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Gibberellic acid, amino acids (glycine and L-leucine), vitamin B_2 and zinc as factors affecting the production pigments by *Monascus purpureus* in a liquid culture using response surface methodology

Fateme Baneshi[1], Majid Azizi[2], Mohsen Saberi[3] and Mohammad Farsi[4]

[1]Horticulture Science, Ferdowsi University of Mashhad, Iran.
[2]Agriculture College, Ferdowsi University of Mashhad, Iran.
[3]Industrial Microorganism Department-ACECR, Mashhad bunch, Iran.
[4]Agriculture College, Ferdowsi University Of Mashhad, Iran.

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The combined effects of zinc, gibberellic acid, vitamin B_2, amino acids (glycine and L-leucine) on pigment production were evaluated in a liquid culture of *Monascus purpureus*. In this study, response surface design was used to optimize each parameter. The data were analyzed using Minitab 14 software. Five parameters were applied by using response surface methodology for pigment production in liquid cultures. Four zinc, 11 gibberellic acids, three vitamins B_2, eleven and fifteen amino acids (glycine and L-leucine) respectively; levels were evaluated. The highest production of pigment was reached with a 10 mg/l vitamin B_2, 50 mg/l gibberellic acid and 50 mg/l glycine amino acid.

Key words: *Monascus purpureus*, response surface methodology, amino acid, gibberellic acid, zinc, vitamin B_2.

INTRODUCTION

Since many kinds of synthetic dyestuffs have been found to be hazardous to human health, only limited kinds of such dyestuffs are permitted to be used in food in many countries, and therefore, there is a need to develop alternative sources of natural food colorants (Lee et al., 2000). *Monascus* is an ascomycetous fungus discovered by Van Tieghem (1884) traditionally used for the production of food colouring, fermented foods and beverages (Alsarrani and Elnaggar, 2005). Pigments synthesized by the fungi *Monascus* spp. have been traditionally used in Asia for colouring and securing a number of fermented foods (Lee et al., 2000). The angkak has long been recognized as a folk medicine for improving food digestion and blood circulation and for treatment of muscle bruising and dysentery. The manufacturing process for angkak and its therapeutic applications are well documented in the ancient Chinese pharmacopoeia (Ben- Taso-Gum-Mu) (Miyake

*Corresponding author. E-mail: baneshi_fa@yahoo.com. Tel: (+98) 9171080023. Fax: (+98) 7112271458.

Abbreviation: ANOVA, analysis of variance; **PDA,** potato dextrose agar.

et al., 2005; Panda et al., 2010). Metals are an integral part of all ecosystems (Raza et al., 2010). Some of them are vital components of living systems and known as essential metal ions (Raza et al., 2010). Secondary metabolisms are affected by the presence or absence of these essential metal ions, as they may be responsible for activation of some of the biosynthetic pathways (Raza et al., 2010). The zinc ions and a particular combination of amino acids (glycine, L- leucine, L-tryptophan) were identified as important components in the richer medium responsible for the increased growth (Johnson and Mchan, 1975). *Monascus purpureus* can synthesize many secondary metabolites including red and yellow pigments, monacolins and gamaaminobutyric acid, which are industrially and medicinally important compounds (Jia et al., 2010). Monascus fungi produce at least six major related pigments which can be categorized into three groups based on color as follows: yellow pigments: monascin ($C_{21}H_{26}O_5$) and ankaflavin ($C_{23}H_{30}O_5$); orange pigments: monascorubrin ($C_{23}H_{26}O_5$) and rubropunctatin ($C_{21}H_2O_5$); and red pigments: monascorubramine (C23H27NO$_4$) and rubropuntamine ($C_{21}H_{23}NO_4$) (Subhasree et al., 2011). Several papers concerning the influence of cultivation media composition on the lovastatin biosynthesis have already been published (Bizukojc et al., 2006).

Considering the biochemical mechanisms of the process, a hypothesis was proposed that the supplementation with chosen B-group vitamins might exert a positive effect on the biosynthesis of mevinolinic acid (Bizukojc et al., 2006). Plant hormones are involved in several stages of plant growth and development (Mukhopadhyay et al., 2004). It might be of interest to investigate if they influence the growth of microorganisms also (Mukhopadhyay et al., 2004). It has been shown that gibberellic acid at an optimum concentration of 10 mg/l increased the cell division rate of different strains of *Hansenula wingei* (Mukhopadhyay et al., 2004).

In the previous study, we have found that the highest production of pigment was reached with a pH value of 3, maltose 250 g/l and a temperature of 25°C (Baneshi et al., 2011). In this work, determination of best conditions for pigment production was investigated on the effect of various zinc, gibberellic acid, vitamin B_2, amino acids (glycine and L-leucine) in a liquid cultured with the best conditions obtained from previous study. Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes (Subhagar et al., 2009). RSM, an experimental strategy was used for finding the optimum combination of mixed substrate for pigment production using *M. purpureus*.

MATERIALS AND METHODS

Microorganism

M. purpureus ATCC 1603 was purchased from the Leibniz Institute DSMZ German Collection of Microorganisms and cell cultures (DS MZ, Germany). *M. purpures* was maintained on the PDA (Merck, Germany) slants at 4°C, and cultured at 37°C for 10 days were used for inoculum preparation (Figure 1).

Culture conditions and inoculum preparation

Ten day-old PDA pure cultures of *M. purpureus* (1 pool standard) were used for inoculation of conical flask containing the fermentation medium: 4% of glucose, 1% of yeast extract and 0.1% of KH_2PO_4 in 30 mL of distilled water, adjusted to pH 6. These cultures were incubated at 25°C for 48 h in a shaking incubator at 100 rpm. The basal medium consisted of: 0.15% of NH_4Cl, 0.1% of KH_2PO_4, 0.05% of $MgSO_4{\cdot}7H_2O$, 0.05% of NaCl, 0.01% of $FeSO_4{\cdot}7H_2O$, 250 g/l of maltose and 150 ml of deionized water, adjusted to pH 3. The content of the flasks were mixed and autoclaved at 121°C at 15 psi for 20 min. After inoculation (7.5%, v/v), the fermentation was carried out at 25°C for 14 days in a shaking incubator at 150 rpm.

Pigment estimation

After fermentation, estimating extracellular pigment using ethanol 96% (20 ml) in each medium of pigment taken. The sample were kept on a rotary shaker at 120 rpm for 2 h, allowed to stand for 15 min and filtered through Whatman GF/C filter paper (47 mm). Ethanol extract of control medium was kept as the blank for pigment and analysis was done using a spectrophotometer (Cecil 2010UV-visible) set at 400 nm for yellow pigment, 460 for orange pigment and 500 nm for red pigment. The results were expressed as optical density units per gram of dried medium multiplied by dilution factor (Lin and Iizuka, 1982; Lee et al., 2007).

Experimental design

The optimum conditions, a response surface design were selected. The involved crucial factors were zinc (X_1), gibberellic acid (X_2), vitamin B_2 (X_3), glycine amino acid (X_4) and L- leucine amino acid (X_5). These factors and the level at which the experiments were carried out are given in Table 1. The low and high levels were coded as -1 and +1; the middle level was coded as 0. A total of 33 runs with 7 central points were generated. The central point of the design arrangement decided on was: Concentrations of each zinc, gibberellic acid, vitamin B_2 and amino acids (L- luecine and glycine) were 10, 25, 5, 37.5 and 25 mg/l, respectively.

Response surface methodology

The data were analyzed using Minitab 14 software (Minitab Inc., USA). The quadratic model for predicting the optimal point was expressed as follows:

$$Y = b_\circ + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_4 + b_5 x_5 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 + b_{44} x_4^2 + b_{55} x_5^2 + b_{12} x_1 . x_2 + b_{13} x_1 . x_3 + b_{14} x_1 . x_4 + b_{15} x_1 . x_5 + b_{23} x_2 . x_3 + b_{24} x_2 . x_4 + b_{25} x_2 . x_5 + b_{34} x_3 . x_4 + b_{35} x_3 . x_5 + b_{45} x_4 . x_5$$

Where, Y is the amount of pigment produced (mg/g dry substrate), and x_1, x_2, x_3, x_4 and x_5 are input variables. X_1, x_2, x_3, x_4, and x_5 indicate zinc, gibberellic acid, vitamin B_2 and amino acids (glycine and L- luecine) concentrations, respectively. B_0 is a constant and B_1, B_2, B_3, B_4 and B_5 are linear coefficients. B_1, B_2, B_3, B_4, and B_5 indicate zinc, gibberellic acid, vitamin B_2 and amino acids (glycine and L- luecine) concentrations, respectively. B_{11}, B_{22}, B_{33}, B_{44} and B_{55} are quadratic coefficients and B_{12}, B_{13}, B_{14}, B_{15}, B_{23}, B_{24},

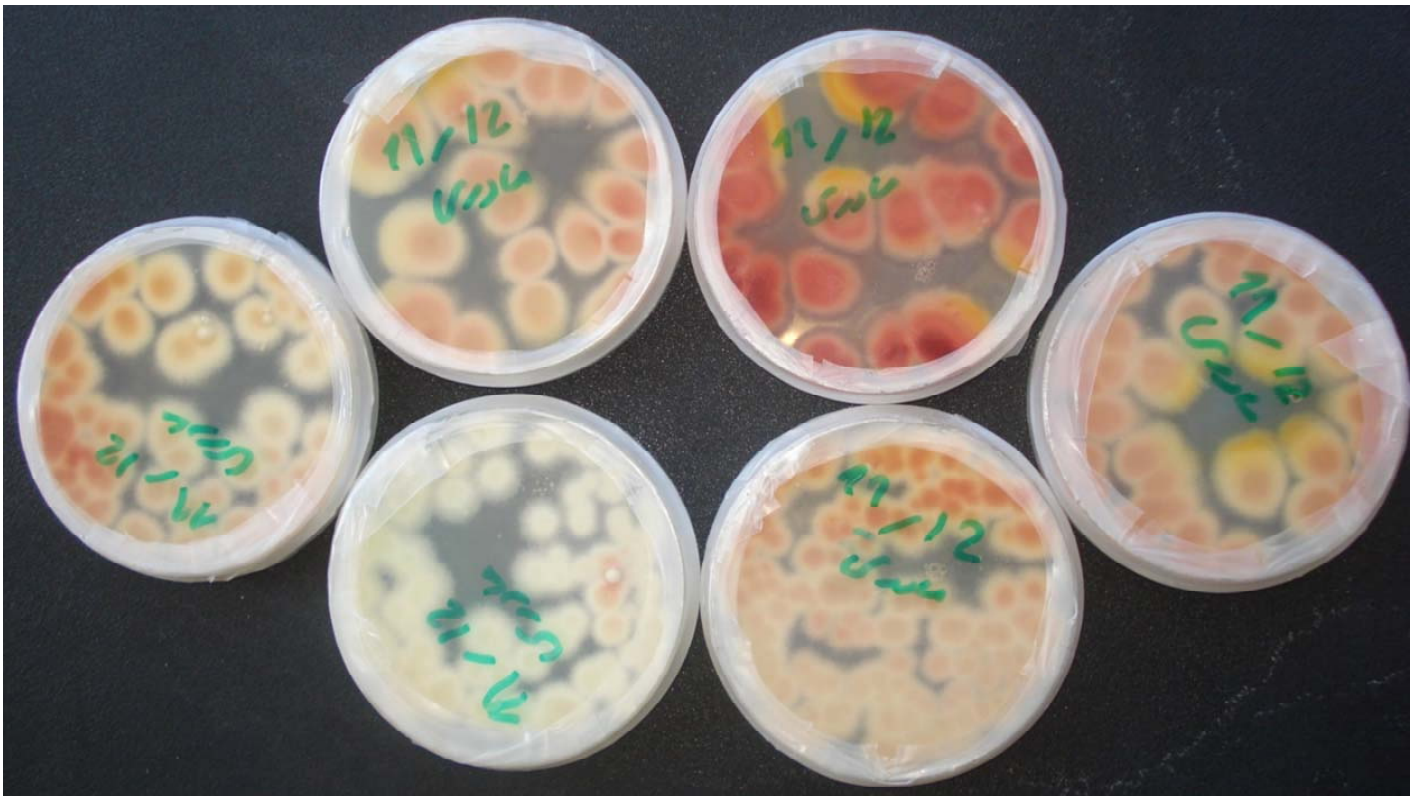

Figure 1. *Monacus purpureus* ATTC 1603 cultivated on potato- dextrose agar for 10 days at 37˚C.

Table 1. Levels of variable used in response surface design.

Variable		Coded-variable level		
	Symbol	-1	0	1
Zinc concentration	X_1	0	10	20
Gibberellic acid concentration	X_2	0	25	50
Vitamin B2 concentration	X_3	0	5	10
Glycine amino acid concentration	X_4	0	25	50
L-luecine amino acid concentration	X_5	0	37.5	75

B_{25}, B_{34}, B_{35} and B_{45} are cross-product coefficients. B_{12}, B_{13}, B_{14}, B_{15}, B_{23}, B_{24}, B_{25}, B_{34}, B_{35}, and B_{45} indicate zinc.gibberellic acid, zinc.vitamin B_2, zinc.amino acid glycin, zinc.amino acid leucine, gibberellic acid.vitamin B_2, gibberellic acid.amino acid glycine, gibberellic acid.amino acid leucine, vitamin B_2.amino acid glycine, vitamin B_2.amino acid leucine and amino acid glycine.amino acid leucine, respectively.

RESULTS AND DISCUSSION

Among the five factors used in the central composite design zinc, gibberellic acid, vitamin B_2 and amino acids (L- leucine and glycine) were used for pigment production. Table 2 shows the results of experimental data and simulated values. Multiple regression analysis of the response surface design for the pigment production:

Yellow Pigment production (A400/g): $0.557+ 0.854\ x_1 + 2.005\ x_2+ 2.433\ x_3 + 0.279\ x_4 +238x_5 – 0.027\ x_1^2 – 0.236x_2^2 – 2.080x_3^2 – 0.012\ x_4^2 + 0.477\ x_5^2 – 1.275\ x_1.\ x_2 – 1.207x_1.\ x_3 – 0.291x_1.\ x_4 – 1.341x_1.\ x_5 – 0.466x_2.\ x_3 – 0.070\ x_2.\ x_4 – 2.546\ x_2.\ x_5 – 0.323\ x_3.\ x_4 – 0.842x_3.\ x_5 – 0.704\ x_4.\ x_5$

Orange pigment production (A460/g): $2.113 – 0.086x_1 + 2.974\ x_2 + 3.014x_3 – 0.642\ x_4 + 1.357\ x_5 + 0.693\ x_1^2 – 1.585\ x_2^2 – 1.343\ x_3^2 + 0.444\ x_4^2 – 0.998x_5^2 – 0.479\ x_1.\ x_2 - 2.395x_1.\ x_3 + 0.798\ x_1.\ x_4 – 1.214\ x_1.\ x_5 – 1.329\ x_2.\ x_3 + 0.563\ x_2.\ x_4 – 1.891x_2.\ x_5 – 1.562\ x_3.\ x_4 – 2.451x_3.\ x_5 + 0.060\ x_4.\ x_5.$

Table 2. Response surface design with actual and predicted pigment production.

Run	Block	Code level					Pigment production (UA400)		Pigment production (UA460)		Pigment production (UA500)	
		X_1	X_2	X_3	X_4	X_5	Actual	predicted	Actual	predicted	Actual	predicted
1	1	-1	-1	-1	-1	1	2.225	2.165	2.530	2.528	2.120	2.121
2	1	1	-1	-1	-1	-1	2.740	2.558	2.900	2.753	2.550	2.475
3	1	-1	1	-1	-1	-1	5.154	5.021	4.710	4.736	2.920	2.994
4	1	1	1	-1	-1	1	4.098	4.130	4.146	4.131	3.174	3.100
5	1	-1	-1	1	-1	1	1.800	1.774	5.304	5.275	1.390	1.471
6	1	1	-1	1	-1	1	2.375	2.514	2.920	2.850	2.570	2.503
7	1	-1	1	1	-1	1	4.362	4.550	5.016	5.119	4.056	4.138
8	1	1	1	1	-1	-1	5.682	5.779	6.465	6.423	4.620	4.625
9	1	-1	-1	-1	1	-1	1.374	1.119	1.192	1.146	0.923	0.931
10	1	1	-1	-1	1	1	2.920	2.830	3.000	2.912	2.630	2.490
11	1	-1	1	-1	1	1	4.428	4.386	4.140	4.226	2.720	2.729
12	1	1	1	-1	1	-1	6.465	6.332	6.090	6.030	4.020	3.952
13	1	-1	-1	1	1	1	2.410	2.475	2.760	2.790	2.240	2.256
14	1	1	-1	1	1	-1	2.940	2.914	3.714	3.599	2.700	2.639
15	1	-1	1	1	1	-1	6.240	6.263	6.135	6.193	4.302	4.390
16	1	1	1	1	1	1	2.380	2.563	2.640	2.657	2.070	2.010
17	1	0	0	0	0	0	4.710	4.692	5.592	5.272	4.074	3.705
18	1	0	0	0	0	0	2.650	4.692	3.690	5.272	2.610	3.705
19	1	0	0	0	0	0	4.962	4.692	5.526	5.272	3.948	3.705
20	1	0	0	0	0	0	5.700	4.692	5.568	5.272	3.762	3.705
21	1	0	0	0	0	0	4.986	4.692	5.646	5.272	3.726	3.705
22	1	0	0	0	0	0	4.902	4.692	5.316	5.272	3.930	3.705
23	2	-1	0	0	0	0	4.494	4.763	5.724	5.498	4.104	3.746
24	2	1	0	0	0	0	5.022	4.997	4.896	5.416	3.552	4.091
25	2	0	-1	0	0	0	3.000	3.465	2.940	3.407	2.250	2.487
26	2	0	1	0	0	0	6.270	6.050	5.538	5.365	3.924	3.868
27	2	0	0	-1	0	0	2.760	3.653	3.852	4.097	2.840	3.105
28	2	0	0	1	0	0	4.338	3.690	4.854	4.903	3.594	3.510
29	2	0	0	0	-1	0	4.890	4.865	5.430	5.606	4.080	4.054
30	2	0	0	0	1	0	4.644	4.914	4.956	5.074	3.594	3.801
31	2	0	0	0	0	-1	4.926	5.561	4.866	5.221	3.492	3.440
32	2	0	0	0	0	1	5.184	4.794	4.164	4.103	2.940	3.173
33	2	0	0	0	0	0	6.120	4.896	6.600	5.131	4.614	3.708

Red pigment production (A500/g): $0.763 + 0.849\ x_1 + 3.242\ x_2 + 1.839\ x_3 - 0.236\ x_4 + 2.288\ x_5 + 0.630\ x_1^2 - 1.589\ x_2^2 - 1.200\ x_3^2 + 0.657\ x_4^2 - 1.203\ x_5^2 - 1.863\ x_1.\ x_2 - 1.781\ x_1.\ x_3 - 0.571\ x_1.\ x_4 - 2.416\ x_1.\ x_5 + 0.736\ x_2.\ x_3 - 0.728\ x_2.\ x_4 - 2.796\ x_2.\ x_5 - 0.408\ x_3.\ x_4 - 1.105\ x_3.\ x_5 - 1.305\ x_4.\ x_5$

The analysis of variance of regression for pigment production was summarized in Table 3. The r^2 values of red pigment level, orange pigment level, and yellow pigment level were 0.88, 0.89, and 0.85, respectively. Also, the test statistics p-value for the overall regression is significant at the 5% level, which means the model is adequate in approximating the response surface of the experimental design. The linear effects of the factors were found to be more significant than the interaction and quadratic effects of the factors. The suitable factors for pigment production, was applied at 10 mg/l vitamin B_2, 50 mg/l gibberellic acid and 50 mg/l glycine amino acid (Figure 2). Figure 3 (A to E) shows response surface plots of the effect of cultivation vitamin B_2, gibberellic acid and glycine amino acid addition on the production of pigment. As shown in Figure 3 (A, B and C) production is optimum at a vitamin B_2 of around 5 mg/l, and increased gibberellic acid addition increased yellow, orange and red pigments content. As shown in Figure 3 (D, E and F), when the vitamin B_2 was around 5 mg/l, the production of pigment gradually increased along with glycine amino acid addition. It was reported that RSM is a reliable and useful statistics methodology for the investigation of the optimal condition (Lee et al., 2007). RSM has some

Table 3. ANOVA for response surface design during pigment production by *M. purpureus.*

Source	Df[a]	Sum of square		
		Red pigment (A500/ g)	Orange pigment (A460/ g)	Yellow pigment (A400/ g)
Regression	20	21.887	48.606	52.088
Linear	5	10.472	27.096	32.985
Square	5	4.659	9.527	8.094
Interaction	10	6.757	11.982	11.008
Residual	11	2.999	5.942	9.330
Lack of fit	6	1.556	3.057	3.950
Pure error	5	1.443	2.885	5.380
Variability explain (r^2)		0.888	0.896	0.859

[a]df: Degree of freedom.

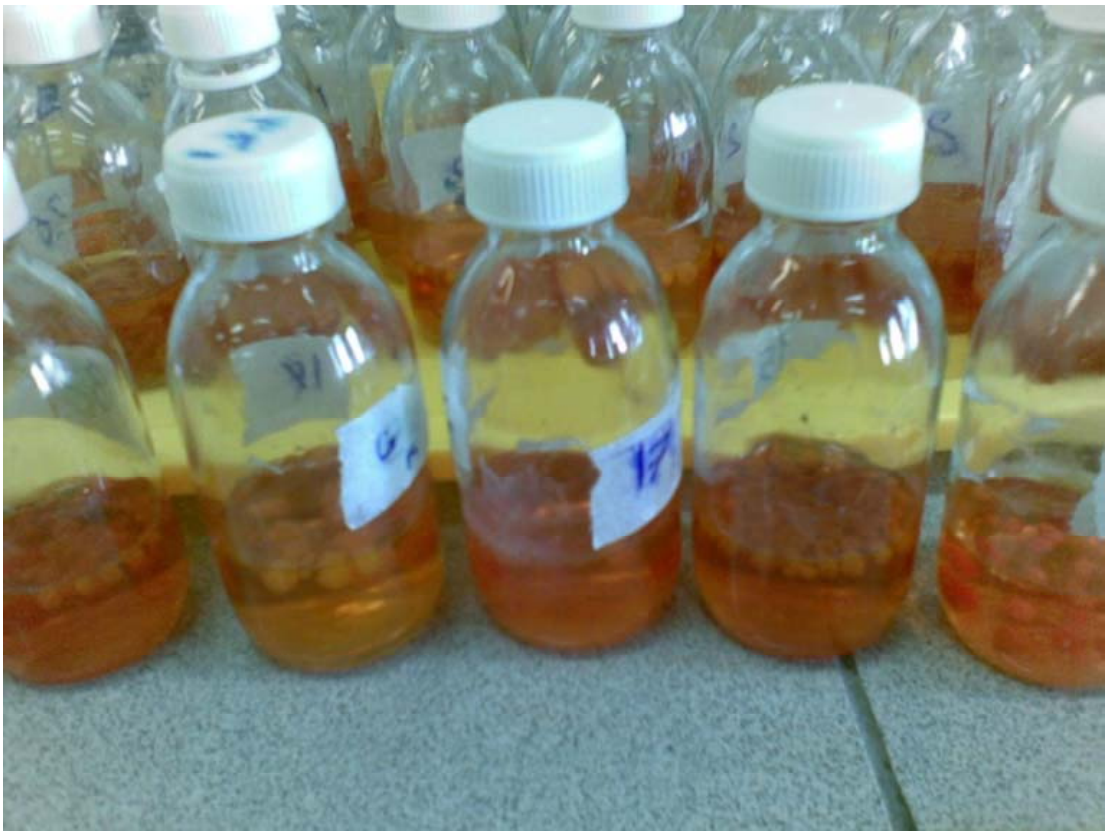

Figure 2. Extracellular pigment production with various zinc, gibberellic acid, vitamin B_2 , amino acids (glycine and L-leucine) concentration in liquid media.

advantages that include fewer experiment numbers, suitability for multiple factor experiments, search for relativity between factors, and finding of the most suitable condition and forecast response (Popa et al., 2007; Panda et al., 2009). This facilitates the determination of optimum values of the factors under investigation and prediction of response under optimized conditions (Panda et al., 2009; Chakravarti and Sahai, 2002).

This study aimed at optimizing the medium composition for higher pigment production in a liquid culture of *M. purpureus* with the best conditions obtained from previous study (Baneshi et al., 2011). The only trace element which was reported to support growth and pigment production by *Monascus* species was zinc and has been reported from different laboratories (Juzlova et al., 1996). Ng et al. (2004) found that the higher certain

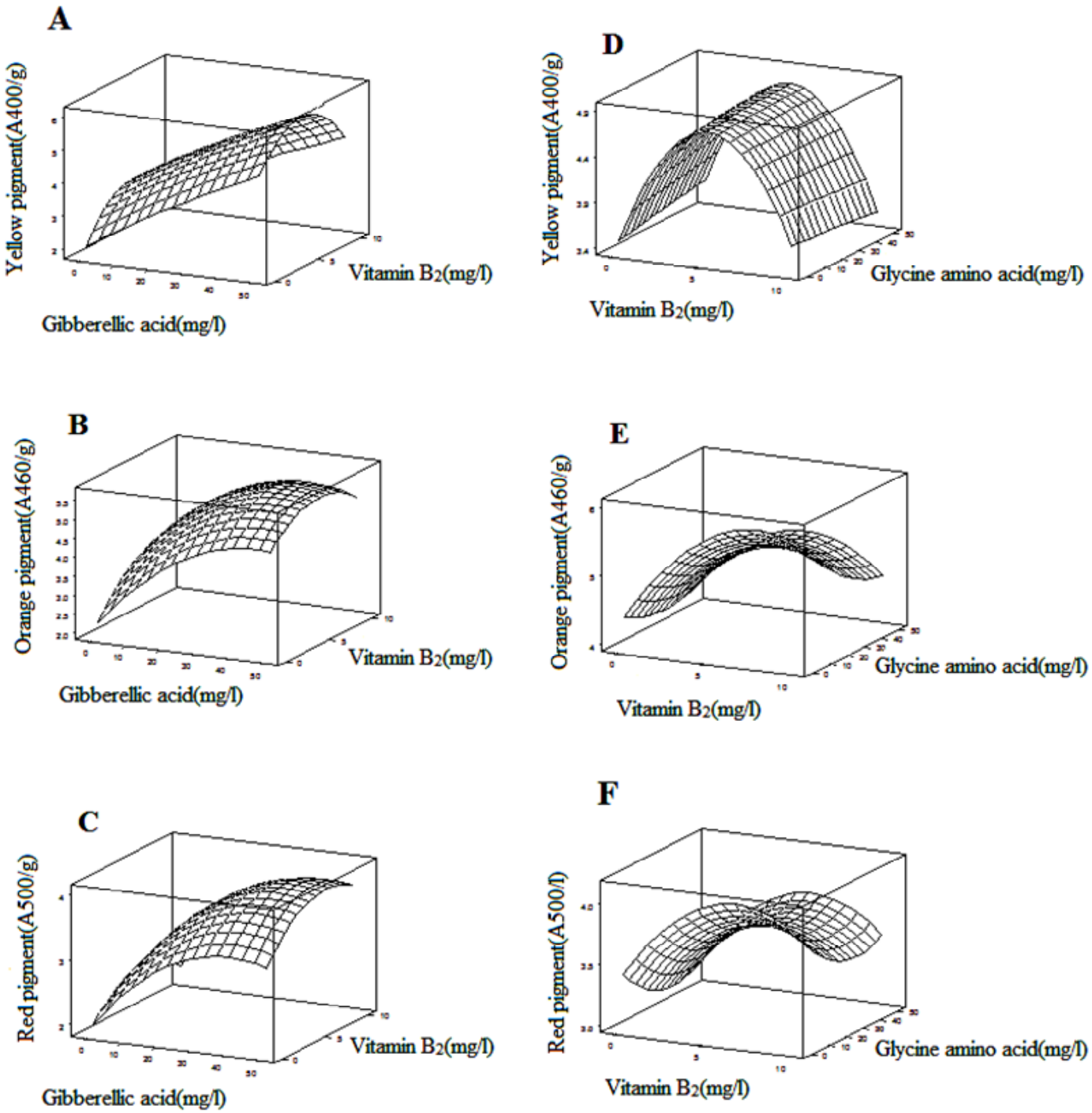

Figure 3. (A-C) Response surface plots showing the effect of gibberellic acid and vitamin B_2 on yellow, orange and red pigments production, respectively, (D-F) Response surface plots showing the effect of glycine amino acid and vitamin B_2 on yellow, orange and red pigments production, respectively while keeping others at constant levels.

zinc concentration, the lower biomass produced but the higher pigment produced. Lee et al. (2001) found that the zinc inhibited red pigment production. Bau and Wong (1979) showed that the growth, pigmentation and antibacterial activity of *M. purpureus* (starch fungus) were affected by zinc. Zinc at concentrations of 2 to 3 mM nearly stopped the growth, pigmentation and antibiotic production of both wild type and strain NI IS in liquid medium. Also, their investigation revealed that the zinc may act as a growth inhibitor and concomitantly as a

stimulant for glucose uptake and for the synthesis of metabolites such as pigments and antibiotics. Timotius and Lestari (1998) proposed that two types of response were observed, first, the higher certain amino acid concentrations, the higher the biomass produced but the production of pigment decreased (Ile, Ala, Leu, Met, Arg, Cys), secondly, the higher certain amino acid concentration, the lower the biomass produced but the higher pigment produced (Tyr, Trp, Thr, Glu, Cys, Gly) of *M. purpureus* UKSW 40. Lin and Demain found that Leucine, valine, lysine and methionine had strong negative effects on the formation of hydrophilic red pigments (Juzlova et al., 1996). Researchers proved that in a nitrogen-deficient medium, the B-group vitamins, both single, especially nicotinamide, pyridoxine and calcium d-pantothenate, and a mixture of thiamine, riboflavin, pyridoxine, calcium d-pantothenate and nicotinamide increased the efficiency of lovastatin biosynthesis (Bizukojc et al., 2006). Mukhopadhyay et al. (2004) suggested that the hormones (indole-3-acetic acid (IAA), gibberellic acid (GA_3) and kinetin (KIN)), at different concentrations, increased the biomass production of *Pleurotus sajor-caju* by 15 to 26%.

Conflict of Interests

The author(s) have not declared any conflict of interests.

Conclusion

In this study, response surface design showed clearly to be a best tool for optimizing pigment production by *M. purpureus* ATCC 1603. RSM results indicated that increasing gibberellic acid and glycine amino acid along with optimum at a vitamin B_2 addition were able to increase the pigment. In conclusion, the optimum conditions of different parameters (10 mg/l vitamin B_2, 50 mg/l gibberellic acid and 50 mg/l glycine amino acid) had an effect on pigment production.

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