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A STATISTICAL APPROACH FOR THE CHITINASE PRODUCTION BY *BACILLUS* SP. AND ITS ANTIFUNGAL POTENTIAL

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Abstract

Chitin is one of the most abundant polysaccharide which is found in marine and terrestrial environment. Chitinase enzyme is very important now days due to its wide range of biotechnological applications. Chitinase producing *Bacillus sp.* CH-2 has been isolated from sample collected from fish market soil. Enzyme production has been optimized by statistical methods (Placket Burmann and Response Surface Methodology). Among 11 factors, 4 factors (pH, chitin, NaCl and casein) were having positive influence on chitinase yield and the interactive effect of pH, chitin, NaCl and casein was analyzed by Response Surface methodology. The chitinase from *Bacillus sp.* CH-2 was optimally active at 40°C. More than 50% of the residual activity was observed at 30°C, 37°C and 40°C upto 8h. Chitinase from *Bacillus sp.* CH-2 was optimally active at pH 6.5. The pH stability profile revealed that chitinase from *Bacillus sp.* CH- 2 was stable between pH 6-8 and retained >60% activity up to 8h. It was stable in non-ionic surfactants and metal ions had little effects on the chitinase activity from *Bacillus sp.* CH-2. Chitinase produced from *Bacillus sp.* CH-2 was showed inhibition on the growth of phytopathogenic strains *Aspergillus niger.* A1 *and Aspergillus niger.* A2.

Keywords: Chitinase, production, stability, surfactants, phytopathogenic

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Introduction

Chitin is a linear polymer of β-1, 4-N-acetylglucosamine (GlcNAC) and is the second most abundant biopolymer in nature after cellulose (Karthik et al 2014). It exists in two allomorphic forms such as α-chitin and β-chitin. These forms vary in packing and polarities of adjacent chains in the succeeding sheets (Bussink et al 2007). Chitin is the major part of sea food waste, it consists of 20-58% of the dry weight of shell fish e.g. crab, krill, and shrimps, lobster (Dahiya et al, 2006). It also found in the exoskeleton of yeasts, algae, fung iand in the internal structures of other invertebrates (Bhattacharya et al, 2007).

Chitinases (E.C 3.2.2.14) are glycosyl hydrolases which catalyze the degradation of chitin. Complete enzymatic degradation of chitin is performed by a diverse group of enzymes that catalyze the hydrolysis of chitin, Endochitinases (E.C 3.2.1.14) and exo-chitinases. The endochitinases cut chitin at internal sites, and forms di- acetylchitobiose, chitotriose, and chitotetraose (Dixon, 1995). The exo- chitinases have been further divided into 2 subcategories: Chitobiosidases (E.C. 3.2.1.29), (Donderski et al, 2000) which release di-acetylchitobiose staby catalyzing at the non-reducing end of the chitin microfibril, and 1-4-β-glucosaminidases (E.C. 3.2.1.30), which cuts the oligomeric products of endochitinases and chitobiosidases, and generating monomers of GlcNAc (Dixon, 1995).

Chitinase can be used for various biotechnological applications e.g. for controling pathogenic fungi, protoplasts isolation from yeast and fungi and synthesis of pharmaceutically important compounds etc. (Hamid et al, 2013). But there are some limitations for their application at commercial scale like low chitinase yields (Stoykov et al, 2014).
The effect of various nutritional factors need to be optimize in the culture medium for the production of chitinase, so it is important to use a statistical technique to design experiments (Ma et al., 2004) such as Plackett-Burman design (Plackett and Buramnn 1946) is a useful statistical tool to evaluate the effect of various factors on a enzyme production.

One of the most important applications of chitinase is its use in the control of plant pathogenic fungi. The fungal phytopathogens cause serious problems in the cultivation of economically important plants. To stop the effect of these phytopathogens chemical fungicides are widely used

in agriculture but excessive use of chemical fungicides in agriculture has led to human health problems, environmental pollution and development of pathogen resistance to fungicide.

Due to this serious problem of fungal disease control, a serious exploration is required to recognize other methods for plant protection, which utilize fewer chemicals and are more environmentally friendly. Therefore, presence of chitin in fungal cell wall, chitinase can be used as an efficient biocontrol agent against phytopathogenic fungi.

Therefore the aim of the present study was to standardize the process for optimal production of chitinase by statistical methods, characterisation and its antifungal potential.

2. MATERIAL AND METHODS

2.1 Strain, media and culture conditions

Bacillus sp. CH-2 used in this study, was isolated from soil collected from the fish market area, Chandigarh, India. Bacterial strain was stored in 25% glycerol at -70°C and with routine culturing on MMHL minimal medium (HSU and Lockwood, 1975) plates.

2.2 Preparation of substrate

Colloidal chitin was made according to modified method as described by (Faramarzi et al, 2009) and used as a substrate in MMHL medium.

2.3 Chitinase production and assay in production medium

20ml MMHL medium (pH 7.0) was inoculated with 1% inoculum of overnight grown cells and incubated at 150rpm at 37°C for 96 h. Supernatant was collected by centrifuge the culture at 10000rpm for 10 min at 4°C. Chitinase activity was measured in amount of N-acetyl-D-glucosamine released from chitin by chitinase as described by Reissig *et al*., 1955 using DMAB as a coloring reagent. Enzyme units were expressed in international units (IU) as micromoles of N-acetyl glucosamine released by 1ml of enzyme in 1 minute under standard assay conditions.

2.4 Statistical optimization of chitinase production

2.4.1 Selection of significant factors

Factors which influence the chitinase production were screened using PB Design Expert 8.0.7.1 (Stat-Ease, Inc., Minneapolis, USA). different physio-chemical factors were selected for the experiments viz.: $FeCl_3$, Casein, Gelatin, pH, Starch, Sucrose, $MnCl_2$, Inoculum size, Chitin, NaCl, $CaCl_2$. Independent variables were evaluated at two levels (high and low). The significant factors were screened in 12 different combinations according to the design matrix and the responses were measured. All the experiments were performed in triplicates and mean of chitinase activity was taken as response. The factors showing highest positive effects were further optimized by response surface methodology.

2.4.2 Central composite design

Central composite design (CCD) at α value as $\pm$ 1.682 was created using Design expert tool to further optimize the levels of the most significant factors identified from the PB design. The experimental design comprised of 30 trials and the four factors were studied at five different levels, low (-2, -1), medium (0) and high (+1, +2) (Table 1). Chitinase activity recorded as response data and all the analysis was performed by the software Design expert. Regression analysis was done on the data obtained after measuring chitinase activity of 30 trials. A second-order polynomial equation was used to fit the data by multiple regression procedure. This resulted in an empirical model that related the response measured to the independent factors of the experiment.

$$Y = \beta 0 + \Sigma \beta i\ Xi + \Sigma \beta ii\ Xi2 + \Sigma \beta ij\ Xi\ Xj \qquad (1)$$

where Y represents response variable (Chitinase $IUml^{-1}$), $\beta 0$ is the interception coefficient, βi, coefficient of the linear effect, βii, the coefficient of quadratic effect and βij, the coefficient of interaction effect.

Table 1: Experimental range and levels of independent factors for RSM design

Codes	Factors	Units	Levels				
			-2	-1	0	+1	+2
A	pH	H	4	5	6	7	8
B	NaCl	%	0.75	1.00	1.25	1.50	1.75
C	Chitin	%	0.25	0.50	0.75	1.00	1.25
D	Casein	%	0.25	0.50	0.75	1.00	1.25

2.5 Characterization of chitinase produced from *Bacillus sp.* CH-2

For understanding the properties and functions of the enzyme, its characterization is very important (Takagi *et al*. 1993). Moreover, for the application of the enzyme, its activity/stability on temperature, pH and also against a number of reagents like detergents, chelating agents, organic solvents etc. needs to be checked (Meena *et al.,* 2015; Aditya *et al.*, 2014; Deepak Kumar *et al.,* 2014; Deepak *et al.,* 2016;).

2.5.1 Temperature optima

The temperature optima for chitinase activity was measuerd by performing standard enzyme assays in the temperature varies from 30-50°C.

2.5.2 Temperature stability

The temperature stability was measured by incubating diluted enzyme in 100 mM phosphate buffer (pH 6.5) at temperatures 30°C, 37°C, 40°C, 45°C, 50°C up to 12 h in the water bath. Samples were withdrawn sequentially at 0h, 2h,4h, 6h, 8h and 12h and enzyme activity was measured.

2.5.3 pH optima

The optimal pH was determined by using different buffers (100mM each) i.e. pH 5.0, 5.5(acetate buffer), 6.0, 6.5, 7.0 (phosphate buffer), 7.5, 8.0, 8.5 (Tris-Cl buffer). 9.0, 9.5 (glycine-NaOH buffer) and performing the assay at 37°C.

2.5.4 pH stability

The pH stability was done by analysing the residual enzyme activity after pre incubating the enzyme in buffers of various pH values (5.0-9.5) at 37°C up to 12 h.

2.5.5 Effect of detergents

The effects of detergents (*cetyl trimethyl ammonium,* tween-20, *sodium dodecyl sulphate, bromide,* triton-X100), at concentration of 0.5% and 0.1%, on the enzyme activity, diluted enzyme was pre-incubated with detergents agents for 1h at 37°C.

2.5.6 Effect of chelating agent

The effect of chelating agents (sodium azide, sodium citrate, urea, ethylene diamine tetraacetic acid) at concentration of 1% and 0.5%, diluted enzyme was pre-incubated with chelating agents for 1h at 37°C.

2.5.7 Effect of metal ions

The effects of metal ions ($HgSO_4$, NH_4SO_4, $MgSO_4$, $MnCl_2$, $CuSO_4$, $FeSO_4$, $ZnSO_4$, $CaCl_2$, $NiSO_4$, $CaSO_4$) at 10mm and 1mm concentrations were studied by incubating for 1h at 37°C.

2.6 Antifungal activity of the bacteria

Chitinase from *Bacillus sp.* CH-2 had been tested for antifungal activity against the laboratory isolates of phytopathogenic fungal strains *Aspergillus niger* A1 and A2.

2.6.1 Antifungal activity on PDA plates

Normal saline dilutions were made using the fungal spores. 10^{-5} dilutions of each fungal strain were used to spread plate for test and control. Wells were made in the centre of test and control plate. 100microlitres of enzyme was added in test and 100microlitre denatured enzyme was added in control. Plates were incubated at 25°C- 28°C and monitored for respective inhibition of fungal growth.

2.6.2 Antifungal activity on PDA broth

Both the fungal strains (10^{-5} dilution) were inoculated in potato dextrose broth tubes. Bacterial enzyme (100microlitres) was added in test and 100microlitre denatured enzyme was added in control. All the tubes were incubated at 25°C-28°C and monitored for inhibition of fungal growth.

3. RESULT AND DISCUSSION

3.1 Screening of parameters by PB design

A set of 12 experiments with the Chitinase yield as a response, are presented in Table.2. In. 12 runs, variation ranging from 2.01IU to 3.18 IU in the yield of chitinase was observed.

Table 2: Plackett Burman design used for the screening of significant factors for chitinase production in submerged fermentation by *Bacillus sp*. CH-2

Run	Factor 1 A :Fecl3 %	Factor 2 B: Casein %	Factor 3 C: Gelatin %	Factor 4 D: pH	Factor 5 E: Starch %	Factor 6 F: Sucrose %	Factor 7 G: Mncl2 %	Factor 8 H: Inoculum size %	Factor 9 J: Chitin %	Factor 10 K: NaCl %	Factor 11 L: CaCl2 %	Response 1 Chitinase Activity Uml^{-1}
1	0.01	0.1	0.1	7	0.1	0	0	0.1	1	0	0	2.28
2	0.01	0.1	0.02	5	0.1	0	0.0001	1	0.5	0.1	0	2.01
3	0	0.1	0.1	5	0.1	0.1	0	1	1	0.1	0	2.49
4	0	0.1	0.02	5	0	0	0	0.1	0.5	0	0	2.64
5	0.01	1	0.1	5	0	0	0.0001	0.1	1	0.1	0	2.94
6	0	1	0.1	5	0.1	0.1	0.0001	0.1	0.5	0	0	2.64
7	0.01	1	0.02	5	0	0.1	0	1	1	0	0	2.76
8	0	1	0.1	7	0	0	0	1	0.5	0.1	0	3.18
9	0.01	0.1	0.1	7	0	0.1	0.0001	1	0.5	0	0	2.4
10	0	1	0.02	7	0.1	0	0.0001	1	1	0	0	3.06
11	0.01	1	0.02	7	0.1	0.1	0	0.1	0.5	0.1	0	3
12	0	0.1	0.02	7	0	0.1	0.0001	0.1	1	0.1	0	2.52

3.2 Screening for the significant parameters and the Statistical analysis of Plackett Burmann analysis.

The effect of various parameters on enzymes yield was measured and casein, chitin, pH and NaCl were found to show the positive effect.

Model significance and parameters influence were analyzed by ANOVA. These results illustrated that the model was significant. Out of the 11 factors, four factors *viz.* Casein, pH, Chitin and NaCl with p values <0.0042, 0.2571, 0.8237 and 0.6576 for chitinase were selected for further optimization using central composite design (CCD) of response surface methodology (RSM).

In case of *Bacillus pumilis* chitin, Yeast Extract, $FeSO_4$, $MgSO_4$ with p values <0.0001, 0.0001, 0.0148 and 0.0268 (Tasharoffi *et al.,* 2011) and in case of *Chitiolyticbacter meiyuanensis SYBC-H1* Urea, Inulin, and Sodium Sulfate with p value <0.0047, 0.0038, 0.0028 have been reported to have positive effect on chitinase production (Hao *et al*., 2012).

3.3 Response surface design

Central Composite design was used to study the interactive effect of factors pH, NaCl, Chitin and Casein at different concentrations. The design resulted in a total of 30 trials. The actual and predicted responses (Chitinase activity) with the residuals are depicted in Table 3.

Table 3: CCD matrix with experimental and predicted values of chitinase production from *Bacillus sp.* CH-2

Factor					**Chitinase Activity Uml^{-1}**		
Run	**A: pH**	**B: NaCl**	**C: Chitin**	**D:Casien**	**Actual**	**Predicted**	**Residual**
1	6	0.40	1.25	0.75	9.80	9.76	0.042
2	5	0.10	1.50	0.50	8.89	8.85	0.042
3	6	0.40	1.25	0.75	9.68	9.76	-0.078
4	5	0.10	1.00	0.50	9.02	8.90	-0.12
5	7	0.70	1.50	1.00	9.25	9.10	0.15
6	6	0.40	1.25	0.75	9.90	9.76	0.14
7	7	0.10	1.00	1.00	8.89	8.79	0.095
8	7	0.70	1.00	1.00	8.76	8.87	-0.11
9	6	0.40	1.25	1.25	8.73	8.92	-0.085
10	7	0.10	1.50	0.50	8.92	8.85	0.069
11	7	0.70	1.50	0.50	8.98	8.96	0.019
12	4	0.40	1.25	0.75	8.05	8.11	-0.061
13	6	0.40	1.25	0.25	8.88	8.89	-6.250E-033
14	5	0.70	1.00	1.00	8.72	8.53	-0.19

15	5	0.70	1.00	0.50	8.05	8.24	-0.19
16	6	0.40	1.25	0.75	9.84	9.76	0.082
17	5	0.70	1.50	0.50	8.89	8.79	0.095
18	7	0.70	1.00	0.50	8.70	8.54	0.16
19	6	0.40	1.25	0.75	9.66	9.76	-0.098
20	6	0.40	1.75	0.75	8.80	9.02	-0.22
21	6	0.20	1.25	0.75	8.97	9.08	-0.11
22	6	1.00	1.25	0.75	8.84	8.92	-0.085
23	7	0.10	1.50	1.00	8.72	8.60	0.12
24	7	0.10	1.00	0.50	8.93	9.04	-0.11
25	5	0.10	1.50	1.00	8.65	0.55	0.099
26	6	0.40	0.75	0.75	8.86	8.84	0.022
27	6	0.40	1.25	0.75	9.90	9.76	0.140
28	5	0.70	1.50	1.00	8.94	8.90	0.044
29	5	0.10	1.00	1.00	8.71	8.79	-0.084
30	8	0.40	1.25	0.75	8.32	8.46	-0.14

By using multiple regression, a predictive quadratic polynomial equation was constructed to describe the correlation between enzymes yield and the four significant factors.

Model equation

(Chitinase Yield)= +9.76 +0.086* A-0.040* B+0.045* C+8.750E-003* D +0.041* A * B-0.033 * A * C+0.011* A * D+0.15* B * C+0.099* B*D-0.047* C * D-0.37* A^2 -0.19* B^2-0.21* C^2-0.21* D^2

The analysis of variance for the response surface quadratic model is depicted in Table 4, for chitinase. The p-values <0.0001 showed that the linear, interactive, and squared terms had significant influence on enzyme yield. The lack of fit was found to be non-significant. For chitinase, the p-value for lack of fit was 0.0903 representing that that this quadratic model adequately fit into the data. The determination coefficient, R^2 0.9367 showed that the predicted and experimental values had perfect coherence with each other. The values of adjusted R^2 0.8776

representing that the variation of 87.76% in the chitinase, was attributed to the independent factors. Only 12.24% of the total variation of chitinase production was not explained by the model.

In a model standardized for chitinase production from *Chitiolytic meiyuanensis* SYBC-H1 (Hao *et al.,* 2012), 15.78% of variation was unexplained whereas only 2% of the variation was unexplained for chitinase production from *Bacillus pumilus* (Tasharoffi *et al.,* 2011).

Table 4: Analysis of variance (ANOVA) of response surface model for chitinase yield

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	
Model	6.14	14	0.44	15.85	< 0.0001	Significant
A-pH	0.18	1	0.18	6.45	0.0227	
B-NaCl	0.038	1	0.038	1.36	0.2620	
C-Chitin	0.050	1	0.050	1.79	0.2010	
D-Casein	1.837-003	1	1.837-003	0.066	0.8002	
AB	0.026	1	0.026	0.95	0.3442	
AC	0.018	1	0.018	0.63	0.4382	
AD	1806E-003	1	1806E-003	0.065	0.8018	
BC	0.38	1	0.38	13.55	0.0022	
BD	0.16	1	0.16	5.71	0.0305	
CD	0.035	1	0.035	1.27	0.2774	
A^2	3.73	1	3.73	134.67	< 0.0001	
B^2	0.98	1	0.98	35.27	< 0.0001	
C^2	1.18	1	1.18	42.62	< 0.0001	
D^2	1.25	1	1.25	45.23	< 0.0001	
Residual	0.42	15	0.028			
Lack of Fit	0.36	10	0.036	3.49	0.0903	Not significant
Pure Error	0.052	5	0.010			
Cor Total	6.56	29				
Model fitting	**C.V%= 17.53**	**R-Sq=0.9367**		**R-Sq (Pred)= 0.6697**	**R-Sq (Adj)=0.8776**	

Interaction between variables

The interactions within two variables are presented in Fig. 1 which was created by the pair-wise combination of the two factors while keeping the others at its optimum level. The response at the central point corresponded to a maximum degree of chitinase yield for the four factors. It was used to draw 3D graphs plots.

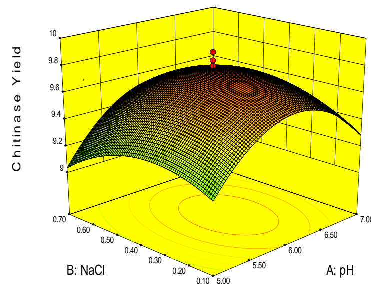
It was analyzed from 3D curves that the maximum response was located inside the design boundary which confirmed tested ranges of the parameters. The spherical curves in Fig. 1 depicted that the interactions between Casein, pH, Chitin and NaCl were significant.

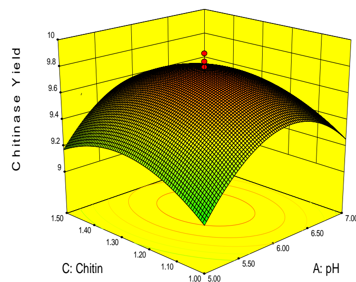
The maximum chitinase yield (9.90 $IUml^{-1}$) was obtained with 0.75% Casein, 1.25% Chitin, 0.40% NaCl and pH 6. The results were internally validated as the maximum response was obtained in the central value.

The enzyme production under unoptimized conditions was 0.31IU which after optimization increased to 9.90 IU resulting in an approximately 31 fold increase in yield.

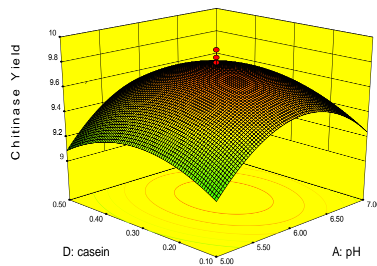
In case of *Bacillus pumilus* (Tasharoffi *et al.,* 2011) even after statistical optimization by RSM only 0.9IU of chitinase yield has been reported, whereas in case of *Chitiolyticbacter meiyuanensis* SYBC-H1(Hao *et al.,* 2012) 5.17IU of chitinase yield could be achieved.

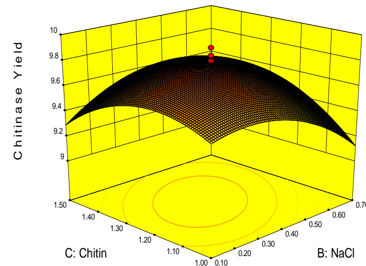
A **B**



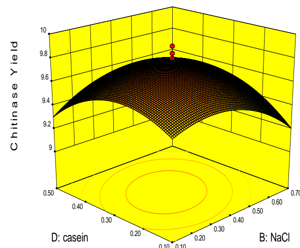


C **D**





E **F**



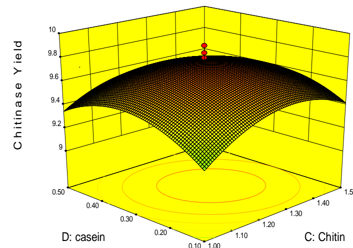


Fig.1: 3D Graph plots for the effect of A) pH and NaCl, B) pH and Chitin, C) pH and Casein, D) Chitin and NaCl , E) Casein and NaCl and F) Casein and Chitin for the yield of Chitinase from Bacterial Isolate CH-2

3.4 Characterization of chitinase from *Bacillus sp.* CH-2

3.4.1 Temperature optima

The chitinase showed maximum activity at 40°C. However, more than 60% activity was observed at 37°C and 45°C also (Fig. 2).

Chitinase from different bacteria have been reported to have optimum activity at different temperatures but most of the times optimum activity is at ambient temperature. In case of *S. marcescens* QM13 1466 (Robertz *et al.,* 1982) maximum enzyme activity was observed at 30 ºC. In case of *Enterobacter sp.* NRG4 (Dhaiya *et al*., 2005) maximum activity was observed at 40ºC.

3.4.2 Temperature stability

At temperature 30°C, 37°C, 40°C more than 50% of the residual activity was observed up to 8h and same was observed at 45°C and 50°C for 6h. (Fig.2). In case of *Bacillus sp.* R2 (Ben *et al.,* 2016) chitinase exhibited a complete temperature stability only for 1h at 40 ºC and retained 50% and 30% of its original activity after 30min at 50ºC and 60ºC respectively. Chitinase from *Enterobacter sp.* NRG4 (Dhaiya *et al.*, 2005) was also stable for only 1h at 45ºC. Chitinase from *Vibrio alginolyticus* TK-22 was stable at 40ºC for 30 min (Ohishi *et al.,* 1996).

3.4.3 pH optima

The pH optima for the chitinase was found to be 6.5. At pH 6.0, 7.0, the enzyme activity was 67%, 81% respectively. Enzyme activity decreases sharply at higher pH value. At pH 8.0, 8.5, 9.0, 9.5 the enzyme activity was less than 50% (Fig. 2). Like temperature chitinase from different organisms have different pH optima. In case of *Serratia marcescens* B4A (Zarei *et al.,* 2011) chitinase showed pH optima at 5. The pH optima for the other bacterial chitinases such as *Aeromonas* sp. No.10S-24 (Ueda *et al*., 1995) and *Bacillus* sp.NCTU2 (Wen *et al.*, 2002) has been reported 4 and 6.3 respectively.

3.4.4 pH stability

The pH stability profile revealed that chitinase in pH range 6-8, retained >60% activity up to 8h and at pH 8.5 to 9.0, retained >50% activity up to 4h (Fig. 2).

In case of *Bacillus sp.* R2 (Ben *et al*.,2016) the enzyme was very stable at pH 7 and 8 and retained more than 90% of its activity but it lost 57% and 62% of its activity at pH 6 and 9 respectively.

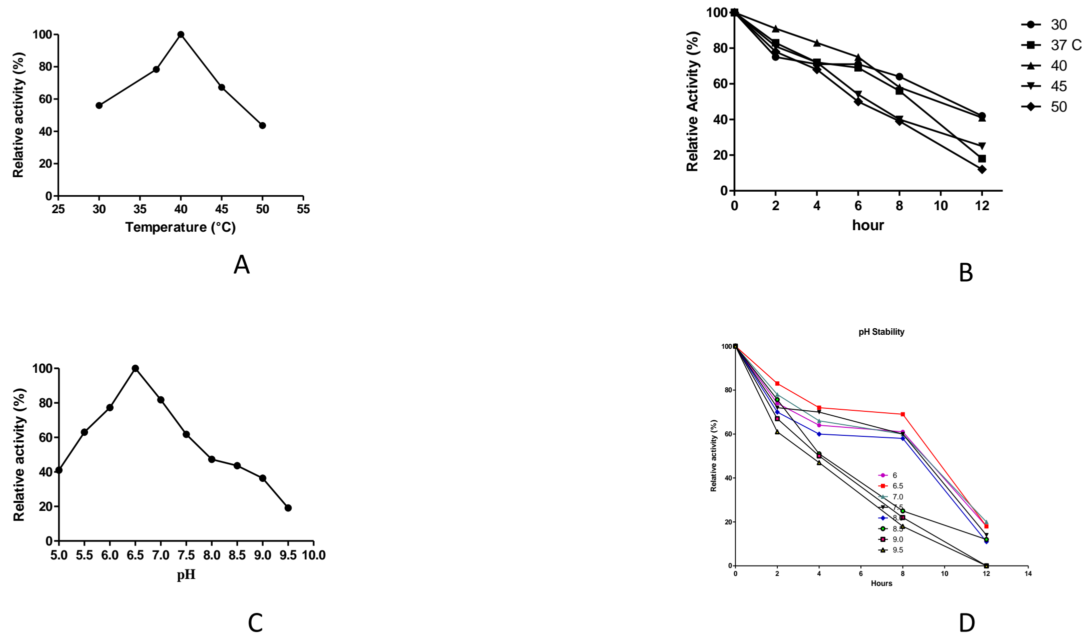


Fig. 2: Temperature and pH effect on chitinase activity from *Bacillus sp.* CH-2:

A: Temperature optima B: Temperature Stability C: pH optima D: pH stability

3.4.5 Effect of detergents

Most of the detergents results in an increase of the enzyme activity marginally at 0.5% as well as at 1% concentration. However the presence of SDS decreased the enzyme activity substantially (Table 5).

Similar to our results, positive effect of detergents like CTAB, tween-20, triton X100 has also been reported in case of chitanase from *Paenibacillus sp.* BISR-047 (Meena *et al*., 2015) and *Alcaligenes xylosoxydans* (Vaidya *et al.,* 2001). Inhibitory effect of SDS has been shown by chitinase produced from *Streptomyces tendae* TK-VL_333 (Kavita and Vijaylakshmi, 2010).

3.4.6 Effect of chelating agent

Chelating agents except EDTA increased the enzyme activity marginally at all concentrations (Table 5). However, strong inhibition of the enzyme was shown by EDTA.

Varied effects of chelating agents on chitinase have been reported in the literature. Singh, 2010 has shown enhancement of enzyme activity with urea in case of *Paenibacillus sp.* D1. However contrary to this Kavita and vijayalakshmi, 2011 showed inhibitory effect of urea in case of chitinase produced by *Streptomyces tendai* TK-VL_333. Similarly, Nawani *et al,* 2001 has reported 10% increase in activity of chitinase produced by *Microbispora sp.*V2 with 1mM EDTA, whereas Ingles and Piberdy has shown reduced activity with EDTA in case of *Ewingella americana*.

3.4.7 Effect of metal ions on *Bacillus sp.* CH-2 Chitinase

Most of metal ions showed little effect on the enzyme activity at both the concentrations except Fe^{2+}, Cu^{2+} and Hg^{+} which inhibited the enzyme activity (Table 5). The inhibition of chitinase by Fe^{2+} has been reported due to binding of these metal ions to amino acids such as aspartic acids and glutamic acids in the active site. Different metal ions have been reported to affect the activity of chitinases from different organisms differently. Chitinases from *A. hydrophila* H-23307 , *Alteromonas sp*. O- 7 28 , *E. americana* , *P. aeruginosa* K-18729, and *Fusarium chlamydosporum* has also been reported to inhibited by Fe^{2+}, Fe^{3+} and/or Cu^{2+} whereas chitinase from *Alteromonas sp.* strain O-737 was activated by Na^{+} and Ca^{2+}. (Milewski *et al*., 1992).

Table 5 Effect of detergents, chelating agents, metal ions on *Bacillus sp.* CH-2 chitinase. Enzyme was incubated at 37°C for 1 h and activity was measured under standard conditions. Enzyme activity without any agents represented the control (100% activity).

Effect of Detergent			
S.No	**Reagent**	**Concentration (%)**	
		0.1	**0.5**
		Residual activity	
1	Control	100%	
2	Tween20	120	133

3	TritonX-100	137	150
4	CTAB	111	133
5	SDS	33	41
6	Tween20	120	133
Effect of Chelating agents			
		Concentration (%)	
		1	**0.5**
		Residual activity	
1	Control	100%	
2	Sodium Citrate	123	132
3	Sodium Azide	135	126
4	Urea	139	121
5	EDTA	30	57
Effect of Metal ions			
		Concentration (mM)	
		1	**10**
		Residual Activity	
1	Control	100%	
2	NH_4SO_4	76	86
3	$FeS0_4$	37	52
4	MgSO4	88	78
5	$CaCl_2$	94	90
6	$ZnSO_4$	82	75
7	$CuSO_4$	50	34
8	$MnCl_2$	70	80
9	$HgSO_4$	44	31

3.5. Antifungal activity of the bacteria

3.5.1 Antifungal activity on potato dextrose agar plates

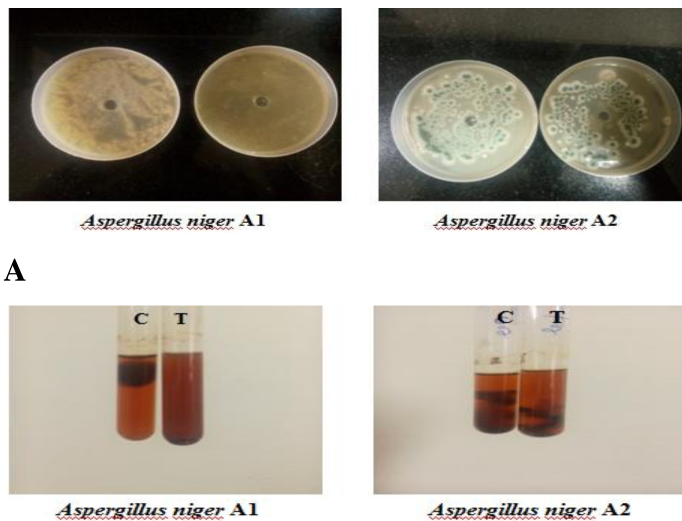
Inhibition of both the phytopathogenic fungal strains *Aspergillus niger* A1 and A2 was observed (Fig.3). However inhibition was much more pronounced in case of *Aspergillus niger* strain A1 than strain A2.

3.5.2 Antifungal activity on potato dextrose broth tubes

Growth of both the strains was inhibited by the enzyme whereas inhibition of *Aspergillus niger* strain1 was more prominent than *Aspergillus niger* strain 2 (Fig. 3). The results correlated with

the inhibition on the plates.These results indicate that chitinase from *Bacillus sp*. CH-2 have potential antagonistic activity against phytopathogenic fungi.

Karunaya *et al*., 2011 had reported antifungal activity against *Penicillium chrysogenum, Aspergillus niger*, and *Aspergillus flavus* by chitinase produced from *Bacillus subtilis*. In case of *Paenibacillus ehimensis* KWN38 (Naing *et al*., 2014) the hyphal morphology of *Rhizoctonia soloni* AG-1, *Fusarium oxosporium,* were significantly destroyed by crude chitinase enzyme.



B

Fig. 3: Inhibition of growth of *Aspergillus niger* A1 and *Aspergillus niger* A2 by chitinase produced by *Bacillus sp.* CH-2: (A) In potato dextrose agar plates after 96 hrs.
(B) In potato dextrose Broth plates after 96 hrs.

4. Conclusion

In this study the optimization of chitinase produced by *Bacillus sp.* CH-2 in a MMHL medium was carried by using statistical methods and the result obtained showed significant yield of the enzyme. Activity/stability of chitinase on temperature, pH and also against a number of reagents had been checked and it showed stability in the presence of non- ionic surfactants. Metal ions had little effects on the activity of chitinase from *Bacillus sp.* CH-2 however Fe^{2+}, Cu^{2+} and Hg^{+} had inhibitory effect on the enzyme activity. After characterization most of the properties of enzyme were found to be useful as antifungal agent. Antifungal activity of the *Bacillus sp.* CH-2

was observed against two phytopathogenic strains *Aspergillus niger.* A1 and *Aspergillus niger.* A2. It showed a significant inhibition against these phytopathogenic strains.

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