Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–6, 2018

www.elsevier.com/locate/jbiosc

Application of microalgae hydrolysate as a fermentation medium for microbial production of 2-pyrone 4,6-dicarboxylic acid

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Received 12 November 2017; accepted 28 December 2017
Available online xxx

Actual biomass of microalgae was tested as a fermentation substrate for microbial production of 2-pyrone 4,6-dicarboxylic acid (PDC). Acid-hydrolyzed green microalgae *Chlorella emersonii* (algae hydrolysate) was diluted to adjust the glucose concentration to 2 g/L and supplemented with the nutrients of Luria–Bertani (LB) medium (tryptone 10 g/L and yeast extract 5 g/L). When the algae hydrolysate was used as a fermentation source for recombinant *Escherichia coli* producing PDC, 0.43 g/L PDC was produced with a yield of 20.1% (mol PDC/mol glucose), whereas 0.19 g/L PDC was produced with a yield of 8.6% when LB medium supplemented with glucose was used. To evaluate the potential of algae hydrolysate alone as a fermentation medium for *E. coli* growth and PDC production, the nutrients of LB medium were reduced from the algae hydrolysate medium. Interestingly, 0.17 g/L PDC was produced even without additional nutrient, which was comparable to the case using pure glucose medium with nutrients of LB medium. When using a high concentration of hydrolysate without additional nutrients, 1.22 g/L PDC was produced after a 24-h cultivation with the yield of 16.1%. Overall, *C. emersonii* has high potential as cost-effective fermentation substrate for the microbial production of PDC.

[**Key words:** Microalgae; *Chlorella emersonii*; Acid hydrolysis; Fermentation; *Escherichia coli*; 2-pyrone 4,6-dicarboxylic acid]

The utilization of bioresources in place of non-renewable resources is of great interest in the field of industrial polymer production. Fossil resources are limited and cause the emission of greenhouse gases to the atmosphere, which leads to climate change and global warming. To circumvent this problem, alternative feedstocks have been investigated to replace the social system based on petroleum to bioresources.

Microalgae are considered as one of the alternative biomasses for producing bio-based fuels and chemicals. An advantage of using microalgae as a feedstock is that they grow with only light and carbon dioxide and without the need for an extraneous carbon source (1–7). Microalgae contain starch and lipids as the main energy reserves under nutrient starvation and variation in growth conditions (8–10). As a feedstock, microalgae contain no lignin, which simplifies the preparation of fermentable sugars without any complicated pretreatment compared with lignocellulosic biomass. Taking advantage of this, microbial production of fuels and chemicals, such as ethanol and succinic acid fermentation by bacteria and yeast, has been conducted using microalgae as a feedstock (11–13). Hydrolyzed microalgae have another possible advantage in that they can be used as bacterial medium without adding any extraneous nutrients. Because bacterial species usually require various nutrients, such as a nitrogen source or trace elements, for growth, it is necessary to add extra nutrients in the medium besides a carbon source. From the view point of exogenous elements, the hydrolyzed microalgae alone could contain sufficient nutrients and trace elements for bacterial growth. *Chlorella* species are one candidate because they contain a large amount of carbohydrates (starch and cellulose), lipids, and other nutrients (14). For example, *Chlorella zofingiensis* can accumulate up to 45%–60% of dry weight of starch and lipid, respectively, under appropriate culture conditions (15). However, the capabilities of *Chlorella* as a feedstock for growing microbes and producing green-chemicals are yet to be investigated.

2-Pyrone 4,6-dicarboxylic acid (PDC) is a dicarboxylic acid with a polar pseudo-aromatic ring with molecular weight of 184.1 (16). It has a similar molecular shape to isophthalic acid, which is currently used for the synthesis of various kinds of polyesters and polyamides, such as polybenzimidazole (17); therefore, as a monomer, PDC has the possibility of producing high-performance polymers. Because PDC cannot be synthesized from petroleum, attempts have been made to bio-conversion to PDC from lignin-derived aromatic

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1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2017.12.026

compounds by using *Pseudomonas putida* (18,19). Recently, fermentative production of PDC from glucose was achieved by introducing genes of the shikimate pathway into *Escherichia coli* cells (20). Five genes were introduced to complete the pathway from glucose to PDC through protocatechuate (PCA). In the recombinant *E. coli*, glucose was first converted to protocatechuate (PCA) by 4-hydroxybenzoate hydroxylase (*pobA*) and dehydroshikimate dehydratase (*qutC*). PCA was then converted to PDC by the action of protocatechuate 4,5-dioxygenase (*ligA* and *ligB*) and 4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase (*ligC*) (20). Although PDC production from pure glucose was successful, it would be ideal to produce the chemical from an actual and cost-effective biomass.

In the present study, to evaluate the potential of algae hydrolysate as a nutrient source for PDC fermentation, starch-accumulated *Chlorella emersonii* was acid-hydrolyzed and the hydrolysate was then applied for PDC production by recombinant *E. coli*.

MATERIALS AND METHODS

Microalgae, bacteria, and plasmids A pure strain of the green microalgae *C. emersonii* (NIES-2151) was purchased from the National Institute for Environmental Studies, Tsukuba, Japan. Bacterial strain, *E. coli* BL21(DE3) harboring three plasmids (pACYC-aroF^{fbr}-aroB, pCDF-ubiC-pobA, and pFT-ligABC-qutC) was used as PDC producer (20).

Cultivation of microalgae Frozen glycerol stock of *C. emersonii* (1 mL) was thawed at room temperature and subsequently pre-cultivated in 500 mL Erlenmeyer flask containing 250 mL of tris-acetate phosphate (TAP) medium (NH_4Cl: 400 mg/L, $CaCl_2 \cdot 2H_2O$: 51 mg/L, $MgSO_4 \cdot 7H_2O$: 100 mg/L, K_2HPO_4: 119 mg/L, KH_2PO_4: 60.3 mg/L, Hutner's trace elements: 1 mL/L, acetic acid: 1 mL/L, tris (hydroxymethyl) aminomethane: 2420 mg/L), according to a previous report (21). The pre-culture in TAP medium was carried out statically at 25 °C under a continuous fluorescent white light (7338 Lux) without aeration.

Next, 10 mL of pre-cultivated microalgae was transferred to a 2 L glass bottle containing 2 L of TAP medium for proliferation of microalgae cell. When the optical density of 540 nm (OD_{540}) reached approximately 2, the algae culture broth was centrifuged (5000 rpm, 10 min) and washed with 0.85% NaCl three times. Subsequently, the cells were transferred to the 2 L glass bottle containing 2 L of TAP medium without sulfur (S-TAP medium) for starch accumulation in the microalgae cells (22). Both the cultures in TAP and S-TAP medium were carried out at 25 °C under continuous fluorescent white light and constant mixing with aeration.

During the culturing in the TAP medium, cell growth was monitored by OD_{540} using a spectrophotometer (DU 730, Beckman Coulter, Inc.). During the culturing for starch accumulation in the S-TAP medium, cell concentration was determined by manual counting using hemocytometer (Bacteria counter A161, Sunlead Glass, Japan).

Acid hydrolysis of microalgae When the microalgae cell entered stationary phase during culturing in S-TAP medium, the cells were harvested by centrifugation (5000 rpm, 10 min). After discarding the supernatant, they were freeze-dried and stored at −25 °C in the dark. The freeze-dried microalgae was hydrolyzed using sulfuric acid, based on the National Renewable Energy Laboratory (NREL/TP-5100-60,957) method (23) which is the standard method for evaluating carbohydrate content in microalgae on the basis of the pretreatment by concentrated acid and the subsequent hydrolysis by diluted acid, where all of carbohydrates (such as cellulose in the cell wall and starch accumulated intercellularly) are supposed to be hydrolyzed into glucose. Briefly, 500 mg dry microalgae was mixed with 250 μL of 72 (wt.%) sulfuric acid. Next, the mixture was incubated at 30 °C for 1 h for the pretreatment. After incubation, the mixture was diluted with 7 mL of water and autoclaved at 121 °C for 1 h for the hydrolysis. Hydrolyzed samples were neutralized with calcium carbonate and filtered (sterile-EO, pore size: 0.2 μm, diameter: 25 mm, Sartorius Stedim Biotech, Germany) to obtain the acid-hydrolyzed microalgae (algae hydrolysate).

Fermentative production of PDC from algae hydrolysate As a fermentation medium, the algae hydrolysate was diluted with ultrapure water to set the initial concentration of the fermentation media at 2, 5, 10, or 16 g/L. This was called as algae hydrolysate medium in the present study. When necessary, the algae hydrolysate medium was supplemented with 10 g/L tryptone and 5 g/L yeast extract as additional nutrients which contains nitrogen, vitamins, and trace elements (24). The concentration of additional nutrients of 10 g/L tryptone and 5 g/L yeast extract was defined as ×1 nutrient in the present study, and the concentration of additional nutrients were changed to ×1/2, ×1/4, ×1/8, and ×0 nutrient (no additional nutrients). As a control medium, pure glucose medium was also prepared by replacing the algae hydrolysate in the algal hydrolysate medium to glucose.

For pre-culture, the PDC-producing *E. coli* was cultured overnight at 37 °C, 135 rpm in a test tube containing 5 mL LB medium (tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L). Antibiotics ampicillin (100 mg/L), streptomycin (10 mg/L), and chloramphenicol (30 mg/L) were added to the medium. After pre-culture, the *E. coli* cells were collected by centrifugation (15,000 rpm, 2 min) and the supernatant was discarded. Next, the *E. coli* cells were inoculated into Sakaguchi flask containing 50 mL fermentation medium at the initial OD_{600} of 0.1. Throughout the fermentation experiment, three antibiotics described above was added to the fermentation medium, and calcium carbonate (25 g/L) was also added to the fermentation medium to control pH during fermentation. Isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 M) was added at 0 h of fermentation as an inducer. Fermentation was carried out at 37 °C with constant stirring at 135 rpm. Samples were collected every 3 h until 12 h and then every 12 h until the end of the fermentation at 48 h.

Analysis for PDC fermentation The glucose concentration in the algae hydrolysate or fermentation medium was determined by the glucose oxidase (GOD) method using the LabAssay Glucose, Mutarotase-GOD method (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Total organic carbon (TOC) and total nitrogen (TN) in the algae hydrolysate or fermentation medium were determined by using a total organic carbon analyzer (TOC-V CPN, Shimadzu Co., Kyoto, Japan). The microelement (Fe^{2+} and Mn^{2+}) concentration in the algae hydrolysate or fermentation medium was determined by using atomic absorption spectrophotometry (AA-7000, Shimadzu Co.).

The *E. coli* cell growth was monitored by OD_{600} using a DU730 spectrophotometer (Beckman–Coulter), and cell concentration was determined using a coefficient of 0.39 g/L per OD_{600}. Before measuring OD_{600}, calcium carbonate in the fermentation broth was solubilized by 0.2 M HCl. PDC concentration in the fermentation broth was determined by using high-performance liquid chromatography (HPLC) using a UV/RI detector (SPD-20A/RID-10A, Shimadzu Co.) and ICSep ICE-ION-300 column. The operating conditions were a flow rate of 0.4 mL min^{-1} of 0.0085 M H_2SO_4 mobile phase with a column temperature of 70 °C.

RESULTS AND DISCUSSION

Carbohydrate content of microalgae and glucose concentration in the hydrolysate Before the fermentation for PDC production by the recombinant *E. coli*, carbohydrate content of microalgae *C. emersonii* and glucose concentration in the acid hydrolysate were evaluated. It was found that the carbohydrate content was 23.6 wt% in the *C. emersonii* obtained via proliferation culture in TAP medium and subsequent starch accumulation culture in S-TAP medium. The algae hydrolysate obtained after the acid-hydrolysis and neutralization contained glucose (16.3 g/L), total organic carbon (19.1 g/L), total nitrogen (4.1 g/L), Mn^{2+} (53.9 mg/L), and Fe^{2+} (0.38 mg/L). This algae hydrolysate was used for the following experiment of microbial fermentation for PDC production by the recombinant *E. coli*.

Effect of glucose source on cell growth and PDC production Firstly, to examine the availability of algae hydrolysate as a substrate for PDC production, the microbial fermentation was carried out using the algae hydrolysate medium or the pure glucose medium (control), where the medium was supplemented with ×1 nutrients and the initial glucose concentration was adjusted to be 2 g/L. As shown in Fig. 1A and B (open and closed circles), glucose was consumed with elapsed time, being depleted at 6 h irrespective of the media. As shown in Fig. 1C (open circles), in the case of the pure glucose medium, the cell concentration reached a saturated value of 1.8 g/L just after the glucose depletion at 6 h. On the contrary, in the case of the algae hydrolysate medium, the cell concentration continued to increase even after the glucose depletion at 6 h, reaching a maximum value of 5.5 g/L at 36 h (Fig. 1D, closed circles). As shown in Fig. 1E (open circles), in the case of the pure glucose medium, the PDC concentration reached 0.19 g/L at 24 h. On the other hand, in the case of the algae hydrolysate medium, the PDC concentration reached 0.43 g/L at 24 h, which was approximately 2.3-fold higher than that in pure glucose medium (Fig. 1F, closed circles). The yield of PDC in the pure glucose medium was 8.6%, whereas the value was increased to 20.1% in the algae hydrolysate medium. It was considered that the nutrients in the algae hydrolysate (such as amino acids) were used as carbon sources for cell growth and PDC synthesis, leading to the higher cell and

Please cite this article in press as: Htet, A. N., et al., Application of microalgae hydrolysate as a fermentation medium for microbial production of 2-pyrone 4,6-dicarboxylic acid, J. Biosci. Bioeng., (2018), https://doi.org/10.1016/j.jbiosc.2017.12.026