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# Enrichment of docosahexaenoic acid from tuna oil via lipase-mediated esterification under pressurized carbon dioxide

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ABSTRACT

This study focused on the use of pressurized $CO_2$ as a reaction medium for the enrichment of docosahexaenoic acid (DHA) from tuna oil fatty acids via lipase-mediated esterification. Of the three lipases tested, Lipozyme RM IM from *Rhizomucor miehei* was selected for further study. Enzyme loading, water addition, and reaction time were also explored. Near-supercritical $CO_2$, prepared at 25 °C and 8.3 MPa, was the most effective reagent tested for enriching DHA from the residual fatty acid fraction. In addition to near-supercritical $CO_2$, optimal conditions included addition of 0.2 wt% (based on total substrates) water, enzyme loading of 5 wt% (based on total substrates), and a reaction time of 18 h. The DHA concentration and recovery yield for the residual fatty acid fraction under these optimal conditions were 75.8 wt% and 81 wt%, respectively.

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## 1. Introduction

Fatty acids such as *n*-3 polyunsaturated fatty acids, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) have specific pharmacological and physiological effects on human health, such as cholesterol reduction and the prevention and treatment of circulatory system problems such as atherosclerosis [1,2]. Many studies of the enrichment of essential fatty acids have been carried out because these purified fatty acids are potentially useful in many fields. Various enzymatic reactions have been studied, including hydrolysis [3,4], transesterification [5], esterification [6–8], alcoholysis [9], and a combination of hydrolysis and esterification [10]. The direct esterification of fatty acids with ethanol has been shown to reduce by-product formation and the complications of regioselectivity and substrate selectivity, unlike transesterification of triacylglycerol with ethanol [11].

Non-aqueous solvents may be appropriate for some enzyme-mediated reactions. These solvents increase the solubilities of hydrophobic compounds and reduce side reactions. They also stabilize enzymes and ease recovery [12]. New supercritical fluid solvents have also been tested for this type of reaction. Pressurized $CO_2$ is an excellent solvent because of its liquid-like solvating ability. Like all gases, it has low viscosity, high diffusivity, and low surface tension, allowing it to penetrate easily throughout macro- and microporous materials [13]. The low viscosity and high diffusivity of pressurized $CO_2$ allow a substrate to diffuse more easily into the pores of an immobilized carrier for contact with an enzyme [14]. Kamat et al. [15] reported that pressurized $CO_2$ below 40 °C readily reacted with the free amino groups on the surface of an enzyme to form a carbamate–enzyme complex via covalent bonding. Muralidhar et al. [16] showed that the pressure could be used to control enzyme activity and stereoselectivity independently. In particular, near-supercritical $CO_2$ could trigger enzyme activation by moving surface groups and creating active sites, thereby enhancing stereoselectivity [17]. Various enzymatic reactions have been attempted in supercritical $CO_2$ because of these advantages [18,19]; of these, several were successful [17,20]. However, value-added fatty acids such as γ-linolenic acid, EPA, and DHA have been primarily produced by supercritical $CO_2$ extraction, based on the effect of the density of supercritical $CO_2$ on solubility differences between fatty acids and fatty acid ethyl esters [21,22]. There have been a few studies of the production of value-added fatty acids using enzymatic reactions in pressurized $CO_2$. In this study, we developed

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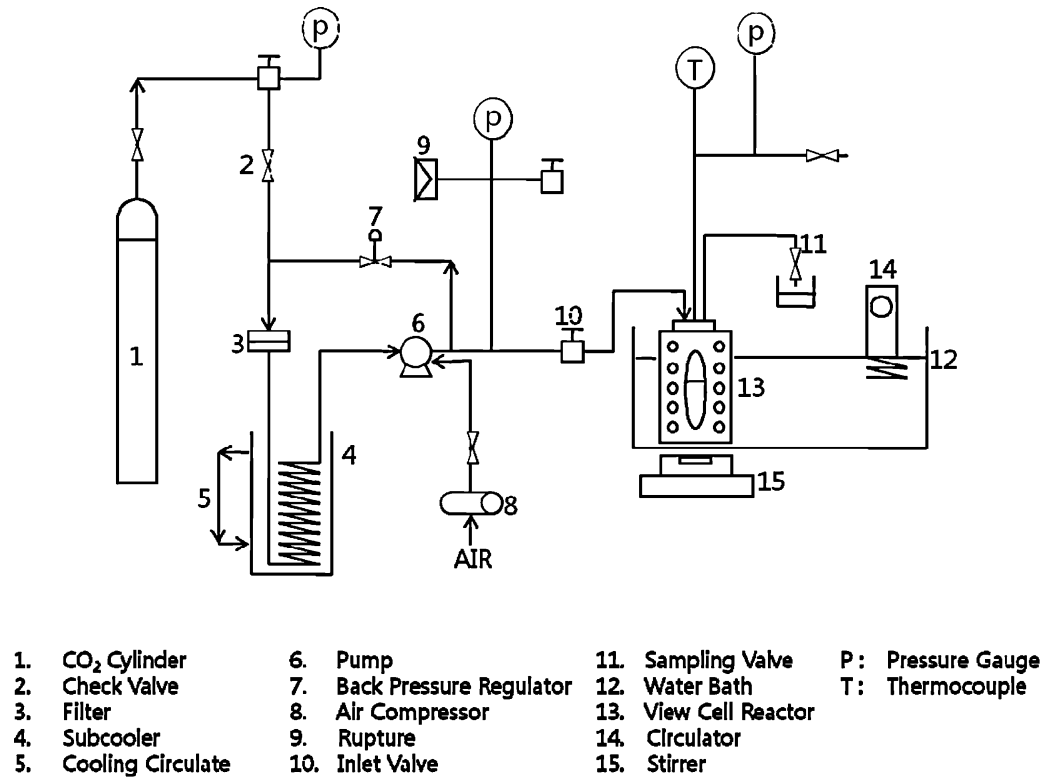


**Fig. 1.** Experimental apparatus for lipase-mediated esterification under pressurized $CO_2$.

improved enzymatic reaction conditions for the enrichment of DHA using tuna oil fatty acids, which have a higher dispersion level than triacylglycerol, under pressurized $CO_2$. We also thoroughly investigated the recovery yields.

In the present study, the effects of the $CO_2$ phase on the reaction medium, the enzyme type, enzyme loading, water addition, and reaction time parameters were explored for the enrichment of DHA by the lipase-mediated esterification of tuna oil fatty acids with ethanol.

## 2. Materials and methods

### 2.1. Materials

Tuna oil was donated by the Il Shinwells Co., Ltd. (Seoul, Korea). Absolute ethanol (>99.9%) was purchased from the Daejung Co., Ltd. (Seoul, Korea). Liquid $CO_2$ (99.99% dry) was purchased from the Shinyang Co., Ltd. (Seoul, Korea). Novozym 435 (*Candida antarctica* lipase immobilized on macroporous acrylic resin), Lipozyme RM IM (*Rhizomucor miehei* lipase immobilized on ion-exchange resin), and Lipozyme TL IM (a silica granulated *Thermomyces lanuginosa* lipase) were purchased from the Novo Nordisk Bioindustry Co., Ltd. (Seoul, Korea); all enzymes used were kept in desiccators with molecular sieves for 48 h. All other solvent and reagents used were of analytical grade.

### 2.2. Preparation of fatty acids from tuna oil

Tuna oil was converted to a fatty acid mixture for use as a substrate. The tuna oil (100 g) was added to a solution of sodium hydroxide (30 g) in distilled water (100 mL) and ethanol (95%, v/v, 300 mL). This mixture was refluxed with stirring at 300 rpm for 30 min. It was transferred to a separating funnel, and water (200 mL) was added to the saponified mixture. The aqueous layer containing the saponifiable matter was acidified by adding 120 mL of concentrated HCl. The resulting lower layer was removed using a separating funnel and discarded. The upper layer containing the fatty acids was extracted with 200 mL of *n*-hexane and washed twice with 100 mL of distilled water. The *n*-hexane layer containing the fatty acids was then dried over anhydrous sodium sulfate. The solvent was then removed using a rotary evaporator at 30 °C.

### 2.3. Lipase-mediated esterification under pressurized $CO_2$

Pressurized $CO_2$ was used as the reaction medium for lipase-mediated esterification of tuna oil fatty acids with ethanol. The experimental apparatus for the lipase-mediated esterification under pressurized $CO_2$ is shown in Fig. 1. A high-pressure reactor (50 mL) with a glass window was used for the reaction. The reactor was preheated to the desired temperature (15–35 °C) using a water circulator. $CO_2$ was then injected into the reactor with a high-pressure pump (PREP PUMP, LabAlliance, USA) to the desired pressure. The substrate and enzyme were mixed, using a magnetic stirrer at 600 rpm. The $CO_2$ pressure was increased to a predetermined value (2.8–13.8 MPa) and it remained constant throughout the reaction. The reaction was started by agitation with a magnetic stirrer, and continued for 24 h. A control reaction was performed in a 50 mL water-jacketed glass vessel under nitrogen gas at atmospheric pressure. The total weight of the substrate used was 18 g, and the molar ratio of tuna oil fatty acids to ethanol was 1:2.

### 2.4. Analysis

Once the reaction was complete, the product mixture was filtered through a membrane microfilter to remove the enzyme. Samples (30 mg) from the reaction mixture were applied to thin-layer chromatography (TLC) plates. The solvent system used to separate the fatty acids and the fatty acid ethyl esters consisted of petroleum ether, diethyl ester, and acetic acid (100:40:1 by volume). Spots on the TLC plates were detected using a 0.2% (w/v)

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