



Increased Water-Soluble Yellow *Monascus* Pigment Productivity via Dual Mutagenesis and Submerged Repeated-Batch Fermentation of *Monascus purpureus*

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Monascus pigments (MPs) have been used in the food industry for more than 2,000 years and are known for their safety, bold coloring, and physiological activity. MPs are mainly yellow (YMPs), orange (OMPs), and red (RMPs). In this study, a mutant strain *Monascus purpureus* H14 with high production of water-soluble YMPs (WSYMPs, λ_{\max} at 370 nm) was generated instead of primary YMPs (λ_{\max} at 420 nm), OMPs (λ_{\max} at 470 nm), and RMPs (λ_{\max} at 510 nm) produced by the parent strain *M. purpureus* LQ-6 through dual mutagenesis of atmospheric and room-temperature plasma and heavy ion beam irradiation (HIBI), producing 22.68 U/ml extracellular YMPs and 10.67 U/ml intracellular YMPs. WSYMP production was increased by 289.51% in optimal conditions after response surface methodology was applied in submerged fermentation. Application of combined immobilized fermentation and extractive fermentation improved productivity to 16.89 U/ml/day, 6.70 times greater than with conservative submerged fermentation. The produced WSYMPs exhibited good tone stability to environmental factors, but their pigment values were unstable to pH, light, and high concentrations of Ca^{2+} , Zn^{2+} , Fe^{2+} , Cu^{2+} , and Mg^{2+} . Furtherly, the produced exYMPs were identified as two yellow monascus pigment components (monascusone B and $\text{C}_{21}\text{H}_{27}\text{NO}_7\text{S}$) by UHPLC-ESI-MS. This strategy may be extended to industrial production of premium WSYMPs using *Monascus*.

Keywords: yellow *Monascus* pigments, stability, mutation, response surface methodology, immobilized fermentation

INTRODUCTION

Monascus pigments (MPs) are polyketide secondary metabolites produced by the genus *Monascus* and have been used as natural edible colorants in Asian food for thousands of years (Feng et al., 2012). Generally, MPs are red (RMPs, such as monascorubramine and rubropunctamine), orange (OMPs, such as rubropunctatin and monascorubrin), and yellow (YMPs, such as monascin

and ankaflavin; Kim and Ku, 2018; Liu et al., 2019a). It has been reported that YMPs are an excellent colorants and are safe, with anti-inflammatory, anti-fatty, antitumor, and antioxidant functions (Wu et al., 2021; Huang et al., 2021b), improving alcoholic liver injury and preventing lipid accumulation (Zhou et al., 2019; Lai et al., 2021), with great prospects for use in food and pharmaceutical industries. However, YMPs are normally hydrophobic or alcohol-soluble intracellular pigments in submerged fermentation, increasing the cost of separation and purification, and limiting their application (Gong and Wu, 2016; Yang et al., 2020). Water-soluble yellow *Monascus* pigments (WSYMPs) and extracellular YMPs (exYMPs) have been studied extensively by researchers.

It has been reported that WSYMPs and exYMPs are not produced on a large scale as a result of their low yield and productivity (Qian et al., 2021; Huang et al., 2021b). Current research efforts often focus on increasing WSYMP/exYMP production through optimization of fermentation broth components, fermentation biotechnology, and chemical modification. Recently, extractive fermentation (EF) with the addition of nonionic surfactants in submerged fermentation of the *Monascus* genus has been widely used. The addition of Triton X-100 can change the mycelium morphology without harming cell growth in the EF of *M. anka* GIM 3.592, and increases exYMP production (Chen et al., 2017a,b). Xiong et al. (2015) intensified the transfer of intracellular YMPs into an extracellular nonionic surfactant micelle aqueous solution using EF and enhanced exYMP production. Huang et al. (2021b) increased WSYMP production by 69.68% compared to conventional fermentation by adding sodium starch octenyl succinate. In addition, high glucose stress can promote YMP biosynthesis and significantly increase extracellular YMP production (Huang et al., 2017c). Changing the extracellular oxidoreduction potential can also enhance WSYMP production in submerged fermentation of *Monascus ruber* CGMCC 10910 (Huang et al., 2017b). Chemical modification was performed by introducing H_2SO_3 into the double bond at the MP sidechain to produce WSYMPs with high stability over a wide pH range (Liu et al., 2020a). However, the application of these strategies, especially EF, to enhance exYMPs has been problematic, increasing the cost of YMP purification.

Breeding of wild strains is most critical in microbial fermentation production of metabolites. Zhou et al. (2009) obtained a mutant strain with high YMP production through physical and chemical mutagenesis. Traditional methods of mutation breeding use chemical agents such as LiCl and nitrosoguanidine (Kodym and Afza, 2003; Sun et al., 2011). However, chemical mutagens can remain in the food industry and are harmful to human health; development of new and powerful mutagenesis methods is required. With the development of breeding biotechnology, atmospheric and room-temperature plasma (ARTP) mutagenesis systems have emerged as a novel methodology for strain breeding (Zhang et al., 2014). In our previous studies, mutant strains including *M. purpureus* M183 with high MP production, *M. purpureus* M630 with high exYMP production, and *M. purpureus* M523 with high rice husk

hydrolysate tolerance were generated using the ARTP screening system (Liu et al., 2019b, 2020b). In addition, heavy ion beam irradiation (HIBI), a novel and more efficient irradiation method for strain mutagenesis, has unique physical and biological advantages due to its high linear energy transfer and relative biological effectiveness, wide mutation spectrum, and high mutation rate (Gao et al., 2020; Guo et al., 2020; Zhang et al., 2022). Previous studies have demonstrated that HIBI produces a high mutation rate, mainly attributed to deletions, nucleotide exchange, and insertions (Chen et al., 2018). However, the combined application of ARTP and HIBI mutagenesis to enhance MP production has not been reported.

Generation of a mutant strain is difficult in industrial WSYMP production using *Monascus*. In this study, we obtained a mutant strain in WSYMP production through dual mutagenesis of HIBI ($^{12}\text{C}^{6+}$) and ARTP. Response surface methodology (RSM), EF, and immobilized fermentation (IF) were used to increase WSYMP yield and productivity.

MATERIALS AND METHODS

Microorganisms and Chemical Materials

Monascus purpureus LQ-6 [CCTCC M 2018600, China Central for Type Culture Collection (CCTCC), Wuhan, China] was isolated from red mold rice obtained from a local market (Changsha, China) as the parent strain. The mutant strain *M. purpureus* H14 was obtained through dual mutagenesis of ARTP and HIBI. Analytical-grade chemicals were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Chemical reagents of vitamins were purchased from Coolaber (Beijing, China). Potatoes used to produce potato dextrose agar (PDA) medium were purchased from a local market (Changsha, China).

Mutagenesis and Screening

Monascus purpureus strains were cultured on PDA medium (200 g/L potato, 20 g/L glucose, and 20 g/L agar) for 7 days at 30°C in darkness. The mycelium on a PDA Petri dish containing a fully grown culture of *M. purpureus* strain was aseptically scraped and inoculated in a 100-ml conical flask containing 20 ml of sterile water and glass beads, and agitated for 3 min to prepare the spore suspension; the concentration was adjusted to approximately 10^7 spores/ml.

Heavy ion beam irradiation was performed using carbon ion beams ($^{12}\text{C}^{6+}$, 80 MeV/u, and 20 keV/ μm) at the Heavy Ion Research Facility in Lanzhou (HIRFL), Institute of Modern Physics, Chinese Academy of Sciences. The spore suspension (1.5 ml) was transferred into a 35-mm irradiation dish. The detailed steps were reported by Gao et al. The irradiation dose was set as 0, 50, 100, 150, 200, 250, and 300 Gy (Gao et al., 2020). The ARTP mutagenesis process was presented in our previous study (Liu et al., 2019b). The conditions were the same as in our previous study, except the variable parameter treatment time T was set to 0, 40, 80, 120, 160, 200, 240, and 280 s. After mutagenesis, 100 μl of the spore suspension was spread onto PDA plates and incubated at 30°C in darkness to generate the mutant strains. The fatality and survival rates

were analyzed by normalizing the colony counts of irradiated spores on PDA plates with those of untreated spores.

The spore suspension (5 ml, 10^7 spores/ml) was inoculated in a 250-ml conical flask containing 45 ml of screening fermentation broth (80 g/L rice flour, 2.5 g/L yeast extract, 2.5 g/L peptone, 5 g/L KH_2PO_4 , and 0.01 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), incubated for 10 days at 30°C, and agitated at 150 rpm in the dark.

Fermentation Biotechnologies

Conventional submerged batch fermentation (SBF) was conducted as follows: 5 ml of the spore suspension (10^7 spores/ml) was inoculated in a 250-ml conical flask containing 45 ml of fermentation broth (50 g/L glucose, 2 g/L malt extract, 10 g/L peptone, 2.5 g/L KH_2PO_4 , 5 g/L NaNO_3 , 1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and pH 3.8), incubated for 10 days at 30°C, and agitated at 150 rpm in the dark.

Recent studies (Hu et al., 2012; Chen et al., 2017a; Lu et al., 2021) have revealed that the concentration of 40 g/L of Triton X-100 is the preferred effective strategy for improving MP production in extractive SBF by parent strain. Thus, Triton X-100 (40 g/L) was added to the optimized conventional submerged batch-fermentation broth (20 g/L glucose, 12.29 g/L malt extract, 15 g/L peptone, 2.5 g/L KH_2PO_4 , 5 g/L NaNO_3 , 0.2 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.32 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.15 g/L CaCl_2 , 0.15 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g/L vitamin B5, and pH 4.8) using RSM to construct the extractive SBF.

Immobilized fermentation was performed according to the method in our previous study, with several modifications (Liu et al., 2019b; Zhang et al., 2022). The spores of mutant strain *M. purpureus* H14 were immobilized in different ratios of sodium alginate (SA) and polyvinyl alcohol (PVA) solution [3% (wt/wt):0% (wt/wt), 2.5% (wt/wt):0.5% (wt/wt), 2% (wt/wt):1% (wt/wt), and 1.5% (wt/wt):1.5% (wt/wt)] instead of in 3% (wt/wt) sodium alginate solution.

The kinetics of SBF and repeated-batch fermentation (RBF) of *M. purpureus* H14 were determined in a 1,000-ml conical flask containing 300 ml of optimized fermentation broth. During fermentation, 10-ml samples were collected to measure metabolite concentrations.

Experimental Design and Statistical Analysis

Response surface methodology was used to optimize four conventional SBF broth components ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, malt extract, and vitamin B5) to enhance WSYMP production after fermentation of *M. purpureus* H14. The Box-Behnken design (BBD) was used to optimize the components. The four independent variables used in the experimental design were $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1–0.5 g/L), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.05–0.2 g/L), malt extract (5–15 g/L), and vitamin B5 (0.3–0.5 g/L); three levels were used to optimize the fermentation process.

Physicochemical Property Assays

To determine the polarity of YMPs produced by the mutant strain *M. purpureus* H14, 1 ml of fermentation medium was collected and centrifuged at 10,000 rpm for 5 min, followed by

the addition of 1 ml of pure water or ethyl acetate to the fermentation medium and ankaflavin-methanol solution, respectively.

As in our previous study, the YMP alcohol solution was generated using the adsorption–separation system with the macroporous adsorption resin LX300C (Liu et al., 2019b). Resin LX300C (2 g) was added to a 100-ml conical flask containing 50 ml of the centrifuged fermentation medium to adsorb the YMPs at 30°C for 24 h and agitated at 150 rpm in the dark. The YMPs were desorbed using 70% (v/v) alcohol eluent in the same conditions. The same volume of pure water was added to the YMPs prepared by freeze-drying to generate the WSYMP solution.

The pH of the WSYMP solution and ankaflavin-methanol solution (the concentration was adjusted to 1 g/L, equal to 30.08 U/ml) was regulated from 1 to 14 with 1 mol/L HCl or NaOH. A full-band scan was performed to determine the maximum absorption wavelength and measure the WSYMPs with different concentrations (1–100 mmol/L) of metal ions added to the WSYMP solution, including Na^+ , Mg^{2+} , K^+ , Ca^{2+} , Zn^{2+} , Cu^{2+} , and Fe^{2+} . Five milliliters of WSYMP solution and ankaflavin-methanol solution was poured into a 60 mm × 20 mm culture dish before irradiation (3,000 lx of light intensity, 30 cm of irradiation distance) in a constant-temperature incubator (adjusted to 30°C) with incandescent LED lamps; the group left in darkness (0 lx) was used as the control. WSYMP solution (5 ml) was treated in an electro-thermostatic water bath at different temperatures (25°C–90°C) to evaluate the pH, metal ions, temperature, and visible light stability.

Composition of YMPs

Yellow *Monascus* pigments were detected by ultra-high-performance liquid chromatography (Dionex Ultimate 3000 UHPLC). The mobile phase was 0.1% formic acid (20%) and methanol (80%) at a flow rate of 0.5 ml/min, with separation carried out using an Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 μm) at 30°C, run time for 15 min and 5 μl of injection volume. Mass spectrometry was carried out by Ultimate 3000 UHPLC-Q Exactive (Thermo Scientific, United States) equipped with an HESI source. The parameters were set as follows: 10 ml/min of aux gas flow rate, 40 ml/min of sheath gas flow rate, capillary temperature at 300°C, 3.8 kv of capillary voltage, and 50% S-Lens. Each sample was analyzed in positive modes with a mass scan range of 100–800 m/z, and resolution was set at 70,000.

Determination of Metabolites and Calculations

The concentrations of glucose, biomass, and YMPs were measured according to our previously described methods (Liu et al., 2019a,b). The residual glucose concentration was determined using the standard 3, 5-dinitrosalicylic acid method. The YMP concentration was analyzed by measuring the absorbance of the supernatant at 370 nm, which included intracellular YMP content (inYMPs) and exMP content.

For the data analysis, F test was applied to evaluate the effect of independent variables on the RSM; the significant results were identified by a value of $p < 0.05$; the fitness of second-order model was evaluated by multiple correlation coefficient (R^2) and adjusted R^2 (R^2_{adj}). Each experiment was repeated at least three times; the numerical data are presented as the mean \pm SD.

RESULTS AND DISCUSSION

Obtaining Mutant Strain

From **Figure 1A**, the fatality rate of parent strain *M. purpureus* LQ-6 was 89.64% (approximately 90%) at an irradiation dose of 200 Gy. Over 250 Gy, the fatality rate exceeded 95.47%; at 300 Gy, the active *M. purpureus* LQ-6 spores were almost completely killed. According to modern theory of breeding and positive/negative mutation rate, a 90% fatality rate was set as the target (Dong et al., 2017). Thus, an irradiation dose of 200 Gy was chosen for further evaluation. The morphology and color of the mutant strains were observed for 7 days during growth on PDA plates. We found three distinct mutant strains among hundreds of potential mutant colonies; mutant strain

No. 2 exhibited a smooth surface (almost no hypha), and the color was light in comparison with others. From the reversed colony on the PDA plate, mutant strain No. 23 exhibited the reddest color, and the hypha was abundant. Mutant strain No. 20 exhibited niveous hypha, but the PDA agar broth was yellow (**Figure 1B**). It remained yellow after inoculation with multiple passages of mutant strain No. 20; thus, this mutant strain (denoted as H1) was selected for further YMP production by ARTP.

From the survival rate curve for mutant strain H1 by ARTP, the mortality rate reached 90.28% at $T = 160$ s (**Figure 1C**), which was different from reported ($T = 180$ s) in our previous study (Liu et al., 2019b), illustrating that the sensitivity of different strains to ARTP mutagenesis is distinct. Subsequently, 68 mutant strains were selected for the determination of the target mutant strain *via* SBF after preliminary investigation of the morphology and color of mutant strains generated by ARTP mutagenesis with $T = 160$ s. **Figure 1D** indicates that the mutant strain *M. purpureus* H14 produced the highest concentration of YMPs (λ_{max} at 370 nm, 21.41 U/ml) by SBF in the screening fermentation broth, which was approximately 3.47 times greater than the concentration produced by the mutant strain *M. purpureus* H1. However, the primary YMPs (λ_{max} at 420 nm),

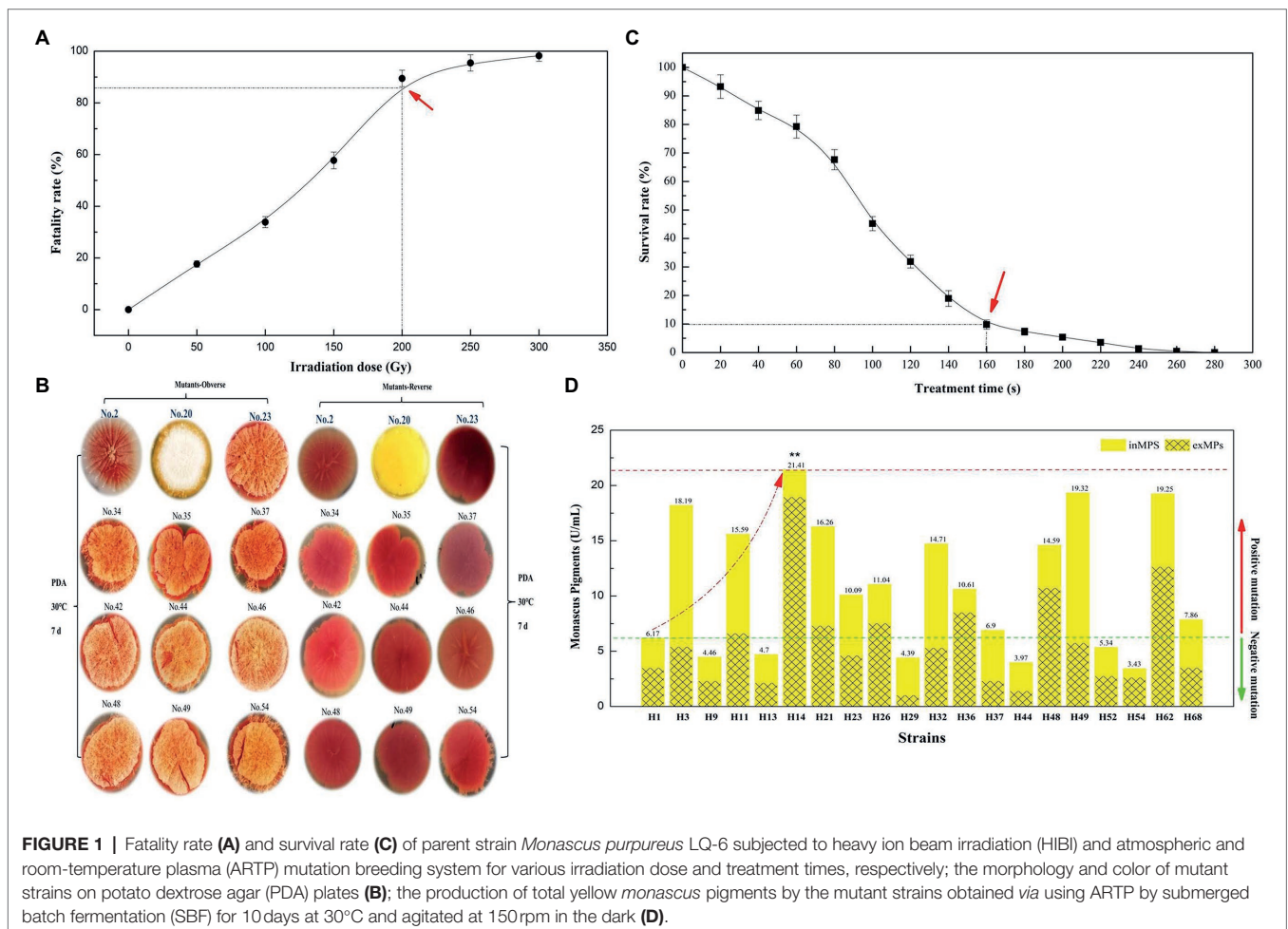


FIGURE 1 | Fatality rate (A) and survival rate (C) of parent strain *Monascus purpureus* LQ-6 subjected to heavy ion beam irradiation (HIBI) and atmospheric and room-temperature plasma (ARTP) mutation breeding system for various irradiation dose and treatment times, respectively; the morphology and color of mutant strains on potato dextrose agar (PDA) plates (B); the production of total yellow *monascus* pigments by the mutant strains obtained *via* using ARTP by submerged batch fermentation (SBF) for 10 days at 30°C and agitated at 150rpm in the dark (D).

OMPs (λ_{\max} at 470 nm), and RMPs (λ_{\max} at 510 nm) were produced by the parent strain *M. purpureus* LQ-6 in these conditions. The ratio of exYMPs to inYMPs was 7.81, indicating that mutant strain *M. purpureus* H14 with high production of exYMPs was obtained through dual mutagenesis of HIBI and ARTP.

Effect of Glucose Concentration and pH on YMP Production

It is known that fermentation parameters, including substrate concentration and pH, play a vital role in targeted product biosynthesis. Pigment production by the genus *Monascus* has been reported to be significantly affected by glucose concentration (Chen and Johns, 1994; Wang et al., 2017a). The composition and color characteristics of *Monascus* pigments can be controlled by pH and nitrogen sources in SBF (Shi et al., 2015). Based on these findings, we determined the optimal original glucose concentration and pH of the fermentation broth for *M. purpureus* H14 in SBF; the initial glucose concentration was increased from 10 to 70 g/L (the raw concentration was 50 g/L), and the pH of the conventional SBF broth was adjusted from 2.8 to 6.8 (the raw pH was 3.8).

Figure 2 shows the effect of initial glucose concentration and pH on YMP production *via* SBF. The total YMP production and the ratio of exYMPs to inYMPs (exYMPs/inYMPs) in SBF for *M. purpureus* H14 were significantly affected by regulating the original glucose concentration and pH. YMP production and the exYMPs/inYMPs ratio were increased by 35.17 and 109.86% (to 45.08 U/ml and 4.47), respectively, when the initial glucose concentration was 20 g/L, compared to a raw concentration of 50 g/L (33.35 U/ml and 2.13). **Figure 2A** also shows that YMP production decreased as the glucose concentration increased beyond 30 g/L. When the glucose concentration was 70 g/L, total YMP production decreased to 15.25 U/ml, but a high exYMPs/inYMPs ratio was maintained. **Figure 2B** shows that YMP production and the exYMPs/inYMPs

ratio increased by 50.22 and 14.55% (to 50.10 U/ml and 2.44), respectively, when the initial pH was adjusted to 4.8, compared with a raw pH of 3.8 (33.35 U/ml and 2.13). The exYMPs/inYMPs ratio decreased when the initial pH was greater than 5.8; the change was insignificant when the pH was less than 4.8. It has been reported that glucose (and pH) can control the oxidoreduction potential (ORP) level, which can further regulate the ratio of NADH/NAD⁺ and intracellular enzyme activity (Wang et al., 2017b). High glucose stress can promote YMP biosynthesis by changing ORP levels (Wang et al., 2017b). High exYMP production was generated by adding 30 g/L glucose in fed-batch fermentation (Hu et al., 2012). Chen and Johns reported that the growth of *M. purpureus* and the biosynthesis of ankaflavin (a type of YMP) were favored at low pH (4.0); the growth and biosynthesis of other pigments were independent of pH (Chen and Johns, 1993).

The optimal initial glucose concentration and pH in SBF broth were determined to be 20 g/L and 4.8, respectively, for maximum exYMP yield and cost-efficiency for industrialized application.

Optimization of Fermentation Medium by RSM

It is known that RSM can effectively increase the yield of a target product using liquid-state fermentation (or submerged fermentation). YMP production in this study using *M. purpureus* H14 with different concentrations of vitamins, nitrogen, and metal ions is shown in **Table 1**. According to the experimental data, vitamin B5 (0.4 g/L), malt extract (10 g/L), ZnSO₄·7H₂O (0.2 g/L), and MnSO₄·H₂O (0.1 g/L) significantly upregulated YMP production (increased by 90.64, 185.31, 153.61, and 123.42%, respectively). In order of importance to YMP production, these can be ranked as malt extract (a representative nitrogen source), ZnSO₄·7H₂O, MnSO₄·H₂O (representative of metal ions), and vitamin B5 (representative of growth cofactors). In addition, we found that the ratio of exYMPs

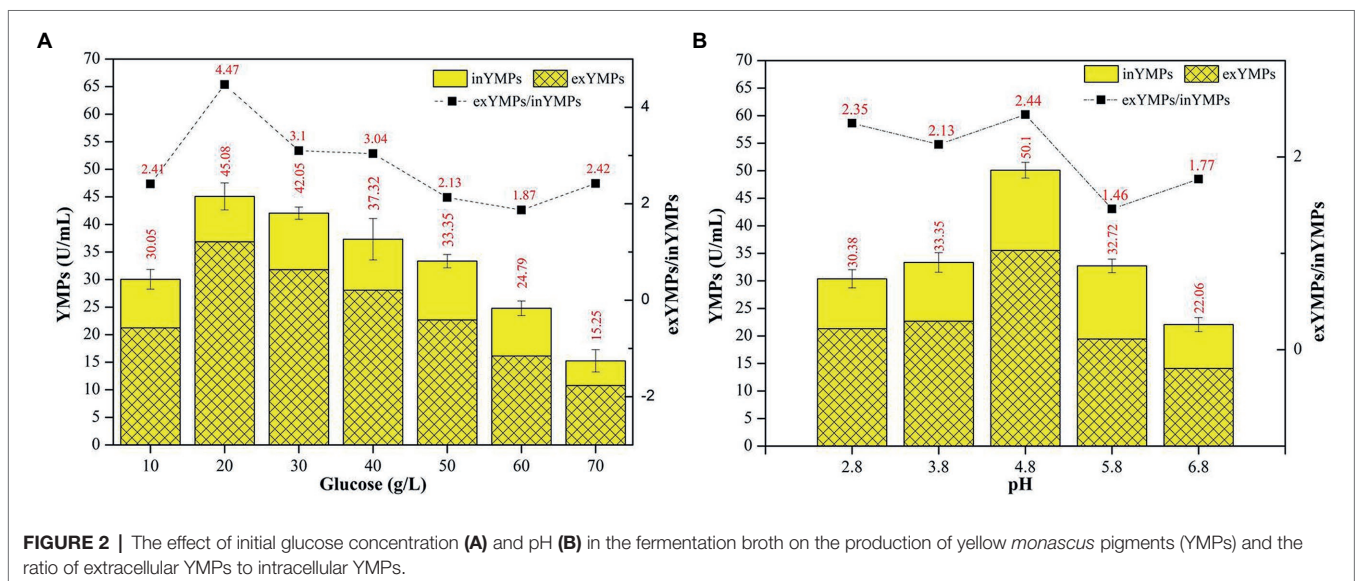


TABLE 1 | The effect of different fermentation medium compounds on the production of yellow *Monascus* pigments in submerged fermentation for 10 days at 30°C and agitated at 150 rpm in the dark.

Composition	Compound	Concentration (g/L)	inMPs (U/ml)	exMPs (U/ml)	T-MPs (U/ml)	exMPs/T-MPs (%)	Increased by (%)	
CK	–	–	10.67	22.68	33.35	68.01	–	
Growth factor	B1	0.2	17.79	29.82	47.61	62.63	42.76	
		0.2	15.06	27.86	42.52	65.52	27.50	
		0.2	15.10	36.53	51.63	70.75	54.81	
		0.2	12.04	28.22	40.26	70.09	20.72	
		0.2	14.57	32.28	46.85	68.90	40.48	
		0.1	10.76	24.84	35.60	69.78	6.75	
		0.2	15.10	36.53	51.63	70.75	54.81	
		0.3	15.28	40.17	55.45	72.45	66.27	
		0.4	19.09	44.49	63.58	69.98	90.64	
		0.5	15.38	33.24	48.62	68.37	45.79	
Nitrogen source	Peptone	5	23.66	36.70	60.36	60.80	80.99	
		10	26.80	46.50	73.30	63.44	119.79	
		15	36.76	52.80	89.56	58.95	168.55	
		20	21.48	46.35	67.83	68.33	103.39	
		5	27.64	54.65	82.29	66.41	146.75	
	Malt extract	10	35.80	59.35	95.15	62.37	185.31	
		15	26.46	48.45	74.91	64.68	124.62	
		20	23.84	44.55	68.39	65.14	105.07	
		NaNO ₃	1	14.45	28.20	42.65	51.24	27.89
			3	19.90	45.65	65.55	69.64	96.55
	5		28.40	51.50	79.90	64.46	139.58	
	Metal ion	Mg ²⁺	7	18.59	39.46	58.05	67.96	74.06
			9	11.85	32.80	44.65	73.46	33.88
			0.05	15.62	29.30	44.92	65.23	34.69
			0.1	15.58	36.73	52.31	70.22	56.85
0.2			17.65	43.27	60.92	71.03	82.67	
Mn ²⁺		0.5	19.59	36.81	56.40	65.26	69.12	
		0.01	13.65	39.12	52.77	74.13	58.23	
		0.05	15.17	48.16	63.33	76.05	89.90	
		0.1	19.63	54.88	74.51	73.65	123.42	
		0.2	17.46	34.08	51.54	66.12	54.54	
Fe ²⁺		0.5	12.35	29.57	41.92	70.55	25.70	
		1	13.20	34.15	47.35	72.13	41.98	
		1.5	13.08	39.76	52.84	75.25	58.44	
		2	16.32	28.04	44.36	63.22	33.01	
		0.5	9.73	24.23	33.96	71.35	1.83	
Ca ²⁺	1	13.01	30.91	43.92	70.38	31.69		
	1.5	17.16	36.92	54.08	68.26	62.16		
	2	11.60	25.30	36.90	68.56	10.64		
	Zn ²⁺	0.05	17.99	47.03	65.02	72.33	94.96	
		0.1	22.41	52.65	75.06	70.15	125.07	
0.2		26.75	57.83	84.58	68.37	153.61		
		0.5	18.78	42.22	61.00	69.22	82.91	

The data were expressed as the mean from three experiments.

to total YMPs was generally approximately 70%, which was equal to the ratio of exYMPs/inYMPs at 2.33 (similar to that with different initial pH). From the glucose and pH regulation findings, we confirmed that *M. purpureus* H14 was a high-production exYMP mutant strain, possibly due to the high cell permeability or the hydrophilia of the YMPs (WSYMPs). Thus, an increase in YMP production by RSM was imminent.

Recently, the use of RSM to increase MP production using *Monascus* spp. has increased. Our previous study showed that the total MPs and exMPs were increased by 33.38 and 150.32%, respectively, via SBF of *M. purpureus* (Liu et al., 2019b). Srivastav et al. (2015) used RSM to optimize a sweet

potato-based medium to increase red MP production using *M. purpureus*. The four medium compounds, vitamin B5, malt extract, ZnSO₄·7H₂O, and MnSO₄·H₂O, were selected for RSM.

According to the BBD for these four compounds with a three-level optimization (−1, 0, and 1), 29 experimental runs were conducted in this study (Table 2), and the experimental YMPs production were marked with arrows in Figure 3. State-ease Design Expert 8.0.6 software was used to analyze the relationship between YMP production (Y) and parameters (X_i) in each experimental run; the following multiple nonlinear regression model (Equation 1) was generated to express the relationship.

TABLE 2 | Box–Behnken design for process parameters of yellow *monascus* pigments production by *M. purpureus* H14 in submerged fermentation for 10 days.

Compounds	Symbol	Coded levels					
		-1	0	1			
ZnSO ₄ •7H ₂ O	A	0.1	0.3	0.5			
MnSO ₄ •H ₂ O	B	0.05	0.13	0.2			
Malt extract	C	5	10	15			
Vitamin B5	D	0.3	0.4	0.5			
Run order	A	B	C	D	YMPs (U/ml)		
					Experimental	Predicted	
1	0	1	0	1	82.06	81.34	
2	-1	0	1	0	98.56	98.76	
3	-1	0	0	1	77.92	76.20	
4	0	0	0	0	103.28	104.69	
5	0	0	-1	1	77.52	76.76	
6	-1	0	-1	0	75.26	77.41	
7	1	0	1	0	91.66	90.82	
8	0	0	0	0	105.88	104.69	
9	0	-1	1	0	97.18	96.90	
10	1	-1	0	0	93.67	91.89	
11	0	1	-1	0	74.38	73.24	
12	0	0	1	-1	91.48	92.65	
13	0	1	1	0	95.71	94.23	
14	0	-1	0	-1	75.37	74.30	
15	0	-1	0	1	79.38	81.77	
16	-1	-1	0	-1	82.94	81.13	
17	1	0	0	-1	73.98	73.98	
18	0	0	0	0	104.56	104.69	
19	1	1	0	0	68.94	71.17	
20	-1	0	0	-1	89.16	88.15	
21	0	-1	0	-1	87.04	89.07	
22	0	0	-1	-1	80.24	79.12	
23	1	0	-1	0	79.54	80.65	
24	0	0	0	0	104.37	104.69	
25	-1	1	0	0	84.45	86.64	
26	1	0	0	1	86.38	85.66	
27	0	-1	-1	0	86.32	86.07	
28	0	0	0	0	105.36	104.69	
29	0	0	1	1	93.21	94.75	

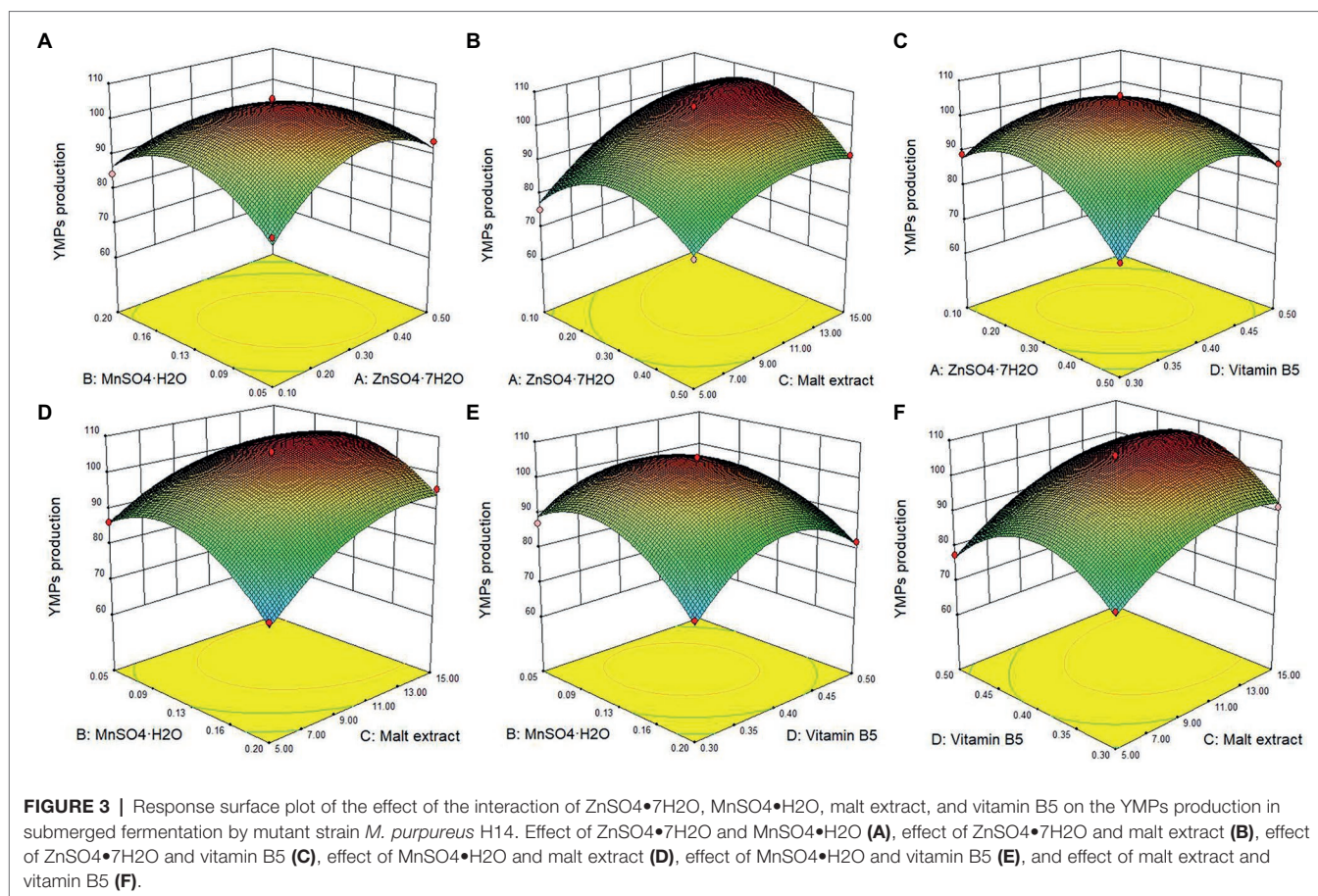
The data were expressed as the mean from three experiments.

$$\begin{aligned}
 Y = & 104.69 - 1.18X_1 - 3.80X_2 \\
 & + 7.88X_3 - 0.067X_4 - 6.56X_1X_2 - 2.80X_1X_3 \\
 & + 5.91X_1X_4 + 2.62X_2X_3 + 3.59X_2X_4 \\
 & + 1.11X_3X_4 - 11.30X_1^2 - 10.68X_2^2 \\
 & - 6.48X_3^2 - 12.39X_4^2
 \end{aligned} \quad (1)$$

where Y represents the response values of YMP production, and X_1 , X_2 , X_3 , and X_4 represent ZnSO₄•7H₂O, MnSO₄•H₂O, malt extract, and vitamin B5, respectively.

The statistical significance of the regression model equation was evaluated by the F test analysis of variance; the associated p -values were lower than 0.001 for YMPs production, indicating that the developed model and the terms were statistically significant; besides, the value of lack of fit was 0.0704, higher than 0.05, demonstrated the precision and the accuracy of the constructed models; what is more, the data show that MnSO₄•H₂O and malt extract were the principal factors on YMPs production (Table 3). Based on the experimental data, there were six response

surface graphs in multiple nonlinear quadratic regression models. The adequacy and accuracy of the generated model were evaluated using coefficient of determination (R^2), adjusted R^2 (R^2_{adj}), predicted R^2 (R^2_{pred}), and coefficient of variation (CV%). In this study, the results of ANOVA for RSM quadratic model showed that the value of R^2 was 0.9842 for YMPs production and R^2_{adj} was 0.9683, which was high and significantly close to the R^2 value. These results indicated that the predicted model was appropriate. In addition, the value of R^2_{pred} (0.9138 for YMPs production) was in reasonable agreement with that of R^2_{adj} , which also demonstrated that the predicted values of YMP productions were accurate. Furthermore, adequate precision (Adeq. Pre.) of 24.113 indicated an adequate signal (greater than 4 is desirable); this model can be used to navigate the design space. Thus, from Figure 3, the YMPs production point prediction was used in the Design Expert software to determine the four medium parameters with optimized values. The optimal values of ZnSO₄•7H₂O, MnSO₄•H₂O, malt extract, and vitamin B5 were determined as 0.32, 0.1, 12.29, and 0.4g/L, respectively. In optimized fermentation conditions



for 10 days, triplicate experiments resulted in a mean YMP production of 104.67 U/ml, which was 98.26% of the predicted value. YMP production and exYMP production increased by 213.85 and 289.51%, respectively, using RSM, and the ratio of exYMPs/inYMPs increased from 2.13 to 5.41. These results indicate that RSM dramatically increased exYMP production; the yield and productivity of exYMPs were 4,417 U/g (glucose) and 8.83 U/ml/day. Subsequently, IF and EF were performed together to increase exYMP productivity.

Improvement of exYMP Productivity via RBF

Extractive fermentation has been proven to efficiently increase the production of *Monascus* pigments in submerged fermentation of *Monascus* spp.; the exMPs increased by 127.48% with the addition of 3 g/L Triton X-100 in submerged fermentation of *M. purpureus* DK (Lu et al., 2021). Our previous study prevented further improvement of the yield and productivity of exMPs by IF (Liu et al., 2019b). In this study, a novel RBF biotechnology was developed by combining EF and IF to significantly increase exYMP production.

It has been reported that the use of Ca-alginate to embed the mycelium of *M. purpureus* C322 resulted in high pigment production in RBF (Fenice et al., 2000). Our previous study investigated the effect of SA concentration on exMP production

and immobilization efficiency and found that the optimal concentration of SA was 3% (wt/wt; Liu et al., 2019b). In this study, we further evaluated the effect of different SA and PVA ratios with a total concentration of 3% on exMP production and immobilization efficiency by immobilizing the spores of *M. purpureus* H14. As in our previous study, with a maximum of seven cycles, a large amount of batch fermentation could not be achieved in RBF (Figure 4A). In addition, although their immobilization efficiencies were good, exYMP production was different with different SA and PVA ratios in RBF with each cycle for 10 days. In descending order of exYMP production, they can be ranked as 2.5% SA and 0.5% PVA, 2% SA and 1% PVA, 1.5% SA and 1.5% PVA, and 3% SA, with mean values of 140.90, 124.90, 107.72, and 106.06 U/ml, respectively. The immobilization efficiency of *M. purpureus* H14 was desirable when the concentration of SA in the immobilized solution was less than 1.5%; it was flocculent and irregular, but not regularly spherical. Concentrations of 2.5% SA and 0.5% PVA in the immobilized solution were selected for immobilization of *M. purpureus* H14 spores in IF.

As shown in Figure 4B, the glucose consumption rate and exYMP biosynthesis rate were increased in IF compared to those in conventional SBF. The residual glucose was consumed at approximately 96 and 108 h, with glucose consumption rates of 0.21 g/L/h (5.04 g/L/day, increased by 16.67%) and 0.18 g/L/h (4.32 g/L/day) in IF and SBF, respectively. The biosynthesis rate

TABLE 3 | ANOVA for the effect of ZnSO₄•7H₂O, MnSO₄•H₂O, vitamin B5, and malt extract on yellow *monascus* pigment production and regression coefficients.

Source	Sum of squares-type III	df	Mean square	F value	p value Prob > F	Significance
Model	3249.06	14	232.08	62.12	<0.0001	**
A (ZnSO ₄ •7H ₂ O)	16.61	1	16.61	4.45	0.0535	–
B(MnSO ₄ •H ₂ O)	173.43	1	173.43	46.42	<0.0001	**
C (malt extract)	744.82	1	744.82	199.36	<0.0001	**
D (vitamin B5)	0.053	1	0.053	0.014	0.9066	–
AB	172.13	1	172.13	46.07	<0.0001	**
AC	31.25	1	31.25	8.36	0.0118	*
AD	139.71	1	139.71	37.40	<0.0001	**
BC	27.41	1	27.41	7.34	0.0170	*
BD	51.48	1	51.48	13.78	0.0023	*
CD	4.95	1	4.95	1.33	0.2690	–
A ²	828.56	1	828.56	221.77	<0.0001	**
B ²	739.81	1	739.81	198.02	<0.0001	**
C ²	272.13	1	272.13	72.84	<0.0001	**
D ²	996.09	1	996.09	266.61	<0.0001	**
Residual	52.31	14	3.74			
Lack of fit	48.33	10	4.83	4.87	0.0704	–
Pure error	3.97	4	0.99			
Corrected total	3301.36	28				
SD	1.93					
Mean	87.79					
CV%	2.20					
R ²	0.9842					
R ² _{adj}	0.9683					
R ² _{pred}	0.9138					
Adep. pre	24.113					

CV, coefficient of variation; R², coefficient of determination; R²_{adj}, adjusted R²; R²_{pred}, predicted R²; and Adep. Pre, adequate precision. **p < 0.01 highly significant. *p < 0.05, significant; –, not significant.

of exYMPs using *M. purpureus* H14 was evenly matched in the mid-early period of fermentation (before 144h) in SBF and IF; subsequently, the biosynthesis rate significantly increased in IF compared to that in SBF, with a maximum exYMP production of 139.75 U/ml at 288 h (a productivity of 11.64 U/ml/day) and 104.13 U/ml at 300 h (a productivity of 8.33 U/ml/day), respectively. In RBF without the addition of Triton X-100 for five cycles, the mean glucose consumption rate and exYMP productivity were 8.23 and 10.28 U/ml/day, respectively, not including the first batch (Figure 4C). However, these values were increased to 8.54 g/L/day and 15.21 U/ml/day, respectively, in RBF with Triton X-100 (Figure 4D). Similarly, Evans and Wang (1984) further increased the maximum *Monascus* pigment yield and production rate through immobilized cell fermentation with the addition of resin, illustrating that a novel RBF with some modifications could increase the yield and productivity of a target product. To evaluate the maximum exYMP production using this novel RBF, we extended the fermentation time to 10 days (the same as conventional SBF) in the last cycle. An exYMP production of 152.08 U/ml was obtained, and the exYMP productivity was almost constant (16.89 U/ml/day). The exYMP yield and productivity increased by 570.55 and 570.04%, respectively, compared with SBF after RSM and RBF (22.68 U/ml and 2.27 U/ml/day).

In addition, we compared the yield and productivity of YMPs produced by *Monascus* strains via SBF. As shown in Table 4, various biotechnologies were carried out to increase the YMPs production, including addition of exogenous

compounds [such as ethanol, sodium starch octenyl succinate (OSA-SNa), and microparticle], pH and sharking speed regulation, temperature control, and response surface methodology, changing oxidation–reduction potential (high glucose stress, addition of H₂O₂ and dithiothreitol). The highest exYMPs production was 209 U/ml in submerged fermentation by *M. ruber* CGMCC 10910 after optimizing the amount of H₂O₂ added and the timing of the addition, with 17.42 U/ml/day of productivity (Huang et al., 2021b), which was similar to the value of that obtained in this study. Besides, it showed that high production of YMPs (328.36 U/ml) was obtained by *M. ruber* CGMCC 10910 under condition of high glucose stress, but the productivity of exYMPs was low (5.22 U/ml/day; Wang et al., 2017b). Although most YMPs productions were much higher than that in this present study, the productivity of exYMPs generated via RBF was advantageous and predominant.

Physicochemical Property of Produced Yellow *Monascus* Pigments

Feng et al. (2012) reviewed the categories, structures, and properties (including solubility, stability, and safety) of MPs in the journal of Applied Microbiology Biotechnology. YMPs (absorption at 420 ± 10 nm), OMPs (absorption at 470 ± 10 nm), and RMPs (absorption at 510 ± 10 nm) are traditionally accumulated in the mycelium during SBF (Kim and Ku, 2018). The four identified MPs, N-glutarylmonascurubramine, N-glutarylrubropunctamine, N-glucosmonascurubramine, and

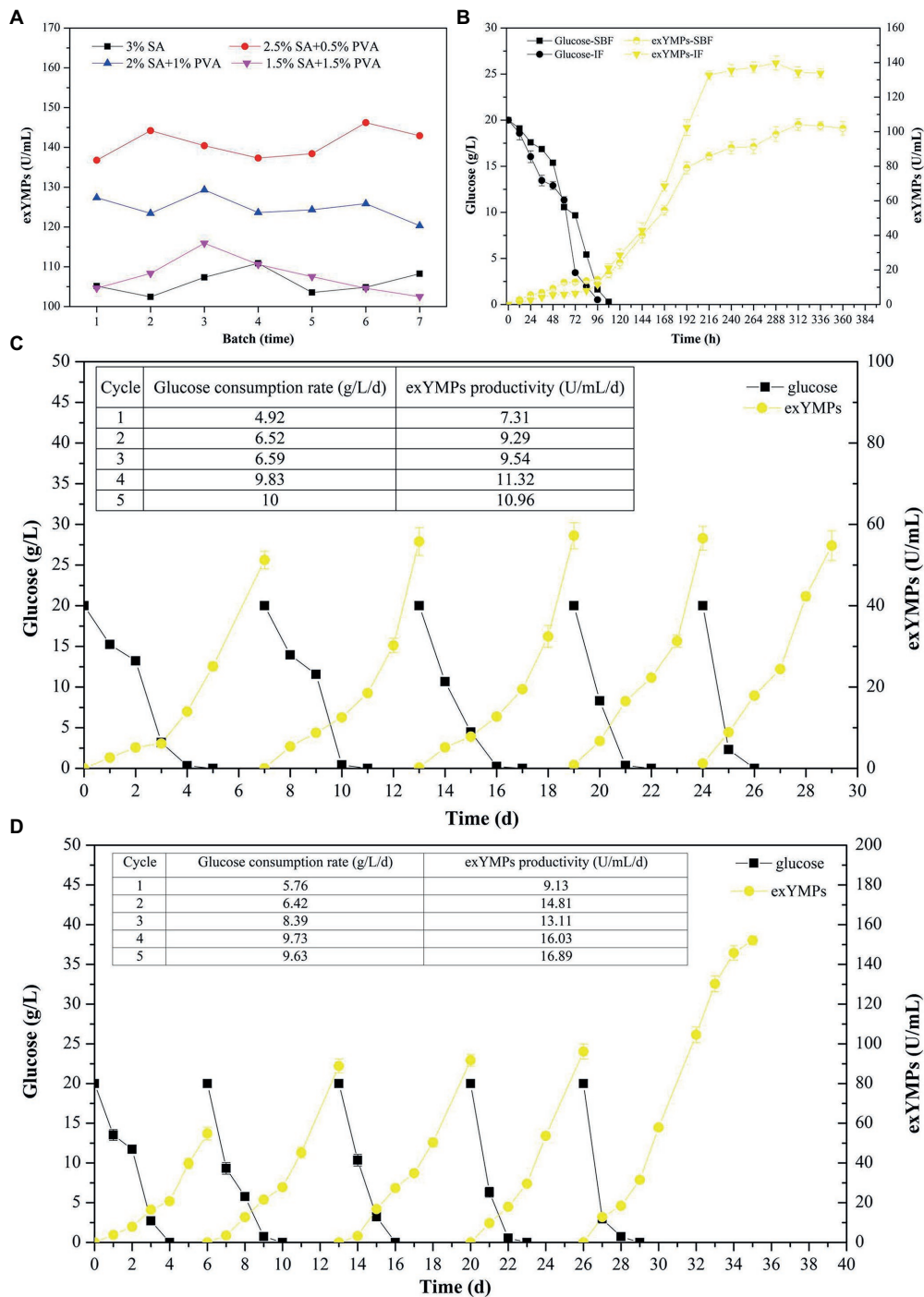


FIGURE 4 | The effect of different ratios of sodium alginate and polyvinyl alcohol solution on the production of extracellular yellow *monascus* pigments via repeated batch fermentation of *M. purpureus* H14 (A); glucose consumption and the biosynthesis of extracellular yellow *monascus* pigments of *M. purpureus* H14 during conventional SBF and immobilized fermentation (B); glucose consumption and the biosynthesis of extracellular yellow *monascus* pigments of *M. purpureus* H14 during repeated-batch fermentation without (C) and with (D) Triton X-100 addition.

N-glucosylrubropunctamine are hydrophilic MPs (exMPs) with a red tone (Chen et al., 2015). In this study, a large number of YMPs produced by the mutant strain *M. purpureus* H14 in SBF were secreted out of the cell; we speculated that the

exYMPs were hydrophilic or water-soluble. From the polarity test (Figure 5A), we found that the exYMPs were soluble in pure water, but were not dissolved in the hydrophobic phase of ethyl acetate. However, ankaflavin, an alcohol-soluble standard

TABLE 4 | Comparison of the production and productivity of yellow *monascus* pigments by *Monascus* strains in submerged fermentation.

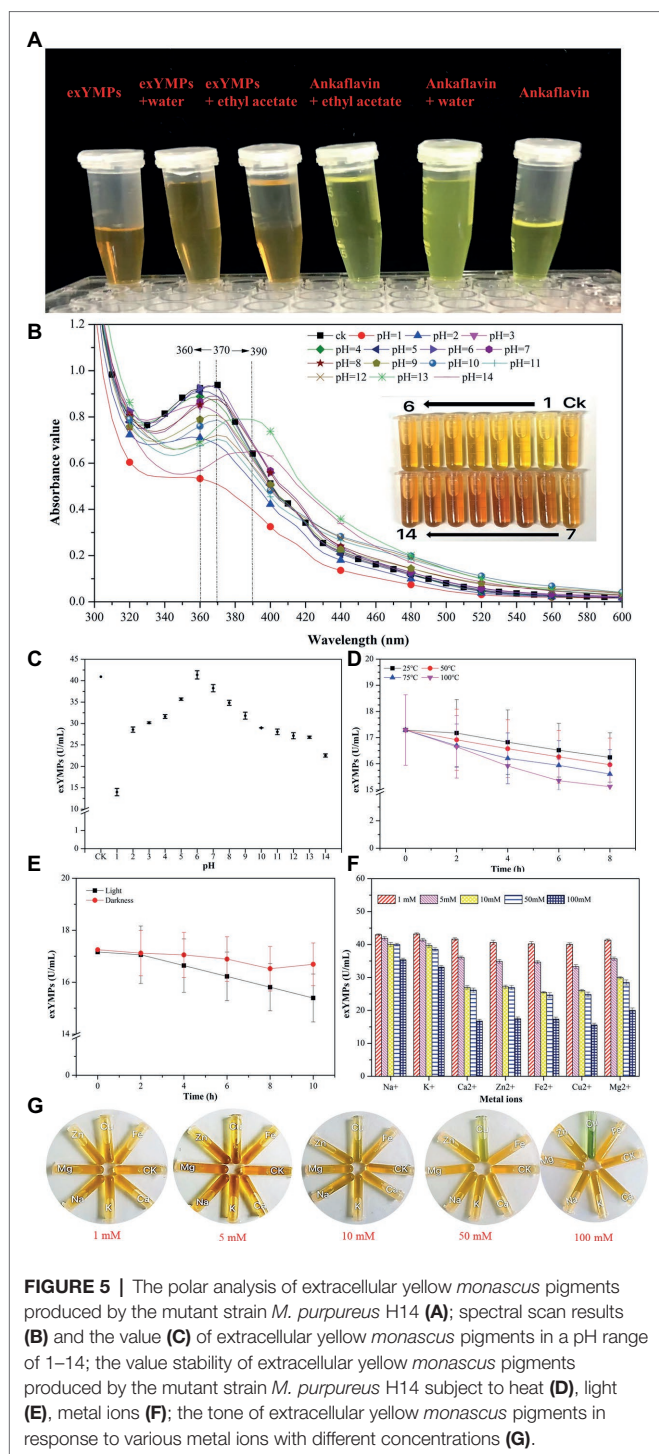
Strain	Biotechnology	exYMPs (U/ml)	T-YMPs (U/ml)	Productivity (U/ml/day)	Reference
<i>M. ruber</i> CGMCC 10910	Temperature control	190	–	23.75 (exMPs)	Huang et al., 2017a
<i>M. ruber</i> CGMCC 10910	Controlling oxidoreduction potential	209	–	17.42 (exMPs)	Huang et al., 2017b
<i>M. ruber</i> CGMCC 10910	High glucose stress	114.80	328.36	5.22 (exMPs)	Wang et al., 2017b
<i>M. ruber</i> CGMCC 10910	Addition of OSA-SNa	131.50	220	16.44 (exMPs)	Huang et al., 2021b
<i>M. anka</i> mutant MYM	Response surface methodology	–	87.24	12.46 (T-YMPs)	Zhou et al., 2009
<i>M. purpureus</i> sjs-6	Adjusting pH and shaking speed	–	401	57.28 (T-YMPs)	Lv et al., 2017
<i>M. purpureus</i> ZH106-E	Addition of ethanol	–	394	56.29 (T-YMPs)	Qian et al., 2021
<i>M. purpureus</i> CH01	Potato pomace as carbon source	–	19.70	2.81 (T-YMPs)	Chen et al., 2021
<i>M. purpureus</i> ZH106-M	Addition of microparticles	–	554.20	79.17 (T-YMPs)	Huang et al., 2021a
<i>M. purpureus</i> H14	Immobilized fermentation	152.08	–	16.89 (exMPs)	In this study

compound, is easily dissolved in the hydrophobic phase. These observations verify that the WSYMP hypothesis was correct. **Figure 5B** shows that the yellow tone of the produced WSYMPs is stable in a pH range of 1–14. However, the maximum absorption peaks are different: 360 nm for pH 1–5, 370 nm for pH 6–12, and 390 nm for pH 13–14. The maximum absorption peaks for WSYMPs were different from those of YMPs. However, the WSYMP values changed with the addition of 1 M HCl or NaOH, decreasing to 13.98 U/ml (65.84%) and 22.52 U/ml (44.97%) from 40.92 U/ml at pH 1 and pH 14, respectively (**Figure 5C**). The produced WSYMPs have high stability in neutral solution environments, decreasing in alkaline and acidic environments. Similarly, the color value degraded at different pH values; degradation was more significant from pH 4–8 (Carvalho et al., 2005). Although the color tone was changeless, **Figure 6A** shows that ankaflavin has relatively high stability in a pH range of 3–8, the value dramatically decreased when pH lower than 2 (decreased by more than 79.15%) and more than 9 (decreased by more than 43.35%). The results stated that the generated WSYMPs by mutant strain *M. purpureus* H14 have higher stability in response to pH than ankaflavin. Besides, in **Figure 5D**, the WSYMPs are almost stable with heat treatment (25–90°C) for 8 h, exhibiting a slight reduction in yellow tone. It has been reported that MPs are generally stable with heat and a wide range of pH values, but are less stable with light (Wang et al., 2017a). However, traditionally the focus is tone stability, without considering the value. Water-soluble RMPs were generated by chemical modification using aminoacetic acid and glutamic acid and exhibited heat discoloration when dissolved in a buffer at pH 3, 7 and 9.2 (Wong and Koeler, 1983). A type of RMP produced by *M. purpureus* using corn bran in SBF produced thermal (sterilization and 40°C–80°C) and pH (4–7) stability for tone and value (Almeida et al., 2021). In this study, the WSYMP value was almost constant in the dark for 10 h, exhibiting a downward trend over time (0–10 h) in light, but remaining nearly constant with an initial value of 17.16 U/ml until 2 h (**Figure 5E**). Similarly, the color value of ankaflavin sharply decreased to 16.84 U/ml for ten hours from 30.08 U/ml in light, but almost constant in the dark (**Figure 6B**). However, the stability of WSYMPs is relatively higher than that of ankaflavin when they are irradiated within 2 h. **Figure 5F** shows that the WSYMPs were stable in Na⁺ and K⁺ solutions, with

low concentrations (<5 mM) of Ca²⁺, Zn²⁺, Fe²⁺, Cu²⁺, and Mg²⁺. However, when the concentration reached 10 mM, the pigment value decreased by 32.60, 32.16, 36.35, 35.03, and 25.30%, and 52.62, 50.63, 50.90, 55.97, and 43.43%, respectively, at a concentration of 100 mM. The pigment tone changed to green when the Cu²⁺ concentration exceeded 50 mM; the YMPs solution with the addition of other metal ions maintained a yellow tone (**Figure 5G**). Traditionally, MPs are stable with a small quantity of Na⁺, K⁺, Mg²⁺, Ca²⁺, Zn²⁺, Al³⁺, and Cu²⁺, but less stable in the presence of Fe²⁺ and Fe³⁺ (Feng et al., 2012; Wang et al., 2017a). These findings illustrated that the WSYMPs showed good stability of tone to environmental factors, including pH, heat, light, and metal ions, in this present study. However, the pigment value was unstable to pH, light, and high concentrations of Ca²⁺, Zn²⁺, Fe²⁺, Cu²⁺, and Mg²⁺.

Characterization of Produced Yellow *Monascus* Pigments

In the past decade, 12 YMP compounds have been investigated (Feng et al., 2012). In this study, we found that the HPLC peak time of produced WSYMPs different to that of ankaflavin and monascin in our laboratory. Thus, we further determined the compound structures of the YMPs through HPLC–MS. From **Figure 7**, a total of five peaks were appeared in the raw fermentation broth (the first tested sample, T1), and the retention time (t_R) was 1.58, 1.95, 2.16, 2.55, 7.16, and 7.59 min, respectively. However, the peaks with t_R of 2.55, 7.16, and 7.59 disappeared in the WSYMPs solution (the second tested sample, T2), and the t_R of residual three peaks occurred rearward shift within the range of recognition, which changed to 1.71, 2.01, and 2.19 min, respectively. Besides, the compounds with t_R of 7.16 and 7.59 min both showed pseudo-molecular [M+H]⁺ ions at m/z 301.14 and 301.14, with the fragment ions in UPLC-ESI-MS² at m/z 149.02343 and 163.03858. The fragment ions of the compound with t_R of 2.55 min in UHPLC-ESI-MS² at m/z 362.32822, 300.28851, and 265.26337. This phenomenon illustrated that some compounds or impurities (not WSYMPs) were cleared away *via* the adsorption–separation system with the macroporous adsorption resin LX300C. Further, we analyzed the peaks with t_R of 2.01 min (or 1.95 min) and 2.19 min (or 2.16 min) by UHPLC-ESI-MS. The peaks of T1-2.16 and T2-2.19 min both showed



pseudo-molecular $[M+H]^+$ ions at m/z 114.09134 (Figure 8). Although a pseudo-molecular $[M+H]^+$ ion with m/z 155.383183 in T1–1.95 min, it disappeared after purification treatment (in T2 2.01 min). In addition, the peak of T1–1.95 min mainly showed pseudo-molecular $[M+H]^+$ ions at m/z 303.723239, 435.33057, and that of T2–2.01 min at m/z 303.723239 and 435.2188. Further, the components of exYMPs solution were separated *via* dynamic desorption of fully

saturated absorbent with glass absorbent columns of 200 mm length and 10 mm diameter. The sample with the darkest yellow color tone was analyzed by Nanjing Sheng Ming Yuan Health Technology Co. Ltd., and the result showed that the molecular weight of component was m/z at 440.1150 of $[M+H]^+$ (Supplementary Figure S1—HPLC and Supplementary Figure S2—MS). Compared with the information in studies, we found that the molecular, monascusone B ($C_{17}H_{18}O_5$), has a weight of m/z 302 [observed m/z at 303.1235 of $(M+H)^+$] and UV λ_{max} value at 375 nm (Jongrungruangchok et al., 2004); compound 7 ($C_{21}H_{27}NO_7S$, a kind of WSYMPs) observed m/z at 435.7 of $[M+H]^+$ (Yang et al., 2018). The results indicated that most possibly only two yellow *Monascus* pigment components were produced by mutant strain *M. purpureus* H14 in submerged fermentation, and the exYMP was identified as monascusone B and $C_{21}H_{27}NO_7S$ (WSYMP; the structures are shown in Figure 9).

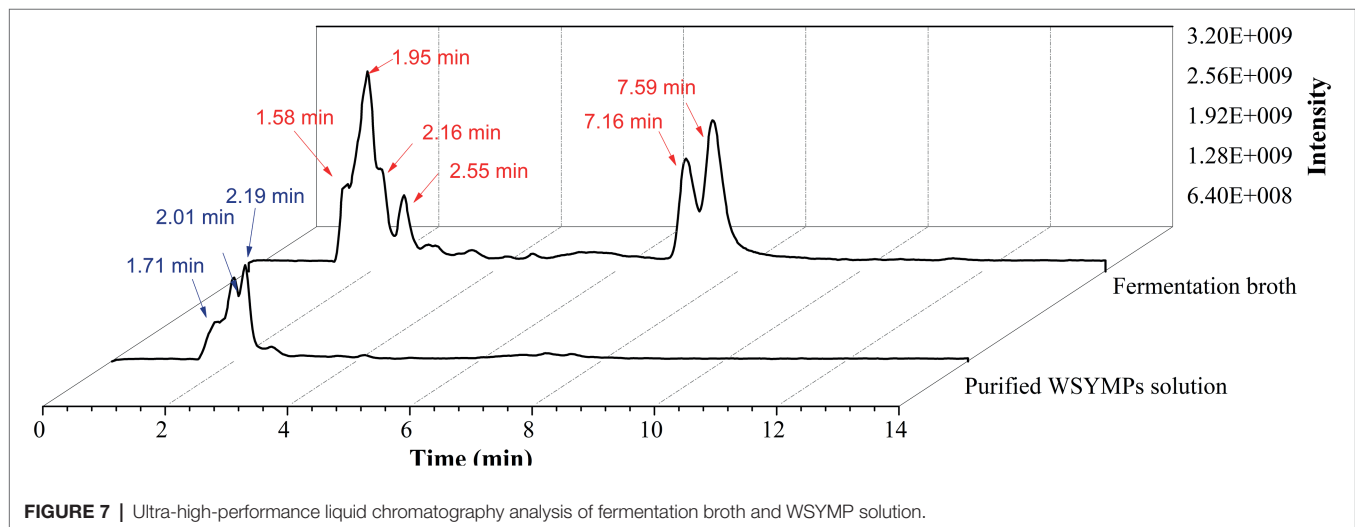
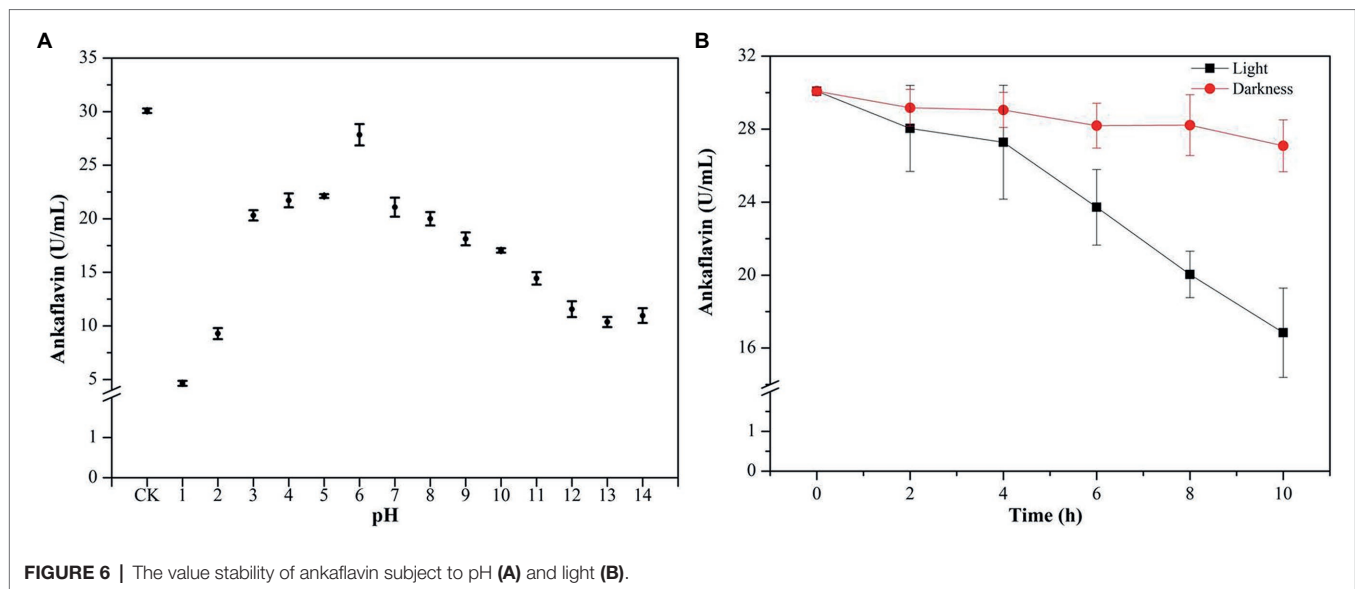
Besides, Jongrungruangchok et al. (2004) have reported that monascusone A (a precursor substance of monascusone B) produced by *M. kaoliang* KB20M10.2 showed no cytotoxicity against breast cancer and human epidermoid carcinoma of cavity cell lines. Monascusone B possesses the same stereochemistry as that of monascin, which exhibited no cytotoxicity against Hep G2 (human cancer cell lines) cells (Su et al., 2005). In addition, the IC₅₀ of ankaflavin on human cancer cell lines is 15 $\mu\text{g}/\text{ml}$, but ankaflavin has no significant cytotoxicity against on normal diploid fibroblast cell lines (Su et al., 2005). These results indicated that the constructed fermentation system (including dual mutagenesis, immobilized fermentation, and extractive fermentation) is beneficial for the production of exYMP in submerged fermentation by *M. purpureus*.

CONCLUSION

Yellow *Monascus* pigments are excellent colorants, are safe, and have several physiological functions. In this study, a mutant strain, *M. purpureus* H14, was generated using dual mutagenesis of atmospheric and room-temperature plasma and HIBI. The physicochemical property of the produced YMP was water-soluble, and two yellow monascus pigment components were identified by UHPLC-ESI-MS. It exhibited good tone stability when subjected to environmental factors including pH, heat, light, and metal ions, but the pigment value was unstable with pH, light, and high concentrations of Ca^{2+} , Zn^{2+} , Fe^{2+} , Cu^{2+} , and Mg^{2+} . Application of IF and EF significantly improved the exYMP yield and productivity.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions. The data presented in this study are available on request from the corresponding author. Requests to access these datasets should be directed to JL, liujundandy@csuft.edu.cn.



AUTHOR CONTRIBUTIONS

JL and QL designed experiments. JB, ZG, and HZ performed experiments. JB wrote the manuscript. JL revised the manuscript. MS collected important background information. FY analyzed the RSM results. JL and BZ analyzed the data. DL and XZ performed heavy ion-beam irradiation. CT analyzed the HPLC-MS data. All authors contributed to the article and approved the submitted version.

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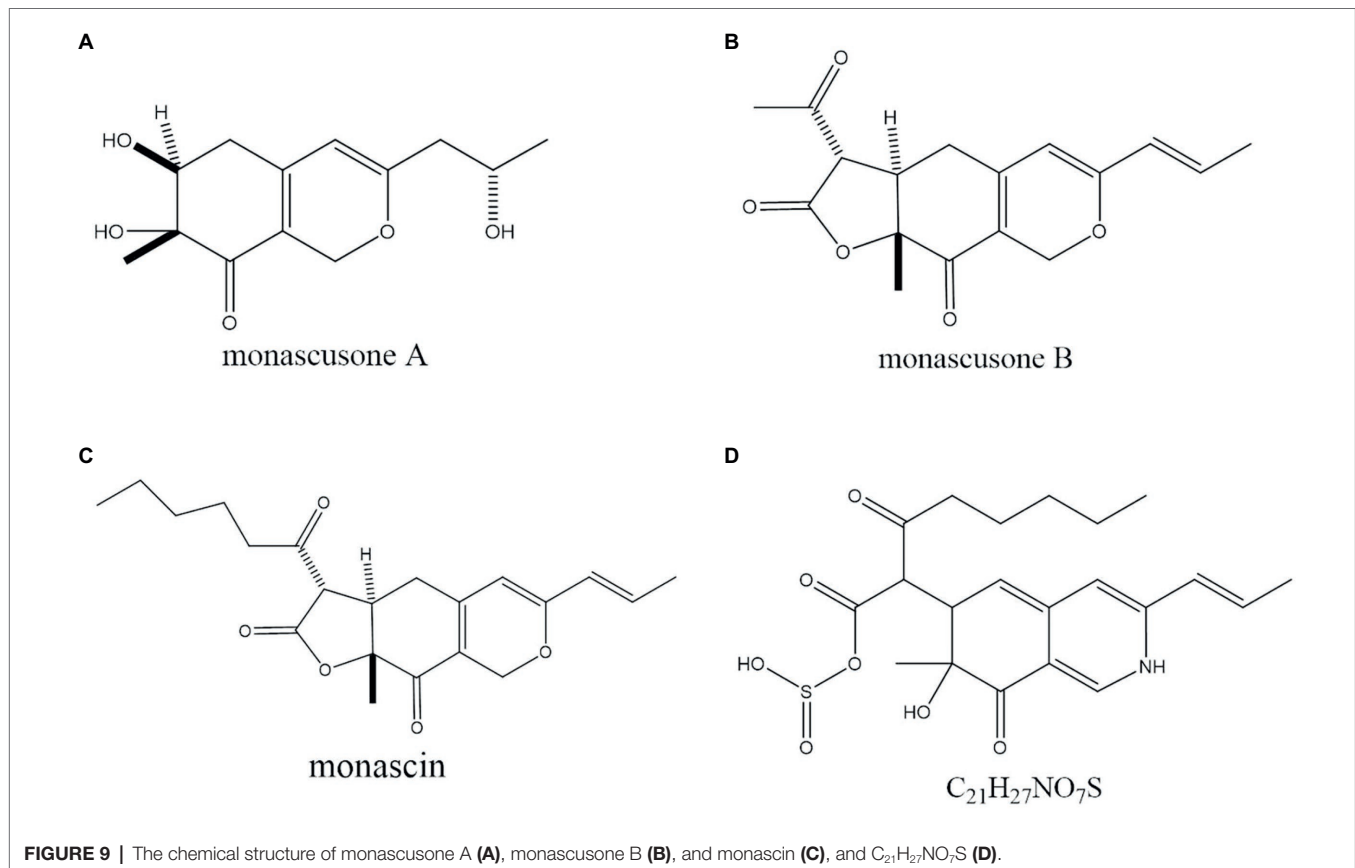
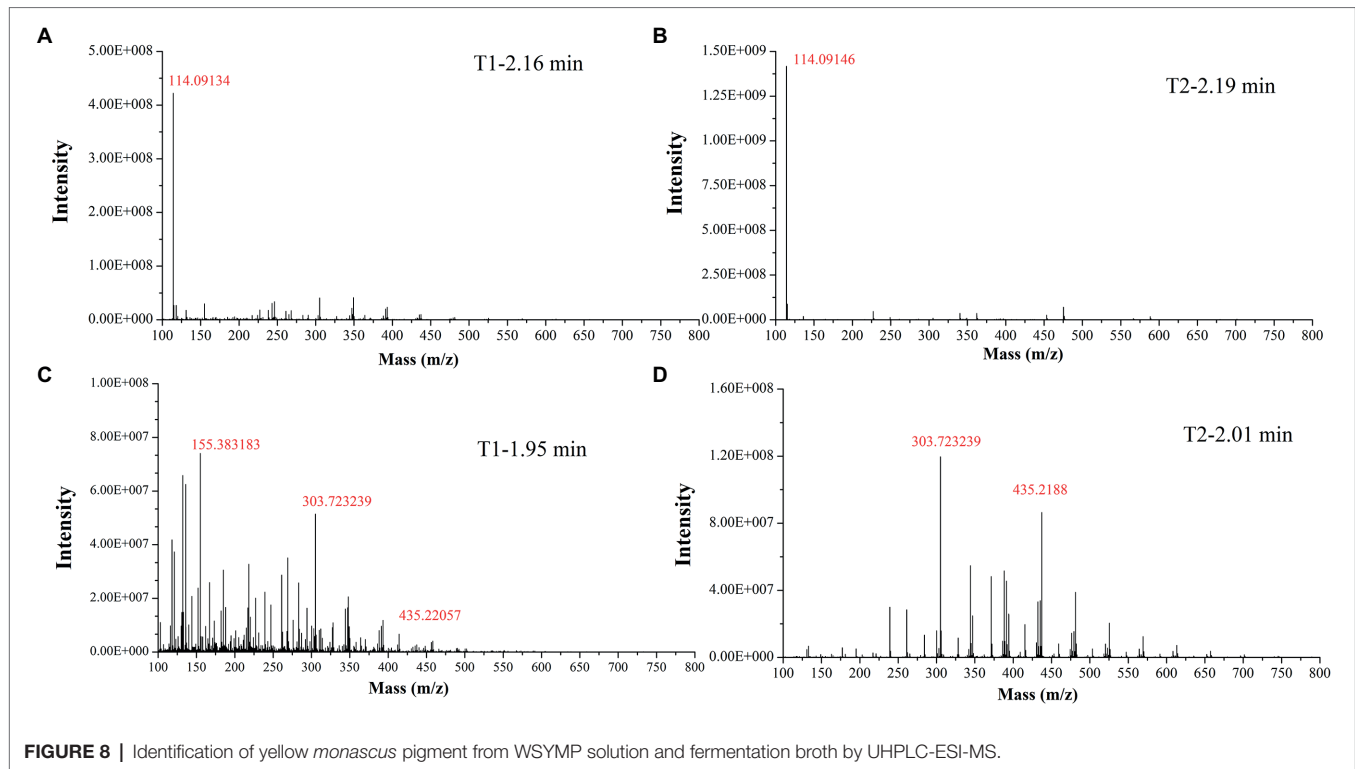
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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.914828/full#supplementary-material>



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Conflict of Interest: CT was employed by Nanjing Sheng Ming Yuan Health Technology Co. Ltd. and Jiangsu Institute of Industrial Biotechnology JITRI Co. Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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