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A N I N T E R N A T I O N A L J O U R N A L

Applications of Plackett–Burman and Central Composite Design for the Optimization of Novel *Brevundimonas diminut*a KT277492 Chitinase Production, Investigation of its Antifungal Activity

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ABSTRACT

Biological control strategy which can damage chitin, a vital component of pathogenic fungi and arthropods promises a safe solution for many fungal problems. And it's more favorable than chemicals which increase health risks and environmental problems. Thus, the chitinase producers appear potential candidates of biological control of pathogenic fungi. Brevundimonus diminuta KT277492 is a new isolate that has been isolated recently from Egyptian soil. Significant factors that affecting the chitinase enzyme production were studied and optimized using Plackett-Burman and Response Surface Methodology (RSM). As a result, maximum production of chitinase enzyme was 832.87 IUL^{-1}, this result presented about 8.767-fold increase in the enzyme production. In the last phase of the study, partially purified chitinase enzyme obtained from B. diminuta KT277492 was tested against two pathogenic fungi and the results showed good inhibitory activity against A. alternata and F. solani with IZD of 31±0.25 and 25±0.91 mm respectively. Finally, obtained results indicated the value of optimization process and the optimized chitinase enzyme could be an excellent choice in application of food and biotechnology as a biofungicide. This reflects the necessity of studying the characteristics and kinetics of the enzyme in the forthcoming study.

Key words: chitinase, *Brevundimonas diminuta* KT277492, optimization, Plackett–Burman, central composite design, antifungal.

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INTRODUCTION

According to [1] Chitin is the second most abundant insoluble biodegradable polymer, which exists naturally in the biosphere as a structural polysaccharide of 1,4-N-acetyl-D-glucosamine. It is highly distributed in nature, as a constituent of insect exoskeleton, shells of crustaceans, fungal cell walls and algae components [2]. Chitinases enzymes (E.C.3.2.1.14) capable of hydrolyzing chitin to its oligo and monomeric components. There are several sources for chitinases as, microorganisms [2], higher plants [3], even lower animals and birds [4].

Bacterial chitinases are believed to be important in the hydrolysis process of chitin for utilization as an energy and carbon sources, therefore, they have been implicated in protection against parasites in fungi, protozoa, invertebrates and especially in breakdown of chitin in the cell wall of fungal pathogens. [5,6]. One of such bacteria are *Brevundimonus sp.* that able to produce the lytic enzymes such as chitinase and has tremendous impact for an industrial scale production [7]. Recently, Ashour et al [8] locally isolated the rhizobacterial strain *Brevundimonus diminuta* KT277492 from rhizosphere of *Trigonella foenum- graecum L* in Egypt. The qualitative colloidal chitin agar plate test suggested its ability for first time to produce chitinase enzyme better than other isolates.

Chitinases are gaining much more attention worldwide because they have wide range of applications in the food, cosmetic industries, medical and fertilizer production areas [9, 10]. Also important in the biocontrol of fungal diseases in plants [11] and as biobestsides [12].

In microorganisms, chitinase production is controlled by a receptor-inducer system; therefore, the composition of the culture medium can affect chitinase production [13]. The conventional method for medium optimization involves changing one parameter at a time while keeping all others constant may be very expensive and time consuming. Furthermore, it is not effective to determine the combined effect of different factors. A number of statistical experimental designs have been used to overcome these problems [13, 14]. One of these methods are full factorial designs which provide more complete information.

The Plackett-Burman design [15], as a two level fractional factorial design, is especially useful in screening studies by estimating the main effects of variables. The variables screened by Plackett-Burman design can be optimized by using statistical and mathematical optimization tools such as response surface methodology (RSM) [16]**.** Recent studies have indicated the use of RSM for analyzing effects of different factors on enzyme activity [17]and optimization of enzyme production [18].

The present study directed towards the improvement and optimization of the valuable newly isolated *Brevundimonus diminuta* KT277492 chitinase enzyme. Depending on the novel statistical designs and estimation of its antifungal activity important for biological control strategies of pathogenic fungi.

MATERIALS AND METHODS

Chemicals

Chitin was purchased from (Sigma-Aldrich-MO-USA). Other reagents were of analytical grade.

Microorganism and maintenance

The rhizobacterial strain *Brevundimonus diminuta* KT277492 used in this study, was locally isolated from rhizosphere of *Trigonella foenum- graecum L*. collected from the green house of Ministry of Agriculture, Giza, Egypt. [8]. The stock culture was maintained on nutrient agar slants at 4 °C and periodically subcultured in the National Research Center, Chemistry of Natural and Microbial Products Dept. [1].

Culture conditions for chitinase enzymes production

Preparation of seed culture

The seed culture was prepared by inoculating single colony in a plate containing the medium composed of (gl^{-1}) glucose 2.0, peptone 4.0, KH_2PO_4 0.7, $MgSO_4.7H_2O$ 0.5, K_2HPO_4 0.3, $FeSO_4 \cdot 7H_2O$, 0.02 and agar 20.0. The plates incubated for 48h at 30°C, after that a loop full of the colony transferred to 50 mL seed liquid medium, initial pH 7 in 250 mL Erlenmeyer flasks and incubated under shaking (200 rpm) for 48 h at 30°C.

Chitinase production medium

Nutrient broth medium (NB) used as basal medium for chitinase production. The medium contained the following components (gl^{-1}): yeast extract 1.5, NaCl 5.0, beef extract 1.5,

supplemented with 0.1% colloidal chitin [19]. 250 mL Erlenmeyer flasks containing 50 mL of NB medium autoclaved at 121°C for 20 min. After cooling, each flask was inoculated with 1 mL containing (1×10^8 spores^{-ml}) of the fresh seed culture and incubated under the same conditions mentioned above. All the experiments were carried out in duplicate and the average values are reported as mean ± SD calculated using MS Excel.

Effect of incubation period on the chitinase production

The time courses of the chitinase production by *B.diminuta* KT277492, using the basal production medium were monitored for 168 h after 24 h intervals, fermentation broth was centrifuged and chitinase activity was determined [1].

Chitinase activity assay

According to [20] chitinase activity was determined by a dinitrosalicylic acid (DNS) method [21] with some modification. This method works on the concentration of *N*-acetyl glucosamine (NAG), which is released as a result of enzymatic action [22]. The 2mL reaction mixture contained 0.5 mL of 0.5% colloidal chitin in acetate buffer (pH 5.5), 0.5 mL crude enzyme extract and 1mL distilled water. The well vortexed mixture was incubated in a water bath shaker at 40°C for 2 h. The reaction was arrested by the addition of 3mL DNS reagent followed by heating at 100°C for 10 min with 40% Rochelle's salt solution. The colored solution was centrifuged at 10,000 rotations per minute for 5 min and the absorption of the appropriately diluted test sample was measured at 530 nm using UV spectrophotometer (UV-160 A, Shimadzu, Japan) along with substrate and enzyme blanks. Colloidal chitin was prepared by the modified method of [23]. One unit (IU) of the chitinase activity is defined as the amount of enzyme that is required to release 1µmol of *N*-acetyl-d-glucosamine per minute from 0.5% of colloidal chitin solution under assay conditions.

Statistical optimization of *B.diminuta* KT277492 chitinase enzyme

Screening of critical media components using Plackett–Burman design

According to Shehata and Abd El Aty [24] Plackett-Burman factorial design was used in our study to select significant medium components affecting the production of chitinase. Eleven medial components (chitin, glucose, starch, fructose, peptone, yeast extract, urea, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium sulphate, sodium sulfate) were studied. Each variable was represented at 2-levels, upper ('high, +') and lower ('low, -') levels of the range covered by each variable and the response **Table (1).** Experimental responses were analyzed by first order model by the following equation $Y=\beta_o+\sum \beta_i x_i$, where Y is the response for chitinase production, B_o is the model intercept and β_i is the linear coefficient, and x_i is the level of the independent variable. According to the Stat-Ease analysis, a first-order model could be obtained from the regression results of fractional factorial experiment. This model describes the interaction among factors and it is used to screen and evaluate important factors that influence the response. The main effect of each variable was determined according to the following equation: $E_{xi}= \frac{(\Sigma M_{i+} - \Sigma M_{i-})}{N}$

Where Exi is the variable main effect, $\Sigma Mi+$ is the summation of the response value at high level; $\Sigma Mi-$ is the summation of the response value at low level, and N is the number of experiments. Statistical analysis of PBD is performed by using Design-Expert® 8 software from Stat-Ease, Inc. **Table (2).**

Table (1): Twelve trials Plackett-Burman experimental design with the response (chitinase activity).

Trial	Carbon source				Nitrogen source			Energy source		Metal ions		Chitinase enzyme activity IUL^{-1}	
	A: Chitin	B: Glucose	C: Starch	D: Fructose	E: Peptone	F: Yeast extract	G: Urea	H: KH_2PO_4	J: K_2HPO_4	K: $MgSO_4$	L: Na_2SO_4	Experimental	Predicted
1	-1 (0.5)	+1(8)	-1(2)	+1(8)	+1(5)	-1(1)	+1(5)	+1(3)	+1(3)	-1(0)	-1(0)	196±0.411	196.292
2	+1(5)	-1(2)	+1(8)	+1(8)	+1(5)	-1(1)	-1(1)	-1(0.5)	+1(3)	-1(0)	+1(5)	165.6±0.007	165.892
3	+1(5)	+1(8)	-1(2)	+1(8)	+1(5)	+1(5)	-1(1)	-1(0.5)	-1(0.5)	+1(5)	-1(0)	295±0.8747	295.292
4	+1(5)	-1(2)	-1(2)	-1(2)	+1(5)	-1(1)	+1(5)	+1(3)	-1(0.5)	+1(5)	+1(5)	207±0.015	206.708
5	-1(0.5)	+1(8)	+1(8)	+1(8)	-1(1)	-1(1)	-1(1)	+1(3)	-1(0.5)	+1(5)	+1(5)	000	000
6	-1(0.5)	-1(2)	+1(8)	-1(2)	+1(5)	+1(5)	-1(1)	+1(3)	+1(3)	+1(5)	-1(0)	175.5±0.005	175.208
7	+1(5)	+1(8)	-1(2)	-1(2)	-1(1)	+1(5)	-1(1)	+1(3)	+1(3)	-1(0)	+1(5)	397±0.008	396.708
8	-1(0.5)	+1(8)	+1(8)	-1(2)	+1(5)	+1(5)	+1(5)	-1(0.5)	-1(0.5)	-1(0)	+1(5)	107±0.02	106.708
9	+1(5)	+1(8)	+1(8)	-1(2)	-1(1)	-1(1)	+1(5)	-1(0.5)	+1(3)	+1(5)	-1(0)	209.9±0.383	209.86
10	-1(0.5)	-1(2)	-1(2)	+1(8)	-1(1)	+1(5)	+1(5)	-1(0.5)	+1(3)	+1(5)	+1(5)	148.8±0.038	149.092
11	+1(5)	-1(2)	+1(8)	+1(8)	-1(1)	+1(5)	+1(5)	+1(3)	-1(0.5)	-1(0)	-1(0)	365±0.159	365.292
12	-1(0.5)	-1(2)	-1(2)	-1(2)	-1(1)	-1(1)	-1(1)	-1(0.5)	-1(0.5)	-1(0)	-1(0)	77.5±0.003	77.2

+1 and −1 represent the coded levels (high and low) of the independent variable. Real values (given in parentheses) are in $gl^{-1}$for carbon and nitrogen sources and energy sources. But in mM for metal ions. Values of response are the mean of duplicate cultivation experiments ±SD.

Table (2): Statistical analysis of Plackett-Burman design.

Source	Sum of Squares	df	Mean Square	F-value	P-value Prob>F
Model	1.430E+005	10	14302.27	14010.39	0.0066significant
A-Chitin	72805.34	1	72805.34	71319.52	0.0024
B-Glucose	357.52	1	357.52	350.22	0.0340
C-Starch	7415.24	1	7415.24	7263.91	0.0075
E-Peptone	226.20	1	226.20	221.58	0.0427
F-Yeast extract	33316.94	1	33316.94	32637.00	0.0035
G-Urea	1262.80	1	1262.80	1237.03	0.0181
H-KH_2PO_4	9447.24	1	9447.24	9254.44	0.0066
J-K_2HPO_4	4852.14	1	4852.14	4753.12	0.0092
K-$MgSo_4$	6160.80	1	6160.80	6035.07	0.0082
L-Na_2SO_4	7178.52	1	7178.52	7032.02	0.0076
Residual	1.02	1	1.02		
Cor Total	1.430E+005	11			

Std. Dev.=1.01, R-Squared =1.0000, Mean=195.36, Adj R-Squared=0.9999, C.V. %=0.52, Pred R-Squared=0.9990, PRESS=147.00, Adeq Precision=409.797.

Optimization of medium with the Response Surface Methodology

Chitin, yeast extract and potassium di-hydrogen phosphate were identified as the key factors that influenced chitinase production from the above experiment, and their suitable concentration ranges were also preliminarily determined. Consequently, response surface methodology of central composite design (CCD) used for statistical optimization of *B.diminuta* KT277492 chitinase enzyme. [25, 26]. The 3–factor–5–level central composite design (CCD) with twenty experiments were carried out to determine the optimal concentration of chitin (A), yeast extract (B) and potassium di-hydrogen phosphate (C) and to develop a mathematical correlation between the three important variables and chitinase activity (Y). All three variables were investigated at low level (-1), zero level (0) and high level (+1), respectively, with $\alpha = 1.682$. Codes and actual values of variables and matrix of CCD along with chitinase activity of each trial are shown in **Table (3).** The behavior of the system was explained by the following quadratic model equation.

$$Y\ (activity) = \beta 0 + \beta 1A + \beta 2B + \beta 3C + \beta 11A2 + \beta 22B2 + \beta 33C2 + \beta 12AB + \beta 13AC + \beta 23BC$$

where Y (activity) was the predicted production of chitinase (IUL^{-1}), β0 intercept, β1, β2 and β3 linear coefficients, β11, β22 and β33 quadratic coefficients and β12, β13 and β23 interactive coefficients. A, B and C were the independent variables corresponding to the concentration of chitin, yeast extract and potassium di-hydrogen phosphate, respectively. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA) **Table (4)** and the quadratic models were represented as contour plots (3D) using Design-Expert® 8 software from Stat-Ease, Inc. [27]. All experiments were carried out in duplicate and the averages of chitinase activity were taken as response.

Partial purification of chitinase enzyme

Fractional precipitation of chitinase with acetone take place. the precipitant was added to the cold culture filtrate in ice-salt bath until the required concentration was achieved. After isolating the precipitated fraction by centrifugation in a refrigerated centrifuge, the supernatant was subjected to further precipitation and the process was repeated. The enzyme fractions obtained (25, 50 and 75% acetone) were dried at room temperature and assayed for chitinase activity [28].

In-vitro estimation of *B.diminuta* KT277492 chitinase antifungal activities

B.diminuta KT277492 chitinase enzyme was screened *in-vitro* against two pathogenic fungi by the agar diffusion technique in Petri dishes according to [28, 29]. The root rot pathogenic fungal isolates (*Fusarium solani* NRC15 and *Alternaria alternata* NRC43) were obtained from the culture collection of the Department of Chemistry of Natural and Microbial Products, National Research Center, Cairo, Egypt. The microorganisms were passaged at least twice to ensure purity and viability. About 200 µl of the partial purified enzyme was applied on each well (10 mm in diameter) made in the solidified inoculated potato dextrose agar plates. The plates incubated for 72 h at 28 ºC. The antifungal effect was evaluated by measuring the inhibition zone diameter (IZD) around wells of the enzyme in (mm) at three different points and the average values are reported as Mean ± SD using MS Excel.

RESULTS

Chitinase enzyme production

Qualitative test of colloidal chitin agar plates showed the ability of *B. diminuta* KT277492 to produce chitinase enzyme, which hydrolyzed chitin forming clear zone around the bacterial colony as shown in **Fig. (1).** Results also demonstrated the ability of the novel isolate to produce chitinase enzyme (95 IUL^{-1}) in nutrient broth medium supplemented with 0.1% chitin better than the other bacterial isolates as mentioned in the previous study. Therefore, *B. diminuta* KT277492 was selected as the best bacterial isolate for further optimization and improvement for chitinase enzyme production [8].

Fig. (1): Photo of colloidal chitin agar plate showing clear hydrolysis zone around the *B.diminuta* KT277492 indicting chitinase production.

Optimization of *B.diminuta* KT277492 chitinase production

Sequential optimization approaches were applied in the present part of the study. The first approach deals with the determination of the optimum incubation period, by one-variable-at-a-time method. The second approach deals with screening for nutritional factors affecting the selected bacterial isolate for chitinase production. The third approach is to optimize the factors that control the enzyme production process.

Determination of the optimum incubation period

One variable-at-a-time method is used to determine the optimum incubation period for chitinase production. Results indicated that at different incubation periods (48, 72, 96, 120, 144 and 168 h) the activity was (59.89, 102.7, 27, 22, 00.00 and 00.00 IUL^{-1}, resp.). The incubation period for 72 h was the most favorable for maximum chitinase production.

Evaluation of the factors affecting chitinase production

Eleven different medium constituents (variables) were chosen to perform the optimization process. The averages of chitinase activity of the different trials (experimental) together with the predicted activity are shown in **Table (1).** The data showed wide variation from 0 to 397±0.008 IUL^{-1} of chitinase activity. This variation reflects the importance of medium optimization to attain c)higher productivity. The main effects of the examined variables on the enzyme activity were calculated and presented graphically in **Fig. (2).** Chitin, yeast extract, KH_2PO_4 showed the higher positive effect on chitinase activity followed by K_2HPO_4, urea and glucose, respectively. On the other hand, starch, fructose, peptone, $MgSO_4$and Na_2SO_4 were contributed negatively. Overall, the percentage contribution of the significant variables indicated that 50.90% was for initial chitin, 23.29% for yeast extract and 6.61% for KH_2PO_4, the remaining 19.2% for the other variables **(Fig. 3)**.The first order model equation developed by PB design showed the dependence of *B. diminuta* chitinase production on the medium constituent: Y (chitinase activity IUL^{-1})=+24.26157+ 34.61852* chitin +1.81944 *glucose -8.28611 * starch -2.17083* peptone +26.34583 * yeast extract +5.12917 * Urea +22.44667 * KH_2PO_4 +16.08667*K_2HPO_4 -9.06333* $MgSO_4$ -9.78333 * Na_2SO_4

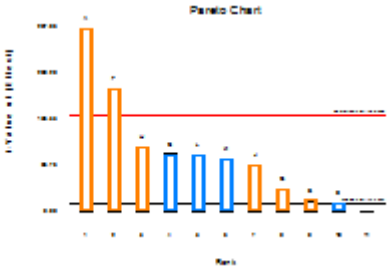


Fig. (2): Pareto chart of eleven-factor standard effects on chitinase production.

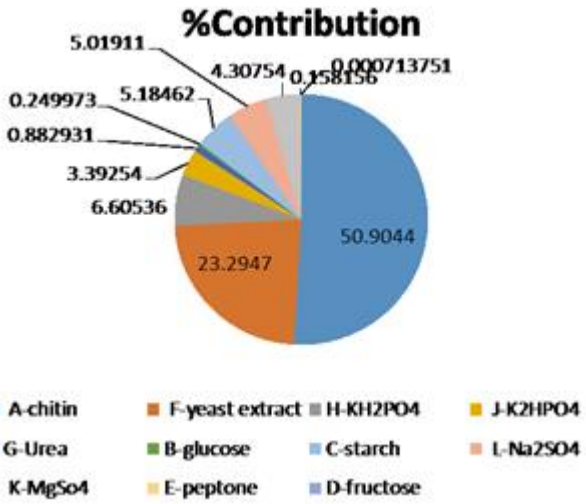


Fig. (3): The percentage contribution of the nutrient components.

Statistical analysis of the responses is represented in **Table (2)** The Model *F* value of 14010.39 implies that the model is significant. Values of

"Prob > *F*" less than 0.0500 indicate that model terms are significant. In this case A, B, C, E, F, G, H, J, K, L are significant model terms. In addition, the predicted R^2 was found to be 0.9990, which is in reasonable agreement with the R^2 of 1.000 and adjusted R^2 of 0.9999. This revealed that there is good agreement between the experimental and the theoretical values predicted by the model. The obtained results showed that varying of the chitin of the medium between 0.5-5 gl^{-1} had high effect on chitinase production by *B. diminuta*. Maximal enzyme activities were obtained only when the chitin contribution in the culture medium was 5 gl^{-1}. According to these results, a medium of the following composition is expected to be near optimum (gl^{-1}): chitin 5, yeast extract 5, K_2HPO_4 3. The enzyme activity measurement on this medium was 397±0.008 IUL^{-1}. This result presented about 4.18-fold increase in the enzyme activity, when compared to the results obtained in basal production medium (95 IUL^{-1}). Variables with less significant effect were not included in the next optimization experiment but instead were used in all trials at their (−1) level and (+1) level, for the negatively contributing variables and the positively contributing variables, respectively.

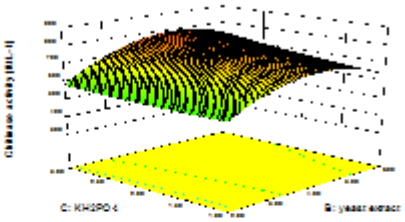
Optimization of the culture conditions by response surface design

Effect of chitin, yeast extract and KH_2PO_4 concentrations on chitinase production were studied by response surface design. The individual and interactive effects of these media components were studied by carrying out the chitinase assay run at different randomly selected level as shown in **Table (3).** The results of experiments performed in this section showed that the maximum average yield of chitinase was 832.87 ±0.282 IUL^{-1}, which obtained under the following optimum conditions of the media (gl^{-1}): chitin 8, yeast extract 6, KH_2PO_4 3, glucose 8, urea 1 and $K_2HPO_4$3. This result presented about 8.767-fold increase in the enzyme activity, when compared to (95 IUL^{-1}), the results obtained in the first basal production medium, without any optimization. And about 2.0979-fold increase when compared with the maximum production yield from plackette-Burman. This reflects the necessity and value of optimization process. The second-order regression equation provided the levels of chitinase activity can be presented in terms of Actual Factors: (Chitinase activity) = -190.18212+139.66876* Chitin +213.58995* yeast extract-58.60884* KH2PO4 +4.47329* Chitin * yeast extract +1.74450* Chitin * KH2PO4 +11.61675* yeast extract-58.60884* KH2PO4 +4.47329* Chitin * yeast extract +1.74450* Chitin * KH2PO4 +11.61675* yeast extract * KH2PO4 -10.92271* $Chitin^2$ -28.87481* $yeast\ extract^2$ ++8.08712* $KH2PO4^2$.

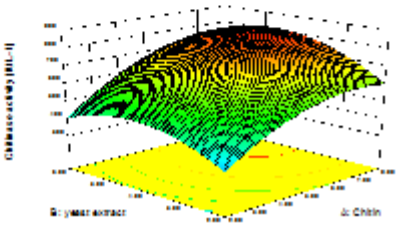
Analysis of variance (ANOVA) for the Quadratic model was shown in **Table (4).** Value of ''Model Prob >F'' less than 0.0050 implied that the model was high significant. In this case A, B, C, AB, AC, BC, A^2, B^2, C^2 are significant model terms. The R^2 coefficient obtained, of 1.0000, suggests that it is a reliable model and the "Pred R-Squared" of 1.0000 is in reasonable agreement with the "Adj R-Squared" of 1.0000. Three dimensional (3D) response surface plots of chitinase production based on the final model are depicted in **Fig. (4)** which were generated in pair-wise combination of the three factors while keeping the other one at its optimum level**.**

The validation was carried out under the optimum conditions of the media and the experimental chitinase production of 832.87 ±0.282 IUL^{-1} was obtained which is closer to the predicted chitinase production of 832.821 IUL^{-1}; this result indicated the validity and the effectiveness of the proposed model.

a)



b)



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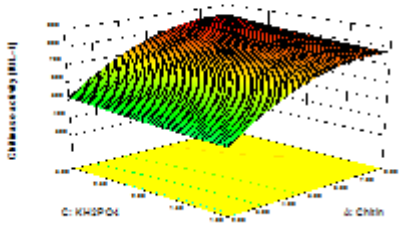

Fig. (4): Three dimensional response surface plot for the effect of (a) chitin and yeast extract, (b) chitin and KH2PO4, (c) yeast extract and KH2PO4

In-vitro antifungal activity of *B. diminuta* KT277492 chitinase enzyme

The antifungal properties of the chitinase enzyme partially purified from *B. diminuta* KT277492 in a petri medium against pathogenic fungi *F. solani*, and *A. alternata* are shown in **Fig. (5).** Results indicated that the enzyme has good antifungal effect against all pathogenic tested fungi causes rot to roots of plants but with different degrees. The tested pathogenic fungi can be arranged according to their degree of susceptibility for *B. diminuta* KT277492 chitinase enzyme as the following order *A. alternata* > *F. solani*, with zones of inhibition 31±0.25 and 25±0.91 mm, respectively.

Fig. (5): The inhibition effect of partially purified chitinase enzyme from *B. diminuta* KT277492on *F. solani* (A) and *A. alternata* (B).

DISCUSSION

Chitin is usually thought as a kind of renewable polysaccharides and organic nitrogenous substance that only second respectively to cellulose and protein in abundance [1]. Several studies stated that chitin derivatives are of interest to people due to their diverse important applications. Therefore, isolating novel chitinase producing microorganisms and production optimization for high productivity are of great importance to the industry. In this context, a novel bacterial isolate *B. diminuta* KT277492 with strong chitinolytic activity was studied for statistical optimization of chitinase production.

In this study, the Plackett-Burman design was used to determine the most important factors influencing *B. diminuta* KT277492 chitinase production. There are many reports describing the use of this method in medium optimization with several microorganisms including *pseudomonas*, *paenibacillus* [13, 30], *Alcaligenes xylosoxydans* [26], *Pantoea dispersa* [31], *Streptomyces* [32], *Azadirachta indica* [33], and *Bacillus circulans* [34] Based on Plackett-Burman design results it was found that among eleven tested components, chitin, yeast extract and KH_2PO_4 exhibit statistically significant effect on chitinase enzyme production. These results agree with several studies that showed chitin is a major inducer in chitinase production by many bacteria such *Stenotrophomonas maltophilia* [35], *paenibacillus* [13, 30] and *Bacillus pumilus* [13].

Table (3): Chitinase activity from the expermental design for the Response Surface Quadratic Model (RSM).

Trial number	Factor levels						Chitinase activity IUL^{-1}	
	Chitin (A, gl^{-1})		**Yeast extract (B, gl^{-1})**		**KH_2PO_4 (C, gl^{-1})**			
	Coded	actual	coded	actual	coded	actual	Actual	Predicted
1	-1	2.00	-1	2.00	+1	3.00	352.295 ±0.022	352.188
2	0	5.00	-1.682	0.64	0	2.00	320.87 ±0.018	320.899
3	0	5.00	+1.682	7.36	0	2.00	510.65 ±0.024	510.547
4	-1.682	0.10	0	4.00	0	2.00	226.235 ±0.03	226.200
5	-1	2.00	+1	6.00	-1	1.00	363.76 ±0.008	363.883
6	0	5.00	0	4.00	0	2.00	742.432 ±0.881	742.714
7	0	5.00	0	4.00	0	2.00	742.432 ±0.141	742.714
8	0	5.00	0	4,00	0	2.00	742.432 ±0.016	742.714
9	+1	8.00	-1	2.00	+1	3.00	619.98 ±0.004	619.919
10	0	5.00	0	4.00	0	2.00	742.432±0.130	742.714
11	0	5.00	0	4.00	-1.682	0.32	716.87 ±0.025	716.625
12	-1	2.00	+1	6.00	+1	3.00	457.76 ±0.255	457.742
13	0	5.00	0	4.00	+1.682	3.68	813.76 ±0.317	813.391
14	+1	8.00	-1	2.00	-1	1.00	597.98 ±0.014	597.051
15	+1	8.00	+1	6.00	+1	3.00	832.87 ±0.282	832.821
16	+1	8.00	+1	6.00	-1	1.00	717.87 ±0.155	717.677
17	0	5.00	0	4.00	0	2.00	742.432 ±0.287	742.714
18	0	5.00	0	4.00	0	2.00	742.432±0.692	742.714
19	+1.682	10.05	0	4.00	0	2.00	725.88 ±0.127	725.839
20	-1	2.00	-1	2.00	-1	1.00	351.163 ±0.237	351.264

Table (4): Analysis of variance (ANOVA) for response surface quadratic model.

Source	Sum of Squares	df	Mean Square	F-value	P-value Prob>F
Model	6.902E+005	9	76684.97	4.623E+006	< 0.0001significant
A-Chitin	3.260E+005	1	3.260E+005	1.965E+007	< 0.0001
B-yeast extract	43415.52	1	43415.52	2.618E+006	< 0.0001
C-KH2PO4	11429.35	1	11429.35	6.891E+005	< 0.0001
AB	5762.98	1	5762.98	3.475E+005	< 0.0001
AC	219.12	1	219.12	13210.68	< 0.0001
BC	4318.36	1	4318.36	2.604E+005	< 0.0001
A^2	1.332E+005	1	1.332E+005	8.028E+006	< 0.0001
B^2	1.925E+005	1	1.925E+005	1.160E+007	< 0.0001
C^2	943.64	1	943.64	56892.90	< 0.0001
Residual	0.17	10	0.017		
Lack of Fit	0.17	5	0.033		
Pure Error	0.000	5	0.000		
Cor Total	6.902E+005	19			

R-Squared =1.0000, Adj R-Squared =1.0000, Pred R-Squared =1.0000, Adeq Precision= 6660.665, Mean = 603.13, umulative variance (C.V. %) = 0.021, PRESS = 1.27, Std. Dev. = 0.13.

It has been suggested that for most microorganisms the optimum chitin concentration for chitinase induction is in the range of 10-20 gl^{-1} [25]. In our investigation, we found that the optimum chitin concentration for chitinase production by *B. diminuta* KT277492 is 8 gl^{-1}, which is considerably lower than the range given above. On the other hand [13] demonstrated that the optimum chitin concentration for chitinase production by *Bacillus pumilus* is 4.76 gl^{-1}, which is considerably lower than the range we demonstrated.

The study of effect of additional nitrogen sources on chitinase production demonstrated that yeast extract as compared to urea and peptone, was found to have positive effect on chitinase production. According to Nawani and Kapadnis [17], this may be due to the presence of chitin or growth factors in yeast extract. On the contradiction some other nitrogen sources including peptone and urea are reported to enhance chitinase production [36] indicating that other mechanisms may be involved. The production of chitinolytic enzymes is also affected by minerals; we examined the effect of KH_2PO_4, K_2HPO_4, $MgSO_4$ and Na_2SO_4. KH_2PO_4 was found to have significant effect, while experiments showed that K_2HPO_4, $MgSO_4$ and Na_2SO_4 had negative effect. [35] work showed that $MgSO_4 \cdot 7H_2O$ and KH_2PO_4 exerts positive effects on chitinase secretion in the presence of chitin. The maximum chitinase production was 832.87 IUL^{-1}. While Khan et al [35] found that, maximum chitinase production from *Stenotrphomonas maltophilia* was 110 IUL^{-1} using medium containing 4.94 gl^{-1}chitin, 5.56 gl^{-1}maltose, 0.62 gl^{-1}yeast extract, 1.33 gl^{-1} KH_2PO_4, and 0.65 $gl^{-1}MgSO_4 \cdot 7H_2O$. The chitinase producers appear potential candidates of biological control of pathogenic fungi. Several bacterial species are being reported as biofungicide. One of such bacteria was *Bacillus subtilis* TV-125 showed antifungal activity against *Fusarium culmorum* which is a pathogenic fungus that cause decomposition of roots of vegetables [37]. These results agree with our findings where the partially purified *B. diminuta* KT277492 chitinase enzyme showed good antifungal activity against pathogenic *F. solani*, and *A. alternata.* According to our findings, chitinase enzyme produced from the novel isolate *B. diminuta* KT277492 is expected to be used in the agriculture against fungal infections as biocontrol agent.

CONCLUSIONS

The evaluation of medium components for *B. diminuta* KT277492 chitinase production was done using the Plackett- Burman statistical method. Out of eleven components chitin, yeast extract and KH_2PO_4 were found to be the most significant variables and further optimization using response surface optimization technique. The enzyme production increased from 95 to 832.87 $IUL^{-1}$and this is presented about 8.767-fold increase. Partially purified chitinase enzyme obtained from *B. diminuta* KT277492 showed good inhibitory effects against two pathogenic fungi.

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