LIPASE CATALYZED ALCOHOLYSIS OF VEGETABLE AND FISH OILS COUPLED WITH SELECTIVE SEPARATION WITH SUPERCRITICAL CARBON DIOXIDE EXTRACTION

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Lipase catalyzed alcoholysis of hoki fish liver and borage seed oils was carried out with ethanol in supercritical (sc) CO_2 in a packed column apparatus. The enzymatic alcoholysis was carried out in a semi-continuous, packed column process using Lipozyme RM-IM. The enzymatic reaction resulted in a complex mixture of lipids consisting of triacylglycerides (TAG), diacylglycerides (DAG), monoacylglycerides (MAG) and fatty acid ethyl esters (FAEE). Most of the saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) were converted into FAEE, whereas the polyunsaturated fatty acids (PUFA) were concentrated in the partial glycerides. The products were extracted from the reaction mixture and fractionated with $scCO_2$ using two separation vessels. The concentration of PUFA in the main product was enhanced by extracting the FAEE from the reaction mixture with $scCO_2$ at 150-200 bar, and by recovering the PUFA-containing partial glycerides from the surface of the enzyme by increasing the extraction pressure to 300 bar. The effect of process parameters including extraction pressure, and ethanol:oil stoichiometric ratio on the efficiency of ethanolysis and on the separation of products was investigated. Between runs the packed column was maintained under a CO_2 atmosphere to prevent deactivation of the enzyme. Under optimum conditions a first separator product with a 2-fold enrichment of PUFA compared to the feed oil was achieved. The second separator products were very rich in FAEEs (90-95 %), with traces of DAG and MAG. The continuous packed column experiments were compared to a batch-wise stirred tank operation.

INTRODUCTION

Although supercritical fluid (SCF) technologies have been utilized in industrial scale operations since the mid 20th century, there are no large scale uses of SCFs in biotechnology. Enzymatic reactions in conventional organic solvents are widely used at large scales in bio-industries for producing pharmaceuticals, vitamins, amino acids and polysaccharides. Carrying out enzymatic reactions in SCFs can eliminate the use of large volumes of organic solvents. Furthermore, fractionation of products can be easily achieved and solvent residue-free products can be produced. SCFs have higher diffusivity and lower viscosity compared to organic solvents making them more attractive as reaction or extraction media. Supercritical CO_2 is of particular interest to the pharmaceutical and food industries because it is non-toxic and non-flammable. In addition, CO_2 can offer a protective, non-oxidative environment and because of its low critical temperature (31.1°C) it is suitable for processing thermally labile materials [1-3].

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Many studies have demonstrated health benefits of polyunsaturated fatty acid (PUFA) intake. These fatty acids are essential as they can be obtained only through diet, since the

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human body is unable to synthesize them. Alpha- (ALA) and gamma-linolenic acids (GLA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids are the most common long carbon chain highly unsaturated fatty acids. These PUFAs are related to homeostasis, pain, inflammation and tumoral genesis. Recently studies show the importance of PUFAs in the extremes of life: pre- and post-natal development and cognitive aging [4-6].

Natural fish oils are used to supply dietary long-chain omega-3 PUFAs in relatively large quantities, in which EPA and DHA with 5-6 *cis* double bonds in the long-chains are the main PUFA compounds. The distribution of these PUFA residues on the glycerol backbone depends on the type of oil. They can be enriched at the *sn-2* position in triacylglycerols from fish oil [3, 7]. Meanwhile linolenic acid (α- and γ-LA) are mainly found in vegetable oils (evening primrose, blackcurrant, borage) in high quantities (40-80%). GLA in borage oil is asymmetrically distributed with preferential location at the *sn-2* and *sn-3* positions on the glycerol backbone [8-9].

Recently, microbial lipases have received attention for the enrichment of long-chain PUFA from TAG oils. Microbial lipases are commercially available in free or immobilized form with different regiospecificities. In this work Lipozyme RM-IM immobilized lipase with 1,3-regiospecificity was used in the selective ethanolysis of hoki fish liver and borage seed oils. The reactions were carried out in $scCO_2$ and the reaction mixture was fractionated with the aim of concentrating PUFA in partial glyceride fraction. The SFA and MUFA were concentrated in a separate FAEE-rich fraction. The FAEE products can be used as biodiesel to replace petroleum based diesel fuels.

MATERIALS AND METHODS

Materials and Chemicals. Refined hoki liver oil (Sealords, New Zealand) was composed almost entirely of TAG and had an EPA, DHA and total PUFA content of 5.5, 12.1 and 25.7 wt %, respectively. Cold-pressed borage seed oil (Oil Seed Extractions Ltd., New Zealand) was composed of 100 % TAG and had GLA and total PUFA content of 20.0, 56.2 wt %, respectively. Lipozyme RM-IM from *Rhizomucor miehei* was supplied by Novozymes A/S, Denmark. Food grade ethanol (Barwell Pacific) was supplied with a purity of 99.8 %. Liquid CO_2 was supplied by BOC (New Zealand) with a purity of 99 %. All materials were used as received without further purification.

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Experimental. Two high-pressure, laboratory-scale apparatuses were used in the experiments. Batch experiments using 10 % enzyme by mass of oil were carried out in a single separator (50-60 bar) operation using a 300 mL stirred tank. Details of the stirred tank apparatus and methodology are given elsewhere [10]. Semi-continuous experiments were carried out in a packed bed column apparatus (400 ml volume), which was connected to two separators in series (Figure 1). The column (water jacket) and separators (water bath) were operated at 40°C. The column was loaded with 50g of enzyme and the top of the bed was covered with glass packings. The CO_2, feed oil and the ethanol were pumped separately and were pre-mixed in a cross piece before entering the packed column. The flow of $scCO_2$ and feed in the column was co-current. The pressures in the first (S1) and second (S2) separators were 100 bar and 50-60 bar, respectively. The CO_2 mass flow rate was measured using a coriolis mass flow meter. Samples were collected from S1 and S2 at regular intervals through sampling valves. The enzyme bed in the packed column was kept under a CO_2 atmosphere between runs and was used repeatedly in order to test enzyme stability.

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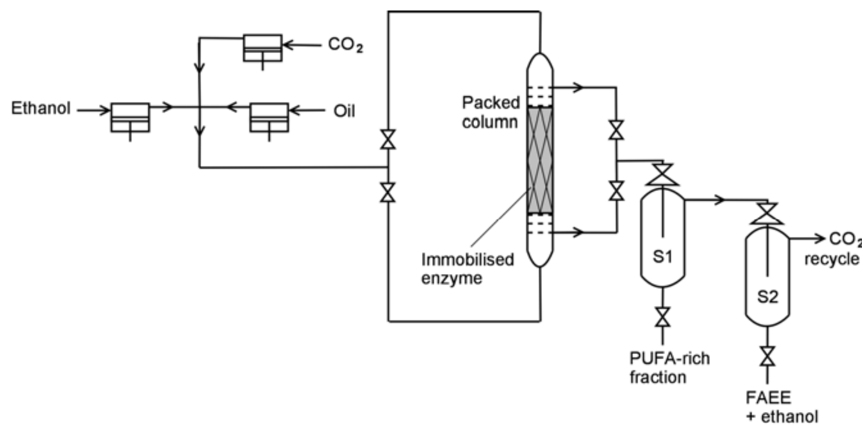

Figure 1. Packed column apparatus for continuous ethanolysis of hoki liver and borage seed oils.

Lipid classes of the fractions were determined by thin-layer chromatography coupled with flame ionization detection (Iatroscan MK-6 apparatus, Iatron Laboratories) and fatty acid profiles were determined by gas chromatography (HP5980 GC using a BP-20 column), after methylation of samples was conducted using the method of Carreau and Dubacq [11].

RESULTS

The enrichment of PUFA from refined hoki liver oil was studied using both the stirred tank and packed column methods. As in previous work [10] the reaction was carried out at 40°C and 150 bar for both methods. In the stirred tank experiment, 100 g hoki oil was used with 10 g Lipozyme RM-IM. Ethanol was added stepwise at a 3:1 ethanol:oil molar ratio [10]. The reaction mixture was then extracted with $scCO_2$ at 150 bar and samples were withdrawn at regular intervals from bottom of the stirred tank and from the separator (S). The stirred tank samples were rich in TAG (90 %) and the S samples were rich in FAEE (83-90%). After extraction at 150 bar the pressure was increased to 300 bar to recover the partial glycerides (comprising ~31% MAG, ~32% DAG, ~23% TAG and ~14% FAEE) from the surface of the enzyme.

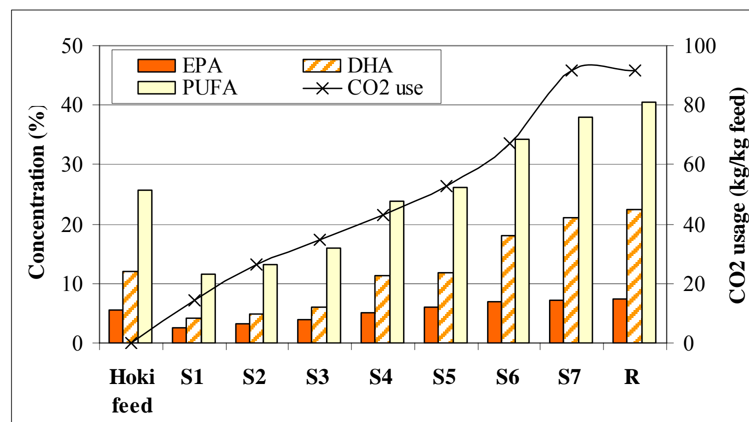

Figure 2. EPA, DHA and total PUFA profiles of the feed oil, separator (S) and raffinate (R) fractions from stirred tank processing of hoki liver oil.

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The EPA, DHA and PUFA profiles of the samples are shown in Figure 2, where R is the raffinate fraction recovered from the stirred tank after extraction at 300 bar. The solvent usage is also shown. The fractions obtained at 300 bar (S6, S7 and R), contained enhanced levels of DHA and total PUFA over the feed (1.8 and 1.6 fold increase for DHA and total PUFA, respectively). The EPA content of these fractions was similar to that of the feed oil. In the stirred tank experiment, 92 kg CO_2/kg oil, with an average flow rate of 4.5-5 kg/hour, was used to produce FAEE-rich and PUFA enriched fractions. The high solvent usage in the batch stirred tank experiments is not practical for industrial scale-up and so a semi-continuous, packed column method was investigated.

The effect of column pressure and ethanol:oil molar ratio on reaction and fractionation efficiency was studied. The enzyme bed was used in consecutive runs in order to test enzyme stability. Figure 3 shows the fatty acid profiles for processing of refined hoki liver oil. The solvent usage is also shown. The reaction was carried out in $scCO_2$ at 150 bar and the FAEE were continually stripped from the reaction mixture at this pressure. The ethanol and oil feeds were stopped after 300g of oil had been fed into the column and extraction was continued at 150 bar until no more FAEE were recovered in S2. The S2 fraction was very rich in FAEE (>90 %).

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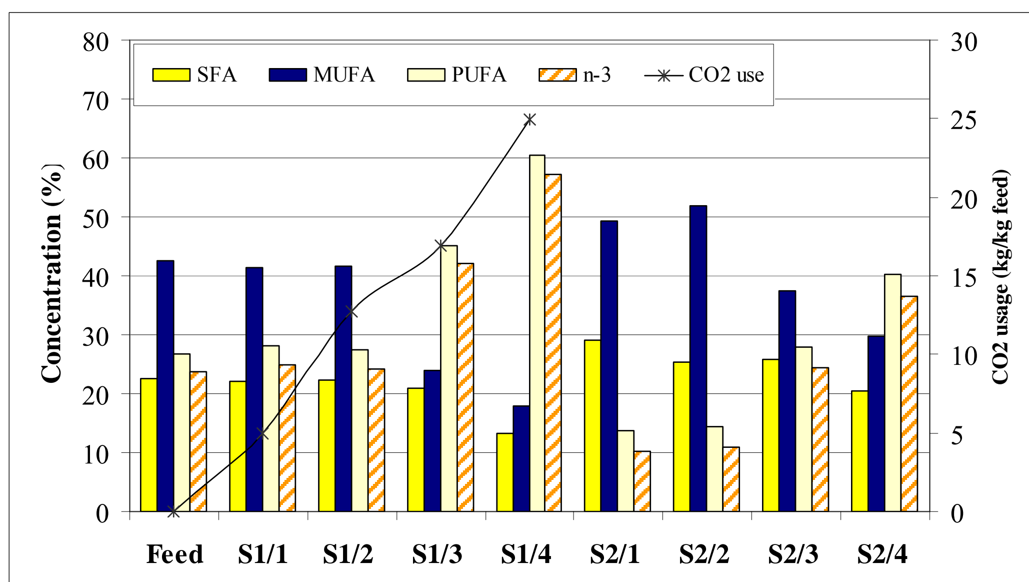

Figure 3. SFA, MUFA, PUFA and omega-3 (n-3) profiles of the feed oil, first (S1) and second (S2) fractions from packed column processing of hoki liver oil.

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The S1 fraction was a mixture of partial glycerides and TAG. The final S1 fraction obtained at 150 bar (S1/3) showed a 1.5 fold enrichment in PUFA and reduced levels of MUFA compared to the feed. After extraction at 150 bar the pressure was increased to 300 bar to recover the PUFA-rich partial glycerides from the packed bed. Extraction at 300 bar increased the concentration of PUFA in S1 (S1/4) to twice that of the feed. The concentrations of DHA and EPA were increased 2.7 and 1.6 fold, respectively, in this fraction. The compositions of the highly PUFA-rich fractions (S1/3 and S1/4) were similar (~12 % TAG, ~41 % DAG and 44-49 % MAG). The undesirable SFA and MUFA were concentrated in the S2 fractions. In this experiment 23 kg CO_2/kg oil, with an average flow

rate of 3 kg/hour, was sufficient to produce FAEE-rich fractions in a semi-continuous process, which is 4 times less than the solvent usage from the stirred tank experiment.

Borage seed oil was also processed using the packed column in a similar manner to that described for hoki liver oil. At the lower reaction pressures (150-200 bar) FAEE-rich fractions were collected in S2 (up to 70 % FAEE) while the S1 fractions contained a mixture of partial glycerides and TAG. After all of the FAEE had been extracted the pressure was increased to 300 bar and a PUFA-rich fraction was collected in S1 with a GLA content of ~35-40 % (*c.f.* 20 % in the feed).

The ethanol:borage seed oil molar ratio was also investigated and was found to have no effect on enzymatic conversion or GLA content of the S1 fractions (Figure 4).

Watanabe *et al*. reported that lipases can be deactivated in the presence of more than a 2:3 molar equivalent of ethanol:total fatty acids in tuna oil [3]. Deactivation of immobilized enzyme in the presence of large quantities of ethanol and using it under solvent free conditions has also been observed in our laboratories [12]. In this work, Lipozyme RM-IM was very stable and performed well with high conversion to FAEE (>80 %) at relatively high ethanol concentrations (9:1 molar ratio of ethanol:oil). No deactivation was observed after 9 cycles of packed column processing. It is believed that keeping the enzyme under a CO_2 atmosphere can prevent enzyme deactivation.

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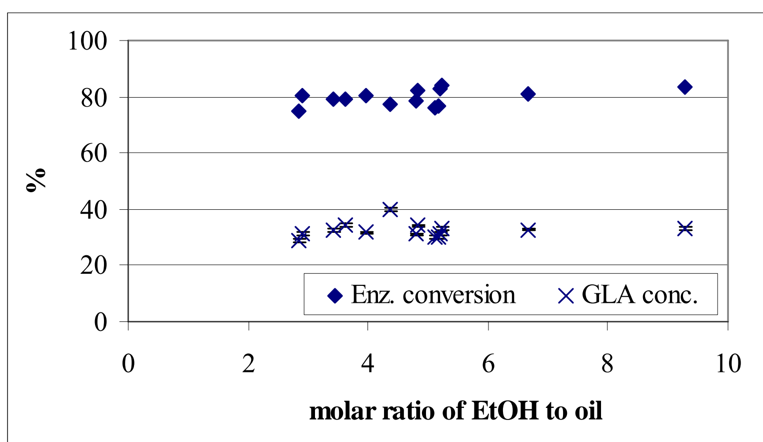

Figure 4. The enzymatic conversion and the concentration of GLA in S1 fractions as a function of ethanol:oil molar ratio from packed column processing of borage seed oil.

CONCLUSION

The enzymatic ethanolysis of refined hoki liver oil and borage seed oil was successfully carried out in $scCO_2$ in a packed column, semi-continuous process at 150 bar, 40°C, with relatively low solvent usage and with high enzymatic conversion. The reaction mixture was easily fractionated with $scCO_2$ to produce fractions rich in PUFA and FAEE. The ethanol:oil stoichiometric ratio had no effect on enzymatic conversion or levels of PUFA in the PUFA-rich fractions. Keeping the enzyme packed column under a CO_2 atmosphere between runs prevented deactivation of the enzyme, even at relatively high ethanol:oil molar ratios. No loss of enzyme activity was observed after 9 semi-continuous, packed column runs. Supercritical packed column processing shows some promise for the production of PUFA-rich and FAEE-rich products for the health food and energy industries.

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