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Efficient biosynthesis of natural yellow pigments by *Monascus purpureus* in a novel integrated fermentation system

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Abstract

Due to the increasing demand for healthy and safe food, *Monascus* spp. have gained much attention as a sustainable source of natural food colorant. In this study, a novel integrated fermentation system consisting of surfactant and *in situ* extractant was established for efficiently producing yellow pigments by *M. purpureus* sjs-6. The maximum production of *Monascus* yellow pigment (669.2 U/mL) was obtained when 40% soybean oil (as extractant) was supplied at the beginning and 5 g/L Span-80 (as surfactant) was supplied at the 72th h, resulting in production 27.8 times of that of the control. Critical factors such as alleviating the product inhibition, increasing the membrane permeability, changing the hyphal morphology and influencing the cell activity have been suggested as the underlying mechanisms. This system is of great significance for the bioprocess which suffered product inhibition and it can serve as a promising step for enhancing the yield of hydrophobic metabolites.

Keywords: *Monascus purpureus*; natural pigment; submerged fermentation; edible and medicinal fungi; hyphal morphology; membrane permeability
Introduction

The genus *Monascus* which belongs to the class Ascomycetes and the family Monascaceae, has been traditionally used in Asian countries for centuries. Natural pigments which are the most important secondary metabolites of *Monascus*, play a significant role in human life and in light industry, in areas such as food additives, textiles, cosmetics, and pharmaceuticals\(^1\). As food additives, *Monascus* pigments are safe and without undesirable toxic effects when compared to synthetic pigments. Moreover, *Monascus* pigments can be harvested in a shorter period by large scale bioreactors and without concerning the vagaries of the weather, when compared to the natural pigments extracted from plants\(^2\).

*Monascus* can produce three kinds of natural pigments: red (monascorubramine and rubropunctamine), orange (monascorubrin and rubropunctatin) and yellow (monascin and ankaflavin)\(^3\). Recently, the biosynthetic pathway of these *Monascus* azaphilone pigments were further elucidated by a combination of targeted gene knockouts, heterologous gene expression, and in vitro chemical and enzymatic reactions\(^4\). Among these pigments, the red ones have been successfully produced in large scale by submerged fermentation\(^5\). However, the yellow and orange pigments are not suitable for industrial production, due to their relatively low production yield. For example, Zhou et al. optimized the culture conditions for *Monascus anka* mutant using response surface methodology and the yield of yellow pigment was 92.45 U/mL in a 5 L agitated bioreactor\(^6\). A two-stage microbial fermentation using
micelle solution was applied to export intracellular *Monascus* pigments and the enhanced production of *Monascus* yellow pigment was about 60 U/mL\(^7\). At present, the main problem existing in the production of yellow pigments is that they are insoluble in the fermentation broth and encounter the phenomenon of product inhibition. Thus, a further improvement in the submerged fermentation process is necessary for enhancing the production of *Monascus* yellow pigment.

To achieve this purpose, eliminating the phenomenon of product inhibition may markedly improve the yield of yellow pigment. The usage of non-aqueous solvents is especially advantageous to transform substrates that are unstable or poorly soluble in water\(^8\). Anvari et al. indicated that using extractive fermentation in submerged fermentation of *Klebsiella pneumoniae* increased the 2,3-butanediol production to 23.01 g/L, compared to 17.9 g/L in conventional fermentation\(^9\). Hu et al. suggested that the application of perstractive fermentation could drive the intracellular pigments to its extracellular broth in submerged fermentation of *Monascus anka*\(^10\). Alternatively, the addition of surfactants can effectively enlarge the membrane permeability to enhance metabolic secretion. For example, Marina et al. indicated that utilization of 1-1.5% Tween 60 or 1% Triton X-100 allowed to solubilize 1 mM fluorene over 150 times more than in water\(^11\). Wang et al. suggested that the maximum production of red pigment (304.3 U/mL) was obtained when 15 g/L Triton X-100 was added at 24\(^{th}\) h of fermentation, corresponding to significant increases of 88.4% compared with that
of the control in submerged fermentation of Monascus purpureus H1102 \textsuperscript{12}.

Although the use of extractants/surfactants has been reported to improve fungal metabolites, the coupling of both is still rare. More importantly, it is necessary to revealing the underlying mechanisms. Therefore, in this study, a novel integrated fermentation system consisting of surfactant and in situ extractant is established to enhance Monascus yellow pigment production in submerged fermentation of Monascus purpureus sjs-6. Furthermore, the underlying mechanisms are revealed from different aspects including the investigation of the change of hyphal morphology, cell activity and membrane permeability.

Materials and methods

Microorganism, inoculum preparation and submerged fermentation conditions

Monascus purpureus sjs-6 is a mutant with high yellow pigment production which is collected by Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University (Wuxi, China). The strain was maintained on potato dextrose agar (PDA) slants and incubated at 30\(^\circ\)C for 7 days and kept at 4\(^\circ\)C.

To prepare the inoculum, 7-days-old mycelia of M. purpureus in PDA slant were transferred to the culture medium by adjusting the concentration of spores at about 2.5\(\times\)10\(^7\) spores/mL. The inoculum was incubated in a 500 mL Erlenmeyer flask containing 50 mL of the culture medium for 3 days in a rotary shaker at 180 rpm at 30 \(^\circ\)C. Then shake-flask
experiments were carried out in 500 mL Erlenmeyer flasks each containing 50 mL of culture medium with 12% (v/v) inoculum.

The culture medium consisted of 60 g/L corn starch, 4 g/L (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 2 g/L NaNO\textsubscript{3}, 1.5 g/L MgSO\textsubscript{4}·7H\textsubscript{2}O, 0.1 g/L CaCl\textsubscript{2}, 2 g/L K\textsubscript{2}HPO\textsubscript{4}·3H\textsubscript{2}O, 2 g/L KH\textsubscript{2}PO\textsubscript{4}·3H\textsubscript{2}O. The initial pH was adjusted to 5.0 with lactic acid. Each shaking-flask culture was incubated at 30 °C for 8 days in a rotary shaker at 180 rpm.

**Determination of the biomass and residual sugar**

For biomass determination, the collected mycelia were filtered through a pre-weighted filter paper under partial vacuum (200 mg Hg), washed with distilled water and dried at 65° C until constant weight. The biomass concentration was expressed as dry weight of mycelia per unit volume of culture medium. Residual sugar in the filtrate was determined by colorimetric method\textsuperscript{13}.

**Determination of total yellow pigment**

The total yellow pigment produced by the mycelia of *M. purpureus* sjs-6 was extracted from the fermentation broth (2 mL) by addition of 70% (w/v) ethanol. They were made up to a total volume of 50 mL and then incubated in a water bath at 50° C for 30 min with intermittent shaking. The yellow pigment was determined spectrophotometrically by measuring the absorbance of the filtrate at 410 nm, with 1 unit of optical density at 410 nm (OD\textsubscript{410}) corresponding to 1 unit of color value\textsuperscript{12}.
Color value (U/mL) = A × (50/2) × dilution factor

where A is the absorbance of the pigment extract at 410 nm, 50 is the total volume of pigment extract and 2 is the volume of fermentation broth used for pigment extraction.

**Isolation and characterization of Monascus yellow pigments**

To isolate and purify the components of *Monascus* yellow pigments, semi-preparative HPLC (Waters1525, USA) equipped with a 2998 DAD detector and a C18 column (19mm×250mm, Xbridge prep, Waters, USA) was used. *Monascus* yellow pigment samples were diluted by ethanol and then filtered through 0.22 µm membrane filter. The separation gradient was ethanol mixed with water (70:30, v/v) over 30 min with flow rate set at 4 mL/min. The injection volume was 100 µL and semi-preparative HPLC was operated under room temperature. The effluent was collected and analyzed on analytical HPLC (Waters e2695, USA) to confirm purity. A C18 column (Symmetry ODS, 5 µm, 250 mm×4.6 mm) was used, and the detection was carried out by a 2998 DAD detector at 410 nm. The mobile phase was methanol mixed up with water (75:25, v/v). The flow rate was 1.0 mL/min and the column temperature was set at 30°C.

LC-MS and NMR analyses were performed to identify and characterize the structure of *Monascus* yellow pigments. By using semi-preparative HPLC, the effluents containing the yellow pigment were gathered and poured into a round-bottomed flask. The sample was rotary-vacuum-dried at 40°C. The concentrated effluent was transferred to a vacuum drying
box and dried for 48h. LC-MS analysis was carried out on Waters LCZ2690XE-996 with ESI in the negative mode. HPLC was carried out on a BEH C18 column (50 mm×2.1 mm, i.d., 1.7 µm) with a gradient elution condition of acetonitrile/water (0.1% formic acid) from 5 to 100% acetonitrile in 12 minutes at a flow rate of 0.3 mL/min. For MS, the ionization conditions were adjusted to 250°C and 3kV for capillary temperature and voltage, respectively. The nebulizer pressure was 450 MPa and the flow rate of nitrogen was 500 L/h.

Negative ions were detected scanning from m/z 20 to 1000 with a total scan duration of 0.5 s and collision energy ranged from 6 to 20 eV. Argon was serving as collision gas with a flow rate at 1.5 mL/min. NMR spectra were recorded at room temperature using Bruker Advance III 400MHZ spectrometer (400 MHz for $^1$H NMR and 100 MHz for $^{13}$C NMR) using CDCl$_3$ as solvent. Chemical shifts were given on δ-scale and were referenced to the solvent and to the TMS as internal standard. The $^1$H NMR and $^{13}$C NMR spectral data of the isolated compound are as follows: $^1$H NMR (CDCl$_3$, 400 MHz): δ0.89 (3H, J=6.8, t, H-U19), δ1.30 (4H, m, H-U18, H-U17), δ1.45 (3H, s, H-U12), δ1.62 (2H, m, H-U15), δ1.87 (3H, J=6.8, d, H-U11), δ2.44 (1H, m, H-U15a), δ2.60 (1H, J=18.0, 7.2, dt, H-U5a), δ2.67 (1H, m, H-U15b), δ3.01 (1H, J=17.8, 7.2, dt, H-U5b), δ3.23 (1H, m, H-U6), δ3.67 (1H, J=13.2, d, H-U13), δ4.72 (1H, J=12.6, d, H-U1a), δ5.06 (1H, J=12.6,d, H-U1b), δ5.27 (1H, s, H-U4), δ5.90 (1H, J=15.4, d, H-U9), δ6.50 (1H, J=15.4, 7.2, dt, H-U10).

$^{13}$C NMR (CDCl$_3$, 100 MHz): δ13.9 (C-19), δ17.7 (C-12), δ18.5 (C-11), δ22.4 (C-18), δ22.8 (C-16), δ29.4 (C-17), δ31.1 (C-5), δ42.9 (C-6, C-15), δ54.9 (C-13), δ63.8
(C-1), δ83.2 (C-7), δ103.3 (C-4), δ114.0 (C-8a), δ124.4 (C-9), δ135.4 (C-10), δ150.8 (C-4a),
δ160.5 (C-3), δ169.5 (C-13a), δ189.8 (C-8), δ202.5 (C-14).

**CTC staining**

The fermentation broth was centrifuged at 4500 g for 10 min to collect the mycelia after
discarding the supernatant. The mycelia were washed twice and then resuspended with saline
(0.9 % NaCl). CTC staining was conducted in a 1.5-mL Eppendorf tube by use of a
Bacstain-CTC rapid staining kit (Dojindo Laboratories, Kumamoto, Japan) for 30 min at
30°C according to the manufacturer’s instructions. 20 µL of stained samples were deposited
on a clean slide and observed under a Leica confocal laser-scanning microscope (TCS-SP8,
Leica Microsystems, Wetzlar, Germany) at the excitation wavelength of 488 nm and
emission wavelength from 620 to 640 nm. Fluorescence intensity was quantified with the
software provided by the manufacturer. A significant number of images were analyzed in a
minimum of three independent culture analyses.

**Scanning electron microscopy (SEM)**

For SEM, samples were fixed in 2% glutaraldehyde in 5 mM piperazine-N,N’bis
(2-ethanesulphonic acid) (PIPS) buffer, pH 6.5, overnight at room temperature, and washed
4 times in PIPES buffer, pH 6.5. The biomass was then dehydrated through a 50-100%
ascending series of ethanol in distilled water, being left for 15 min at each stage. Three
transfers were made in 100% ethanol, and the samples were dried by the critical point method.
The samples were mounted using double-sided carbon tape on 7-mm diam aluminum stubs. For SEM, the samples were sputter-coated for 5 min using a Polaron E5100 series II 'cool' sputter coater fitted with an Au/Pd target. The hyphal morphology was then observed by SEM.

**Determination of cell integrity**

To evaluate the cell integrity, the LIVE/DEAD Bac-Light Bacterial Viability Kit L-13152 (Invitrogen detection technologies, California, USA) containing two nucleic acid staining dyes, propidium iodide (PI), and SYTO 9 was used. The SYTO9 is a green fluorescent stain which enters all the cells, those with intact membranes as well as those with damaged ones. In contrast, PI only penetrates dead cells with damaged membranes. However, PI has a higher affinity for the nucleic acids and displaces SYTO 9 in dead cells. Therefore, in the presence of both stains, mycelial cells with intact membranes appear to be fluorescent green, whereas mycelial cells with damaged membranes appear red. The fermentation broth was centrifuged at 4500 g for 10 min to collect the mycelia after discarding the supernatant. The mycelia were washed twice and then resuspended with saline (0.9 % NaCl). The two stains were prepared and mixed together (1:1, v/v). Equal volume (20 µL) of the stain mixture and culture samples were mixed on a clean slide and left in the dark for at least 10 min. Afterwards, the sample was covered with a slip and analyzed under a Leica TCS-SP8 confocal laser-scanning microscope, which was sequentially excited at wavelengths of 488
and 568 nm and observed at emission wavelengths of 530 nm (green) and 630 nm (red), respectively\(^{16}\).

**Determination of fatty acid composition of cell membrane**

Determination of fatty acid profiles was carried out using an AutosystemXL series gas chromatograph (GC) equipped with a flame ionization detector. A Supelco Omegawax 250 fused silica capillary column (30 m × 0.25 mm × 0.25 µm) was used. The oven temperature program was as follows: initial temperature 205°C and hold for 10 min; temperature rise at 6°C/min to 240°C and hold for 9 minutes\(^{17}\).

**Statistical analysis**

Each experiment was performed at least in triplicate and the results were expressed as mean ± standard deviation. All statistical analyses were performed by using the software SPSS Statistics 17.0 (SPSS, Chicago, Illinois). All the data obtained were analyzed by one-way ANOVA, and tests of significant differences were determined by using Tukey multiple comparison or Student’s t-test at \(p<0.05\).

**Results and discussion**

**Effect of different in situ extractants**

The novel integrated fermentation system consisting of surfactant and \textit{in situ} extractant can enhance the production of fungal metabolites by submerged fermentation\(^{18}\). Hence, in
order to set up an integrated fermentation system, five kinds of oils were applied in the
cultivation as *in situ* extractants and the results of mycelial biomass and yellow pigment
production are shown in Table 1. Addition of various oils increased mycelial growth in the
submerged fermentation of *M. purpureus* sjs-6. Vegetable oil, which has higher oxygen
solubility than water, could be used as oxygen carrier for improving oxygen transfer rates in
submerged fermentation\(^{19-20}\). Sufficient oxygen is beneficial for the mycelial growth of
aerobic fungi such as *Monascus* sp.

On the other hand, the addition of all tested oils led to a significant increase in the
production of yellow pigment. It might be because the added extractant can remove
hydrophobic metabolites from its production site as soon as it is formed, thus alleviating the
product inhibition and resulting in enhanced productivity. Song et al. suggested that oils
could serve as either a carbon source or a stimulator during submerged culture\(^{21}\). More
interestingly, the stimulatory extent of the five kinds of oils are totally different, although all
of them have a certain positive effect on the biomass and yellow pigment production. The
different composition and contents of fatty acids present in these vegetable oils can partly
explain the phenomenon\(^{22}\). Among the tested oils, medium chain triglycerides (MCT)
increased the mycelial biomass by 49.8% and maximized the yellow pigment synthesis 16.7
times.

Hence, the effect of different concentrations and addition time of MCT on mycelial
biomass and yellow pigment production was further investigated in submerged culture of *M. purpureus* sjs-6. As shown in Fig 1a, the production of mycelial biomass and yellow pigment increased significantly (*p* < 0.05) as the concentration of MCT increased from 10% to 40%, beyond which no significant increase was observed. Accordingly, the maximum amounts of mycelial biomass (38.2 g/L) and yellow pigment (465.2 U/mL) were obtained when 40% (v/v) MCT was added to the medium (Fig 1a).

To better understand the effect of addition time of MCT on the mycelial growth and yellow pigment production, 40% (v/v) MCT was added at different periods of fermentation. As shown in Fig 1b, addition of MCT at the earlier stage resulted in significantly higher biomass and yellow pigment production when compared with the addition at later stage. These results suggested that the timely addition of MCT was crucial for stimulating mycelial growth and eliminating the product inhibition of yellow pigment. Hence, addition of MCT at the beginning of fermentation was preferred in the subsequent experiments.

**Effect of different surfactants**

After successfully establishing a biphasic fermentation system by the addition of oils, the use of surfactants may further accelerate the production of *Monascus* yellow pigments. As shown in Table 2, all the tested surfactants attributed to the decrease of mycelial biomass of *M. purpureus* sjs-6, which might be resulted from the toxicity and low biocompatibility of
these five surfactants. Remarkably, when coupling of MCT, only the addition of Span 80 significantly enhanced the production of Monascus yellow pigment, whereas the other four kinds of surfactants exerted negative effects. These results indicated that the effect of surfactant on the yellow pigment production probably had no direct correlation with the effect on mycelial growth.

Various amounts of Span 80 were added into fermentation broth and the results were shown in Fig. 2a. It was interesting to notice that the biomass of M. purpureus sjs-U6 decreased along with the increase of the concentration of Span 80. This may be attributed to the excessive damage to mycelial cells by high concentration of Span 80\textsuperscript{23}. The inhibitory effect of surfactant on the mycelial growth might be due to the damage to the cell membrane or their detrimental interaction with other bio-components in cells\textsuperscript{23}. By contrast, that addition of surfactants in the culture broth proved to be an effective strategy for improving the extraction of Monascus yellow pigment. The increase in cell membrane permeability has been suggested as the possible mode of action of the surfactants\textsuperscript{24}.

Moreover, although the mycelial growth was inhibited when Span-80 was added, a late addition time was favorable for the mycelial growth (Fig. 2b). This suggests that the inhibitory effect of Span 80 on mycelial growth was attenuated with the delay of addition. However, the promoting effect of Span 80 on the yellow pigment production decreased when added at the later period of submerged fermentation. This indicated that the release of
intracellular pigment was not effective if Span 80 was not added in timely manner. Therefore, 
the maximal Monascus yellow pigment production (669.2 U/mL) was obtained when 5 g/L 
Span 80 was added at the 72nd h of the submerged fermentation.

Isolation and characterization of Monascus yellow pigments

Although the absorbance at a selected wavelength has been generally utilized to measure the concentration of different Monascus pigments, a further isolation and characterization of Monascus yellow pigments would make it clear about the accurate structure and possible changes under different conditions. With the help of analytic HPLC, it was found that only one yellow pigment component was produced by submerged fermentation of M. purpureus sjs-6 in the control and it was the same in the integrated system (Fig. 3). The Monascus yellow pigment isolated from both control and treatment group (MCT+Span 80) was further characterized by LC-MS and NMR analyses. The chemical structure of the Monascus yellow pigment was elucidated as 3-hexanoyl-9a-methyl-6-((1Z)-prop-1-en-1-yl)-3a,4,8,9a-tetrahydro-2H-furo[3,2-g] isochromene-2,9(3H)-dione. Compared with the information in literatures, the compound completely matched with monascin. This result indicated that the novel integrated fermentation system was beneficial for the production of Monascus yellow pigment while it didn’t affect the structure.
Mechanistic study on the effect of the extractant and surfactant

After successfully establishing an integrated fermentation system coupling of surfactant and in situ extractant, the underlying mechanisms were further revealed from different aspects including the investigation on the change of hyphal morphology, cell activity and membrane permeability.

Changes in hyphal morphology

The hyphal morphology was significantly influenced by the fermentation conditions and closely related to the production of secondary metabolites. Fig. 4 displayed the SEM of *M. purpureus* sjs-6 in the three different culture conditions (control, with MCT and with MCT+Span 80). The hyphae maintain their complete, regular morphology without the addition of Span 80, which indicated the integrity of cell wall structure and function (Fig. 4a&b). Thicker and multi-branched hyphae of *M. purpureus* sjs-6 were obtained with the addition of MCT while slender and less-branched hyphae were obtained in control. This observation further supports the hypothesis that the swelling of the hyphal tips may result in high metabolite production. It's worth noting that with the addition of Span 80, a large number of vesicles emerged at the hyphal surface besides the phenomenon of swelling and multi-branching (Fig. 4c). The hyphal morphology of Fig. 4c corresponded to the highest *Monascus* yellow pigment production, which indicated that a certain extent of hyphal damage was beneficial for the release of yellow pigment. Hence, further experiments were conducted
to study the relationship of cell viability, membrane permeability and *Monascus* yellow pigment production.

**Time course and the respiratory activity of mycelia**

As shown in Fig. 5a, mycelial biomass increased continuously until the 96th h and afterwards reached a stationary phase or even a slight decline in all three cases. Although the trend of mycelial growth was the same, the addition of MCT resulted in the highest biomass (37.4 g/L) at the 120th h of fermentation. Furthermore, it was found that the consumption of sugar corresponded well with the mycelial growth in the three cases (Fig. 5b). A more rapid and greater extent of utilization of sugar existed when MCT or MCT-Span 80 was added in the fermentation broth, compared to that of control. In order to display clearly the time course of biosynthesis and extraction of *Monascus* yellow pigment, the extracellular and intracellular yellow pigments were measured separately, and the results were shown in Fig. 5c & 5d. It was interesting to note that the intracellular yellow pigment concentration was almost the same in different conditions, whereas the extracellular yellow pigment concentration was much higher in both MCT and MCT-Span 80 group than in the control. These findings suggested that the addition of extractant and surfactant could significantly promote the secretion of intracellular metabolites such as *Monascus* yellow pigment. Accumulating these metabolites into the extractant phase could effectively alleviate the product inhibition.
Furthermore, 5-cyano-2,3-tolyl-chlorotetrazole (CTC) staining was used for measuring the respiratory activity of mycelia. Reduced by respiring cells, CTC was transformed to CTC formazan, which has a red fluorescence when excited by blue light. Therefore, the respiratory activity of mycelia could be evaluated by fluorescence intensity. As shown in Fig. 6, the respiratory activity of mycelia in three cases all decreased gradually, which may be caused by the aging of mycelia. In comparison, the average respiration rate of mycelia with the addition of MCT was 39.2% higher than that of the control, while the average respiration rate of mycelia in the group MCT-Span 80 was 56.6% lower than that of the control in 6 days. Although almost all mycelial cells lost respiration activity in the 7th and 8th day, it appeared earlier in the group with addition of Span 80. These results indicated that MCT had a promoting effect on the mycelial respiratory activity, while the addition of Span 80 exerted a detrimental effect.

Cell membrane permeability

Cell membrane permeability is critical for the utilization of supplied nutrients, and also for the secretion and biosynthesis of intracellular metabolites. The composition and properties of the cell membrane may exert a significant effect on its permeability, which represents an important factor in the adaptation to different conditions. Herein, two different approaches were applied to evaluate the change in cell membrane permeability and its effect on the mycelial growth and the yield of yellow pigment. The first method used of confocal
laser scanning microscopy for testing of cell integrity by fluorescent dye staining. The second method detected the changes of fatty acid composition in the cell membrane by GC analysis. The fluorescent dyes SYTO 9 and PI were used to stain the Monascus cells grown in different culture conditions, which were further observed by confocal laser scanning microscopy. The green fluorescence indicated the integrity of the cell membrane while the red one showed the defective membrane. As shown in Fig. 7, most of the mycelia displayed in green in control and MCT group in the first 6 days, and gradually turned red till the end of fermentation. In contrast, with the addition of Span 80, some red fluorescent parts appeared from the 3rd day and afterwards more red zones were observed in the mycelia, indicating serious damage of the cell membrane. This phenomenon was in accordance with the results that some surfactants can serve as a promising pre-treatment step for extracting metabolites from intact cell. In addition, an interesting phenomenon appeared in both control and MCT group (Fig. 7a & 7b), in which Monascus mycelia generally presented in aggregated forms. However, with the addition of Span 80 (Fig. 7c), the mycelia mainly existed in freely dispersed form, which may be correlated with the higher production of yellow pigment, but this is still without substantial evidence of proof.

The composition and proportion of fatty acids, including saturated and unsaturated, can directly affect the cell membrane fluidity and permeability. The change of fatty acid composition is an important mechanism of cell membrane in response to external
environmental changes. The major fatty acid components of *M. purpureus* sjs-6 produced by submerged fermentation were palmitic acid (C16:0), stearic acid (C18:0), arachidic acid (C20:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3), no matter whether MCT and Span 80 were added or not (Table 3). However, with the addition of MCT, the unsaturated/saturated fatty acid ratio increased significantly from 3.39 to 4.81 and with addition of Span 80 the value increased further to 6.48. It should be noted that the IUFA value was also much higher when Span 80 was added in the fermentation broth. These results suggested that the addition of Span 80 could partially destroy the integrity of the cell membrane, and hence promote both uptake of extracellular nutrients and exit of intracellular metabolites through modification of membrane permeability.\(^{35}\)

In this study, a novel integrated fermentation system coupling the use of surfactant and *in situ* extractant was successful in establishing and enhancing production of natural *Monascus* yellow pigment 27.8 times of that of the control. Detailed mechanistic study showed that the novel integrated fermentation system can efficiently promote the secretion of yellow pigment and effectively alleviate the product inhibition, by partially damaging the cell membrane with Span 80 and accumulating the yellow pigment with MCT. Further in-depth investigation at molecular levels are underway to provide more support for the findings of this study.
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**Figure Captions**

Fig. 1 Effects of the concentration (a) and addition time (b) of MCT on the biomass and production of yellow pigment in submerged fermentation of *M. purpureus* sjs-6.

Conditions: (a) various concentrations of MCT were added at the 72th h of the fermentation. (b) 40% (v/v) of MCT added at different time of the fermentation. Each culture was carried out at 30°C for 8 days at 180 rpm (values are mean ± standard deviation, n=3).

Fig. 2 Effects of the concentration (a) and addition time (b) of Span 80 on the biomass and production of yellow pigment in submerged fermentation of *M. purpureus* sjs-6.

Conditions: (a) various concentrations of Span 80 were added at the initial of the fermentation. (b) 5 g/L of Span 80 added at different time of the fermentation. Each culture was carried out at 30°C for 8 days at 180 rpm (values are mean ± standard deviation, n=3).

Fig. 3 Chemical structure of Monascus yellow pigment (monascin), as deduced from LC-MS and NMR spectroscopy

Fig. 4 SEM of the hyphae of *M. purpureus* sjs-6 in different conditions after 8-days’ cultivation
Conditions: (a) Control (b) 40% MCT added at the beginning of fermentation (c) 40% MCT added at the beginning and 5 g/L span 80 added at beginning of the fermentation.

Fig. 5 Time course of (a) biomass, (b) sugar consumption, (c) extracellular yellow pigment production and (d) intracellular yellow pigment production in submerged fermentation of *M. purpureus* sjs-6 in different culture conditions

Fig. 6 Confocal laser scanning microscopy images of mycelial viability staining with 5-cyano-2,3-tolyl-chlorotetrazole (CTC) in the submerged fermentation of *M. purpureus* sjs-6.

Conditions: (a) Control (b) 40% MCT added at the beginning of fermentation (c) 40% MCT and 5 g/L span 80 added at the beginning of fermentation.

Fig. 7 Confocal laser scanning microscopy images of mycelial integrity staining with SYTO9 and PI in the submerged fermentation of *M. purpureus* sjs-6

Conditions: (a) Control (b) 40% MCT added at the beginning of fermentation (c) 40% MCT and 5 g/L span 80 added at the beginning of fermentation.
Table 1 Effects of various oils on the biomass and production of Monascus yellow pigments in submerged fermentation of M. purpureus sjs-6

<table>
<thead>
<tr>
<th>Kinds of oils</th>
<th>Biomass (g/L)</th>
<th>Yellow pigments (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.1±0.5\textsuperscript{a}</td>
<td>24.3±3.0\textsuperscript{A}</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>35.1±0.8\textsuperscript{be}</td>
<td>347.8±7.2\textsuperscript{B}</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>32.1±1.0\textsuperscript{d}</td>
<td>328.0±8.1\textsuperscript{C}</td>
</tr>
<tr>
<td>Corn oil</td>
<td>30.1±0.8\textsuperscript{c}</td>
<td>379.0±5.4\textsuperscript{E}</td>
</tr>
<tr>
<td>Colza oil</td>
<td>34.1±0.6\textsuperscript{b}</td>
<td>272.1±3.3\textsuperscript{F}</td>
</tr>
<tr>
<td>Medium chain triglycerides (MCT)</td>
<td>36.1±1.1\textsuperscript{e}</td>
<td>406.7±5.5\textsuperscript{D}</td>
</tr>
</tbody>
</table>

Conditions: various oils were added at a concentration of 20% (v/v) at the beginning of fermentation. Each culture was carried out at 30 °C for 8 days at 180 rpm. Values (mean ± standard deviation, n = 3) within each column having different lowercase/capital letters have significant differences (ANOVA Tukey’s test: p < 0.05).
Table 2 Effects of various surfactants on the biomass and production of yellow pigment in submerged fermentation of *M. purpureus sjs-6*

<table>
<thead>
<tr>
<th>Kinds of surfactants</th>
<th>Biomass (g/L)</th>
<th>Yellow pigments (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.1±0.5</td>
<td>24.3±3.0</td>
</tr>
<tr>
<td>MCT</td>
<td>36.8±1.2</td>
<td>441.3±6.7</td>
</tr>
<tr>
<td>MCT+Span 20</td>
<td>33.0±1.5</td>
<td>319.2±3.4</td>
</tr>
<tr>
<td>MCT+Span 80</td>
<td>31.0±0.7</td>
<td>518.4±18.0</td>
</tr>
<tr>
<td>MCT+Tween 20</td>
<td>27.3±0.9</td>
<td>261.9±7.5</td>
</tr>
<tr>
<td>MCT+Tween 80</td>
<td>25.4±0.8</td>
<td>236.2±4.4</td>
</tr>
<tr>
<td>MCT+TritonX-100</td>
<td>30.1±1.3</td>
<td>383.2±8.3</td>
</tr>
</tbody>
</table>

Conditions: various surfactants were added at a concentration of 3 (g/L) at the beginning of fermentation. Each culture was carried out at 30 °C for 8 days at 180 rpm. Values (mean ± standard deviation, n = 3) within each column having different lowercase/capital letters have significant difference (ANOVA Tukey’s test: p < 0.05).
Table 3 Fatty acid composition (% total fatty acid) of cell membrane of *M. purpureus sjs-6* in submerged fermentation

<table>
<thead>
<tr>
<th>Fatty acid composition</th>
<th>Control</th>
<th>MCT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MCT-Span 80&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated fatty acid %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>C18:0</td>
<td>18.20</td>
<td>16.50</td>
<td>11.10</td>
</tr>
<tr>
<td>C20:0</td>
<td>4.31</td>
<td>2.46</td>
<td>2.15</td>
</tr>
<tr>
<td><strong>Unsaturated fatty acid %</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:1</td>
<td>25.01</td>
<td>28.38</td>
<td>29.19</td>
</tr>
<tr>
<td>C18:2</td>
<td>48.22</td>
<td>50.32</td>
<td>53.50</td>
</tr>
<tr>
<td>C18:3</td>
<td>4.01</td>
<td>2.21</td>
<td>4.81</td>
</tr>
<tr>
<td><strong>Unsaturated/saturated fatty acid ratio &lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td>3.39</td>
<td>4.81</td>
<td>6.48</td>
</tr>
<tr>
<td><strong>IUFA (index of unsaturated fatty acid) &lt;sup&gt;d&lt;/sup&gt;</strong></td>
<td>133.48</td>
<td>135.65</td>
<td>150.62</td>
</tr>
</tbody>
</table>

**Conditions:**

<sup>a</sup> 40% MCT added at the beginning of fermentation

<sup>b</sup> 40% MCT added at the beginning of fermentation and 5 g/L Span 80 added at 72th h of the fermentation.

<sup>c</sup> Unsaturated/saturated fatty acid ratio = (C18:1+C18:2+C18:3)/(C14:0+C16:0+C18:0)

<sup>d</sup> IUFA (index of unsaturated fatty acid) = C18:1 + 2×C18:2 + 3×C18:3
Fig. 1
Fig. 2
Fig. 4
Fig. 5
Fig. 7
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