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Export of intracellular *Monascus* pigments by two-stage microbial fermentation in nonionic surfactant micelle aqueous solution

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ABSTRACT

Microbial fermentation of intracellular product is usually limited by high intracellular product concentration inhibition and complex downstream product processing. Perstractive fermentation of intracellular *Monascus* pigments in the nonionic surfactant Triton X-100 aqueous solution was studied in the present work, in which the intracellular product was exported from the intracellular to the extracellular aqueous solution and consecutively extracted into the nonionic surfactant micelles. After the second stage perstractive fermentation in the two-stage operation mode, biomass increased from 5 to 24 g/l DCW. The corresponding extracellular concentrations of yellow, orange, and red pigments were 60, 49 and 26 AU. The increase of cell density and the final pigment concentration were difficult to occur in a conventional aqueous medium using the two-stage fermentation. This positive effect of perstractive fermentation was ascribed to low intracellular pigment density, which eliminated the product inhibition and prevented the product from further degradation. The high efficiency of perstractive fermentation was further confirmed by fed-batch operation mode, in which the final biomass reached 28 g/l DCW and the corresponding extracellular concentrations of yellow, orange, and red pigments were 130, 84 and 47 AU.

1. Introduction

The current microbial industry of intracellular product fermentation usually follows a basic procedure, i.e., culture of microbes to achieve its maximum intracellular product concentration, collection of the biomass, and finally separation of the intracellular product by destruction of the microbial cells. There are at least three limits in this process. The first is the high intracellular product concentration, which inhibits further formation of product in the microbial culture process (Chen, 2007). Occasionally intracellular enzymes can also degrade the high concentration of intracellular product. The second is the destruction of cell structure, which makes the repeated utilization of the biomass impossible (Brodelius and Nilsson, 1983). The third also pertains to the destruction of cell structure, which makes the downstream process become more complex.

Extractive fermentation of extracellular product in a water-organic solvent two-phase system has been studied extensively (Leon et al., 1998; Daugulis, 2001; Fernandes et al., 2003; Straathof, 2003). The organic solvent is used to enhance the solubility of hydrophobic substrate, eliminate product inhibition, prevent the product from further degradation by microbial enzymes, and simplify downstream processing. Most of the microbes are active in the presence of hydrophobic organic solvents (biocompatibility), but are inactive in the presence of hydrophilic organic solvents (toxicity), which is the famous log *P* criterion (Laane et al., 1987) (log *P* is the partitioning coefficient of an organic compound in the water-octanol two-phase system). For example, hydrophobic solvents (log *P* > 5.5) are biocompatible with *Nannochloropsis* sp. while hydrophilic solvents (log *P* < 5.5) are toxic to *Nannochloropsis* sp. (Zhang et al., 2011). However, the extraction capacity of a hydrophobic organic solvent to most of organic compounds, especially to an organic compound with a moderate log *P*, tends to be very limited (Meyer et al., 2006). Thus, the biocompatibility of an organic solvent to the microbes and the extraction capacity of the organic solvent to the extracellular product are usually in conflict.

"Milking processing", i.e., cultivation of microalgae in a water-organic solvent two-phase system, has been proposed to production of intracellular products (Leon et al., 2003; Hejazi and Wijffels, 2004; Kleinegris et al., 2011a), in which the intracellular product is "milked" into the organic solvent phase of a water-organic solvent two-phase system. Milking processing is a perstractive fermentation process which consists of permeabilization of intracellular product across the cell membrane into the extracellular broth, followed by consecutive extraction of the

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product into the nonaqueous solvent phase (Hu et al., 2012). In a perstractive fermentation process, biocompatibility of microbes in an organic solvent and the adjustment of the cell membrane permeability by the organic solvent for secretion of intracellular product are simultaneously required. Only a few reports about perstraction of intracellular product in microalgae can be found (Leon et al., 2001; Hejazi et al., 2004; Mojaat et al., 2008; Kleinegris et al., 2011a,b; Zhang et al., 2011). Screening biocompatibility and permeability of different organic solvents to the microalgae *Dunaliella salina* reveals that the secretion of intracellular β-carotene does not solely depend on the organic solvent affinity with β-carotene, but also on the organic solvent concentration incorporated in the cellular membrane (Mojaat et al., 2008). Cultivation of *D. salina* in the water-decane two-phase system shows that β-carotene is "milked" into the organic solvent phase, in which the biomass grows very slow while the extracellular β-carotene concentration continues to increase (Hejazi et al., 2004). Further research indicates the increase of extracellular β-carotene concentration is partially ascribed to the microalgae autolysis. The no change of biomass is a result of the equilibrium between microalgae growth and microalgae autolysis (Kleinegris et al., 2011b). Thus, biocompatibility and permeability of the organic solvent becomes the critical issue of perstractive fermentation in a water-organic solvent two-phase system. Immobilized *Anabaena* (blue-green alga) is also used to improve the biocompatibility of perstractive fermentation in a water-organic solvent two-phase system (Leon et al., 2001).

Surfactant forms micelle pseudophase in an aqueous solution at the surfactant concentration above its critical micelle concentration (CMC). The surfactant micelle aqueous solution is a two-phase system where one phase is an aqueous solution and the other one is a micelle pseudophase. A nonionic surfactant micelle aqueous solution above a certain temperature (cloud point) forms another two-phase system, where one is a surfactant micelle aqueous solution and the other is a coacervate phase (surfactant-rich phase). This two-phase system is known as a cloud point system, which has been extensively studied for the extraction of metal ions, organic compounds and biomaterials (Hinze and Pramauro, 1993). Application of nonionic surfactant micelle aqueous solution (Kolomytseva et al., 2009; Cameotra and Makkar, 2010; Xue et al., 2010; Dhamole et al., 2012), or cloud point system (Wang et al., 2004) as a novel method for extractive microbial fermentation has also been developed. The advantages of extractive fermentation in the novel two-phase system, such as extractive fermentation of relatively higher polar product L-phenylacetylcarbinol (Xue et al., 2010) and 1-butanol (Dhamole et al., 2012), have been reviewed recently (Wang and Dai, 2010). An examination of *Saccharomyces cerevisiae* growth in the nonionic surfactant Triton X-100, or polymeric nonionic surfactant Pluronic F68 aqueous solution, indicates that Pluronic F68 exhibits nearly no effect on microbial growth whereas Triton X-100 leads to a relatively longer lag phase. However, both nonionic surfactants improve the secretion of intracellular alcohol dehydrogenase into their fermentative broth (Laouar et al., 1996). The biocompatibility and permeability of nonionic surfactant Triton X-100 to *Monascus* cells are confirmed by the perstractive fermentation of *Monascus* pigments, in which the *Monascus* maintains growth while the intracellular *Monascus* pigments exports into the extracellular micelle aqueous solution (Hu et al., 2012). Thus, the microbial fermentation of intracellular *Monascus* pigments can be carried out in the same manner as that of extracellular product, which provides a novel strategy to intensify its bioprocess efficiency.

The genus *Monascus* belongs to the class *Ascomycetes* and the family *Monascaceae*. This fungus is a source of various secondary metabolites of polyketide structure, including six main pigments, i.e., two kinds of yellow pigments, two kinds of orange pigments, and two kinds of red pigments. Recently, a novel red pigment and a novel yellow pigment production by *Monascus* sp. were also isolated and identified (Compoy et al., 2006). The *Monascus* pigments as a traditional food additive are produced by microbial fermentation with a relatively lower productivity. It has been demonstrated that strains, culture media and operation conditions are important factors that affect the production of *Monascus* pigments (Juzlova et al., 1996a). In the present work, microbial fermentation of intracellular *Monascus* pigments in a chemical defined medium was set up as a model. Perstractive fermentation in the nonionic surfactant Triton X-100 micelle aqueous solution was examined comparatively to that of aqueous solution using batch fermentation, two-stage fermentation and fed-batch fermentation mode. The microbial growth, the glucose consumption, the production of *Monascus* pigments and the partitioning of *Monascus* pigments between intracellular and extracellular were determined. The positive effect of perstractive fermentation on high cell density and high final product concentration were examined.

2. Materials and methods

2.1. Microorganism and cultivation media

Monascus anka (China Center of Industrial Culture Collection, CICC 5013) was maintained on potato dextrose agar (PDA) medium (potato dextrose 200 g, glucose 20 g and agar 15–20 g/l of water) and preserved at 4 °C. In every three weeks a sub-culture was carried out at 30 °C for 3 days.

The 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 $FeSO_4{\cdot}7H_2O$ 0.01 g/l of tap water. Nos. 1–4 medium were defined to clarify the different fermentation media used in the following text, respectively. No. 1 medium consisted of glucose 30 g, $(NH_4)_2SO_4$ 3 g, KH_2PO_4 5 g, $CaCl_2$ 0.1 g, and $FeSO_4{\cdot}7H_2O$ 0.01 g/l of tap water. No. 2 medium consisted of the same substrates as no. 1 medium does but without $(NH_4)_2SO_4$. No. 3 medium also consisted of the same substrates as no. 1 medium does except for the addition of 40 g/l Triton X-100. No. 4 medium consisted of the same substrates as no. 3 medium does but without $(NH_4)_2SO_4$. The initial pH of the fermentation culture medium was adjusted to 4 with 10% (V/V) hydrochloric acid and was left unaltered throughout the culture process.

2.2. Microbial cultivation and two-stage fermentation mode

All microbial cultivation was carried out aerobically at 30 °C. The seed cultivation was carried out in a 250 ml Erlenmeyer flask with 25 ml of the seed culture medium. The flask was shaken at 180 rpm for 2 days.

Batch fermentation was carried out in an aqueous solution (no. 1 medium) and a nonionic surfactant micelle aqueous solution (no. 3 medium), respectively. 25 ml of no. 1 medium was added to every 250 ml Erlenmeyer flask. After the seed cultivation, 2 ml of seed culture broth were withdrawn and added into the flasks where the seed broth and the no. 1 medium were combined. The flasks were shaken at 180 rpm for at least 4 days. Every day two flasks were withdrawn for independent analysis of pigment concentration and biomass. The rest of the flasks were left for continuous cultivation. For perstractive fermentation, no. 1 medium was replaced with no. 3 medium and microbial cultivation was carried out under the same condition.

Two-stage fermentation consists of the first stage fermentation of *Monascus* in no. 1 medium and the second stage (perstractive) fermentation in different media (nos. 1–4 medium). The first stage fermentation was carried out in no. 1 medium for 4 days under the same condition as the batch fermentation in no. 1 medium as described above. After the first stage cultivation, the fermentation broth was subjected to centrifugation at 5000 rpm for 10 min.