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Sequential statistical optimization of red pigment production by *Monascus purpureus* (MTCC 369) using potato powder

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ABSTRACT

The present work attempts to utilize the potato powder as a low cost carbon source and statistically evaluate the medium composition for red pigment production by *Monascus purpureus*. Plackett–Burman (PB) design was successfully used to screen the significant medium variables and potato powder was found more significant than other components. Central composite design (CCD) of response surface methodology (RSM) was used to optimize the four significant medium components (Potato powder, K_2HPO_4, $ZnSO_4·7H_2O$ and MSG) selected from PB experiment. ANOVA results showed that model was significant and R^2 value of 0.833 demonstrated that experimental results were fitted well with predicted values. Maximum pigment absorbance of 7.18 ODU/ml was predicted at optimal level of potato powder 2.50% (w/v), K_2HPO_4 0.480% (w/v), $ZnSO_4·7H_2O$ 0.0013% (w/v) and MSG 0.60% (w/v). Model accuracy was found to be 96.65% from the data obtained from validation experiment.

1. Introduction

In recent years, development of alternate sources for the production of natural pigments has been focused to overcome the unlimited usage of synthetic pigments, which found to be hazardous to human health and environment. Natural colorants can be obtained from plants and microbial sources have found to be an alternative to synthetic pigments. Plant derived pigments have limited usage because of low water solubility, instability against light and heat. Nowadays, pigments from microbial origin gain more importance in food industry applications. Various microorganisms such as *Monascus* sp. (Lin, 1973; Lee et al., 2001; Zhou et al., 2009), *Serratia* sp. (Kim et al., 1998), *Streptomyces* sp. (Ryu et al., 1989), *Bacillus* sp. (Kim et al., 1995), *Achromobacter*, *Rhodoturla*, *Yarrowia* sp. (Carreira et al., 2001) and *Paecilomyces* sp. (Cho et al., 2002) were reported for fermentative production of pigments. *Monascus purpureus* belongs to Monascaceae family and produces pigments (Silveira et al., 2011), lovastatin (Panda et al., 2010) and fatty acids (Juzlova et al., 1996) by secondary metabolism. The pigments produced by *M. purpureus* are classified into at least six types of pigments based on the color (1) red pigment (rubropunctamin, $C_{21}H_{26}NO_4$ and monascurubramin, $C_{23}H_{27}NO_4$); (2) orange pigment (rubropunctatin, $C_{21}H_{22}O_5$ and monascorubrin, $C_{23}H_{26}O_5$) and (3) yellow pigment (monascin, $C_{21}H_{26}O_5$ and ankaflavin, $C_{23}H_{30}O_5$) (Babitha, 2009). *Monascus* pigments are used traditionally for natural coloring of oriental foodstuffs in Asian countries and also in textile dyeing process (Santis et al., 2005; Velmurugan et al., 2010). Recent studies on *Monascus* pigments reported that it possess anti-tumor activity (Hsu and Pan, 2012).

Substrates for the bioproduct production were highly influenced by the cost of the bioprocess. So, there is a need to select cheap and efficient substrates for producing the bioproducts economically. Various agricultural products and byproducts such as corn cob (Velmurugan et al., 2011), sugarcane bagasse (Silveira et al., 2011), grape waste (Silveira et al., 2008), jackfruit seed (Babitha et al., 2006), corn steep liquor (Hamano and Kilikian, 2006), wheat substrates (Dominguez-Espinosa and Webb, 2003) and cassava (Yongsmith et al., 1993) were successfully utilized for the production of Monascus pigments. Potato powder was used as a low cost substrate in our study and previous literature on this substrate was not reported so far.

Medium optimization is one of the important processes for getting maximum product yield and it involves several factors such as medium components, operating conditions, pH, temperature, aeration and agitation, etc. Classical way of one factor at a time optimization is accepted well but it has many disadvantages like more number of experimental runs, laborious, need more time, etc. Statistical approach of screening and optimization has gained importance for medium and process optimization of different metabolites production. Plackett–Burman design was

Abbreviations: MSG, monosodium glutamate; PB, Plackett–Burman; OFAT, one factor at a time; RSM, response surface methodology; CCD, central composite design; ANOVA, analysis of variance; DF, degrees of freedom; SS, sum of squares; MS, mean square; *F*, Fisher test value; *p*, probability value.

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employed by several researchers for screening of significant factors involved in a particular process (Yu et al., 1997). Response surface methodology (RSM) also successfully applied for optimization of medium components and fermentation conditions with less number of experiments (Anita and Narsi, 2012; Anuradha and Valli Nachiyar, 2011; Mamatha et al., 2008). Also, it is used to investigate the interaction and quadratic effects among the variables in the process.

The main objective of this study was to screen and optimize the medium variables for red pigment production by *Monascus purpureus* MTCC 369 using potato powder as a carbon source. The effects of each medium variables and their interaction were analyzed by PB and CCD methods.

2. Materials and methods

2.1. Microorganism and media

Fungi culture *Monascus purpureus* (MTCC 369) procured from MTCC, IMTECH Chandigarh, India, was used in this study. It was maintained on potato dextrose agar (PDA) medium preserved at 4 °C and sub-cultured once in every three weeks.

2.2. Preparation of potato flour

Potato obtained from a local market in Chennai was used as substrate. After peeling off the brown arils of potato, it was cut into small pieces, sun dried for 3 days and grinded well for making very fine particles. Sieving was done by using 0.09 mm size sieve and the fine potato powder was used as substrate.

2.3. Fermentation conditions

Ten milliliters of Tween 20 (0.1%, v/v) in sterile distilled water was added to fully sporulated (7–8 days old) PDA agar slope cultures. The spores were scraped off under aseptic conditions to produce a spore suspension to be used as the inoculum (1×10^{-6} spores/ml). Optimization studies were carried out in 250 ml conical flask each containing 50 ml of production medium as per experimental design (Tables 1 and 3). Five percent spore suspension was used to inoculate the flasks and kept in temperature controlled rotary shaker at 150 rpm at 30 °C for 7 days. Samples were taken at every 24 h interval and analyzed for pigment, biomass, and total reducing sugars.

2.4. Pigment estimation

Pigment estimation was done as described by Tseng et al. (2000) in which the optical density at its absorbance maxima was expressed as the concentration of pigment produced extracellularly. The analysis of pigment production was done by measuring absorbance maxima (480 nm) of pigment extract by using a UV–vis spectrophotometer and multiply with the dilution factor. Pigment yield was expressed as OD units at its maximum wavelength per ml of clarified fermented broth (Chiu and Poon, 1993).

2.5. Biomass estimation

The fermentation broth was filtered through Whatman No. 1 filter paper and the mycelia pellets were washed twice with the distilled water. It was dried at 80 °C in hot air oven to constant weight and measured as biomass.

2.6. Residual sugar estimation

Reducing sugar was estimated by the dinitrosalicylic acid (DNS) method (Miller, 1959).

2.7. Plackett–Burman optimization method

Plackett–Burman (PB) experimental design was used to evaluate the relative importance of various nutrients for pigment production in submerged culture (Plackett and Burman, 1946). Seven medium variables (%, w/v), Potato powder (A), MSG (B), KH_2PO_4 (C), K_2HPO_4 (D), $MgSO_4{\cdot}7H_2O$ (E), KCl (F), and $ZnSO_4{\cdot}7H_2O$ (G) were chosen for screening experiment. Experimental design matrix was prepared with the help of Minitab 14 software. Each medium variable were tested at two levels: high (+) and low (−). Range of variable levels and experimental design was shown in Table 1. A simple polynomial model (Eq. (1)) was generally used for explaining the relation between dependent and independent variables

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j \qquad (1)$$

where Y is the dependent variable, β_0, β_j are regression coefficients for the intercept and linear effects respectively and X_j is coded independent variable.

2.8. Optimization by response surface methodology

Statistical optimization of red pigment production by response surface methodology was performed by using Minitab 14 software. Four factors (Potato powder, K_2HPO_4, $ZnSO_4{\cdot}7H_2O$ and MSG) were selected for the optimization at 5 levels (+2, +1, 0, −1, −2) and range of variable levels and experimental design were given in Table 2. A second order polynomial model given in Eq. (2) was used to estimate the response.

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum\sum \beta_{ij} X_i X_j \qquad (2)$$

where Y is the response, β_0, β_j, β_{jj} and β_{ij} are regression coefficients for the intercept, linear, quadratic and interaction effects respectively and X_i and X_j are coded independent variables.

3. Results and discussion

3.1. Screening of significant factor by PB design

Twelve experimental runs were carried out according to the combination of medium components given in Table 1 and response for each run was also represented. Regression analysis of the experimental results was done to estimate the coefficient and the main effect of the each medium variable. The estimated regression coefficient, main effect, t and p value were given in Table 2. Five medium variables (potato powder, K_2HPO_4, $MgSO_4{\cdot}7H_2O$, KCl and $ZnSO_4{\cdot}7H_2O$) were showed positive effect on pigment production whereas negative effect was shown by MSG and KH_2PO_4. A simple polynomial model (Eq. (3)) was constructed by using regression coefficients (in coded units) to represent pigment production.

$$\text{Red pigment (ODU/ml)} = 4.21 + 0.708\,A - 0.541\,B + 0.075\,C - 0.625\,D - 0.175\,E + 0.125\,F + 0.592\,G \qquad (3)$$

The significance of the medium variable can be evaluated by p-value of the each variable shown in Table 2. p-Value less than 0.05 was generally used to represent the statistical significant of the variable with confidence level higher than 95%. From Table 2 potato powder