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Hydrothermal-acid treatment for effectual extraction of eicosapentaenoic acid (EPA)-abundant lipids from *Nannochloropsis salina*

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HIGHLIGHTS

- Hydrothermal acid treatment was proposed to extract EPA.
- Optimal conditions for EPA extraction from *N. salina* were determined.
- Sulfuric acid was a better acid catalyst than nitric acid for EPA extraction.

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ABSTRACT

Hydrothermal acid treatment, was adopted to extract eicosapentaenoic acid (EPA) from wet biomass of *Nannochloropsis salina*. It was found that sulfuric acid-based treatment increased EPA yield from 11.8 to 58.1 mg/g cell in a way that was nearly proportional to its concentration. Nitric acid exhibited the same pattern at low concentrations, but unlike sulfuric acid its effectiveness unexpectedly dropped from 0.5% to 2.0%. The optimal and minimal conditions for hydrothermal acid pretreatment were determined using a statistical approach; its maximum EPA yield (predicted: 43.69 mg/g cell; experimental: 43.93 mg/g cell) was established at a condition of 1.27% of sulfuric acid, 113.34 °C of temperature, and 36.71 min of reaction time. Our work demonstrated that the acid-catalyzed cell disruption, accompanied by heat, can be one potentially promising option for ω-3 fatty acids extraction.

1. Introduction

Eicosapentaenoic acid (hereafter: EPA), one type of ω-3 polyunsaturated fatty acids (PUFAs), plays an important role in the regulation of biological functions; it is involved in the prevention and treatment of a number of human diseases such as heart and inflammatory diseases (Wen and Chen, 2003). EPA also takes part in many membrane functions and serves as a precursor of a variety of lipid regulators in cellular metabolism (Gill and Valivety, 1997). This fatty acid, essential as it is, is not synthesized by the human body (Nettleton, 1995); it can be supplemented via transformation from short-chain ω-3 fatty acids (e.g., α-linolenic acid found in vegetables), but the bodily conversion efficiency is below 5% (Gerster, 1997; Brenna, 2002). It is this reason that there has been considerable interest in developing processes for its commercial production (Belarbi et al., 2000).

Marine fish oil is the richest source for EPA; however, its purification remains challenging because of its peculiar taste, odor, and complexity (Barclay et al., 1994; Belarbi et al., 2000). Not only that, marine fish stocks are subjected to seasonal and climatic variations (Gill and Valivety, 1997). The fish, just like human, is unable to synthesize EPA, but rather takes it up from microalgae consumed (Falk-Petersen et al., 1998).

Since microalgae are microbes almost solely able to anabolize PUFAs including EPA, substantial efforts are being made to develop technologies to realize their potential (Lebeau and Robert, 2003; Molina Grima et al., 2003). Dewatering from EPA-producing microalgae is one of key steps, particularly critical for minimum use of chemicals needed for the downstream process of lipid extraction. Unfortunately, however, it is energy-intensive, increasing the cost of EPA. That was why direct oil extraction from wet microalgae has been thought of as the ultimate way to go (Catchpole et al., 2010; Park et al., 2014). Microalgal lipids, specifically EPA-containing neutral lipids (triglyceride), are not readily extractable without some kinds of cell disruption (Lee et al., 2014; Lee and Han, 2015), mainly on account of their being enveloped in the rugged cell wall like cellulose. Most commonly employed methods for cell disruption include bead-beating, microwaving, sonication, osmotic shock, high-pressure

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homogenization, and supercritical carbon dioxide (Halim et al., 2011, 2013; Lee et al., 2010). These effective physico-chemical means, however, further heighten the overall energy consumption (Harun and Danquah, 2011).

Acid-catalyzed cell disruption, accompanied by heat, is another noteworthy alternative avenue. This thermochemical method, which is most widely practiced for the pretreatment of cellulosic biomass, bases on the acid hydrolysis of sugar polymers in cell walls, which can only take place and/or be accelerated at elevated temperatures (Hsu et al., 2010; Kim et al., 2014; Mosier et al., 2005). It is reasonable to believe that microalgae cells respond at least similarly if not more sensitively, as many of species are known to have cellulose-containing cell walls (Hu et al., 1998; Nguyen et al., 2009). It is highly likely that the acid-treated algae cells become vulnerable to a subsequent solvent extraction, releasing oils to a highest possible degree (Kates, 1986). In this study, sulfuric acid and nitric acid were selected as acid catalysts. Sulfuric acid is the most commonly used acid catalyst due to its high effectiveness and also low cost. Commercial unit price of sulfuric acid (185 $/ton) is lower than other inorganic acids, such as nitric acid (260 $/ton), phosphoric acid (400 $/ton), and hydrofluoric acid (300 $/ton) (Lee et al., 2014). Nitric acid is equally potent (Kim et al., 2014; Lee et al., 2014), and in theory, can be produced in an economical manner from NO_X in flue gases using an innovative technology developed by our research group (Kim and Han, 2013).

The primary aim of this study was therefore to explore if the hydrothermal acid pretreatment was applicable to the extraction of EPA-abundant lipids. To this end, sulfuric acid and nitric acid were selected and tested for their effectiveness, in terms of lipid extractability and EPA content. Response surface methodology was employed to establish the optimal pretreatment conditions for maximum EPA yield in terms of acid concentration, reaction time, and temperature.

2. Methods

2.1. Microalgae cultivation and harvesting

Nannochloropsis salina (*N. salina*), a microalgae species known to be a potent EPA producer (Sukenik, 1991). *N. salina* was maintained in a medium composed of $NaNO_3$ (200 mg/L), $NaH_2PO_4{\cdot}2H_2O$ (25 mg/L), $MnCl_2{\cdot}4H_2O$ (0.6 mg/L), $FeCl_3{\cdot}6H_2O$ (0.15 mg/L), $ZnCl_2$ (0.06 mg/L), and $CoCl_2{\cdot}6H_2O$ (0.015 mg/L). Mass cultivation was conducted in an outdoor raceway pond with a flow rate of 10 ton/d from the Jiyang lake water (Hadong, Korea). The cultured algal cells were harvested by ultrafiltration so that its final concentration became 20 g/L of algae slurry.

Total lipids were extracted from lyophilized biomass using a mixed solvent containing the following mixture pairs: hexane (96%), hexane:methanol (99.6%) = 7:3 (v/v), and chloroform (99%): methanol = 2:1 (v/v), and the ratio of biomass-to-solvent was adjusted to 5 wt.%. The mixture was stirred at 1000 rpm for 6 h at room temperature, and then separated into the bottom cell debris layer and the top organic-solvent layer by 4000 rpm centrifugation for 10 min. Finally, the solvent was removed from the lipids-containing organic-solvent layer using a vacuum evaporator (EZ2 PLUS, Genevac, UK), and the lipids were recovered. The total lipid content was determined by measuring the residues, and its detailed fatty acid composition is summarized in Table 1.

Table 1
Total fatty acid content of *Nannochloropsis salina*.

Fatty acid composition	Amounts of fatty acids (mg/g)
Myristic acid (C14:0)	8.49 ± 0.86
Palmitic acid (C16:0)	25.47 ± 2.11
Palmitoleic acid (C16:1)	40.39 ± 3.04
Stearic acid (C18:0)	0.54 ± 0.08
Oleic acid (C18:1)	9.59 ± 0.52
Linoleic acid (C18:2)	1.85 ± 0.13
α-Linolenic acid (C18:3)	0.82 ± 0.38
Eicosapentaenoic acid (C20:5)	70.66 ± 4.02
Others	31.62 ± 2.97
Total fatty acid/microalgae	189.43 ± 14.11

2.2. Pretreatment and lipids extraction

Acid pretreatment was carried out at acid concentrations from 0.1% to 2.0% (v/v) using 70% sulfuric acid (Samchun, Korea) or 70% nitric acid (Samchun, Korea). Mixtures of acid and algae biomass were heated to 120 °C for 20–60 min using autoclave (AC-14, Jeio-tech, Korea). All experiments were done three times to minimize any random errors.

Algal lipids were extracted from the pretreated samples using a modified method of Hara and Radin (1978). Each pretreated algal sample was mixed with 96% hexane (Junsei, Japan) for 1 h in a 250-mL Erlenmeyer flask with a screw-cap. The extracted hexane–lipid complex was blended with deionized water to a 1:1 ratio to remove any impurities and to clearly distinguish the layers by density. The pure lipid fraction isolated in hexane was recovered by evaporating only the solvent using a vacuum evaporator. Lipid yield (g extracted lipid/g total lipid content × 100 in wt.%) of each pretreatment was measured using the weight of the recovered lipid.

2.3. EPA analysis

EPA contained in the extracted lipids underwent transesterification, and its resulting fatty acid methyl esters (FAMEs) were analyzed using a modified method of Cho et al. (2011). The extracted lipid (10 mg) was separated and placed in a 10 mL culture tube, to which chloroform–methanol (1.98 mL; 2:1, v/v) was added. The mixture was then vigorously agitated using a vortex mixer (Vortex genie 2™, Fisher Scientific Inc., USA) for 10 min. One milliliter of chloroform containing heptadecanoic acid (0.5 mg/L) as an internal standard, 1.02 mL of methanol, and 0.3 mL of sulfuric acid (95.0%, Samchun, Korea) were added to the tube in sequence. The tube was vortex-mixed for 10 min, and then immersed in water at 100 °C for 10 min using a water bath (WB-11, Daihan Scientific, Korea). After cooling at room temperature, 1 mL of deionized water was supplemented to the tube, and vortex-mixed for 5 min. To clearly segregate the layer of solution clearly, the tube was centrifuged at 4500 rpm for 10 min. The organic phase of bottom layer was taken using a syringe with a 0.2 μm PVDF syringe filter (Millex-GV; Millipore, USA). FAMEs content containing EPA were analyzed using a gas chromatograph (GC; Agilent 7890A, Agilent technologies, USA) equipped with a flame ionized detector and a 30 m × 320 μm × 0.5 μm capillary column (HP-INNOWax, Agilent technologies, USA). GC was operated as follows: starting temperature (50 °C), condition of temperature increase (until 200 °C at a ratio of 15 °C/min for 9 min; until 250 °C at a rate of 2 °C/min for 2 min).

2.4. Experimental design

Response surface methodology (RSM) was used to optimize the hydrothermal acid pretreatment conditions influencing on EPA yield (Y). The key variables were sulfuric acid concentration (X_1), pretreatment temperature (X_2), and reaction time (X_3). The experimental conditions for the RSM were as follow: acid concentrations