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Use of orange peel extract for mixotrophic cultivation of *Chlorella vulgaris*: Increased production of biomass and FAMEs

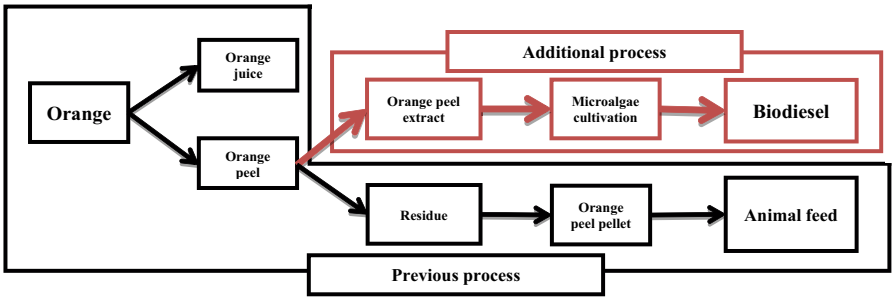
Won-Kun Park [a], Myounghoon Moon [a], Min-Su Kwak [a], Seungjib Jeon [a], Gang-Guk Choi [a], Ji-Won Yang [a,b], Bongsoo Lee [a,*]

[a] Department of Chemical and Biomolecular Engineering, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea
[b] Advanced Biomass R&D Center, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon 305-701, Republic of Korea

HIGHLIGHTS

- Orange peel extract was used for microalgae cultivation.
- This extract contains nutrients that support mixotrophic cultivation of microalgae.
- Cells cultured in OPE produced significantly increased biomass and FAME.

GRAPHICAL ABSTRACT



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ABSTRACT

Mass cultivation of microalgae is necessary to achieve economically feasible production of microalgal biodiesel, but the high cost of nutrients is a major limitation. In this study, orange peel extract (OPE) was used as an inorganic and organic nutrient source for the cultivation of *Chlorella vulgaris* OW-01. Chemical composition analysis of the OPE indicated that it contains sufficient nutrients for mixotrophic cultivation of *C. vulgaris* OW-01. Analysis of biomass and FAME production showed that microalgae grown in OPE medium produced 3.4-times more biomass and 4.5-times more fatty acid methyl esters (FAMEs) than cells cultured in glucose-supplemented BG 11 medium (BG-G). These results suggest that growth of microalgae in an OPE-supplemented medium increases lipid production and that OPE has potential for use in the mass cultivation of microalgae.

1. Introduction

Global warming is caused by the continuing use of fossil fuels, and is one of the most significant environmental challenges world-wide. Moreover, limited oil deposits and increasing oil prices have increased the search for alternative energy sources as replacements for petroleum-based fuels. Biomass can be used for sustainable biodiesel production, and there is increasing interest in microalgae, rather than oil crops and land plants, as a biodiesel source because microalgae have higher photosynthetic efficiency, faster growth rate, and sustainable biomass production (Chisti, 2007). Despite these advantages, the high cost of commercial biomass production from microalgae has been a major limitation (Demirbas and Fatih Demirbas, 2011).

The production of microalgal-based biodiesel consists of four steps: cultivation, harvesting, lipid extraction, and conversion to biodiesel. Increased efficiency of mass cultivation is necessary for commercial production of microalgal-based biodiesel. A recent review suggested that significant cost reductions in cultivation

* Corresponding author. Tel.: +82 42 350 3964; fax: +82 42 350 3910.
E-mail address: bongsoolee@kaist.ac.kr (B. Lee).

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may be achieved if water and nutrients can be obtained at low cost (Slade and Bauen, 2013). Thus, one approach is to use agricultural and industrial wastewaters for cultivation of microalgae (Moon et al., 2014; Yang et al., 2011). An additional advantage is that cultivation of microalgae using wastewater can provide simultaneous wastewater treatment (removal of toxic materials and nutrients, particularly N and P) and biodiesel production (Cho et al., 2011).

However, there are some limitations in using wastewater for the cultivation of microalgae. A continuous supply of wastewater is needed to maintain constant nutrient levels for microalgal cultivation, but the supply and suitability of a wastewater source may not be guaranteed over time (Cho et al., 2011). Moreover, research on cultivation using wastewater has emphasized the benefit of cost-effective cultivation, rather than maximization of biomass production. For example, *Chlorella minutissima* can grow cost-effectively in municipal sewage wastewater, but its growth was maximized under mixotrophic conditions (with added carbon source) with a biomass yield of 379 mg L^{-1} after 10 days growth, compared to a biomass yield of 73.03 mg L^{-1} under photoautotrophic conditions (no added carbon source) (Bhatnagar et al., 2010). Another study indicated that mixotrophic growth of three microalgae (*Chlamydomonas globosa*, *C. minutissima*, and *Scenedesmus bijuga*) from industrial wastewater resulted in 3–10 times more biomass production than growth under phototrophy (Chinnasamy et al., 2010). These results indicate cultivation in wastewater does not simultaneously maximize biomass production and reduce costs. In particular, it has been established that mixotrophic cultivation is the most efficient method to maximize production of microalgal biomass (Cheirsilp and Torpee, 2012). However, mixotrophic cultivation requires cost-intensive carbon sources. Therefore, the use of wastewater and a low-cost organic carbon source may be another option to maximize production of microalgal biomass and reduce the costs of biodiesel production.

It has long been proposed that food waste, such as water extracts of soy bean waste, could be used for cultivation of microalgae (Wong, 1985). Recent experiments showed that food waste alone or food waste combined with wastewater can be used for heterotrophic and mixotrophic cultivation of microalgae (Pleissner et al., 2013; Yan et al., 2011).

The present study assessed the use of an extract of orange peel waste as nutrient source for mixotrophic cultivation of *Chlorella vulgaris* OW-01. In particular, this study evaluated the use of orange peel extract (OPE) for mixotrophic cultivation of microalgae, the chemical properties of the OPE, and the biomass and FAME yields of microalgae cultivated with OPE.

2. Methods

2.1. Algal strain and culture conditions

Axenic *C. vulgaris* strain OW-01, kindly provided by Dr. Hee-Sik Kim of the Korea Research Institute of Bioscience and Biotechnology (KRIBB), was used for these experiments. Cultures were maintained on Tris–Acetate–Phosphate (TAP) agar plates (375 mg NH_4Cl, 100 mg $MgSO_4{\cdot}7H_2O$, 50 mg $CaCl_2{\cdot}2H_2O$, 108 mg K_2HPO_4, 54 mg KH_2PO_4, 2.42 g Tris, 1 mL glacial acetic acid, and 1 mL Hutner's trace elements per liter). For testing the effect of pH (4.4–8.5), different amounts of 3 M KOH were added.

Experiments that evaluated the effect of OPE on growth and FAME content employed BG 11 medium (1.5 g $NaNO_3$, 40 mg K_2HPO_4, 75 mg $MgSO_4{\cdot}7H_2O$, 36 mg $CaCl_2{\cdot}2H_2O$, 6 mg citric acid H_2O, 6 mg ferric ammonium citrate, 1 mg $Na_2EDTA{\cdot}2H_2O$, 20 mg Na_2CO_3, 2.86 mg H_3BO_3, 1.81 mg $MnCl_2{\cdot}4H_2O$, 0.22 mg $ZnSO_4{\cdot}7H_2O$, 0.39 mg $Na_2MoO_4{\cdot}2H_2O$, 0.079 mg $CuSO_4{\cdot}5H_2O$, and 0.0494 mg $Co(NO_3)_2{\cdot}6H_2O$ per liter) or BG 11 medium with 5 g L^{-1} of glucose (BG-G). Cells were grown to the exponential phase in 250-mL baffled flasks with a filter cap (DURAN), with 200 mL of growth medium under continuous light from five 55 W cool white fluorescent lamps (160–180 μmol photons m^{-2} s^{-1}) at 26 °C and 150 rpm. There were more than four replicates for each treatment.

2.2. Pretreatment of OPE and analysis methods

Orange peel waste was obtained from an orange juice manufacturing facility (C.J. Cheiljedang) in Korea, stored in a freezer, and used after thawing. This waste product was homogenized by chopping and blending in a 1:5 ratio (w:v) with distilled water using a commercial blender (Type HR 2011, Philips, USA). The mixture was then subjected to extraction at room temperature for 0.5–24 h. After extraction, the blended waste was removed using a sieve (0.2 mm) and then a 0.7 μm GF/F glass microfiber filter (Whatman, USA).

To confirm the suitability of OPE as a culture medium, nitrogen, phosphorous, carbon, and trace elements were analyzed. Nitrate, nitrite, phosphate, and various organic acids were quantified by ion chromatography (883 Basic IC plus, Metrohm, Switzerland) with a Metrosep A Supp 5-150/4.0 anion column, a Metrosep C4-150 cation column, and a Metrosep Organic Acids-250/7.8 column. A YSI 2900 Biochemistry Analyzer (YSI, USA) was used to determine carbohydrate concentrations. Trace elements were analyzed with an inductive coupled plasma atomic emission spectrometer (OPTIMA 7300 DV, Perkin-Elmer, USA).

2.3. Growth measurements

Cell growth was measured by three methods: optical density, dry cell weight, and chlorophyll analysis. Optical density was measured at 682 nm using a UV/Vis spectrometer (Beckman coulter, USA). Dry cell weight was measured by harvesting 100 mL of algal culture, washing twice with distilled water, and freeze drying for 2 days. Chlorophyll (Chl) was extracted into methanol and measured as described (Park et al., 2013), in which the equations for Chl *a* and *b* were:

$$\text{Chl } a \ (\mu g \ ml^{-1}) \approx -8.0962 \ OD_{652} + 16.5169 \ OD_{665} \quad (1)$$

$$\text{Chl } b \ (\mu g \ ml^{-1}) \approx 27.4405 \ OD_{652} - 12.1688 \ OD_{665} \quad (2)$$

2.4. Biochemical analysis

The absolute analysis of protein was performed using 2 mg of a dry microalgal sample with an elements analyzer (FLASH 2000 series, Thermo Scientific, USA). Measured values of nitrogen were multiplied by 6.25 (nitrogen-to-protein conversion factor) to estimate the protein content (Park et al., 2013). Starch content was determined using the anthrone method (Park et al., 2013) and was quantified by multiplying the glucose concentration by 0.9, with glucose concentration calculated as:

$$\text{Glucose } (mg \ L^{-1}) = 1.496 \times (OD_{625})^2 - 0.0298, \ R^2 = 0.9997 \quad (3)$$

2.5. FAME analysis

Microalgal biomass was harvested by centrifugation (7000 rpm, 3 min) and washed twice with distilled water. The cell pellet was freeze-dried at −40 °C for 3 days and then used for lipid extraction. Total lipids were extracted from 0.05 g of lyophilized biomass with chloroform–methanol (2:1, v/v), followed by the modified Folch method (Park et al., 2013). Extracted lipids were then converted into fatty acid methyl esters (FAMEs) *via* transesterification, and