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RESEARCH ARTICLE

OPTIMIZATION OF RED PIGMENT PRODUCTION BY ENDOPHYTIC *PEZICULA* SP. BDF9/1 THROUGH OVAT AND RSM METHODOLOGY

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ABSTRACT

Optimization of initial pH of the medium, suitable carbon, nitrogen sources, temperature, metal ions, phosphate source and C/N ratio for improvement in red pigment production by Pezicula sp. BDF 9/1 was carried out. Carbon sources like fructose and glucose were found important for growth and pigment production. Among the nitrogen sources glycine was found suitable for growth whereas maximum pigment production occurred with $NaNO_3$. The pronounced formation of red pigment was found after the incubation time 20 days, initial medium pH 5.0 and at 23 °C. C/N ratio 3: 0.8 was found suitable for both mycelial growth and pigment production. RSM optimization based on Box-Behnken Design (BBD) revealed that most influencing interaction of the variable on pigment production is in between temperature and glucose concentration followed by pH and temperature, and incubation time and pH. Furthermore, RSM study stated that maximum biopigment production (1.695 OD at 500nm) can be achieved when 20.15 days incubation time, 23.2 °C temperature, 5.07 pH and 2% glucose were provided. The experimental result also very close to the predicted optimized response which validates the employment of the RSM in this case.

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INTRODUCTION

Pigments are those chemical which absorb lights belong to the visible wavelength. Pigments are mainly two types, synthetic and natural. Synthetic pigments are widely used as colouring agents in industries for the production of commercial products. Some of the synthetic pigments are toxic, carcinogenic, teratogenic, allergic effects, and cause severe damage of vital organs (Duran *et al.,* 2002, Fabre *et al.,* 1993, Merlin *et al.,* 1987). Living organisms such as plants, animals, fungi, and other microorganisms produce natural pigments. Number of micro-organisms which have the ability to produce pigments in high yields, including species of *Monascus* (Yongsmith *et al.,* 1994; Hajjaj *et al.,* 2000), *Serratia* (Williams *et al.,* 1971; Kim *et al.,* 1998) and *Streptomyces* (Oshima *et al.,* 1981) and among them *Monascus* have special attention because they have the capability of producing different coloured pigments showing high chemical stability (Mak *et al.,* 1990, Hajjaj *et al.,* 2000). In this way the pigments from microbial sources are a good alternative that could easily be produced in high yields and capability of producing different coloured pigments.

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Process optimization is considered very essential in any microbial metabolite production as product yield massively varies between pre and post optimization (Banerjee *et al.,* 2009). Several reports have indicated that pigment production in submerged culture is affected by numerous environmental factors, likely carbon source and medium pH etc. (Carels and Shepherd, 1997; Chen and John, 1993; Hamdi *et al.,* 1996; Hamdi *et al.,* 1997; Unagul *et al.,* 2005; Visalakchi *et al.,* 2009), and hence optimization of production conditions is necessary. In this connection, the present study deals with the optimization of pigment production by newly isolated endophytic fungi employing classical one variable at a time (OVAT) and statistical approach (RSM).

MATERIALS AND METHODS

Microorganism and Culture condition

Pezicula sp. BDF 9/1 (Gen Bank Accession Number KP234255) an endophytic fungi which have potentiality to produce pigment was newly isolated from plant sample of Paschim Medinipur District, West Bengal, India. The stock culture was maintained on potato dextrose agar slant. The organism was initially grown on Potato Dextrose agar medium at 23°C for 7 days and then transferred to the liquid Potato

Dextrose broth culture medium. The culture was grown in 250 ml flasks containing 50 ml of Potato Dextrose broth medium at 23^{o}C on a rotary shaker (150 rpm) for 7 days. Experiments were performed at in triplicate to ensure reproducibility.

Optimization

The physico-chemical condition for fungal bio-pigment production was optimized by following one variable at a time (OVAT) and Response Surface Methodology (RSM) approach sequentially.

One variable at a time OVAT Optimization

During OVAT optimization, effect of incubation time (4-24 days), incubation temperature (30-35 °C), pH (3-7), effect of additional carbon and nitrogen source, metal ion source, phosphate source were optimized in step wise manner. In each step only one parameter was considered for variation keeping rest of the parameters at a fixed level, and after optimization of the tested parameter, the level kept constant in subsequent steps. After OVAT optimization, the four most influencing factors are chosen and the levels of the factors are optimized through RSM.

RSM optimization through Box Behnken Design (BBD)

Optimization of maximal pigment production by the fungal strain through statistical approach was carried out by employing Box-Behnken Design (BBD) and Response Surface Methodology (RSM). The statistical package used was Design expert 8.0. The design was used for creating the quadratic response model, where each independent variable in the design are at three different levels,-1,0,+1 representing the lower, middle and higher levels respectively. A design model with 29 runs was generated and experiment was carried out accordingly. The pigment production values recorded were taken as the dependent variable or response (Y). Regression analysis was performed and data were then used to fit a quadratic equation by multiple regression procedure (Mahapatra and Banerjee, 2013). This resulted in an empirical model that related the response measured to the independent variables of the experiment. The performance of the system is explicated by the subsequent second order polynomial equation:

$$Y= \beta_o + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2$$

Where Y is the predicted response or dependant variable, xi and xj are independent factor, β_o is the intercept of the regression equation, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient. Analysis of variance (ANOVA) was performed and 3-dimentional response surface were plotted to study the interaction among various physico-chemical factors employed. In order to practically validate the response surface model, a experiment in triplicate was carried out by providing the RSM optimized conditions.

Isolation and estimation of the red pigment

The culture broth was filtered and centrifuged at 10,000 xg for 20 min. and the supernatant was filtered through a filter paper (No.2; Whatman). The concentration of red pigment was estimated measuring the absorbance of the culture filtrate at 500 nm using Spectrophotometer. Blank was prepared with uncultured broth.

RESULTS AND DISCUSSION

Effect of Incubation time on the mycelial growth and red pigment production

To investigate the effect of incubation time on mycelial growth and pigment production, *Pezicula* sp. BDF 9/1 was cultivated in the potato dextrose broth medium for varied time (4 to 24 days) at 23 ^{o}C in shake flask culture (150 rpm). Maximum pigment production and mycelia growth was noticed after 20 days incubation (Fig. 1). It was found that there was a smooth increase in mycelia growth and pigment production from 4 to 20 days and a slight decrease from 20 to 24 days. Mycelia growth (5.567±0.379 g/l) and pigment production (OD 1.052±0.054) reached the highest level after 20 days of cultivation.

Table 1. Effect of C/N ratio on the pigment production of *Pezicula sp.* BDF 9/1

Glucose (%)	$NaNO_3$ (%)			
	0.2	0.6	0.8	1.2
1	0.552±0.012	0.592±0.008	0.721±0.023	0.781±0.015
2	0.892±0.019	1.466±0.031	1.498±0.027	1.212±0.005
3	1.102±0.013	1.512±0.004	1.592±0.018	1.507±0.025
5	0.612±0.028	0.689±0.011	0.712±0.035	0.521±0.021

Table 2. Effect of C/N ratio on the mycelial growth of *Pezicula sp.* BDF 9/1

Glucose (%)	$NaNO_3$ (%)			
	0.2	0.6	0.8	1.2
1	5.4±0.005	5.6±0.017	5.7±0.023	5.9±0.032
2	6.2±0.015	6.6±0.011	6.8±0.026	6.5±0.031
3	6.4±0.029	6.85±0.016	7.01±0.018	6.92±0.009
5	5.5±0.016	5.5±0.025	5.8±0.009	4.9±0.027

Table 3. Minimum and Maximum ranges for the factors selected in Box-Behnken Design for optimization of pigment production by endophytic *Pezicula* sp. BDF9/1

S.No.	Factors	Level	
		Minimum (-1)	Maximum (+1)
1	Incubation time (day)	15	25
2	pH	3	7
3	Temperature (^{o}C)	15	31
4	Glucose (%)	1	3

After 20 days the fungal strain was entered its death phase and as a consequence, biomass decreased. Amongst the several fungal physiological properties, the incubation time usually plays an important role in fungal development (Glazebrook *et al.,* 1992; Bae *et al.,* 2000). Gunasekaran *et al.,* 2008 reported that *Penicillium* sp. was cultivated in the optimal medium with different inoculums ages from 2 to 6 days old culture at 30 ^{o}C in shake flask cultures. Earlier Cho *et al.,* (2002) reported that *Paecilomyces sinclairii* produced maximum pigment after 3 days of fermentation. Their findings were quite dissimilar with our experimental result.

Table 4. Effect of Individual variable on pigment production by *Pezicula* sp. BDF9/1 studied using Box-Behnken Design experiment

Run	Time(day)	pH	Temp (degree)	Glucose (%)	Pigment OD at 500 nm	
					Actual	Predicted
1	20	5	31	3	0.925	0.909
2	20	3	31	2	0.810	0.780
3	15	5	15	2	0.550	0.525
4	20	7	15	2	0.760	0.758
5	25	5	15	2	0.620	0.612
6	15	5	31	2	0.580	0.597
7	25	5	31	2	0.570	0.604
8	25	5	23	3	0.680	0.653
9	15	5	23	1	0.578	0.574
10	20	3	23	3	0.740	0.765
11	20	5	23	2	1.695	1.692
12	20	5	23	2	1.712	1.692
13	20	5	23	2	1.680	1.692
14	15	3	23	2	0.560	0.566
15	20	5	23	2	1.675	1.692
16	15	5	23	3	0.635	0.629
17	25	5	23	1	0.670	0.644
18	25	3	23	2	0.650	0.660
19	20	7	23	3	0.825	0.813
20	20	7	31	2	0.715	0.719
21	15	7	23	2	0.615	0.624
22	20	3	15	2	0.712	0.676
23	20	5	23	2	1.701	1.692
24	20	5	15	3	0.570	0.602
25	20	7	23	1	0.760	0.744
26	20	3	23	1	0.750	0.771
27	20	5	15	1	0.810	0.845
28	20	5	31	1	0.615	0.602
29	25	7	23	2	0.610	0.623

Table 5. ANOVA for Response Surface Quadratic regression model using Box-Behnken design for pigment production by *Pezicula* sp BDF9/1

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	4.4702429	14	0.319303061	395.301112	1.10301E-15
A-Time	0.006627	1	0.006627	8.204307398	0.012492055
B-pH	0.0003308	1	0.00033075	0.409472563	0.532570177
C-Temperature	0.0031041	1	0.003104083	3.842893294	0.070168329
D-Glucose	0.003072	1	0.003072	3.803173733	0.071470207
AB	0.0022563	1	0.00225625	2.793265213	0.116854523
AC	0.0016	1	0.0016	1.980819653	0.181117613
AD	0.0005523	1	0.00055225	0.683692283	0.422187857
BC	0.0051123	1	0.00511225	6.329028293	0.02470268
BD	0.0014063	1	0.00140625	1.740954773	0.208190008
CD	0.075625	1	0.075625	93.62467889	1.40256E-07
A^2	2.4225986	1	2.422598602	2999.206825	9.81083E-18
B^2	1.38755	1	1.38755	1717.803944	4.74323E-16
C^2	1.5974422	1	1.597442163	1977.653018	1.78184E-16
D^2	1.3495629	1	1.349562926	1670.775479	5.75194E-16
Residual	0.0113085	14	0.000807746		
Lack of Fit	0.0103873	10	0.001038725	4.510312636	0.079813065
Pure Error	0.0009212	4	0.0002303		
Cor Total	4.4815513	28			
C.V. %			3.3270315		
R-Squared			0.997476666		
Adj R-Squared			0.994953332		
Pred R-Squared			0.986328409		
Adeq Precision			57.0922815		

Effect of incubation temperature on the mycelial growth and red pigment production

A change in growth temperature had noteworthy effect on mycelia growth and pigment production. *Pezicula* sp. BDF 9/1 was cultivated at different temperatures (20-35^{o}C) for testing of pigment production and growth. The optimal temperature for pigment production was found to be 23^{o}C (OD 1.187±0.065), while the mycelium grew (6.150±0.078 g/l) better at 26^{o}C (Fig. 2). It was found that the organism was able to grow at a temperature range 20-35 ^{o}C. These findings are correlated to secondary metabolite production in mesophilic fungi that have optimal temperature range for growth different from that of the secondary metabolite production (Griffin, 1994; Hajjaj *et al.,* 1999, Unagul *et al.,* 2005). Unagul *et al.,* (2005) reported optimum temperature for pigment production by *Cordyceps unilateralis* was at 30-32^{o}C, with an optimal

temperature for growth of 26°C. It might be due to the fact that temperature played a key role in regulation of different enzyme activity responsible for mycelia growth and pigment production.

Effect of medium pH on the Mycelial growth and red pigment production

To investigate the effect of pH on mycelia growth and pigment production by *Pezicula* sp. BDF 9/1, it was grown at different initial pH values (3-7) in shake flask culture. The maximum pigment production (OD 1.206±0.023) and mycelia growth (6.250±0.066 g/l) was found at initial medium pH value of pH 5 (Fig. 3). Below the pH 5 and above the pH 5 pigment production was strongly inhibited. Many investigators claimed that the different morphology of fungal mycelia under a different initial pH value was the critical factor in biomass accumulation and pigment production (Duarte *et al.,* 2003, Unagul *et al.,* 2005). The medium acidic pH may affect cell membrane function, cell morphology and structure, the solubility of salts, the ionic state of substrates, the uptake of various nutrients and product biosynthesis. In general, cell can only grow within a certain pH range, and metabolite formation is also often affected by pH (Kim *et al.,* 2003). Earlier Suhr *et al.,* (2002) and Cho *et al.,* (2002) reported maximum pigment production at pH 4 with *Penicillium caseifulvum* and *Paecilomyces sinclairi* respectively. Several other reports also concluded that different fungi needs acidic pH during submerged fermentation for pigment production (Bae *et al.,* 2000). It was also reported that fungi require more acidic pH optima for growth in submerged culture and that an increase in initial pH was found to stimulate the accumulation of metabolites (Wang and McNeil, 1995; Shu and Lung, 2004).

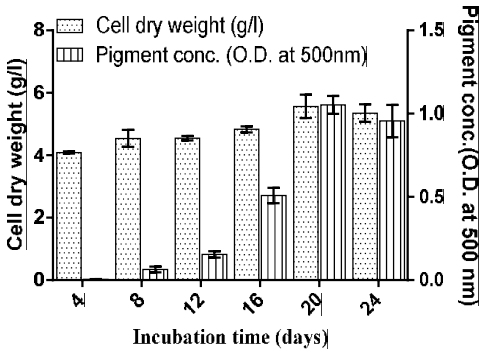


Fig. 1. Effect of Incubation time on the mycelial growth and red pigment production by *Pezicula* sp. BDF9/1

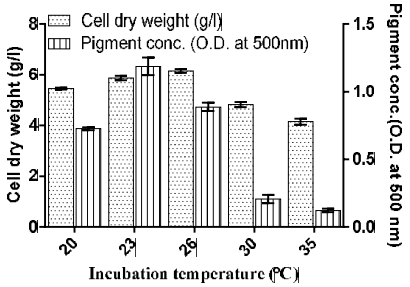


Fig. 2. Effect of incubation temperature on the mycelial growth and red pigment production by *Pezicula* sp. BDF9/1

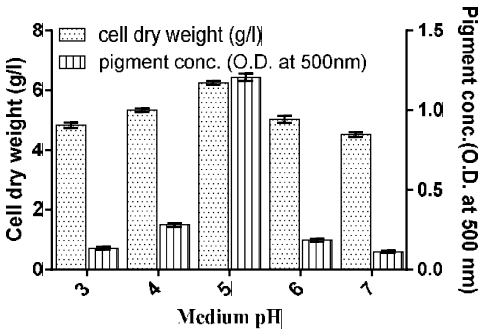


Fig. 3. Effect of medium pH on the mycelial growth and red pigment production by *Pezicula* sp. BDF9/1

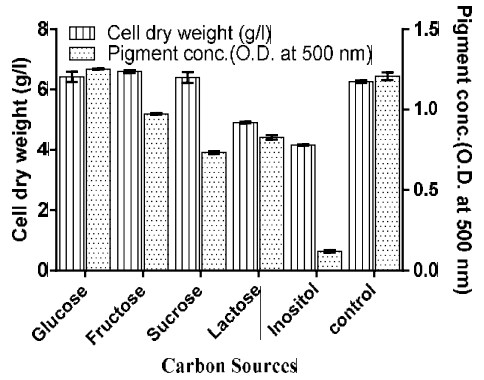


Fig.4. Effect of additional carbon source on the mycelial growth and red pigment production by *Pezicula* sp. BDF9/1

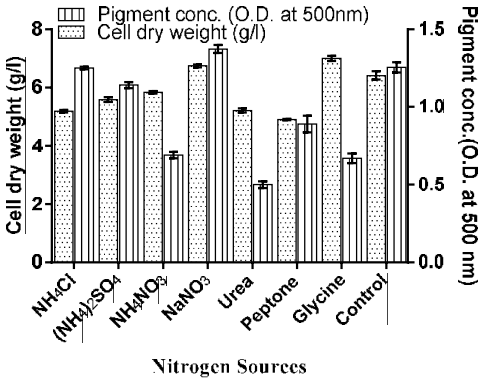


Fig. 5. Effect of additional nitrogen source on the mycelial growth and red pigment production by *Pezicula* sp. BDF9/1

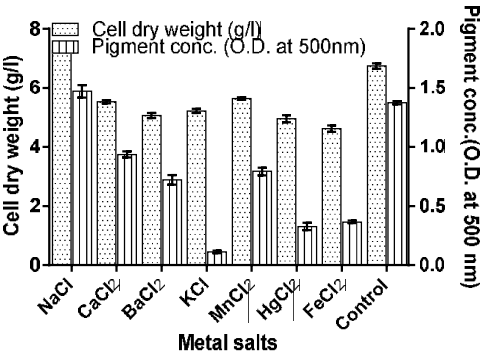


Fig. 6. Effect of metal salts on the mycelial growth and red pigment production by *Pezicula* sp. BDF9/1

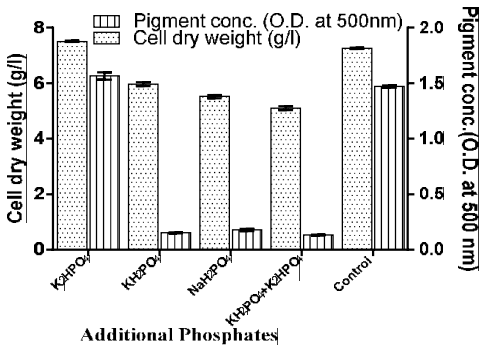
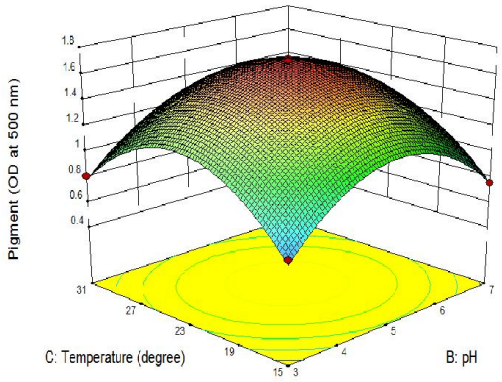
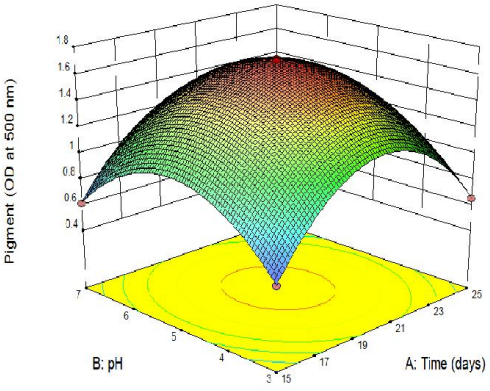


Fig. 7. Effect of additional phosphate source on the mycelial growth and red pigment production by *Pezicula* sp. BDF9/1

(a)



(b)



(c)

Fig. 8. Effect of (a) temperature (^{0}C) and glucose (%) (b) temperature (^{0}C) and pH (c) time (days) and pH on pigment production by *Pezicula* sp. BDF9/1

Effect of additional carbon source on the mycelial growth and red pigment production

Carbon source is the important factor for microbial growth as well as pigment production. In our experiment *Pezicula* sp. BDF 9/1 was cultivated in the potato dextrose broth medium containing various additional carbon sources (2%). Among the five carbon sources tested, glucose, fructose and sucrose were found more favourable for mycelia growth of *Pezicula* sp. BDF 9/1. Maximum pigment was produced (OD 1.252±0.002) in medium containing glucose as a carbon source (Fig. 4). Glucose, usually an excellent carbon source for growth, interfered with the biosynthesis of many secondary metabolites. These results suggested that the suitable concentration of glucose for pigment production has 20 g/l. Earlier Cho *et al.* (2002) reported maximum pigment production with soluble starch medium from *Paecilomyces sinclairii*, whereas Tseng *et al.* (2000) reported fructose as a suitable carbon source for maximum pigment production from *Monascus purpureus*.

Effect of additional nitrogen source on the mycelial growth and red pigment production

It is well known that utilization of different nitrogen sources in fermentation had effect on mycelial growth and pigment production. A stimulatory effect of additional nitrogen source on pigment production has been reported (Hamdi *et al.,* 1997). For the determination of a suitable additional nitrogen source for the red pigment production and mycelia growth, *Pezicula* sp. BDF 9/1 was cultivated in the potato dextrose broth medium containing various nitrogen sources. Among the nitrogen sources glycine was found suitable for growth (6.997±0.087 g/l) whereas maximum pigment production (OD 1.373±0.026) occurred with $NaNO_3$ (Fig.5). As shown in the Fig.5, organic nitrogen sources yielded higher mycelia growth compared with the other inorganic nitrogen sources. It has been reported that various kinds of amino acids containing organic nitrogen sources are essential for secondary metabolite biosynthesis (Jung *et al.,* 2003). Our findings were quite different from Cho *et al.* (2002), Chen and Johns (1993) reported that various types of organic nitrogen supported greater pigment production in many kinds of pigment producing fungi.

Effect of metal salts on the mycelial growth and red pigment production

Different metal ions (mostly chloride salt) were added in potato dextrose broth medium to study their effects on the mycelia growth and pigment production in several microorganisms (Bau and Wong, 1979; An *et al.,* 2001). The maximum pigment production (OD value 1.472±0.052) and mycelia growth (7.260±0.032 g/l) was found when sodium chloride was added in the medium (Fig. 6). It has been reported that bio-elements are one of the important factors affecting pigment production in several microorganisms (Fogarty *et al.,* 1996). An interaction between pigment production and metal ions were reported by Fogarty and Tobin (1996). Metals such as Fe^{2+}, Mg^{2+}, Zn^{2+} ions played a significant role in the increase of naphthoquinine type red pigment formation (Mendentsev *et al.,* 1998). Bau and Wong

(1979) reported a detrimental effect of zinc ions on *Monascus* pigment. According to the observations of An *et al.,* (2001), iron decreased astaxanthin production and its composition in *P. rhodozyma.*

Effect of additional phosphate source and C/N ratio on the mycelial growth and red pigment production

Different inorganic phosphates like K_2HPO_4, KH_2PO_4 and their mixture were supplemented separately to investigate their effect on the mycelia growth and pigment production. It was observed that K_2HPO_4 was found suitable for growth (7.507±0.026 g/l) as well as pigment production (OD 1.565±0.032) (Fig. 7). C/N ratio usually influenced the mycelia growth and pigment production. The effect of the C/N ratio on pigment production was investigated using glucose-$NaNo_3$ containing potato dextrose broth medium. C/N ratio 3: 0.8 was found suitable for both mycelia growth (7.01 ± 0.018 g/l) (Table 2) and pigment production (O.D 1.592 ±0.018) (Table 1). Nam and Rhee (1991), reported that the carotenoid content of the pink pigment decreased as the C/N ratio increased.

Optimization by Box-Behnken design

For optimization, conventional OVAT approach had some limitations, especially for interpretation about interactions among different significant factors. To resolve this type of problem, a coupled OVAT and RSM was strongly suggested by different researchers (Feng *et al.,* 2010; Xiao *et al.,* 2010). In fermentation technology, RSM was applied adequately as it considered most effective, economic and reasonable design for integrate test analysis and mathematical modelling with suggested values of most probable optimum conditions of significant variables and their corresponding product yield level (Zhang *et al.,* 2012). Here, after OVAT, RSM was adopted for optimization of pigment production by *Pezicula* sp. BDF9/1. A three level Box-Behnken design of four factors Table 3 (Incubation time, pH, Temperature, Glucose concentration) with five replicates at the centre point of each factor was implicated as model for analysis of pigment production. Table 4 showed the experimental design including predicted and measured values of pigment production than any others. The predicted response Y for pigment production obtained from multiple regression analysis on the experimental data was described as follows:

$$Y_{PIGMENT} = 1.6926 + 0.0235A + 0.00525B + 0.0160833C + 0.016D - 0.02375AB - 0.02AC - 0.01175AD - 0.03575BC + 0.01875BD + 0.1375CD - 0.611133A^2 - 0.462508B^2 - 0.496258C^2 - 0.456133D^2$$

Where $Y_{PIGMENT}$ is the predicted pigment yield; A, B, C, D are coded factors of incubation time (day), pH, incubation temperature (°C), and glucose (g%), respectively. A regression analysis was performed to analysis of goodness of fit of the RSM with the experimental output (Table 5). The F- test data was checked and the model F-value of 395.30 for pigment indicated that the model was significant. The adjusted determinant coefficient (R^2_{ADj}) of the polynomial model was measured for testing the goodness-of-fit of the regression equation. The value of R^2_{ADj} was determined as 0.9949 for pigment which indicated that there was a high degree of correlation between the experimental and predicted values, and 99.49% variations in the response could be explained by that second order polynomial prediction equation. Adequate precision measured the signal-to-noise ratio and value greater than 4 is desirable. In the present experimental model the ratio 57.092 indicated an adequate signal and the model could be used to navigate the design space. The lack of fit F-value of 4.51 implies the lack of fit was not significant relative to the pure error and the fitness of the model was good. The resulted model P-value indicated that the model equation was suitable to describe the response of experiment pertaining to pigment production. The P-value of lack of fit for the equation 4.5103 was higher than 0.05 which indicated a high degree of accuracy and consistency of the experimental values. The P-value of prob> F less than 0.05 indicated model terms were significant. Furthermore, the interactions among the variables for the production of pigment were also analyzed. Based on the constructed model, three dimensional response surface plots were constructed to get better understanding of the effects of the variables and their interactions on the yield of pigment production. The 3D plots visualized interactions between the variables and helped to rapidly determine the optimum values of each variable for maximum pigment production. The most sound interaction was found between temperature (^oC) and glucose concentration (g%); pH and temperature (^oC); incubation time (day) and pH for pigment production ($P<0.05$) (Fig. 8). After analysis, the model predicted that maximum production of pigment (1.692 O.D. at 500nm) can be realized if incubation time of 20.15 days, temperature of 23.2 ^oC, pH 5.07, glucose concentration 2% were provided. Validation of the predicted optimized conditions was carried out in laboratory flask in triplicate. Experimental result revealed that pigment production of 1.695 O.D. at 500nm was practically realized which is very close to the predicted value.

Conclusion

Optimization of initial pH of the medium, incubation temperature, incubation time and suitable carbon, nitrogen sources, metal ions, phosphate source, and C/N ratio greatly influenced the red pigment production by *Pezicula* sp. BDF 9/1. Both OVAT and RSM successively applied to optimize the pigment production condition. At optimized condition 1.695 (O.D. at 500 nm) red pigment was produced.

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