Enhanced production of natural yellow pigments from *Monascus purpureus* by liquid culture: The relationship between fermentation conditions and mycelial morphology

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Natural yellow pigments produced by submerged fermentation of *Monascus purpureus* have potential economic value and application in the food industry. In the present study, the relationships among fermentation conditions (in terms of pH and shaking/agitation speed), mycelial morphology and the production of *Monascus* yellow pigments were investigated in both shake-flask and scale-up bioreactor experiments. In the shake-flask fermentation, the highest yield of the *Monascus* yellow pigments was obtained at pH 5.0 and a shaking speed of 180 rpm. Microscopic images revealed that these results were associated with the formation of freely dispersed small mycelial pellets with shorter, thicker and multi-branched hyphae. Further investigation indicated that the hyphal diameter was highly correlated with the biosynthesis of the *Monascus* yellow pigments. In a scaled-up fermentation experiment, the yield of yellow pigments (401 U) was obtained in a 200-L bioreactor, which is the highest yield to the best of our knowledge. The present findings can advance our knowledge on the conditions used for enhancing the production of *Monascus* yellow pigments in submerged fermentation and facilitate large-scale production of these natural pigments.

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[Key words: *Monascus purpureus*; Natural yellow pigment; Mycelial morphology; Submerged fermentation; Bioreactor]

Color pigments/colorants are widely used as additives in foods to make them more appealing to the consumers. Food pigments can be classified into synthetic colorants such as quinoline yellow (1) and tartrazine (2), as well as natural ones, such as lycopene (3) and curcumin (4). The global demand for food pigments is estimated to reach up to US$27.5 billion in 2018. At present, natural pigments comprise 31% of the market, as compared to 40% for the synthetic ones and others are semi-synthetic (5). However, due to undesirable mutagenicity and potential carcinogenicity, the number of permitted synthetic colorants has decreased. Nowadays, more attention has been paid to the pigments originating from natural sources, such as plants, animals and microorganisms (6). Among these pigments, those produced by microorganisms possess several advantages: they have a good quality for harvest, scale-up of production is easier and they are not subject to the vagaries of nature (7). However, prior to food use, toxicological assessments must be conducted because some fungal species producing pigments are also myco-toxicogenic producers (8).

*Monascus* spp. produce three kinds of natural pigments, mainly including red (monascusorubramine and rubropunctamine), orange (monascorubrin and rubropunctatin) and yellow (monascin and ankaflavin) ones (9). Among these, the red pigments have been widely used in Asia for centuries as food colorant and now have been successfully produced by fermentation (10). However, the yellow and orange pigments are still not suitable for industrial production, due to their relatively low production and purity. By controlling pH and nitrogen sources, the yield of *Monascus* yellow pigment was obtained approximately at 100 U (11). Zhou et al. (12) optimized the cultural conditions for *Monascus anka* mutant using response surface methodology and the yield of yellow pigment was 92.45 U in 5 L agitation bioreactor. Moreover, a novel approach of two-stage microbial fermentation in nonionic surfactant micelle aqueous solution was used to export intracellular *Monascus* pigments and the production of *Monascus* yellow pigment was about 60 U (13). Hence, great efforts on the optimization of the fermentation conditions are necessary for enhancing the large-scale production of *Monascus* orange and yellow pigments.

One of the main challenges of using filamentous fungi such as *Monascus* for production of fungal metabolites is the control of the morphology of the hyphae. This is of crucial importance, as morphology affects the yield of target products during submerged fermentations (14,15). The hyphae of filamentous fungi can exist in three different forms, which are commonly classified as free hyphae, aggregated forms (pellets/flocs), and artificially bound/entrapped forms (16–18). The hyphal morphology is significantly influenced by the fermentation conditions, such as carbon sources, pH, agitation rate, design of sparger, and rate of aeration (19). For example, the
most desirable hyphal morphology for lactic acid production in a bubble column reactor was a free dispersion of small pellets, which the dispersion was obtained at initial pH 6.0 and with potato starch as the carbon source (14). Likewise, ergothioneine production during submerged fermentation of the edible mushroom *Lentinula edodes* was associated with a larger pellet size, which was linked with a lower agitation rate and a higher inoculum volume (20). As another example, a decrease in the hyphal length during submerged fermentation of *Penicillium chrysogenum* due to high agitation rate reduces penicillin synthesis (21). For production of *Monascus* red pigment, maximum pigment yield was obtained under high agitation rate (22), which induced shorter branches in the mycelia (22).

However, in-depth mechanistic studies into the relationships among the submerged fermentation conditions, mycelial morphology and the biosynthesis of the other *Monascus* pigments are rare.

In our preliminary experiments, the pH and shaking speed influenced the mycelial morphology of *Monascus* as well as the yield of its yellow pigments. Therefore, the aim of this work was to systematically investigate the effect of culture conditions (pH and shaking speed) on the mycelial morphology and production of the yellow pigments in *Monascus purpureus* sjs-6. Furthermore, the present study also demonstrated the successful scaled-up production of *Monascus* yellow pigments in a 200-L bioreactor by controlling the appropriate culture conditions and mycelial morphology. The findings of this work advance our understanding of pigment biosynthesis during submerged fermentation in *Monascus* and could contribute to making the industrial production of *Monascus* yellow pigments feasible.

**MATERIALS AND METHODS**

Microorganism  *M. purpureus* sjs-6 could be obtained from the collection of the Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University (Wuxi, China). The specific strain *M. purpureus* sjs-6 in this study was obtained by strain screening and mainly produced yellow pigments. The strain was maintained on potato dextrose agar (PDA) slants which was incubated at 30 °C for 7 days and kept at 4 °C.

Inoculum preparation and submerged fermentation conditions  To prepare the inoculum, a 7-day-old mycelium of *M. purpureus* in a PDA slant was washed by 6 mL distilled water and then transferred to the culture medium by adjusting the concentration of suspension spores at about 2.5 × 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 under 30 °C. Then shake-flask experiments were carried out in 500-mL Erlenmeyer flasks containing 50 mL of culture medium with 12% (v/v) inoculum.

The culture medium consisted of 60 g/L corn starch, 4 g/L (NH₄)₂SO₄, 2 g/L NaNO₃, 1 g/L MgSO₄·7H₂O, 0.1 g/CaCl₂, 2 g/L KH₂PO₄, 3H₂O, 2 g/L KH₃PO₄, 3H₂O. The initial pH was adjusted to 5.0 with lactic acid. Each shake-flask culture was incubated at 30 °C for 8 days in a rotary shaker at 180 rpm.

Effect of pH and shaking speed  The relationship between morphology and pigment production in *M. purpureus*, was investigated using different initial pH values (initial pH adjusted to 3, 4, 5, 6, 7 and 8 by lactic acid) and shaking speeds (60, 120, 180, 240 and 300 rpm).

**Fermentation in bioreactors**

For scaled-up fermentations in a 200-L agitated bioreactor (ABEC 200-L, ABEC, Allentown, PA, USA), the mycelial inoculum of *M. purpureus* was firstly prepared in a 15-L agitated bioreactor (15L Bio Bench, Applikon Biotechnology, Delft, The Netherlands) and cultivated at 30 °C for 24 h. The agitation speed was 150 rpm and the aeration rate was 1.0 vvm. Afterward, the 5% (v/v) inoculum was transferred to the 200-L agitated bioreactor containing 130 L of culture medium at 30 °C for 8 days. The bioreactor was equipped with an upper marine impeller and a lower disk turbine impeller. The initial agitation speed was set at 100 rpm in the first day, then gradually increased to 200 rpm over the following 5 days, and finally maintained at 200 rpm. The initial pH was adjusted to 5.0 with lactic acid and the pH was monitored throughout the fermentation. The dissolved oxygen concentration of the culture medium in the bioreactor was monitored by using a galvanic dissolved oxygen electrode.

**Determination of the biomass**

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 mycelial dry weight per unit volume of culture medium.

**Determination of total yellow pigment**

The total yellow pigment produced by *M. purpureus* sjs-6 was extracted from the fermentation broth (2 mL) by addition of 70% (v/v) ethanol and made up to a total volume of 50 mL and then the mixture was 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 (OD₄₁₀) corresponding to 1 unit of color value (23). The calculation was shown as Eq. 1:

\[ \text{OD}_410 = 0.530 \times \text{A} \]

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

**Image analysis**

The morphology of mycelial pellets, mycelia and individual hyphae were monitored by an optical microscope (Olympus CX31, Olympus, Tokyo, Japan). Generally, the mycelial pellet was formed by *Monascus* mycelia and a mycelium is made up of hyphae.

The mean diameter of 20 randomly selected mycelial pellets was determined using an optical microscope (Olympus CX31) with the image analysis software Image Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) while the mean hyphal diameter was measured using the image analysis software TSVview 7 (Tucson, Fuzhou, China).

**Statistical analysis**

Each experiment was performed at least in triplicate and the results were expressed as mean ± standard deviation (n = 3). All statistical analyses were performed 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**

The production of *Monascus* yellow pigment is greatly influenced by the viscosity of the fermentation broth, which depends on the cell concentration and hyphal morphology (7). Therefore, the effects of two important factors (initial pH and shaking speed) were systematically investigated in this work.

**Effect of pH**

The effects of different initial pH values on the biomass and production of yellow pigments were investigated in the shake-flask (Table 1). The biomass and production of yellow pigments increased as the pH increased from 3.0 to 5.0, above which significant inhibition (p < 0.05) was observed. The optimal initial pH for mycelial growth was 5.0, with the biomass reaching 33.7 g/L, while the highest yellow pigment concentration (644.3 U) (p < 0.05) was obtained at the same pH value. The trends of yellow pigment yield (unit/g biomass) along with the different initial pH were the same as the yellow pigment.

**TABLE 1. Effects of different pH on the biomass and yellow pigment production in submerged fermentation of *Monascus* sjs-6.**

<table>
<thead>
<tr>
<th>pH</th>
<th>Biomass (g/L)</th>
<th>Yellow pigment concentration (U)</th>
<th>Yellow pigment yield (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>29.2 ± 0.5</td>
<td>339.1 ± 4.9^a</td>
<td>11600</td>
</tr>
<tr>
<td>4</td>
<td>31.1 ± 0.5^b</td>
<td>591.9 ± 8.3</td>
<td>19030</td>
</tr>
<tr>
<td>5</td>
<td>31.7 ± 0.3^c</td>
<td>644.3 ± 10.5^c</td>
<td>19110</td>
</tr>
<tr>
<td>6</td>
<td>21.2 ± 0.4^d</td>
<td>331.8 ± 4.0^d</td>
<td>14300</td>
</tr>
<tr>
<td>7</td>
<td>21.3 ± 0.2^e</td>
<td>280.7 ± 5.6^e</td>
<td>13180</td>
</tr>
<tr>
<td>8</td>
<td>20.2 ± 0.8^f</td>
<td>217.3 ± 4.1^f</td>
<td>10760</td>
</tr>
</tbody>
</table>

Conditions: Each shake-flask culture was incubated at 30 °C for 8 days in a rotary shaker at 180 rpm, the yellow pigment units were measured at OD₄₁₀. Biomass and yellow pigments (mean ± standard deviation, n = 3) having different capital/ lowercase letters, respectively, have significant difference (ANOVA Tukey’s test; p < 0.05).
concentration. These results indicated that *Monascus* mycelia tend to grow better at an acidic pH than a near neutral pH and the higher biomass coincide with higher yield of yellow pigment.

The morphology of the mycelial pellets was affected when *M. purpureus* sjs-6 was cultivated with different initial pH values (Fig. 1a). At acidic pH (3.0–5.0), freely dispersed small mycelial pellets were formed, while larger and clumpy pellets were formed at near neutral pH (pH 6.0–8.0) (Fig. 1a). Furthermore, the production of yellow pigments significantly increased ($p < 0.05$) with the decrease of pellet diameter from 4300 μm to 250 μm (Fig. 1c), which indicated that the biosynthesis of yellow pigments was strongly associated with the development of the mycelial pellets. The formation of freely dispersed small pellet was most favorable for the production of yellow pigments.

The influence of pH on mycelial pellet formation has been closely linked to its effect on the surface properties of the fungal cells (24,25): the pH affects the fungal cell wall structure and alters the conformation of proteins protruding from the plasma membrane, which also exert an impact on the lipid organization and function of cellular membranes (26). Culture broth with fungi in pellet form was less viscous than in filamentous form, which allowed more efficient mixing and aeration (27). Large mycelial pellets had lesser growth than smaller pellets due to poor oxygen diffusion (28). Furthermore, insufficient oxygen transfer inside the large mycelial pellets could lead to the development of elongated and delicate hyphae, which might induce the low productivity of yellow pigments.

As shown in Fig. 1b, the *Monascus* mycelia grown under the lower pH (pH 3.0–5.0) consisted of highly branched and wider hyphae while the near neutral pH (pH 6.0–8.0) promoted the formation of elongated and thinner hyphae. Among the tested pH range, the shorter, thicker and multi-branched hyphae at pH 5.0 corresponded to the highest yellow pigment production (Fig. 1c). In filamentous fungi, the biomass increases through tip extension and the extension of new tips by branching can facilitate the utilization of the nutrients (29,30), which can explain the observation that multi-branched hyphae result in a higher yellow pigment production.

Although the exactly underlying mechanism is still not clear, multi-branched and swollen hyphae indicate favorable growing status, which is due to a rapid growth rate associated with reduced pellet size and an increased yield of yellow pigment production.

**Effect of shaking speed** The rate of cell growth and metabolite formation in shake-flask fermentation can be affected by the shaking speed, which influences mixing and oxygen transfer. Different shaking speeds (60, 120, 180, 240 and 300 rpm) were applied in the submerged fermentation process for investigating their effects on the fungal biomass and yellow pigment production. Higher biomass was obtained with increasing shaking...
speed from 60 to 180 rpm (Table 2). However, further increase of shaking speed resulted in inhibition on cell growth, which might be caused by the intense force at high shaking speed. The impact of shaking speed directly on cell growth is complicated because it is a combined result of substrate mixing and oxygen transfer. Meanwhile, the maximum yellow pigment production ($p < 0.05$) was achieved at the shaking speed of 180 rpm. A lower or higher shaking speed both led to a decline in the yellow pigment concentration. Moreover, the yellow pigment yield (unit/g biomass) changed along with the different shaking speed, which was in the same tread as the yellow pigment concentration. Although fungi may suffer greater shear damage as the shaking speed is increased, the resulting higher oxygen transfer might give a higher yield of the metabolites (21).

As shown in Fig. 2a, large circular mycelial pellets were obtained at the lower shaking speeds (60 and 120 rpm), which corresponded to a lower production of the yellow pigments. The average pellet diameter significantly decreased from 4000 µm to 150 µm when the shaking speed was increased from 60 to 300 rpm. These results were consistent with previous report showing that lower agitation intensity led to an increase in the extent of large pellet formation which could be attributed to the hydrodynamic conditions in the flask (25). However, when the shaking speed is too high, the liquid medium will be out of phase with the movement of the flask, thus prevent pellets from forming (31). It appears that the shaking speed primarily affects the mycelial structure and consequently the size of the mycelial pellets (32). A less dense small pellet is a preferable morphological form for fungal cell growth and product formation because oxygen/nutrient transfer can take place more easily through the less dense pellets via turbulent diffusion and convective flow (14).

### TABLE 2. Effects of different shaking speed on the biomass and yellow pigment production in submerged fermentation of *M. purpureus* sjs-6.

<table>
<thead>
<tr>
<th>Shaking speed (rpm)</th>
<th>Biomass (g/L)</th>
<th>Yellow pigment concentration (U)</th>
<th>Yellow pigment yield (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>15.2 ± 0.4^a</td>
<td>54.6 ± 3.6^a</td>
<td>3600</td>
</tr>
<tr>
<td>120</td>
<td>27.8 ± 0.3^b</td>
<td>186.8 ± 4.1^b</td>
<td>6700</td>
</tr>
<tr>
<td>180</td>
<td>32.2 ± 0.5^c</td>
<td>595.2 ± 6.3^c</td>
<td>18480</td>
</tr>
<tr>
<td>240</td>
<td>32.7 ± 0.4^d</td>
<td>388.6 ± 5.0^d</td>
<td>11880</td>
</tr>
<tr>
<td>300</td>
<td>25.4 ± 0.6^e</td>
<td>264.5 ± 4.4^e</td>
<td>10410</td>
</tr>
</tbody>
</table>

Conditions: Each shaking-flask culture was incubated at 30°C for 8 days in a rotary shaker at different shaking speed, the yellow pigment units were measured at OD$_{410}$. Biomass and yellow pigments (mean ± standard deviation, $n = 3$) having different capital/lowercase letters, respectively, have significant difference (ANOVA Tukey’s test; $p < 0.05$).

**FIG. 2.** Effect of different shaking speeds on (a) the morphology of the mycelial pellets of *M. purpureus* sjs-6 at the 8th day, (b) the morphology of the hyphae of *M. purpureus* sjs-6 at the 8th day, and (c) the pellet diameter, the average hyphal diameter and the yield of yellow pigments. Conditions: Each shaking-flask culture was incubated at 30°C for 8 days in a rotary shaker at different shaking speeds, the yellow pigment units were measured at OD$_{410}$. 
In order to observe the mycelial morphology in more detail, images of the *Monascus* hyphae under different shaking speed are shown in Fig. 2b. It was obvious that multi-branched and thicker hyphae existed under relatively high shaking speed (180 rpm). However, speeds above 180 rpm could seriously damage the hyphal structure and caused extensive fragmentation of the hyphae to occur at the shaking speed of 300 rpm. Moreover, the average hyphal diameter increased from 1.02 μm at 60 rpm to 9.61 μm at 180 rpm, and then dropped back to 3.37 μm with the increase of shaking speed to 300 rpm (Fig. 2c). Accordingly, the highest pigment production was 595.2 U at 180 rpm and a lower or higher shaking speed resulted in a sharp decrease in the pigment yield (Fig. 2c). These findings were in accordance with a previous report showing that high rate of agitation would lead to the formation of fungal hyphae with shorter filaments and higher branching [21]. It has also been demonstrated that the extent of increase in the total number of branches in the hyphae was equal to the specific growth rate of the fungus which can be used as an indicator of the cell growth status [29]. Moreover, the highly branched hyphae were less susceptible to breakage and they could reduce the viscosity of the fermentation broth, which was beneficial for the oxygen transfer and the cell growth [33].

More interestingly, the present results exhibited a highly positive correlation between hyphal diameter and the yellow pigment production, irrespective of the different initial pH values or shaking speeds (Fig. 3). Based on these results, the color values of the final *Monascus* yellow pigments can be predicted by the observation of mycelial morphology and the determination of hyphal diameter. However, the inherent relationship among fermentation conditions, mycelial morphology and the production of fungal secondary metabolites is still largely unknown. We are currently investigating the underlying mechanistic study of culture conditions on the enhanced production of natural yellow pigments from *Monascus* by submerged fermentation.

**Scaled-up production of Monascus yellow pigments in a 200-L agitated bioreactor**

The above findings were applied in a pilot scale for *Monascus* yellow pigment production conducted in a 200-L agitated bioreactor. In a preliminary fermentation process in a 200-L agitation bioreactor, the production of *Monascus* yellow pigment was only 109.1 U with a lower biomass (25.4 g/L), compared to the results of shake-flask. The unsatisfactory result might be caused by the inappropriate constant agitation rate of 100 rpm in the bioreactor, which was too low and resulted in the formation of large size pellets in the fermentation broth (data not shown). It was in accordance with previous report that large pellets develop at very low agitation intensity caused by sparging of air at low aeration rate [34].

The problem of low agitation encountered in the preliminary experiment was overcome through the use of a gradual increase agitation approach. As shown in Fig. 4a, a successful scale-up of production of *Monascus* yellow pigment was conducted in a 200-L agitated bioreactor with an initial pH of 5.0 and an aeration rate of 1 vvm. The agitation was at low speed (100 rpm) in the first day, gradually increasing to 200 rpm in the following 5 days, and maintaining at 200 rpm until the end of the fermentation. During the whole fermentation process, there were four growth phases including a slow growth phase (0–24 h), a logarithmic phase (24–72 h), a stationary phase (72–144 h), and a decline phase (144–192 h) observed from the growth curve of *M. purpureus* sjs-6. In the first 72 h, although biomass of *M. purpureus* sjs-6 grew quickly, the yellow pigment accumulation in this period was relatively slow. Subsequently, a rapid accumulation of yellow pigments was accompanied with the mycelial growth reaching the stationary phase. The yellow pigment concentration reached the maximum level of 401 U after 7 days. Afterwards, the *Monascus* yellow pigment concentration decreased, probably due to decomposition of the yellow pigments or their transformation into other metabolites during the decline phase. At the same time, the fungal biomass also decreased rapidly, probably due to shortage of nutrient (e.g., sugar) and oxygen supply.

**FIG. 3.** The correlation of the hyphal diameter of *M. purpureus* sjs-6 and the yellow pigment production (the yellow pigment units were measured at OD410).

**FIG. 4.** (a) Time course of biomass, sugar consumption, pH change and yellow pigment production in a scale-up submerged fermentation of *M. purpureus* sjs-6 in a 200-L bioreactor (the yellow pigment units were measured at OD410). (b) SEM of the hyphae of *M. purpureus* sjs-6 after 7-day cultivation.
Hyphal concentration and morphology greatly influence the viscosity of the fermentation broth. At a high cell concentration, the viscosity of culture medium was high and the supply of oxygen for the cells was insufficient, thus led to the cell death (35). Increasing agitation speed could maintain dissolved oxygen concentration at high levels, which was the key parameter for high yield of Monascus yellow pigments in the 200-L agitated bioreactor fermentation. Meanwhile, the viscosity of culture broth should be kept relatively low in the mid and later stages of cultivation, which could be achieved via the control of cell morphology through varying the agitation speed (22). It has been reported that substrates diffuse freely inside the less dense pellet and mass transfer may occur via turbulence and convective flow, which could be induced by proper agitation speed (29).

During the whole fermentation period, if the agitation speed was not increased over time, weaker and less branched mycelia were observed, which might be due to the problem of viscosity and nutrient transfer/oxygen transfer. Sufficient transfer of oxygen and nutrients can be maintained with increasing agitation speed during the fermentation to allow thicker hyphae to form in the later period. Fig. 4b shows the thicker and multi-branched hyphae (401 U, Fig. 4a). This observation further supports our previous hypothesis that the swelling of the hyphal tips can result in higher metabolite production.

The mechanism of morphological change at various conditions might be explained by the integration of physiochemical properties of the strain, surface thermodynamics, and the rheology of culture broth (36). In this study, the higher yields of biomass and of Monascus yellow pigments were due to the formation of the freely dispersed small mycelial pellets which were composed of shorter, stronger and multi-branched hyphae. Consequently, a high yield of Monascus yellow pigment (401 U) was achieved in the 200-L agitated bioreactor, which is the highest yield to the best of our knowledge.

In conclusion, the mycelial morphology of Monascus purpureus sjs-6, including the pellet size and hyphal diameter, was significantly influenced by the culture conditions such as the initial pH and shaking speed, which further exerted great impact on the production of yellow pigments. Although the regulatory mechanisms of the biosynthesis of Monascus pigments are not yet clear, the relationship between the culture conditions and the fungal morphology has been revealed in this study for enhanced production of natural yellow pigments. The present findings will provide useful information for the scaled-up production of valuable metabolites by filamentous fungi such as Monascus in submerged fermentation.

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