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Synergistic effects of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* for enhancement of biomass and lipid yields

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HIGHLIGHTS

- Biomass and lipid yields achieved a theoretical model of 1+1>2 by mixed culture.
- A double system bubble column photo-bioreactor was designed for mixing cultivation.
- Growth curves of yeast and alga were confirmed in mixing cultivation system.
- Real-time online detection of off-gas showed synergistic effects on O_2/CO_2 balance.
- Analysis of metabolite variations proved synergistic effects on substance exchange.

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ABSTRACT

The optimal mixed culture model of oleaginous yeast *Rhodotorula glutinis* and microalga *Chlorella vulgaris* was confirmed to enhance lipid production. A double system bubble column photo-bioreactor was designed and used for demonstrating the relationship of yeast and alga in mixed culture. The results showed that using the log-phase cultures of yeast and alga as seeds for mixed culture, the improvements of biomass and lipid yields reached 17.3% and 70.9%, respectively, compared with those of monocultures. Growth curves of two species were confirmed in the double system bubble column photo-bioreactor, and the second growth of yeast was observed during 36–48 h of mixed culture. Synergistic effects of two species for cell growth and lipid accumulation were demonstrated on O_2/CO_2 balance, substance exchange, dissolved oxygen and pH adjustment in mixed culture. This study provided a theoretical basis and culture model for producing lipids by mixed culture in place of monoculture.

1. Introduction

Oleaginous microorganisms, involving bacterium, yeasts, moulds and algae, have been extensively studied because the microbial lipids were very similar to soybean lipids to promise potential feedstock for biodiesel production (Cheirsilp et al., 2011). Among oleaginous microorganisms, the yeast *Rhodotorula glutinis*, as a high-yielding lipid strain, could utilize some wastewater for lipid production (Xue et al., 2006, 2010a). In particular, it has been reported that light irradiation could stimulate the synthesis of pigment which resists light damage, and affect cell growth rate and lipid content (Zhang et al., 2014; Yen and Zhang, 2011a; Yen and Yang, 2012). Also, *R. glutinis* has a great growth rate in aerobic condition wherein large amount of O_2 must be required and CO_2 be emitted (Li et al., 2007). Yen and Zhang (2011b) reported that low dissolved oxygen (DO) could retard cell growth and enhance lipid accumulation. However, under enough nutrients and low DO conditions, partial yeast cells would be anaerobic respiration to produce a large number of volatile organic acids (Chen and Gutmanis, 1976), which decreased the pH value of cultivation system. The optimum initial pH for the growth rate of *R. glutinis* is 6.0 (Bhosale and Gadre, 2001). Undissociated organic acid could lower intracellular pH following translocation across the yeast plasma membrane so that yeast growth is inhibited (Nguyen et al., 2001).

Microalgae are considered as another attractive source for biodiesel production due to their high lipid content, photosynthesis efficiency and CO_2 reduction efficiency (Eugenia, 2012; Xiong et al., 2010). Some algal cells can grow not only in photosynthesis system, but also in heterotrophic system or mixotrophy when the culture contains both inorganic and organic substrates (Zhao et al., 2012). *Chlorella vulgaris*, as a kind of mixotrophy algal species, could utilize the high concentration N, P and other ions of waste-

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water in photosynthesis for lipid production meanwhile release O_2 (Li et al., 2010; Liu et al., 2008). Moreover, *C. vulgaris* grows better at pH 6.5–7.0 and accumulates lipids at pH 7.0–8.5 (Wang et al., 2010). However, when CO_2 is consumed by microalgae, the pH becomes more alkaline (Eugenia, 2012). Therefore, the algal growth will be inhibited after a period of culture due to the increasing pH value.

In theory, there are synergistic effects on gas, substance exchange and pH adjustment in the mixed culture system of oleaginous yeast *R. glutinis* and microalga *C. vulgaris* based on the above mentioned researches. However, the reports of lipid production by mixed culture mainly focused on the different alga species (Su et al., 2012; Fradinho et al., 2013; Olguín et al., 2013; Chen et al., 2014) and the mixed culture of yeast and alga based on the mutually beneficial relationship of gas exchange (Shu et al., 2013; Santos et al., 2013).

In this study, one of the aims was to confirm that mixed culture of both microorganisms could significantly enhance biomass and lipid production. In order to demonstrate the synergistic effects of the mixed culture, the authors designed a double-system bubble column photo-bioreactor consisting of an association of two cultivation systems, in which one was used for *R. glutinis* cultivation and the other was for *C. vulgaris*. A polyester fabric filter (2.6 μm) was fixed between the two systems, which could achieve the exchange of substances and gases and keep microorganisms grow independently.

2. Methods

2.1. Strains

The yeast *R. glutinis* (CGMCC No. 2258) was supplied by the China National Research Institute of Food and Fermentation Industries and kept at Beijing University of Chemical Technology. The strain was maintained on yeast extract, peptone and dextrose (YPD) agar slant at 4 °C.

The microalga *C. vulgaris* was stored in National Energy R&D Center for Biorefinery at Beijing University of Chemical Technology after being provided by Institute of Hydrobiology, Chinese Academy of Sciences. The strain could conduct mixotrophy in the culture containing both inorganic and organic substrates and was maintained on alga medium BG-11 (described below) agar slant with glucose (2 g/L) at 4 °C.

2.2. Culture media and conditions

Seed media of *R. glutinis* and *C. vulgaris* were described as follow. Yeast medium composition (Xue et al., 2008): glucose 40 g/L, yeast extract 1.5 g/L, $(NH_4)_2SO_4$ 2 g/L, KH_2PO_4 7 g/L, $NaSO_4$ 2 g/L, $MgSO_4{\cdot}7H_2O$ 1.5 g/L. The initial pH was adjusted to 5.5. Alga medium BG-11 composition (Zhao et al., 2012): citric acid 6.0 mg/L, ferric ammonium citrate 6.0 mg/L, EDTA 1.0 mg/L, $NaNO_3$ 1.5 g/L, $K_2HPO_4{\cdot}2H_2O$ 0.051 g/L, $MgSO_4{\cdot}7H_2O$ 0.075 g/L, $CaCl_2$ 0.024 g/L, Na_2CO_3 0.02 g/L, A5 trace mineral solution 1.0 mL/L. The composition of A5 was: H_3BO_4 2.86 g/L, $MnCl_2{\cdot}4H_2O$ 1.81 g/L, $ZnSO_4{\cdot}7H_2O$ 0.222 g/L, $Na_2MoO_4{\cdot}2H_2O$ 0.391 g/L, $CuSO_4{\cdot}5H_2O$ 0.079 g/L, $Co(NO_3)_2{\cdot}6H_2O$ 0.049 g/L. The initial pH was adjusted to 6.5–7.5. The mixotrophic culture medium was the alga medium containing 5 g/L of glucose. Mixed medium were adducts of yeast and alga media adjusting the concentration of glucose to 20 g/L (Xue et al., 2010b). Yeast and alga were respectively incubated from agar slant culture to 500 mL Erlenmeyer flasks containing 100 mL seed media. The flasks were placed in a rotary shaker at 180 rpm, 30 °C and around 80 μmol/m^2 s. Light intensity was measured by a light-meter (TES-1339, Taiwan Taishi) on the outside surface of flasks. Mixed culture was taken in 500 mL flask or double system bubble column photo-bioreactor using logarithmic phase cultures as seed.

The monocultures of yeast and alga at the log-phase with 10% (v/v) were transferred into 260 mL double system bubble column photo-bioreactor containing 100 mL mixed medium for measuring the change of gases and substances (Fig. 1). During the mixed culture, the aeration rate was 0.8 L air/min. Aseptic air filter (0.22 μm) was accessed to the bottom of bioreactor through a pipeline and was dispersed by an air distributor. Temperature was controlled by the insulation layer circulating water from electric-heated thermostatic water bath (MPE-40C, China). The bioreactor was illuminated with white fluorescent light tubes assembled outside surface of bioreactor, which supplied a light intensity of 100 μmol/m^2 s.

2.3. Measurement of glucose, biomass and lipid content

Glucose concentration was measured using a glucose biosensor (SBA-40C, Biological Institute of Shandong Academy of Sciences). The biomass concentrations of yeast and alga were determined by optical density reading at 600 nm (OD_{600}) and 680 nm (OD_{680}), respectively. A calibration curve of yeast dry cell weight corresponded to OD_{600} value as follows: Biomass (g/L) = 1.052 × OD_{600} + 0.041, R^2 = 0.9982, and that curve of alga at OD_{680} as follows: Biomass (g/L) = 0.324 × OD_{680} + 0.0099, R^2 = 0.9991. Biomass concentration of mixed culture was carried out following the method of Zhao et al. (2012) by cell dry weight. The lipid content was determined by sulfo-phospho-vanillin method (Izard and Limber, 2003).

2.4. Measurement of off-gas, dissolved oxygen and pH value

Real-time online detection of the different gas concentrations (O_2, CO_2 and N_2) in off-gas was performed by using the Industrial Gas Analyzer (MAX300-LG, Extrel, USA). The online dissolved oxygen (DO) and pH value were determined by using DO meter (OXYFERM FDA 225, Hamilton) and pH meter (M-10, AMER), respectively.

2.5. Analysis of extracellular metabolites

Extracellular metabolites were analyzed by the method of gas chromatography–mass spectrometry (GC–MS) (GC–MS-QP2010, SHIMADZU, Japan) according to the modified procedure of Coutinho et al. (2013) and Cai et al. (2007). Samples (1 mL) taken from mixed culture and controls were centrifuged at 12,000 rpm for 10 min, then dried the supernatant to get the metabolite powder by centrifugal concentrator (HR/T16MM, China) at 30 °C. A volume (100 μL) of methoxyamine pyridine solution (2 g/L) was added into the metabolite powder for suspension and oximation reaction at 30 °C for 2 h, and then 100 μL of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) agent was added into the reaction system for derivatization at constant temperature of 30 °C for 6 h. After filtered through filter paper (0.22 μm), sample of 1 μL was injected into GC–MS with a column of 30 mm × 0.25 mm × 0.25 μm.

3. Results and discussion

3.1. The mixed culture of R. glutinis and C. vulgaris

3.1.1. Directly mixed culture

Because microalga grew slower than yeast, the increasing initial amount of algal cells were attempted to mixed culture with constant amount of yeast cells. Even so, the biomass in the mixing