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Research Article

Inducible accretion of extra cellular chitinolytic enzyme through media optimization confers enhanced biocontrol trait in *Pseudomonas aeruginosa* fp 183

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ABSTRACT: Extracellular chitinolytic enzymes play a significant role to stipulate the bio-efficacy level of several *Pseudomonas* isolates against phytopathogenic fungus such as *Fusarium udum* and insect pests such as *Spodoptera litura.* Present exertion is to confirm and evaluate whether extracellular chitinase production is considered as an inducible biocontrol trait in *Pseudomonas* sp. or not. *Pseudomonas aeruginosa* fp 183 showed higher chitinase activity (3.75 U/mL) in modified King's B broth amongst all chitinase positive native isolates of Anand, Gujarat. Enhanced chitinase activity by 28.0%, 15.2% and 8.0% was observed after 84 hours when broth was amended with 1.5% of colloidal chitin, sterilized dry powder of *F. udum* and *S. litura* (third instar) respectively. The components of King's B medium were modified and optimized by using crude glycerol (byproduct in biodiesel production) via Plackett-Burman design and Response Surface Methodology for the responses such as colony count, chitinase activity and antagonistic (biocontrol) activity. Screening of media components and media optimization showed altered affects on all the responses. Production of extracellular chitinolytic enzyme increased by up to 27.2% and exhibited 27% more inhibition diameter against *F. udum*. The efforts revealed the thriving use of crude glycerol (4.7%), colloidal chitin (3.9%) and peptone (2.69%) in production media to enhance the chitinase secretion by *P. aeruginosa* fp 183.

KEY WORDS: *Pseudomonas aeruginosa*, chitinolytic enzyme, *Fuzarium udum*, *Spodoptera litura,* media optimization, Plackett-Burman design, Response Surface Methodology

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INTRODUCTION

Convectional synthetic chemical pesticides have long served as agents for reducing the incidence of plant disease; however, they are costly, can cause environmental pollution and may induce resistance in pathogens. Microbial products are especially valuable because their toxicity to non target organisms (animals and human being) is extremely low. They are safe for both the user and consumer of treated crops compared to other commonly used chemicals (Prakob *et al.,* 2009). Fluorescent *Pseudomonas*, particularly *Pseudomonas fluorescens*, *P. putida* and *P. aeruginosa,* which are commonly isolated from the plant rhizosphere, have been shown to protect plants from nematodes, insect attack and fungal infection. Studies have shown that certain *P. aeruginosa* can be potential agent against many biotic threats like *Drosophila melanogaster* (Lau *et al*., 2003), *Galleria mellonella* (Mostakim *et al*., 2012), *Meloidogyne javanica* (Siddiqui *et al*., 2001), *Fuzarium udum* (Badri and Sariah, 2009) and *Pythium* (Buysens *et al*., 1994).

Chitinase activity is considered as an important trait for the biocontrol ability of *Pseudomonas* sp. (Fallahzadeh *et al.,* 2010). *Pseudomonas aeruginosa* strains are reported to produce extracellular chitinase enzyme as they harbor conserved chitinase genes like *chiC* (Folder *et al.,* 2001). The expression level of such chitinolytic enzymes can be increased by involving inducers and optimal media conditions. Present study was performed to evaluate the usability of crude glycerol (byproduct from biofuel production process) with preliminary studies for screening of optimal media components for chitinase production and to heighten biocontrol trait.

MATERIALS AND METHODS

Microorganism and cultural conditions

Pseudomonas aeruginosa fp 183, a native biocontrol strain was isolated from disease suppressive rhizospheric soil of Anand, Gujarat, India. It was identified by 16S rDNA sequence analysis (accession – KF647773). Bacterial

culture was multiplied overnight in King's B broth medium (KB) at pH 7 and 29°C. For fungal powder preparation and inhibition assay *Fuzarium udum* Butler was obtained from ICRISAT, Hyderabad, which was cultured for mycelial growth on potato dextrose agar (HiMedia) for seven days at 29°C. Lignocellulose agar sporulation media was used to harvest its spores after 10 days in 0.5 M tris HCl.

Media components preparation

Chitin powder

Chitin, insect and mycelia powders were prepared for the alternative media preparation by modified practices. Chitin powder (1 gm) was added slowly into 18 ml of concentrated HCl under vigorous stirring and the mixture was then added to 100 ml of ice cold ethanol with rapid stirring, kept overnight at 25°C. The precipitate was collected by centrifugation at 8000 x g (Beckman Coulter Allegra 64R) for 10 min at 4°C and washed with sodium phosphate buffer until it was neutralized (pH 7), stored at –20 °C and used for further applications (Hackman and Goldberg, 1974).

Insect and mycelia powder

Harvested mycelia and *S. litura* were dried in oven at 45°C until constant weight. They were than subjected to grinding and subsequently 0.5% tris HCl (pH 7.5) was added and allowed to stand overnight at room temperature. The mixture was centrifuged at 8000 x g for 10 min. at 4°C. The supernatants were discarded and pellets of insect and mycelial powder were tyndallised in 0.5% tris HCl pH 7.5. The procedure of sterilization in buffer was repeated for three consecutive days followed by centrifugation. The pellets were dried, stored at –20°C and used for further application.

Crude glycerol: byproduct from jatropha biodiesel plant

Modification of the basic King's B medium was done so as to enable it to be more economical. Crude glycerol was used as alternative carbon source for chitinase production in place of purified glycerol. Crude glycerol a byproduct of biodiesel production from jatropha (*Jatropha curcas* L.) was obtained from biodiesel production plant, Department of Food Processing Technology and Bio-Energy, AAU Anand, Gujarat. It was autoclaved and analyzed for the specifications like glycerol, moisture per cent and colour etc. (Table 1).

Quantification assays

In order to check optimal growth, chitinase assay and antagonistic activity, two sets of experiments having 4 subsets each were designed. The first set utilized King's B medium with pure glycerol while in second set pure glycerol was replaced by crude glycerol.

Table 1. Crude glycerol specifications: an alternative carbon source

Sl. No.	Parameter	Extent
1	Water	8%
2	Glycerol	15%
3	Methanol	2%
4	pH	6.1
5	Colour	Dark brown
6	Density (gcm^{-3})	1.2

Each set was supplemented with colloidal chitin, fungal powder and insect powder and inoculated with *P. aeruginosa* fp 183. All the subsets were checked for growth, chitinase activity and antifungal activity.

Chitinase assay (UmL^{-1})

Chitinase (N-acetyl-â-glucosaminidase) activity was checked in different media system by a modified approach to the method described by Roberts and Selitrennikoff (1988). Each sample was centrifuged at 8000 × g for 5 min and the supernatant was used for enzyme activity. One mL supernatant was used for direct estimation of N-acetyl-d-glucosamine (GlcNAc). In second part, 1 mL of supernatant was incubated with 1 mL of 1% colloidal chitin in a 0.05M phosphate buffer, pH 7.0 at 37°C for 1 hr., centrifuged at 10000 x g for 15 min. The amount of N-acetyl-d-glucosamine released in the supernatant was determined by the standard method (Lingappa and Lockwood, 1962) using GlcNAc as a standard.

Microbial biomass (log cfu/mL)

Culture grown at 29°C for 72 and 82 hrs was compared. After centrifugation at 10,000 x g for 15 minutes, pellets were washed with sterile distilled water and re-suspended in 5 mL King's B basal medium. The colonies were calculated and transformed to logarithmic value (Thomson, 1996) for quantification. Optical density (OD) of concentrated (4X) cultured broth were determined by UV-visible spectrophotometer (Bekman Coulter DU® 730) at 600 nm and used for determination of the colony forming unit (cfu) by comparing the standard graph of OD verses log cfu/mL.

Antagonistic activity (mm)

The antifungal activity of cultured broth supernatant was assayed for *in vitro* inhibition of *F. udum* on

Sabouraud's Dextrose Agar (SDA) medium (Dennis and Webster, 1971 and Velusamy *et al.,* 2011). Fungal pathogen was pre inoculated by pouring 5 mL of soft agar with 10^3 spores/mL. 0.2 mL supernatant (filtered with 0.2µm Advavantec® cellulose acetate membrane filter) of 72 and 84 hrs culture grown broth was added in 10 mm well. Sterile distilled water was added to the wells of control plates. Diameter of the zone of inhibition was measured and expressed in millimeter (mm) after seven days of incubation at 26°C.

Effects of amendment and media alteration on responses

King's B medium with 2% crude glycerol (KBCG) and second set with 1% of pure glycerol were used. Twenty mL broth was prepared for chitinase production in 50 mL sugar tubes. Both media with and without supplementation of 1.5% of colloidal chitin, fungal powder and insect powder (eight treatments and three repetitions) were inoculated with 0.2 mL overnight grown culture and assayed for chitinase activity after 84 hrs of incubation. Further KBCG amended with colloidal chitin was used for screening and optimization of media components by using statistical designs.

Experimental designs

Plackett-Burman design

It is an efficient way to identify the important factors among a large number of variables (Abedin and Taha, 2008) and used to screen the important variables that significantly influenced the response (like chitinase production) (Plackett and Burman, 1946). Total number of trials to be carried out according to Plackett-Burman is *k* + 1, where *k* is number of variables.

For the selection of the key ingredients which could significantly affect the responses, six nutrient factors were considered (Table 2). Apart from these, five dummy variables were also used which were $\mathbf{E_{(xi)} = (ÓMi^+ – ÓMi)/N}$ denoted as D_1 to D_5 respectively. In this study, 11-run Plackett-Burman design was applied to evaluate selected factors. The entire variable had two levels with lower (L) and higher (H) concentration. The number of H and L per trial were (*k*+1)/2 and (*k*-1)/2 respectively. The main effect figure with an 'H' indicated that high concentration of this variable was nearer to optimum and an 'L' indicated that low concentration of this variable was nearer to optimum. The principal effect of each on chitinase production was estimated as the difference between both averages of measurements made at the higher level and at the lower level. The main effect of each variable (Table 2) was determined with the following equation:

Table 2. Variables showing process parameters used in Plackett-Burman design

Variables	Media components	(+) Values (High) (g/L or ml/L)	(–) Values (Low) (g/L or mL/L)
X_1	Crude glycerol	50	5
X_2	Colloidal chitin	30	3
X_3	Peptone	50	5
X_4	Tryptone	50	5
X_5	K_2HPO_4	5	0.5
X_6	$MgSO_4.7H_2O$	5	0.5

Where, E(xi) is the variable main effect, Mi^+ and Mi^- are the responses value in trails; where the independent variable (xi) was present in high and low concentrations, respectively, and N is the number of trails divided by 2.

Using Microsoft Excel, statistical *t*-values for equal unpaired sample were calculated for determination of significant variable. Experimental error was estimated by calculating the variance (V_{eff}) among the dummy variables as described by the formula.

$$V_{eff} = \Sigma(E_d)^2 / n$$

Where, V_{eff} is the variance of the concentration effect, E_d is the concentration effect of dummy variable and *n* is the number of dummy variables. The standard error (S.E.) of the concentration effect was the square root of the variance of an effect and the significance level (P value) of each concentration effect was determined using Student's test [$t_{(xi)}$].

$$t_{(xi)} = E_{(Xi)}$$

Where, $E_{(Xi)}$ is the effect of variable X_i.

Response Surface Methodology (RSM)

In order to describe the nature of response surface in the experimental region and to elucidate the optimal concentrations of the most significant independent variable, a Central Composite Design (CCD) (Box and Behnken, 1960) was applied, which is a RSM. As presented in Table 7, factors of highest confidence levels namely; Crude glycerol (A), peptone (B) and colloidal chitin (C) were tested at five levels (–á, +á, 0, +1 and –1). According to the design, 20 treatment trial combinations were executed. For predicting the optimal point, the following second order polynomial model was fitted to correlate relationship between independent variables and response:

$$Y = âo + Ó âixi + Ó âijxixj + Ó âiixi^2$$

Where Y is predicted response, $\hat{a}_0$ is a constant, *âi* is the linear coefficient, *âii* is squared coefficient, *âij* is the cross product coefficient, x_i is the dimensionless coded value of (X_i). The above equation was solved by using the software Design-Expert (Version 7.0.2, State ease inc., USA). A 2^5 factorial design with five replicates at the centre point with a total number of 20 trials was employed (Table 5).

2.5.3 Validation of optimization of media

For validation study, periodic analysis of chitinase activity up to 5 days was performed by taking six separate 20 mL fermentation systems for un-optimized fermented KBCG broth with 1.5% colloidal chitin v/v at 29°C and compared with periodical responses in optimized media provided by the design. Log cfu/mL and antifungal activity of the supernatant of the cultured broth at the time period of maximum chitinase activity were also analyzed for comparison of growth level.

RESULTS AND DISCUSSION

Effects of crude glycerol and inducers

In the present study, chitinase assay (UmL^{-1}) was performed from supernatants of cultured broth for 84 hrs. Among all the eight combinations, which were amendments in basal King's B (KB) medium and King's B medium with crude glycerol (KBCG), supernatant of cultured broth in presence of colloidal chitins showed significantly higher 3.51 and 2.72 UmL^{-1} chitinase activities respectively. Our culture showed high chitinase activity as compared to *P. aeruginosa* K 187 (Wang and Chang, 1997) which produced 0.6 to 0.8 U/mL of extracellular chitinase in culture supernatant.

Highest inhibition zone of 20 mm (Table 3) was exhibited by the supernatant of KB amended with colloidal chitin followed by KBCG + CC (14 mm). KB produced lowest antagonistic properties as 2.69 mm. Microbial biomass obtained was highest with KBCG + CC (9.5 log cfu/mL). Amongst all the treatments, colloidal chitin proved as most significant inducer for chitinase production in *P. aeruginosa* fp 183 after 84 hrs. In the presence of altered carbon source as crude glycerol in King's B medium showed higher chitinase activity and inhibition diameter and exhibited significantly higher microbial biomass. It has been reported that the presence of chitin in bacterial growth media had induced the production of extracellular chitinase (Viswanathan and Samiyappan, 2001). In an analogous approach, Nandakumar *et al.* (2007) reported 31.2% enhanced chitinase activity after the amendment 1% colloidal chitin in King's B medium, grown for 48 hrs with *Pseudomonas* sp. PB.

In many reports, crude glycerol has been explored as a carbon source for bacterial multiplication as a value added process (Anand and Saxena, 2011) such as Yang *et al.* (2012) used crude glycerol for the multiplication of *Rhodopseudomonas palustri.*

Table 3. Effects of media amendments on chitinase production, growth and *in vitro* antagonistic activity against *Fusarium udum* Butler

Treatment No.	Treatments	Chitinase activity (U/mL)	Bacterial biomass x (log cfu/mL)	Inhibition zone (mm)
T_1	KB	2.73	4.5 (2.9×10^4)	2.69
T_2	KB + FP	3.16	7.5 (3.1×10^7)	11.00
T_3	KB + CC	3.51	5.5 (3.2×10^5)	20.00
T_4	KB + IP	2.96	4.5 (3.5×10^4)	9.00
T_5	KBCG	2.00	4.5 (4.1×10^4)	6.00
T_6	KBCG + FP	2.10	5.0 (9.5×10^4)	14.00
T_7	KBCG + CC	2.72	9.5 (2.9×10^9)	13.00
T_8	KBCG + IP	2.23	6.5 (3.1×10^6)	10.00
S. Em. ±	0.08	0.22	0.06	
CD (P=0.05)	0.25	0.63	0.20	
CV %	5.64	6.42	4.27	

(KB = King's B medium, FP = fungal powder, CC = colloidal chitin, IP = insect powder, KBCG = KB with crude glycerol and the figure in parentheses are log retransformed values as cfu/mL)

To enhance the production of chitinase, Plackett-Burman Design was employed as statistical approach for the screening of suitable media components.

Table 2 represents the independent variables and their respective high and low concentrations used in the optimization study, while Table 4 shows the Plackett-Burman experimental design for 12 trials with two levels of concentrations for each variable and the corresponding chitinase activity in terms of (UmL^{-1}) of the culture media. The chitinase activities ranged between 0.35 and 3.00 U/mL during the screening studies.

Table 5 represent the effect, standard error, $t_{(xi)}$, and P values of each process variable for chitinase production. The significant process variables were screened at probability value ($P \leq 0.05$). During the present investigation, the probability value of crude glycerol, colloidal chitin and peptone ($P < 0.05$) for chitinase production were considered as significant positive variables as they showed 99.96%, 99.52% and 99.72% confident level at 5 mL/L, 3 and 5 gm/L respectively.

However, tryptone, K_2HPO_4 and $MgSO_4.7H_2O$ showed no significant influence on chitinase production. In the similar way Narasimhan and Shivakumar (2012) considered Plackett-Burman design as a prevailing tool for identifying the media factors which had significant influence on extracellular metabolite (chitinase) production. They exploited *Bacillus* sp. and confirmed carboxy methyl cellulose, incubation period, and temperature as significant factor for the chitinase production in the culture supernatant.

3.3 Optimization of screened media components using Central Composite Design (CCD)

CCD was used to determine the optimal concentration (level) of the media components. A total of 20 experiments with three variables (components of the media) and five coded levels (five concentrations) were performed (Table 6). Model was run without any transformation to optimize the quantity of 3 important media components (crude glycerol, colloidal chitin and peptone), which were found significant in Plackett-Burman experimental design. As they had positive influence, increased concentrations was studied for their optimization. The other components of the production media were not significant and hence, their concentrations were set at their middle level in CCD. The other cultural conditions were: temperature 29°C, 0.2% inoculums size, agitation speed (140 rpm), incubation period 84 hrs and pH 7.2. Chitinase activity ranged between 1.35-3.47 UmL^{-1} (Table 6) and the ratio of maximum to minimum was found 2.57. Table 6 represents the experimental design matrix for CCD along with the experimental results of predicted responses for chitinase activity in broth. The experimental values for the regression coefficient were obtained by

Table 4. Plackett-Burman design matrix with six process variables (X_1–X_6) and five dummy variables (D_1–D_5) along with observed response (chitinase activity)

Run no.	X_1	X_2	X_3	X_4	X_5	X_6	D_1	D_2	D_3	D_4	D_5	Chitinase activity (UmL^{-1})
1	H	H	L	H	H	H	L	L	L	H	L	2.63
2	L	H	H	L	H	H	H	L	L	L	H	2.71
3	H	L	H	H	L	H	H	H	L	L	L	1.11
4	L	H	L	H	H	L	H	H	H	L	L	2.08
5	L	L	H	L	H	H	L	H	H	H	L	1.35
6	L	L	L	H	L	H	H	L	H	H	H	0.51
7	H	L	L	L	H	L	H	H	L	H	H	1.00
8	H	H	L	L	L	H	L	H	H	L	H	2.84
9	H	H	H	L	L	L	H	L	H	H	L	3.00
10	L	H	H	H	L	L	L	H	L	H	H	2.06
11	H	L	H	H	H	L	L	L	H	L	H	1.49
12	L	L	L	L	L	L	L	L	L	L	L	0.35

(X = media components as variables, D= dummy variables, L and H represents low and high concentration respectively)

Table 5. Statistical analysis of process parameters for chitinase activity

Factors	Fermentation parameters	Chitinase activity (UmL^{-1})			
		Effect [$E_{(xi)}$]	Standard error (S.E.)	$t_{(xi)}$	P value
X_1	Crude glycerol	12.07	0.66	4.58	0.00593
X_2	Colloidal chitin	15.32		14.48	0.00003
X_3	Peptone	11.72		3.52	0.01695
X_4	Tryptone	9.88		-2.09	0.09131
X_5	K_2HPO_4	11.26		2.12	0.08785
X_6	$MgSO_4.7H_2O$	11.15		1.78	0.13487

Table 6. Central Composite Design (CCD) matrix of independent variables and the corresponding experimental and predicted values

Run No.	Factors						Response	
	A: Crude glycerol (mL/L)		B: Colloidal Chitin (g/L)		C: Peptone (g/L)		Chitinase activity (UmL^{-1})	
	Coded	Actual	Coded	Actual	Coded	Actual	Observed	Predicted
1	0	55.0	0	27.5	0	28.75	2.18	2.29
2	–1	32.5	1	38.75	1	39.38	2.42	2.47
3	0	55.0	0	27.50	0	28.75	2.29	2.29
4	–1	32.5	1	38.75	–1	18.13	2.69	2.72
5	1	77.5	–1	16.25	–1	18.13	2.31	2.25
6	0	55.0	–1.68	8.58	0	28.75	2.30	2.39
7	1	77.5	1	38.75	1	39.38	2.20	2.23
8	–1.6	17.1	0	27.50	0	28.75	1.55	1.53
9	0	55.0	0	27.50	0	28.75	2.29	2.29
10	0	55.0	0	27.50	0	28.75	2.05	2.29
11	0	55.0	0	27.50	0	28.75	2.32	2.29
12	1	77.5	–1	16.25	1	39.38	2.06	2.02
13	0	55.0	1.68	46.42	0	28.75	3.55	3.47
14	0	55.0	0	27.50	0	28.75	2.18	2.29
15	0	55.0	0	27.50	–1.68	10.88	2.00	2.03
16	–1	32.5	–1	16.25	–1	18.13	1.68	1.64
17	1	77.5	1	38.75	–1	18.13	2.40	2.41
18	–1	32.5	–1	16.25	1	39.38	1.37	1.35
19	1.6	92.8	0	27.50	0	28.75	1.80	1.83
20	0	55.0	0	27.50	1.68	46.62	1.63	1.62

quadratic polynomial equation, where only significant coefficients (P <0.05) were considered.

The smaller P values indicate the higher significance of the corresponding coefficient. The insignificant coefficients were not omitted from the equations, since it was a hierarchical model. The predicted response (Y) for the chitinase activity was obtained as follow:

$$Y= 2.29 + 0.090*A + 0.32*B - 12*C – 0.23*A*B + 0.016*A* C + 0.011* B*C – 0.22*A^2+ 0.23*B^2 – 0.17*C^2$$

Where Y is the chitinase activity (UmL^{-1}) and A, B, C are the coded values of the independent variables (crude glycerol, colloidal chitin and peptone respectively).

The statistical significance of the quadratic model for the experimental responses was evaluated by the analysis of variance (ANOVA). According to the ANOVA results (Table 7), the model was significant with an *F* test of a very low probability value [($P > F$) < 0.0001]. The goodness of fit for the model was expressed by the coefficient of determination R^2 and the value was found 0.977. The value of R^2 indicates that the experimental values were significantly in agreement with the predicted values and also suggested that the model was suitable and practicable.

For the interaction interpretations model generated 3D and contour plots which illustrated the response over a region of interesting factor levels, the relationship between the response and experimental levels of each variable and the type of interactions between the test variables in order to deduce the optimal composition of the culture media. A comparison between the actual data and model predicted data was analyzed by using the SPSS software (version 13.0, SPPS Inc.).

The result showed in the Fig. 2 confirmed that experimental value was in good agreement with the predicted one and suggested that the model was satisfactory and hence model run showed that the predicted and obtained response values were with minimum alteration as shown in Fig. 2. Fig. 3 depicts the two-dimensional (2D) contour plot showing the effects of crude glycerol and colloidal chitin on extracellular chitinase activity; while other variable, peptone was fixed at the 17.5 g/L. An increasing trend was observed for chitinase activity with low concentration of colloidal chitin and increasing crude glycerol (Fig. 4).

When colloidal chitin was higher, higher chitinase production was obtained at lower values of both the

Table 7. Analysis of variance (ANOVA) for the experimental results of the CCD

Source	Sum of Squares	Degree of freedom	Fvalue	P value Prob > F	Remark
Model	4.119	9	47.0175	< 0.0001	
A: Crude glycerol	0.110	1	11.3875	0.0071	
B: Colloidal chitin	1.412	1	145.1031	< 0.0001	
C: Peptone	0.199	1	20.5334	0.0011	
AB	0.418	1	42.9998	< 0.0001	Significant
AC	0.002	1	0.2169	0.6513	
BC	0.001	1	0.1040	0.7537	
A^2	0.672	1	69.0752	< 0.0001	
B^2	0.735	1	75.5607	< 0.0001	
C^2	0.399	1	41.0462	< 0.0001	

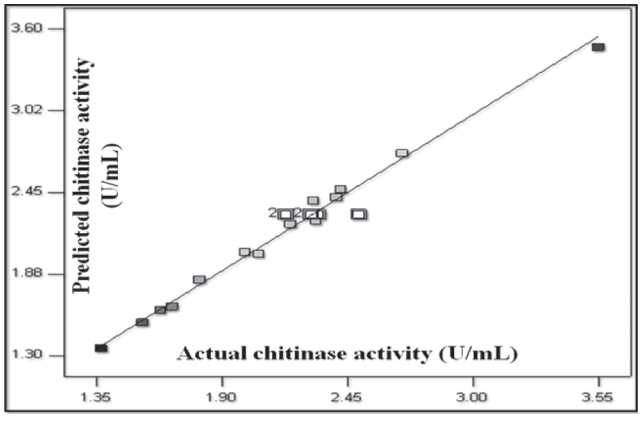

Fig. 2. Deviation in the predicted and observed response

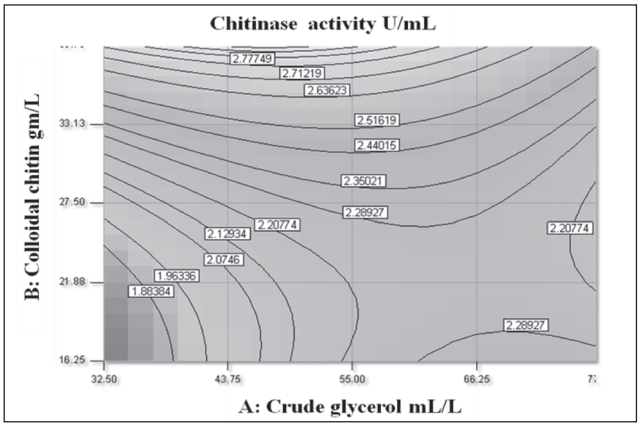

Fig. 3. Interaction effects of crude glycerol and colloidal chitin

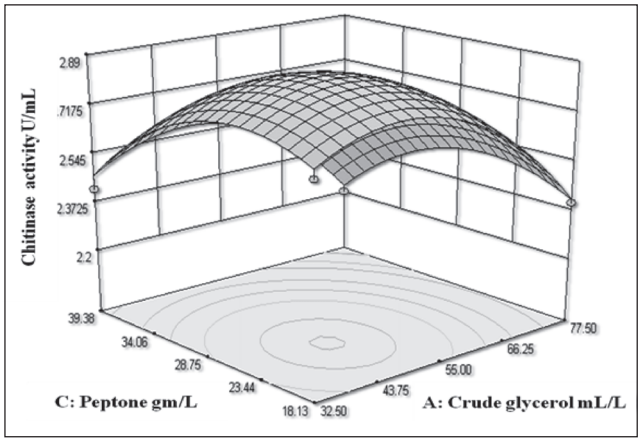

Fig. 4. Effects of peptone and crude glycerol on chitinase production

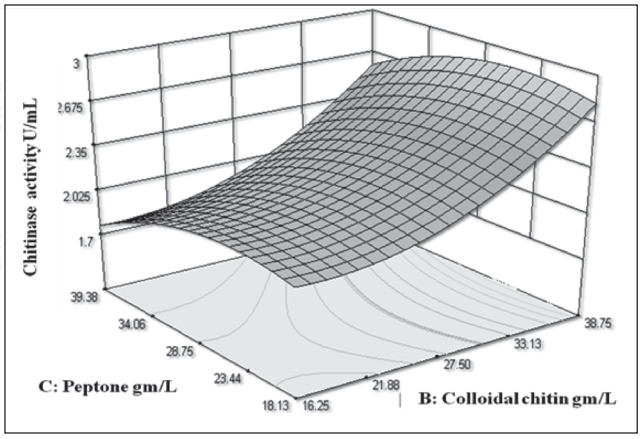

Fig. 5. Effect of peptone and colloidal chitin

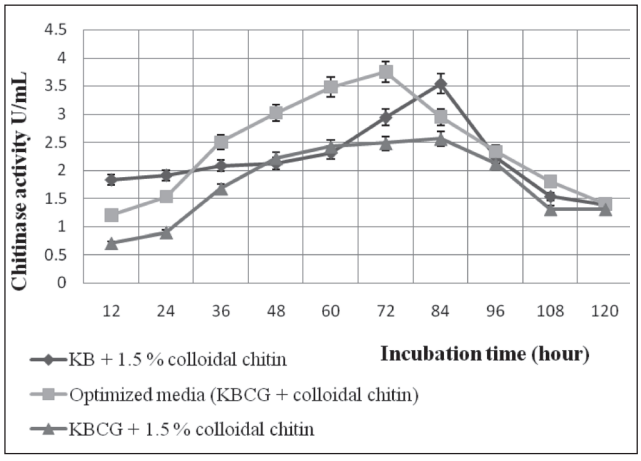

Fig. 6. Validation of optimization for difference responses

variables. There was maximum production of chitinase at lower peptone and highest colloidal chitin (Fig. 5).

Study of 3D surface plots, contour plots and the equations obtained from the multiple regression analysis helped to determine the optimal concentration of the media components.

The model predicted that the chitinase production (2.88 UmL^{-1}) would be located at the actual values: crude glycerol = 47.70 mL/L, colloidal chitin = 38.75 mL/L and peptone = 25.59 gm/L.

The predicted values of the response were obtained and their corresponding concentration of media components varies accordingly. Thus, graphical optimization of the overall desirability function was performed to determine the best possible combination for each response simultaneously.

3.4 Validation of optimal media composition for actual and other responses

To validate optimized media components for chitinase activity, periodical production curve with 0.2% culture inoculation up to 120 hrs was carried out and compared with both un-optimized KBCG and KB in presence of 1.5% of colloidal chitin.

It was found that optimal media components were responsible for premature production peak (72 hour) with 3.75 U mL^{-1}, which was 12 hour earlier as well as 46.4%, 30.20% and 5% higher than un-optimized KBCG, predicted optimized response and KB respectively (Fig. 6). Effects on other responses such as log cfu/mL and *Fusarium* growth inhibition (mm) was performed at 72 and 84 hours and found log cfu value (10 log cfu/mL) and inhibition (14 mm) value were higher at 84 hours as compared to 9.5 log cfu/mL and 11 mm respectively at 72 hours. The result suggested that inhibition of *F. udum* exhibited by extracellular chitinase in combination with other metabolites in *Pseudomonas* sp. fp 183.

Present result was analogous with the findings of Hassanein *et al.,* 2009, who suggested that hydrogen cyanide and siderophores (pyocyanin) were produced by *Pseudomonas* sp. conferred the antagonistic activity in the broth. There are several reports on the optimization of media composition for the production improvement using statistical approaches as it was found to be a reliable methodology to obtain reproducible results (Dong *et al.,* 2012). In an analogous study Folder *et al.* (2001) confirmed the secretion of chitinase from *Pseudomonas* sp. as an inducible trait and Chakrabortty *et al.* (2012) optimized the physical and media parameters for the production of chitinase from bacterial isolate and achieved 10.3% increase in chitinase yield.

This study confirmed that carbon source in King's B medium can be substituted with byproduct such as crude glycerol for the production of bacterial biocontrol agents (*Pseudomonas* sp.). Statistical model such as Plackett-Burman design and response surface model can optimize

the growth parameters (media or diet components); hence it can be established as a tool for enhanced *in vitro* production of biocontrol agents. Biocontrol potential via chitinase production in *P. aeruginosa* fp 183 can be enhanced by using colloidal chitin and optimizing the media components.

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