Journal of Functional Foods 21 (2016) 330–337

Available online at www.sciencedirect.com

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Preparation of high purity docosahexaenoic acid from microalgae oil in a packed bed reactor via two-step lipase-catalysed esterification

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ARTICLE INFO

Article history:
Received 5 September 2015
Received in revised form 10 December 2015
Accepted 11 December 2015
Available online 31 December 2015

Keywords:
Crypthecodinium cohnii
Docosahexaenoic acid (DHA)
Esterification
Lipase
Microalgae
Rhizomucor miehei

ABSTRACT

Highly pure docosahexaenoic acid (DHA) was produced successfully in a packed bed reactor via the two-step lipase-catalysed esterification using the fatty acid from microalgae (from *Crypthecodinium cohnii*) and ethanol as substrates. Lipozyme RM IM from *Rhizomucor miehei* was employed as a biocatalyst. In the first step, a temperature of 40 °C, a molar ratio of 1:4 (fatty acid to ethanol), and a water content of 0.6 wt% (based on the total substrate weight) were selected as optimum conditions. A maximum DHA concentration of ca. 90 wt% was achieved in the fatty acid fraction with an 89 wt% yield after 15 min. In the second step, the fatty acids from the first step were used as the substrate and a recursive reaction was conducted using identical optimized conditions. Following this second step, a DHA concentration of 100 wt% was achieved in conjunction with an 83.2 wt% yield after 25 min.

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1. Introduction

Docosahexaenoic acid (DHA, C22:6) is an *n*-3 polyunsaturated fatty acid (PUFA) with numerous physiological benefits (Broadhurst et al., 2002). There has been considerable interest in its health benefits, especially with regard to cardiovascular health (Lopez-Huertas, 2010; Williams & Burdge, 2006). The importance of DHA consumption has been recognized on the basis of increasingly strong evidence of its connection with the early visual and neurological development in neonates (Agostoni, Riva, Trojan, Bellu, & Giovannini, 1995; Birch, Garfield, Hoffman, Uauy, & Birch, 2000; Carlson, Werkman, Rhodes, & Tolley, 1993; Makrides, Neumann, Simmer, Pater, & Gibson, 1995). For these

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http://dx.doi.org/10.1016/j.jff.2015.12.020
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reasons, concentration of DHA in natural sources, such as fish oil, has been widely studied using various methods including fractional distillation, low-temperature crystallization, urea complexation, and selective enzymatic method. Among these, selective enzymatic reactions are ideally suited to the fractionation of DHA because of the mild reaction, eco-friendly nature, regiospecificity, and minimal formation of side reaction product (Patil, 2014).

There are several reports in the enrichment of *n*-3 PUFA via lipase-catalysed esterification of fatty acids from fish oil with different alcohols. Shimada et al. (1997) reported that DHA was enriched to ca. 73% from ca. 20% of starting material via *Rhizopus delemar* lipase-catalysed selective esterification of the fatty acid from tuna oil with lauryl alcohol. Halldorsson, Kristinsson, Glynn, and Haraldsson (2003) reported that ca. 71% DHA enrichment was obtained via the esterification of fatty acids from tuna oil with glycerol using *Rhizomucor miehei* lipase. For several decades, fish oil has been the most readily accessible DHA source, although its appeal is limited because of its unfavourable smell and taste. In addition, the complex fatty acid profile of fish oil makes it difficult to obtain DHA solely, because other fatty acids, such as eicosapentaenoic acid (EPA, C20:5 *n*-3), are concentrated together with DHA during the purification process. This can be especially problematic when producing DHA for use in infant formulas, since EPA is reportedly linked with neonate growth retardation (Carlson et al., 1993). On the contrary, microalgae single cell oil is increasingly considered as a promising vegetarian DHA source that may overcome the disadvantages of fish oil. As an example, DHA single cell oil (DHASCO) from *Crypthecodinium cohnii* microalgae contains more than 40% of DHA and less than 1% of EPA, thus allowing DHA to be readily isolated in high purity (Behrens & Kyle, 1996; Harrington & Holz, 1968).

Packed bed reactor (PBR) systems have several advantages over reactions in a batch mode. PBR system can be operated continuously and the removal of enzyme from the product is not necessary (Chen, Huang, Lin, & Shang, 2010; Laudani, Habulin, Knez, Della Porta, & Reverchon, 2007). In addition, these systems are cost-effective since they can increase the stability of the enzyme (Du, Li, Sun, Chen, & Liu, 2008). Therefore, PBR systems are suitable for lipase-catalysed esterification in the industrial applications (Xu, Balchen, Hoy, & Adler-Nissen, 1998). There are several studies on the enrichment of *n*-3 PUFA from microalgae oil using various methods. Medina et al. (1995) reported that DHA was enriched efficiently up to ca. 92.2% from the oil in *Isochrysis galbana* microalgae by using urea complexation combined with preparative high performance liquid chromatography. Senanayake and Shahidi (2000) reported that high purity DHA concentrate (ca. 97.1%) was prepared by urea complexation of fatty acids of microalgae oil from *C. cohnii*. Fajardo et al. (2006) concentrated EPA up to 71% from oil in *Phaeodactylum tricornutum* microalgae by *Candida rugosa* lipase-catalysed esterification. EPA and arachidonic acid (C20:4 *n*-3) were also concentrated from the oil in *Porphyridium cruentum* microalgae by the combination of urea complexation and argentated silica gel column chromatography (Guil-Guerrero, Belarbi, & Rebolloso-Fuentes, 2000). However, to the best of our knowledge, there has been little study on the preparation of highly purified DHA (purity > 99%) from microalgae oil via only enzymatic method in a continuous reactor system.

In the present study, pure DHA was obtained from DHASCO in a PBR system via the two-step lipase-catalysed esterification of the fatty acid with ethanol. The PBR employed in this study was a mini scale system specially made in our laboratory. In the first step, fatty acids from DHASCO were esterified with ethanol by using Lipozyme RM IM (from *R. miehei*) as a biocatalyst. The effects of temperature, molar ratio (fatty acid to ethanol), and water content were investigated with regard to the resulting DHA concentration and yield. As a second step, the DHA-enriched fatty acids from the first step were further purified by a recursive esterification with ethanol.

2. Materials and methods

2.1. Materials

DHASCO is purchased from Martek Biosciences (Boulder, CO, USA). Absolute ethanol (≥99.9%) was obtained from Daejung Chemicals & Metals Co., Ltd (Cheongwon, Republic of Korea). The immobilized lipase of Lipozyme RM IM (from *R. miehei*) was purchased from Novozymes (Seoul, Republic of Korea). The activity and type of carrier of the enzyme was 48 U/mg and macroporous anion exchange resin. All of the other solvent and reagents used in this study were purchased as the analytical grade unless otherwise noted.

2.2. Preparation of fatty acids from DHASCO

DHASCO was initially converted to the fatty acids and used as a substrate. DHASCO (150 g) was added to a solution of sodium hydroxide (60 g) in a mixture of distilled water (150 mL) and ethanol (99%, 450 mL). The mixture was refluxed with stirring at 500 rpm and 65 °C for 1 hour under nitrogen stream and then transferred to a 2 L separatory funnel, following which distilled water (300 mL) was added to the saponified mixture. The aqueous layer containing the saponifiable matter was acidified by adding 180 mL of 6 N HCl and then adjusted to pH 1 to generate the free fatty acids. The resulting lower layer was discarded and the upper layer containing the fatty acid was extracted into 300 mL of *n*-hexane. The hexane layer was washed twice with 150 mL of distilled water and dried over anhydrous sodium sulfate. Then *n*-hexane was removed from the fatty acid by evaporation in a rotary vacuum evaporator at 40 °C. Any residual hexane in the fatty acid was removed completely by nitrogen flushing in a water bath of 40 °C. Thin Layer Chromatography (TLC) was applied to ensure complete saponification, and only free fatty acid spot was detected. The oil recovery after saponification was ca. 85 wt%.

2.3. Lipase-catalysed esterification in a PBR

A small stainless steel column (Swagelok, Salon, OH, USA) was used as a tubular PBR. The dimensions of the reactor were 6.5 cm (length) × 4.65 mm (internal diameter), and Lipozyme RM IM from *R. miehei* (0.39 g) was packed manually into the reactor. A syringe pump (Model 200; KD Scientific, New Hope, PA, USA) was used to feed the substrate mixtures continuously into the PBR, and the PBR was immersed in a water bath

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