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Production of *Monascus* pigments using bread waste as a substrate in SSF

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Abstract

The present study aimed to investigate the production of pigments by *Monascus purpureus* using solid state fermentation (SSF). The starch-rich substrates were used for the production of pigments. Among the various solid substrates used, bread waste yield maximum production, hence bread waste was chosen for further production. Because lots of bread is being wasted across the western countries, so it is chosen as a waste valorization substrate. Further, response surface methodology (RSM) was applied for optimization of process variables using bread waste as a substrate. RSM was applied to determine the optimal parameters for initial moisture content, temperature and inoculum size. In validation experimentation, a maximum amount of pigments 65.71UA_{410} was obtained with bread waste using *M. purpureus* strain.

Keywords: Pigments, solid state fermentation, *Monascus purpureus*.

Introduction

Natural colorants and flavors mainly derived from plants and chemosynthetic compounds are used by the food industry to replenish and sometimes raise the genuine stock (Pandey *et al*. 2001). *Monascus* is known to produce at least six molecular structures of pigment which can be

classified into three groups depending on their color. They include yellow pigments monascin ($C_{21}H_{26}O_5$) and ankaflavin ($C_{23}H_{30}O_5$), the orange pigments monascorubrin ($C_{23}H_{26}O_5$) and rubropunctatin ($C_{21}H_{22}O_5$), and the red pigments monascorubramine ($C_{23}H_{27}NO_4$) and rubropuntamine ($C_{21}H_{23}NO_4$) (Pattanagul *et al*. 2007; Kim *et al*. 2006). The red pigment has been increasing interest to the food industry because products are extra cellular and water soluble making them easy to use. A synthetic red pigment such as azorubin or tartrazin causes allergic reactions (Fabre *et al*. 1993). *M. purpureus* having the ability to produce secondary metabolites like statins as well as pigments. Since the number of permitted synthetic colorants has decreased because of undesirable toxic effects, including mutagenicity and potential carcinogenicity, interest focuses on the development of food pigments from natural sources (Vidyalakshmi *et al*. 2009). Though many natural colors are available, microbial colorants play a significant role as a food coloring agent, because of its flexibility in production and ease downstream processing. Among the various pigment producing microorganisms, *Monascus* was reported to produce non-toxic pigments, which can be used as a food colorant. The pigment of *Monascus* improves the coloring appearance of foods and their organoleptic characters. The demand for highly edible coloring agents increased, one of which is the *Monascus* pigment (Francis, 1987). *Monascus* pigments have been a long-established food ingredient for Asian consumers. *Monascus* rice products are gaining importance as a dietary supplement in the United States and many Asian countries, due to its anti-cholesterol activity (Silveira *et al*. 2008). The main aim of the present study was to produce pigments from bread waste using RSM. Roughly one third of the food produced in the world for human consumption every year, approximately 1.3 billion tonnes gets lost or wasted. Every year, in Austria, an estimated amount of 60,000 to 65,000 tons of bakery goods are thrown away 2009 reports. In the present study bread waste was used as a substrate for

the optimization studies for the production of pigments. RSM is a powerful and efficient mathematical approach widely applied in the optimization of the fermentation process. The effects of process parameter were investigated such as the initial moisture content, temperature and inoculum volume using RSM.

Materials and Methods

Media components

Potato dextrose agar (PDA), glucose, malt extract, magnesium sulfate, and manganese sulfate were purchased from Hi-Media Limited, India. All the substrates for SSF were purchased from local markets of Andhra Pradesh, India. Ethanol was purchased from Rankem, New Delhi, India. All the chemicals used were of analytical grade Mumbai, India.

Culture maintenance

M. purpureus used in the present study were obtained from the Institute of Microbial Technology (MTCC 369), Chandigarh, India. The cultures were maintained on potato dextrose agar slants at 25 ˚C for 14 d and the slants were sub-cultured every 30 d. A spore suspension 10^6 spores/mL prepared from such slants was used to inoculate 100 mL of sterile seed medium in 250 mL flasks at 25 ˚C, 120 rpm for 2 d in incubator shaker.

Fermentation procedure

Experiments were carried out in 250 mL Erlenmeyer flasks containing 5 grams of substrate (bread waste) and supplement solution was added to it with initial moisture content being 66% (w/w). 5 mL of nutrient solution consists of glucose (5%, w/v), malt Extract (1%, w/v), $MgSO_4$ (0.07%, w/v) and $MnSO_4$ (0.2%, w/v). The contents of the flasks were mixed and autoclaved at 121 ˚C at 15 pa for 20 min. The seed medium was inoculated with 10^6 spores/mL and incubated

at 30 ˚C for 48 h. Five percent of this preculture was used to inoculate the production medium. Fermentation was carried out at 30 ˚C; incubation time for *M. purpureus* is 14 d.

Pigment extraction

Five grams of a fermented solid substrate was taken for pigment extraction using 25 ml of 95% ethanol (Carvalho *et al*. 2005), with shaking on a rotary shaker at 200 rpm for 1 hr. The extracts were allowed to settle at room temperature and then filtered through the Mira cloth membrane (Calbiochem). Ethanol extracts of unfermented substrates were kept as blanks.

Pigment estimation

Pigment estimation was done using optical density and its absorbance maxima were expressed as the concentration of pigment produced (Tseng *et al*. 2000). The analysis of pigment production was done by measuring absorbance maxima (ʎ max) of pigment extract by spectral analysis (Lin and Demain, 1992) using a double beam spectrophotometer (Shimadzu UV 1601) taking into consideration the dilution factor of the sample (Chiu and Poon, 1993). Only extracellular pigments were considered in this study. Pigment yield was expressed as OD at its ʎ max per gram dry fermented matter (Johns and Stuart, 1991).

Results and Discussions

Screening of various solid substrates

Among the Eleven different solid substrates tested, pigment production was high in the substrates, bread waste, wheat (powder) and sweet potato with 21.71,9.91 and 8.60 UA_{410} respectively as shown in Table 1. Among the substrates tested, bread waste was found to be the most suitable substrate; further optimization experimentation was carried out with bread waste. Pigments absorption maxima was obtained at 410 nm which indicate that yellow pigments

production was maximum than red pigments. High-through put a system for screening of *M. purpureus* high-yield strain in pigment production (Tan *et al*. 2014).

Response surface methodology

The effect of various process parameters such as initial moisture content, incubation temperature and inoculum size was studied. To optimize the process variables for enhancing pigments production, RSM was applied. These process parameters mostly influence fungal growth and secondary metabolite production. RSM is a rapidly and efficiently along a path of improvement toward the general vicinity of the optimum. To identify optimum levels for different process parameters influencing pigments production, SSF was carried out in conical flasks containing optimized nutrients. The individual and interactive effects of these process variables were studied by conducting the fermentation run at randomly selected and different levels (Table 2) of all three factors. Six replicates at the center with 20 experiments used to optimize the process parameters. The optimum levels of the selected variables were obtained by solving the regression equation using MATLAB software. Table 2 gives the coded values and the levels of the variables temperature, moisture content, and inoculum size. The experimental and the predicted values were presented along with the central composite design (CCD) experimental design in Table 3.

The analysis of variance of the quadratic regression model demonstrated that Eq. (1) was a highly significant model, as was evident from the Fisher's *F*-test with a very low probability value [(*P* model> *F*) = 0.001]. The student's *t*-test and *P*-values were used as a tool to check the significance of each coefficient, which also indicated the interaction strength between each independent variable. The larger the magnitude of the *t*-value and smaller the *P* value, the more significant is the corresponding coefficient. Analysis of variance for pigments production by *M. purpureus* was shown in Table 4. The linear effect of x_3 and the squared effect of x_1^2, $x_2^{2,}$ and x_3^2

were found to be significant as the *P*-value is less than 0.05 for pigments as shown in Table 4. The goodness of fit of the model based on RSM can be checked by the coefficient of determination (R^2), which provides a measure of how much variation in the observed response values can be explained by the experimental factors and their interactions. The closer the R^2 value is to 1, the stronger the model is, and the better it predicts the response. In this case, the value of the determination coefficient (R^2=89.21%) indicated that only 10.79% of the total variations were not explained by the model for pigments.

Validation of the models

The validation experiment was carried out in 250 mL Erlenmeyer flask under the optimum combination of the process parameters predicted by the polynomial model. The optimum values predicted by the model for pigments are, temperature-29.23 ^{o}C, moisture content-4.52 mL, and inoculum size-4.82 mL. The maximum responses of 65.02 UA_{410} of pigment production were predicted by the model. The pigment production of 65.71UA_{410} was obtained, which is nearly equal to the predicted pigment production, thereby validating the proposed model.

Conclusion

The most significant outcome of this study was various starchy rich substrates were tested for the production of pigments using *M. purpureus*. Bread waste substrate was found to be ideal for *M. purpureus*. A highly significant quadratic polynomial obtained by the CCD was very useful for determining the optimal process parameters that have significant effects on pigments production. The operation parameters for the SSF process were as follows: 60%-66% initial moisture content, pH 6.5, incubation temperature 30 ^{o}C for and incubation time 14 d respectively. The above-mentioned conditions results in a maximum amount of pigments 65.71UA_{410}. This paper

evaluates a cheap waste material is used for the production of pigments, which could be a great industrial application.

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Table 1. *Monuscus purpureus* pigments at 410 nm using various substrates

S.No	Substrates	U/g
1.	Sweet potato	8.60
2.	Potato	7.97
3.	Topiaco (powder)	0
4.	Banana skin	2.62
5.	Bread waste	21.71
6.	Corn kernel (powder)	4.94
7.	Wheat (powder)	9.91
8.	Oats (powder)	5.95
9.	Beet root	7.17
10.	Coconut cake	4.94
11.	Elephant foot yam	1.09

Table 2. Experimental ranges and the levels of the independent variables

Variables with designate	Coded values				
	-2	-1	0	+1	+2
Initial moisture content (mL)	3	4	5	6	7
Temperature (^{0}C)	28	29	30	31	32
Inoculum size (mL)	3	4	5	6	7

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Table 3 Central composite design matrix of three variables in coded and natural units along with the observed responses

Runs	Initial moisture (mL)	Temperature (^{0}C)	Innoculum size (mL)	Pigments U/g Experimental	Pigments U/g Predicted
1	-1	-1	-1	31.01	28.12
2	1	-1	-1	19.20	22.44
3	-1	1	-1	14.41	22.11
4	1	1	-1	20.12	25.66
5	-1	-1	1	21.19	42.09
6	1	-1	1	11.61	11.54
7	-1	1	1	24.63	68.55
8	1	1	1	12.90	23.42
9	-1.682	0	0	48.70	18.33
10	1.682	0	0	18.74	10.99
11	0	-1.682	0	22.65	24.91
12	0	1.682	0	42.91	29.85
13	0	0	-1.682	43.63	39.23
14	0	0	1.682	23.63	17.24
15	0	0	0	64.74	64.05
16	0	0	0	62.98	64.05
17	0	0	0	64.91	64.05
18	0	0	0	64.25	64.05
19	0	0	0	63.44	64.05
20	0	0	0	62.12	64.05

Table 4 Analysis of variance for pigment production by *Monuscus purpureus*.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	99733272	99733272	11081475	19.97	0.000
Linear	3	7145485	7145485	2381828	4.29	0.034
A	1	1012180	1012180	1012180	1.82	0.207
B	1	294258	294258	294258	0.53	0.483
C	1	5839047	5839047	5839047	10.52	0.009
Square	3	90939986	90939986	30313329	54.63	0.000
A*A	1	47887669	59852713	59852713	107.86	0.000
B*B	1	19943387	24216808	24216808	43.64	0.000
C*C	1	23108930	23108930	23108930	41.65	0.000
Interaction	3	1647801	1647801	549267	0.99	0.436
A*B	1	426195	426195	426195	0.77	0.401
A*C	1	847277	847277	847277	1.53	0.245
B*C	1	374329	374329	374329	0.67	0.431
Residual Eror	10	5549004	5549004	554900		
Lack-of-Fit	5	5489852	5489852	1097970	92.81	0.000
Pure Error	5	59152	59152	11830		
Total	19	105282276				

DF- Degree of freedom, **SS**- Sum of squares, **MS**- Mean square, **F**- *F*-value, **P**- *P*-value