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Optimization of Monascus Pigment Production on Date Waste Substrates Using Solid State Fermentation

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Abstract

Background and Objective: *Monascus purpureus* can produce pigments with light yellow to dark red colors. It includes several health properties. In this study, a low-cost process has been developed for the production of natural pigments from filamentous fungi through solid-state fermentation using cheap substrates.

Material and Methods: In this study, cultivation conditions were optimized for the production of red *Monascus* pigment by *Monascus purpureus* ATCC16362 using response surface methodology. Incubation time (7-24 days), date waste syrup concentrations (1-69%) and NaCl contents (7-13.75 $g.l^{-1}$) were analyzed base on central composite design.

Results and Conclusion: The maximum production of red *Monascus* pigment (5.10 $AU.g^{-1}$) by *Monascus purpureus* was achieved using 55% date waste syrup concentration, 7 $g.l^{-1}$ NaCl and incubation time of 21 days. At optimum conditions, μ_{max} of 6.2×10^{-3} ($mg.g^{-1}.h^{-1}$), pigment efficiency of 0.238 ($AU.g^{-1}.day^{-1}$), conversion factor of biomass in red pigments of 0.25 ($AU.mg^{-1}.g^{-1}$), glucose utilization of 93% were achieved. Results showed that use of date waste syrup and wheat straw as substrates were successful in solid state cultivation for the production of red pigments by *Monascus purpureus*.

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1. Introduction

Color is one of the most important characteristics of food ingredients. The term "bio-colors" means natural colorant compounds derived from the plant, animal and microorganism sources. As synthetic colors can cause hyperactivity, allergic reactions and cancers in humans, their replacement with bio colors has been interested [1,2]. *Monascus purpureus* belongs to *Ascomycota* class and *Monascosa* family [3]. This fungus is capable of producing polyketidic compounds, lovastatin, antioxidants and proteins [4]. It is able to produce six various types of colors from yellow to red. Pigments produced by this fungus are highly stable at high temperatures, a moderate range of pH (2-10) [5] and darkness [6]. These bio pigment compounds offer antimicrobial [5], antioxidant [7], lipid-lowering [8], anti-obesity [9] and anti-inflammatory [10] characteristics. Furthermore, *Monascus* pigments (Mps) are used as nitrite or nitrate replacements in meats [11]. Two types of fermentation methods are used for the growth and production of microorganisms, solid state fermentation (SSF) and submerged fermentation [1]. Benefits of SSF are comparatively higher than those of submerged fermentation, including more efficiency, decreased bacterial contamination, decrea-

sed substrate costs and need of less equipment. Due to high costs of artificial culture media, efforts have been carried out to use low-cost agricultural wastes for the production of pigments through solid-state fermentation. Several studies have investigated production of microbial metabolites on solid substrates (especially on agricultural wastes) such as production of cellulase by *Asparagillus niger* on corncobs [12], production of erythromycin by *Saccharopolyspora erythraea* on sugar beet roots [13], citric acid production by *A. niger* on sugarcane bagasses [14] and Mp production by *M. purpureus* on jackfruit seeds [15].

Date is widely produced in the Middle East. A large quantity of date is wasted during picking, packing and processing. Based on its high content of carbohydrates, minerals and vitamins as well as low prices, date is as an appropriate source of fermentation processes [16,17]. Use of date wastes as a substrate for the production of lactic acid by *Lactobacillus* KCP01 [18], baker's yeast by *Saccharomyces cerevisiae* [19], citric acid by *Aspergillus niger* PTCC 5010 [20] and arachidonic acid by *Mortierella (M.) alpine* [21] has been studied. Temperature, pH, incubation time, carbon and nitrogen sources and nutrients are the factors affecting red Mp production and growth of *M. purpureus* [1]. Although use of various wastes as carbon sources has been studied for the pigment production [15,22], no efforts have been carried out to use date wastes as substrates to produce red Mps during SSF. Hence, the aim of this study was to develop a potential fermentation process for the production of red Mps from *M. purpureus*. Furthermore, effects of significant factors during SSF were studied using response surface methodology (RSM). These factors included date waste syrup concentration (1-69%), NaCl concentration (5.25-13.75 $g.l^{-1}$) and incubation time (7-24 day).

2. Materials and Methods

2.1 Preparation of fungal strains

The microbial strain used in this study was *Monascus purpureus* ATCC 16362 from Persian Type Culture Collection (Iran, Tehran). The stock culture was maintained on Yeast powder- soluble starch agar (YpSs) slant, including yeast extract, 4 g; soluble starch, 15 g; $MgSO_4,7H_2O$, 0.5 g; K_2HPO_4, 1 g; and agar, 15 g per 1 l distilled water [15]. Theinoculum was prepared using 7-day-old *M. purpureus* spores. Number of spores was adjusted at 10^5 $spore.ml^{-1}$ under aseptic conditions using hemocytometer.

2.2 Solid-state media and fermentation conditions

Date waste was purchased from a local market. Date waste was mixed with distilled water at a ratio of 1:3 ($w\ v^{-1}$) using blender. Ten grams of the wheat straw, date waste (1-69%) and nutrient solution (3 ml) (K_2HPO_4, 2g; $MgSO_4$, 1g; KCl, 1 g; $FeSO_4$, 0.02 g; $NaNO_3$, 6 g; and yeast extract, 8 g in 100 ml) were transferred into a flask [16]. Initial moisture content and pH of the substrate were adjusted to 50% ($w\ w^{-1}$) and 6.0, respectively. Then, this was autoclaved at 121˚C for 20 min and inoculated with *M. purpureus* under aseptic conditions. Then, mixtures were prepared using various levels of the substrate (Table 1) and incubated at 30 ˚C [22].

2.3 Extraction and estimation of red Monascus pigments

Five grams of the fermented solid material was mixed with 50 ml of ethanol 70% ($v\ v^{-1}$) and transferred to an ultrasonic bath (Parsonic, Iran) for 30 min at 25 ℃. Mixture was rotated at 180 rpm for 1 h using rotary shaker. The ethanolic extract was filtered and then absorbance of the supernatant was measured at 500 nm using spectrophotometer (Spectronic UNICO2100, USA). Values were expressed as $AU.g^{-1}$ [16].

2.4 Biomass assessment

The total solid medium (0.5 g) was mixed with 2 ml of sulfuric acid and incubated at 25 ℃ for 24 h. This was autoclaved and filtered through Whatman no. 1 filter papers. Acetone (1ml) was added to the supernatant and the solution was transferred into a water bath for 20 min. After cooling, ethanol (6 ml) was added to the solution, followed by addition of Ehrlich reagent (1 ml) and incubated at 65 °C for 10 min. After cooling, absorbance of the solution was measured at 530 nm using spectrophotometer (UNICO 2100, USA). Various concentrations of N-acetyl glucosemine were used as standard. Results were expressed as $mg.g^{-1}$ [23].

2.5 Glucose assessment

Anthrone reagent (4 ml) was mixed with 1 ml of the fermented solid material extracts. Mixture was incubated for 10 min using boiling water bath. After cooling to room temperature, absorbance was measured at 625 nm. Generally, glucose was used as standard [24].

2.6. Experimental design

The central composite design (CCD) with 20 experimental sets (alpha 1.7) was used for the optimization of Mp production from *M. purpureus* using RSM. Independent variables, including incubation time (A), NaCl content (B), and date waste syrup content (C), were studied at three various levels of low, medium and high (Table 1). Dependent variables (response) included red Mp ($AU.g^{-1}$) and biomass ($mg.g^{-1}$) productions. Second-order polynomial equation was used to describe effects of variables of linear, quadratic and cross-product terms. Analysis of variance and regression analysis were carried out and a response surface plot was drawn using Design-Expert Software v.7.0.0 at 95% confidence interval. After validation of models, μ_{max} (Eq. 1), pigment efficiency (P_M) (Eq. 2), conversion factor of biomass in red pigments ($Y_{P/X}$) (Eq. 3), conversion factor of substrate in red pigments ($Yp/_S$) (Eq. 4), conversion factor

of substrate in biomass ($Yx_{/S}$) (Eq. 5), average cell productivity (P_{cells}) (Eq. 6) and proportion of glucose utilization (Eq. 7) after 21 days of incubation were calculated [25].

$$\mu_{Max}=\frac{lnX-lnX0}{(t-t_0)} \quad \text{Eq.1}$$

$$P_M\ (AU\ g^{-1}\ day^{-1}) =\frac{P_{max}-\ P_0}{(t_{p_{max}}-t_{p_0})} \quad \text{Eq.2}$$

$$Y_{P/X}(AU\ g\ g^{-1}) = \frac{P_{Max-}P_0}{X_{Max-}X_0} \quad \text{Eq.3}$$

$$Y_{P/S}(AU\ gr.gr^{-1})=\frac{PMax}{\Delta S} \quad \text{Eq.4}$$

$$Yx_{/S}(mg.gr^{-1}) =\frac{XMax}{\Delta S} \quad \text{Eq.5}$$

$$P_{cells}\ (g\ l^{-1}day^{-1}) = \frac{X_{max}-X_0}{(t-t_0)} \quad \text{Eq.6}$$

$$\Delta S=\frac{S0-S}{S0}\times 100 \quad \text{Eq.7}$$

Where, P_{Max} was the maximum production of red pigments at time $t_{P_{Max}}$ (AU ml^{-1}), P_0 was the quantity of red pigments at t_0(AU ml^{-1}), $t_{P_{Max}}$ was the time to reach the maximum production of red pigments (day^{-1}), t_0 was the initial cultivation time, X_{max} was the maximum biomass formation at time t (g l^{-1}) and X_0 was the biomass formation at time t_0 (g. l^{-1}).

3. Results and Discussion

3.1 Regression model

Results of the CCD with three factors in 20 treatments have been shown in Table 1. Red Mp and biomass productions varied 1.41-4.9 (AU.g^{-1}) and 8.52-48.02 (mg.g^{-1}), respectively. Multiple regression of CCD was used to analyze main and interaction effects of the red Mps (AU.g^{-1}) and biomass (mg.g^{-1}) productions (Table 2).

Table 1. The central composite design using experimental data

No	Variables			Responses	
	A: Iincubation time (day)	B:NaCl content (g l^{-1})	C:Date waste syrup content (%)	Red *Monascus* pigment (AU g^{-1})	Biomass production (mg g^{-1})
1	11	7	15	2.32	18.41
2	21	7	15	2.02	41.3
3	11	12	15	1.71	10.92
4	21	12	15	1.67	48.02
5	11	7	55	2.87	22.4
6	21	7	55	4.9	8.52
7	11	12	55	1.5	21.29
8	21	12	55	2.97	8.86
9	7.5	9.5	35	1.45	19.53
10	24.5	9.5	35	2.9	20.65
11	16	5.25	35	3.5	20.43
12	16	13.75	35	1.41	27.28
13	16	9.5	1	1.62	17.21
14	16	9.5	69	3	17.211
15	16	9.5	35	2.97	24.41
16	16	9.5	35	1.97	22.55
17	16	9.5	35	2.02	14.82
18	16	9.5	35	2.5	20.6
19	16	9.5	35	1.7	18.11
20	16	9.5	35	2.5	30.52

Table 2. Analysis of variance for response surface design assessment of red *Monascus* pigment (Y_1) and Biomass production (Y_2)

Source	Y_1 Red *Monascus* pigments production (AU g^{-1})				Y_2 Biomass production (mgg^{-1})			
	df	Squares Sum of	F-Value	p-value	df	Squares Sum of	F-Value	p-value
Model	5	12.67	22.84	0.01>	3	1263.43	13.32	0.01
A-Incubation Time	1	2.30	20.69	0.01	1	91.89	2.91	0.11
B-NaCl	1	4.43	39.93	0.01>				
C-Date	1	3.42	30.83	0.01>	1	240.58	7.61	0.01
AC	1	1.84	16.61	0.01	1	930.96	29.45	0.01>
BC	1	0.68	6.17	0.02				
Residual	14	1.55			16	505.85		
Lack of Fit	9	0.48	0.25	0.96	11	358.67	1.11	0.48
Pure Error	5	1.07			5	147.18		
Corrected total	19	14.23			19	1769.29		
R^2			0.89					0.71

Backward elimination of insignificant terms ($p > 0.05$) was carried out and polynomial equations (7 and 8) were derived from the regression analysis. To assess quality of the models and to measure how well the suggested models fit the experimental data, F-value, lack of fit and R^2 were used (Table 2) [25]. As shown in Table 2, F-value of the red Mp and biomass productions was significant and lack of fit of the models was insignificant ($p > 0.05$), which showed that the models were significant. The value of R^2 showed significant relationships between the experimental and predicted values and verified significance of the models. Based on the results in Table 2, red Mp production (Y_1) was significantly affected by the linear effects of the three variables and the interactions of A × C and B × C ($p < 0.05$) (Eq. 7). Incubation time showed strong linear effects on biomass production.

Using multiple regression analysis on the experimental results, a final model (Eqs. 8 and 9) was derived explaining the role of each variable (incubation time (A), NaCl content (B) and date waste syrup content (C) and their interaction in red Mp (Y_1) and biomass (Y_2) production.

$Y_1 = (+3.102) - (0.086A) - (0.022B) + (0.0036C) + (0.0048AC) - (0.0058BC)$ Eq.8

$Y_2 = (-39.259) + (4.292A) + (1.517C) - (0.107AC)$ Eq.9

The three-dimensional (3D) response surface curves were plotted base on the polynomial models, showing relationships between the response and the independent variables (2). The 3D diagram is shown in Figs. 1a, b, c.

3.2 Interaction between the affecting factors

The 3D diagram plots were generated for the responses [red MP (a, b) and biomass production (c)] at any of the two independent variables, while setting the others at the middle levels. Thus, three response surface plots were achieved by considering all the significant combinations (Fig. 1). The response surface plot (Figs. 1a, b) demonstrated that red Mp production increased with the increases in content of the date waste syrup. Therefore, date waste content could stimulate pigment production as shown by Kim et al. [9], who reported that increasing of glucose concentration increased production of the pigment. In fact, amplification of the carbohydrates in culture media was achieved by increases in quantity of the date wastes. Therefore, the higher content of carbon in solid state media increased accessibility of the element to *M. purpureus*, which resulted in increases in the microbial growth, biomass production (Fig. 1c) and red Mp production (Fig. 1a) [26]. Moreover, higher contents of carbon in solid state media caused intensification of osmotic pressure on the microorganism cells, which developed leak of the pigment by the fungi. Moreover, increases in incubation time increased biomass and red Mp productions by *M. purpureus*, (Figs. 1a, c), as previously verified by Rashmi and Padmavathi [27]. They assessed red Mp production by *M. purpureus* in potato dextrose broth media within 16 days and reported that the Mp production was started on Day 4 with a peak on Day 16. Naturally, *M. purpureus* needs several days to adapt to the environment (delayed phase), which is longer in SSF.

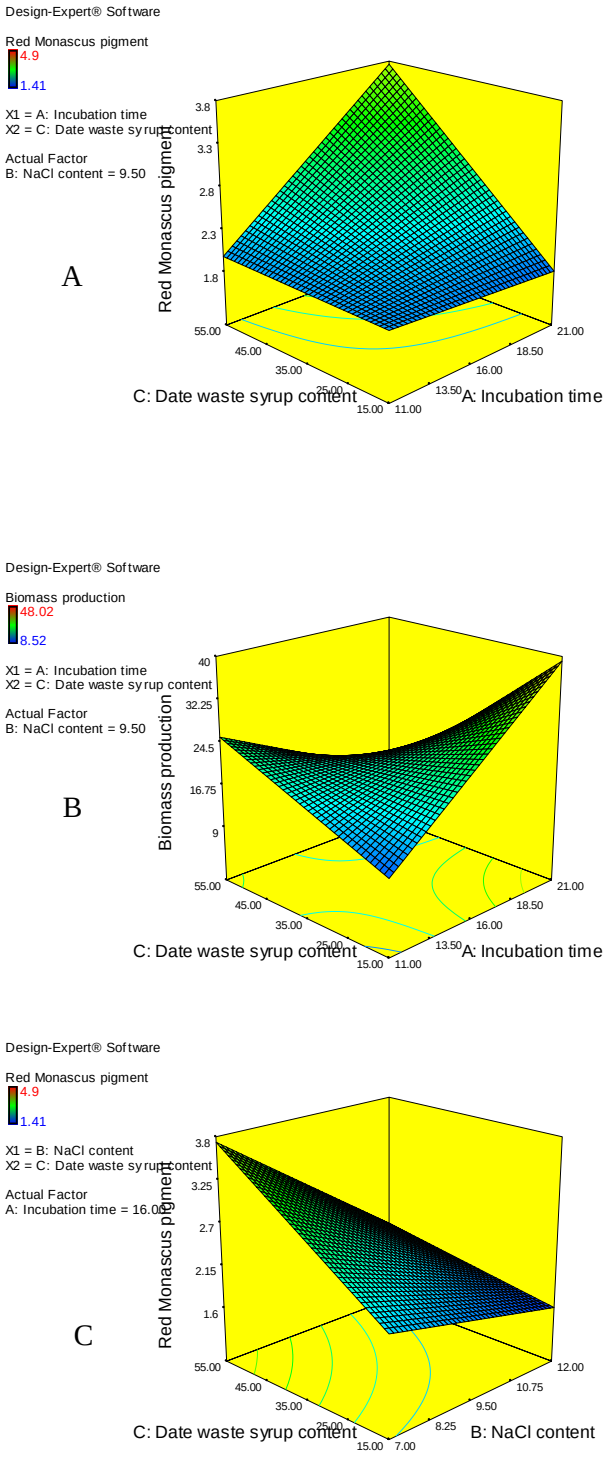

Figure 1. The 3D diagram plots for red *Monascus* pigment (A, B) and biomass (C) production at various incubation time (A), NaCl content (B) and date waste content (C)

Table 3. Biomass and red *Monascus* pigment production by *Monascus purpureus* under optimized conditions using solid state fermentation

Time (day)	Red *Monascus* pigment production (AU.g^{-1})	Biomass production (mg.g^{-1})
0	0^{e}±0	0.53^{e}±0
3	0.85^{de}±0.29	0.82^{e}±0.18
6	1.63^{cde}±0.07	5.43^{de}±2.19
9	2.12^{cd}±1.46	8.48^{d}±0.7
12	2.91^{bc}±0.76	17.11^{c}±2.34
15	3.51^{abc}±0.07	21.05^{bc}±2.15
18	4.35^{ab}±0.3	25.01^{ab}±1.4
21	5.01^{a}±0.43	26.51^{a}±1.69

However, growth of mycelia has increased with time.

The response surface plot (Fig. 1b) showed that higher concentrations of NaCl included inverse effects on red Mp production, in contrast to findings by Babita et al. [28]. These researchers reported that higher NaCl concentrations stimulated red, orange and yellow Mp productions due to increases in osmotic pressure. In the present study, increases in date waste syrup and NaCl content of wheat straw media in SSF might disrupt osmotic balance of the fungal cells, which drastically decreased the microbial growth and pigment production. The highest pigment production was observed in formulations containing 7 g.l^{-1} of NaCl and 55% of date wastes syrup.

3.3 Validation of the model under optimized conditions

The optimum conditions for higher red Mp and biomass productions (as suggested by the software) included date waste syrup concentration (55%), NaCl concentration (7 g.l^{-1}) and 21 days of incubation, respectively corresponding to 4.63 AU.g^{-1} and 39.65 mg.g^{-1} as predicted values. For the validation of these conditions, three replicates were carried out to achieve the highest experimental value of red Mp and biomass productions. The highest red Mp and biomass productions respectively included 5.01 AU.g^{-1} ±0.43 and 26.51 mg.g^{-1} ±1.69, which were supported by the model (Eqs. 7 and 8) with an acceptable accuracy. Biomass and red Mp productions in SSF by *M. purpureus* at optimized conditions within 21 days were investigated (Table 3). Results showed that the growth of *M. purpureus* from Days 0 to 6 was not significant (lag phase). This time was needed for the fungi to adapt to the environment and break the substrate. Up to Day 15, the microbial growth significantly increased (logarithmic phase); then, growth of *M. purpureus* decreased (stationary phase). Based on the results from other studies, these data showed good growth of microorganisms [29,30,31]. From Day 15, growth was restricted due to the culture media and their carbohydrate contents [32]. The maximum specific growth, pigment efficiency, conversion factor of biomass in red pigments, conversion factor of substrates in red pigments, conversion factor of substrates in biomasses, cell productivity and glucose utilization of the optimized samples were 6.2 × 10^{-3} mg g^{-1} h^{-1}, 0.23 AU g^{-1} day^{-1}, 0.25 AU l g^{-1}, 0.04 AU g g^{-1}, 0.36 g l^{-1} day^{-1}, 1.237 g l^{-1} day^{-1} and 93%, respectively. Results of a study by Farhan et al. [30] on fungi and other species of *Monascus* demonstrated that increases in incubation time increased production of the microorganism-linked metabolites. The pigment production efficiency of *Monascus* on potato dextrose agar was lower [33] than that of this study, when date waste syrup was used. The substrate conversion rate was 93%, which was nearly similar to the rate of a study by Farhan et al. [30] (99-97%). The maximum specific growth μ_{max} on submerge media was 0.1 h^{-1} [34] because of the differences in biomass assessment method for solid and submerge cultures. Mousa et al. investigated production of red pigments on various substrates, including rice bran, cob corn, potato peel and wheat bran, with values of 1.30 ±0.03, 0.128 ±0.02, 2.636 ±0.04, 1.193 ±0.02 (AU.gr), respectively [34].

4. Conclusion

The current study was carried out to assess the effects of three important variables of incubation time (7-24 days), date waste syrup concentration (1-69%) and NaCl content (7-13.75 g.l^{-1}) on red pigment production by *M. purpureus* in SSF using RSM. Results showed that use of date waste syrup increased red pigment production. Using selected levels of the process variables, a relatively high quantity of the red Mp was achieved with 55% date waste syrup concentration, 7 g.l^{-1} NaCl and 21 days of incubation in SSF. In general, this study has successfully used CCD for the production red Mps by *Monascus purpureus* ATCC 16362 using date waste and wheat straw as a substrate in SSF to decrease culture media costs at large scales. Furthermore, the current study can promote commercialization of red Mp production. Successful uses of date waste and wheat straw in SSF correctly address problems of other studies for their higher costs of the recently optimized media due to the use of expensive carbon sources for the production of red Mps.

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6. Conflict of Interest

The authors report no conflicts of interest.

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بهینه‌سازی تولید رنگدانه موناسکوس با تخمیر حالت جامد رشدمایه‌های ضایعات خرما

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چکیده

سابقه و هدف: *موناسکوس پورپورئوس* توانایی تولید رنگدانه‌هایی به رنگ‌های زرد روشن تا قرمز تیره را دارد، که خواص سلامتی بخش دارند. این مطالعه، فرایندی کم هزینه برای تولید رنگدانه‌های طبیعی از قارچ‌های رشته‌ای در شرایط تخمیر حالت جامد با استفاده از رشدمایه‌های[1] ارزان‌قیمت به‌کار گرفته شد.

مواد و روش ها: در این مطالعه، شرایط کشت تولید رنگدانه قرمز موناسکوس توسط *موناسکوس پورپورئوس* ATCC13632 به‌روش روش سطح پاسخ، بهینه‌سازی شد. زمان گرمخانه‌گذاری (۷-۲۴ روز)، غلظت شربت ضایعات خرما (۶۹٪-۱) ومیزان سدیم کلرید ($g.l^{-1}$ ۱۳.۷۵-۷) بر اساس طرح مرکب مرکزی مورد آنالیز قرار گرفت.

یافته‌ها و نتیجه‌گیری: بیشینه تولید رنگدانه موناسکوس ($AU.g^{-1}$ ۵/۱۰) توسط *موناسکوس پورپورئوس*، در غلظت ۵۵ درصد شربت ضایعات خرما، غلظت $g.l^{-1}$ ۷ سدیم کلرید و زمان گرمخانه گذاری ۲۱ روز به‌دست آمد. در شرایط بهینه، بیشینه سرعت رشد (μ_{max}) معادل $mg.g^{-1}.h^{-1}$ ۶/۲×۱۰$^{-۳}$، کارایی رنگدانه $AU.g^{-1}.day^{-1}$ ۰/۲۳۸، ضریب تبدیل رشدمایه در رنگدانه‌های قرمز $AU.mg^{-1}.g^{-1}$ ۰/۲۵ و ضریب مصرف گلوکز ۹۳٪ حاصل شد. نتایج نشان داد که استفاده از شربت ضایعات خرما و ساقه گندم، به‌عنوان رشدمایه، در شرایط کشت در حالت جامد برای تولید رنگدانه‌های قرمز از *موناسکوس پورپورئوس* موفقیت آمیز بود.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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- شربت ضایعات خرما
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- رنگدانه قرمز
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