Effects of Oxygen Supply on Growth and Carotenoids Accumulation by *Xanthophyllomyces dendrorhous*

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The effects of oxygen supply on growth and carotenoids accumulation by *Xanthophyllomyces dendrorhous* were studied. Initial volumetric oxygen transfer coefficients (K_La) within the range 21.5–148.5 h^{-1} had significant effects on growth and carotenoids accumulation, and an increase of the initial K_La value led to higher carotenoids, astaxanthin and biomass yields by *X. dendrorhous*. At an initial K_La value of 148.5 h^{-1}, a maximal cell concentration of 19.37 g l^{-1} and optimal carotenoids and astaxanthin productions of 18.1 and 14.5 mg l^{-1} were obtained, as well as a maximal astaxanthin content of 0.8 mg g DCW^{-1}, respectively. A higher oxygen supply was advantageous to astaxanthin biosynthesis and the ratio of astaxanthin in the total carotenoids. An increasing initial K_La value gave stronger fluorescence intensities by *X. dendrorhous*, resulting in the maximal intensity of fluorescence at the K_La value 148.5 h^{-1}. The cell growth of *X. dendrorhous* was significantly inhibited when dissolved oxygen tension (DOT) was controlled at ~20% air saturation, which was due to the oxygen limitation in broth. The astaxanthin yield and content at ~50% DOT were higher than those at ~20% DOT.

Key words: Volumetric Oxygen Transfer Coefficient, Total Carotenoids, *Xanthophyllomyces dendrorhous*

Introduction

The red yeast *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) is one of the most promising microorganisms for the commercial production of carotenoids and astaxanthin (3,3′-dihydroxy-*β*,*β*′-carotene-4,4′-dione) (Cruz and Parajó, 1998). Astaxanthin is an interesting carotenoid owing to its high market price and the growing demand. It is the main carotenoid pigment found in aquatic animals, such as lobster, crab, shrimp, trout, and salmon (Johnson and Lewis, 1979). It is known to play a role in delaying or preventing degenerative diseases, to enhance the pigmentation of egg yolks of poultry as well as fish such as farmed salmon, and to be a more powerful scavenger of singlet oxygen ($^1O_2$) and peroxide radicals (O_2^{2-}) than *β*-carotene, cantaxanthin (*β*,*β*′-carotene-4,4′-dione) and zeaxanthin (3,3′-dihydroxy-*β*-carotene) due to its special structure. Furthermore, astaxanthin may exert antitumour activities through the enhancement of immune responses (Lai *et al.*, 2004).

To improve the astaxanthin production of *X. dendrorhous*, some research dealed with the optimization of fermentation methodologies (Ramírez *et al.*, 2001), mutagenesis (Sun *et al.*, 2004), chemical stimulants (Gu *et al.*, 1997), chemical and biological elicitors (Wang *et al.*, 2006; Wang and Yu, 2007), genetic and metabolic engineering (Visser *et al.*, 2003). But little work focused on oxygen supply, growth and carotenoids accumulation by *X. dendrorhous*. Oxygen affects the cell growth, cellular morphology, nutrients uptake, and metabolite biosynthesis. There were some studies reporting that a high oxygen transfer rate (OTR) and sufficient oxygen supply could result in an increase in the specific growth rate and a positive effect on the production rate (Ishmetentskii *et al.* 1981; Rau *et al.* 1992).

In the present study, we investigated the impacts of the initial volumetric oxygen transfer coefficient (K_La) and dissolved oxygen tension (DOT) on growth and carotenoids accumulation of *X. dendrorhous* in order to obtain useful information for large-scale production of bioactive compounds by the bioprocess.

Material and Methods

Maintenance and preculture of X. dendrorhous

P. rhodozyma AS 2.1557 was obtained from China General Microbiological Culture Collection Center (CGMCCC, Beijing, China). It was maintained on slants of yeast malt (YM) agar at 4 °C and transferred monthly. *X. dendrorhous* used in this work was an astaxanthin-overproducing mutant from *P. rhodozyma* AS 2.1557 (Wang *et al.*, 2005). The slant was inoculated and incubated at 22 °C for 2 d in YM medium. The components of YM medium were: 10 g glucose, 5 g bactopeptone, 3 g yeast extract, 3 g malt extract, and 20 g agar (for plates) in 1 l distilled water. The preculture medium consisted of the following components: 30 g glucose, 10 g yeast extract, 2 g KH_2PO_4, 1 g Na_2HPO_4, and 2 g $MgSO_4 \cdot 7H_2O$ in 1 l tap water (initial pH value was 5.0). For the first preculture, 30 ml medium with an initial pH value of 5.0 were prepared in a 250-ml flask, then 3 ml yeast suspension from a slant culture were inoculated followed by 2 d of incubation at 22 °C on a rotary shaker (200 rpm). 5% (v/v) of this preculture were used to inoculate the second one in an 1-l flask, under the same conditions as the first preculture. 10% (v/v) of this preculture were used to inoculate in the 5-l bioreactor.

Experiments with different initial K_La values in a 5-l bioreactor

The bioreactor used was a 3-l (working volume) agitated bioreactor (Biostat 5, B. Braun, Germany). Fermentation was conducted at 22 °C for 108 h. The components of the fermentation medium were: 50 g glucose, 10 g yeast extract, 2 g KH_2PO_4, 1 g Na_2HPO_4, and 2 g $MgSO_4 \cdot 7H_2O$ in 1 l tap water (initial pH value was 5.0). The cultures were conducted at the same aeration rate (1000 ml min^{-1}), and the agitation speed was set at 100, 200, 300 and 400 rpm to obtain the desired initial K_La values of 21.5, 43.7, 95.1, and 148.5 h^{-1}, respectively. Each experiment was performed twice at the same time to check the reproducibility.

Experiments with different dissolved oxygen tensions

To study the effects of the DOT on growth and carotenoids formation by *X. dendrorhous*, the cultivation process was conducted for 4 d at pH 5.0, 20 °C, and an aeration rate of 1000 ml min^{-1} in a 10-l bioreactor (Dashen Fermentation Equipment Co., Ltd, Jiangsu Province, P. R. China). The DOT in the culture broth was monitored using a polarographic DO probe and regulated by changing the aeration rate (100–1000 ml min^{-1}) and agitation speed (50–300 rpm) during fermentation.

Analytical procedures

For sampling, about 20–30 ml of broth were taken once from each reactor. 5-ml samples were centrifuged (8000 rpm, 8 min), and pellets were washed twice with 5 ml sodium chloride solution in deionized water and centrifuged again. Aliquots of cell pellets were dried at 105 °C for 24 h in order to allow the calculation of the biomass concentration on a dry weight basis. The wet aliquots were used for astaxanthin and β-carotene analysis by the DMSO method (Sedmark *et al.*, 1990). Astaxanthin and β-carotene standards (purchased from Sigma Chemical Co., USA) were used as external standards. The residual sugar concentration was measured by the DNS method (Miller, 1959). The initial K_La value was determined using the dynamic gassing-in and gassing-out method (Wang and Zhong, 1996). The cellular distribution of carotenoids in *X. dendrorhous* was examined by an Olympus BX60 fluorescence microscope equipped with a Sensys 1401E CCD camera based on the improved method of An *et al.* (2000).

Results and Discussion

Effects of the initial K_La value on growth, DOT and sugar consumption by X. dendrorhous

The effects of the initial K_La value on *X. dendrorhous* cultures were studied by choosing various initial K_La values. All cell cultures were conducted at the same aeration rate of 1000 ml min^{-1}, and the agitation speeds were adjusted over a range of 100–400 rpm to produce the desired initial K_La values from 21.5 to 148.5 h^{-1} (as shown in Fig. 1). Fig. 1A shows the cell growth kinetics at initial K_La values of 21.5, 43.7, 95.1, and 148.5 h^{-1}. Compared to cultures at lower K_La values, at an initial K_La value of 148.5 h^{-1} the lag phase was shortened substantially from 24 to 8 h and the yeast quickly reached the maximal biomass after cultivation for 56 h, whereas it was 80 h or longer in the other cases. The initial K_La value affected the biomass level, and its peak value of 19.37 g DCW^{-1} (dry cell weight) was obtained at an initial

K_La value of 148.5 h^{-1}. The results indicated that the initial K_La value has a significant effect on cell growth during fermentation and a higher initial K_La value seems to be better for cell growth of *X. dendrorhous*. The dynamic changes of the DOT are shown in Fig. 1B. The DOT at the later stage of culture at an initial K_La value of 148.5 h^{-1} was relatively high. As we know, the DOT in the fermentation broth is directly related with the OTR and oxygen uptake rate (OUR). During fermentation, the K_La value may change with the change of cellular morphology, cell number, viscosity of fermentation broth, and so on. Time courses of the residual glucose concentration are compared in Fig. 1C. At an initial K_La value of 148.5 h^{-1}, the glucose consumption rate was much higher than in other cases, while it was the lowest at an initial K_La value of 21.5 h^{-1}.

Effects of the initial K_La value on astaxanthin accumulation by X. dendrorhous

From Fig. 2A a clear positive influence of the initial K_La value on astaxanthin yields is visible; the higher its K_La value was, the higher the production was. From 8 to 72 h, a rapid increase of the astaxanthin concentration was observed at all K_La

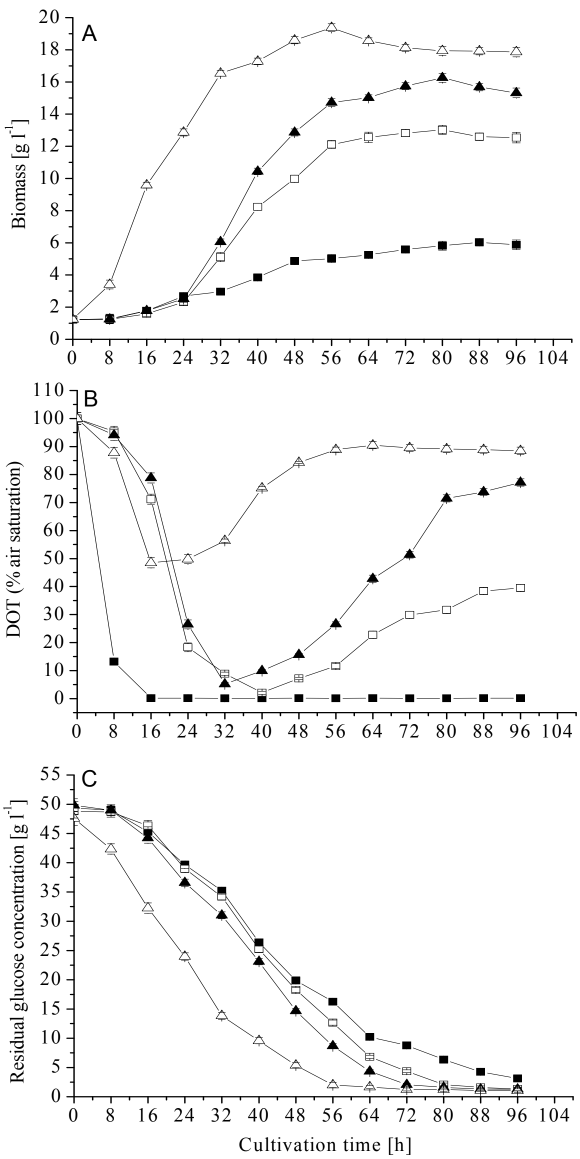

Fig. 1. Time profiles of (A) biomass, (B) dissolved oxygen tension, and (C) residual glucose concentration of *X. dendrorhous* at various initial K_La levels in a 5-l fermentor. Symbols for initial K_La values (h^{-1}): 21.5 (–■–); 43.7 (–□–); 95.1 (–▲–); 148.5 (–△–).

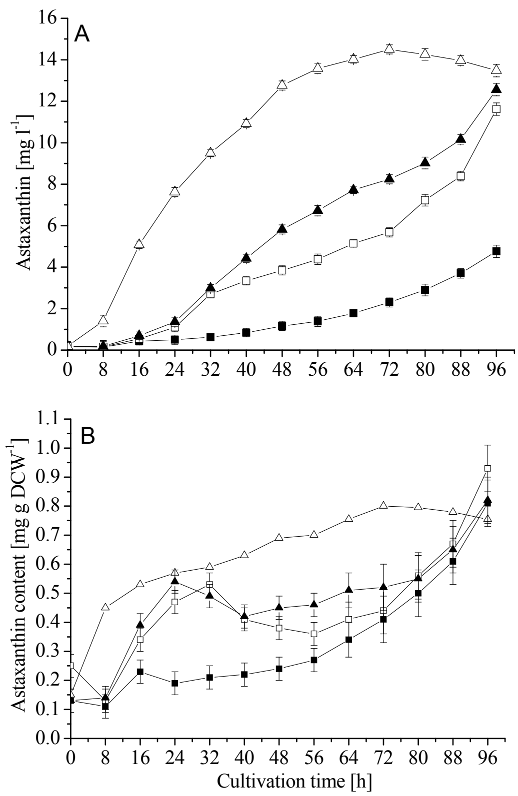

Fig. 2. Effects of initial K_La values on (A) astaxanthin yields and (B) astaxanthine contents of *X. dendrorhous* in a 5-l fermentor. Symbols for initial K_La values (h^{-1}): 21.5 (–■–); 43.7 (–□–); 95.1 (–▲–); 148.5 (–△–).

values, but from 72 h to the end of culture (96 h), its accumulation level showed a slight decrease at the K_La value of 148.5 h^{-1}. The maximum astaxanthin yield of 14.5 mg l^{-1} was attained at an initial K_La value of 148.5 h^{-1}, while the lowest astaxanthin concentrtion was obtained at an initial K_La value of 21.5 h^{-1}. The K_La value also influenced the astaxanthin contents remarkably (as shown in Fig. 2B). An increase in the initial K_La value led to an increased production and content. At K_La values of 43.7 and 95.1 h^{-1}, the astaxanthin content declined after 32 h while, after 56 h, there was a rapid increase of the astaxanthin content, but at K_La values of 21.5 and 148.5 h^{-1} there was a steady increase within the whole cultivation time. Before 72 h the astaxanthin content increased slowly at K_La values of 21.5 and 148.5 h^{-1}, but after 72 h the astaxanthin content decreased slowly at the K_La value of 148.5 h^{-1} and the astaxanthin content rose very quickly at the K_La value of 21.5 h^{-1}. The results indicated that the higher K_La would yield the better results, and they are similar to the conclusion of Johnson and Schroeder (1995).

Effects of the initial K_La value on carotenoids accumulation by X. dendrorhous

There are several carotenoids accumulated such as astaxanthin, β-carotene and lycopene in the astaxanthin biosynthesis of *X. dendrorhous*. As shown in Fig. 3, the initial K_La value had a significant influence on carotenoids accumulation. With the increase of the K_La value, the total carotenoids and astaxanthin yields enhanced quickly, while the β-carotene yield raised very slowly. At the K_La value of 148.5 h^{-1} the optimal total carotenoids and astaxanthin yields were 18.1 and 14.5 mg l^{-1}, respectively. There was a steady increase of the ratio of astaxanthin in total carotenoids along with the K_La value. When the K_La value was 21.5 h^{-1} the ratio of astaxanthin in total carotenoids was only 46%, but it climbed rapidly up to 80.6% at the K_La value of 148.5 h^{-1}, while the ratio of β-carotene in total carotenoids decreased dramatically from 44.7% (at K_La = 21.5 h^{-1}) to 17.9% (at K_La = 148.5 h^{-1}). It presented that the higher oxygen supply not only was propitious to total carotenoids accumulation, but also was more advantageous to the β-carotene being transformed to astaxanthin.

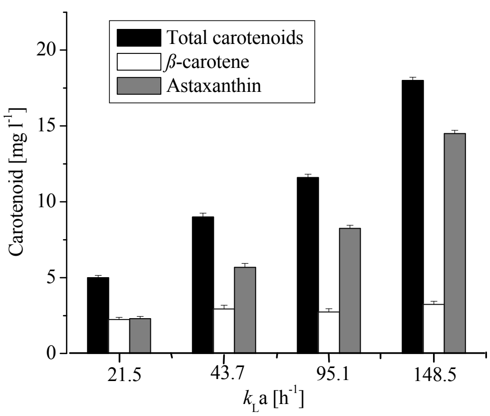

Fig. 3. Effects of initial K_La values on different carotenoids accumulation of *X. dendrorhous* in a 5-l fermentor.

Effects of the initial K_La value on autofluorescence intensity and cell morphological change by X. dendrorhous

The autofluorescence intensities of cellular carotenoids in *X. dendrorhous* (after 72 h of cultivation) were examined by laser confocal fluorescence microscopy (shown in Fig. 4). An *et al.* (2000) reported that the autofluorescence intensity within *P. rhodozyma* was caused by carotenoids. As we can see there were the steadily increasing autofluorescence intensities of cellular carotenoids (mainly astaxanthin) along with the different K_La values, and at the K_La value of 148.5 h^{-1} the maximal intensity of fluorescence was obtained. From the pictures we can see that the yeast pellets were small at the K_La value of 21.5 h^{-1}, and the cells became much bigger at the K_La value of 148.5 h^{-1} than those at other lower K_La values. The results also indicated that an increase of the initial K_La value was favourable for more carotenoids (mainly astaxanthin) accumulation and cell growth of *X. dendrorhous*.

Effects of the DOT on growth, glucose consumption and carotenoids formation by X. dendrorhous

Fig. 5 shows two different DOT profiles during the submerged fermentation in a 10-l bioreactor. Compared with a low DOT (~20% air saturation), the yeast grew more quickly if DOT was kept at a higher level (~50% air saturation) (Fig. 5A). The maximum cell densities were 6.88 and 18.57 g l^{-1} at ~20 and ~50% of DOT, respec-

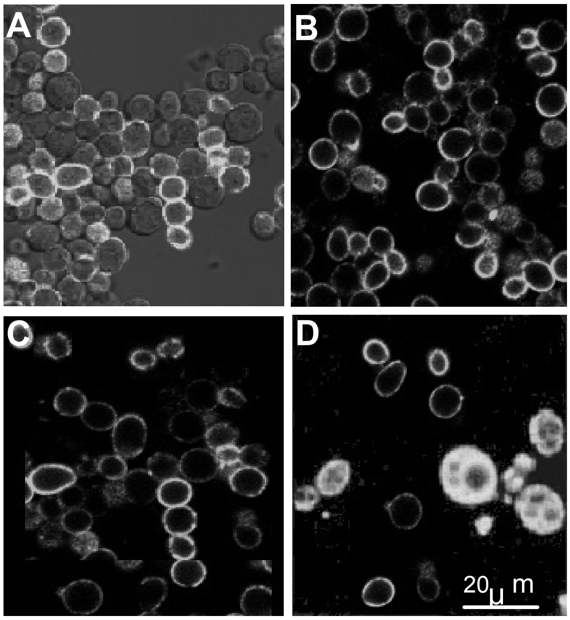

Fig. 4. Effects of initial K_La values on the fluorescence intensity of *X. dendrorhous* at a cultivation time of 72 h. Digital images were obtained with an Olympus BX60 fluorescence microscope equipped with a Sensys 1401E CCD camera. Symbols for initial K_La values (h^{-1}): (A) 21.5; (B) 43.7; (C) 95.1; (D) 148.5.

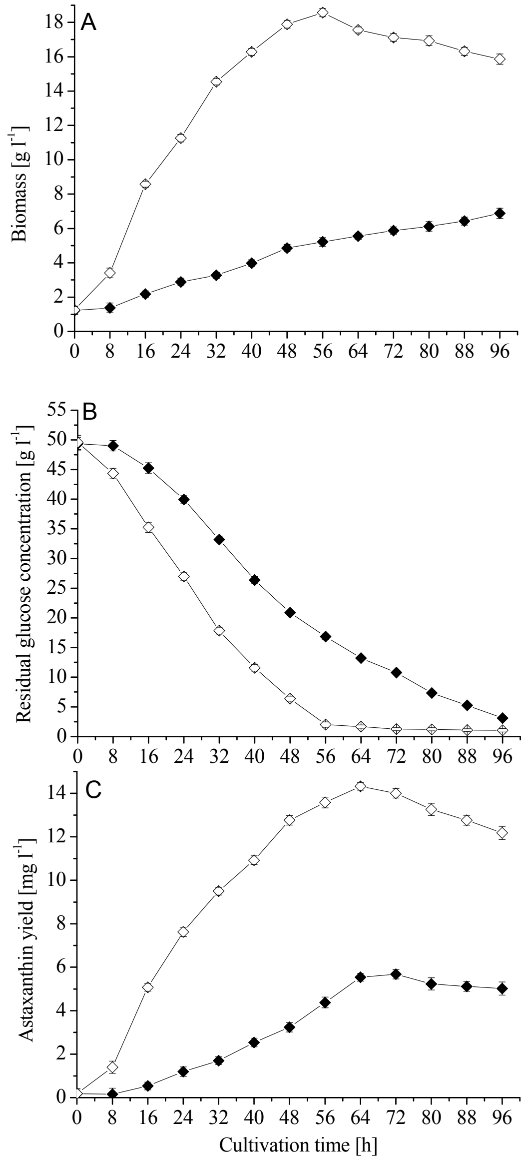

Fig. 5. Effects of DOT on (A) growth, (B) glucose consumption, and (C) astaxanthin yield of *X. dendrorhous* in a 10-l bioreactor. Symbols for DOT: ~20% air saturation (—◆—); ~50% air saturation (—◇—).

tively. The results indicated that the cell growth of *X. dendrorhous* was significantly inhibited when the DOT was controlled at ~20% air saturation. Time courses of residual sugar are compared in Fig. 5B. The glucose consumption corresponded well to the cell growth. Compared with the low DOT (~20%), the yeast consumed glucose more quickly when the DOT was controlled at ~50%. Around 20% and 50% of DOT, almost all the glucose was utilized at the end of fermentation (96 h). An increase of the DOT led to a higher glucose consumption rate and a higher cell yield against glucose, which was similar to the results of Yamane *et al.* (1997). Kinetics of astaxanthin accumulation is indicated in Fig. 5C. After inoculation, a rapid increase of the astaxanthin level was observed. The DOT level affected remarkably the final production of astaxanthin. The astaxanthin production (14.32 mg l^{-1}) at ~50% of DOT was much higher than that at ~20% of DOT (5.68 mg l^{-1}). The results suggested that abundant oxygen supply was beneficial for the metabolic flux towards the astaxanthin biosynthesis.

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