Article

Colaconema formosanum, *Sarcodia suae*, and *Nostoc commune* as Fermentation Substrates for Bioactive Substance Production

Meng-Chou Lee [1,2,3,*], Chin-Yi Huang [1], Chin-Ling Lai [1], Han-Yang Yeh [1], Jing Huang [1], Wei Qing Chloe Lung [1], Po-Tsang Lee [1] and Fan-Hua Nan [1,3]

[1] Department of Aquaculture, National Taiwan Ocean University, Keelung City 20224, Taiwan; thormars@gmail.com (C.-Y.H.); 10532074@email.ntou.edu.tw (C.-L.L.); 20833001@mail.ntou.edu.tw (H.-Y.Y.); haungjim31336@gmail.com (J.H.); chloelung98@gmail.com (W.Q.C.L.); leepotsang@email.ntou.edu.tw (P.-T.L.); fhnan@mail.ntou.edu.tw (F.-H.N.)

[2] Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung City 20224, Taiwan

[3] Center of Excellence for Ocean Engineering, National Taiwan Ocean University, Keelung City 20224, Taiwan

* Correspondence: mengchoulee@email.ntou.edu.tw; Tel.: +886-2-246222192 (ext. 5239) or +886-9-78586589; Fax: +886-2-24635441

Abstract: Bioactive compounds extracted from natural renewable sources have attracted an increased interest from both industry and academia. Recently, algae have been highlighted as promising sources of bioactive compounds, such as polyphenols, polysaccharides, fatty acids, proteins, and pigments, which can be used as functional ingredients in many industrial applications. Therefore, a simple green extraction and purification methodology capable of recovering biocompounds from algal biomass is of extreme importance in commercial production. In this study, we evaluated the application of three valuable algae (*Colaconema formosanum*, *Sarcodia suae*, and *Nostoc commune*) in combination with *Pseudoalteromonas haloplanktis* (type strain ATCC 14393) for the production of versatile compounds. The results illustrate that after 6 h of first-stage fermentation, the production of phycobiliproteins in *C. formosanum* was significantly increased by 156.2%, 188.9%, and 254.17% for PE, PC, and APC, respectively. This indicates that the production of phycobiliproteins from algae can be enhanced by *P. haloplanktis*. Furthermore, we discovered that after *S. suae* and *N. commune* were fermented with *P. haloplanktis*, mannose was produced. In this study, we describe a feasible biorefinery process for the production of phycobiliproteins and mannose by fermenting marine macroalgae with cyanobacteria. We believe it is worth establishing a scale-up technique by applying this fermentation method to the production of phycobiliproteins and mannose in the future.

Keywords: *Pseudoalteromonas haloplanktis* ATCC 14393; *Colaconema formosanum*; *Sarcodia suae*; *Nostoc commune*; bioactive compounds; phycobiliproteins; one-stage fermentation; fragmentation

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1. Introduction

In response to the increasing requirement for non-toxic and environmentally friendly products, algae have been considered and are recommended as a good source of naturally derived compounds because they contain multiple bioactive components, such as polysaccharides, pigments, fatty acids, polyphenols, and peptides, which can be used for functional food and in nutraceutical fields [1]. Notably, the cell wall of algae is constituted by complicated cellulosic compounds, and their compositions differ depending on species, e.g., green algae mainly consist of cellulose, pectins, xyloglucans, xylans, extensins, and lignins [2], whereas red algae consist of cellulose, mannans, xylans, sulfated polysaccharides, and lignins [3], and the brown algae produce fucose-containing polysaccharides [4,5].

The diversity in the structure and rigidity of algal cell walls blocks the development of cell disruption for downstream processing. Traditionally, physical high-pressure and chemical solvent extraction methods are used to extract biologically active compounds, for instance, solvent systems, sonication, ultrasound, microwave, enzymatic, super-critical

fluid, and subcritical water. However, most of the abovementioned methods are associated with high energy cost and are environmentally unfriendly. Specifically, the main goal of extraction techniques is to achieve a high yield of desired compounds, although it is also desirable to preserve coproducts, minimize energy consumption, investigate recycling methods to minimize waste generation, optimize the process, and increase the production scale [6].

A promising strategy for overcoming the impediment of algal cell walls involves a series of processes called consolidated bioprocessing, which includes lysis enzymes, hydrolysis of biomass, and fermentation; then, the manufacturer finally acquires the desired products [7]. As the digestive capacity of working bacteria species used in fermentation is affected by both material (algae) and fermenting conditions, production consumes and outputs various bioactive substances, such as peptides, phenolics or phenolic compounds, and fucoidan.

In brief, fermentation is a metabolic conversion activity achieved by micro-organisms. Previous studies illustrated that multiple potential micro-organism, such as *Saccharomyces cerevisiae*, *Pichia stipitis*, *Kluyveromyces fragilis*, *K. marxianus*, *Escherichia coli*, *Klebsiella oxytoca*, *Zymomonas mobilis*, and *Pseudoalteromonas haloplanktis*, have a positive effect on the algal fermentation process [8,9]. Among those, *P. haloplanktis* is a marine bacterium that can degrade the cell walls of algae by secreting beta-glucosidase and is therefore regarded as an ideal micro-organism to enable consolidated bioprocessing [10].

Notably, a water-soluble protein, phycobiliprotein, is the oldest photosynthetic light-harvesting pigment present in red algae, cyanobacteria, and cryptomonads. This protein has become increasingly important in the research, clinical, pharmaceutical, and aquaculture industries, owing to its multiple functions and efficacy, as described below. First, these proteins have the potential to be used as a natural dye, and a number of investigations have shown that they also have health-promoting properties and a broad range of pharmaceutical applications [11]. Phycobiliproteins are widely used in clinical and immunological research laboratories as probes in fluorescence flow cytometry, fluorescence microscopy, and fluorescence immunoassays [12]. Hence, it is valuable to develop an efficient and ecofriendly way to mass produce phycobiliprotein.

To improve the extraction efficiency of bioactive compounds, the objective of this study was to determine the optimal microbial fermented conditions to extract protein and polysaccharides contained marine algae. We used the bacteria *Pseudo-alteromonas haloplanktis* (type strain ATCC 14393) to ferment three algae (*Colaconema formosanum*, *Sarcodia suae*, and *Nostoc commune*) to obtain the valuable pigments and polysaccharides.

2. Materials and Methods

2.1. Algae and Bacterial Strain

The marine macroalgae *C. formosanum* and *S. suae* used in the experiment were collected on the Pingtung County coast, southwest Taiwan (January 2017), and cultured in fiberglass tanks in a greenhouse for two years to ensure similar conditions for all samples to avoid any biases. *N. communes* were collected from Pingtung, South Taiwan, in March 2018. Fresh seaweed was washed twice with seawater and distilled water to remove any visible surface contaminants. The biomass was harvested, washed with seawater, drained with a spinner, and stored at −20 °C until use. This biomass, defined as the wet weight, was used for fermentation. The strain of bacteria used in this fermentation was *P. haloplanktis* (type strain ATCC 14393), which was purchased from the Bioresource Collection Research Center (BCRC), Food Industry Research and Development Institute (Hsinchu, Taiwan). Before experiments, *P. haloplanktis* was cultured in Marine Broth 2216 medium (Difco Laboratories, Detroit MI, USA) with a shaking speed of 240 rpm at 22 °C for 12 h to reach the log phase of the strain. (The growth curve of bacteria stain *P. haloplanktis* ATCC 14393 used in this study can be found in Appendix A). Before experiments, the algal samples were cultured in vessels with sterile media and a clean growth chamber. During cultivation, pollution by other algae or bacteria did not occur due to sterilization and because the algae were

fermented in a fresh situation. For the process, we hypothesized that the main fermentation reaction would be contributed by the bacteria *P. haloplanktis*, as it is the dominant species in the environment. For fermentation, the conditions were adjusted to 22 °C for 0 to 78 h according to experimental design and harvested with 5000× *g* centrifugation (Allegra X-30R Centrifuge, Beckman counter) for 10 min. The cultural broth was washed out twice with distilled water before application.

2.2. Fermentation of the Three Types of Algae

Fermentation experiments were carried out using shaking incubators under controlled conditions (temperature and shake speed). Fermentation experiments were assigned to the three types of algae. Approximately 37.5 g of milled algae samples (*S. suae* and *N. commune*) and an un-milled algae sample (*C. formosanum*) were mixed with 187.5 mL sterilized sea water. Next, 75 mL precultured *P. haloplanktis* ATCC 14393 was added to the mixture, which was continuously shaken at 150 rpm and held at 22 °C during the 78 h fermentation. After fermentation, the mixture was centrifuged at 10,000× *g* for 20 min at 4 °C. The supernatant was collected and used to determine the water-soluble polysaccharide and pigment contents.

2.3. Two-Stage Fermentation

C. formosanum underwent a strong fragmentation using a FastPrep-24 5G (MP Biomedicals, LLC, Santa Ana, CA, USA) under the conditions 4.0 $m \cdot s^{-1}$ and 5 s for 6 cycles. After fragmentation, the mixture was centrifuged at 10,000× *g* for 20 min at 4 °C. The supernatant was then collected, and the algal precipitate was used in the subsequent fermentation. For the first phase of fermentation, approximately 30 g of extracted *C. formosanum* was mixed with 135 mL sterilized sea water. Next, 45 mL precultured *P. haloplanktis* ATCC 14393 was added to the mixture, which was continuously shaken at 150 rpm and held at 22 °C during the 6 h fermentation. After this first phase of fermentation, the mixture was centrifuged at 10,000× *g* for 20 min at 4 °C; then, some of the supernatant was collected and used to determine the concentration of phycobiliprotein, chlorophyll *a* and reducing sugar, and the rest was kept under the same conditions for subsequent fermentation. The second phase of fermentation followed, in which 25 g fermented algae from the first phase was mixed with 112.5 mL sterilized seawater and 37.5 mL *P. haloplanktis* ATCC 14393. The reaction was allowed to continue at 22 °C for 12, 24, 30, 36, 48, 54, and 60 h. A sufficient amount of fermented product was sampled every 6 h for a fermentation time 24 and 48 h (the important time points for industry utilized) during the second phase of fermentation. Except for the sampled amount of product, the rest was subsequently fermented under the same conditions.

2.4. Determination of Algae Moisture Content

The algae moisture content was determined by heating algae samples using an electronic moisture analyzer (MOC63, Shimadzu, Kyoto, Japan). The results shown by the analyzer were % moisture and weight in g.

2.5. Determination of Phycobiliproteins

Phycobiliprotein samples were evaluated using a spectrophotometer (Ultrospec 8000; Biochrom US, Holliston, MA, USA) to determine phycobiliprotein concentrations. In brief, 1 mL fermentation mix was collected at different time points at 6 h or 12 h intervals over the 72 h fermentation cycle. The mixture was centrifuged at 10,000× *g* for 20 min at 4 °C; then, the supernatants were collected and used to determine the contents at every check point. The absorbance of the supernatant was taken to determine PE (at 562 nm), PC (at 615 nm), and APC (at 652 nm) concentrations. The calculations of phycobiliprotein concentrations were based on the formulae described by Allen and Lawrence (1973):

$$PE\ (mg \cdot mL^{-1}) = [A562 - 2.41(PC) - 0.849(APC)]/9.62 \quad (1)$$