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Full Length Research Paper

Response surface methodology for optimizing process parameters for the mass production of *Beauveria bassiana* conidiospores

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***Beauveria bassiana* is an insect pathogenic fungus and is currently being exploited as an effective commercial mycoinsecticide. Solid state fermentation is the most efficient way to mass produce *B. bassiana* conidiospores on solid substrates. Statistical optimization strategy was adopted to maximize the conidia production through solid state fermentation. Four substrates viz., rice (polished), crushed sorghum, wheat bran and rice bran at variable moisture content and yeast extract concentration were used. A full factorial central composite design was used to design the experiment and response surface method was used to study the optimal parameters required for large scale production. Optimization of the two most important factors like moisture content and yeast extract concentration at varied levels conferred best conidial yield of 28.8 × 10^9 /gm for the mixed substrate rice + wheat bran at 35% moisture content and 1.5% yeast extract concentration.**

Key words: *Beauveria bassiana,* central composite design, conidiospores, response surface methodology, solid state fermentation.

INTRODUCTION

The strong impetus to find an effective alternative to chemical pesticides, focused on a more environment friendly sustainable approach that exploits natural predators of insect pests, the entomopathogenic fungi. *Beauveria bassiana*, a natural soil borne entomopathogenic fungus can be used efficiently for the suppression of insect pests and as a potential biocontrol agent (Inglis et al., 2001). A considerable effort has been made to establish *B. bassiana* as a commercial mycopesticide by using solid state fermentation (SSF) as a suitable method for mass production (Shah and Pell, 2003). SSF can be defined as growth of a microorganism on solid substrate in the absence or near absence of free liquid, but the substrate must posses enough moisture to support fungal growth (Moo-Young et al., 1983). This production process involves a biphasic system where a liquid culture is obtained at first by mycelium harvesting and then using the liquid culture as an inoculum for the solid substrate (Mendonca, 1992). Rice is the most popular solid substrate for conidia production of entomopathogenic fungi due to its complex nutritional source (Posada-Flórez, 2008).

A comparative study shows that production of 10^{13} conidia, costs the same as chemical insecticides used per hectare and hence high production efficiency leads to the development of consciousness to mass produce *B. bassiana* conidia in a cost effective way (Inglis et al., 2001). It involves a lower operating cost and a copious prospect of processing agro - industrial residues like

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declassified potato flour, rice flour as solid substrate for conidia production of entomopathogenic fungi *B. bassiana* (Soccol et al., 1997). Water alone is the most suitable and humidifying agent. Grajek and Gervais (1987) reported the efficiency of water for supporting conidia formation on wheat bran, rather than other liquid mineral medium. Im et al. (1988) have reported the dependence of vegetative growth for four entomo-pathogenic fungi on yeast extract, a complex nutritional source of nitrogen and vitamins.

Response surface methods are empirical statistical tools that are being used for modeling and analyzing problems where the response is influenced by several independent variables and the aim is to maximize the process variables to get an optimum response (Montgomery, 2001). Optimization of the variables in a fermentation process can give information about the main effects of the variables and also the interaction between variables in varying level. The main advantage of using response surface methodology includes the reduction in number of experiments saving time, chemicals and labor and also its rapid and reliable prediction of response made it a lucrative option to explore. This current study illustrates the optimization of two important factors: moisture content and yeast extract concentration for conidial production through SSF of entomopathogenic fungus *B. bassiana*. Both the variables were tested for four different substrates: rice, crushed sorghum, rice + wheat bran and crushed sorghum + rice bran to observe the effect of each substrate and their correlation on the conidial yield.

MATERIALS AND METHODS

Fungal culture and inoculum preparation

The fungal isolate, *B. bassiana* USDA-ARS (2034) was obtained from ARSEF (USDA-ARS Plant Protection Unit, Ithaca NY) and the culture was grown on Sabouraud dextrose agar slants at 28°C for 8 d. Fungal spores at a concentration of 1×10^7 conidia /ml were inoculated in Sabouraud dextrose broth and incubated for three days at 28°C and 180 rpm for generating adequate quantity of inoculum for solid-state fermentation.

Media preparation and solid state fermentation

Rice (polished) and crushed sorghum (crushed in grinder) were used as single substrates and two mixed substrates were used: rice + wheat bran and crushed sorghum + rice bran, mixed in equal amounts (w/w). Single substrate, rice and crushed sorghum (60 g each) were weighed and taken into poly propylene bags separately and for mixed substrates (rice + wheat bran and crushed sorghum + rice bran), thirty grams of each substrate was taken into poly propylene bags (25 × 30 cm), respectively. All of these different substrates were supplemented with appropriate amounts of water [20 - 80 % (v/w)] and yeast extract [1 - 2 % (w/v)] (Table 1). The range of moisture content and yeast extract concentration for each substrate was decided based on previous experiments (data not published). The mouth of the poly propylene bags had cotton plugged PVC pipes (5 inches length) which aided in ventilation. The bags were then autoclaved at 121°C (15 psi) for 20 min and bags were cooled overnight. All the substrates were inoculated with 5 ml of the cultivated mycelium in SD broth and substrate clumps if any were broken before inoculation to provide more surface area for fungal growth. Bags were then incubated for 15 d at 28°C. Subsequently, after 15 days of incubation, one gram of each solid fermented substrate was mixed with 5 ml of sterile 0.01% Tween-80 solution and vortexed thoroughly. The spore concentrations were determined using a Haemocytometer and this was repeated thrice for each bag and the average counts were taken into consideration and the conidial yield determined. Each run (experiment) were carried out in triplicates and average conidial yield was taken into consideration.

Experimental design and optimization

Central composite design is widely used statistical design for fitting second order models. A central composite design generally consists of 2^n factorial design, where n is the number of variables used in the experiment with 2n axial runs and n center runs that gives a total of $N = 2^n + 2n + n$ runs. In this experiment 2^2 central composite designs with 5 center points, 4 cube points and 4 axial points was used giving 11 trials (ignoring 2 replicates at centre point) per substrate.

Response surface methods are useful statistical techniques to analyze and optimize the response from different variables. The test variables were coded according to the following equation:

$$x_i = (X_i - X^{\circ}_i) / X \qquad \text{(i)}$$

Where, x_i is the coded variable and X_i is the natural value of independent variable, X°_i is the value of the variable at the center point and X is the step change value. The details of the range and levels of each variable for each substrate are given in Table 1.

A second order polynomial equation was then fitted to the response data.

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\Sigma\, \beta_{ij} X_i X_j + \varepsilon \qquad \text{(ii)}$$

Where Y is the response variable, β_0 is the intercept term, β_{ii} is the squared effect and β_{ij} is the interaction between Xi and Xj. All thestatistical calculations, design and graphical analysis were done by Minitab statistical software (release15) and Design Expert (version 7.1.5).

RESULTS AND DISCUSSION

The mycelial growth on the different substrates was visible from the fifth day onwards and sporulation started on seventh day after incubation at 28°C. The predicted response along with the experimental data on the four substrates presented in Tables 2 and 3 reveal a close correspondence between the values. Silva and Loch (1987) reported conidial production on polished rice grain. Single substrate rice at 40% moisture content yielded the second best conidial yield of 16.2×10^9 conidia/gm (Table 2). The mixed substrate, rice + wheat bran was the most effective substrate for conidia production by SSF. A conidial yield of 28.80×10^9 conidia/gm was obtained at 35% moisture content and 1.5% yeast extracts (Table 2). Moisture content plays an important role in the final conidial yield, and optimization

Table 1. Range and levels of variables for the CCD design experiment.

Symbols	Independent variables	Substrates	Range and Levels				
			-α	-1	0	+1	α
A	Moisture content	Rice	31.72	40	60	80	88.28
		Crushed Sorghum	11.72	20	40	60	68.28
		Crushed Sorghum + Rice bran	11.72	20	40	60	68.28
		Rice + Wheat bran	13.79	20	35	50	56.21
B	Yeast extract concentration	Rice	0.79	1	1.5	2	2.21
		Crushed Sorghum	0.79	1	1.5	2	2.21
		Crushed Sorghum + Rice bran	0.79	1	1.5	2	2.21
		Rice + Wheat bran	0.79	1	1.5	2	2.21

Table 2. Experimental design with observed and predicted values using rice and rice + wheat bran as substrates.

Trials	Variables		Substrate		Variables		Substrate	
	Moisture content (%)	Yeast extract (g)	Rice		Moisture content (%)	Yeast extract (%)	Rice + Wheat Bran	
			Observed response $\times 10^9$	Predicted response (Y)$\times 10^9$			Observed response $\times 10^9$	Predicted response (Y) $\times 10^9$
1	60.0000	1.50000	7.95	8.43	50.0000	1.00000	19.50	17.90
2	60.0000	1.50000	8.15	8.43	35.0000	1.50000	26.50	27.00
3	80.0000	1.00000	15.90	15.60	35.0000	0.79289	17.10	17.30
4	60.0000	0.79289	15.60	16.60	13.7868	1.50000	17.50	15.60
5	40.0000	1.00000	16.20	15.20	35.0000	1.50000	25.80	27.00
6	88.2843	1.50000	12.80	12.10	56.2132	1.50000	15.50	17.50
7	60.0000	1.50000	9.20	8.43	20.0000	1.00000	14.40	15.60
8	80.0000	2.00000	8.93	10.20	20.0000	2.00000	15.90	17.30
9	40.0000	2.00000	13.20	13.80	35.0000	1.50000	28.80	27.00
10	31.7157	1.50000	14.00	14.40	50.0000	2.00000	19.00	17.60
11	60.0000	2.20711	13.00	11.80	35.0000	2.20711	18.20	18.20

of this factor for each substrate is essential (Jenkins et al., 1998). Although high moisture content aid in fungal sporulation, very high moisture content in SSF could create aeration problems as the interparticle spaces are filled with water (Moo-Young et al., 1983). A reduced amount of moisture content (20%) was optimum for substrate sorghum (crushed) and the conidial yield was 9.63×10^9 conidia/gm although yeast

Table 3. Experimental design with observed and predicted values using crushed Sorghum and crushed Sorghum + Rice bran as substrates.

Trials	Variables		Substrates			
	Moisture content (%)	Yeast extract (g)	Crushed Sorghum		Crushed Sorghum+ Rice bran	
			Observed response $\times 10^9$	Predicted response (Y) $\times 10^9$	Observed response $\times 10^9$	Predicted response (Y) $\times 10^9$
1	40.0000	0.79289	7.43	7.89	4.98	5.01
2	40.0000	1.50000	7.80	7.70	6.25	6.16
3	60.0000	1.00000	7.40	7.25	5.73	5.93
4	40.0000	2.20711	8.28	8.11	5.78	4.97
5	60.0000	2.00000	5.40	5.69	4.18	4.97
6	11.7157	1.50000	8.05	8.50	2.43	2.21
7	40.0000	1.50000	8.00	7.70	5.98	6.16
8	20.0000	2.00000	9.63	9.49	3.18	3.73
9	68.2843	1.50000	5.73	5.56	5.83	5.27
10	40.0000	1.50000	7.30	7.70	6.25	6.16
11	20.0000	1.00000	8.20	7.62	2.88	2.84

extract concentration was higher compared to other three substrates. On the contrary, mixed substrate crushed sorghum + rice bran showed poor sporulation at 20% moisture content and required moisture content level of 40% for maximum conidial yield of 6.25×10^9 conidia/gm and the yeast extract concentration was 1.5% (Table 3). Low moisture content results in poor nutrient diffusion and on the other hand high moisture content can lead to particle clumping and reduced gas transfer (Moo-Young et al., 1983). Addition of yeast extract helped in increased vegetative growth and sporulation for *Alternaria* sp. (Rajderkar, 1966). Nelson et al. (1996) observed that *Beauveria brongniartii* conidiospores were efficiently produced on rice supplemented with 5% yeast extract. Ye et al. (2006) reported 2.4×10^{12} conidia/kg rice was harvested from the seven day cultures of *B. bassiana* using SSF. Crushed sorghum aided with 1% yeast extract was found to be effective for the conidia production of entomopathogenic fungus *Nomuraea rileyi* (Vimala et al., 2000). Bharati et al. (2007) investigated the conidial yield of *Metarhizium anisopliae* on different crushedcereals and agro wastes. Among the cereals tested, crushed sorghum supplemented with 1% yeast extract produced 13.68×10^8 conidia/gm and rice bran supplemented with 10% molasses was the best agro waste for conidia production. In this study, yeast extract concentration ranging from 1 - 2% was sufficient to support the vegetative growth and yielded excellent sporulation. Similar results were reported by Bhanu et al. (2007), for solid substrate rice and sorghum.

The responses of the statistical design were fitted into the second order polynomial equation (equation ii). The significance and adequacy of the model was checked by Fishers F-test value of Analysis of Variance (Table 4). The F value is the variance ratio between group and within group variability (S_B^2 / S_W^2) that actually measures the ability of factors to describe the variation in the data about its mean. The model F value for mixed substrate rice + wheat bran was 10.91 and low probability value of 0.010, accounts for the high significance of the model. The model F and P values for other substrates also correspond to high significance of the respective models. The multiple regression analysis is also represented in Table 4. The coefficient of determination (R^2) for mixed substrate rice + wheat bran was 0.9160, which indicates almost 92% variability in the response is explained by the model. The coefficient of determination (R^2) for rice, crushed sorghum and crushed sorghum + rice bran was 0.9291, 0.9134, and 0.9021, respectively, which describes goodness of fit of the model. These models can be used to navigate the design space.

The significance of the linear, squared and interaction terms were determined by the Student's t test and p values (Table 5). The larger the t value and lower the p value ($p < 0.1$), more significant are the coefficients. For the model using rice as a solid substrate, only linear term B that is, yeast extract concentration and squared terms, A^2 (moisture content × moisture content) and B^2 (yeast extract × yeast extract) are significant. For substrate sorghum (crushed), linear term A and interaction term AB are the significant model terms only and this implies that moisture content and interaction between moisture content and yeast extract have significant effect on response, that is, conidial yield. In case of other two mixed substrates, that is, crushed sorghum + rice bran and rice + wheat bran; linear term A, that is, moisture content, squared term A^2 (moisture content × moisture content) and squared terms A^2, B^2 were significant model terms, respectively. Although yeast extract is essential in the vegetative growth of the fungus but except for

Table 4. ANOVA table and regression analysis.

Substrate	Source	SS (Sum of square)	MS (Mean of square)	F (Freedom)	P (Probability)	Regression equation (in terms of coded factors)	R^2	Adjusted R^2	Adequate precision (signal to noise ratio>4)
Rice	Model	93.51	18.70	13.11	0.007	$Y = 8.43-0.78A-1.70B-0.99AB+2.41A^2+2.87B^2$	0.92	0.86	9.23
	Residual	7.13	1.43						
	Total	100.64							
Crushed sorghum	Model	12.63	2.53	10.55	0.011	$Y = 7.70-1.04A+0.079B-0.86AB-0.33A^2+0.15B^2$	0.91	0.83	10.87
	Residual	1.19	0.24						
	Total	13.83							
Crushed sorghum + Rice bran	Model	18.78	3.76	9.22	0.015	$Y = 6.16+1.08A-0.015B-0.46AB-1.21A^2-0.58B^2$	0.90	0.80	8.37
	Residual	2.04	0.41						
	Total	20.82							
Rice + Wheat bran	Model	2.20	0.44	10.91	0.010	$Y = 27.03+0.67A+0.32B-0.50AB-5.24A^2-4.66B^2$	0.92	0.83	7.69
	Residual	0.20	0.04						
	Total	2.41							

substrate rice, it did not exert any significant effect except for the interaction terms (AB).

Contour plots and three dimensional response surface plots were used to determine the relationship between two predictor variables and also the optimum moisture content and yeast extract concentrations. 3D plot of conidial yield for substrate rice shows that decreasing both the variables increases the response, that is, conidial yield, giving highest response value at 1% yeast extract concentration (Figure 1).

The contour plot with minimax approach or saddle point for substrate crushed sorghum shows that simultaneous increase or decrease of both the variables leads to a decrease in the response whereas decreasing one variable and increasing the other at the same time increases the response variable. In Figure 2, it was noticed that decreasing the moisture content to around 20% and increasing the yeast extract concentration to about 2% showed the highest response. For the substrate crushed sorghum + rice bran, continuous increase in both factors increases the response, showing optimum conidial yield at 40% moisture content and 1.5% yeast extract (Figure 3).

Mixed substrate rice + wheat bran showed maximum conidial yield almost at design center point, that is, at 35% of moisture content and 1.5% of yeast extract concentrations (Figure 4). Studies on shelf life of the conidia produced by SSF are also important as the bioactivity of the conidia is to be preserved and should retain viability over an extended period of storage. Research is in progress in order to study the shelf life of the conidia produced by SSF and this would enhance and stabilize the performance of the conidia as a commercialized product to be based in the integrated pest management programs.

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Table 5. Model coefficients estimated by multiple linear regressions.

Substrate	Terms	Coefficient	T-value	P- value
Rice	Constant	8433333333	12.242	0.000
	A	-783382034	-1.857	0.122
	B	-1701449990	-4.033	0.010
	A^2	2408645833	4.797	0.005
	B^2	2864895833	5.706	0.002
	AB	-992500000	-1.664	0.157
Sorghum	Constant	7700000000	27.253	0.000
	A	-1038871933	-6.005	0.002
	B	79010191	0.457	0.667
	A^2	-333750000	-1.621	0.166
	B^2	148750000	0.722	0.502
	AB	-857500000	-3.505	0.017
Sorghum + Rice bran	Constant	6.15833	16.712	0.000
	A	1.08229	4.796	0.005
	B	-0.01483	-0.066	0.950
	A^2	-1.20729	-4.495	0.006
	B^2	-0.58229	-2.168	0.082
	AB	-0.46250	-1.449	0.207
Rice + Wheat bran	Constant	27033333333	23.295	0.000
	A	671446609	0.945	0.388
	B	319454365	0.450	0.672
	A^2	-5235416667	-6.190	0.002
	B^2	-4660416667	-5.510	0.003
	AB	-500000000	-0.498	0.640

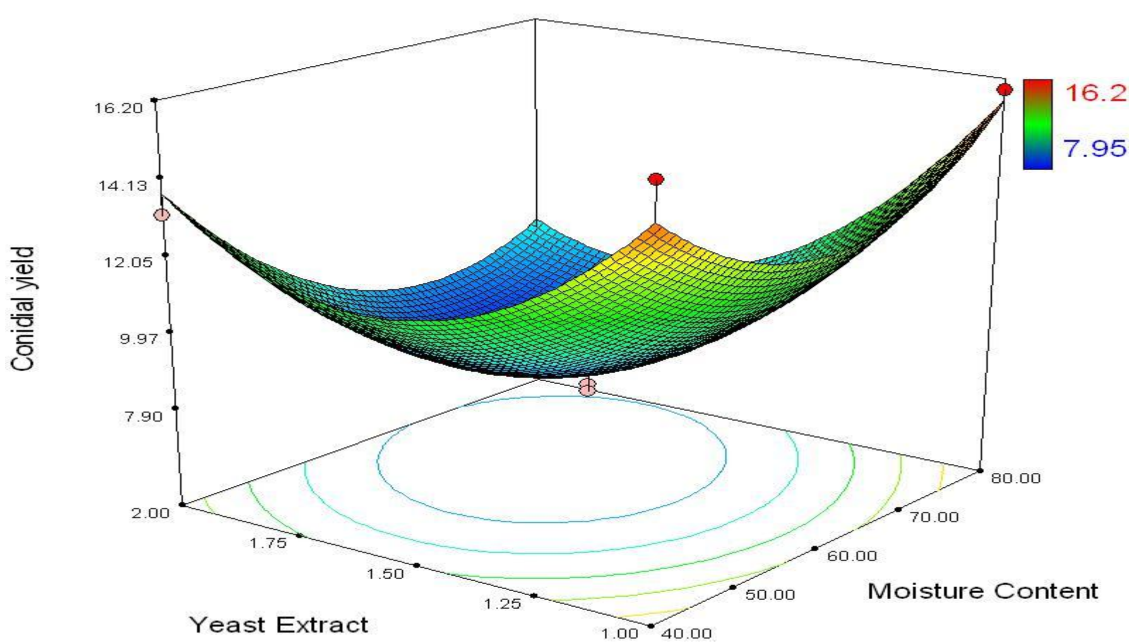

Figure 1. Response surface and contour plot showing the effect of yeast extract and moisture content on the conidial yield of *B. bassiana* using rice as substrate. (Conidial yield represented to be multiplied by 10^9).

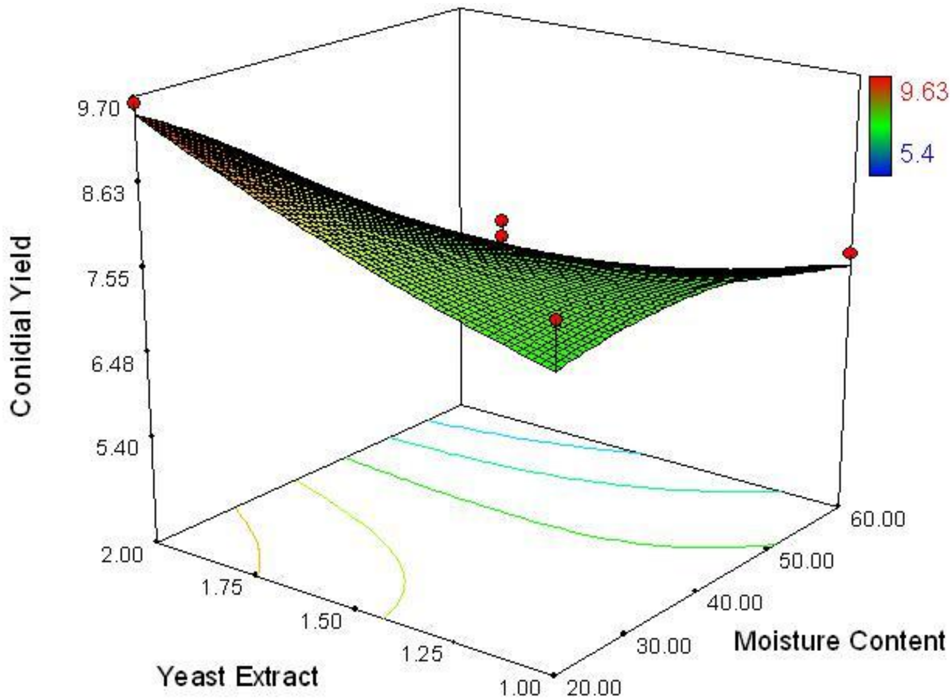

Figure 2. Response surface and contour plot showing the effect of yeast extract and moisture content on the conidial yield of *B. bassiana* using crushed sorghum as substrate. (Conidial yield represented to be multiplied by 10^9).

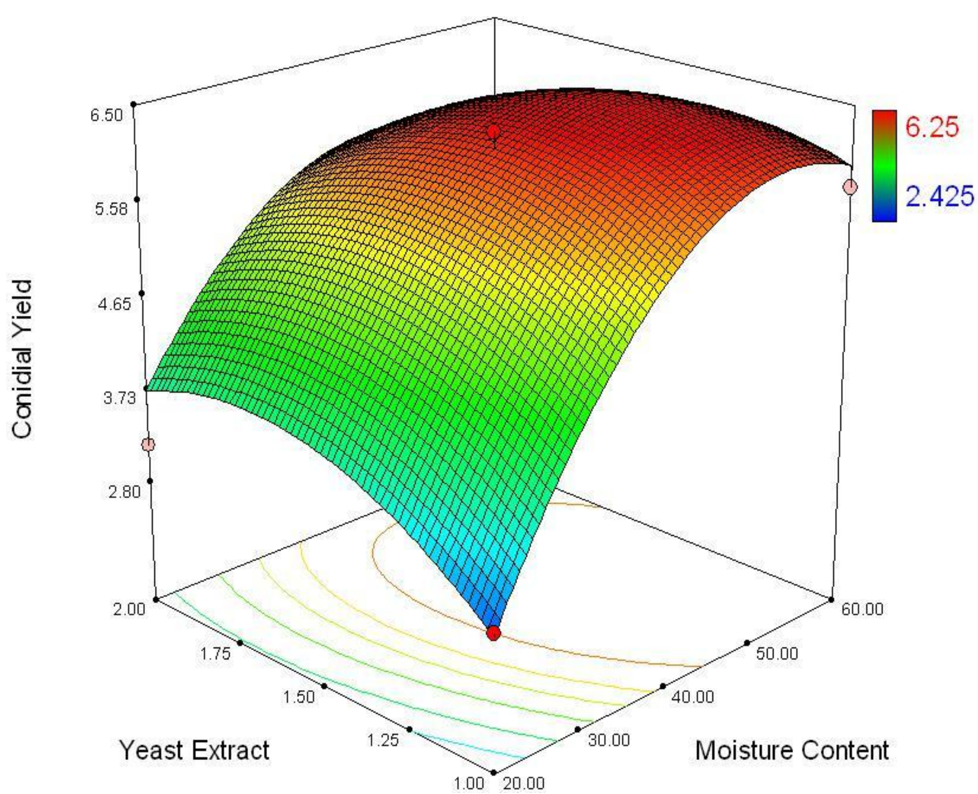

Figure 3. Response surface and contour plot showing the effect of yeast extract and moisture content on the conidial yield of *B .bassiana* using crushed sorghum+ rice bran as substrate. (Conidial Yield represented to be multiplied by 10^9).

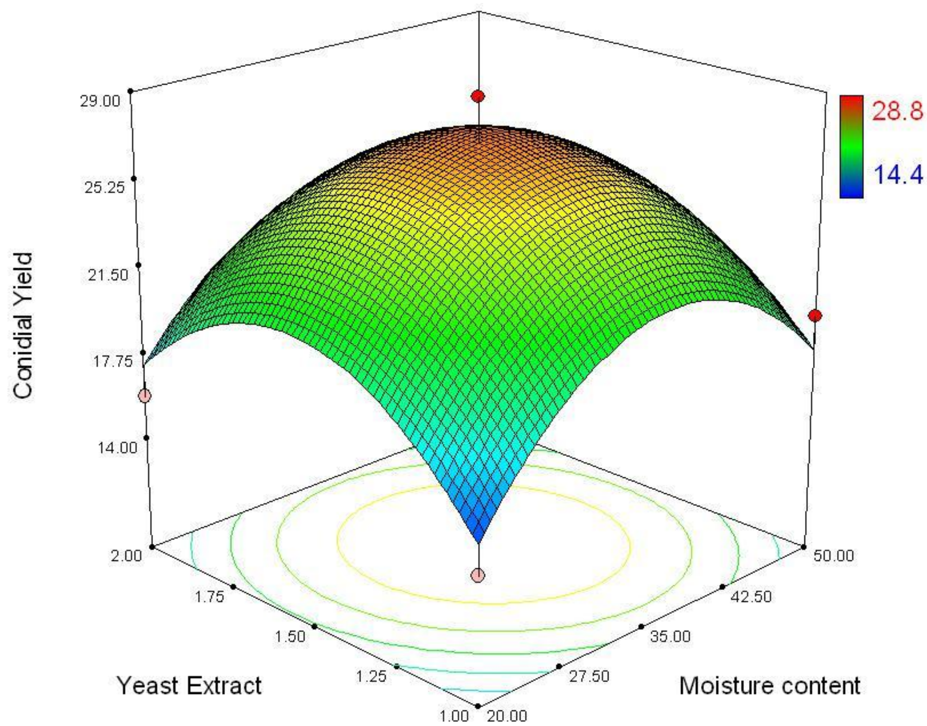

Figure 4. Response surface and contour plot showing the effect of yeast extract and moisture content on the conidial yield of *B. bassiana* using rice+ wheat bran as substrate (Conidial Yield represented to be multiplied by 10^9).

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