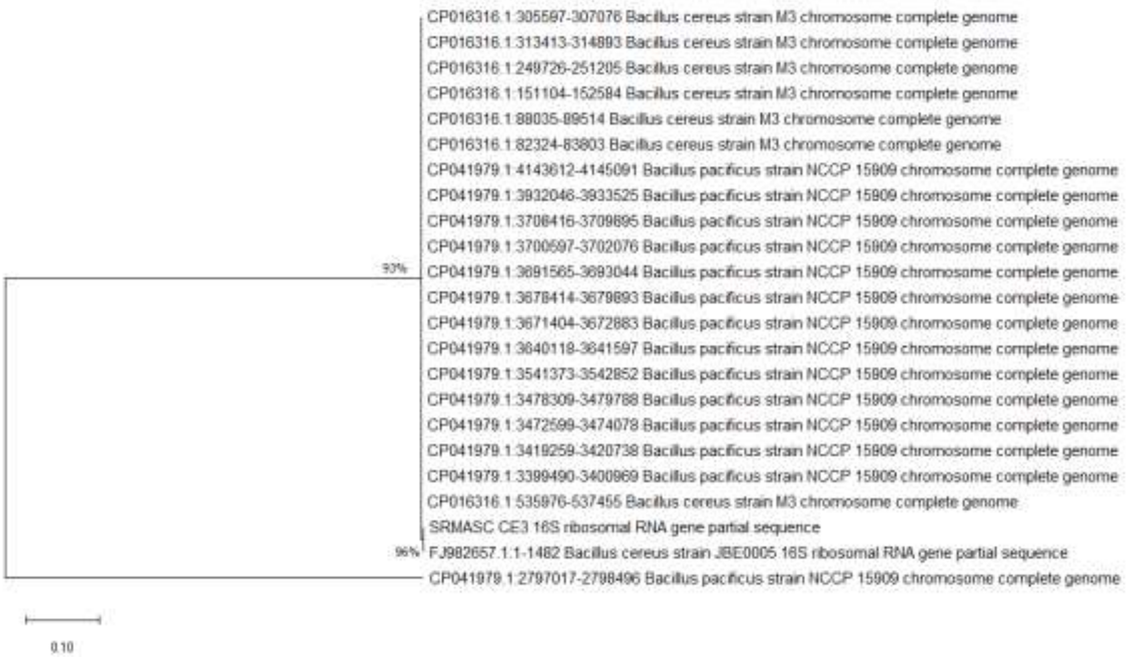

Fig. 3: Phylogenetic tree of *Bacillus cereus* strain CE3

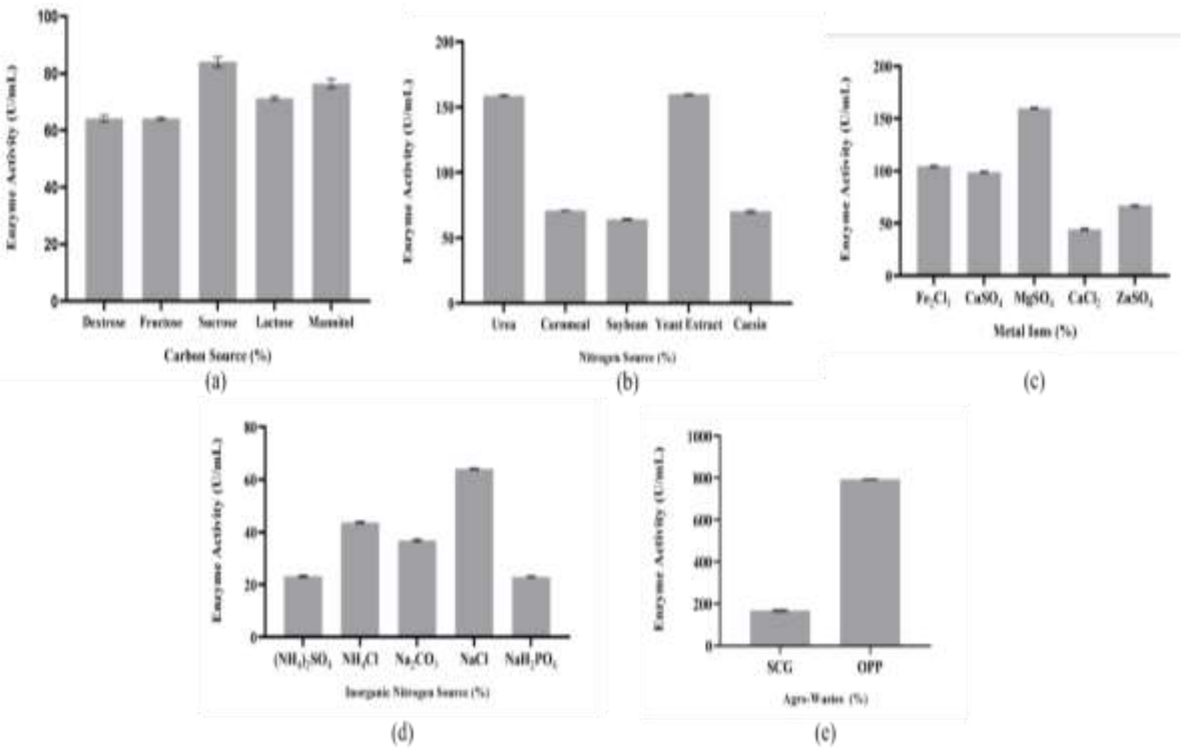

Fig. 4: One factor at a time approach to evaluate the effect of various nutritional factors on chitinase production at optimum temperature (37°C) and pH (8)

the bacteria to abide to the fermentation conditions. The maximum chitinase production was achieved at pH 8.0 with an activity of 110 U mL^{-1}. These results are similar to the earlier reports on chitinase production by other species *Bacillus laterosporus* (Shanmugaiah *et al*., 2008), *B. licheniformis*, *B. pabuli*, (Gomaa, 2012) and *Streptomyces pratensis* (Shivalee *et al*., 2018). Also, temperature plays a major role in withstanding the climatic and fermentation conditions for chitinase synthesis. The study showed that the bacterium could withstand upto 37°C which is similar to the earlier reports of chitinase synthesis by *Bacillus* sp. (Kumar *et al*., 2017; Shivalee *et al*., 2018; Poria *et al*., 2021). The fermentation period varies with the inherent ability of a microbe to utilize a substrate and other parameters. In present study the fermentation and chitinase production was maximum in 24-72 h. The bacterium had the ability to produce maximum chitinase till 72 h which is in line with Poria *et al*. (2021).

Selection of significant variables by PB method

The significant variables required for chitinase were selected based on design matrix. For 12 different two factorial variable concentrations with the enzyme activity as response (Y) are given in Table 1. The variables that had the most significant effect were determined by t-test whose p-value (<0.05) was 0.0001. Yeast extract and fermentation period with a probability value of 0.0002 was the most significant factor, followed by OPP (0.1630) [Table 2]. The lower probability values are the most significant factors that influenced the chitinase production. Also, the model F-value of 28.85 implies that the design matrix is significant. The predicted R-square value (0.8096) is in reasonable agreement with the adjusted R-square value 0.8837. Thus, the significant variables showing positive effect on the chitinase production were selected and fitted in first order linear model equation as follows:

$$\text{Enzyme activity} = +315.49 + 82.09 * B + 19.71 * D + 84.32 * E \qquad (3)$$

Where B = Yeast extract, D = OPP, and E = Fermentation period

Statistical optimization using RSM:CCD

On the basis of PB design, CCD experimental was conducted for the variables that showed significant impact on chitinase production. The observed values of the response (Y) determining the chitinase

Table 1: Design model and response (Y) for the Plackett-Burman design

Run	Sucrose (A)	Yeast extract (B)	NaCl (C)	Orange peel powder (D)	Fermentation period (E)	pH (F)	Temperature (G)	Enzyme activity (U mL^{-1}) (Y)
1	0.5	0.5	0.25	0.5	48	6	25	125.3
2	0.5	1.0	0.50	1.0	48	6	25	291.6
3	1.0	0.5	0.25	0.5	72	6	37	251.3
4	1.0	0.5	0.50	1.0	48	8	37	223.0
5	0.5	0.5	0.50	0.5	72	8	25	326.0
6	1.0	1.0	0.25	0.5	48	8	25	277.2
7	0.5	0.5	0.25	1.0	48	8	37	172.7
8	1.0	0.5	0.50	1.0	72	6	25	302.1
9	1.0	1.0	0.25	1.0	72	8	25	460.0
10	0.5	1.0	0.50	0.5	72	8	37	497.7
11	0.5	1.0	0.25	1.0	72	6	37	561.8
12	1.0	1.0	0.50	0.5	48	6	37	297.2

activity are given in Table 3. Maximum enzyme activity was 469.23 U mL^{-1} in design matrix. The experimental data was analysed using ANOVA. The p-value for our model was <0.0001 confirming the model was significant for medium optimization study (Table 4).

Table 2: Analysis of variance table for PB design

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Model	1.709E+005	3	56950.18	28.85	0.0001	Significant
B: Yeast extract	80865.22	1	80865.22	40.97	0.0002	-
D: Orange peel powder	4660.23	1	4660.23	2.36	0.1630	-
E: Fermentation period	85325.09	1	85325.09	43.23	0.0002	-
Residual	15791.20	8	1973.90	-	-	-
Corrected total	1.866E+005	11	-	-	-	-

These numbers are calculated by the design expert software using the obtained results.

Table 3: Experimental design and response (Y) for central composite design

Run	A: Yeast extract (%)	B: OPP (%)	C: Fermentation period (h)	Enzyme activity (U mL^{-1})
1	0.50	0.50	72	327.714
2	0.75	0.75	60	467.260
3	0.50	1.00	72	379.465
4	1.00	1.00	48	317.933
5	0.75	0.75	60	437.980
6	0.75	0.75	60	436.53
7	0.75	0.75	39.8185	192.632
8	0.3295	0.75	60	364.831
9	1.00	1.00	72	469.223
10	0.75	1.1705	60	420.120
11	0.50	0.50	48	308.119
12	0.75	0.75	60	437.850
13	1.1705	0.75	60	432.620
14	1.00	0.50	48	351.662
15	0.50	1.00	48	282.425
16	0.75	0.3295	60	418.389
17	1.00	0.50	72	395.507
18	0.75	0.75	60	433.250
19	0.75	0.75	60	438.790
20	0.75	0.75	80.1815	254.309

OPP =orange peel powder

It was found that the A, C, BC, A^2 and B^2 are significant model terms. The lack of fit F value of p-value = 0.1137 indicating not significant determining the design model is fit for the study. On the calculation of enzyme activity as response (Y). All experimental data was correlated and analysed with quadratic second-order polynomial equation as follows:

Table 4: Analysis of variance table.

Source	Sum of squares	df	Mean square	F value	p-value Prob > F	
Model	1.077E+005	9	11967.58	36.14	< 0.0001	Significant
A: Yeast extract	9001.10	1	9001.10	27.18	0.0004	
B: Orange peel powder	348.16	1	348.16	1.050	0.3294	
C: Fermentation period	12641.16	1	12641.16	38.17	0.0001	
AB	24.26	1	24.26	0.073	0.7922	
AC	770.28	1	770.28	2.33	0.1582	
BC	4273.04	1	4273.04	12.90	0.0049	
A^2	2295.08	1	2295.08	6.93	0.0251	
B^2	414.27	1	414.27	1.25	0.2895	
C^2	80161.67	1	80161.67	242.04	< 0.0001	
Residual	3311.87	10	331.19	-	-	
Lack of fit	2523.65	5	504.73	3.20	0.1137	Insignificant
Pure error	788.22	5	157.64	-	-	-
Corrected total	1.110E+005	19	-	-	-	-

Table 5: Purification and yield of chitinase from *Bacillus cereus* CE3

Purification steps	Total protein (mg mL^{-1})	Total activity (U mL^{-1})	Specific activity (U mg^{-1})	Recovery (%)	Purification fold
Crude extract	658	2556.0	3.88	100	1.00
Ammonium sulphate precipitation	333	2151.2	6.46	84.16	1.66
Dialysis	245	1820.7	7.43	71.23	1.91
Ion-exchange chromatography	164	1288.9	7.85	50.42	2.02
Sephadex G-100	95	1146.9	12.07	44.87	3.11

$$\text{Enzyme activity} = 441.536 + 25.6728^{*}\,A + 5.04913^{*}\,B + 30.4241^{*}\,C + 1.74125^{*}\,AB + 9.8125^{*}\,AC + 23.1113^{*}\,BC + -12.6196^{*}\,A^2 + -5.36154^{*}\,B^2 + -74.5816^{*}\,C^2 \qquad (4)$$

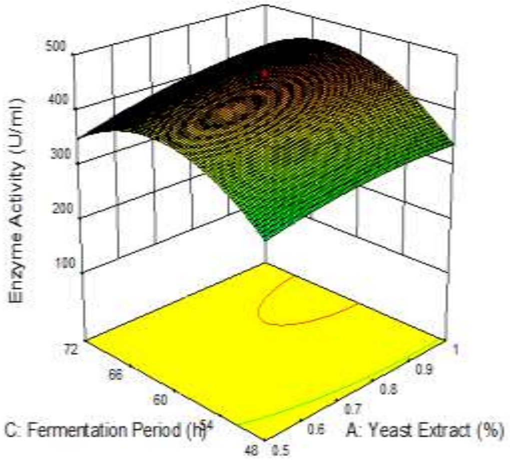

Fig. 5: CCD analysis depicting 3D representation of interaction between the variables yeast extract and fermentation period showing elliptical plot revealing the significance

The 3D surface plots showed the impact of the interaction of two variables for enzyme production while the third one was kept at zero coded level (Fig. 5). Among the variables used in CCD, yeast extract and fermentation period significantly affected enzyme production as compared to the other variables. Also, the perturbation plot represented that the factors yeast extract, OPP and fermentation period have a vital role in enzyme production. The predicted R square (0.8157) was reasonably in agreement with the adjusted R square of 0.9433. Hence comparing the previous OFAT (110 U mL^{-1}) optimization, the enzyme's synthesis increased four-fold to the enzyme activity of 469.23 U mL^{-1} in RSM.

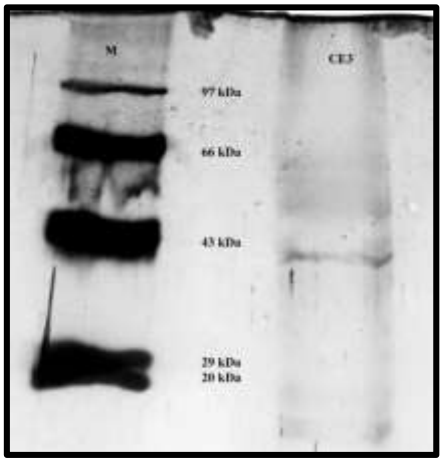

Fig. 6: Molecular weight determination of partially purified chitinase from *Bacillus cereus* CE3; M (protein marker); CE3 (purified chitinase)

Partial purification of chitinase from Bacillus cereus CE3

Enzymes are to be purified prior to their analysis for biochemical function, stability and activity. Various studies on chitinase production have shown a yield of 18.4-21.6% chitinase with ~4.5 purification fold (Poria *et al.*, 2021b). In this study, the activity, specific activity and yield was calculated at each step of partial purification of extracellular chitinase enzyme obtained from *B. cereus* CE3 (Table 7). The overall enzyme yield was 44.87% with 3-fold enzyme production. The molecular weight of partially purified chitinase was ~43 kDa (Fig. 6). The molecular mass of chitinase from bacterial species generally range from 20 to 80 kDa. *Bacillus* sp. reportedly has partially purified chitinase of molecular weight 60 to 62 kDa (Shivakumar *et al.*, 2014).

Enzyme stability at optimal conditions

The efficiency and stability of purified chitinase was analysed and its enzyme stability and activity studied at variable pH and temperature. The enzyme was found active in the temperature range of 4-60°C with optimal activity at 45°C (Fig. 7b). The chitinase purified from *B. cereus* CE3 may be classified as a mesophilic enzyme. Earlier works have shown that the chitinase purified from *B. amyloliquefaciens* has optimum temperature of 50°C and *B. cereus* 108 has shown stability at optimum temperature upto 65°C (Lestari *et al.*, 2017). The pH affected chitinase enzyme stability with optimum enzyme activity at pH 8. This confirmed that enzyme is alkaline in nature. These findings are similar to the those of Akeed *et al.* (2020) who reported the chitinase synthesized from *Bacillus* sp., to be alkaline in nature.

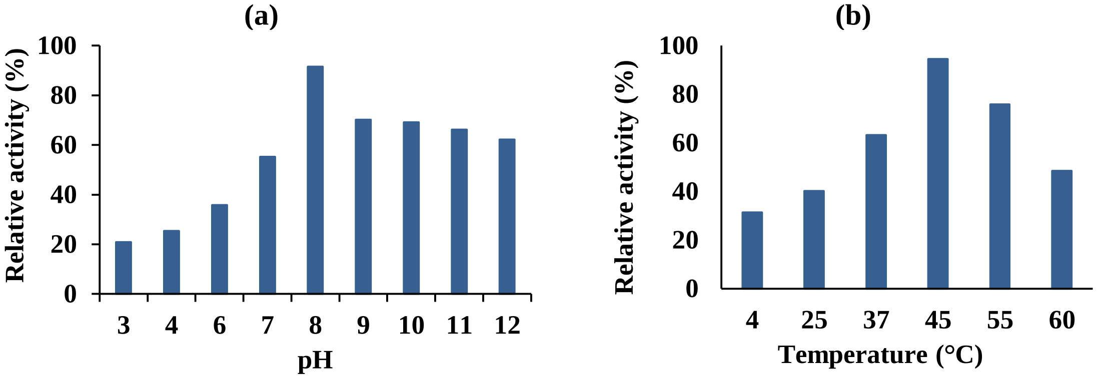

Fig. 7: Effect of pH and temperature on the efficiency and stability of chitinase enzyme

Antimicrobial property of partially purified chitinase

The diseases caused by bacteria, fungi, etc. greatly reduce crop yields. More than 70% plant diseases are caused by fungi (Gomaa, 2021). Therefore, chitinase enzymes having the ability to degrade fungal chitinous cell wall and insect exoskeletons have great significance in disease management (Poria *et al.*, 2021).The partially purified chitinase was evaluated against *Aspergillus* and *Fusarium* and the result revealed that the partially purified chitinase from endophyte *B. cereus* CE3 had maximum growth inhibition in *Fusarium* sp., with 18 mm zone of inhibition as compared to 1 mm in *Aspergillus* sp. The results are line with those of Pandya *et al*. (2014) who observed growth inhibition in *Fusarium graminearum* and *B. sorokiniana* s when chitinase from *B. subtilis* and *B. pumilus* were used.

Conclusion: Chitinase is a commercially important enzyme extracellularly produced by bacteria, fungi and insects and can be exploited in various processes like single cell protein production, protoplast creation, waste management system and pest and disease management. The chitinase produced by endophyte *Bacillus cereus* CE3 was non-toxic, harmless and symbiotic. The endophytic chitinase enzyme though similar to other environmental bacterial chitinase showed some variation at genetic level and also exhibited biocontrol activity. The partially purified chitinase from *B. cereus* CE3 was a stable extracellular alkaline mesophilic enzyme which can be effectively used agriculture, pharmaceuticals, and biotechnology.

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