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Ultrasound-assisted *in-situ* transesterification of wet *Aurantiochytrium* sp. KRS 101 using potassium carbonate

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ABSTRACT

A new *in-situ* transesterification method was developed for wet biomass: K_2CO_3 was used as an alkaline catalyst and, *Aurantiochytrium* sp. KRS 101 as oleaginous DHA-producing microalgae. It was found that the presence of water greatly impaired the overall efficiency even with the powerful catalyst that had worked surpassingly well with dry biomass, and thus a mechanical aid like ultrasonication was needed to make advantage of full potential of the alkaline catalyst. The total fatty acid ethyl ester (FAEE) recovery yield of 94.6% was achieved with sonication at 100 g/L of biomass (40% moisture), 3% of K_2CO_3, 70 °C and 30 min. All these suggest that the ultrasound assisted *in-situ* transesterification can offer a feasible means for FAEE recovery and it was so by way of overcoming the physical limitation of mass transfer caused the presence of water and providing effective contacts between reactants.

1. Introduction

Daily intake of omega-3 fatty acids like docosahexaenoic acid (DHA) is proven to be beneficial for promoting health and it is particularly effective with arthritis, asthma, dementia, depression, migraine headaches, and schizophrenia (Hur et al., 2002; Turunen et al., 2013). Its commercial production relies almost exclusively on fish; this likely poses a problem in terms of securing the source and is especially so in view of rapidly growing demand of the product (Puri, 2017). In addition, the current products, because of being from fish, always have a possibility of being contaminated with heavy metals: and this acts as a hurdle to more rapid and wider spread of consumption of the otherwise almost ideal food supplement.

Microalgae, free from all such limitations, would be a perfect replacement for fish. What is better, microalgae-derived DHAs are known to be highly stable to oxidation and less toxic (Sijtsma and De Swaaf, 2004). *Aurantiochytrium* sp., a heterotrophic microalgae species, is uniquely suited for the purpose, as it is a superb oleaginous microbe (56.8% of total lipids) with a high proportion of DHA (a functional component) and palmitic acid (a best fatty acid for biodiesel) (Chang et al., 2013; Kim et al., 2016). The production of the aimed products either a fuel of fatty acid alkyl ester or FAAE (i.e., FAEE with ethanol) or DHA-enriched fatty acid alkyl ester, DHA-AE) from microalgae including *Aurantiochytrium* sp. is comprised of several steps: namely, cultivation, harvesting (dewatering), lipid extraction, and oil conversion. The whole process is fairly expensive (Kim et al., 2015a). Since the downstream process from harvesting to oil conversion accounts for 60% of the total production cost (Kim et al., 2015b), the heterotrophic cultivation is advantageous in that it produces highly concentrated biomass and thus the burden of the harvesting/dewatering steps can be reduced to a substantial degree. *In-situ* transesterification, which is to process lipid extraction and oil conversion in a simultaneous way, is another way of further reducing the process cost, and thus there have been a good many studies with some interesting results (Kumar et al., 2014; Park et al., 2015; Velasquez-Orta et al., 2012). Resulting products of this transformation include the desired DHA alkyl esters (DHA AE), which is a DHA form approved by the US Food and Drug Administration (FDA), and has been utilized as prescription drugs for severe hypertriglyceridemia treatment (Lopez-Toledano et al., 2017).

Preferred catalysts for *in-situ* transesterification of microalgae are acids such as sulfuric acid (Ehimen et al., 2010; Johnson and Wen, 2009; Kim et al., 2015b; Im et al., 2014) and hydrochloric acid (Kim et al., 2015a), largely because microalgae oils contain high contents of free fatty acids which the *in-situ* method tends to cause to undesired saponification (Dong et al., 2013). As long as triglyceride is a predominant form of lipid as in the case of *Aurantiochytrium*, however, alkaline catalysts are surpassingly better, in terms of fast reaction rate, mild reaction condition and less inhibitor formation (Mosier et al., 2005; Kim et al., 2013). Even more, the alkaline catalyst was previously reported to be better tolerable towards water (Harvey et al., 2008), which is a particularly important property in the wet-based transformation. K_2CO_3 is poised to be the most promising alkaline catalyst of

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all: it is so because soap formation is further limited due to bicarbonate formation instead of water during transesterification reaction (Ejikeme et al., 2010; Sung and Han, 2016).

In our previous study, *in-situ* transesterification of dry *Aurantiochytrium* sp. KRS101 yielded an impressive recovery of fatty acid methyl esters (FAMEs) with K_2CO_3 as an alkaline catalyst and methanol as alcohol (Sung and Han, 2016). To make this potent transformation method more practical in terms of economic feasibility, dry biomass, which was used in the previous study, was replaced with wet biomass in this study; it eliminates the cost for biomass drying cost. Also, ethanol was substituted for methanol and reasons are as follows. First, ethanol, due to its lower polarity and thus better miscibility with both water and lipid than methanol, is capable of extracting lipids especially from wet biomass in a more effective manner (Kim et al., 2015b). In addition, ethanol, particularly along with a high concentration of K_2CO_3, causes to form two separate layers: a solvent-rich phase with limited salts and a water-rich phase with low alcohol, a phenomenon called salting-out (Salabat and Hashemi, 2006). This salting-out effect allows the lipids extracted from microalgae to remain in the organic phase containing very low salts, and thus saponification, which is a critical issue for the alkaline-based conversion for microalgae oils, can effectively be prevented.

Ultrasound was also employed to facilitate the critically impaired mass transfer for wet microalgae treatment. The presence of water interfered with mass transfer of the extraction solvent, resulting in a substantial decrease in yield (Halim et al., 2012). The ultrasound method has been adopted and verified for its effectiveness in the *in-situ* transesterification in previous studies. Guldhe et al. (2014) reported that ultrasound assisted transesterification showed higher conversion yield of *Scenedesmus* sp. as compared with microwave at low reaction temperature. Veljković et al. (2012) in their review article concluded that ultrasound-assisted process showed similar or better performance, in terms of yield and reaction time, than other methods including mechanical stirring, microwave, and hydrodynamic cavitation.

The purpose of this study was therefore to establish a new condition of the K_2CO_3-based *in-situ* transesterification of wet *Aurantiochytrium* sp. KRS 101 and apply ultrasound method to maximize the efficiency of wet *in-situ* transesterification.

2. Materials and methods

2.1. Microorganism preparation

Aurantiochytrium sp. KRS 101 was obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Republic of Korea) and cultivated in a nutrient medium containing the following ingredients: 50 g/L of glucose, 10 g/L of yeast extract, 9 g/L of KH_2PO_4, 5 g/L of sea salt, and 10 mg/L of tetracycline, in a 500 L bioreactor for 3 days at 28 °C and 0.5 v/v/min of air. The pH of the culture was 6. After cultivation, microalgae biomass was harvested by centrifugation and lyophilized to maintain cell condition during storage and experiment periods.

2.2. In-situ transesterification

Wet biomass solution was prepared using lyophilized biomass with water, and potassium carbonate and ethanol were mixed with the biomass slurry. *In-situ* transesterification was proceeded as follows: (1) stirring at 800 rpm in a 250-mL Erlenmeyer flask with a screw-cap, and (2) sonicated with an operating frequency of 20 kHz, power of 750 W, and amplitude of 20% (Cole-Parmer model 04177-40). To ascertain uniform irradiation, the ultrasonic processor probe was submerged in the center of the biomass mixture in the reactor, with the top part sealed to prevent solvent volatilization during treatment. Operational variables were as follows: 20–80% (w/w) of water content in the biomass solution, 25–90 °C of temperature, 10–60 min of reaction time, and 0–10% (w/v) of alkaline catalyst concentration in the ethanol. Experiments were conducted in triplicate. The chemicals used were methanol (99.9% purity, Duksan Chemicals, Korea), ethanol (99.5% purity, Samchun Chemicals, Korea), hexane (95.0% purity, Samchun Chemicals, Korea), chloroform (99.5% purity, Samchun Chemicals, Korea), sulfuric acid (95.0% purity, Sigma Aldrich, USA), potassium carbonate (99.5% purity, Junsei Chemicals, Japan), which were all of analytical grade.

2.3. Determination of FAEE recovery yield of the in-situ transesterification from gas chromatography (GC) analysis

After the *in-situ* transesterification, a treated sample was cooled to room temperature and mixed with hexane at a volumetric ratio of 5:4 (v/v) at 800 rpm for 1 hr. Afterwards each mixture was washed with distilled water and then centrifuged at 4000 rpm for 5 min to separate phases. The upper layer containing fatty acid ethyl esters (FAEEs) was lastly analyzed to measure FAEEs by a gas chromatography (GC) (HP5890; Agilent, CA). The recovery yields of FAEEs were calculated by the following equation.

$$\text{FAEE recovery yield} = \frac{\text{The amount of obtained FAEE}\left(\text{mg}\frac{\text{FAEE}}{\text{mg}}\text{cell}\right)}{\text{The saponifiable lipid content of algal cell}\left(\text{mg}\frac{\text{saponifiable lipid}}{\text{mg}}\text{cell}\right)} \times 100(\%)$$

The original saponifiable lipid content of dry *Aurantiochytrium* sp. was analyzed by the GC according to Sung and Han (2016), and the value was 418.1 mg saponifiable lipid/g cell.

3. Results and discussion

3.1. Effects of water content and temperature on FAEE yield

To investigate the effect of water content for *in-situ* transesterification of wet microalgae, 0 to 80% of water content was tested with other parameters fixed: 50 g/L of biomass concentration, 3% of K_2CO_3/ethanol, 60 °C of reaction temperature, and mixing by stirring at 800 rpm for 60 min (Fig. 1(a)). Dry biomass control failed to produce a high yield of fatty acid ethyl esters (FAEEs) that was easily achieved with methanol. This was because K_2CO_3 was basically insoluble in ethanol and therefore ethoxides ($CH_3CH_2O^-$) was not formed to a sufficient extent. With ethanol, water proved to behave cooperatively, rather than detrimentally: its existence, along with mixing, aided in generating ethoxides, thereby giving rise to *in-situ* transesterification without soap formation. The FAEE recovery yield was found to be highest at 40% of water content (almost 80% FAEE recovery yield) and at higher contents of water decreased. This result was quite interesting, as it has been generally known that water of the biomass has a negative impact on the catalyzed *in-situ* transesterification reaction (Ehimen et al., 2010).

The effect of reaction temperature on the FAEE yield from wet *Aurantiochytrium* sp. was examined with temperature varied from 25 to 90 °C (Fig. 1(b)). Conversion of pure oil to FAME is typically proceeded at temperature lower than 60 °C (Veljković et al., 2012). It is not the case for microalgae cells and even more so for wet biomass: bound water molecules protect the already hard microalgae cells in a way that makes it difficult for catalyst and reactants to penetrate them and reach lipids (Cao et al., 2013). The FAEE recovery yield increased dramatically from 40 to 60 °C and above 70 °C, remained at maximum levels. It was found that the optimal temperature was 70 °C, at which 76.7% FAEE recovery yield resulted. Considering only room temperature was needed for the dry biomass (Sung and Han, 2016), the existence of water indeed interfered with the cell disruption and/or oil extraction and/or FAME conversion.