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In situ carbon dioxide fixation in the process of natural astaxanthin production by a mixed culture of *Haematococcus pluvialis* and *Phaffia rhodozyma*

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Abstract

In order to fix CO_2 generated by microbial fermentation, two astaxanthin over-producing microorganisms, the green alga *Haematococcus pluvialis* and the red yeast *Phaffia rhodozyma*, were mix-cultivated in the same media. CO_2 from *P. rhodozyma* fermentation was fixed by *H. pluvialis* simultaneously in the process of photosynthesis, while O_2 produced by *H. pluvialis* in photosynthesis stimulated astaxanthin formation in *P. rhodozyma*. As a result, both concentrations of biomass and astaxanthin increased significantly compared to the pure cultures of the two species. The maximum concentrations of biomass and astaxanthin, 5.70 g/l and 12.95 mg/l, were obtained by the mixed culture. This culture strategy provides a novel way for improving the yield of higher valued bio-product with in situ CO_2 fixation.

Keywords: CO_2; Fixation; Astaxanthin; *Haematococcus pluvialis*; *Phaffia rhodozyma*; Mixed culture

1. Introduction

Carbon dioxide is considered as a major greenhouse gas causing the global warming problem. Mankind has now recognized both the necessity and increasing urgency to reduce the CO_2 content in the atmosphere. To find a feasible solution, many attempts have been made through out the world, among which, biological CO_2 fixation (BCF) is an environment friendly way to remove CO_2 using micro-algal photosynthesis.

Micro-algae are unique and valuable microscopic algae containing chlorophyll and other photosynthesis-related antenna pigments such as carotenoids, which enable them to absorb and utilize CO_2 as the principal carbon source in the growth process. These microscopic algae usually are unicellular and are found growing nearly every biotope due to their ecological diversity and physiological adaptability, especially in water system. The major reason for the utilization of micro-algae in CO_2 fixation is that they can tolerate up to 12% CO_2 at a temperature of 35 °C, while most plants can only live with up to 0.1% CO_2. Unlike higher plants, micro-algae cannot capture CO_2 directly from the air. Therefore conventional technique for micro-algae cultivation needs to transfer CO_2 and/or air into the culture broth, resulting in lower CO_2 conversion rate [1], but higher concentration of O_2, which inhibits the growth of algal cells [2].

Up to now, micro-algae biotechnologies have been successfully practiced to convert CO_2 emitted from power plant [3] and lime production plant [4] into algal biomass. In the yeast fermentation, nearly 50% of the input carbon substrate, typically glucose is used for cell growth and maintenance, releasing high level of CO_2 into the environment [5]. Nevertheless, no information is available regarding conversion of CO_2 derived from microbial fermentation.

Furthermore, it is also economical to combine CO_2 fixation with useful metabolite production. There are over ten thousands species of micro-algae that have been recognized, however, up to date, only a few are commercially cultivated: including *Spirulina*, a filamentous blue green alga, *Chlorella*, *Dunaliella* and *Haematococcus*, the last three are unicellular green algae. The first two species

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are used for food supplements, while the last two for their pigment content, especially beta-carotene and astaxanthin [6].

Astaxanthin is a high value carotenoid (US$ 2500 kg) and strong biological antioxidant, which has widespread applications in nutraceutical, cosmetic, food and feed industries. *Haematococcus pluvialis* and *Phaffia rhodozyma* are the two main microorganisms used in the natural astaxanthin production and have attracted attention through out the world. Of the two strains, *H. pluvialis* is a ubiquitous unicellular green alga that utilizes CO_2 and produce O_2 in photosynthesis, as the algal cells synthesize and accumulate astaxanthin in response to environmental stress such as high irradiance, high temperature [7], deficiency of nitrogen or phosphate [8–10]. While, *P. rhodozyma* is a sort of red yeast that can use a vast variety of organic materials as fermenting substrates, in particular carbohydrates, generating CO_2 and organic acids [11,12], which restrain both cell growth and astaxanthin formation of the red yeast. On the contrary, O_2 is required both for the primary metabolic activity and astaxanthin synthesis in *P. rhodozyma* [13]. Thus, *H. pluvialis* and *P. rhodozyma* are good candidates for symbiosis. Nevertheless, at present, both of the two species are always cultivated purely in separated cultures in academic and commercial astaxanthin production [14–17], and under the traditional separate culture strategy, astaxanthin production declines due to the changes of pH, CO_2 and O_2 concentrations in the broth throughout the culture process.

Mixed cultures of microorganisms are common in natural ecology system. In recent years, exploration of mixed cultures became critical to many key biochemical processes [18]. Mixed cultures have been used in various aspects, such as complementary biotransformations, multistep biotransformations, in situ enzyme regeneration, in situ oxygen generation, waste degradation and remediation. In the present study, in order to combine CO_2 fixation with astaxanthin production, we have, for the first time, mix-cultivated two different astaxanthin-producing strains, *H. pluvialis* and *P. rhodozyma*, on a simple well-defined medium to make good use of the complementary effects between the two independent microbial metabolic activities.

2. Experimental

2.1. Microorganisms

H. pluvialis and *P. rhodozyma* (AS2-1557) were obtained from Institute of Hydrobiology and Institute of Microbiology of Chinese Academy of Science respectively. *H. pluvialis* was maintained at 4 °C in a liquid BBM medium (Bold's Basal Medium) [19]; *P. rhodozyma* was maintained at 4 °C on a slant of yeast malt agar (YM agar, Difco) containing (per liter) 10 g of glucose, 5.0 g of Bacto peptone, 3.0 g of malt extract, 3.0 g of yeast extract, and 20 g of agar.

2.2. Culture media and conditions

The seed culture of *H. pluvialis* was prepared by transferring 5 ml of the alga liquid culture into a 500 ml flask containing 100 ml BBM medium. The BBM recipe is as follows: $NaNO_3$, 250 mg/l; $MgSO_4{\cdot}7H_2O$, 75 mg/l; NaCl, 25 mg/l; K_2HPO_4, 75 mg/l; KH_2PO_4, 175 mg/l; $CaCl_2{\cdot}2H_2O$, 25 mg/l; $ZnSO_4{\cdot}7H_2O$, 8.82 mg/l; $MnCl_2{\cdot}4H_2O$, 1.44 mg/l; MoO_3, 0.71 mg/l; $CuSO_4{\cdot}5H_2O$, 1.57 mg/l; $Co(NO_3)_2{\cdot}6H_2O$, 0.49 mg/l; $FeSO_4{\cdot}7H_2O$, 4.98 mg/l; H_3BO_3, 11.4 mg/l; KOH, 31 mg/l; $EDTANa_2$, 50 mg/l. The seed culture of *P. rhodozyma* was prepared by inoculating the yeast from the fresh slant into a 500 ml flask containing 100 ml YM medium (glucose, 10 g/l; malt, 3 g/l; peptone, 5 g/l; yeast extract, 3 g/l). Both cultures were shaken at 110 rpm in an orbital shaker (with top cool white fluorescent lamps) at 23.8 °C under the light intensity of 15 μmol photons/m^2 s for 48 h. Pure cultures of *H. pluvialis* and *P. rhodozyma* were conducted by transferring 3 ml seed cultures into 250 ml-flasks containing 30 ml culture media respectively. Mixed cultures were conducted in the same way except that the inoculum contained 1.5 ml of each seed culture. The flasks were incubated under15 μmol photons/m^2 s constant light intensity (adjusted to 90 μmol photons/m^2 s after 48 h) provided by top cool white fluorescent lamps at 23.8 °C and 110 rpm in a rotary shaker for 120 h. Culture media were prepared from BBM by supplementing it with glucose. The glucose concentrations were as follows: 3, 5, 10, 15, 20 and 25 g/l, respectively.

2.3. Biomass measurement (dry cell weight)

Five milliliters samples of each culture broth was centrifuged at 5000 rpm for 10 min, the supernatants were transferred into 50 ml tubes for residual sugar and nitrogen analysis, the cell pellets were rinsed twice with distilled water, then dried in a electrical oven at 105 °C for 2 h to constant weight.

2.4. Determination of residual sugar

Residual sugar concentration in the culture broth was determined with 3,5-dinitrosalicylic acid [20].

2.5. Determination of residual nitrogen

Residual nitrogen in the culture broth was quantified spectrophotometrically using the standard method [21].

2.6. Astaxanthin extraction and quantification

The pure culture of *H. pluvialis* and mixed culture: cells were harvested by centrifuging 5 ml samples at 5000 rpm for

10 min. The supernatant was discarded. The cell pellets were re-suspended in 3 ml 30% (v/v) methanol containing 5% KOH (w/v) and heated at 70 °C 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 2 ml 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 (HP1100) using a 250 mm × 4.6 mm Ultrasphere C_{18} column (Waters). 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.

3. Results and discussion

In order to evaluate the effect of mixed culture on CO_2 fixation and astaxanthin formation, mixed cultures were compared with pure cultures of *H. pluvialis* and *P. rhodozyma* on five aspects: biomass concentration, cell yield, glucose conversion rate, nitrogen conversion rate as well as astaxanthin production.

3.1. Biomass concentration

Since all the cultures were conducted under the same conditions and carbon dioxide could only be fixed in the mixed cultures, the comparison of biomass concentrations between the mixed cultures and the pure cultures of *P. rhodozyma* could reflect the effects of carbon dioxide fixation by *H. pluviallis* in the mixed cultures. Therefore, in this article, we use biomass concentration to represent the effect of carbon dioxide fixation.

As shown in Table 1, the biomass concentrations in the mixed cultures of *H. pluvialis* and *P. rhodozyma* were 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 3–5 g/l, and increased as the glucose concentration rose. Similar trend was also found in the pure cultures of *P. rhodozyma* when the glucose concentration was lower than 15 g/l. In contrast, biomass concentrations in the pure cultures of *H. pluvialis* were rather low, showing small variation within the glucose concentrations tested.

Table 1
Biomass concentrations of cultures at different glucose concentrations

Glucose (g/l)	Biomass concentration (g/l)		
	Mixed culture	*H. pluvialis*	*P. rhodozyma*
3	1.56	0.54	1.12
5	2.45	0.61	1.42
10	3.32	0.69	3.22
15	4.82	0.68	5.11
20	5.32	0.65	5.08
25	5.7	0.62	5.02

It was previously reported that the cell growth in *P. rhodozyma* was promoted at lower glucose concentrations but inhibited at higher concentrations [22]. This conclusion is also confirmed in the present study (Table 1), in which, biomass concentration declined when the glucose concentration was higher than 15 g/l. The reason for this may be due to the Crabtree effect (the glucose effect), which commonly presented in fermentations. Furthermore, the possible mechanism of Crabtree effect in the present study may root in two factors: One is the accumulation of the waste metabolites. The main waste metabolites detected in this study were carbon dioxide and pyruvate (data not shown). The accumulation of these two metabolites resulted in a lower pH level, which in turn inhibited the growth of *P. rhodozyma*. To cope with these negative effects caused by lower pH, usually glucose or NaOH were added into the broth continuously or semi-continuously [13]. The other is insufficient supply of oxygen. Oxygen is the most important gaseous substrate for microbial metabolism. Its solubility is rather low. Only 0.3 mM (9 mg/l) O_2 dissolves in 1 l of water at 20 °C in an air/water mixture. Although the glucose concentration (no more than 20 g/l) involved in the present research did not affect the dissolved oxygen significantly, the yeast metabolism within the culture broth became more and more active due to the increased cell number (biomass), and the dissolved oxygen might be depleted in a few seconds by the concentrated red yeast population. Since the Crabtree effect could be repressed by a high oxygen concentration [22] and the oxygen supply in the flask cultures was limited, oxygen deficiency was a limiting factor in the red yeast cultures with higher glucose concentrations.

As to *H. pluvialis,* the principal carbon source is carbon dioxide. Unlike other algae, it can also utilize some organic carbon source in small amount, and acetate is commonly used as a complementary organic carbon source in the previous investigations [23]. It only possessed a limited ability to metabolize glucose showing low biomass production levels (Table 1) and low glucose conversion rate (Fig. 1). It may be due to the lack and/or low enzyme activities involved in the glucose metabolism through

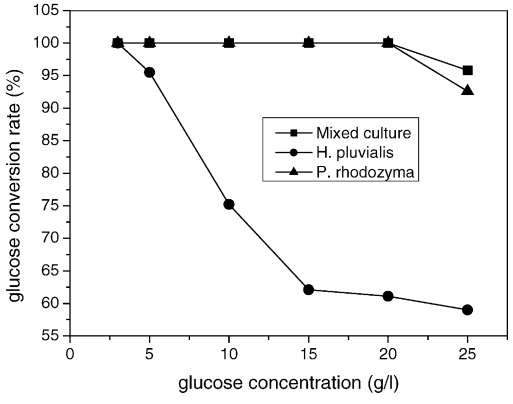

Fig. 1. Glucose conversion rates of cultures at different glucose concentrations.

Embden–Meyerhof–Parnas (EMP) pathway and tricarboxylic acid (TCA) cycle within the algal cells. In fact, glucose was rarely used in the cultivation of *H. pluvialis* due to the easily contamination by other microorganisms. However, no information is available concerning the metabolic mechanism of *H. pluvialis* on glucose.

Contrary to the trends from pure cultures of *H. pluvialis* and *P. rhodozyma*, biomass concentrations (Table 1) increased with greater glucose concentration beyond 15 g/l in the mixed culture. The reason for this may be attributed to the following factors:

(a) CO_2 evolved in *P. rhodozyma* fermentation was absorbed by *H. pluvialis* for photosynthesis. As shown in Fig. 2, the red yeast *P. rhodozyma* metabolizes glucose via EMP pathway and TCA cycle (Fig. 2A), in which glucose was converted into CO_2. On the contrary, the green alga *H. pluvialis* absorb CO_2 to synthesize glucose through Calvin–Benson cycle (Fig. 2B) in photosynthesis. In previous investigations, in order to provide sufficient CO_2 to meet the needs of algal growth, usually air and/or pure CO_2 were pumped into the high-cell-density microalgal cultures [24–27]. In this way, however, CO_2 conversion rate is not high. While in the mixed cultures, both the two metabolic reactions of carbon dioxide release and uptake took place in the same broth. Therefore, we conclude that in the mixed culture system, the two metabolic activities were combined and complementary. Carbon dioxide generated by the red yeast provides an excess carbon source for the growth of algal cells.

Further information confirming CO_2 fixation by the green alga within the mixed culture is provided by an additional experiment at a glucose concentration of 4 g/l, in which, cultures in the light was compared to the sets in the dark by covering the flasks with black plastic sheets. As results shown in Table 2, the biomass concentration obtained by the mixed culture in the light was nearly two-fold higher than that in the dark, which suggested that the elevated biomass concentration level was attributed to CO_2 fixation by *H. pluvialis* in the photosynthetic activity, because without light energy, Photosystem I (PS I) and Photosystem II (PS II) did not work at all. And, photosynthesis in the algal cells ceased due to the lack of energy. CO_2 evolved by the red yeast could not be absorbed and converted into biomass. With regard to the two pure cultures grown in the light, the biomass concentrations presented a small increase in *H. pluvialis* but a little decrease in *P. rhodozyma* in comparison with the cultures in the dark. These results are in agreement with the previous reports [23,28].

Table 2
Biomass and astaxanthin concentrations of cultures grown under light and dark

	Biomass (g/l)		Astaxanthin	
	Light	Dark	Light	Dark
Mixed culture	2.12	1.39	10.26	2.28
H. pluvialis	0.56	0.22	3.11	0.96
P. rhodozyma	1.31	1.36	1.28	1.12

(b) The growth condition was optimized for both *H. pluvialis* and *P. rhodozyma* in the mixed cultures simultaneously. Since high concentrations of CO_2 and O_2 are deleterious to the yeast and alga, respectively, in order to remove carbon dioxide from the yeast fermentative broth and oxygen from the alga culture

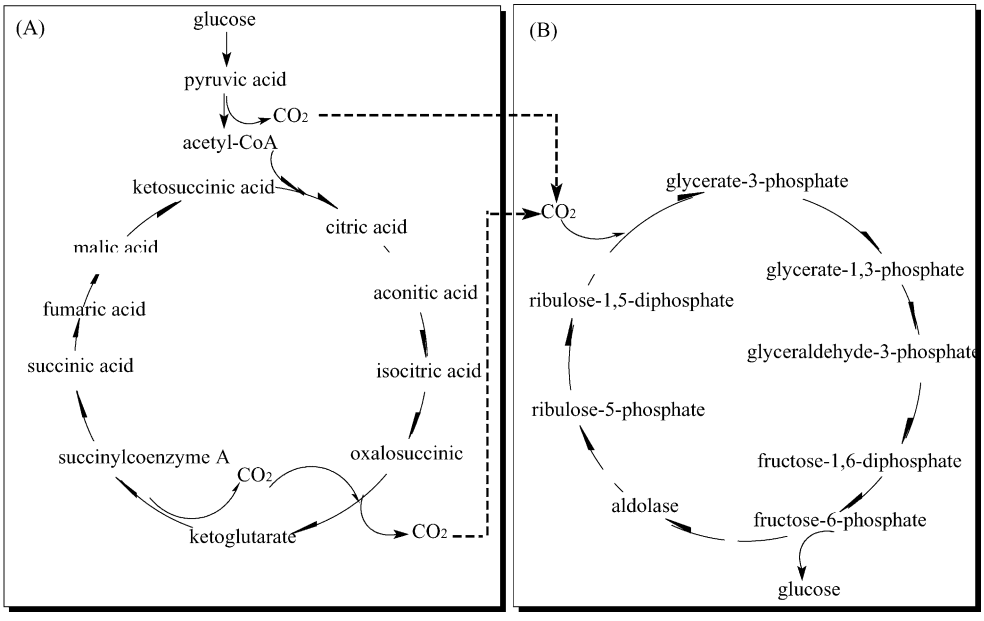

Fig. 2. CO_2 exchanges between *P. rhodozyma* (A) and *H. pluvialis* (B) in the mixed culture. CO_2 produced by *P. rhodozyma* in glucose oxidation through EMP pathway and TCA cycle (A) was absorbed by *H. pluvialis* to synthesize glucose via Calvin–Benson cycle (B).

broth, common strategies are (1) increasing turbulence and (2) O_2 and/or CO_2 stripping with air. However, both approaches imply an "unresolved dilemma" in the reactor system. Although an intensive search for membranes suitable for gas exchange is underway, no breakthrough has yet been reported. At present, sufficient O_2 and/or CO_2 transitions on membranes can be provided only under pressure. Otherwise, the transition proceeds slowly along the gradient [4]. While, in the mixed cultures, gas exchange of carbon dioxide and oxygen between the red yeast and the green alga respectively took place in the same culture broth, which may alleviate and/or eliminate the stresses caused by carbon dioxide on the red yeast and oxygen on the green alga, and optimize the growth conditions for both of the two strains simultaneously.

(c) Carbon dioxide released by the red yeast may be beneficial to keep the balance between CO_2 and O_2 in term of photosynthesis. When the green alga reaches a higher photosynthesis rate, it is better to keep CO_2/O_2 balance in a way that the prime carboxylating enzyme, Rubisco, furnishes CO_2 for the Calvin cycle but does not use O_2 for photorespiration. Since Rubisco is located on the surface of the thylakoid membranes, and O_2 competes with CO_2 for binding to the Rubisco active site (Rubiso is actually an acronym for Ribulose Bisphosphate Carboxylase Oxygenase), the CO_2 and O_2 concentration levels may serve as a trigger for photosynthesis and photorespiration. In algal cultures with high cell densities, sufficient CO_2 should be available, while O_2 produced has to be removed before reaching inhibitory concentrations. Nevertheless, it is difficult to keep CO_2/O_2 balance by common strategy, and the complete avoidance of photorespiration remains unresolved [2,4]. Whereas, in the mixed culture of *P. rhodozyma* and *H. pluvialis*, *P. rhodozyma* produce carbon dioxide but absorb oxygen, which may be helpful to keep CO_2/O_2 balance.

3.2. Glucose conversion rate and cell yield

The glucose conversion rate and cell yield are the two important parameters in evaluating the fermentation efficiency. Of which, the glucose conversion rate means the percentage of the total glucose used by the microorganism; while, the cell yield indicates the percentage of glucose being converted into biomass. Therefore, for the goal of carbon dioxide fixation, the best fermentation/cultivation mode should exhibit not only a highest glucose conversion rate, but also a maximum cell yield. As shown in Fig. 1, the glucose conversion rates of the mixed cultures are nearly the same as the pure cultures of *P. rhodozyma*, and both are much higher than the pure cultures of *H. pluvialis*, in which, glucose could be used completely when its concentration was less than 20 g/l. In contrast, in the pure cultures of *H. pluvialis*, glucose could be utilized efficiently only at a lower glucose concentration of 3 g/l.

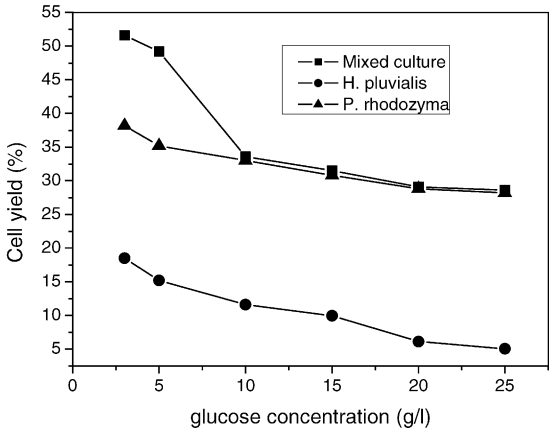

Fig. 3. Cell yields of cultures at different glucose concentrations.

On the other hand, the cell yields (Fig. 3) obtained by the mixed cultures were higher than those of the two pure cultures in particular when the glucose concentration was no more than 5 g/l. The maximum cell yield of 52% was achieved by the mixed culture at the lowest glucose concentration of 3 g/l, which was 1.5- and 3-fold higher than the pure cultures of *P. rhodozyma* and *H. pluvialis*, respectively. Since glucose conversion rates of mixed cultures and pure cultures of *P. rhodozyma* were all the same of 100% when the glucose concentration was below 20 g/l, the higher cell yields reached in the mixed cultures provided a further confirmation about the carbon dioxide fixation by the green alga within the mixed cultures.

Furthermore, it should be noted that different from the biomass production, the cell yields of the mixed cultures decreased as the glucose concentration increased. The reasons for this may come from two aspects: First, the amount of carbon dioxide and organic acids generated by *P. rhodozyma* increased as the glucose concentration elevated, leading to lower pHs (5–6, data not shown). Since the suitable pH for the cell growth of *H. pluvialis* is 7.0–7.8 [8], consequently, the algal cell proliferation was restrained. Second, higher glucose concentrations resulted in relatively higher biomass concentrations. That is to say, the cell number became larger as the glucose concentration increased. Accordingly, light could hardly penetrate the thick culture broth and its intensity attenuated dramatically. In turn, the amount of light received by the algal cells reduced. The photosynthetic activity declined remarkably. As a result, carbon dioxide evolved by the red yeast could not be converted into biomass efficiently. Therefore, we conclude that for the purpose of CO_2 fixation, lower glucose concentration should be maintained within the broth by the fed-batch technique.

In addition, the pure cultures of *P. rhodozyma* and *H. pluvialis* shared a similar variation trends with mixed

Table 3
Astaxanthin concentrations of cultures at different glucose concentrations

Glucose (g/l)	Astaxanthin concentration (mg/l)		
	Mixed culture	*H. pluvialis*	*P. rhodozyma*
3	12.95	3.68	1.09
5	8.86	2.99	1.61
10	6.5	2.34	1.95
15	5.62	2.02	2.05
20	2.15	1.96	1.51
25	2.1	1.71	1.36

cultures on cell yield, which may be attributed to the Crabtree effect as described above.

3.3. Astaxanthin production

As shown in Table 3, astaxanthin concentration levels of the mixed cultures were much higher than those of the two pure cultures, and the maximum volumetric astaxanthin yield of 12.95 mg/l was obtained by mixed culture when the glucose concentration was 3 mg/l, which were 3.5- and 11-fold higher than those of *H. pluvialis* and *P. rhodozyma* pure cultures, respectively.

Astaxanthin concentration in the pure cultures of *H. pluvialis* decreased as the glucose concentration increased, while the pure cultures of *P. rhodozyma* had a similar trend as the glucose concentration was higher than 15 g/l but an opposite trend with glucose concentrations lower than 15 g/l.

Like *H. pluvialis*, astaxanthin production levels reached in the mixed cultures also presented a negative relationship to the glucose concentrations. This may be attributed to the variation on the ratio of cell number between *H. pluvialis* and *P. rhodozyma*. Since *H. pluvialis* synthesizes and accumulates more astaxanthin than *P. rhodozyma*, the highest astaxanthin content reported were up to 4% by dry cell weight in *H. pluvialis* [29] but only around 0.5% in *P. rhodozyma* under the optimal conditions [30]. And, higher astaxanthin yields also presented in the green alga compared to the red yeast in this study (Table 3). As shown in Fig. 4, when glucose concentration was low (3–5 g/l), the microbial population within the culture broth was dominated by the green alga, while as the glucose concentration increased, in turn, the red yeast became the dominant species. Based on these results, we conclude that the higher astaxanthin yields in mixed culture at lower glucose concentrations may be mainly derived from the green alga, and the lower astaxanthin production levels at higher glucose concentrations may come from the red yeast. Supporting this conclusion is the fact that mixed culture in the light presented a higher astaxanthin production than that in the dark (Table 2). Since the ratio of various species in the mixed culture is critical, sudden shifts in the composition of the population can lead to failure of the unit to meet the objectives [31]. Therefore, for the purpose of obtaining a higher astaxanthin production, it is critical to keep the *H. pluvialis* as the predominant strain by adjusting the glucose concentration within the culture broth.

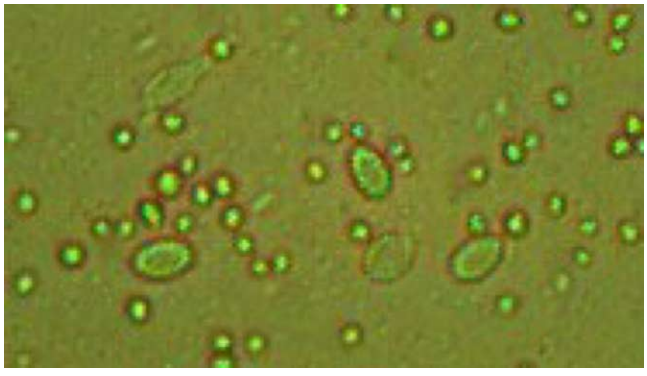

Fig. 4. Microscopic image of the mixed culture of *H. pluvialis* and *P. rhodozyma* (400×). The large ones are the cells of *P. rhodozyma*, and the small ones are *H. pluvialis*.

The reasons for the elevated astaxanthin production levels reached in the mixed cultures may come from the following aspects:

(a) O_2 produced by *H. pluvialis* in the photosynthetic activities promoted astaxanthin synthesis of *P. rhodozyma*. It has been well studied that astaxanthin synthesis requires oxygen and NADH [32–33], which may be due to the conversion reactions from β-carotene to astaxanthin catalyzed by β-carotene hydroxylase and β-carotene ketolase requiring oxygen [34]. A mathematical model indicates that 21 mol of oxygen are required per mol of astaxanthin synthesized [35]. When *P. rhodozyma* grew under fermentative conditions with limited oxygen, the astaxanthin production decreased remarkably. Whereas, when the red yeast grew under aerobic conditions, the astaxanthin production increased with the increasing oxygen uptake [36,37]. Algae have been used for in situ oxygen generation in previous investigations, in which, the green algae *Chorella vulgaris* and *Chlorella pyrenoidosa* were coimmobilized with the bacterium *providencia* and the fungus *Cephalosporium acremonium* respectively to provide oxygen for the two species to produce isocaproic acid and Cephalosporin [38,39]. Since oxygen supply from an external source has limitations in the mixed cultures, oxygen generated by *H. pluvialis* may provide an excess supply to stimulate astaxanthin synthesis in *P. rhodozyma*.

(b) Lower nitrogen concentrations in the mixed cultures may be propitious to astaxanthin synthesis. As described above, *H. pluvialis* produce astaxanthin in response to nitrogen deficiency [8]. When the culture broth was deficient in nitrogen, algal growth was restricted but astaxanthin synthesis greatly stimulated. As shown in Fig. 5, contrary to the glucose conversion rate, the nitrogen conversion rates in the pure cultures of

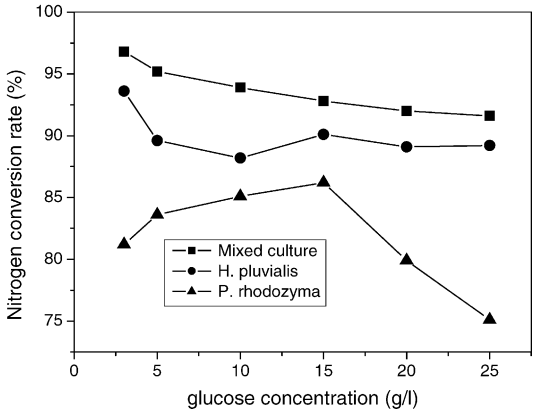

Fig. 5. Nitrogen conversion rate of cultures at different glucose concentrations.

H. pluvialis were higher than the pure cultures of *P. rhodozyma*, the reason may be that nitrogen was used not only for protein synthesis but also for chlorophyll formation within the cells of *H. pluvialis*. In the mixed cultures, the residual nitrates concentrations were lower than those of the two pure cultures due to the higher nitrogen conversion rates (Fig. 4), which may enhance astaxanthin formation in *H. pluvialis*. Furthermore, lower nitrate concentrations in the mixed culture resulted in a relatively higher carbon/nitrogen ratio (C/N), which may also promote astaxanthin synthesis in the red yeast [22,40].

(c) The constant pH (7.0) in the mixed culture was beneficial to astaxanthin synthesis in *H. pluvialis*. As described above, pH in the culture broth of *P. rhodozyma* decreased dramatically due to the secretion of organic acid and carbon dioxide. On the contrary, in the culture broth of *H. pluvialis*, pH increased owing to the utilization of inorganic salts. Though the selected buffer solution of K_2HPO_4/KH_2PO_4 was applied in the present study, however, pHs in the two pure cultures still presented variations (data not shown). While pH in mixed culture was kept constant at 7.0 (data not shown) due to the interaction between *H. pluvialis* and *P. rhodozyma*, which was beneficial to astaxanthin synthesis in the green alga than pHs of 6.0, 8.0, and 9.0 [41].

4. Conclusions

Mixed culture of *H. pluvialis* and *P. rhodozyma* presents a new strategy for combining CO_2 fixation with high value co-product (astaxanthin) formation, promoting glucose and nitrogen conversion rate, keeping pH constant as well as increasing cell yield and astaxanthin production. This culture scheme lays a ground for developing a new technology using carbon dioxide fixation.

Acknowledgements

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