



Optimization of biomass production by *Monascus purpureus* in culture medium containing dairy sludge

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ABSTRACT

Cheap compounds and wastes of various industries have a set of nutritious compounds that can be used as a culture medium for different strains to produce metabolites with high nutritional value and expensive. Pigment production from microbial source is of interest because it is safe for human health. In this study, with the aim of producing red pigment from the mold *Monascus purpureus*, firstly, the effect of culture medium factors, potato dextrose broth, yeast extract-sucrose, dairy sludge, soybean meal, sugar cane molasses, whey, temperature, glucose, monosodium glutamate and ammonium sulfate on production biomass (a measure of pigment production) was evaluated through Plackett-Burman design. Then the factors were optimized based on the central square design. Based on the obtained results, dairy sludge base culture medium, glucose, monosodium glutamate and temperature had a positive effect on growth and biomass production (pigment production). With optimization, biomass production in 10% dairy sludge, 0.999% monosodium glutamate, 27°C temperature and 9.83% glucose were obtained with the production of 26.15 g/l of biomass as the optimal state. The results show the capability of dairy sludge waste in the growth of microbial strains and production of significant products.

1- Introduction

In recent years, the demand for the consumption of natural colors has increased, which is due to the increased concern about the harmful effects of synthetic colors in food on human and animal health and the chemicals used in their preparation [1]. Many researchers have proven the medicinal effects of the metabolic products obtained from the fermentation of *Monascus* species, among them, it is possible to mention the reduction of blood pressure, the reduction of plasma cholesterol and the reduction of blood sugar. The production of red pigment by the fungus with reduced citrinin can expand the market for the use of this strain [2]. *Monascus* belongs to the *Monascus* family and is considered one of the real molds. More than 20 species of *Monascus* have been presented in research, but only 9 species of this genus are recognized internationally, which are *Monascus argentinensis*, *Monascus eremophilus*, *Monascus floridanus*, *Monascus lunisporas*, *Monascus pallens*, *Monascus pilosus*, *Monascus purpureus*, *Monascus ruber*. and *Monascus sanguineus*. Among these species, *Monascus pilosus*, *Monascus purpureus* and *Monascus ruber* are the most important and widely used species in the food industry [3]. One of the necessities for production sectors is to reduce the final cost of production. Using food industry waste to produce valuable products such as bioactive compounds seem to be a suitable solution to reduce production costs and waste of food resources. [4, 5]. Dairy industries are one of the largest producers of wastewater, whose separator wastewater is called dairy sludge and contains high amounts of organic compounds such as carbohydrates, proteins, etc., which can be used as a source of carbon and nitrogen for microorganisms [6]. Dairy sludge has a volume between 0.5 and 1% of the volume of milk (about 1000 tons per year). Therefore, this compound can be used as a cheap culture medium for the growth of

microorganisms and the production of various products such as amino acids, exopolysaccharides, enzymes, organic acids, etc. [7]. Pigment production using dairy sludge has not been done so far, and dairy sludge has been used in the production of lactic acid [7] and gamma-aminobutyric acid (GABA) [8]. Molasses is a byproduct of the sugar factory, which is mainly used for animal feed. Due to its high nitrogen content, along with essential amino acids and various minerals, this material can be used as an effective compound on the production of metabolites in the formulation of culture medium [6]. Soybean meal is a byproduct of oilseeds after oiling and solvent extraction, which contains more than 43 proteins. Until now, soybean meal has been used in various fermentation processes as a substrate for the production of secondary metabolites from microorganisms, including fumaric acid by *Rhizopus oryzae*, ethanol by *Saccharomyces cerevisiae* and *Zymomonas mobilis* [6, 9, 10]. Pigment synthesis through fermentation of *M. purpureus* including starch, Saba banana peel, beer residue, cheese, soybean meal, lokot waste kernels, date waste substrates and whey have been evaluated [11]. In this study, with the aim of producing the maximum amount of biomass (a measure of production pigment) along with reducing the cost of fermentation medium, 10 combinations of culture medium and temperature were first screened using Placket-Burman and then the most effective variables were optimized using statistical design.

2- materials and methods

2-1- Preparation of strain

M. purpureus mold was obtained from the collection center of industrial microorganisms and grown on a solid culture medium containing yeast powder, soluble starch and agar and placed in a incubator for 7 days at a temperature of 30°C. Then, it was stored in the refrigerator until use, and preculture was prepared from it once every two weeks [12].

2-2- Screening the type of culture environment and operating conditions affecting biomass production by *M. purpureus* mold through Plackett-Burman

11 variables including potato dextrose broth culture medium, yeast extract-sucrose culture medium, dairy sludge base culture medium, soybean meal base culture medium, sugarcane molasses base culture medium, whey base culture medium, temperature, glucose, monosodium glutamate and sulfate ammonium was screened based on the Plackett-Burman design in the form of 12 test steps. One week before the experiment, a few pieces of mycelium together with the spores grown from the primary culture (mother culture) were transferred to the solid culture medium of starch yeast powder and agar solution and kept in a incubator for 7 days at a temperature of 30°C until the mycelium grew and produce spores. The spores produced from the 7-day culture of *M. purpureus* were washed from the surface of the culture medium with the help of sterile phosphate buffered saline. Based on McFarland's 0.5 standard, a suspension with a concentration of 1.5×10^6 cells/ml was prepared; Then, 400 μ l of the spore suspension with the adjusted concentration was transferred to a 250 ml flask containing 40 ml of different culture media with 5 or 10% glucose and kept for 14 days in a shaker incubator with a certain temperature according to the statistical plan and at 60 rpm was kept in a dark environment. The initial pH of the culture medium was set to 6 to force the microorganism to produce red pigment [10].

2-3- Optimizing the cultivation environment to increase biomass production

Based on the results of the screening test of monosodium glutamate factors, temperature and carbon source were selected as the most effective variables and were optimized using the central composite design (CCD). In the optimization stage,

10% of dairy sludge was used in all treatments due to its positive role.

2-4- Measurement of biomass

Measuring the amount of biomass produced as a response and a measure of pigment production in the tests was considered as a response. The fungal biomass was estimated by determining the amount of N-acetylglucosamine released by acid hydrolysis of chitin present in the mycelium cell wall. Chitin hydrolysis was performed using 10 M hydrochloric acid in an autoclave at 130°C for 2 h. The hydrolyzed mixture was neutralized to pH = 7, then mixed with acetylacetone reagent, followed by Ehrlich's reagent. Finally, the light absorption at 530 nm (against pure nitrogen) was measured. After that, the sample was centrifuged and the supernatant was dried on special plates, and after calculating the percentage of moisture and subtracting it from 100, we considered the dry biomass weight as the answer [13].

3-Results and discussion

3-1- Plackett Burman

In the first part of the study, the basic culture medium (potato dextrose broth, yeast-sucrose extract, dairy sludge culture medium, soybean meal medium, sugarcane molasses medium, whey medium), temperature, monosodium glutamate, ammonium sulfate and glucose were screened (Table 1) and the amount of biomass was considered as the response. Based on the obtained results shown in Figure 2, the basic medium of potato dextrose broth (PDB), basic culture medium of dairy sludge, monosodium glutamate (MSG), glucose and temperature had a positive effect on growth and pigment production. Among these factors, dairy sludge culture medium with a fixed concentration of 10% was considered for the fermentation medium and three factors temperature, glucose and monosodium glutamate were selected for optimization in the plan. The combination of ammonium sulfate was not used because, based on the

studies, it had a lesser effect on biomass production than other variables. Also, the potato dextrose broth culture medium is used as the base culture of the fungus, which was used as a control in the screening, but it was not used in order to investigate the effect of dairy sludge on the production in the fermentation medium in the optimization. Then, the concentration of monosodium glutamate substrates, the carbon source and the temperature used in

the *M. purpureus* mold cultivation environment for the purpose of pigment production were optimized and fully investigated using a statistical scheme. In this part, biomass index was measured as a criterion for pigment production by *M. purpureus* mold. Due to its origin from milk, dairy sludge has a set of nutrients required by microorganisms for growth and production of metabolites [7].

Table 1. Plackett-Burman design of *M. purpureus* fermentation medium.

Ru n	A: PD B (%)	B: Yeas t extra ct (%)	C: Saccharo se (%)	D: Dair y slud ge (%)	E: Soybe an (%)	F: Molasses (%)	G: Whe y (%)	H: Gluco se	J: MS G (%)	K: Ammoniu m sulfate (%)	L: Temperat ure (°C)	Respons e: Biomass (g/l)
1	-1	-1	-1	1	-1	1	1	-1	1	1	1	14.3
2	1	1	-1	1	1	1	-1	-1	-1	1	-1	7.2
3	1	-1	1	1	-1	1	1	1	-1	-1	-1	13.9
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	3.2
5	-1	1	1	-1	1	1	1	-1	-1	-1	1	4.2
6	-1	1	1	1	-1	-1	-1	1	-1	1	1	10.3
7	-1	-1	1	-1	1	1	-1	1	1	1	-1	6.6
8	1	1	-1	-1	-1	1	-1	1	1	-1	1	14.5
9	1	-1	1	1	1	-1	-1	-1	1	-1	1	13.5
10	1	-1	-1	-1	1	-1	1	1	-1	1	1	15.2
11	1	1	1	-1	-1	-1	1	-1	1	1	-1	2.3
12	-1	1	-1	1	1	-1	1	1	1	-1	-1	2.4

Design-Expert® Software

Biomass

Shapiro-Wilk test
W-value = 0.846
p-value = 0.182

- A: PDB
- B: Yeast extract
- C: Saccharose
- D: Dairy sludge
- E: Soybean meal
- F: Molasses
- G: Whey
- H: Glucose
- J: MSG
- K: Ammonium sulfate
- L: Temperature

- Positive Effects
- Negative Effects

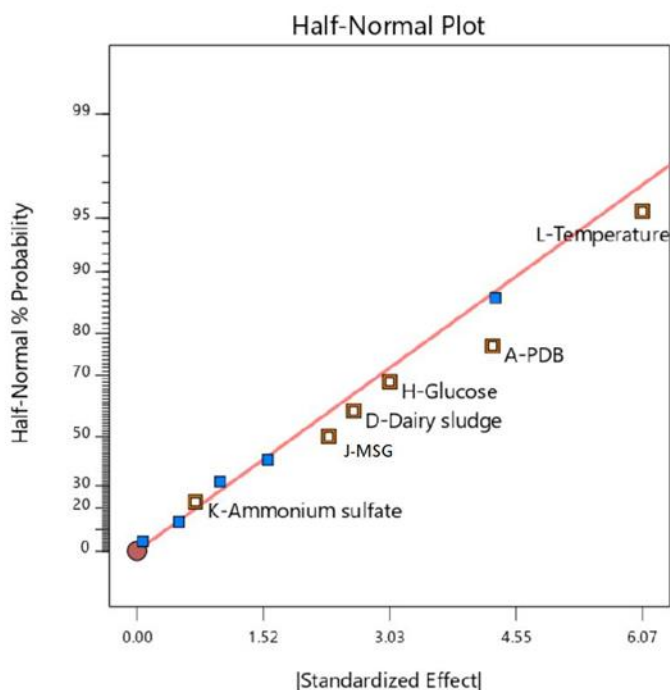


Fig. 1. Plot of the actual values versus the predicted values of the Plackett–Burman design.

3-2- Optimization

Optimization of the factors affecting the amount of biomass was done through RSM in 20 tests (Table 2). The highest and lowest amount of biomass was obtained in test number 12 (25.96 g/l) and 3 (11.95 g/l), respectively. Based on the results obtained, in conditions with 10% dairy sludge, 0.999% monosodium glutamate, 27°C temperature, and 9.83% glucose, the

optimal conditions were selected, which produced 26.15 g/l of biomass. The pictures of 20 *M. purpureus* strain plates are shown in Figure 2. The equation obtained from the design is stated below:

$$\text{Biomass} = +15.07 + 2.66A - 0.6210B + 2.28C - 0.4850AB + 0.0525AC - 1.74BC + 0.0391 A^2 - 0.8009 B^2 + 4.38 C^2$$

Table 2. Tests obtained through optimization.

Run	A: Monosodium glutamate (%)	B: Temperature (°C)	C: Glucose	Biomass (g/L)
1	1	37	10	20.73
2	0.5	32	5	15.04
3	0	32	5	11.95
4	0.5	32	5	14.68
5	0.5	32	5	16.03
6	0.5	32	10	22.71
7	0.5	32	0	15.97
8	1	37	5	19.71
9	0.5	32	5	16.87
10	0.5	32	5	14.08
11	0.5	32	5	14.20
12	1	27	10	25.96
13	1	27	0	18.75
14	1	32	5	18.04
15	0	27	10	20.14
16	0.5	27	5	14.93
17	0	37	10	16.07
18	0	27	0	12.36
19	0	37	0	16.04
20	0.5	37	5	13.38

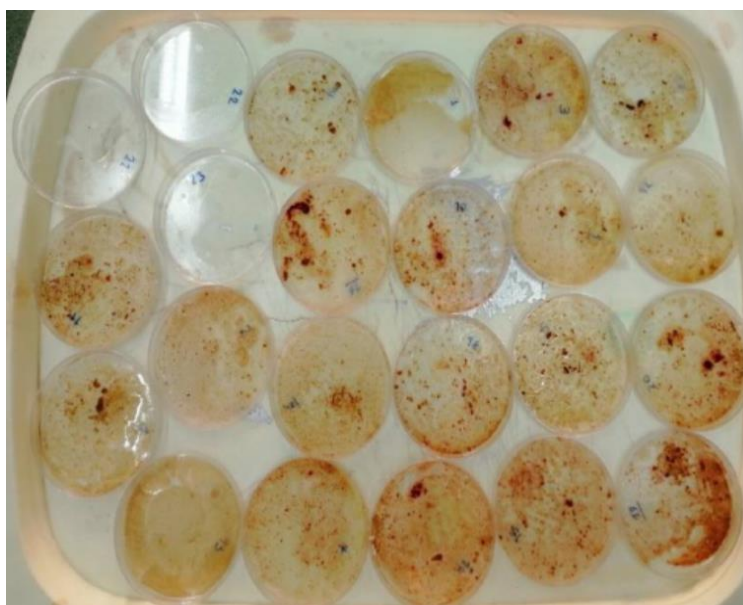


Fig. 2. Images of plates related to *M. purpureus* mold in 20 test run.

The analysis of variance related to the plan is given in Table 4. The results show that the effect of monosodium glutamate and glucose percentage factors on biomass production was quite significant at the significance level of 5%, but the effect of temperature was not significant. In general, the value of the p-value of the model is less than 0.05, which indicates the significance of the model and also the lack of fit was insignificant. The amount of R^2 and R_{Adj}^2

for the model is 95.95% and 92.31%, respectively, which indicates that 95.95% of the data responses can be predicted through the model (Table 5). The predicted R^2 of 0.827 is in reasonable agreement with the adjusted R^2 of 0.923 and the difference is less than 0.2. Adeq Precision measures the signal-to-noise ratio, and a ratio greater than 4 is desirable, and in your study, 20.49 indicates sufficient signal.

Table 3. Analysis of variance table (ANOVA) of biomass production.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	231.95	9	25.77	26.33	< significant 0.0001
A-Monosodium glutamate	70.92	1	70.92	72.45	< 0.0001
B-Temperature	3.86	1	3.86	3.94	0.0753
C-Glucose	51.89	1	51.89	53.01	< 0.0001
AB	1.88	1	1.88	1.92	0.1957
AC	0.0221	1	0.0221	0.0225	0.0437
BC	24.29	1	24.29	24.82	0.0006
A ²	0.0042	1	0.0042	0.0043	0.9491
B ²	1.76	1	1.76	1.80	0.2091
C ²	52.86	1	52.86	54.00	< 0.0001
Residual	9.79	10	0.9789		
Lack of Fit	3.83	5	0.7655	0.6421	0.6807 not significant
Pure Error	5.96	5	1.19		
Cor Total	241.74	19			
Std. Dev.	0.9894			R²	0.9595

Mean	16.88	Adjusted R²	0.9231
C.V. %	5.86	Predicted R²	0.8275
		Adeq	20.4933
		Precision	

The three-dimensional graphs of examining the interaction of variables that had a significant effect on biomass production are given in Figure 3. From the fully significant graphs of the model, the greater effect of monosodium glutamate and carbon source variables than temperature on biomass production is clearly evident. Also, in both graphs, more biomass has been produced by increasing the concentration of glucose in the other constant value. In Figure 3a, with the increase of both variables, the amount of biomass production has increased to a greater amount. Also, in figure 3b, the amount of biomass has decreased with the increase of temperature and glucose simultaneously. In recent years, food product waste is used as one of the valuable substrates in the production of various metabolites. Potato pulp has also been used as an efficient carbon source for pigment production. In particular, the use of potato pulp as a substrate for *M. purpureus* has been investigated and shows its superiority in pigment production. However, it was observed that high concentration of slag inhibits both mycelium growth and pigment production [14]. Other low-cost substrates such as corn starch, banana peel, whey, soybean meal, loquat kernel waste and date palm waste substrates have been evaluated for pigment synthesis through *M. purpureus* fermentation and have yielded positive results. Notably, combining corn starch with oils significantly increased red pigment production by *M. purpureus* NRRL 1992, while enrichment of the medium with sesame oil significantly increased pigment production [15]. Production of extracellular pigments and high biomass in submerged fermentation with glucose-based medium was achieved by *M. purpureus*. Also, the results showed that *M. purpureus* produces 8.61 U/mL and 20.86 U/mL in rice straw-based medium alone or in combination with glucose fermentation medium, respectively [5]. The amount of

biomass of 16.23 g/L has been reported by *M. purpureus* in culture medium based on whey and monosodium glutamate (5 g/L) [16]. The amount of UA500 pigment was 22.25 in the culture medium containing monosodium glutamate, nitrogen and waste [10]. Studies have shown that when some of the cultivation conditions such as inoculum size, temperature, initial pH, hot house time and oxygen concentration and nutritional components (nitrogen, carbon and mineral source) are optimized, *Monascus* species can produce more biomass and pigments. [17, 18]. Similar to the results of this study, in the study on the effect of different carbon sources (sucrose, maltose, glycerol, lactose, glucose and soluble starch), it was observed that in the environment containing glucose as a carbon source compared to other sources, the amount More biomass (4.22 g/l) has been produced [3]. *Monascus* pigments are divided into three groups based on their color, yellow pigments (monascin and enkaflavin), orange pigments (monascorobrine and rubopunctuation) and red pigments (monascorobramin and rubopontamine). The main producers of these pigments are *M. purpureus*, *M. ruber* and *M. pilosus* [19]. These pigments, especially red and yellow pigments, are used as natural colors in the food industry. In addition to color properties, it is known that these pigments have anti-cancer, antimicrobial, anti-diabetic and anti-obesity properties [3]. According to previous studies, glucose concentration has a significant effect on the production of yellow and red pigment. Observed at high concentrations of glucose (120 g/L), the maximum absorption changes from yellow to red and shows the tendency to produce more red pigments at higher concentrations. This may be due to carbon to nitrogen ratio and culture conditions. They also emphasized that using glycerol as a carbon source produces more yellow color than red [3, 4]. According to the results of this study, optimizing the production

of gamma-aminobutyric acid in the culture medium containing dairy sludge has increased the production of gamma-aminobutyric acid [8]. Also, the use of dairy sludge in the culture

medium with the aim of producing lactic acid has increased the production efficiency of this metabolite [7].

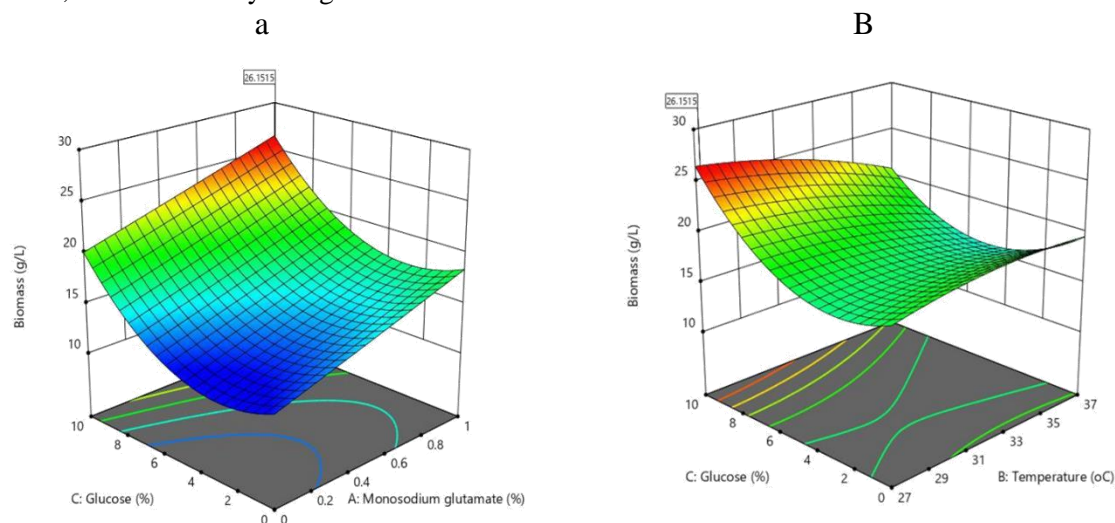


Fig. 3. The contour plot related to the effect of (a) AC and (b) BC affecting biomass production.

4- Conclusion

Total resulting and future perspective, applications and significant progress has been achieved in the optimization of microbial culture methods for the production of natural pigments. Biodegradable waste has been used as a promising substrate for *Monascus* fermentation and discovery of pigment biosynthesis pathways. In this study, according to the obtained results, the selection of culture media and culture methods were done correctly and a significant amount of biomass was produced in the non-specific culture medium containing dairy sludge, monosodium glutamate and glucose. As a result, the production of biomass and color in this non-specific cultivation environment has a bright prospect for industrial production and use in food.

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بهینه سازی تولید زیست توده توسط *Monascus purpureus* در محیط کشت حاوی لجن لبنی

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چکیده	اطلاعات مقاله
<p>ترکیبات ارزان قیمت و ضایعات صنایع مختلف دارای مجموعه‌ای از ترکیبات مغذی هستند که می‌توان از آن‌ها به عنوان بستر کشت سویه‌های مختلف جهت تولید متابولیت‌هایی با ارزش غذایی بالا و گران قیمت تولید کرد. تولید رنگدانه از منبع میکروبی به دلیل ایمن بودن برای سلامتی انسان مورد توجه است. در این مطالعه با هدف تولید رنگدانه قرمز از کپک <i>Monascus purpureus</i> ابتدا تاثیر فاکتورهای محیط کشت پوتیتو دکستروز برات، عصاره مخمر- ساکارز، لجن لبنی، کنجاله سویا، ملاس نیشکر، آب پنیر، دما، گلوکز، مونوسدیم گلوتامات و سولفات آمونیوم بر تولید زیست توده (معیاری از تولید رنگدانه) از طریق طرح پلاکت-برمن مورد بررسی قرار گرفت. سپس بهینه سازی فاکتورها براساس طرح مربع مرکزی انجام شد. براساس نتایج بدست آمده محیط کشت پایه لجن لبنی، گلوکز، مونوسدیم گلوتامات و دما تاثیر مثبتی بر رشد و تولید بیومس (تولید رنگدانه) داشتند. با بهینه سازی، تولید زیست توده در لجن لبنی ۱۰ درصد، مونوسدیم گلوتامات ۰/۹۹۹ درصد، دمای ۲۷ درجه سانتی گراد و ۹/۸۳ درصد گلوکز با تولید مقدار ۲۶/۱۵ گرم بر لیتر زیست توده به عنوان حالت بهینه بدست آمد. نتایج نشان دهنده قابلیت ضایعات لجن لبنی در رشد سویه میکروبی و تولید محصول قابل توجه می باشد.</p>	<p>تاریخ های مقاله :</p> <p>تاریخ دریافت: ۱۴۰۲/۱۱/۱۸</p> <p>تاریخ پذیرش: ۱۴۰۳/۲/۱۹</p> <p>کلمات کلیدی:</p> <p>زیست توده، <i>Monascus purpureus</i> بهینه سازی، لجن لبنی</p> <p>DOI: 10.22034/FSCT.21.150.182.</p> <p>* مسئول مکاتبات: Morteza@um.ac.ir</p>