SIMULTANEOUS CARBON DIOXIDE FIXATION IN THE PROCESS OF NATURAL ASTAXANTHIN PRODUCTION BY A MIXED CULTURE OF *HAEMATOCOCCUS PLUVIALIS* AND *PHAFFIA RHODOZYMA*

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Introduction

Carbon dioxide is considered as a major greenhouse gas causing the global warming problem. To find a solution, many attempts have been made through out the world, among which, biological CO_2 fixation (BCF) is thought to be an environment friendly way to remove CO_2 using microalgal photosynthesis. Up to now, microalgae biotechnologies have been practiced to convert CO_2 emitted from power plant [1] and lime production plant [2] into algal biomass. However, there still exist some problems remaining unsolved, such as lower CO_2 conversion rate, high concentration O_2 evolved in photosynthesis, which cause inhibitory effects on cell growth.

Astaxanthin is a kind of high value carotenoid (US$2500/kg) possessing strong biological antioxidation. *Haematococcus pluvialis* and *Phaffia rhodozyma* are the two main different species of microorganisms used in the production of astaxanthin through biotechnology. *H. pluvialis* is a green alga that absorbs CO_2 and evolves O_2 in photosynthesis; While, *P. rhodozyma* is a sort of red yeast which can use a variety of organic materials as fermenting substrates, generating CO_2 and carboxylic acids[3-4], which restrain both cell growth and astaxanthin formation of the red yeast. On the contrary, O_2 is required for astaxanthin synthesis in *P. rhodozyma* fermentation [5]. However, at present, both of the two species are all cultured purely, and under this culture regime, astaxanthin production declines due to the changes of pH, CO_2 and O_2 concentrations in the media through out the culture process. In this study, the mixed culture method for *H.pluvialis* with *P. rhodozyma* was used trying to fix CO_2 released in red yeast fermentation simultaneously and also to stimulate astaxanthin production.

Materials and Methods

Microorganisms. *H. pluvialis* and *P. rhodozyma(AS2-1557)* were obtained from Institute of Hydrobiology and Institute of Microbiology , Chinese Academy of Science respectively. *H. pluvialis* was maintained at 4℃ in a liquid BBM medium; *P. rhodozyma* was maintained on a slant of yeast malt agar (YM agar, Difco) at 4℃.

Culture Media and Conditions. The seed culture of *H. pluvialis* was prepared by transferring 5ml of the alga liquid culture into a 500ml-flask containing 100ml BBM medium (supplemented with 0.25g/l acetate). The seed culture of *P. rhodozyma* was prepared by inoculating the yeast from the fresh slant into a 500ml flask containing 100ml YM medium. Both cultures were shaken at 110 rpm in an orbital shaker (with top cool white fluorescent lamps) at 23.8℃ under light intensity 15μmol photons/m^2.s for 48h. Pure cultures of *H. pluvialis* and *P. rhodozyma* were conducted by transferring 3ml seed cultures into 250ml-flasks containing 30ml culture media respectively. Mixed cultures were conducted in the same way except that the inoculum contained 1.5ml of each seed culture. The flasks were incubated under12μmol photons/m^2.s constant light intensity (adjusted to 30μmol photons/m^2.s after 48h) provided by top cool white fluorescent lamps at 23.8℃, 110rpm in a rotary shaker for 120h.Culture media were prepared from BBM by supplementing it with glucose, the glucose concentrations (g/l) were as follows: 3, 5, 10, 15, 20, 25 respectively.

Biomass Measurement (dry cell weight). 5ml samples of each culture were centrifuged at 5000rpm for 10 min, the supernatants were transferred into 50ml tubes for residual sugar and nitrogen analysis, the cell pellets were rinsed with distilled water twice, then dried in a electrical oven at 105℃ for 2h to constant weight.

Determination of Residual Sugar. Residual sugar in the culture media was quantified by DNS method.

Astaxanthin Extraction and Quantification. *H. pluvialis* pure culture and mixed culture: cells were harvested by centrifuging 5ml samples at 5000 rpm for 10 min. The supernatant was discarded, the cell pellets were re-suspended in 3ml 30%(v/v) methanol containing 5% KOH (w/v) and heated at 70℃ in a water bath for 5 min to destroy the chlorophyll. The mixture was centrifuged and the supernatant discarded, the remaining cell pellets were extracted with 2ml dimethyl sulphoxide and 5 ml acetone. All the steps were carried out in the dark. For *P. rhodozyma* pure culture, the extraction process was conducted in the same way with an exception of chlorophyll destroying step. Astaxanthin was quantified by HPLC using a 250×4.6 mm Ultrasphere C_{18} column, the eluting solvent was ethyl acetate: methanol: water=5:18:2 (v/v/v) with a flow rate of 1 ml/min, peaks were measured at 480 mm.

Results and Discussion

Mixed cultures were compared with pure cultures of *H. pluvialis* and *P. rhodozyma* on four aspects: biomass, glucose utilization efficiency, glucose conversion rate, and astaxanthin production, just trying to find out the feasibility of combining CO_2 fixation with astaxanthin production.

Table 1. shows that the biomass concentrations of mixed cultures of *H. pluvialis* and *P. rhodozyma* were much higher than those of the two pure cultures, which were nearly the sum of the two pure cultures when glucose concentration was in the range of 3-5g/l, and increased as the glucose concentration rose. On contrast, biomass concentrations of pure cultures of *H. pluvialis* and *P. rhodozyma* decreased when glucose concentrations were higher than 10g/l and 15g/l respectively.

The reason for higher biomass production of mixed cultures may be as follow: CO_2 released through *P. rhodozyma* fermentation could be absorbed by *H. pluvialis* for photosynthesis, since the dissolved CO_2 in the culture medium is very low and could not meet the need of *H. pluvialis*, so supplementing CO_2 or sodium acetate are required. To deal with this problem, usually air or pure CO_2 were pumped into the high-cell-density microalgal cultures. In this way, however, CO_2 conversion rate is not high. While, in the mixed cultures of *H. pluvialis* and *P. rhodozyma*, CO_2 releasing and uptaking, both processes took place in the same medium, gas exchanged much easily and more efficiently. This conclusion is also confirmed by the results of glucose utilization efficiencies, as shown in Figure 1. For mixed culture and *P. rhodozyma* pure culture, glucose could be utilized totally when its concentration was below 20 g/l, but for *H. pluvialis*, glucose could not be used efficiently when the concentration was higher than 5g/l. Taking biomass concentrations and glucose conversion rates of the three cultures into account, the glucose conversion rates of mixed cultures were higher than both of the pure cultures of the two species, and lower glucose concentrations resulted in higher utilization efficiencies and conversion rates, especially when glucose concentration was between 3-5g/l (Figure 2), this is

because higher glucose concentration inhibits both *H. pluvialis* and *P. rhodozyma* growth. Therefore we can conclude that CO_2 evolved from the red yeast *P. rhodoxyma* fermentation was absorbed or fixed rapidly by the green alga *H. pluvialis* in the process of photosynthesis, the highest glucose conversion rate (52.0%) was obtained by mixed culture when glucose concentration was 3g/l, which indicates, for CO_2 fixation purpose, lower glucose concentration is more favorable and fed-batch culture techniques should be employed

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Table 1. Biomass production of Different Cultures.

	Biomass production (DCW g/l)		
Glucose (g/l)	Mixed culture	H. pluvialis	P. rhodozyma
3	1.56±0.01	0.54±0.04	1.12±0.13
5	2.45±0.02	0.61±0.12	1.42±0.08
10	3.32±0.35	0.69±0.47	3.22±0.07
15	4.82±0.19	0.68±0.22	5.11±0.11
20	5.32±0.31	0.65±0.11	5.08±0.02
25	5.70±0.28	0.62±0.13	5.02±0.01

. Data are expressed as mean ± standard deviation of three replicates

Table 2. Astaxanthin Production of Different Cultures.

	Volumetric astaxanthin content (mg/l)		
Glucose (g/l)	Mixed culture	H. pluvialis	P. rhodozyma
3	12.95±0.11	3.68±0.16	1.09±0.03
5	8.86±0.09	2.99±0.12	1.61±0.13
10	6.50±0.15	2.34±0.17	1.95±0.11
15	5.62±0.12	2.02±0.13	2.05±0.07
20	2.15±0.08	1.96±0.04	1.51±0.02
25	2.10±0.10	1.71±0.03	1.36±0.04

. Data are expressed as mean ± standard deviation of three replicates

Table 2. summarizes the astaxanthin production of different cultures. Mixed culture could remarkably promote astaxanthin production comparing with the two pure cultures, which are more than the sum of the two single cultures when glucose concentration was in the range of 3-15g/l. Maximum volumetric astaxanthin production 12.95 mg/l was obtained by mixed culture when glucose concentration was 3mg/l, which was 3.5 and 11 times that of *H. pluvialis* and *P. rhodozyma* pure cultures respectively.

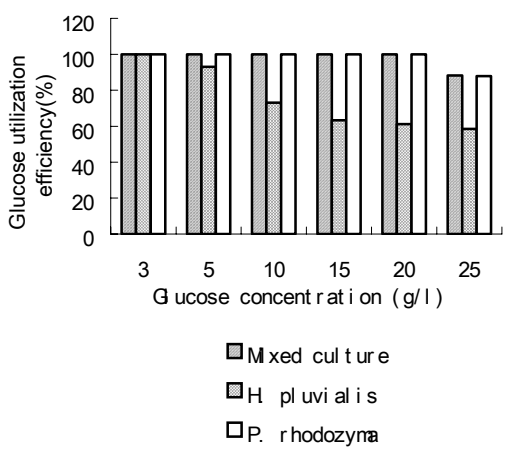

Figure 1. Glucose utilization efficiency of cultures at different glucose concentrations

The reasons for this may come from three aspects: (1) O_2 released by *H. pluvialis* in photosynthesis enhances the astaxanthin production of *P. rhodozyma*, because the conversion of carotene to astaxanthin catalyzed by ß-carotene hydroxylase and ß-carotene ketolase is oxygen-required, if O_2 supplying rate is under 30mmol/h, cell growth and astaxanthin production decline evidently, and astaxanthin production increases as O_2 concentration in the medium rises. (2) Considering biomass, nitrogen and glucose utilization efficiencies, under mixed culture regime, glucose and nitrogen were utilized efficiently, and higher biomass concentration was obtained so as to increase astaxanthin production. Furthermore, higher nitrogen utilization efficiency resulted in lower nitrogen concentration in the media, which is beneficial to astaxanthin formation both for *H.pluvialis* and *P.rhodozyma*. (3) pH in mixed culture keeps constant at 7.0 due to the interaction between *H. pluvialis* and *P. rhodozyma* which is more favorable for astaxanthin synthesis than pHs are 6.0, 8.0, and 9.0 for *H. pluvialis*.

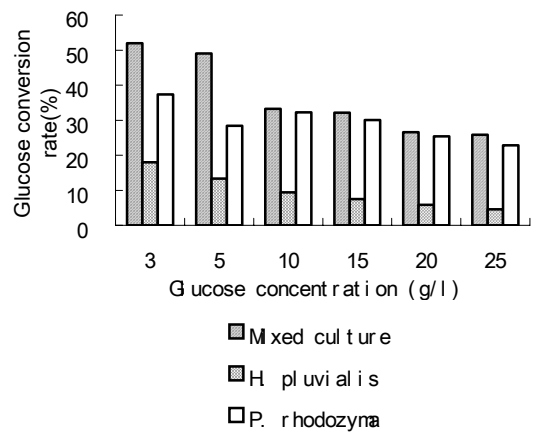

Figure 2. Glucose conversion rate of cultures at different glucose concentrations

Conclusions

Mixed culture of *H. pluvialis* and *P. rhodozyma* presents a new way for combining CO_2 fixation with high value co-product (astaxanthin) formation, promoting glucose utilization efficiency and conversion rate, keeping pH constant as well as increasing astaxanthin production. This culture regime lays a foundation for developing a new technique to put yeast or bacterium fermentation together with algae cultivation.

Acknowledgement. This project was supported by the National Natural Science Foundation of China (Grant No. 20036010).

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