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Optimization of Bioactive Compounds Production by Endophytic *Chaetosphaeronema* sp. (KY321184) Using Experimental Design Method

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THE PRODUCTION of bioactive compounds by endophytic *Chaetosphaeronema* sp. (KY321184) has been optimized using the experimental design methods. Among seven fermentation parameters, Plackett-Burman design revealed that potato extract concentration, glucose concentration and inoculum size are three significant variables positively affecting the production process. The levels of these variables have been optimized using Box-Behnken design. The best productivity was estimated under potato extract concentration, glucose concentration and inoculum size of 190gL^{-1}, 15gL^{-1} and 5 fungal discs (0.8cm diameter), respectively. The model was validated experimentally, and it showed a deviation error of 4.3% and 5.3% from the predicted values of Design Expert's model and the Excel solver, respectively, which considered as an acceptable error. Moreover, it has been observed that, trails of Plackett-Burman design stimulated the production of different compounds and one of them is produced in a partially pure form. This observation suggests that, this type of experimental design not only can be employed for optimization purpose but also may represent a method to modify the production pattern of bioactive compounds in a trial to obtain them in a partially pure form to save the cost and effort exerted in the purification process.

Keywords : *Chaetosphaeronema* sp. KY321184, Endophytic, Bioactive compounds, Statistical optimization, Plackett-Burman, Box-Behnken.

Introduction

Endophytic fungi can be defined as fungi that colonize living plant tissues without causing any immediate overt negative effects (Stone et al., 2004). Plant endophytic fungi have been recognized as an important and novel source of natural bioactive products where endophytes synthesize many metabolites to compete with epiphytes and pathogens to colonize the host, also to regulate the host metabolism in a balanced association (Chandra, 2012). Recently, many endophytes have been reported to produce novel antibacterial, antifungal, antiviral, anti-inflammatory and antitumor compounds (Kumar et al., 2013). Optimization of fermentation conditions for endophytic fungi represents an important method to overcome the supply problems of such compounds and may play a role to develop an economically effective production method. Optimization process is traditionally performed by the one factor at time approach (OFAT) (Anderson & Whitcomb, 2004). In such technique one variable is studied at a time while the other variables are kept constant (Stowe & Mayer, 1966). This method is a time-consuming method where the effect of only one variable is monitored at one time thus the variables must be optimized one by one (Zacharis & Tzanavaras, 2004). Moreover, such approach cannot detect the interaction of factors which likely found in many fermentation processes. Therefore, quality professionals prefer the use of statistically-based methods known as design of experiment (DOE) (Anderson & Whitcomb, 2004). These methods not only investigate the main effect of a large number of factors in a relatively small number of experiments, but also detect the interaction between factors (Quinn & Keough, 2002 and Rakić

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et al., 2012). Experimental designs for optimization process begins with a screening design to evaluate the vital few factors affecting the process from a large number of factors, for example; Plackett-Burman (PB) experimental design. (Anderson & Whitcomb, 2004 and Rakić et al., 2012). Such design only determines the main effect of factors but the information about factors interaction is missing (Rakić et al., 2012). This step is followed by the optimization design which generates a response surface map and moves the process to the optimum location (Anderson & Whitcomb, 2004). Moreover, it predicts the interaction effects among the variables, for example; Box–Behnken (BB) design (Li et al., 2008). Finally, the design must be validated by a confirmatory run to ensure that the model produces accurate predictions for the responses of interest (Anderson & Whitcomb, 2004).

In this study the production of bioactive compounds by endophytic *Chaetosphaeronema* sp. (KY321184) has been optimized using the experimental designs methods in a trail to produce such compounds by an economically effective method.

Materials and Methods

Microorganism

The fungus under this investigation is an endophytic fungus isolated from *Nepeta septemcrenata* bark samples which have been collected from Saint Catherine, South Sinai, Egypt, in May 2014 by the method described by Garyalis et al. (2013).

Assessment of bioactive compounds production

Standard fermentation process

Four fungal discs (disc diameter =0.8cm) were inoculated to 75ml potato dextrose broth medium on 250ml conical flask and incubated at 25°C for two weeks under static conditions.

Extraction method

The culture medium was filtered by Whatman filter paper no.1 to separate the mycelium. The filtrate and the grinded mycelium were extracted by equal volume methylene chloride, then separated and evaporated at 35°C using rotary vacuum evaporator. The dry residues were re-dissolved in minimal volume of methanol to collect the crude extracts.

Assessment of bioactive compounds contents

The crude extracts were tested for their antibacterial activity against *Staphylococcus aureus* ATCC 25923 to evaluate the bioactive compounds production. The crude extracts were dissolved in 6ml acetone and 50µl were inoculated in 2ml nutrient broth inoculated with 100µl bacterial suspension (0.5O.D), then incubated at 37°C for 24h. The antibacterial activity was evaluated by measuring the density of bacterial growth at 600nm and calculation of the inhibition percent as following :

% of inhibition= (Ab. of control – Ab. of sample/Ab. of control) × 100.

Note: Control was inoculated with 50µl acetone instead of the inoculum of extract.

Experimental designs for optimization of bioactive compounds production

Screening for the vital factors affecting the bioactive compounds production by Plackett-Burman (PB) experimental design

Seven variables representing the basal medium components and fermentation conditions were tested at low (-1) and high (+1) levels as shown in Table 1. Fifteen runs were organized according to the Plackett–Burman design as indicated in Table 2. This design matrix contains three replicated center points to avoid error. All the trials were performed in duplicates using 250ml Erlenmeyer flasks and the final data were calculated as the mean of duplicate.Plackett-Burman experimental design is based on the first-order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i$$

where Y is the response, β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variable (Talukdar et al., 2016). The variables significant at 95% level ($P<0.05$) were considered to have significant effect.

Optimization of bioactive compounds productions by response surface methodology using Box-Behnken design (BBD)

To enhance the production of the bioactive metabolites, the three most significant factors positively affecting the production have been selected according to Plakett-Burman experimentand optimized by employing Box-Behnken design. These factors were studied at three different levels, low (-1), high (+1) and

intermediate (0) (Table 3). The experiment was carried out in fourteen trials (Table 4) with two central points. All the trials were performed in duplicates using 250ml Erlenmeyer flasks and final response value was calculated as the mean of duplicate. Results were fitted with a second-order polynomial equation. The general form of the second-order polynomial equation is:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ii} X_i^2$$

where Y is the predicted response, β_0 is the intercept term, β_i is the linear coefficient, β_{ij} is the quadratic coefficient, β_{ii} is the interaction coefficient, and $X_i X_j$ represent the independent variables (Jose et al., 2013). Regression analysis was performed on the data obtained using software package "Design Expert Software" (Version 7.0) and confirmed by Microsoft Excel 2016 (Moghannem et al., 2017). The accuracy of polynomial model equation was evaluated by determination of R^2 and adjusted R^2. The fitted polynomial equation was expressed in the form of three-dimensional response surface plots. The Design Expert numerical optimization method and Excel solver were employed to optimize the level of each variable for maximum response.

Experimental validation

To confirm the model, one predicted solution for maximum bioactive compounds production which has been estimated by the Design Expert numerical optimization and the Excel solver was selected as a check point and tested experimentally to calculate the percent of deviation.

Chemical evaluation of PB trails products

Identity and purity of compounds produced by different PBD trails has been investigated by TLC, HPLC and H^1NMR. HPLC was carried out for trail number 15, where 60µg dissolved in acetone was injected in reversed phase column (ZORBAX SB-C18 5µm, 250 x 9.4mm), eluted with methanol:water 1:1 and detected at λ = 254 and 280nm. H^1 NMR for trail number 15 was carried out in CDCl3 using a BRUKER 400MHz in Microanalytical Unit -Faculty of Pharmacy, Cairo University, Egypt.

TABLE 1. Factors and coded levels examined in the Plackett–Burman design experiment.

Factor	Symbol	Units	Low level (-1)	High level (+1)	Central point
Potato extract conc.	A	gL^{-1}	0	200	100
Glucose conc.	B	gL^{-1}	0	20	10
Temperarure	C	°C	20	28	24
pH	D	-	4.5	8.5	6.5
Incubation period	E	Week	1	3	2
Inoculum size	F	Fungal disc*	2	6	4
Aeration	G	ml/250ml flask	25	75	50

*Fungal disc Diameter =0.8cm

TABLE 2. Plackett-Burman design matrix for evaluating significant factors affecting bioactive compounds production by *Chaetosphaeronema* sp. (KY321184).

Trial no.	A	B	C	D	E	F	G	H*	J*	K*	L*	Response (% inhibition)
1	0	0	0	0	0	0	0	0	0	0	0	89.7
2	0	0	0	0	0	0	0	0	0	0	0	85.3
3	1	1	1	-1	-1	-1	1	-1	1	1	-1	3.6
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	7.8
5	1	1	-1	-1	-1	1	-1	1	1	-1	1	90.6
6	1	-1	-1	-1	1	-1	1	1	-1	1	1	23.1
7	0	0	0	0	0	0	0	0	0	0	0	85.3
8	-1	1	-1	1	1	-1	1	1	1	-1	-1	7.8
9	1	-1	1	1	-1	1	1	1	-1	-1	-1	2.9
10	-1	1	1	1	-1	-1	-1	1	-1	1	1	2.6
11	-1	-1	1	-1	1	1	-1	1	1	1	-1	4.6
12	1	1	-1	1	1	1	-1	-1	-1	1	-1	8.1
13	-1	1	1	-1	1	1	1	-1	-1	-1	1	35
14	-1	-1	-1	1	-1	1	1	-1	1	1	1	9.4
15	1	-1	1	1	1	-1	-1	-1	1	-1	1	16.8

*: H, I, J, K are Dummy variables.

TABLE 3. Levels of the factors selected for Box-Behnken design.

Factor	Units	Low level (-1)	High level (+1)	Intermediate level (0)
Potato extract conc.	gL^{-1}	0	200	100
Glucose conc.	gL^{-1}	0	20	10
Inoculum size	Fungal disc	2	6	4

*Fungal disc diameter =0.8cm.

TABLE 4. Box-Behnken design matrix for optimizing bioactive compounds production by *Chaetosphaeronema* sp. (KY321184).

Trail no.	Potato extract conc.	Glucose conc.	Inoculum size	Response (% inhibition)
1	0	-1	1	30.9
2	-1	-1	0	0.15
3	1	0	-1	88.7
4	-1	0	-1	8.2
5	0	-1	-1	33.6
6	1	0	1	91.4
7	0	0	0	63.4
8	1	1	0	91.7
9	-1	1	0	10.9
10	0	0	0	76.4
11	-1	0	1	10.7
12	1	-1	0	58.5
13	0	1	1	61.9
14	0	1	-1	40.4

Results

Plackett–Burman design(PB)

The significance of seven factors (potato extract concentration, glucose concentration, temperature, pH, incubation period, inoculum size and aeration) on the production of bioactive compounds by *Chaetosphaeronema* sp. (KY321184) was investigated by PB design. The analysis depends on the inhibition percent exerted by the trails against *Staphylococcus aureus* ATCC 25923 (Table 2) as the main response. It is found that, potato extract concentration, glucose concentration, temperature, pH, inoculum size and aeration are significant model terms ($P< 0.05$). The effect, standard error, P value, and significance of each model term were represented in Table 5. Only potato extract concentration, glucose concentration and inoculum size were found to have a positive significant effect on the bioactive compounds production but temperature, pH, aeration were found to negatively affect the process and that was illustrated in Pareto chart (Fig. 1). The ANOVA results showed that, the model P- value = 0.0103, Table 6. Correlation coefficient (R^2) value equals 0.9981 and the adjusted R^2 value equals 0.9877.

TABLE 5. Statistical analysis of effects of tested factors on bioactive compounds production by PBD.

Variable	Symbol	Effect (Coefficient)	Standard error	P value	Significance
Potato extract conc.	A	6.49	0.73	0.0125	Significant
Glucose conc.	B	6.92	0.73	0.0110	Significant
Temperarure	C	-6.78	0.73	0.0115	Significant
pH	D	-9.79	0.73	0.0056	Significant
Incubation period	E	-1.79	0.73	0.1345	Non-significant
Inoculum size	F	7.41	0.73	0.0097	Significant
Aeration	G	-4.06	0.73	0.0311	Significant

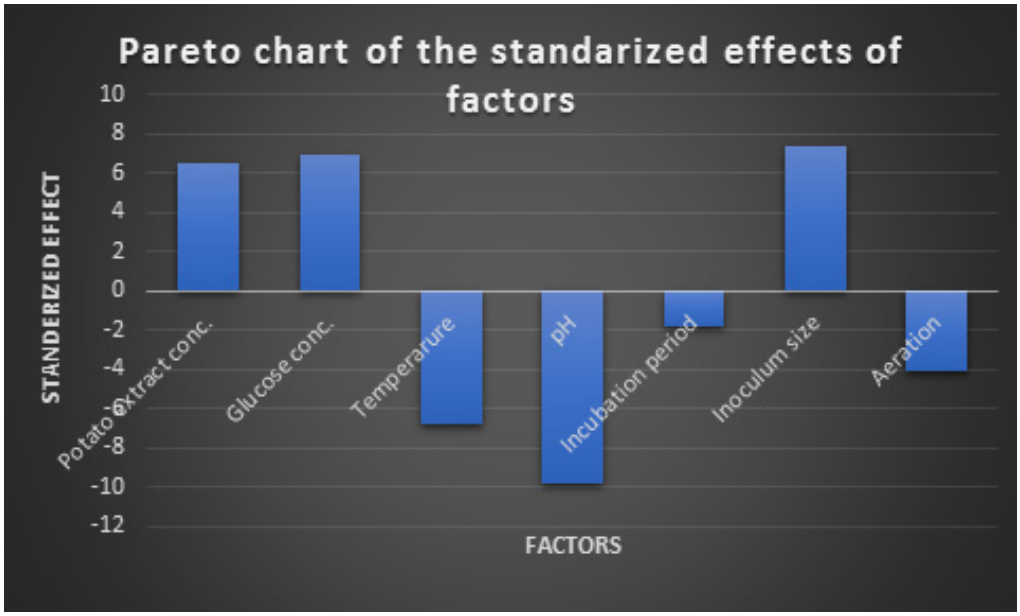


Fig. 1. Pareto chart showing the effect of different factors on bioactive compounds production by *Chaetosphaeronema* sp. (KY321184) according to Plackett-Burman design analysis.

TABLE 6. Regression statistics and model P-value for PBD.

Model P value	**0.0103**
Model R^2	0.9981
Model adjusted R^2	0.9877

Box-Behnken design(BBD)

Based on the Plackett–Burman design results, response surface methodology using Box-Behnken design was employed to determine the optimal levels of the three significant factors positively affecting the bioactive compounds production (potato extract concentration, glucose concentration and inoculum size) to enhance the production process. The analysis depends on the inhibition percent exerted by the trails against *Staphylococcus aureus* ATCC 25923 as the main response (Table 4). The ANOVA analysis of model (Table 7) showed that, the model P-value was 0.0018, the model F-value was 35.48 and the Lack of Fit is not significant (The "Lack of Fit F-value" was 0.35). Moreover, it has been reported that, A, B, A^2, B^2 are significant model terms where $P< 0.05$. The model correlation coefficient (R^2) value equals 0.9876 while the adjusted R^2 equals 0.9598 and the predicted R^2 was 0.8745 (Table 8). The second-order polynomial equation estimated by the model regression analysis was as the following;

$$\text{Inhibition percent} = 69.90 + 37.54\ A + 10.22\ B + 3.00\ C + 5.61\ AB + 0.050\ AC + 6.05\ BC - 10.77A^2 - 18.82\ B^2 - 9.38C^2$$

where A, B and C are the coded factors of potato extract concentration, glucose concentration and inoculum size, respectively. The main effect of factors and the interaction between them are illustrated by the perturbation and the three-dimensional response plots. Perturbation plot (Fig. 2) shows the main effect of factors on bioactive compounds production. The plot showed that potato extract has the major effect followed by the glucose concentration. The three-dimensional response plots (Fig. 3, 4 and 5) were obtained using two factors, while keeping the other factor set at the zero level (intermediate value) to evaluate both the main and the interactive effects of factors. There was insignificant mutual interaction between the tested factors. It is observed that, the increase in potato extract concentration leads to an increase in bioactive compounds production but the increase in glucose concentration above $15gL^{-1}$ has inhibitory effect. The maximum bioactive compounds production (which yields the maximum inhibition percent) was calculated by the aid of Design Expert's numerical optimization

which predicts to be 98.28% when potato extract, glucose concentrations and inoculum size equal 190.23gL^{-1}, 14.56gL^{-1} and 5.16, respectively (Fig. 6) while the Excel solver predicts the maximum inhibition percent to be 99.4% when potato extract, glucose concentrations and inoculum size equal 193.3gL^{-1}, 15.5gL^{-1} and 4.7, respectively.

TABLE 7. Statistical analysis of effects of tested factors on bioactive compounds production by BBD.

Variable	Effect (coefficient)	Standard error	P-value	Significance
A	37.54	2.33	< 0.0001	Significant
B	10.22	2.33	0.0118	Significant
C	3.00	2.33	0.2666	Non-significant
AB	5.61	3.29	0.1632	Non-significant
AC	0.050	3.92	0.9886	Non-significant
BC	6.05	3.29	0.1397	Non-significant
A^2	-10.77	3.68	0.0429	Significant
B^2	-18.82	3.68	0.0069	Significant
C^2	-9.38	3.68	0.0632	Significant
Model	69.90	4.65	0.0018	Significant

A= Potato extract conc., B= Glucose conc., C= Inoculum size..

TABLE 8. Regression statistics for BBD.

Model R^2	**0.9876**
Model adjusted R^2	0.9598
Model predicted R^2	0.8745

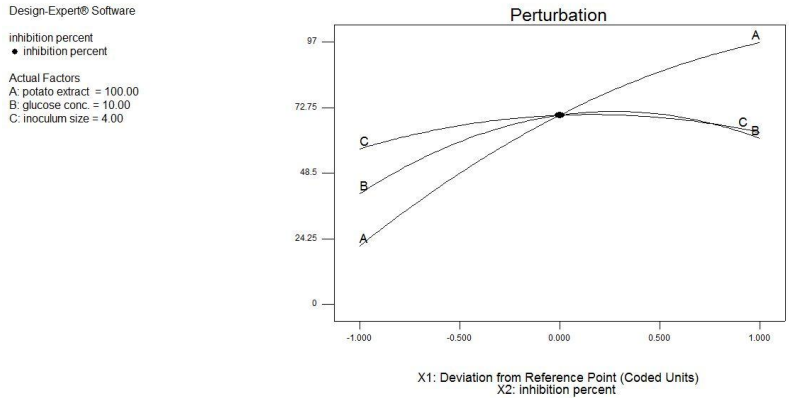


Fig. 2. Perturbation plot showing the main effect of: A= Potato extract conc., B= Glucose conc., C= Inoculum size on bioactive compounds production (%inhibition).

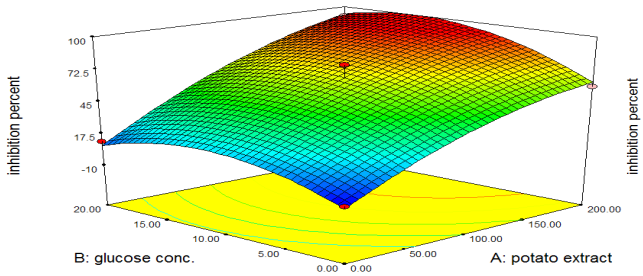


Fig. 3. 3-D. Response surface plot representing the interaction between the glucose conc. and potato extract conc. on bioactive compounds production (%inhibition).

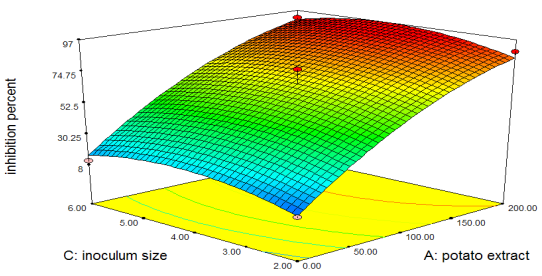


Fig. 4. 3-D. Response surface plot representing the interaction between the inoculum size and potato extract conc. on bioactive compounds production (%inhibition).

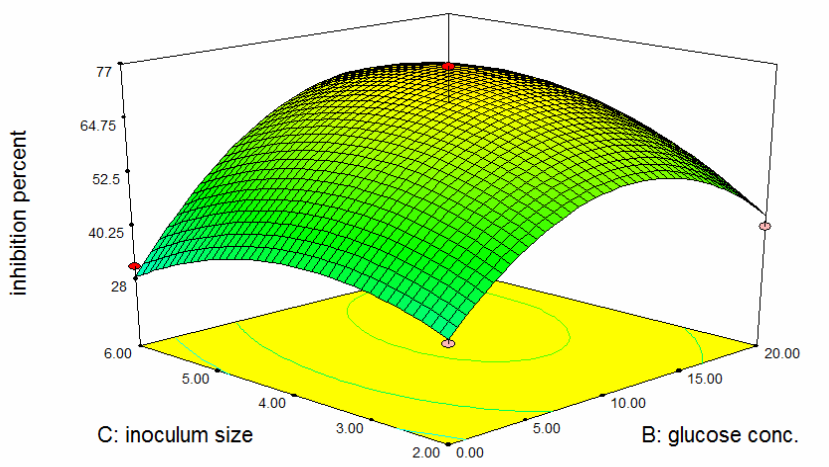


Fig. 5. 3-D. Response surface plot representing the interaction between the inoculum size and glucose conc. on bioactive compounds production (%inhibition).

Fig. 6. Predicted solution for maximum bioactive compounds production (maximum inhibition percent) by BBD numerical optimization.

Validation of model

The approximated values of the previous predicted levels were tested experimentally as a check points for model validation. It is reported that, inhibition percent of 94.1% was obtained when the potato extract, glucose concentrations and inoculum size were 190gL^{-1}, 15gL^{-1} and 5 discs, respectively, giving a deviation of 4.3% and 5.3% from the predicted values of Design Expert model and Excel solver, respectively (Table 9).

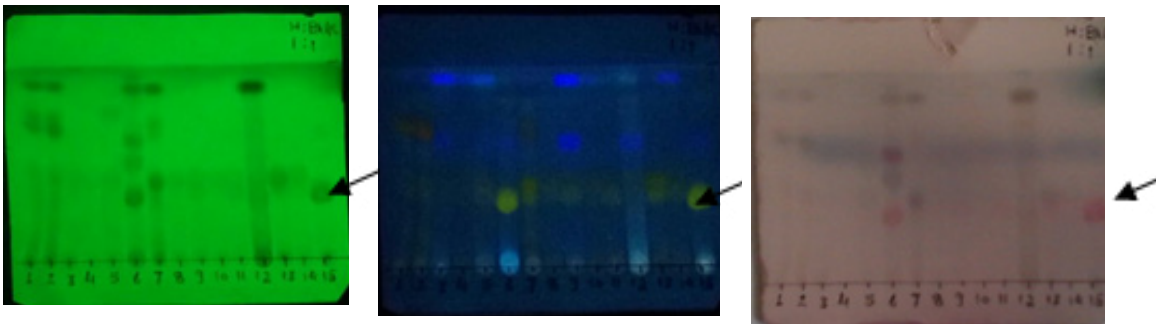
Chemical analysis for PB trails products

It has been observed that, the different trails of PB design stimulated the production of different compounds, TLC chromatogram (Fig. 7). Moreover, trail number 15 was reported to stimulate the production of a bioactive compound in a considerable pure form. HPLC chromatogram for this trail showed one major beak corresponding to this compound (Fig. 8) and the general chemical identity was elucidated by ^{1}HNMR (Fig. 9).

TABLE 9. Model validation results (predicted and actual values for maximizing the main response).

Method	Variable level			Response (% inhibition)	Deviation
	Potato extract conc. (gL^{-1})	Glucose conc. (gL^{-1})	Inoculum size*		
Design Expert model prediction	190.23	14.56	5.16	98.28	4.3%
Excel solver prediction	193.3	15.5	4.7	99.4	5.3%
Experimental value (tested/actual)	190	15	5	94.1	

*Inoculum size= Fungal disc diameter= 0.8cm.



Under short UV **Under long UV** **Sprayed with p-anisaldehyde agent**

Fig. 7. TLC chromatogram of PB trails

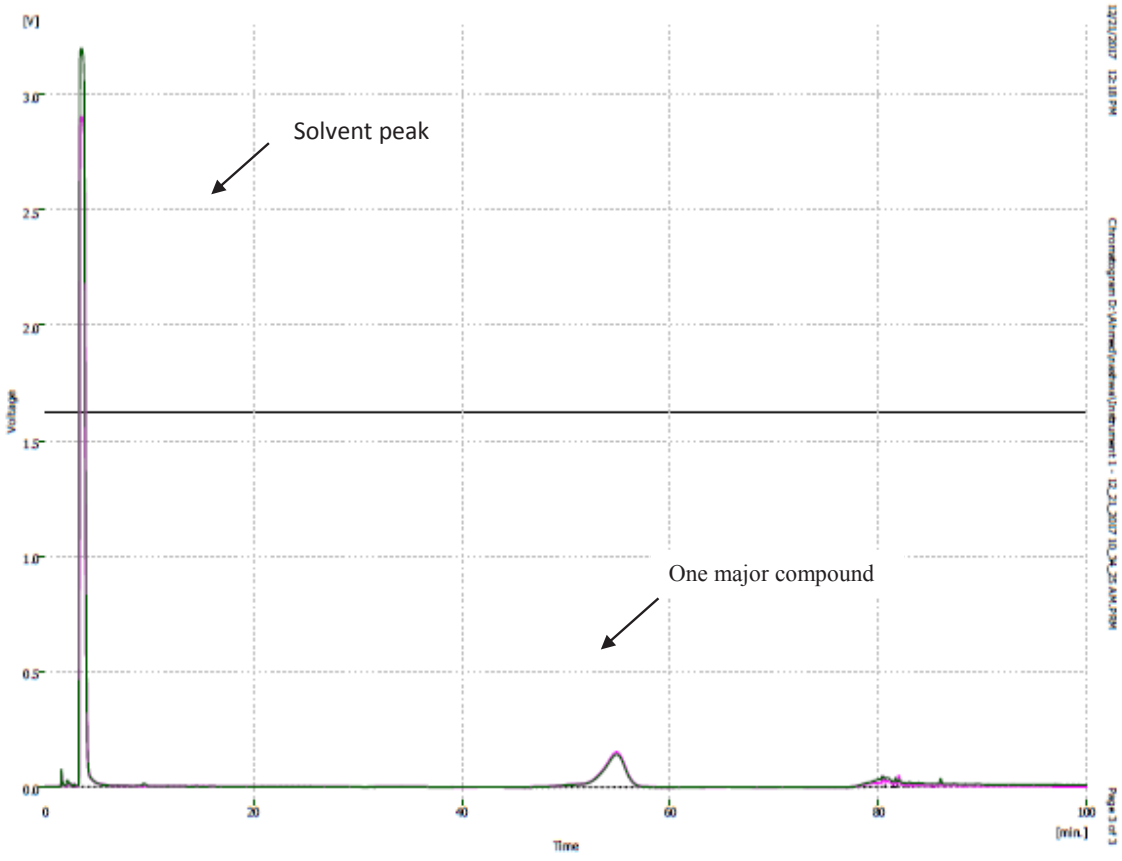


Fig. 8. HPLC chromatogram of PB trail number 15.

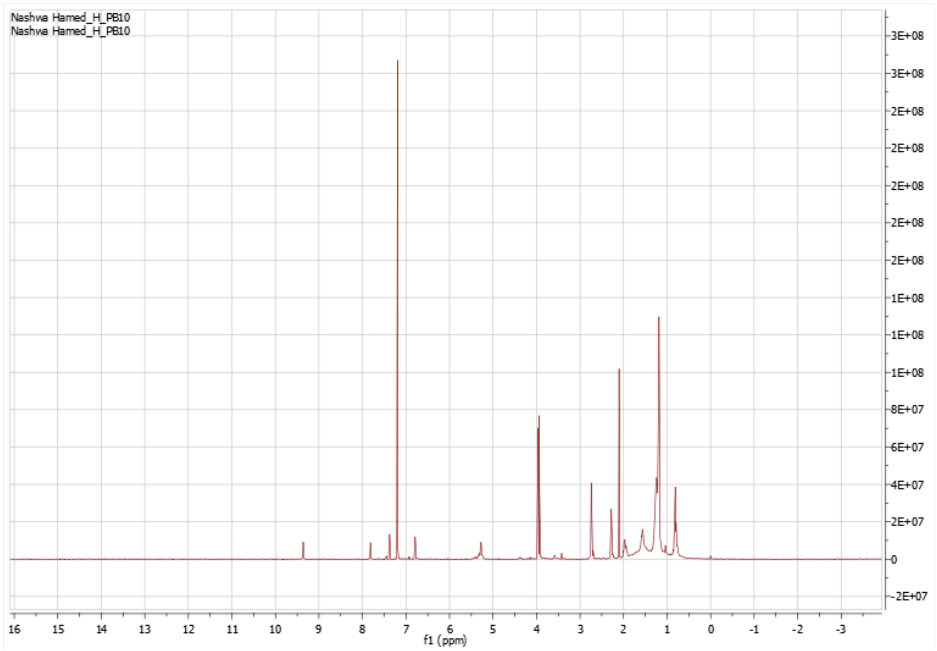


Fig. 9. ^{1}HNMR for compound produced in trail number 15 in PBD.

Discussion

Endophytic fungi have been characterized as a reliable source for promising bioactive compounds. The optimization of fermentation conditions for these fungi will play a role in the production of such compounds by an economically effective method. Experimental design methods for optimization process represent competitive and more efficient methods than the traditional 'one factor at time' method. The first step in this optimization study was a fast screen for the fermentation factors to determine the factors significantly affecting the bioactive compounds production process. That was maintained by employing Plackett-Burman (PB) experimental design. PB method has been introduced since 1946, it represents an economically approach that determines the main effects of factors (Analytical Methods Committee, 2013). The ANOVA results showed that the employed model was highly significant where the model P-value equals 0.0103. Correlation coefficient (R^2) value equals 0.9981 which implies that the model equation could explain 99.81% of the total variation which suggested a good agreement between predicated values and experimental data. R^2 is the coefficient of variance of response under test and whose values are always between 0 and 1; closer the value of R^2 to 1, the stronger is the statistical model and better is the prediction (Baadhe et al., 2014). The adjusted R^2 value was 0.9877. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model (Baadhe et al., 2014). That value of the adjusted R^2 is also good, supporting the significance of this developed model. According to the model results, potato extract concentration, glucose concentration, temperature, pH, inoculum size and aeration are significant model terms in antibacterial compounds production by *Chaetosphaeronema* sp. (KY321184). However, only potato extract concentration, glucose concentration and inoculum size were found to have a positive significant effect on the production process. Based on these results, Box-Behnken design was employed as a response surface methodology (RSM) method, to determine the optimal levels of these three factors to enhance the production process.

Response surface methodology (RSM) answers the question of how to select the levels for the applied factors to obtain the desirable value of the response in a reduced number of experiments (Bai et al., 2015). Moreover, it has been widely used to evaluate and understand the interactions between the different process parameters (Baadhe et al., 2014). Box-Behnken design is a RSM technique which has been widely studied by many researchers as an established and promising method for the optimization and formulation of various types

of processes (Ding et al., 2016). The ANOVA analysis of the employed model showed that, the model was significant where the model P- value was 0.0018, the model F-value was 35.48 and the Lack of Fit is not significant (The "Lack of Fit F-value" was 0.35). The model correlation coefficient (R^2) value equals 0.9876 while the adjusted R^2 equals 0.9598 which reflects the very high correlation and supports the significance of model. The Predicted R^2 was 0.8745 which is in reasonable agreement with the Adjusted R^2.

According to results of this model, potato extract concentration and glucose concentration are significant model terms in bioactive compounds production by *Chaetosphaeronema* sp. (KY321184) where potato extract has the major effect followed by the glucose concentration. The increase in potato extract concentration leads to an increase in bioactive compounds production but the increase in glucose concentration above $15gL^{-1}$ has inhibitory effect. There was insignificant mutual interaction between the tested factors.

Design Expert numerical optimization predicted the maximum bioactivity to be 98.28% when potato extract, glucose concentrations and inoculum size equal $190.23gL^{-1}$, $14.56gL^{-1}$ and 5.16 fungal discs, respectively, the calculation by Excel solver predicts the maximum inhibition percent to be 99.4% when potato extract, glucose concentrations and inoculum size equal 193.3 gL^{-1}, $15.5gL^{-1}$ and 4.7 fungal discs, respectively. Thus, the approximated values of these predicted levels were tested experimentally as a check points for model validation. When potato extract, glucose concentrations and inoculum size were $190gL^{-1}$, $15gL^{-1}$ and 5 fungal discs, respectively, the inhibition percent of 94.1% was obtained giving a deviation of 4.3% and 5.3% from the predicted values of Design Expert model and Excel solver, respectively and that is considered as an acceptable error range.

Apart from the optimization aim, it has been observed that, the different trails of Plackett–Burman model have stimulated the production of different compounds and one of them was produced in a considerable pure form (trail number 15) as indicated from the HPLC chromatogram. That reflects the possibilities of utilizing such statistical models in modifying the production pattern of bioactive compounds by microorganisms trying to produce them in a partially pure form to save the cost and the effort exerted in the purification process.

Conclusion

Endophytic fungi have been characterized as a promising source for bioactive compounds. The optimization of fermentation conditions for these fungi will play a role in the production of such compounds by an economically effective method. Experimental design methods for optimization process represent a competitive and more efficient methods than the traditional 'one factor at time' method where the experimental design methods not only estimate the effects of many factors and optimize their levels using a fewer number of experiments but also can illustrate the interaction between them. Moreover, in the present study the trails of Plackett-Burman design have been found to stimulate the production of different bioactive compounds. Some of these compounds are produced in a partially pure form. That represents an initial guide for more studies in this point to evaluate the effectiveness of PB design runs in modifying the production pattern of bioactive compounds by microorganisms.

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تحسين إنتاجية المواد النشطة حيوياً بواسطة الفطر الداخلي *Chaetosphaeronema* sp. (KY 321184) بإستخدام طرق تصميم التجارب الإحصائية

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تهدف هذه الدراسة إلى تحسين إنتاجية الفطر *Chaetosphaeronema* sp. (KY321184) (احد الفطريات الداخلية المعزولة من نبات *Nepeta septemcrenata*) للمواد النشطة حيوياً. تم إستخدام الطرق الإحصائية لتصميم التجارب لتحقيق هذا الغرض حيث تم مسح تأثير عدد من العوامل والظروف المستخدمة فى عملية الإنتاج بواسطة إستخدام تصميم بلاكت بيرمان ثم تعيين أفضل قيم للعوامل ذات التأثير الإيجابي وهى تركيز كلاً من مستخلص البطاطس والجلوكوز إلى جانب حجم الحقنة الفطرية بواسطة إستخدام تصميم بوكس بينكين. وقد أظهرت هذه التجربة أن إنتاجية المواد النشطة حيوياً تتحسن مع الزيادة فى تركيز مستخلص البطاطس ولكن الزيادة فى تركيز الجلوكوز فوق قيمة 15جرام للتر تؤدى إلى نقص فى إنتاجية هذه المواد. وقد أوصت النتائج إحصائياً باستخدام تركيز 190جرام للتر من مستخلص البطاطس وتركيز 15جرام للتر من الجلوكوز والحقن بخمس دسكات فطرية لتحقيق أعلى إنتاجية. وللتحقق من صحة هذه التوقعات تم أجراء تجربة إضافية بإستخدام هذه القيم و قد حققت نتائج جيدة بنسبة حيود 4.3% و 5.3% فقط عن القيم المتوقعة. علاوة على هذا فقد تم ملاحظة استحساس إنتاج مركبات مختلفة وأحدهم أنتج بصورة شبة نقية من خلال تصميم البلاكت بيرمان والذى قد يمثل إحدى طرق تغيير إنتاجية المركبات النشطة حيوياً لأنتاجها فى صورة نقية لتوفير الجهد المبذول فى عمليات التنقية.