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Optimization of carotenoid pigment production by yeast *Sporobolomyces* sp. using response surface methodology

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Abstract
The Response Surface Methodology (RSM) technique was performed to increase the carotenoid pigment production of *Sporobolomyces* sp. The Central composite design involving the variables pH (A), fermentation time (B) and incubation temperature(C) for the production of carotenoid pigment by *Sporobolomyces* sp was standardized. Data analyzed using a second order polynomial equation resulted in the optimized process condition for pigment production was 120 h growth at 25 °C in a Yeast extract dextrose broth of pH 5.0. Using this experimental design, the total carotenoid production yield increased from 674.34 to 990.87 µg g^{-1}. The model showed that the value of R^2 0.9986 was high and p- value of interaction of variance was <0.0001. Hence the model can said to be significant.

Keywords: Carotenoid pigment, optimization, response surface methodology, *Sporobolomyces* sp

1. Introduction
Now a days biological sources of carotenoid pigment received major focus because of the stringent rules and regulations applied to chemically synthesized /purified pigments. Compared with the extraction from vegetables or chemical synthesis the microbial production of carotenoid pigment is of paramount interest, mainly because of overcoming the problems of seasonal and geographic variability in the production and marketing of the colourants of plant origin and because of the economic advantages of microbial processes using natural low-cost substrates. Yeasts are more convenient than algae or molds for large scale production in fermenters, due to their unicellular nature and high growth rate (Frengova and Beshkova, 2009) [5]. Several yeast species belonging to the genera *Rhodotorula* and *Phaffia* are considered to be as potential pigment sources.
Designing a fermentation medium is a critical and important process as the medium composition can significantly affect the product yield. Important medium variables are screened by response surface methodology (RSM) (Kennedy and Krouse, 1999) [6]; (Panda *et al*., 2007) [11]. The response surface methodology (RSM) can be used to evaluate the relative significance of several affecting factors and is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously. RSM has been increasingly used for an optimization process in fermentation (Buchanan and Philips, 1990) [2]. Thus, RSM experimental design is an efficient approach to deal with a large number of variables and there are several reports on application of RSM for the production of primary and secondary metabolites through microbial fermentation (Ergun and Mutlu, 2000) [4]; (Li *et al*., 2001) [8] such as enzymes, biomass and spores production (Xu *et al*., 2003) [13]; (Yu *et al*., 1997) [14].

2. Materials and Methods
2.1 Microorganism and Culture Conditions: The microorganism used in this study was isolated from phyllosphere surface of rice plant collected from wet land, Tamil Nadu Agricultural University, Coimbatore (India). Stock culture was maintained on yeast malt extract agar slants at 4ºC after being incubated at 25-30ºC for 4-5 days. The nutrient broth for liquid culture contained 30.0 g glucose, 2.5 g $(NH_4)_2SO_4$, 1.0 g K_2HPO4, 0.5 g $MgSO_4.7H_2O$ and 4.0 g yeast extract (per litre).

2.2 Flask cultivation: Optimization experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of cultivation medium. Seed culture was transferred to growth medium and for RSM experiment the yeast was grown in flask for three different pH (4,5 and 6) time intervals (96, 120 and 144 h) at three different temperature (20, 25, 30 ^{0}C) based on experimental design.

2.3 Extraction of carotenoid pigment: The yeast culture was inoculated on nutrient broth and incubated at 28±1ºC for 5 days. A known amount (500mg) of freeze-dried red yeast was hydrolyzed with 1 ml of 1N hydrochloric acid in water bath at 70 °C for one and half hour. After removal of excess acid by washing with water, the cells were soaked overnight in acetone: methanol (1:1) solution. The pigment was extracted with acetone until the entire colour was leached out from the cells. Acetone extracts were transferred to light petroleum (20ml) at (40 – 60 °C) in a separating funnel and washed thrice with distilled water. The absorbance of the light petroleum phase was documented at 474 nm. The carotenoid yield is reported on the basis of cell mass (µg g^{-1} dried cell weight) (Latha *et al*., 2005) [7].

2.4 Response Surface Methodology: Central composite design was performed for optimizing the pigment production from *Sporobolomyces* sp. Three important factors, namely pH(A), fermentation time (B) and Temperature (C), considered as operating (independent) parameters, were selected to study their effect on pigment production. Table 1 states the actual values and the coded values of the variables employed. Coded values of +1, 0 and -1 correspond to high, medium and low values of variables, respectively. Pigment yield was regarded as the response or output variable (r). The central composite design (CCD) was used to access the effects of the three input independent parameters on the desired responses and build a second order (quadratic) model for the response variable (r). The statistically designed experiments comprised 8 factorial points, 6 axial points and 6 replicates at the centre points resulting in a total of 20 experiments. The pigment yield was observed from each of the 20 experiments analysed by Analysis of Variance (ANOVA) to determine the optimum conditions. The regression analysis was performed to fit the response.

3. Results and discussion

The three independent variables (pH, temperature and time) and their concentrations at different coded and actual levels of the variables employed in the design matrix are shown in Table 1. Three level central composite design matrix and the experimental responses of the dependent variable (pigment yield) are listed in Table 2. The data obtained were used to develop models in which each dependent variable was obtained as the sum of the contributions of the independent variables through second order and interaction terms (Fig 1). Data from 20 experiments were used for the following equations, where the coded values were obtained from regression analysis for the carotenoid pigment production.

Table 1: Natural levels, codes and intervals of variation of the independent variables in the design of experiments for *Sporobolomyces* sp. pigment production

Factors	Codes	Levels					Interval of variation
		-1.681	**-1**	**0**	**+1**	**+1.681**	
pH	*P*	3.318	5	6	7	6.681	1
Temperature (ºC)	*T*	16.591	20	25	30	33.409	5
Incubation Time (h)	*I*	79.637	72	96	120	160.363	24

3.1 Optimization of carotenoid pigment production

Based on the experimental response, the carotenoid pigment produced by *Sporobolomyces* sp. ranged from 674.34 to 990.87 µg g^{-1} run 9 and 17 had the minimum and maximum pigment production respectively. The ANOVA results of quadratic regression model for pigmentation in *Sporobolomyces* sp. yield is described in Table 3. ANOVA of the regression model for pigment yield demonstrated that the model was significant due to an F- value of 2514.32 and very low probability value (p models > F- 0.0001). ANOVA for the model explained the response of thedepends variable. Table 4 showed that the experimental yields fitted the second order polynomial equation as well as indicated by R^2 value 0.9986

Table 2: Central composite design matrix of physical parameters of independent variables and their corresponding experimental and predicted yield of pigment

Run No.	Independent variables			Pigment yield (µg g^{-1})	
	pH	**Temperature (ºC)**	**Time (h)**	**Observed**	**Predicted**
1	4	20	96	748.22	749.68
2	6	20	96	880.35	877.46
3	4	30	96	703.06	705.96
4	6	30	96	753.21	753.64
5	4	20	144	698.01	697.38
6	6	20	144	880.44	881.34
7	4	30	144	730.11	732.80
8	6	30	144	840.32	838.66
9	3.31	25	120	674.34	675.88
10	6.68	25	120	868.99	870.81
11	5	16.59	120	931.44	931.93
12	5	33.40	120	857.04	857.59
13	5	25	79.63	691.90	693.05
14	5	25	160.36	721.44	720.57
15	5	25	120	989.47	991.12
16	5	25	120	980.87	989.47

17	5	25	120	990.87	989.47
18	5	25	120	989.08	989.47
19	5	25	120	987.59	989.47
20	5	25	120	990.65	989.47

Table 3: Analysis of variance (ANOVA) for optimization of physical parameters for pigmentation in *Sporobolomyces* sp.

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	Significance –*F*
Regression	2.754E+005	9	30600.94	2514.32	<0.0001
Residual	121.71	10	12.17	-	-
Cor total	2.755E+005	19	-	-	-
$R^2 = 0.9986$, adjusted $R^2 = 0.9982$					

The regression co-efficient along with the corresponding p values for the models of carotenoid pigment is presented in Table 4. It showed that the regression coefficient of all the quadratic coefficient of P, T, t were significant at 0.05 per cent level. The 3D response surfaces based on independent variables were obtained using the same software package indicated that a local optimum exists in the area experimentally investigated. Fig. 1 show three dimensional (3D response) surface plots of the effect of pH and temperature on the pigment production. As the pH increased from 4 to 5 the curve gradually increased *i.e.,* when the temperature was around 25 °C, the production of carotenoid pigment increased along with increasing incubation time.

The optimum levels in coded values for pH, temperature and incubation time were 0 and +1. The variable pH and temperature, pH and incubation time indicated that mutual interactions between these set of variables had a significant effect on the pigment yield. When the third independent variable fermentation time was kept constant at 120 h (Fig. 1), the interaction between the two variables (temperature and pH) showed that the pigment yield was sensitive even when pH and temperature were subject to small alterations.

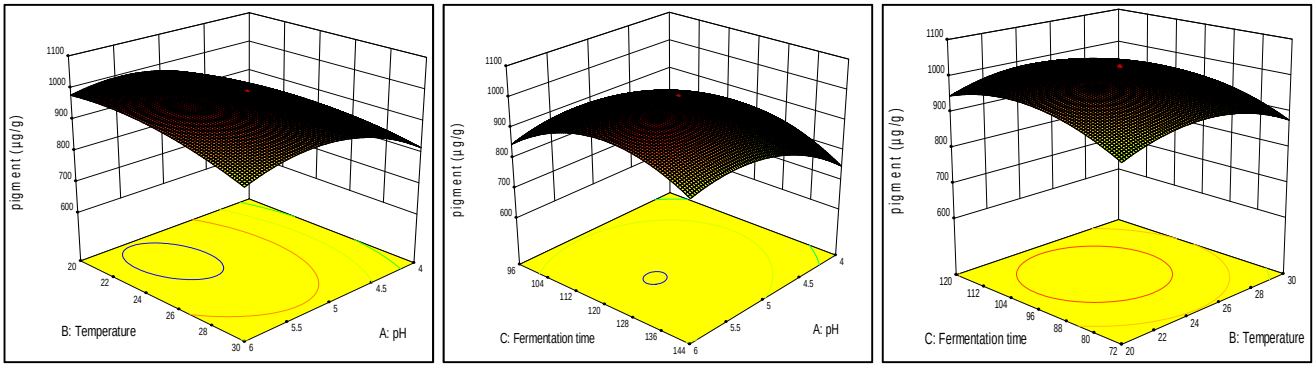

Fig 1: Response surface curve showing the physical parameters on the pigment production by *Sporobolomyces* sp.

Table 4: Regression coefficient for optimization of physical parameters for pigmentation in *Sporobolomyces* sp.

Factor	Regression Co efficient	Standard error	P- value
Intercept	989.47	1.42	< 0.0001
A-P	58.41	0.94	< 0.0001
B-T3	-22.10	0.94	< 0.0001
C-t	8.18	0.94	< 0.0001
AB	-19.52	1.23	< 0.0001
AC	14.05	1.23	< 0.0001
BC	20.29	1.23	< 0.0001
A2	-76.68	0.92	< 0.0001
B2	-33.49	0.92	< 0.0001
C2	-99.94	0.92	< 0.0001

The other pair of the independent variables pH and incubation time showed similar effects while keeping the third independent variable, temperature as a constant at 25 °C. The 3D surface response for temperature and fermentation time on the yield of pigment (Fig.1). The results showed that as the values of process variables increased, the yield also increased but only upto the mid point range of variables and thereafter the yield decreased even though the values of variables increased. The pigment yield was significantly effected by pH, temperature and incubation time where pH produced greater effect. Based on the model, the optimal working conditions were obtained to attain high carotenoid pigment yield.

In the present study, the independent variables *viz.,* pH, temperature and fermentation time were selected for maximization of carotenoid pigment production. The effect of physical parameters on pigment production was performed with the quadratic model expressed by determination of R^2, which was found to be 0.9986 respectively. The optimal point of pH 5, temperature 25 °C and fermentation time 120 h was found to be best for maximum carotenoid pigment production (990.87 µg g^{-1}). The 3D response surface plots described by the regression model were drawn to illustrate the effects of interaction of each independent variable (pH, temperature and time) on the response variable (Bocchini *et al.,* 2002) [1]. The shape of the corresponding contour plots indicated that the mutual interaction between the independent variable were orientation of principle axes of contour plots (Cui *et al.,* 2005) [3]. Sanjay *et al.* (2007a) [12] also studied the same parameters (pH, temperature and time) for maximization yield of xanthin pigment by *Aspergillus carbonarious.* They reported that it could be achieved at the conditions when pH of growth media was 3.3, temperature 29.3 °C and fermentation time of 46.7 h. Malisorn and Suntornsuk (2008) [9] studied a face centered central composite design which was applied to optimize a cultivation condition for improved β-carotene production by

R. glutinis using fermented radish brine as solid substrates. In that study pH, temperature and dissolved oxygen were considered as independent variable. Optimum condition for β-carotene was at 30 °C; pH 6 and 80 per cent dissolved oxygen to yield 201µg g^{-1} of β-carotene. Manowattana *et al*. (2012) [10] employed Placket-Burman design and RSM to enhance carotenoid production by *Sporobolomyces pararoseus* using waste glycerol as carbon source. In that study pH, temperature and waste glycerol were considered as independent variables. Optimum condition for carotenoid pigment production were 5.64 pH, 23.90 temperature and 34 g l^{-1} waste glycerol to yield 16.55 mg l^{-1} of carotenoid pigment. (Yu *et al.,* 1997) [14].

4. Conclusion

In the present study, physical parameters were identified as most influencing components for enhancing pigment production of *Sporobolomyces* sp. The RSM allowed a rapid screening of the important influence factors and development of a polynomial model to optimize the process parameters for enhancing pigment yield. Data obtained from experiment were analysed with RSM software gave the optimum pigment yield 990.87µg g^{-1} was obtained at the optimum condition of temperature 25 °C, pH 5 and 120 h after fermentation. It was evident that the systematic methods had the advantage of identifying the most significant medium composites through that identifying their optimal levels and also useful for operating the fermentation towards the accumulation of wanted metabolite

5. References

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