Journal of Biotechnology 170 (2014) 28–34

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Innovative use of *Mucuna monosperma* (Wight) callus cultures for continuous production of melanin by using statistically optimized biotransformation medium

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ARTICLE INFO

Article history:
Received 25 July 2013
Received in revised form 22 October 2013
Accepted 19 November 2013
Available online 27 November 2013

Keywords:
Biotransformation
Continuous production
Melanin
Mucuna monosperma
Response surface methodology

ABSTRACT

Melanins are predominantly indolic polymers which are having extensive applications in cosmetics, agriculture and medicine. In the present study, optimization of nutritional parameters influencing melanin production by *Mucuna monosperma* callus cultures was attempted using the response surface methodology (RSM). Standardization of four factors was carried out using the Box–Behnken design. The optimized levels of factors predicted by the model include tyrosine 0.978 g L^{-1}, pH 5.85, SDS 34.55 mg L^{-1} and copper sulphate 21.14 mg L^{-1} tyrosine, which resulted in highest melanin yield of 0.887 g L^{-1}. The optimization of medium using RSM resulted in a 3.06-fold increase in the yield of melanin. The ANOVA analysis showed a significant R^2-value (0.9995), model *F*-value (1917.72) and probability (0.0001), with insignificant lack of fit. Optimized medium was used in the laboratory scale column reactor for the continuous production of melanin. Uninterrupted flow column exhibited maximum melanin production rate of 250 mg L^{-1} h^{-1} which is the highest value ever reported using plant as a biotransformation source. Melanin production was confirmed by spectrophotometric and chemical analysis. Thus, this study demonstrates the production of melanin by *M. monosperma* callus, using a laboratory scale column reactor.

1. Introduction

Melanins is a group of high molecular weight, black and brown pigments that are produced from oxidation and polymerization of polyphenols and are widely distributed in plant and animal kingdom and are also reported to be synthesized by microorganisms (Plonka and Grabacka, 2006). Coloration of seeds, berries, flowers, human skin, or hair is essentially due to the presence of melanin (Nicolaus, 1968). Melanins are among the most stable, insoluble and resistant to biochemical materials and could enhance the survival and competitive abilities of organisms in environments (Bell and Wheeler, 1986).

Melanins are widely employed in cosmetics, photo protective creams, eyeglasses as well as protective agents in *Bacillus thuringenesis* insecticidal crystals (Zhang et al., 2007). Microorganisms producing melanin have been used for the immobilization of radioactive waste of uranium (Turick et al., 2008), for screening recombinant bacterial strains (Adham et al., 2003), for AIDS treatment (Montefiori and Zhou, 1991), and for the treatment of human metastatic melanoma (Dadachova et al., 2008). In addition to pharmaceutical applications, melanins also have high commercial value in food industries as natural additives and colorants. Synthetic colorants are frequently perceived as undesirable or harmful which uphold the craze for natural colorants and food additives among consumers (Kannan and Ganjewala, 2009).

Plants are always considered to be the best source of natural pigments such as melanin and others. Wang et al. (2006) have isolated and characterized melanins from seeds of *Osmanthus fragrans* and studied its biological properties to be used as food colorants. Extensive studies have been carried out regarding biological properties of melanin pigment from different plant sources (Nicolaus, 1968; Zherebin et al., 1982). However, to fulfill the ever-growing demand of the melanin, these sources are not sufficient and necessitated development of a cost effective process such as biotransformation of L-tyrosine to melanin.

The plant under present study is *Mucuna monosperma*; seeds of which are proved to be a good source of L-DOPA and possess sufficient level of tyrosinase, an enzyme required for biotransformation of L-tyrosine to melanin (Inamdar et al., 2012). Considering this background, callus cultures raised from seeds are expected to provide a continuous source for biotransformation which could be further augmented using fermentation process. Medium optimization and physical conditions in fermentation have been traditionally performed using one-factor-at-a time method which is time consuming, laborious and expensive; in addition, it fails to determine the combined effect of different factors on the contrary,

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0168-1656/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jbiotec.2013.11.012

response surface methodology (RSM) could provide a substantial information based on only a small number of experiments (Aghaie-Khouzania et al., 2012).

Against this background, the objective of the present work is to establish callus cultures of *M. monosperma* for efficient biotransformation of L-tyrosine to melanin and optimization of process by using Box–Behnken design of RSM. Literature survey revealed that, this is the first report concerning continuous production of melanin by callus cultures using statistically optimized biotransformation medium.

2. Materials and methods

2.1. Tissue culture and callus initiation

Seed explants of *M. monosperma* were surface sterilized and were inoculated onto media containing different concentrations of hormones on full-strength MS medium (Murashige and Scoog, 1962). Sucrose (3.0%) was used as a carbon source and 0.2% (w/v) Clarigel as a solidifying agent and incubated at 25 °C under a photoperiod of 12/12 h. Plant hormones mainly NAA and 2,4-D as well as glutamine at different concentrations were added for callus initiation and proliferation. First subculturing was done after 24 h and then after every 20–25 days.

2.2. Biotransformation of L-tyrosine to melanin

Potential of biotransformation of L-tyrosine to melanin was exploited in 50 mL citrate phosphate buffer (0.1 M, pH 5.0) containing 1.0 g L^{-1} tyrosine in presence of different activators such as $CuSO_4$ and SDS. Reaction mixture was incubated at 120 rpm on rotary shaker for 48 h and produced melanin was analyzed spectrophotometrically at 475 nm using a calibration curve of standard synthetic melanin (Sigma–Aldrich, St. Louis, USA) (Hoti and Balaraman, 1993; Surwase et al., 2012).

2.3. Purification and analysis of melanin

Melanin was purified from cell-free extract by previously described methods (Zhang et al., 2007). The chemical characterization of purified pigment was carried out using tests described in earlier reports (Zhang et al., 2007; Shrishailnath et al., 2010).

2.4. Experimental design

Based on the results obtained in previous experiments and literature survey, concentrations of tyrosine, $CuSO_4$, SDS and pH were found to influence melanin production significantly (Surwase et al., 2012; Shrishailnath et al., 2010; Lagunas-Muñoz et al., 2006). Hence, these variables were optimized using Box–Behnken design for maximum biotransformation. Four variables at three levels were used to fit a polynomial model (Box and Behnken, 1960) and the boundary conditions for each parameter are as depicted in supplementary data (Table S1). The Design Expert software (version 8.0, Stat-Ease Inc., Minneapolis, USA) was used in the experimental design and data analysis. A quadratic model was designed such that the variance of Y is constant for all points equidistant from the center of the design. Response surface graphs were obtained to understand the effect of the variables, individually and in combinations, and to determine their optimum levels for maximum melanin production. All experiments were performed in triplicate, and the average yield was used as response *Y*. The significance of the model equation and model terms was evaluated by '*P*' value and *F*-test. The quality of the quadratic model equation was expressed by determination coefficient R^2 and adjusted R^2. Analysis of variance (ANOVA) was applied to evaluate the statistical significance of the model. Model fitting was confirmed with 'Lack of Fit' test. Adequacy of the predicted model was evaluated with Normal probability plot and Box–Cox analysis. The optimal values were obtained by solving the regression equation and analyzing 3-dimensional response surface plots and contour plots.

2.5. Laboratory scale column reactor for melanin production

Continuous production of melanin was achieved using a laboratory scale column (3 cm $\Phi \times$ 30 cm) with a provision for aeration at the bottom. Medium was allowed to pass through bottom of the column using peristaltic pump and produced melanin was recovered from the top of the column. Sterile air was introduced in to the column using aerator and bacteria proof filter. Flow rate was controlled using peristaltic pump.

3. Results and discussion

3.1. Induction of callus cultures

M. monosperma seeds are a good source of L-DOPA, intermediate of melanin synthesis pathway, and possess tyrosinase activity (Inamdar et al., 2012). Hence, callus cultures were established using endosperm as explants on medium supplemented with NAA (1.0 mg L^{-1}) and 2,4-D (1.0 mg L^{-1}) glutamine (500 mg L^{-1}) for callus initiation and proliferation. Callus formed was regularly subcultured on same medium and used for biotransformation.

3.2. Medium optimization by response surface methodology

Melanins have wide applicability in various fields and bacteria and fungi have been extensively used for the melanin production. However, plant sources are underutilized for this purpose. In addition, response surface methodology has not been utilized for melanin production by biotransformation approach using callus or suspension cultures. The ranges of studied variables are provided in supplementary Table 1. The variables L-tyrosine, pH, and $CuSO_4$ were selected based on previous reports of Surwase et al., 2012; Lagunas-Muñoz et al., 2006 and Shrishailnath et al., 2010. In addition, the SDS is known to induce tyrosinase activity in *M. monosperma* seeds (Inamdar et al., 2012) which will in turn enhance the melanin yield. Therefore, effect of SDS was assessed in initial experiments in which SDS incorporation significantly increased the melanin production (Supplementary Fig. S1). Hence, to achieve maximum production of melanin we optimized the biotransformation medium using the Box–Behnken design with significant components like L-tyrosine (A), pH (B), SDS (C), and $CuSO_4$ (D). Table 1 represents the design matrix and the results of the 29 experiments carried out using the Box–Behnken design consisting of 24 trials plus 5-center points. The results obtained were analyzed by ANOVA using the Design expert software (version 8.0, Stat-Ease Inc. USA), and the regression model was given as:

$$\begin{aligned}\text{Melanin} = {} & -5231.85 + 7749.87 \times \text{Tyrosine} + 569.70 \times \text{pH} \\ & +43.68 \times \text{SDS} + 85.59 \times \text{Copper sulphate} - 113.33 \\ & \times \text{Tyrosine} \times \text{pH} + 5.70 \times \text{Tyrosine} \times \text{SDS} + 4.40 \times \text{Tyrosine} \\ & \times \text{Copper sulphate} + 0.83 \times \text{pH} \times \text{SDS} + 6.22 \times \text{pH} \\ & \times \text{Copper sulphate} - 0.05 \times \text{SDS} \times \text{Copper sulphate} - 4962.80 \\ & \times \text{Tyrosine}^2 - 54.80 \times \text{pH}^2 - 0.84 \times \text{SDS}^2 - 2.87 \\ & \times \text{Copper sulphate}^2 \end{aligned} \quad (1)$$