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RESEARCH

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Statistical optimization of chitinase production by *Streptomyces rubiginosus* SP24 and efficacy of purified chitinase to control root-knot nematode infection in *Vigna radiata* under controlled conditions

Sneha Jha* and H. A. Modi

Abstract

Background: *Actinomycetes* are known to produce various lytic enzymes such as chitinase which have different biotechnological aspects. Plackett–Burman design (PBD) was applied to evaluate significant components of medium to improve chitinase enzyme production. Response surface methodology was implemented to select those variables which are highly fitted in the model and helped in increasing enzyme production. A second experiment was performed as one of the major applications of statistically optimized and purified chitinase (method and result of purification not shown) for reducing root-knot nematode infection in roots known for causing diseases in plants. Among them, *Meloidogyne* sp. is one which is responsible for decreasing plant growth and fruit yield. For this, *Meloidogyne* sp. (1000 J_2 stage) was used to infect *Vigna radiata* (mung) plant which was previously coated with formulations of purified chitinase and organic manure ratio—1:1 (w/w).

Results: From ANOVA results of PBD data analysis, it was confirmed that colloidal chitin, xylose, peptone and dihydrogen potassium phosphate were found to be significant components for chitinase enzyme production. The best combination of factors (gm) to accomplish the optimum response was found to be colloidal chitin: 1.0, xylose: 2.0, peptone: 0.6 and dihydrogen potassium phosphate: 0.25 for predicted response of 3.81. Seeds coated with purified chitinase (5 ml) isolated from *Streptomyces rubiginosus SP24* helped in enhancing plant growth promotion and decreasing root-knot nematode infection.

Conclusion: Statistically optimized seeds of mung coated with purified chitinase was used for suppressing plant-parasitic nematodes which cause severe harm to the production of crops in terms of plant growth and fruit yield.

Keywords: Chitinase, Plackett–Burman design, Response surface model, Statistical approach, Optimization, *Streptomyces rubiginosus* SP24, *Meloidogyne* sp.

Background

Chitinase (EC 3.2.1.14) belongs to family of glycosyl hydrolase that catalyzes the degradation of chitin, an insoluble linear β-1,4-linked polymer of *N*-acetylglucosamine [10, 52]. They are found in a wide range of microorganisms including bacteria [10, 12], fungi [10, 12], and *Actinomycetes* [10, 36, 20, 54], but their role is diverse. Nowadays, the focus is mainly on *Actinomycetes* which have ample capacity for various types of metabolite production, such as those of antibiotics and extracellular enzymes [22]. The molecular weight of chitinases vary widely in their size, ranging from as low as 20 kDa to about 90 kDa [4, 26]. Chitinase activity was found to be present over a wide range of temperature (psychrophilic

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to thermophilic) and pH (generally, neutral to basic), depending on the microbe isolated. Chitinolytic enzymes can be divided broadly into two categories: endochitinases (EC 3.2.1.14), which cleave the internal β-1,4-glycosidic bond, and exochitinases, which are further sub-divided into two categories, i.e., chitobiosidases (EC 3.2.1.29) which acts only at the non-reducing end of the chitin chain, and β-*N*-acetylglucosaminidases (EC 3.2.1.30), which cleaves *N*-acetylglucosamine units sequentially from the non-reducing end of the substrate [25, 47].

Optimization process is significant in improving the ultimate production of enzymes and to understand the influence of each variable on the response [38]. In the conventional method for the optimization of enzyme production, the "one factor at a time" approach is used, which involves changing one factor at a time while keeping all the other factors constant [7]. Statistical-based design considers the interaction between independent variables, on the basis of which response is generated. An alternative and more efficient approach is the use of statistical methods. The Plackett–Burman design has been used for screening the main factors and this in turn can be preserved for further optimization [17]. RSM is a collection of statistical techniques that are useful for designing experiments, building models, evaluating the effects of different factors and searching for optimal conditions of the studied factors for desirable responses [17]. In our previous study, optimization using OFAT was done, which was further taken to statistically optimize the medium components for enhanced chitinase production [26] in the presence of all the required nutrients.

Root, stem and leaves of many plants are infected by nematodes causing various morphological as well as physiological changes in plants and in turn leading to disease conditions such as wilting, chlorosis, growth reduction and depletion of the entire plant, ultimately leading to huge economic loss [1, 29, 33, 64]. To control nematodes, anti-nematicidal agents are used worldwide. These anti-nematicidal agents are very much toxic to human health and environment. Among these, root-knot nematode (*Meloidogyne* spp.) is one of the major pathogens that is widespread, most dangerous and difficult to control [13, 28, 53]. Mung (*Vigna radiata*) plants have been reported to be infected by different species of nematodes causing serious damage to plant yield worldwide [62]. Different measures for control of nematode infection in plant have been applied by researchers such as soil amendment with oil cakes of neem, castor, mustard and other plant products for control of plant-parasitic nematodes [2, 3, 9, 32, 35, 50, 57, 61, 57]. The life cycle of nematodes consists of five stages, starting from eggs which develop into root-infecting J_2 juveniles that further invades host by attaching to its root and develop permanent feeding sites [51]. There was considerable decrease in root length, shoot length, fresh and dry weight of plants with increase in inoculum level of nematodes (Haseeb and Butool [19]; Pandey [42]). As a biological agent, chitinase can be used to degrade the nematode cell wall, which is made of chitin [6, 11]. With the use of actinomycetal chitinase as a potential weapon, it is comparatively easy to decrease root-knot nematode disease, which affects total yield, production and quality of crops.

The aim of this research was to apply the Plackett–Burman design as well as Box–Behnken design for screening the media components to enhance the chitinase enzyme production from *Streptomyces rubiginosus SP24* under submerged fermentation. The work was novel as no data on chitinase production from *Streptomyces rubiginosus* was available till date. The efficacy of the optimized as well as purified chitinase thus produced must be utilized for its application studies. Hence, seeds (*Vigna radiate*) coated with purified chitinase in formulation with organic manure was assessed for various plant growth parameters and the number of galls in the root system of the plant to decrease root-knot nematode disease.

Results

Chitinase production by submerged fermentation using Plackett–Burman design

Plackett–Burman design [43] was employed to determine the effect of individual parameters influencing chitinase production by *Streptomyces rubiginosus* SP24 under submerged fermentation (SmF). Factors with $p < 0.05$ were considered to have significant effects on the production of chitinase enzyme and were therefore selected for further optimization studies. Table 1 gives the actual and coded levels of independent variables tested in the design matrix. The design matrix for the screening of significant variables for maximum enzyme production and the corresponding responses (actual and predicted chitinase activity) are shown in Table 2. Optimizing response helps in identification of various significant factors and their desirability.

From Table 2, it was observed that the variation in actual chitinase activity was 0.78–2.36 U/ml. The two values of each variable [maximum (+) and minimum (−)] were chosen such that the difference between the two values (+and −) was large enough to ensure that it included the peak area for the maximum enzyme production. The effect of the eight nutrient components of submerged fermentation for chitinase production by *Streptomyces rubiginosus* SP24 was studied by the Plackett–Burman design. Among them, colloidal chitin, xylose, peptone and dihydrogen potassium phosphate showed positive effect for chitinase production in the

Table 1 Actual and coded levels of independent variables used in the Plakett–Burman design

Factor	Name	Units (g/100 ml)	Low actual	High actual	Low coded	High coded
A	Colloidal chitin	%	0.1	1	− 1	1
B	Peptone	g	0.1	2	− 1	1
C	Beef extract	g	0.1	2	− 1	1
D	Xylose	g	0.2	2	− 1	1
E	Sucrose	g	0.2	2	− 1	1
F	Magnesium sulfate	g	0.1	0.5	− 1	1
G	Dihydrogen potassium phosphate	g	0.1	0.4	− 1	1
H	Calcium carbonate	g	0.1	0.5	− 1	1

Table 2 Optimization of variables using Plakett–Burman design for production of chitinase showing the actual and predicted values in terms of U/ml

Run	Colloidal chitin	Peptone	Beef extract	Xylose	Sucrose	Magnesium sulfate	Dihydrogen potassium phosphate	Calcium carbonate	Actual chitinase activity	Predicted chitinase activity
1	1	− 1	1	− 1	1	1	− 1	− 1	1.78	1.57
2	1	− 1	− 1	1	− 1	1	− 1	1	2.02	2.06
3	− 1	1	1	− 1	− 1	1	− 1	1	1.28	1.38
4	1	1	− 1	1	1	− 1	− 1	− 1	2.36	2.29
5	− 1	1	− 1	1	− 1	1	1	− 1	1.36	1.38
6	− 1	1	1	1	1	− 1	− 1	1	1.96	1.88
7	1	− 1	1	1	− 1	− 1	1	1	1.36	1.56
8	− 1	− 1	− 1	− 1	− 1	− 1	− 1	− 1	0.96	1.16
9	− 1	− 1	− 1	− 1	1	− 1	1	1	0.78	0.66
10	1	1	1	− 1	− 1	− 1	1	− 1	1.42	1.3
11	− 1	− 1	1	1	1	1	1	− 1	1.28	1.15
12	1	1	− 1	− 1	1	1	1	1	1.16	1.3

Level (− 1 and 1) defines low and high coded values corresponding to the variable (colloidal chitin—0.1 and 1 gm, peptone—0.1 and 2, beef extract—0.1 and 2.0, xylose—0.2 and 2, sucrose—0.2 and 2, magnesium sulfate—0.1 and 0.5, dihydrogen potassium phosphate—0.1 and 0.4, calcium carbonate—0.1 and 0.5)

tested range of concentration. The Pareto chart gives idea regarding the order of significance of the components. After the initial selection, effects below the *T* value limit are likely to be less significant (Fig. 1). The Model *F* value of 17.50 implies that the model is significant as mentioned in Table 3. There is only a 0.09% chance that a "Model *F* value" this large could occur due to noise. Values of "Prob > *F*" < 0.0500 indicate that the model terms are significant.

Optimization of significant variables using the Box–Behnken design

Depending on the Box–Behnken design, four independent variables including colloidal chitin, xylose, peptone, and dihydrogen potassium phosphate were selected for the production of chitinase enzyme (Table 4). The predicted and observed responses are mentioned in Table 5. The results obtained from the Box–Behnken design were then analyzed by standard analysis of variance (ANOVA), and the quadratic regression equation was applied for the prediction of chitinase enzyme production (Table 6). Based on the model, it appeared that colloidal chitin ($p \leq 0.0001$), xylose ($p = 0.0009$), and peptone ($p = 0.0015$) were most significant and dihydrogen potassium phosphate ($p = 0.1670$) was less significant.

The ANOVA result (Table 6) discloses that the quadratic model was highly significant, from "*F*" value of 9.35 and a probability "*p*" value of < 0.0001. There is only a 0.01% chance that a "Model *F* value" this large could occur due to noise. The large value of *F* shows that most of the difference in the response is given by the regression equation. Values of "Prob > *F*" < 0.0500 indicate that model terms are significant. The "lack of fit *F* value" of 0.24 implies that the lack of fit is not significant relative to the pure error. There is a 98.10% chance that a "lack of fit *F* value" this large could occur due to noise.

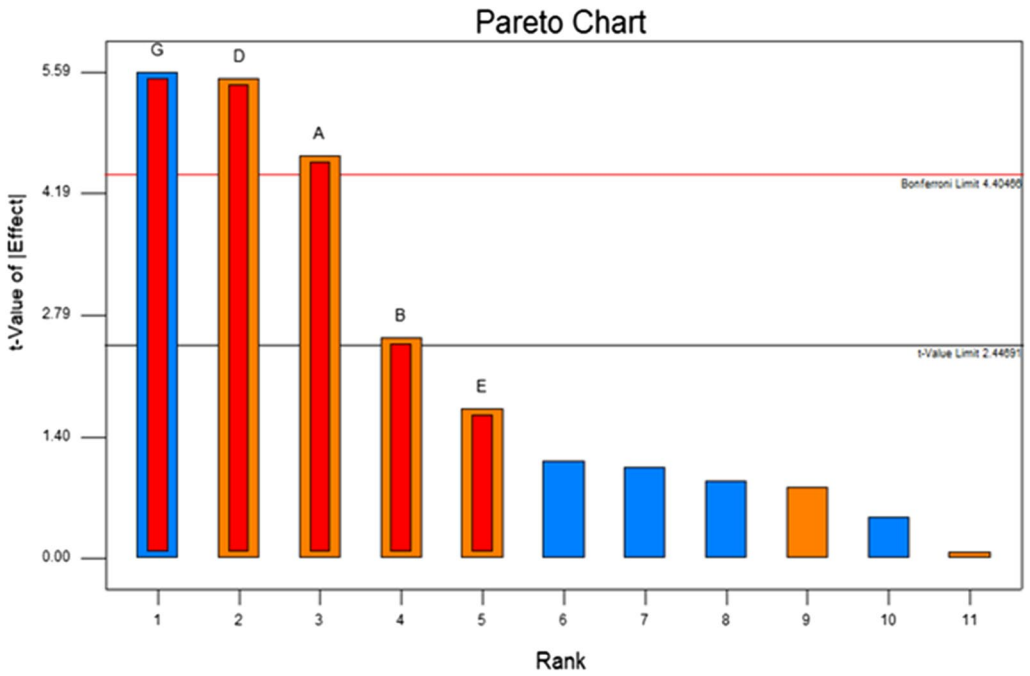

Fig. 1 Pareto chart of independent variables having significant effects. Pareto chart indicates the order of significance of components. After the initial selection, the effects below the T value limit are likely to be less significant. Alphabetical numbering on Pareto chart indicates the significance of variables coded with actual variables: A colloidal chitin; B peptone; D xylose; E sucrose; G dihydrogen potassium phosphate

Table 3 Results of ANOVA for selected factorial model

Source	Sum of squares	*df*	Mean square	*F* value	*p* value prob > *F*
Model	2.1468	4	0.5367	17.50109	0.0009
A colloidal chitin	0.512533	1	0.512533	16.71304	0.0046
B peptone	0.154133	1	0.154133	5.026087	0.0599
D xylose	0.730133	1	0.730133	23.8087	0.0018
G dihydrogen potassium phosphate	0.75	1	0.75	24.45652	0.0017
Residual	0.214667	7	0.030667		
Cor total	2.361467	11			

R^2 0.9091, Adj R^2 0.8572, Pred R^2 0.7329, Adeq Precision 14.449, *df*-degree of freedom

Table 4 Independent variables and their levels for Box–Behnken design

Factor	Name	Low actual	High actual	Low coded	High coded
A	Colloidal chitin	0.1	1	−1	1
B	Xylose	0.2	2	−1	1
C	Peptone	0.2	1	−1	1
D	Dihydrogen potassium phosphate	0.1	0.4	−1	1

Table 5 Chitinase activity from the experimental design for the response surface quadratic model

Std. order	Colloidal chitin	Xylose	Peptone	Dihydrogen potassium phosphate	C.A[a]	C.A[b]
1	0.1	1.1	1	0.25	2.32	2.13
2	0.55	0.2	1	0.25	2.76	2.49
3	0.55	2	0.6	0.1	2.92	2.82
4	0.55	1.1	0.6	0.25	2.12	2.52
5	0.55	0.2	0.6	0.1	1.56	1.88
6	0.55	1.1	0.6	0.25	2.18	2.52
7	0.55	1.1	0.2	0.4	1.96	2.25
8	1	1.1	1	0.25	3.96	3.78
9	0.1	1.1	0.6	0.1	1.68	1.52
10	1	0.2	0.6	0.25	2.38	2.87
11	0.55	0.2	0.2	0.25	1.28	1.61
12	0.1	1.1	0.6	0.4	1.76	1.86
13	0.1	1.1	0.2	0.25	1.62	1.25
14	0.55	2	1	0.25	3.52	3.43
15	0.55	2	0.6	0.4	2.98	3.16
16	0.55	1.1	0.6	0.25	1.92	2.52
17	0.1	0.2	0.6	0.25	1.38	1.22
18	1	2	0.6	0.25	4.28	3.81
19	0.55	2	0.2	0.25	2.78	2.55
20	1	1.1	0.2	0.25	2.56	2.9
21	0.1	2	0.6	0.25	1.24	2.16
22	1	1.1	0.6	0.1	2.72	3.17
23	0.55	1.1	1	0.4	2.82	3.13
24	0.55	0.2	0.6	0.4	2.72	2.22
25	0.55	1.1	0.2	0.1	2.62	1.91
26	0.55	1.1	0.6	0.25	3.48	2.52
27	0.55	1.1	1	0.1	2.72	2.79
28	0.55	1.1	0.6	0.25	2.82	2.52
29	1	1.1	0.6	0.4	4.02	3.51

C.A[a] = actual chitinase activity (U/ml) C.A[b] = predicted chitinase activity (U/ml)

Non-significant lack of fit is required to fit the model which is obtained if $p > 0.1$.

Final equation in terms of actual factors:

$$\begin{aligned}\text{Chitinase activity} = {} & +2.52 + 0.83 \times \text{A} + 0.47 \\ & \times \text{B} + 0.44 \times \text{C} + 0.17 \times \text{D} \\ & + 0.51 \times \text{A} \times \text{B} + 0.18 \times \text{A} \times \text{C} \\ & + 0.30 \times \text{A} \times \text{D} - 0.18 \times \text{B} \times \text{C} \\ & - 0.28 \times \text{B} \times \text{D} + 0.19 \times \text{C} \times \text{D},\end{aligned}$$

where A is colloidal chitin, B is xylose, C is peptone and D is dihydrogen potassium phosphate.

The three-dimensional (3D) plots give an idea of the combined effect of two independent variables for chitinase enzyme production, when other variables were kept constant (Fig. 2a–d). The perturbation plot showed the outcome of all factors at a point in the design space (Fig. 3). Among the variables used for RSM, xylose had a significant effect on chitinase enzyme production compared with other variables. The perturbation graph shows that the factor colloidal chitin had a significant role in enzyme production. Before optimization, the enzyme production predicted was 0.66 U/ml, and threefold increased enzyme production was achieved after optimizing the medium by RSM. The predicted maximum chitinase enzyme activity was estimated to be 3.81 U/ml, while that of the actual chitinase enzyme activity obtained was 4.28 U/ml.

Table 6 Results of ANOVA for response surface quadratic model

Source	Sum of squares	*df*	Mean square	*F*-value	*p* value Prob > *F*
Model	15.64013333	10	1.56401333	9.352075121	< 0.0001
A colloidal chitin	8.200533333	1	8.20053333	49.03539	< 0.0001
B xylose	2.6508	1	2.6508	15.85055588	0.0009
C peptone	2.3232	1	2.6508	13.89165965	0.0015
D Dihydrogen potassium phosphate	0.3468	1	0.3468	2.073703326	0.1670
AB	1.0404	1	1.0404	6.221109979	0.0226
AC	0.1225	1	0.1225	0.732493245	0.4033
AD	0.3721	1	0.3721	2.224985605	0.1531
BC	0.1369	1	0.1369	0.818598574	0.3775
BD	0.3025	1	0.3025	1.808809851	0.1954
CD	0.1444	1	0.1444	0.863445099	0.3651
Residual	3.010266667	18	0.16723704		
Lack of fit	1.364346667	14	0.09745333	0.236836136	0.9810
Pure error	1.64592	4	0.41148		
Cor total	18.6504	28			

R-Squared 0.838595, Adj R-Squared 0.748926, Pred R-Squared 0.662642, Adeq Precision 11.52083

Biocontrol of root-knot nematode disease (*Meloidogyne* sp.) using purified chitinase in formulation

Under pot study, it was noted that there was significant increase in the number of leaves in set-1 which was inoculated with purified chitinase: organic manure (1:1) [22], and set-2, which was inoculated with purified chitinase and nematodes (10 ml) at the beginning of experiment [22]. But, it was found on comparison that the number of leaves was less and equal to that found in the control, which received only water [16], and set-3 [16], which was inoculated with commercially available nematicidal agent (fenamiphos) and nematodes (10 ml) at the beginning of the experiment (pot—9, 10 and 11) as described in Fig. 4.

There was an increase found in the overall plant growth parameters in case of set-1 compared to the other three sets. Plant shoot length was found to be higher (14.83) in set-1 and lowest in the control (9.36), while plant root length was found to be higher in set-1 (6.83) and lowest in set-2 (5.13). Fresh shoot weight was found to be higher in set-1 (0.444) and lowest in set-3 (0.224), while fresh root weight was found to be higher in the control (0.04) and lowest in set-3 (0.01). Dry shoot weight as well as dry root weight was found to be higher in set-1 (0.093, 0.018) and lowest in set-3 (0.018, 0.0053). Chlorophyll a was found to increase in set-2 (0.3) and chlorophyll b was found to increase in set-1 (0.495), while carotenoid content was found to be slightly higher in set-1 (0.138) compared to that in set-3 (0.137). There was statistical difference found between them according to Duncan's test, within the column in Table 7.

The inoculum level (10 ml) of *Meloidogyne* sp. was found to affect plant growth directly by affecting plant growth parameters such as plant height, fresh and dry weight of shoot and root, chlorophyll *a* and *b* as well as carotenoid content and the number of galls/root system. As the nematode density (1000) increased, the corresponding root-knot index was found to increase (set-2 and set-3) as mentioned in Table 8. There was comparatively less number of galls/plant in set-2 as compared to set-3 which showed the ability of purified chitinase to decrease infection.

Discussion

Optimizing response helps in identification of various significant factors and its desirability. Chitin is an important factor influencing chitinase production by *Streptomyces lividans* and *Streptomyces* sp. Da11 [18, 60]. Supplementation of glucose in colloidal chitin medium was found to decrease chitinase production in *Streptomyces* sp. [37]. Excluding chitin, other carbon sources such as sucrose, glucose, maltose, lactose and arabinose were not found to be significant in increasing chitinase production [16, 46, 58]. According to Gangwar et al. [14], additional carbon sources such as maltose, xylose, fructose, and lactose enhanced chitinase yield in *Streptomyces violascens* NRRL B2700 by 1.6 to sixfold and our results were similar to the above statement, as it was found that xylose as a carbon source was found to enhance chitinase production. Comparative optimization between organic and inorganic nitrogen sources revealed that organic nitrogen sources such as yeast extract and peptone were significant factors influencing chitinase activity as compared to inorganic sources, according to Vaidya et al. [59]. This is in accordance with

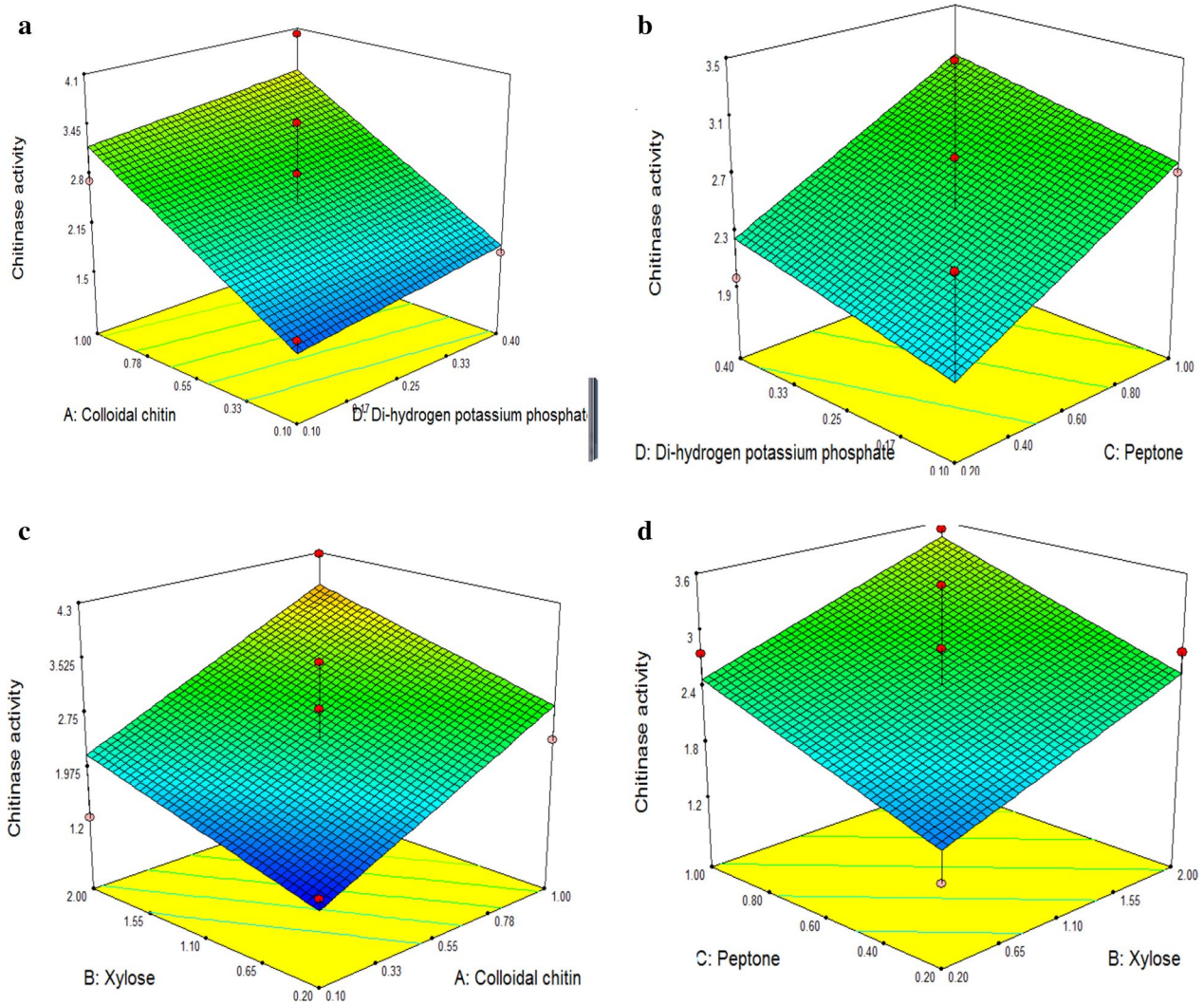

Fig. 2 **a**–**d** 3D plot showing the effect of two variables against chitinase activity while all other factors are kept constant

our result showing that the addition of yeast extract and peptone to the medium enhanced chitinase activity, leading to increased production from *Streptomyces rubiginosus* SP24. Enhanced production of chitinase was obtained in strains of *Streptomyces* sp. NK1057, NK528 and NK951 by 29, 9.3 and 28%, respectively, as described by Sarrafzadeh and Hussein [49] under optimized conditions. As reported by Sandhya et al. [48], optimum chitin concentration for chitinase induction is in the range of 10–20 g/1000 ml in case of microbes and our results are in agreement with this. During the course of the present study, the optimum colloidal chitin concentration for higher chitinase production by *Streptomyces rubiginosus* SP-24 is 1.0 g/100 ml, which is considerably lower than the range studied by Sandhya et al. [48]. Tasharrofi et al. [56] reported that the optimum chitin concentration for chitinase production by *B. pumilus* was 4.76 g/1000 ml, which was considerably higher than the present investigation. The minimum use of substrate with higher production of enzyme may be favorable, as this may be an added advantage for industrial use. Lower concentrations of chitin sometimes fail to increase chitinase production in microbes, and as an alternative some other nitrogen sources such as peptone and urea are reported to increase chitinase production [59], indicating that other mechanisms may be involved. Production of chitinase is increased on addition of minerals such as KBr and $MgSO_4$ to the production medium as described by Gohel et al. [15]. From the model, it is clear that dihydrogen potassium phosphate exerts positive effect by increasing chitinase activity, in turn leading to increase in the production of chitinase.

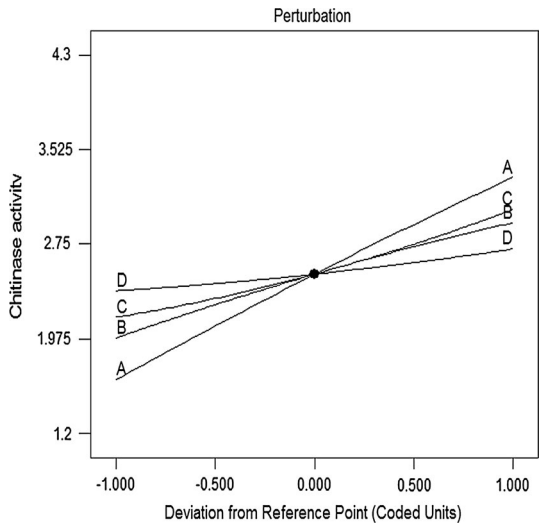

Fig. 3 Perturbation curve showing the maximum chitinase production by factor A (colloidal chitin)

Biocontrol of root-knot nematode disease *(Meloidogyne* sp.) using purified chitinase in formulation

A characteristic study of chitinase treatment for nematicidal activity was studied for *Paenibacillus illinoisensis KJA-424* by Jung et al. [27]. The present study was, therefore, conducted to determine if purified chitinase produced by *Streptomyces rubiginosus SP24* was associated with the inhibition of egg hatching of root-knot nematode with a hypothesis that there was an association between microbes and plant-parasitic nematodes. From the results of plant growth parameters, it can be concluded that purified chitinase indirectly showed inhibition of the egg hatching of *Meloidogyne* sp. by showing significant increase in plant growth parameters (Table 7). As reported by Cronin et al. [8], in vitro studies resulted in 70% inhibition of egg hatching by *Globodera rostochiensis* (Ro1), when purified chitinase was used. Production of chitinase by *Pseudomonas aureofaciens* was able to attack the egg shell rather than the cuticle of *Criconemella xenoplax* [63]. Another significant factor that helped in increased plant growth is the number of leaves which significantly increased when chitinase in formulation with organic manure was coated on mung seeds compared to the control (Fig. 4). This might have occurred due to the direct damage of chitinase to the juvenile nematodes (J_2) caused by chitinase activity. *Actinomycetes* are known for the production of various enzymes having lytic property such as lipase, protease, chitinase, amylase, xylanase, cellulase, and mannanase [5] and might be capable of affecting eggshells of root-knot nematode [33–41]. As reported by Mercer et al. [34], chitinase interferes with the egg

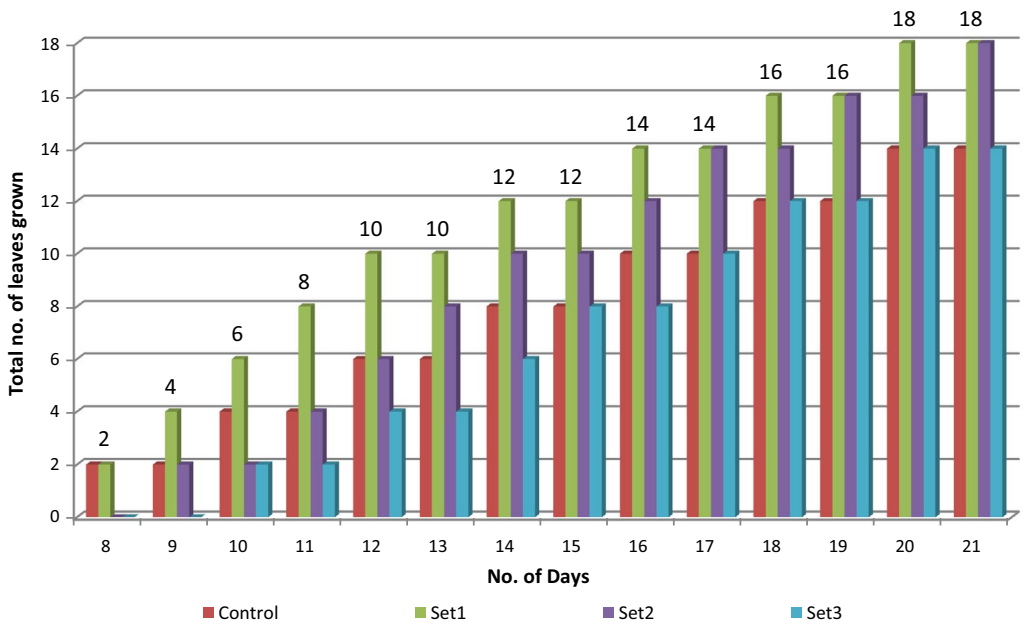

Fig. 4 Growth of leaves in mung plant (*Vigna radiata*) during the experimental period (day-1 to day 21). Results shown here in bar graph are the mean replicates of three. There was no growth of leaves from day 1 to day 7. Hence, it is not shown in the graph. Control—addition of only water; set-1 contained only chitinase:organic manure formulation); set-2 contained chitinase:organic manure formulation as well as 10 ml of inoculum volume of nematode suspension added near the roots of the plant; set-3 contained fenamiphos:organic manure formulation as well as 10 ml of inoculum volume of nematode suspension added near the roots of plant at the beginning of the experiment

Table 7 Influence of purified chitinase and commercially available anti-nematicidal agent on plant growth promotion (*Vigna radiata*) under controlled conditions

Plant growth parameters	Control	Set-1	Set-2	Set-3
Shoot length	9.36^{b}	14.83^{b}	13.2^{b}	12.46^{b}
Root length	6.6^{c}	6.83^{c}	5.13^{c}	6.32^{c}
Total plant height	15.96^{a}	21.66^{a}	18.33^{a}	18.83^{a}
Shoot weight	0.408^{de}	0.444^{d}	0.308^{de}	0.224^{de}
Root weight	0.04^{f}	0.029^{f}	0.011^{g}	0.01^{f}
Total plant fresh weight	0.448^{d}	0.474^{d}	0.319^{de}	0.234^{de}
Shoot weight	0.038^{f}	0.093^{f}	0.028 ^{g}	0.018^{f}
Root weight	0.009^{f}	0.018^{f}	0.007 ^{g}	0.0053^{f}
Total plant dry weight	0.048^{f}	0.111^{f}	0.035 ^{g}	0.023^{f}
Chlorophyll *a*	0.119^{f}	0.27^{e}	0.3d^{e}	0.29^{de}
Chlorophyll *b*	0.225^{ef}	0.495^{d}	0.482^{d}	0.477^{d}
Carotenoid	0.085^{f}	0.138^{f}	0.132^{ef}	0.137^{f}

Control (2 pots), set-1, 2 and 3 are replicates of three pots, and each set is the mean average of three seedlings/pot. Control—addition of only water (pots 1 and 2)

Set-1 contained only chitinase:organic manure formulation (pots 3, 4 and 5)

Set-2 contained chitinase:organic manure formulation as well as 10 ml of inoculum volume of nematodal suspension added near roots of plant (pots 6, 7, and 8)

Set-3 contained fenamiphos: organic manure formulation as well as 10 ml of inoculum volume of nematode suspension added near the roots of the plant at the beginning of the experiment (pots 9, 10, and 11)

Statistical analysis results showed values with the same letters (a, b, c, d, e, f, g) within the column were not significantly different and values with different letters within the column were significantly different from each other according to Duncan's test

hatching of *M. hapla*. Endo-chitinase production by isolate KJA-424 led to a change in the shape of egg as well as its rupturing [27]. The results of pot study showed that chitinase caused lysis of the eggshell of *Meloidogyne* sp. that resulted in egg hatching inhibition. The lysis of the eggshell by chitinolytic activity plays an important role in the control of root-knot nematode (*Meloidogyne* sp.), as seen with increased chlorophyll a and b content as well as carotenoid content of the plant (Table 7). The inoculum level (10 ml) used in formulation with purified chitinase significantly decreased the infection to the plant (Table 8) as compared with a formulation containing anti-nematicidal agent.

Table 8 Different nematode inoculum volumes affecting mung plant (*Vigna radiata*) in the pot study

Sr. no.	Nematode density	Inoculum volume (ml)	No. of galls/root system	Root-knot index
Control/set-1	0	0	0	0
Set-2	1000	10	5	2
Set-3	1000	20	9	2

Control (2 pots), set-1, 2 and 3 are replicates of three pots, and each set is the mean average of three seedlings/pot

Control—addition of only water (pots 1 and 2)

Set-1 contained only chitinase:organic manure formulation (pots 3, 4 and 5)

Set-2 contained chitinase:organic manure formulation as well as 10 ml of inoculum volume of nematode suspension added near the roots of the plant (pots 6, 7, and 8)

Set-3 contained fenamiphos:organic manure formulation as well as 10 ml of inoculum volume of nematode suspension added near the roots of the plant at the beginning of the experiment (pots 9, 10, and 11)

The number of galls per root system was counted as described by Taylor and Sasser [55], where gall index 1 was on a scale of 0–5: 0 = 0 galls; 1 = 1–2 galls; 2 = 3–10 galls; 3 = 11–30 galls; 4 = 31–100 galls; 5 > 100 galls

Conclusion

In the present study, through Plakett–Burman design, it was found that higher chitinase activity resulted due to four major variables (colloidal chitin, xylose, peptone and dihydrogen potassium phosphate). The statistical model as designed by the Box–Behnken tool of the Design-Expert software has showed significant elevation in chitinase activity in the presence of selected variables of the defined media. It was confirmed that supplementation (g/100 ml) with colloidal chitin 1.0, xylose 2.0 and peptone 0.6 helped in enhancing chitinase production. Similar optimization process may be used to improve the chitinase activity, in turn leading to its improved production from novel isolate without any extra expenditure. Plant-parasitic nematodes are found in all agricultural sections of the world. They severely influence and cause harm to the productivity of crops, where environmental factors support their survival and dispersal particularly in tropical and sub-tropical regions of the world. The damage caused by nematodes to plants is directly proportional to the number of nematodes in soil and their reproduction rate on the plant. Results showed that purified chitinase used in formulation had good property to decrease the disease and increase plant growth-promoting traits.

Methods

Microorganism and culture conditions

Streptomyces rubiginosus SP24 (accession number KT198721) isolated from the rhizosphere of *Gossypium* sp. was selected, as it was able to produce extracellular chitinase [26].

Experimental culture medium

50 ml seed culture medium used for growth of *Streptomyces rubiginosus SP24* contained the following composition (gm/100 ml): colloidal chitin, 1%; sodium nitrate, 0.2; magnesium sulfate, 0.1; dihydrogen potassium phosphate, 0.1; potassium chloride, 0.05; calcium carbonate, 0.1; iron sulfate heptahydrate, 0.001; pH 7.0 at 30 °C under shaking conditions (100 rpm) for 72 h in orbital shaker (REMI Model no. 396LAG).

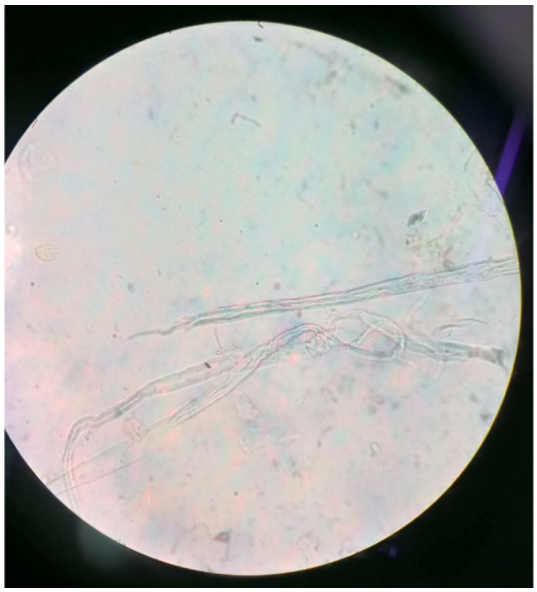

Fig. 5 Stereomicroscopic view. Inoculum J_2 stage (*Meloidogyne* sp.) selected for biocontrol of root-knot nematode disease

Chitinase assay

The reaction mixture contained 1 ml of 1% colloidal chitin in phosphate buffer (0.05 M, pH 7.0) and 1 ml culture filtrate was incubated at 37 °C for 1 h. Suitable substrate and enzyme blanks were included. Chitinase activity was assayed by following the spectrophotometric method of Reissig et al. [45]. The reaction was terminated by adding 0.1 ml of 0.08 M potassium tetraborate (pH 9.2) to 0.5 ml of the reaction mixture. It was then boiled in a water bath for 3 min. Then 3 ml of diluted *p*-dimethylaminobenzaldehyde [10 g of DMAB dissolved in 100 ml of glacial acetic acid containing 12.5% 10 N (v/v) hydrochloric acid] reagent was added and again incubated at 37 °C for 15 min. The released product in the reaction mixture was read at 585 nm in a visible spectrophotometer (Systronics). Chitinase activity was determined using *N*-acetylglucosamine (Hi-media) as the standard. One unit of chitinase activity is defined as the amount of enzyme, which produces 1 µ mol of *N*-acetylglucosamine in 1 ml of reaction mixture under standard assay condition [31].

Optimization of process parameters

Plackett–Burman design: An efficient method for selection of medium components was used to determine the factors that have significant influence on chitinase production (Plackett and Burman [43]).

Experimental Design: Carbon sources, nitrogen sources, colloidal chitin, and inorganic ions were selected for increasing the production of chitinase. The design was formulated according to Plackett–Burman design as well as Box–Behnken design using Design-Expert 7.0.0 (Stat-Ease). Plackett–Burman screening method was designed with a two-level factorial design (−1) for low level and (+1) for high level for the identification of important medium components with consideration of their main effects.

The design was developed to evaluate the interactions of various process variables with one another. The variables that had the most significant effect, required for maximal chitinase production, were investigated by screening k variables in $k+1$ experiments. Eight independent variables were screened in 12 runs in one block according to the design. The most important effect of each variable was calculated as the difference between the average of measurements made at the high level (+1) and the average of measurements observed at low level (−1) of that factor The significance of the variables was determined by calculating the p value (Prob > F) through standard regression analysis which indicates whether the model is significant or not. The dependent (response) variable studied was chitinase activity. After completion of the experiment, the results entered were compared to that of the predicted value and data obtained were subjected to the analysis of variance (ANOVA).

Box–Behnken design

After estimating the comparative significance of independent variables, four most significant variables were selected for further determination of their optimal level with respect to chitinase activity as a response. For this reason, the Box–Behnken design was applied. The effects of colloidal chitin, xylose, peptone and dihydrogen potassium phosphate on chitinase activity were evaluated. The levels of these factors were optimized for maximum chitinase production (the response). A 29-trial experimental design was used, where each variable was tested at two different levels: low (−1) and high (+1). The significance of variables was determined by calculating the p value (Prob > F) through standard regression analysis, which indicates whether the model is significant or not.

Software and data analysis

Design-Expert version 7.0 trial version (Stat-Ease Inc., Minneapolis, MN, USA) as a statistical software was used for analysis and interpretation of experimental design.

Purification of optimized chitinase enzyme

Four methods of purification (ammonium sulfate precipitation, dialysis, concentrators and Sephadex G-50 gel permeation chromatography) were used for isolation of chitinase from *Streptomyces rubiginosus SP24* and has already been published in our previous work (Jha

and Modi [23]). Hence, the methods and results are not described here.

Biocontrol of root-knot nematode disease *(Meloidogyne* sp.) in unsterilized soil by *Streptomyces rubiginosus* SP24

Experimental design for pot study

For the screening experiment as well as inoculation and termination of experiment, modified method of Rajeswari and Ramakrishnan [44] was used. Pot study was carried out in triplicates under controlled conditions with 1:4 soil–sand ratio (now referred to as soil). Seeds of *Vigna radiata* were surface sterilized by gently shaking with 70% ethanol (5 min) and 20% sodium hypochlorite solution, followed by rinsing with sterile distilled water. Seeds were allowed to soak in a Petri dish with sterile distilled water overnight [24]. 5 gm of organic manure was mixed with 5 ml of purified chitinase, and 4.28 U/ml (chitinase thus obtained after applying RSM was further purified with molecular techniques—ammonium sulfate precipitation, dialysis, using concentrators and with Sephadex G-50 column chromatography) enzyme was used for coating seeds of *Vigna radiate* using 1% CMC as an adhesive. Three seeds were planted in each pot and watered on a regular basis. Control sets were maintained, with no treatment as well as no nematodes added. Set-1 contained only chitinase:organic manure formulation, set-2 contained chitinase:organic manure formulation as well as 10 ml of inoculum volume of nematode suspension added near the roots of the plant and set-3 contained fenamiphos:organic manure formulation as well as 10 ml of inoculum volume of nematode suspension added near the roots of the plant. Each treatment was replicated three times and arranged in a completely randomized block design. Plants were watered appropriately and maintained. The number of leaves was counted each day till termination of the experiment. The plants were examined 21 days after sowing and then uprooted for determining fresh as well as dry weights of shoot, root, chlorophyll a and b content, carotenoid content, nematode population in roots and number of galls/root system.

Preparation of nematode inoculum

Second-stage juveniles (J_2) of the root-knot nematode were used as inoculum in the present investigation. 100 gm of infected soil sample was strained on a kitchen strainer, washed with water and collected in a 2000 ml beaker. The strained water was further collected and sieved through a 400-mesh size strainer and the collected debris containing the soil sample on the strainer was transferred to a centrifuge tube. A small amount of water was added and the solution centrifuged at 3500 rpm for 3 min. The supernatant was discarded and the pellets obtained in the centrifuge tube were collected and added to sucrose solution (45.4 gm/100 ml). The resulting suspension was centrifuged at 3500 rpm for 3 min. Egg masses were placed in a coarse sieve fitted with double-layered tissue paper in Petri plates. These Petri plates were then placed in an incubator at 23 ± 2 °C. The hatched juveniles (J_2) were collected in a beaker every 24 h (Fig. 5) and observed under stereomicroscope to identify J_2-stage juveniles. The average number of juveniles was then calculated to represent the number of juveniles per 10 ml and 20 ml of the suspension.

Inoculation of Meloidogyne sp. in pot

For inoculation, the suspension containing J_2 was taken in a micropipette and added near the roots of the seedlings. The holes were covered with soil after inoculation. Nematode inoculation density consisted of a uniform quantity of suspension containing either 0 or 1000 (10 ml) freshly hatched second-stage juveniles (J_2)/pot.

Termination of the experiment

The plants were uprooted 21 days after inoculations and the following parameters studied. The following growth-related parameters were determined for each treatment of the different experiments.

(1) Plant fresh weight (root and shoot), (2) plant dry weight (root and shoot), (3) number of leaves/plant, (4) chlorophyll a and b content, and (5) number of root galls/root system.

Plant fresh and dry weight

Fresh as well as dry weights of shoots and roots were recorded for each treatment. Plants of each treatment were taken out from the pots and soil particles adhering to roots were removed by washing in tap water and labeled appropriately. In the laboratory, fresh weights of shoot and root were determined with the help of a weighing balance. For determining the dry weights, plants from each treatment were wrapped in blotting paper sheets, labeled and then dried in a hot air oven at 60 °C for 24 h before weighing.

Estimation of chlorophyll and carotenoids

The leaves of the mung bean plants were homogenized with 80% acetone. The homogenate was filtered and centrifuged at 3000 rpm for 15 min at 4 °C. The supernatant was collected and the volume was made up to 25 ml with 80% acetone. Chlorophylls and carotenoid contents were estimated in accordance with Maclachlan and Zalik [30]. The absorbance of the supernatant was recorded at 645 and 663 nm for chlorophyll contents, and at 480 and 510 nm for carotenoid contents, on a spectrophotometer. The amount of chlorophylls and carotenoids was expressed as mg/gm fresh weight.

Number of root galls per root system

Roots of uprooted plants were washed under the tap water and examined for the presence of root galls. The number of galls per root system was counted as described by Taylor and Sasser [55], where Gall index 1 was on a scale of 0–5: 0 = 0 galls; 1 = 1–2 galls; 2 = 3–10 galls; 3 = 11–30 galls; 4 = 31–100 galls; 5 > 100 galls.

Statistical analysis

The experiment was designed in such a way that three replicates were obtained for each plant growth parameter and the mean results were analyzed by performing analysis of variance test (ANOVA), followed by Duncan's post hoc test using software IBM SPSS 23.0 trial version [21] (IBM Corp. Released 2015).

Abbreviations

PBD: Plackett–Burman design; RSM: response surface methodology; Mung: *Vigna radiata*; SmF: submerged fermentation; Coded values: maximum (+) and minimum (−); 3D: three dimensional; ANOVA: analysis of variance; J_2: second-stage juveniles.

Authors' contributions

SJ designed and carried out the experiments and wrote the manuscript. HM supervised and directed the research outcomes as well as overall improved and finalized the manuscript. Both authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Software Design-Expert version 7.0 (Stat-Ease Inc.) Trial version of software was used as a tool for statistical analysis and hence did not require any licence key for activation of software.

Consent for publication

We did not include any images which required prior permission. Consent for publication of images was not required as it included only research findings dealing with plants and nematodes.

Ethics approval and consent to participate

The study was approved by the RDC committee of Gujarat University. The certificate was not required as there were no human/animal trials involved during the course of the study.

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