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Monascus Yellow Pigment Production by Coupled Immobilized-Cell Fermentation and Extractive Fermentation in Nonionic Surfactant Micelle Aqueous Solution

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Abstract: Microbial fermentation with immobilized cells possesses many advantages. However, this fermentation mode is restricted to the production of extracellular products. Our previous study demonstrated that the extractive fermentation of *Monascus* spp. in nonionic surfactant micelle aqueous solution can export *Monascus* pigments that are supposed to be mainly intracellular products to extracellular culture broth and, in the meantime, extracellularly enhance the production of yellow pigments at a low pH condition; consequently, this makes the continuous production of yellow pigments with immobilized *Monascus* cells feasible. In this study, immobilized-cell fermentation and extractive fermentation in Triton X-100 micelle aqueous solution were successfully combined to continuously produce *Monascus* yellow pigments extracellularly. We examined the effects of cell immobilization and Triton X-100 on cell growth, pigment production, and pigment composition. In the repeated-batch extractive fermentation with immobilized cells, the biomass in Ca-alginate gel beads continued to grow and reached 21.2 g/L after seven batches, and dominant yellow pigments were produced extracellularly and stable for each batch. The mean productivity of the extracellular yellow pigments reached up to 22.31 $AU_{410\,nm}$/day within the first four batches (13 days) and 19.7 $AU_{410\,nm}$/day within the first seven batches (25 days). The results also provide a new strategy for producing such intracellular products continuously and extracellularly.

Keywords: *Monascus* yellow pigments; extractive fermentation; immobilized cells; nonionic surfactant

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1. Introduction

Monascus pigments are an azaphilone mixture, generally including red, orange, and yellow components, which have been widely used as natural food colorants with a long history in East and Southeast Asian countries, such as China, Japan, Korea, and so on [1,2]. Unlike synthetic pigments, natural *Monascus* pigments possess higher security and favorable physiological functions [3,4]. Moreover, compared with other natural pigments from plants and animals, the production process of *Monascus* pigments is not limited by the vagaries of nature [5]. Solid state fermentation and submerged fermentation are two major conventional methods for producing *Monascus* pigments. Compared with solid state fermentation, submerged fermentation is more attractive because it allows convenient control of fermentation parameters, is easy to scale up, has a shorter fermentation time, results in lower production costs and leads to higher product quality.

For microbial fermentation, product feedback inhibition is the major problem limiting the yield of targeted products [6]. Therefore, "taking away the aimed products" in

the fermentation process is one of the most feasible strategies to overcome this problem. Fermentation using in situ product removal based on resin and water–organic solvent two-phase systems are two common strategies for striping away aimed products during the fermentation process, in order to alleviate feedback inhibition [7–9]. After removing the targeted products, the yield of extracellular products can be increased, such as the enhancement of antibiotics (esperamicin A_1 and teicoplanin) [10,11] and other natural products (thailandepsin A) [12] production using neutral resin Diaion HP-20, the enhancement of 2-phenylethanol production using resin HZ818 [13], and the enhancement of biobutanol production using a water–organic solvent two-phase system in acetone–butanol–ethanol (ABE) fermentation [7,14]. However, these strategies are more favorable for the production of extracellular products. For those products that are restricted to the intracellular environment or are only excreted extracellularly at very low concentrations, a water–organic solvent two-phase system may enhance product release through permeabilization, but at the cost of low cell viability or cell death [15]; to a great extent, this limits the application for in situ product removal if the used organic solvent cannot possess both good biocompatibility and extraction ability for the aimed products.

Monascus pigments are mainly hydrophobic intracellular products, and solvent extraction with aqueous ethanol is the most common method for their downstream processing. Previous studies have reported that the extractive fermentation of *Monascus* spp. in nonionic surfactant (such as Triton X-100, Triton X-114, Brij 35 and Tween 80) micelle aqueous solution can promote the export of intracellular pigments to extracellular culture broth through the permeabilization of nonionic surfactant, and they enhance the production of extracellular pigments through the solubilization of pigments in nonionic surfactant micelles [16,17]; this was assimilated to the "milking processing" to alleviate product feedback inhibition. Triton X-100 shows both good biocompatibility and extraction ability for intracellular *Monascus* pigment. In addition, aqueous Triton X-100 micelles can further prevent the degradation of *Monascus* pigments in the fermentation broth [18,19]. Moreover, at a certain temperature above the cloud point of the nonionic surfactant micelle aqueous solution, it would form a cloud point system, which consists of a surfactant-rich phase and a dilute phase. Hydrophobic *Monascus* pigments were concentrated in the surfactant-rich phase, which would further simplify the downstream processes [17]. More interestingly, *Monascus* yellow pigments were the dominant pigments in extractive fermentation in Triton X-100 micelles aqueous solution, while *Monascus* orange pigments were the dominant pigments in conventional submerged fermentation at a low pH condition [19,20]. Currently, *Monascus* yellow pigments have drawn more attention because of the increasing demand for yellow pigments in the food industry.

Immobilized-cell fermentation, which is derived from enzyme immobilization, is attractive due to its many advantages, including the continuous reuse of microbial cells, higher volumetric productivities, shorter fermentation period, ability to endure a more open fermentation process, and consequent lower cost. Moreover, continuous immobilized-cell fermentation can also alleviate the adverse influence caused by the feedback inhibition of products and substrates [21]. However, similar to fermentation using in situ product removal, this fermentation mode is also restricted to the production of extracellular products and cannot be well employed for the production of *Monascus* pigments, most of which are mainly accumulated intracellularly. Various methods have been tested to solve this problem, such as using *Monascus* strains that can produce a large number of water-soluble extracellular pigments [22,23], using the fermentation media containing rice flour with a nearly neutral pH [22,24], adding adsorbent resin or active carbon [25,26] to promote the secretion of intracellular pigments, or collecting intracellular pigments from the cells falling from immobilization carriers (the results revealed that the obtained pigments were still intracellular products from the falling cells) [27]. Fortunately, the aforementioned extractive fermentation in Triton X-100 micelle aqueous solution can efficiently export intracellular hydrophobic pigments to extracellular culture broth, providing feasibility for the direct and continuous production of *Monascus* pigments coupled with immobilized-cell fermentation.

In this study, we attempted to combine extractive fermentation in nonionic surfactant (Triton X-100) micelle aqueous solution and immobilized-cell fermentation for the continuous production of Monascus yellow pigments. The effects of cell immobilization and Triton X-100 on cell growth, as well as on the yield and characteristics of pigments, were examined. Repeated-batch extractive fermentation with immobilized cells was conducted to continuously produce yellow pigments.

2. Materials and Methods

2.1. Microorganism and Cultivation Media

Monascus anka GIM 3.592 (deposited in the publicly accessible culture collection GDMCC/GIMCC, Guangdong Culture Collection Centre of Microbiology, Guangdong, China) was maintained on potato dextrose agar (PDA) medium (potato dextrose 200 g, glucose 20 g and agar 15–20 g, per liter of double-distilled water) and preserved at 4 °C. Sub-culture was carried out at 30 °C for seven days every month.

Seed culture medium consisted of glucose 20 g, $(NH_4)_2SO_4$ 4 g, peptone 10 g, KCl 0.5 g, KH_2PO_4 4 g and FeSO4·$7H_2O$ 0.01 g, per liter of double-distilled water. Conventional fermentation medium consisted of glucose 50 g, $(NH_4)_2SO_4$ 1 g, KH_2PO_4 1 g, KCl 0.5 g, $MnSO_4 \cdot H_2O$ 0.03 g, $ZnSO_4 \cdot 7H_2O$ 0.01 g, $MgSO_4 \cdot 7H_2O$ 0.5 g and $FeSO_4 \cdot 7H_2O$ 0.01 g, per liter of double-distilled water. Extractive fermentation medium consisted of the same substrates as conventional fermentation medium plus 40 g/L of Triton X-100. The initial pHs of the conventional and extractive fermentation media were adjusted to 4 with 10% (*v*/*v*) hydrochloric acid.

2.2. Fermentation

Free-cell fermentation and immobilized-cell fermentation in conventional or extractive fermentation media were conducted according to the procedures shown in Figure 1.

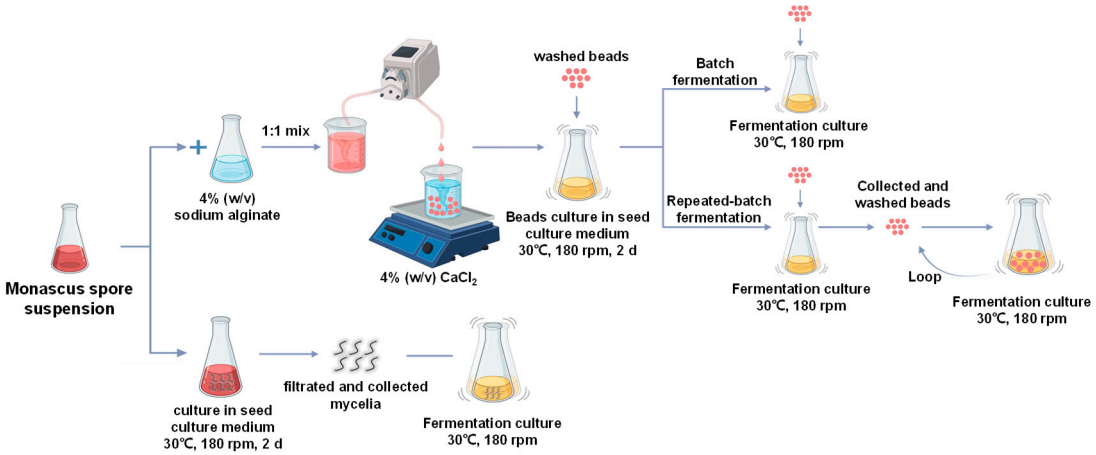

Figure 1. Procedures of free-cell fermentation and immobilized-cell fermentation.

2.2.1. Free-Cell Fermentation

Monascus spores from a seven-day old culture on potato dextrose agar were suspended in sterile 0.9% (*w*/*v*) NaCl solution. An amount of 5 mL of the spore suspension (approximate 2×10^5 spores/mL) was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of seed culture medium and incubated for 2 days at 30 °C in an orbital shaker set at 180 rpm. After the seed cultivation, the mycelia were collected by filtration and then transferred to a 250 mL Erlenmeyer flask containing 50 mL of the conventional or extractive fermentation media. The cultivation was conducted at 30 °C in an orbital shaker set at 180 rpm for five days. At the end of fermentation, the biomass and pigments were analyzed.

2.2.2. Immobilized-Cell Fermentation

An amount of 5 mL of spore suspension was mixed with an equal volume of 4% (*w*/*v*) sodium alginate solution and then added dropwise to sterile 4% $CaCl_2$ solution using an small peristaltic pump (Longer Pump, Beijing, China). Ca-alginate gel beads containing spores formed, since Na^+ was replaced by Ca^{2+}. Approximately 40 beads could be obtained by 2 mL of the mixture. After calcification for 3 h, all the beads (approximate 2.5 mm in diameter) were decanted and repeatedly rinsed with double-distilled water, and then they were transferred to 50 mL seed culture medium. After being cultured at 30 °C and 180 rpm for two days, the collected beads were transferred to a 250 mL Erlenmeyer flask containing 50 mL of the conventional or extractive fermentation medium and cultured at the same conditions with free-cell fermentation.

2.2.3. Repeated-Batch Extractive Fermentation with Immobilized Cells

Extractive fermentation was performed for several consecutive batches by cultivating immobilized cells in extractive fermentation medium. At the end of each batch, the beads were decanted and washed with sterile double-distilled water to remove the loose mycelia, and then transferred into the fresh medium. The first batch started after the seed culture. The first, fifth, sixth, and seventh batches lasted for four days, and the second, third, and fourth batches lasted for three days.

2.3. Analysis Methods

For free-cell fermentation, the fermentation broth was filtered under vacuum through 0.8 μm of mixed cellulose esters membrane. The filtrate was appropriately diluted to determine the residual glucose concentration and extracellular pigment concentration. The mycelia (filter cakes) were soaked in 50 mL of 70% (*v*/*v*) ethanol aqueous solution (pH 2.0) for 2 h; then, they were separated from the ethanol extract by filtration and dried to a constant weight at 60 °C to determine DCW (dry cell weight). The ethanol extract was subjected to intracellular pigment concentration determination.

When immobilized cells were used, the Ca-alginate gel beads and free cells (leaked cells from the beads) were separated from the fermentation broth by sequentially filtrating them through a 12-mesh screen (Shangyu Huafeng Hardware Instrument, Shaoxing, China) and filter membrane. The pigments in the immobilized cells (beads) and free cells were extracted as mentioned above and measured, respectively. The immobilized biomass was determined as follows: the beads were dissolved in 5% (*w*/*v*) sodium citrate solution, and the mycelia were collected and repeatedly rinsed with double-distilled water; then, the dry weight was determined as described above. In addition, 2 mL of fermentation broth collected from repeated-batch fermentation was subjected to centrifugation at 10,000 rpm for 5 min, and then the supernatant was used for analysis.

Residual glucose was quantified by the standard 3, 5-dinitrosalicylic acid (DNS) method. Pigment concentration was analyzed by a spectrophotometer [28,29]. The absorbance was measured at 410, 470 and 510 nm, corresponding to yellow, orange, and red pigments, respectively. Pigment concentration was calculated by multiplying the absorbance values by the dilution factor. An HPLC analysis was conducted according to the previous method [20] using Alliance e2695 with a 2998 photo-diode array detector (PDA) (Waters, Milford, CT, USA). The detector's wavelength was set as 210–600 nm. The absorption peak of the spectrum of each pigment was recorded by extracting the spectrum from HPLC data. The HPLC profile of intracellular and extracellular pigments was presented at a 310 nm detection wavelength.

2.4. Statistical Analysis

The results are expressed as the mean of three replicates ± standard deviations. A Student's *t*-test and one-way analysis of variance (ANOVA) test were performed to check the significant differences.

3. Results and Discussion

3.1. Effects of Cell Immobilization and Triton X-100 on Cell Growth, Pigment Production and Pigment Composition

As shown in Figure 1, immobilized-cell and free-cell fermentations were conducted in the conventional and extractive fermentation media. In the immobilized-cell fermentation, the mycelia predominantly grew in the outer layer of the Ca-alginate gel beads and formed a "mycelia coat" due to the limitation of nutrients and oxygen transfer [25,30]. Immobilized-cell growth could generate tension on the Ca-alginate gel beads, and K^+, Mg^{2+} and some chelating agents—such as phosphates, lactate, citrate and EDTA—in the medium could gradually rupture the beads [31,32]; this would decrease the mechanical strength of the beads and result in cell leakage. Therefore, free cells normally exist in immobilized-cell fermentation, and these free cells, serving as an indicator of cell leakage, were approximately one seventh of the total cells in biomass (Figure 2). The total biomass in immobilized-cell fermentation, including free cells and immobilized cells, showed no significant difference (immobilized-cell conventional fermentation vs. free-cell conventional fermentation, $p = 0.23$; immobilized-cell extractive fermentation vs. free-cell extractive fermentation, $p = 0.29$) with that in the free-cell fermentation, which indicated that the immobilization of *Monascus anka* by Ca-alginate had no obvious influence on the production of the final biomass. No matter if the cells were immobilized or not, the final biomass in extractive fermentation was lower (immobilized-cell extractive fermentation vs. immobilized-cell conventional fermentation, $p = 0.03$; free-cell extractive fermentation vs. free-cell conventional fermentation, $p = 0.02$) than that in conventional fermentation. This suggests that the nonionic surfactant Triton X-100 has a slightly negative effect on the final biomass, which is consistent with previous reports [16].

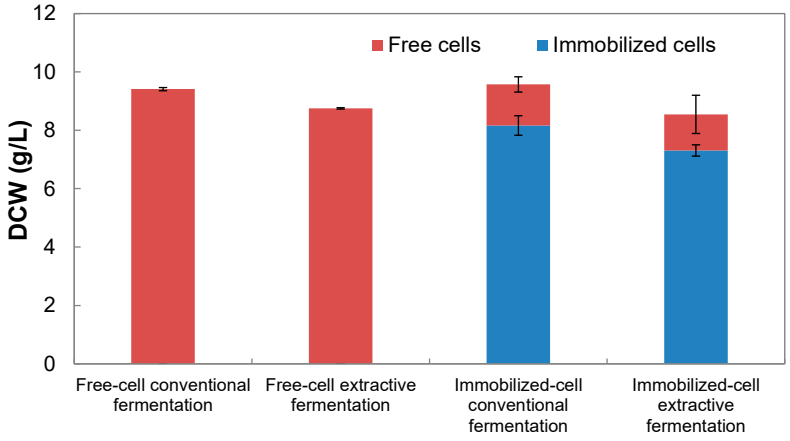

Figure 2. Effects of cell immobilization and Triton X-100 on cell growth. Leaked cells from the beads in immobilized-cell fermentation were recorded as free cells.

The effects of cell immobilization and Triton X-100 on the yield of intracellular and extracellular pigments are shown in Figure 3A,B. Compared with free-cell fermentation, immobilized-cell fermentation produced less pigments. This might be caused by oxygen or nutrients insufficiency and/or production inhibition due to the limitation of mass transfer in the immobilized carrier, which is considered a potential drawback of cell immobilization.

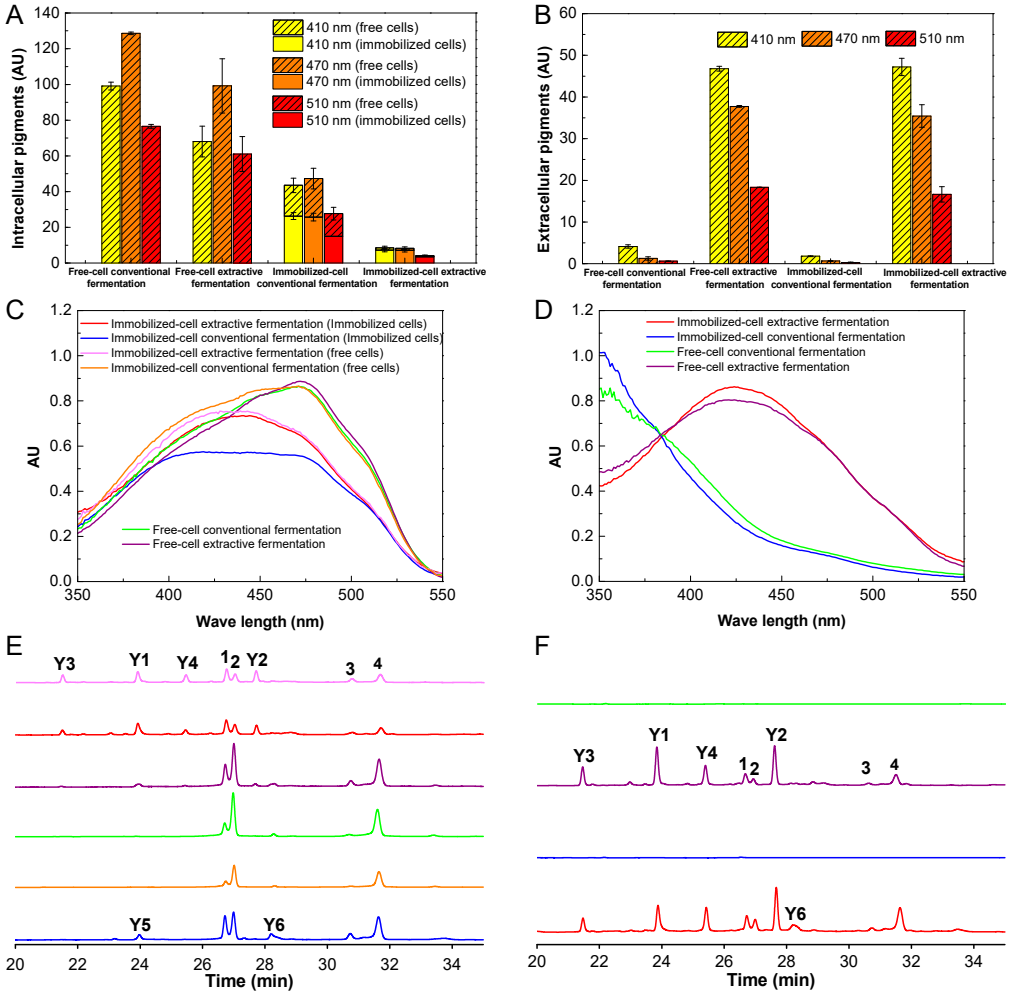

Figure 3. Effects of cell immobilization and Triton X-100 on pigment production. (**A**): Intracellular pigment concentration; (**B**): extracellular pigment concentration; (**C**): visible spectra of intracellular pigments; (**D**): visible spectra of extracellular pigments; (**E**): HPLC profile of intracellular pigments (its legend was the same as (**C**)); (**F**): HPLC profile of extracellular pigments (its legend was the same as (**D**)).

In conventional fermentation, pigments were primarily produced intracellularly, and very few were produced extracellularly (Figure 3A,B). The consumption of $(NH_4)_2SO_4$ could cause the decrease in pH; further, intracellular pigments can only be marginally exported into extracellular culture broth or form water-soluble red pigments through the ammoniophilic reaction between the hydrophobic orange pigments and amino acids at a low pH [33,34], so extracellular pigment concentration is very low. However, the extracellular pigment concentrations in free-cell and immobilized-cell extractive fermentation were very similar, both of which were much higher than that in conventional fermentation. This suggests that the presence of Triton X-100 led to the export of intracellular pigments into extracellular culture broth regardless of immobilized-cell and free-cell fermentation. This has been well explained in previous studies, i.e., that Triton X-100 can modify cell membrane structure to facilitate the export of intracellular pigments to extracellular culture broth [16,35], and its micelles in aqueous solution can solubilize hydrophobic pigments [18].

Extracellular pigment concentration was lower than intracellular pigment concentration in free-cell extractive fermentation, which is inconsistent with previous reports [17,36]. The solubilization of pigments in nonionic surfactant micelles strongly affects the final concentration of extracellular pigments [18]. Since the solubilization of micelles depends on the type of nonionic surfactant, the super-molecule assembly structure of nonionic surfac-

tant in an aqueous solution and the nonionic surfactant concentration [18,37], it is possible that 40 g/L of Triton X-100 was not enough to solubilize the pigments produced by the high-yield *Monascus anka* strain in this study. However, we did not further increase the concentration of Triton X-100, as this would cause high viscosity of the system, inconvenience in products separation and high production costs.

The effects of cell immobilization and Triton X-100 on the visible spectra and composition of pigments are shown in Figure 3C–F. Based on the fact that the visible spectra of different individual *Monascus* pigments present the maximum absorption at different wavelengths [38,39], the visible spectra of mixed pigments, which are caused by an overlap of every individual pigment spectrum, are usually used to approximately reflect the pigment composition [19,33,34]. In the meantime, the HPLC profile was also used to further reflect the pigment composition [20]. In this study, using an HPLC analysis, the pigments were also identified and are shown in Table 1 based on the characteristic absorption peaks of the spectra of the individual pigments and the previous results [20,35]. The spectra of intracellular pigments in free-cell extractive fermentation were similar to that in free-cell conventional fermentation, with an absorption peak at approximate 470 nm, in keeping with the previous results (Figure 3C). In addition, the spectra of intracellular pigments in the immobilized cells in immobilized-cell conventional fermentation also showed an absorption peak at approximately 470 nm. This spectrum characteristic suggested that these intracellular pigments consisted of dominant orange pigments (compounds 2 and 4) and a small amount of yellow pigments (compounds 1 and 3), which was further verified by the pigment profile detected by HPLC (Figure 3E). There was a small difference between the intracellular pigment profile of free-cell extractive and conventional fermentation that a trace amount of compounds Y1–Y4 were only found in free-cell extractive fermentation. In the previous study, compounds Y1–Y4—with an absorption peak at approximately 430 nm—turned out to be new yellow pigments that are dominant in extracellular pigments in extractive fermentation and not found in conventional fermentation [20]. Similarly, in this study, yellow pigments Y1–Y4 were dominant in extracellular pigments in the free-cell and immobilized-cell extractive fermentation, but not detected in either extracellular or intracellular pigments in the conventional fermentation (Figure 3E,F). Therefore, the spectra of extracellular pigments in free-cell and immobilized-cell extractive fermentation showed an absorption peak at approximately 430 nm (Figure 3D). The intracellular pigments in the immobilized and free cells in immobilized-cell extractive fermentation also contained a high proportion of yellow pigments (Y1–Y4) and presented spectra with a 430 nm absorption peak. Different from free-cell fermentation, the spectrum of intracellular pigments in the immobilized cells in immobilized-cell conventional fermentation was flat from 410 nm to 470 nm, and the HPLC profile indicated that the intracellular pigments consisted of a relatively high proportion of yellow pigments (compounds 1 and 3) and yellow pigments (compounds Y5 and Y6) with a 420 nm absorption peak, which have not been reported before. In fact, in immobilized-cell fermentation, the microenvironment of immobilized cells was different from that of free cells, which led to some differences in product biosynthesis [40]. The cells entrapped in Ca-alginate gel beads did not have sufficient access to oxygen due to mass transfer limitation. In the biosynthesis of *Monascus* pigments, the orange pigments (2 and 4) are formed from a precursor by a FAD-dependent oxidoreductase, and the yellow pigments (1 and 3) are formed from the same precursor by a reductase [41]. Interestingly, compared with the pigment composition in the free cells, a relatively high proportion of intracellular yellow pigments (1 and 3) was found in the immobilized cells in both immobilized-cell extractive and conventional fermentation. We speculated that the sufficient oxygen supply for the immobilized cells might have facilitated the formation of a reductive intracellular microenvironment, which then increased the biosynthesis of the yellow pigments (1 and 3).

Table 1. Individual *Monascus* pigments detected by HPLC-DAD.

No.	Pigments	HPLC Retention Time (min)	Characteristic Absorption Peaks (nm)	Molecular Weight [a]
1	Monascin	26.68	230, 291, 388	358
2	Rubropunctatin	26.94	214, 247, 286, 471	354
3	Ankaflavin	30.62	230, 293, 388	386
4	Monascorubrin	31.51	214, 274, 285, 471	382
Y1	Not reported	23.84	219, 310, 430	480
Y2	Not reported	27.62	219, 310, 430	508
Y3	Not reported	21.64	218, 310, 430	467
Y4	Not reported	25.40	218, 310, 430	495
Y5	Not reported	23.98	278, 420	
Y6	Not reported	28.20	278, 420	

[a] According to the previous report [20].

In free-cell and immobilized-cell conventional fermentation, the absorbance of extracellular pigments in conventional fermentation decreased from 370 nm to 550 nm (Figure 3D). Yellow pigments were the main extracellular pigments produced in both conventional and extractive fermentation; however, the composition and yield of the yellow pigments were very different. Very few hydrophilic yellow pigments were produced extracellularly in conventional fermentation, whereas a large number of hydrophobic yellow pigments (Y1-Y4) were the major components in extractive fermentation (Figure 3F). Extractive fermentation for yellow pigments in Triton X-100 micelle aqueous solution can be well combined with the immobilized-cell system.

3.2. Yellow Pigment Production in Repeated-Batch Extractive Fermentation with Immobilized Cells

As extractive fermentation in Triton X-100 micelle aqueous solution can export intracellular pigments into extracellular culture broth, a combination of extractive and immobilized-cell fermentation provides feasibility to continuously produce *Monascus* pigments extracellularly, which are supposed to be accumulated intracellularly in conventional fermentation. Moreover, the yield of extracellular pigments was very high (47 $AU_{410\,nm}$) and yellow pigments occupied a large proportion in the immobilized cell extractive fermentation (Figure 2B,D,F). Repeated-batch extractive fermentation was then carried out with immobilized cells for extracellular yellow pigments. For the immobilized cells, the lag phase was long, and the growth rate was slow at the seed culture stage due to mass transfer limitation in immobilization carriers [42]. Hence, we extended the period of seed culture to three days. Repeated-batch extractive fermentation was sustained for at least seven batches. After the seventh batch, the beads began to swell and rupture.

The process of extracellular pigment production in the first five batches showed the same trend (Figure 4A–E). The absorption peak of the spectrum of the extracellular pigments shifted from 470 nm to 410 nm along with the fermentation process (Figure 4F, data of other batches were similar and are not shown), indicating that the proportion of yellow pigments (Y1–Y4) increased during the fermentation process and that yellow pigments became the dominant component after three days in each batch. At the end of each batch, all the spectra of the extracellular pigments showed the same absorption peak at around 430 nm (Figure 5). The color characteristics of the extracellular pigments at the end of each batch are shown in Table 2. The AU_{410}/AU_{510} values of the extracellular pigments were greater than 2.2; they were approximately 1.2, indicating that the pigments exhibited a yellow hue. The yield and productivity of extracellular pigments in the second to fifth batches were relatively high but gradually decreased after the fifth batch (Table 2). The maximum batch yield (81.3 AU_{410}) and productivity (27.1 $AU_{410\,nm}$/day) were found in the fourth batch. In the seventh batch, the batch yield and productivity only reached approximately 70% and 52% of the maximum (the fourth batch), respectively. This result is consistent

with previous reports [23,27] that the capacity of pigments production firstly increased but started to decrease as the fermentation batch continued, even though cell immobilization created a suitable microenvironment. This might be related to the increased biomasses in Ca-alginate gel beads (Figure 6) and the decreased immobilized-cell activities. Glucose was quickly consumed in the first three batches, but the rate of glucose consumption slowed down from the fourth batch. More than 10 g/L of residual glucose remained in the medium at the end of the sixth and seventh batches. Such glucose consumption suggests that the immobilized-cell activities gradually declined, which might be caused by the long-term effects of Triton X-100, oxygen deficiency and an increasing number of dead cells.

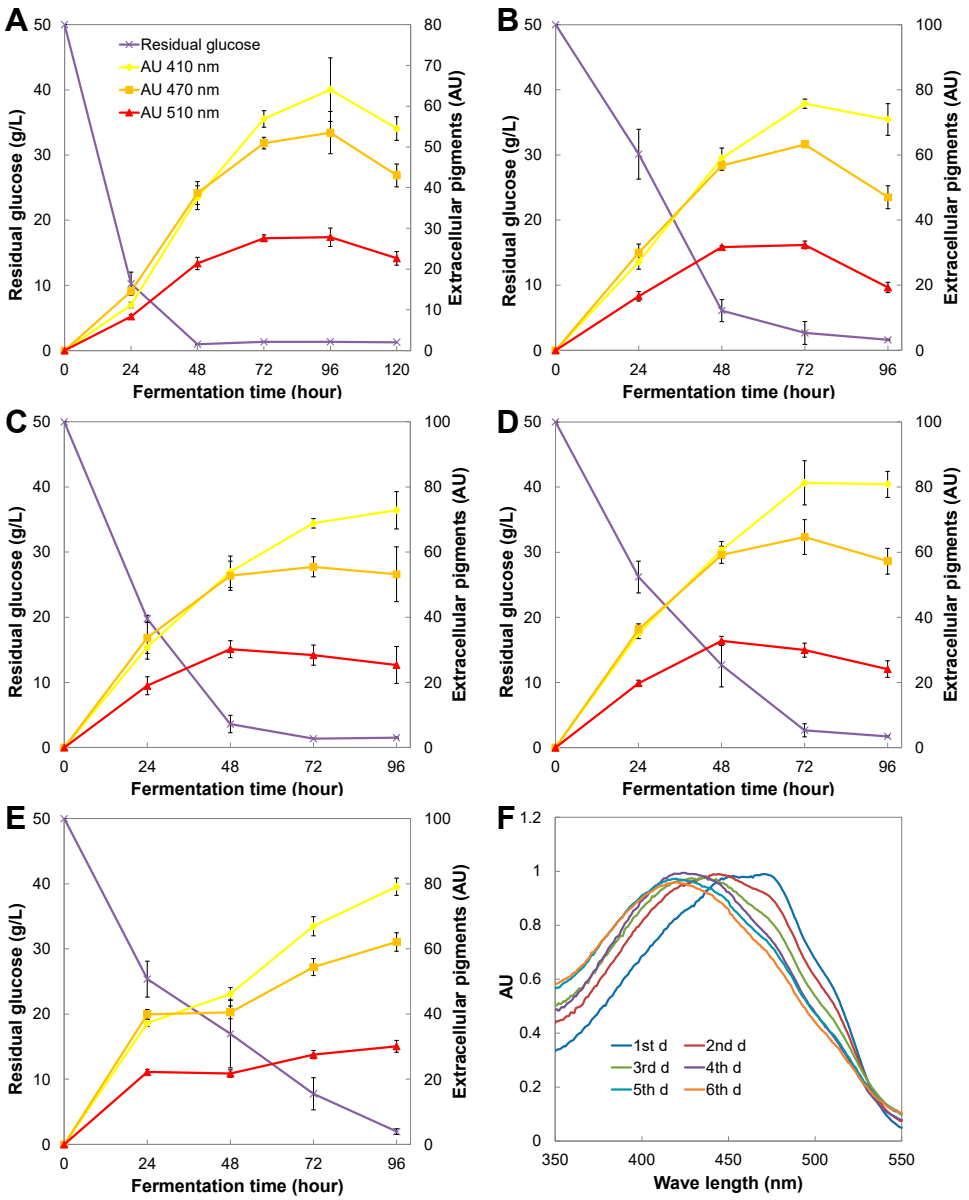

Figure 4. Extracellular pigment production and glucose consumption in repeated-batch extractive fermentation with immobilized cells. (**A**): First batch; (**B**): second batch; (**C**): third batch; (**D**): fourth batch; (**E**): fifth batch; (**F**): visible spectrum shift of extracellular pigments in the process of the first batch.

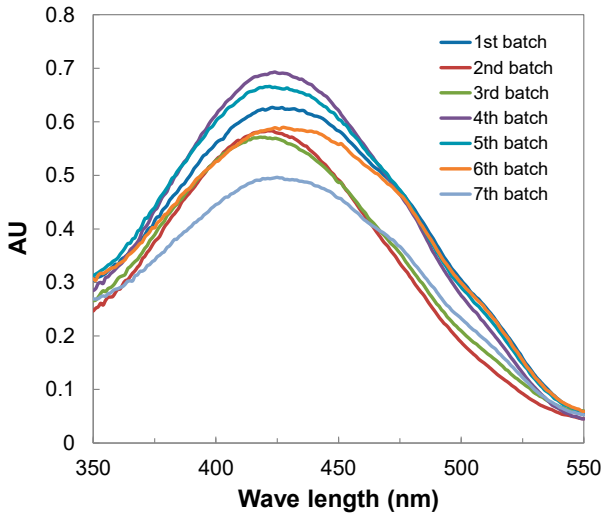

Figure 5. Spectra of extracellular pigments at the end of each batch in repeated-batch extractive fermentation with immobilized cells.

Table 2. Production of extracellular pigments in repeated-batch extractive fermentation with immobilized cells.

Batch	Cultivation Time (day)	Color Characteristic		Batch Yield ($AU_{410\ nm}$)	Batch Productivity ($AU_{410\ nm}$/Day)	Cumulative Yield ($AU_{410\ nm}$)	Mean Productivity ($AU_{410\ nm}$/Day)
		$AU_{410\ nm}$/ $AU_{510\ nm}$	$AU_{410\ nm}$/ $AU_{470\ nm}$				
1	4	2.30	1.20	64.06	16.01	64.06	16.02
2	3	2.34	1.20	75.77	25.26	139.82	19.97
3	3	2.43	1.24	68.84	22.95	208.67	20.87
4	3	2.71	1.26	81.30	27.10	289.97	22.31
5	4	2.63	1.27	79.08	19.77	369.05	21.71
6	4	2.26	1.16	66.85	16.71	435.90	20.76
7	4	2.46	1.23	56.63	14.16	492.53	19.70

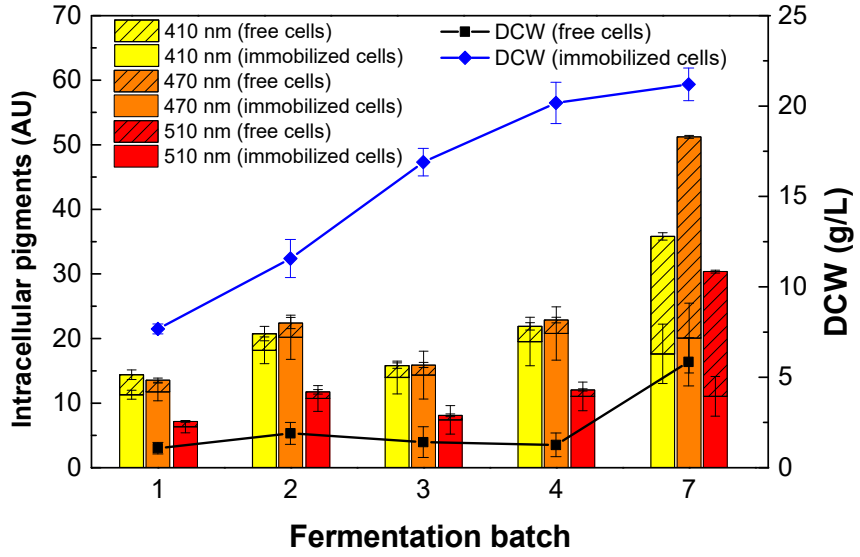

Figure 6. Biomass and intracellular pigment concentration at the end of each batch in repeated-batch extractive fermentation with immobilized cells.

Based on the above results, the duration of the total fermentation process should be limited to five or six batches in order to obtain high mean productivity. The highest

mean productivity could reach up to 22.31 $AU_{410\,nm}$/day within the fermentation of the first four batches (13 days) and 19.7 $AU_{410\,nm}$/day within that of the first seven batches (25 days), which was significantly higher than that reported previously (Table 3). Although the productivities reported in the literature could not be accurately compared because the calculation methods for pigment concentration in the literature are quite inconsistent, we can be certain that in most previous studies, immobilized *Monascus* cells mainly produced red pigments, and that in this study, immobilized *Monascus* cells mainly produced yellow pigments. However, the immobilized-cell system only performed well for a few batches, which might be further improved by optimization of the cell immobilization method and culture conditions.

Table 3. Comparison of repeated-batch fermentation for *Monascus* pigments with immobilized cells.

Monascus Stain	Immobilization Carrier	Number of Batch and Time	Major Pigments	Mean Productivity
M. anka SCUT1123	Ca-alginate	5 batches, 20 days	Yellow	15.38 AU_{410}/day (this study)
M. purpureus C322	Ca-alginate	6 batches, 37 days	Orange	4.7 AU_{470}/day [27]
Monascus F4018	Ca-alginate	12 batches, 36 days	Red (uncertainty) [a]	15~15.33 (AU_{420} + AU_{520})/day [22]
M. purpureus AS3.972	Ca-alginate	5 batches, 20 days	Red (uncertainty) [a]	14.5~15 (AU_{420} + AU_{520})/day [24]
Monascus purpureus M183	Ca-alginate	7 batches, 44 days	uncertainty [a]	4.76 (AU_{420} + $AU470_{470}$ + AU_{505}) [43]
M. purpureus	Polyelectrolyte complex	14 batches, 10.33 days	Red	3.79 AU_{500}/day [23]
Monascus M101	Polyvinyl alcohol	12 batches, 60 days	Red	9.25 AU_{520}/day (without active carbon)36.34 AU_{520}/day (with active carbon) [26]

[a] There is no clear description about the major pigments in the article, but red components may be the major pigments according to their culture conditions.

3.3. Cell Growth and Intracellular Pigments Metabolism in Repeated-Batch Extractive Fermentation with Immobilized Cells

In the process of repeated-batch extractive fermentation, the biomass of immobilized cells continued to grow (Figure 6). The accumulation of biomass in the beads could account for the initial increase in yield and productivity of both extracellular and intracellular pigments (Table 2, Figures 4 and 6). The biomass increase in immobilized cells gradually slowed down, and at the end of the seventh batch the biomass reached 21.2 g/L, which appeared to be the saturated biomass in the beads. The concentration of 40 g/L of Triton X-100 exhibited acceptable biocompatibility in the whole process. The biomass of free cells, which was measured as cell leakage from immobilized carriers, was lower than 2 g/L in the first four batches; however, it increased by nearly three times in the seventh batch due to the saturated space of beads for the growing cells.

The intracellular pigment concentration in the immobilized cells at the end of each batch was relatively stable from the second batch (Figure 6), but the intracellular pigment concentration in the immobilized cells per dry cell weight decreased due to the increase in biomass, which indicated a decline in the immobilized cells' capacity for pigments production. In the first four batches, yellow pigment concentration in the free cells was less than 3 $AU_{410\,nm}$, whereas in the seventh batch this increased to 18.2 $AU_{410\,nm}$ (Figure 6) due to the saturated biomass in the beads and the increasing cell leakage. The distribution ratio between intracellular and extracellular pigments might be influenced by many factors, such as the concentration of Triton X-100, the physiological state of cells and the biomass. Triton X-100 could export and solubilize not only the pigments, but also other intracellular hydrophobic substances, such as lipids, proteins, and so on [37]. These exported substances

would increase and then compete with the pigments in Triton X-100 micelles when the biomass became large. The solubilization capacity of triton X-100 was also limited to a great extent by the concentration of Triton X-100, resulting in the intracellular pigments in the free cells possibly not being effectively exported in the later batches.

4. Conclusions

In this study, immobilized cell fermentation and extractive fermentation were successfully combined to continuously produce *Monascus* yellow pigments extracellularly. This system showed good biocompatibility for *Monascus* cells. The immobilized cells could be reused for at least seven batches. The biomass in Ca-alginate gel beads continued to increase and finally, reached 21.2 g/L. Most intracellular pigments were exported into extracellular culture broth, and hydrophobic yellow pigments accounted for a large and stable proportion in the extracellular pigments of each batch. The mean productivity reached up to 22.31 $AU_{410\,nm}$/day within the first five batches (13 days) and 19.7 $AU_{410\,nm}$/day within the first seven batches (25 days). Our results also provide a novel strategy to continuously and extracellularly produce such intracellular products.

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References

1. Chen, W.; He, Y.; Zhou, Y.; Shao, Y.; Feng, Y.; Li, M.; Chen, F. Edible filamentous fungi from the species Monascus: Early traditional fermentations, modern molecular biology, and future genomics. *Compr. Rev. Food. Sci. Food Saf.* **2015**, *14*, 555–567. [CrossRef]
2. Feng, Y.; Shao, Y.; Chen, F. Monascus pigments. *Appl. Microbiol. Biotechnol.* **2012**, *96*, 1421–1440. [CrossRef] [PubMed]
3. Adin, S.N.; Gupta, I.; Panda, B.P.; Mujeeb, M. Monascin and ankaflavin—Biosynthesis from Monascus purpureus, production methods, pharmacological properties: A review. *Biotechnol. Appl. Biochem.* **2022**, 1–11. [CrossRef] [PubMed]
4. He, J.; Jia, M.; Li, W.; Deng, J.; Ren, J.; Luo, F.; Bai, J.; Liu, J. Toward improvements for enhancement the productivity and color value of Monascus pigments: A critical review with recent updates. *Crit. Rev. Food Sci. Nutr.* **2021**, *62*, 7139–7153. [CrossRef] [PubMed]
5. Fabre, C.E.; Santerre, A.L.; Loret, M.O.; Baberian, R.; Pareilleux, A.; Goma, G.; Blanc, P.J. Production and food applications of the red pigments of *Monascus ruber*. *J. Food Sci.* **1993**, *58*, 1099–1102. [CrossRef]
6. Sanchez, S.; Demain, A.L. Metabolic regulation and overproduction of primary metabolites. *Microb. Biotechnol.* **2008**, *1*, 283–319. [CrossRef]
7. Stark, D.; von Stockar, U. In situ product removal (ISPR) in whole cell biotechnology during the last twenty years. *Adv. Biochem. Eng. Biotechnol.* **2003**, *80*, 149–175.
8. Santos, A.G.; de Albuquerque, T.L.; Ribeiro, B.D.; Coelho, M.A.Z. In situ product recovery techniques aiming to obtain biotechnological products: A glance to current knowledge. *Biotechnol. Appl. Biochem.* **2021**, *68*, 1044–1057. [CrossRef]
9. Van Hecke, W.; Kaur, G.; De Wever, H. Advances in in-situ product recovery (ISPR) in whole cell biotechnology during the last decade. *Biotechnol. Adv.* **2014**, *32*, 1245–1255. [CrossRef]

10. Lam, K.S.; Gustavson, D.R.; Veitch, J.A.; Forenza, S. The effect of cerulenin on the production of esperamicin A_1 by *Actinomadura verrucosospora*. *J. Ind. Microbiol.* **1993**, *12*, 99–102. [CrossRef]
11. Lee, H.B.; Kim, C.J.; Lee, J.C.; Kim, Y.B.; Park, H.R.; Park, D.J. Improved production of teicoplanin using adsorbent resin in fermentations. *Lett. Appl. Microbiol.* **2003**, *37*, 196–200. [CrossRef]
12. Bing, L.; Hui, J.; Cheng, Y.Q.; Zhang, X. Extractive fermentation for enhanced production of thailandepsin A from *Burkholderia thailandensis* E264 using polyaromatic adsorbent resin Diaion HP-20. *J. Ind. Microbiol. Biotechnol.* **2012**, *39*, 767–776.
13. Hua, D.; Lin, S.; Li, Y.; Chen, H.; Zhang, Z.; Du, Y.; Zhang, X.; Xu, P. Enhanced 2-phenylethanol production from L-phenylalanine via in situ product adsorption. *Biocatal. Biotransform.* **2010**, *28*, 259–266. [CrossRef]
14. Outram, V.; Lalander, C.A.; Lee, J.G.; Davies, E.T.; Harvey, A.P. Applied in situ product recovery in ABE fermentation. *Biotechnol. Prog.* **2017**, *33*, 563–579. [CrossRef]
15. Kleinegris, D.M.; Janssen, M.; Brandenburg, W.A.; Wijffels, R.H. Two-phase systems: Potential for in situ extraction of microalgal products. *Biotechnol. Adv.* **2011**, *29*, 502–507. [CrossRef]
16. Wang, Y.; Zhang, B.; Lu, L.; Huang, Y.; Xu, G. Enhanced production of pigments by addition of surfactants in submerged fermentation of *Monascus purpureus* H1102. *J. Sci. Food Agric.* **2013**, *93*, 3339–3344. [CrossRef]
17. Hu, Z.; Zhang, X.; Wu, Z.; Qi, H.; Wang, Z. Perstraction of intracellular pigments by submerged cultivation of *Monascus* in nonionic surfactant micelle aqueous solution. *Appl. Microbiol. Biotechnol.* **2012**, *94*, 81–89. [CrossRef]
18. Kang, B.; Zhang, X.; Wu, Z.; Qi, H.; Wang, Z. Solubilization capacity of nonionic surfactant micelles exhibiting strong influence on export of intracellular pigments in *Monascus* fermentation. *Microb. Biotechnol.* **2013**, *6*, 540–550. [CrossRef]
19. Xiong, X.; Zhang, X.; Wu, Z.; Wang, Z. Accumulation of yellow *Monascus* pigments by extractive fermentation in nonionic surfactant micelle aqueous solution. *Appl. Microbiol. Biotechnol.* **2014**, *99*, 1173–1180. [CrossRef]
20. Shi, K.; Tang, R.; Huang, T.; Wang, L.; Wu, Z. Pigment fingerprint profile during extractive fermentation with Monascus anka GIM 3.592. *Bmc Biotechnol.* **2017**, *17*, 1–11. [CrossRef]
21. Gao, H.; Lu, J.; Jiang, Y.; Fang, Y.; Tang, Y.; Yu, Z.; Zhang, W.; Xin, F.; Jiang, M. Material-mediated cell immobilization technology in the biological fermentation proces. *Biofuels Bioprod. Biorefining* **2021**, *15*, 1160–1173. [CrossRef]
22. Li, C.; Wang, C.; Zhang, H.; Li, T. Study on water-soluble pigment from fermentation of immobilized *Monuscus*. *China Food Addit.* **2008**, *2*, 106–108.
23. Liu, J.; Ren, Y.; Yao, S. Repeated-batch Cultivation of Encapsulated *Monascus purpureus* by Polyelectrolyte Complex for Natural Pigment Production. *Chin. J. Chem. Eng.* **2010**, *18*, 1013–1017. [CrossRef]
24. Yi, X.; Hou, H.; Li, T.; Shao, W. Optimization of fermentation condition for the immobilization of *Monascus purpureus* cell with RSM. *China Brew.* **2008**, *9*, 80–83.
25. Evans, P.J.; Wang, H.Y. Pigment production from immobilized *Monascus* sp. utilizing polymeric resin adsorption. *Appl. Environ. Microbiol.* **1984**, *47*, 1323–1326. [CrossRef]
26. Wang, K.; Zhang, S. Study of production of *Monascus* pigment by PVA immobilized *Monascus* cell. *J. Yantai Univ. (Nat. Sci. Eng.)* **1998**, *11*, 306–309.
27. Fenice, M.; Federici, F.; Selbmann, L.; Petruccioli, M. Repeated-batch production of pigments by immobilised *Monascus purpureus*. *J. Biotechnol.* **2000**, *80*, 271–276. [CrossRef]
28. Babitha, S.; Soccol, C.R.; Pandey, A. Effect of stress on growth, pigment production and morphology of *Monascus* sp. in solid cultures. *J. Basic Microbiol.* **2007**, *47*, 118–126. [CrossRef]
29. Shi, K.; Chen, G.; Pistolozzi, M.; Xia, F.; Wu, Z. Improved analysis of Monascus pigments based on their pH-sensitive Uv-Vis absorption and reactivity properties. *Food Addit. Contam. Part A* **2016**, *33*, 1396–1401. [CrossRef]
30. Pashova, S.; Slokoska, L.; Sheremetska, P.; Krumova, E.; Vasileva, L.; Angelova, M. Physiological aspects of immobilised *Aspergillus niger* cells producing polymethylgalacturonase. *Process Biochem.* **1999**, *35*, 15–19. [CrossRef]
31. Zhu, Y. Immobilized cell fermentation for production of chemicals and fuels. In *Bioprocessing for Value-Added Products from Renewable Resources*; Yang, S.-T., Ed.; Elsevier: Amsterdam, Netherlands, 2007; pp. 373–396.
32. Bucke, C.; Brown, D.E. Immobilized cells [and discussion]. *Philos. Trans. R. Soc. Lond. Ser. B* **1983**, *300*, 369–389.
33. Kang, B.; Zhang, X.; Wu, Z.; Qi, H.; Wang, Z. Effect of pH and nonionic surfactant on profile of intracellular and extracellular *Monascus* pigments. *Process Biochem.* **2013**, *48*, 759. [CrossRef]
34. Shi, K.; Song, D.; Chen, G.; Pistolozzi, M.; Wu, Z.; Quan, L. Controlling composition and color characteristics of *Monascus* pigments by pH and nitrogen sources in submerged fermentation. *J. Biosci. Bioeng.* **2015**, *120*, 145–154. [CrossRef]
35. Chen, G.; Bei, Q.; Huang, T.; Wu, Z. Tracking of pigment accumulation and secretion in extractive fermentation of Monascus anka GIM 3.592. *Microb. Cell. Fact.* **2017**, *16*, 1–13. [CrossRef]
36. Hu, Z.; Zhang, X.; Wu, Z.; Qi, H.; Wang, Z. Export of intracellular *Monascus* pigments by two-stage microbial fermentation in nonionic surfactant micelle aqueous solution. *J. Biotechnol.* **2012**, *162*, 202–209. [CrossRef]
37. Chen, G.; Bei, Q.; Shi, K.; Tian, X.; Wu, Z. Saturation effect and transmembrane conversion of Monascus pigment in nonionic surfactant aqueous solution. *Amb Express* **2017**, *7*, 1–11. [CrossRef]
38. Zheng, Y.Q.; Xin, Y.W.; Guo, Y.H. Study on the fingerprint profile of Monascus products with HPLC–FD, PAD and MS. *Food Chem.* **2009**, *113*, 705–711. [CrossRef]
39. Teng, S.S.; Feldheim, W. Analysis of anka pigments by liquid chromatography with diode array detection and tandem mass spectrometry. *Chromatographia* **1998**, *47*, 529–536. [CrossRef]

40. de Alteriis, E.; Zueco, J.; Rico, H.; Sentandreu, R.; Parascandola, P. Electrophoretic mobility of external invertase from free and gel-immobilized yeast cells. *Res. Microbiol.* **1995**, *146*, 217–225. [CrossRef]
41. Chen, W.; Feng, Y.; Molnár, I.; Chen, F. Nature and nurture: Confluence of pathway determinism with metabolic and chemical serendipity diversifies Monascus azaphilone pigments. *Nat. Prod. Rep.* **2019**, *36*, 561–572. [CrossRef]
42. Junter, G.; Coquet, L.; Vilain, S.; Jouenne, T. Immobilized-cell physiology: Current data and the potentialities of proteomics. *Enzyme Microb. Technol.* **2002**, *31*, 201–212. [CrossRef]
43. Liu, J.; Guo, T.; Luo, Y.; Chai, X.; Wu, J.; Zhao, W.; Jiao, P.; Luo, F.; Lin, Q. Enhancement of Monascus pigment productivity via a simultaneous fermentation process and separation system using immobilized-cell fermentation. *Bioresour. Technol.* **2019**, *272*, 552–560. [CrossRef]