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Effect of glycerol as a sole carbon source on *Monascus* sp. for pigment production

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Abstract

In the field of natural pigment production, *Monascus* sp. is a well known name. These pigments have their applicability as food colorants. In this study, two *Monascus* sp. namely *Monascus purpureus* procured from MTCC, Chandigarh, India and *Monascus sanguineus* isolated in-house, were compared for pigment production in submerged fermentation. Glycerol was used as sole carbon source and malt extract as sole nitrogen source in synthetic defined media. These media were optimized by employing factorial design and response surface techniques. It was observed from the study that *Monascus purpureus* was able to grow on these media but pigment yield was less compared to *Monascus sanguineus*. The maximum value of pigment yield was observed around 9.2 CVU/mL with glycerol concentration of 36.96 g/L and malt extract concentration of 8 g/L for *M. purpureus.* For the isolated culture, this value was observed as 26.98 CVU/mL at 8 g/L of malt extract concentration and 64 g/L of glycerol concentration with both the variables showing the impact. The biomass also showed similar trend with both the strains.

Introduction

The pigments produced by *Monascus* sp. have been traditionally used as natural colorants for foodstuffs, but recent studies show that some of the *Monascus* pigments have important biological functions such as anti-tumor activity etc. Traditionally, Monascus pigment is produced through solid state fermentation (Babitha *et al.*, 2007). Submerged fermentation for Monascus pigment production has also been studied to minimize the problems of space, scale and process control (Hamdi *et al*., 1996; Vendruscolo *et al*., 2010). The *Monascus* sp. is basically known to produce red color during growth in substrates. These red colors are due to the formation of rubropunctamine and monascorubramine pigments. These pigments are formed by the chemical modification of orange pigments, rubropunctatin and monas-corubrine, respectively which are synthesized in the cytosol from acetyl Co-A by the multienzyme complex polyketide synthase (Dahale *et al.*, 2010). It is important to find out the specific composition of pigments produced by *Monascus* sp. under different culture conditions for the safe and successful application of these pigments in the food and pharmaceutical industries (Miyake *et al.*, 2008). Several physical factors like pH, temperature, agitation and aeration etc. and nutrients especially source of carbon and nitrogen is known to influence the pigment production by *Monascus* sp. (Silvana *et al*., 2008; Hajjaj *et al.*, 2000). Best experimented substrates for *Monascus* pigment are glucose, celobiose, maltose and fructose, whereas sucrose is not suitable for pigment yield (Juzlova *et al*., 1996). An alternative substrate is glycerol, which can be obtained as the main residue from the production of biodiesel. The high cost in the production of natural pigments can be minimized by using organic waste at low cost (Meinicke *et al*., 2012).

The aim of present study was to analyze the potential of glycerol and malt extract as sole carbon and nitrogen sources respectively by using two factorial designs for pigment production for two different *Monascus* strains (*Monascus purpureus* and *Monascus sanguineus*).

Materials and Methods

Culture

Wild strain of *Monascus* was isolated from pomegranate (*Punica granatum*). The strain was maintained on Potato Dextrose Agar (PDA) medium and incubated at 28-30°C for 7 days, preserved at 4°C, and sub-cultured once every 4 weeks (Rashmi and Padmavathi, 2012).

Source of reference

Monascus purpureus MTCC 410 was obtained

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Table 1. Variables along with maximum pigment yield and biomass for both the strains

Run	Glycerol (g/L)	Malt Extract (g/L)	Maximum Pigment yield (CVU/mL)		Biomass (g/L)	
			Isolated	MTCC	Isolated	MTCC
1	8	1	7	2.5	7.6	6.2
2	8	4	11	3.7	6.4	9.2
3	8	8	21	9	7	7.2
4	32	1	14	2.2	7.4	7.2
5	32	4	19	6	10.4	10
6	32	8	17	10	9.2	14.8
7	64	1	21	3	14.8	8.4
8	64	4	23	10	12	12.4
9	64	8	29	8	12.2	10.8
10	32	4	20	8	8.2	9

from the Microbial Type Culture Collection, IMTECH, Chandigarh, India and used as reference strain (Rashmi and Padmavathi, 2012).

Inoculum preparation

One loop of sporulated (6-day old) agar slope culture was diluted in distilled water. The spores were scraped off under aseptic conditions to produce a spore suspension to be used as the inoculum (Babitha *et al*., 2007).

Submerged fermentation

The synthetic growth medium containing (g/L), 5 g K $2HPO_4$, 5 g KH_2PO_4, 0.1 g $CaCl_2$, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.01 g $ZnSO_4.7H_2O$ and 0.03 g $MnSO_4.H_2O$ was prepared. In this media glycerol as a sole carbon source and malt extract as a sole nitrogen source was used (Meinicke *et al.*, 2012). The amount of glycerol and malt extract (ME) tested in this work is presented in Table 1. 50 mL of media was prepared in 100 mL flask and autoclaved at 121°C for 20 minutes. Medium pH was adjusted to 5.5. After cooling, this media was inoculated with 0.5 mL of both cultures and incubated for 16 days in static condition (Rashmi and Padmavathi, 2012).

Dry cell weight

The mycelia was separated by filtration (Whatmann No. 1) from the broth and weighed on an analytical scale. It was then vacuum filtered through pre-weighed membrane filters, cleansed with distilled water and dried in an oven at 50°C. The results were presented in grams per liter (Mukherjee and Singh, 2010).

Pigment Estimation

Filtrate from both the strains was centrifuged at 10000 × g for 15 minutes. Pigment estimation was done using colorimeter at 510 nm. The following formula was used to convert the absorbance values into pigment units:

Color value = O.D. × dilution × volume of extracts / Amount of sample (mL) (Ratana and Toshima, 1987).

Experimental Design

The influence of the concentrations of glycerol and malt extract on pigment production by both the strains was evaluated using a second order factorial design (2^2). In the statistical model, the coded variables were defined as follows: glycerol (X1) and malt extract (X2), the dependent variable being pigment production (Y). Table 1 shows the two independent variables and their concentrations at the different coded levels of the factorial design experiments as well as the response evaluated (Meinicke *et al*., 2012).

Statistical Analysis

Statistical analysis has been done with MATLAB® software Version 7.5.0.342 (R2007b) from The Math Works, USA.

Results and Discussion

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_1X_2 + b_4X_1^2 + b_5X_2^2 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

The above equation shows the generalized relation and the real equation for pigment yield for both the strains is shown below

$$\text{Pigment yield of } M.\,purpureus \left(\frac{CVU}{mL}\right) = -0.9235 + 0.0713 \times \text{Glycerol} + 1.9930 \times \text{Malt Extract} - 0.0053 \times \text{Glycerol} \times \text{Malt Extract} - 0.0004 \times \text{Glycerol}^2 - 0.0991 \times \text{Malt Extract}^2 \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

$$\text{Pigment yield of } M.\,sanguineus \left(\frac{CVU}{mL}\right) = 3.6193 + 0.222 \times \text{Glycerol} + 2.3118 \times \text{Malt Extract} - 0.0132 \times \text{Glycerol} \times \text{Malt Extract} + 0.0005 \times \text{Glycerol}^2 - 0.0736 \times \text{Malt Extract}^2 \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

A grid of 30 points each for both the variables was generated. The effects of variables on pigment yield (CVU/mL) for both the strains were studied by plotting 3D surface curves. This provides with 900 data points for investigation. These 3D plots with the contours of the calculated response for the variables from equation 1 (900 data points) are shown in Figure 1 to Figure 4. The higher t-value for the malt-extract and its lower p-value shows that the linear effect of the malt-extract is the only significant variable in the model for pigment yield for Monascus purpureus. However for the isolated culture the linear effects of both the variables were notable. These are also reflected in the 3D and contour plots for the pigment yield of both the strains. For the MTCC culture, it can be seen from the Fig. 1 that the variation in the pigment yield was not so significant with glycerol but it varied significantly and positively with the change in the concentration of malt extract. The maximum value was observed to be around 9.2 CVU/mL with a

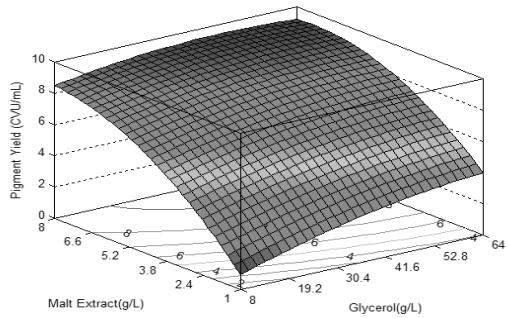

Figure 1. 3D plots with contours for the calculated response of pigment yield for *M. purpureus*

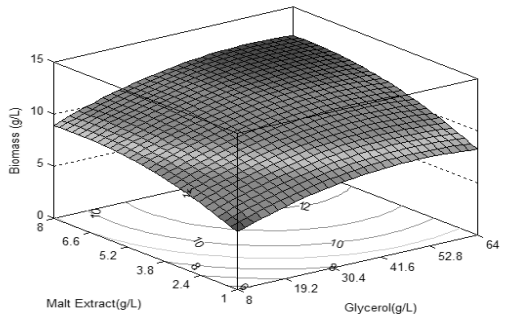

Figure 3. 3D plots with contours for the calculated response of biomass for *M. purpureus*

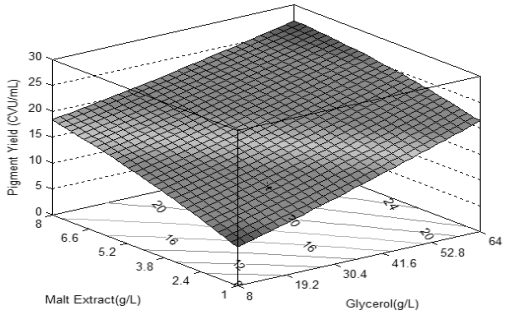

Figure 2. 3D plots with contours for the calculated response of pigment yield for *M. sanguineus*

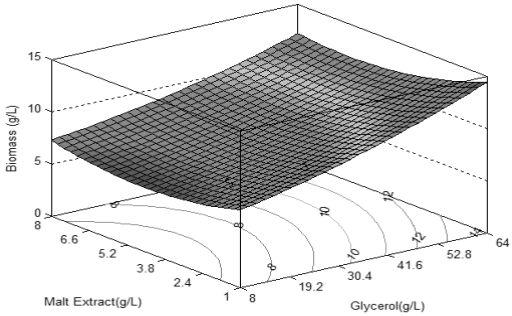

Figure 4. 3D plots with contours for the calculated response of biomass for *M. sanguineus*

glycerol concentration of 36.96 g/L and malt extract concentration of 8 g/L. For the isolated culture in Fig. 2, both the variables showed an impact and the maximum pigment yield of 26.98 CVU/mL was observed at 8 g/L of malt extract concentration and 64 g/L of glycerol concentration

$$\text{Biomass of } M.purpureus \left(\frac{g}{L}\right) = 7.6445 + 0.0749 \times \text{Glycerol} - 0.8767 \times \text{Malt Extract} - 0.0052 \times \text{Glycerol} \times \text{Malt Extract} - 0.0008 \times \text{Glycerol}^2 - 0.0995 \times \text{Malt Extract}^2 \quad \ldots\ldots\ldots\ldots\ldots (4)$$

$$\text{Biomass of } M.sanguineus \left(\frac{g}{L}\right) = 4.6152 + 0.1042 \times \text{Glycerol} + 1.1112 \times \text{Malt Extract} + 0.0001 \times \text{Glycerol} \times \text{Malt Extract} - 0.0011 \times \text{Glycerol}^2 - 0.0641 \times \text{Malt Extract}^2 \quad \ldots\ldots\ldots\ldots\ldots (5)$$

For biomass also the trend was similar. It can be seen from Fig. 3 for the MTCC culture that the variation in the biomass was neither so significant with glycerol nor with malt extract, though it varied positively with the change in the concentration of malt extract. The maximum value was observed to be around 11.9 g/L with a glycerol concentration of 46.62 g/L and malt extract concentration of 8 g/L. For the isolated culture in Fig. 4, the change in malt extract concentration didn't show much variation, but the biomass varied significantly and positively with the glycerol concentration. The maximum value of biomass was observed as 14.45 g/L with 1 g/L of malt extract concentration and 64 g/L of glycerol concentration.

Silvana *et al*. (2008), reported pigment yield with grape waste as a substrate was significantly affected by the concentration of nitrogen. The used nitrogen sources were peptone and monosodium glutamate with peptone having the greatest effect. Glycerol waste derived from biodiesel production has a high potential for pigments production. The variables evaluated were glycerol and monosodium glutamate concentrations and the responses were pigment and biomass production. The monosodium glutamate concentration was the most significant variable for pigment production (Meinicke *et al.*, 2012). Gradual increase in both pigment yield and biomass was observed with the increase in glycerol concentration for *Monascus purpureus* LPB97 (Babitha *et al*., 2007).

The statistical analysis of the maximum pigment yield and biomass is shown in Table 2. The t-values clearly indicate the positive and the negative effects with magnitude of the components on the output. From the analysis of variance for both the strains, the fitting of the model for both the production of red pigment and biomass was calculated by coefficient of determination (R^2) (Subhagar *et al*., 2010). R^2 value was 0.8636 for red pigment yield for isolated culture, which indicated that 86.36% of the total variation in the observed response value could be explained by the model. The rest (13.64%) of the total variation remains unexplained by the model. For MTCC standard culture this value was found to be 90.42% (Table 3). For biomass R^2 for the isolated culture was estimated as 0.9882 (Table 3). This accuracy of

Table 2. Regression coefficient results for pigment yield and biomass for both the strains

Term	*Output*	*Monascus purpureus* MTCC 410			*Monascus sanguineus*		
		Standard error	t - value	p - value	Standard error	t - value	p - value
Constant	P. Yield	1.9370	-0.4767	0.6584	5.0820	0.7122	0.5157
	Biomass	0.6343	12.0517	0.0003	1.7034	2.7094	0.0536
Glycerol	P. Yield	0.0905	0.7874	0.4751	0.2376	0.9346	0.4029
	Biomass	0.0297	2.5275	0.0648	0.0796	1.3090	0.2607
Malt Extract	P. Yield	0.7244	2.7514	0.0513	1.9004	1.2165	0.2907
	Biomass	0.2372	-3.6958	0.0209	0.6370	1.7445	0.1560
Glycerol × Malt	P. Yield	0.0067	-0.7812	0.4783	0.0176	-0.7499	0.4950
Extract	Biomass	0.0022	-2.3699	0.0768	0.0059	0.0179	0.9865
Glycerol ×	P. Yield	0.0011	-0.3533	0.7417	0.0030	0.1758	0.8690
Glycerol	Biomass	0.0004	2.0471	0.1101	0.0010	-1.1081	0.3299
Malt Extract ×	P. Yield	0.0727	-1.3633	0.2445	0.1907	-0.3861	0.7191
Malt Extract	Biomass	0.0238	4.1811	0.0139	0.0639	-1.0026	0.3728

Table 3. Analysis of Variance (ANOVA) for pigment yield and biomass for both the strains

Strain	Output	SS	DF	f-value	p-value	Mean Square	R^2	Adj. R^2
M. purpureus	P. Yield	7.0449	5	7.5521	0.0364	1.7612	0.9042	0.7845
	Biomass	5.4479	5.0000	3.8873	0.1064	1.3620	0.8293	0.6160
M. sanguineus	P. Yield	48.4923	5	5.0665	0.0706	12.1231	0.8636	0.6932
	Biomass	0.7555	5.0000	67.0746	0.0006	0.1889	0.9882	0.9735

98.82% further substantiates our experiment and its findings though this accuracy was 82.93% for the MTCC culture.

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