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Economical DHA (Docosahexaenoic acid) production from *Aurantiochytrium* sp. KRS101 using orange peel extract and low cost nitrogen sources

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ABSTRACT

The commercial production of microalgae-derived docosahexaenoic acid (DHA) has been expanding due to several advantages of algal over fish oil produced DHA. While the DHA production technology from microalgae is already economically competitive, alternatives to pure glucose and yeast extract still need to be explored to reduce the costs and increase the profits further. In the present study, orange peel waste (OPW) and various nitrogen sources were investigated as alternative nutrient sources for the economic cultivation of *Aurantiochytrium* sp. KRS101. The utilization of orange peel extract (OPE) supplemented with $NaNO_3$ showed a higher DHA yield than that supplemented with NH_4Cl or urea, and pH 5.5 was found to be the optimum initial condition for *Aurantiochytrium* sp. KRS101. OPE optimized with supplemental $NaNO_3$ (1.2 g/L) resulted in a DHA yield of 0.63 g/L, which was 2.5 fold greater than the yield obtained using a conventional basal medium containing a similar amount of total nitrogen and 67% greater total carbon sources. This result implied not only that the conventional use of glucose and yeast extract have lower efficiency levels during nutrient metabolism but also that the types of carbon and nutrient sources have a significant effect on the DHA yield. The addition of supplemental glucose further enhanced the biomass, fatty acid methyl esters (FAME), and DHA yields, which unveiled the high C:N ratio requirement of *Aurantiochytrium* sp. KRS101. These results suggest that the development and optimization of microalgae fermentation using OPE and $NaNO_3$ is a possible route for the economical production of DHA and for the additional utilization of food waste.

1. Introduction

The commercial production of DHA (docosahexaenoic acid) has been on the rise due to the growing awareness of DHA as an important dietary compound. It is known that regular uptake of DHA provides various health benefits; it prevents arteriosclerosis and coronary heart disease and can alleviate inflammatory conditions, asthma, depression, and rheumatoid arthritis [1–5]. The majority of DHA for human consumption is derived from fish oil sources (e.g., salmon, tuna, sardines) [6]. However, obtaining DHA from these fish has a number of disadvantages. First, fish oil from certain sources is associated with potential sustainability issues. For example, some claim that EPA (eicosapentaenoic acid) and DHA sourced from krill oil may have a negative impact on the food chains in the Antarctic Ocean. A stable supply of fish oil against increasing needs is not straightforward due to seasonal and climatic variations, over-harvesting, and population declines [7]. The most serious concerns, however, are heavy metals (e.g., mercury) and toxic environmental pollutants (e.g., polychlorinated biphenyls (PCBs) and dioxins), which are present at high levels in some fish oils [8]. Lastly, some reports complain over distinctive odors within fish oil, and purely vegetarian individuals may find its consumption problematic [9,10].

For these reasons, the production of microalgae has received much attention as an alternative means of procuring DHA for human consumption. In general, the production of polyunsaturated fatty acids (PUFA) from fish requires additional steps for separation and purification to meet industrial standards for DHA production. On the other hand, as primary producers, microalgae are positioned at relatively low trophic levels compared to fish and, hence, have much simpler fatty acid profiles for easier separation of PUFA [11]. More importantly, DHA in microalgae are more readily absorbed by humans, as this type of DHA tends to be more abundant in the polar fraction among microalgal lipids, such as phospholipids and free fatty acids [11].

Among microalgae, heterotrophic strains classified as the genus

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Thraustochytrium or *Schizochytrium* have been intensively studied, as heterotrophic cultivation typically results in higher biomass and PUFA productivity levels compared to photoautotrophic microalgae cultivation [12]. The growth media used in the fermentation industry always consists of a mixture of carbon, nitrogen, and micronutrient sources. As a carbon source, purified glucose and glycerol are used in the industry for the fermentation of high-value products, such as microalgal DHA [13,14]. The production cost of microalgal DHA is, thus, highly dependent on the price of the carbon sources used, as the carbon source is the most expensive component in fermentation media [15]. In conventional heterotrophic fermentation using eukaryotic organisms, glucose alone can account for 80% of the total cost [16]. Due to the prices of sugar reaching historic levels in the recent years, combined with the gradually decreasing market price of algal DHA, many large-scale algal DHA-producing companies, such as DSM, have been exploring the use of cruder forms of carbon sources, such as unrefined corn syrup [17]. In addition to glucose, yeast extract, commonly obtained via the processing of spent yeast from the brewery industry, is widely used in the large-scale fermentation industry as a major nitrogen source [18,19]. Therefore, the development of low-cost carbon and nitrogen sources can greatly increase the profitability of algal DHA production. In an effort to increase DHA production using low-cost growth substrates, empty palm fruit [20], sweet sorghum juice [21], and coconut water [22] have been investigated as alternative carbon sources. Studies of the heterotrophic cultivation of microalgae using organic waste may allow researchers to achieve the cost-efficient production of biomass and DHA. In the same context, orange peel waste also has great potential as a nutrient source for heterotrophic microalgae cultivation given that it also contains many other nutrients, such as amino acids and micronutrients that can support microalgae growth [23]. In fact, a previous study demonstrated the successful mixotrophic cultivation of *C. vulgaris* OW-01 using orange peel extract (OPE) [24].

Oranges, the most widely consumed fruit in the juice industry, are produced at a magnitude of 51.1 million metric tons per year worldwide, with the USA accounting for 7.33 million metric tons per year [25]. Among these amounts, oranges used for the manufacturing of orange juice constitute 12.25% of production. Approximately half of the fruit's biomass, which consists of solids such as pulps and peel, are generated as byproducts after juice production. Hence, millions of tons of orange peel are potentially available to be used as a substrate for microalgae growth [25,26]. However, despite the potential of OPE, the utilization of orange peels is limited, this far, to being used as low-cost cattle feed in the livestock industry [24].

The purpose of the present study is to assess the potential of OPE as a nutrient substrate for cost-efficient biomass and DHA production from the oleaginous microalgal strain *Aurantiochytrium* sp. KRS101. In addition, the performance capabilities of various nitrogen sources are assessed based on the DHA yield from *Aurantiochytrium* sp. KRS101 for the first time. The culture condition of *Aurantiochytrium* sp. KRS101 that yields the greatest amounts of biomass, FAME, and the highest DHA productivity rate using OPE was investigated through the optimization of the pH, the optimization of nitrogen in the medium and the additional supplementation of glucose.

2. Materials and methods

2.1. Algal strain and pre-culture conditions

Aurantiochytrium sp. KRS101, kindly provided by Dr. Jeong-Woo Seo (Korea Research Institute of Bioscience and Biotechnology, KRIBB), was used in this study. *Aurantiochytrium* sp. KRS101 cells were maintained in a modified basal medium [27], which consisted of 60 g/L glucose, 10 g/L yeast extract, 9 g/L KH_2PO_4, 15 g/L sea salt (Sigma Aldrich, USA) and 10 mg/L tetracycline [28]. Cells were cultivated in 250 mL baffled flasks with a 100 mL working volume at 28 °C, while shaking at 120 rpm. For long-term storage, 900 μL of an *Aurantiochytrium* sp. KRS101 cell suspension was mixed with an equivalent volume of 50% glycerol in a 2 mL cryotube (SPL, Korea). The tubes were directly stored at − 80 °C in a freezer.

2.2. Preparation of orange peel extract

Orange peel waste was obtained from a squeezing juice factory (CJ Cheiljedang) in Korea, stored in a deep-freezer and used after thawing. Based on the size of the particles and the physical properties of the mixture, 200 g of orange peel waste was homogenized using a commercial blender (Type HR 2011, Philips, USA) with 1 L of 50% sea water (500 mL distilled water and 500 mL natural sea water). After blending, the mixture was subjected to extraction at room temperature for 24 h, and solid waste was removed using a sieve (0.2 mm) and a 0.7 μm GF/F glass microfiber filter (Whatman, USA).

2.3. Nitrogen source screening and optimization of the nitrate concentration

In order to find a suitable nitrogen source that can replace the yeast extract of a basal medium, various nitrogen sources were tested. Three nitrogen sources (1.5 g/L of sodium nitrate, 0.994 g/L of ammonium chloride and 0.53 g/L of urea) containing equivalent concentrations of nitrogen (17.64 mM) were added into the OPE. The pH was then fixed at 5.5 using 1 M KOH. Deeply frozen stock cells were thawed in an ice bucket and 1 mL of the cell suspension was inoculated into 100 mL of the OPE medium and cultivated at 28 °C with agitation at 120 rpm for 3 days. In addition, nitrogen source screening with fixed pH was also conducted. A photobioreactor (CNS, Korea) was used for fixed pH experiments. As for the nitrogen source, 1.5 g/L of sodium nitrate, 0.994 g/L of ammonium chloride and 0.53 g/L of urea were added into each photobioreactor containing 2 L OPE. The pH was fixed between 5 and 5.25 using 2 N KOH and HCl. The seed culture was prepared with the same method as above, and cultivation was conducted at 28 °C and 120 rpm for 4.5 days.

2.4. pH optimization

Using 1.5 g/L sodium nitrate, pH optimization was conducted. The pH of the orange peel extract was adjusted to 5.5, 7.0, and 8.5 with 1 M KOH. The cell inoculation method and the culture conditions were identical to those used in the nitrogen source screening experiment.

2.5. Optimization of the nitrate concentration

The concentration of sodium nitrate was optimized to achieve the highest DHA yield. Amounts of 0.9, 1.2, and 1.5 g/L of sodium nitrate were added to each OPE and the pH was adjusted to 5.5 using 1 M KOH. The cell inoculation method and culture conditions were identical to those used in the nitrogen source screening experiment.

2.6. Additional supplementation of glucose

In order to further optimize the C:N ratio, additional amounts of 2.5 and 5 g/L of glucose were supplemented into the OPE media containing 1.5 g/L sodium nitrate. The initial pH was adjusted to 5.5 using 1 M KOH. The cell inoculation method and culture conditions were identical to those used in the nitrogen source screening experiment.

2.7. Growth analysis

Cell growth was determined by measuring the optical density (OD) and dry cell weight (DCW). OD600 values were measured by a UV/visible spectrometer (Beckman Coulter, USA). The DCW was estimated by harvesting 50 mL of the cell culture, washing it twice with PBS buffer, re-harvesting at 1719 × *g* for 5 min, freeze-drying for 2 days and weighing the culture on a microbalance (CP224S, Sartorius, Germany).