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ORIGINAL PAPER

Comparative Study of Sterol Ester Synthesis using *Thermomyces Lanuginosus* Lipase in Stirred Tank and Packed-Bed Bioreactors

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Abstract A comparative study was done on the production of different sterol esters using a stirred tank batch reactor (STBR) and packed bed reactor (PBR) using *Thermomyces lanuginosus* (Lipozyme TLIM) enzyme, a commercially immobilized lipase. Different oils were used as the sources of particular fatty acids, e.g., fish oil for n-3 polyunsaturated fatty acids (n-3 PUFA), linseed oil for alpha linolenic acid (ALnA) and mustard oil for erucic acid. Reaction parameters, such as substrate molar ratio, reaction temperature and enzyme concentration, were standardized in the STBR and maintained in the PBR. To provide equal time of residence between the substrate and enzyme in both the reactors for the same amount of substrates, the substrate flow rate in the PBR was maintained at 0.27 ml/min. Thin layer chromatography was used to monitor the reaction, and column chromatography was used to determine the product yields. Fatty acid compositions of the esters were determined by gas chromatography. The study showed that the packed bed bioreactor was more efficient than the batch reactor in sterol-ester synthesis with less migration of acyl groups.

Keywords Phytosterol · Sterol-ester · Lipase-catalyzed transesterification · Packed bed reactor · *Thermomyces lanuginosus* (Lipozyme TLIM)

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Introduction

Sterol esters are currently gaining importance because of their recent recognition and application in the food and nutraceutical industries. Phytosterol esters have an advantage over phytosterols, naturally occurring antioxidants, with better fat solubility and compatibility [1]. Recently, steryl and stanyl esters have been added to special margarines that are commercially available as functional foods with the ability to reduce both total and LDL cholesterol levels [2–4]; especially the esters of phytosterols with eicosa-penta-enoic acid (20:5; EPA) and docosa-hexa-enoic acid (22:6; DHA) have proven their importance by showing a synergistic action and added benefits against different diseases [5]. The efficacy of encapsulated phytosterol ester administration was also examined by Earnest et al. [6] by having hypercholesterolemic adults ingest it, and encouraging results were obtained when lipid indices were measured.

Synthesis of sterol esters using biocatalysts in the presence of organic solvents and molecular sieves or other drying agents has been reported in various studies [7–11]. Moreover, a method has also been described for the preparation of sterol esters of polyunsaturated fatty acids in an aqueous system by Shimada et al. [12]. In most of the above methods, the extent of steryl ester formation is rather moderate. In their two consecutive works, Weber, Weitkamp and Mukherjee showed that plant steryl and stanyl esters can be efficiently prepared from sterols and stanols via lipase-catalyzed esterification with fatty acids and by transesterification with fatty acid methyl esters or triacylglycerols [5, 13].

For lipase-catalyzed esterification reactions, a nonpolar environment with a low water content is required to shift the thermodynamic equilibrium in favor of esterification/

transesterification over hydrolysis. Therefore, most of the studies dealing with lipase-catalyzed ester synthesis are carried out in organic solvents [14, 15]. It also offers the advantages of high solubility of the substrate and product and controlling microbial contamination [16, 17]. Therefore, the use of an immobilized enzyme is an obvious choice as it also provides enzyme reusability and hence reduces operational costs. It also reduces enzyme contamination and facilitates easy separation of products. The probability of using a continuous packed bed reactor (PBR) could also be feasible using immobilized enzymes.

The aim of this study was to investigate the utility of PBR to synthesize sterol esters of various fatty acids by a transesterification reaction using *Thermomyces lanuginosus* (Lipozyme TLIM) lipase and to compare it with a stirred tank batch reactor (STBR) regarding yield and composition. Different oils were used as the sources of different fatty acids.

Experimental Procedures

A standard β-sitosterol sample was procured from Fluka Chemicals and analyzed at the laboratory by gas chromatography (GC).

Fish oil (Mega-Shelcal capsules from Elder Pharmaceuticals, India) was used as the source of eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA), and the GC analysis of the fish oil showed that the oil contained 32% EPA and 22% DHA.

Refined and bleached linseed oil procured from V.K.V.K. Oil Limited, Kolkata, India, was used as the source of alpha linolenic acid (ALnA), and the GC analysis of the oil showed the presence of 54% ALnA in the oil.

Mustard oil (solvent extracted, refined and bleached in the laboratory) was used as a source of erucic acid (EA: $C_{22:1,}$ n-9), and the GC analysis of the oil confirmed the presence of 49% erucic acid in the oil.

Thermomyces lanuginosus lipase (Lipozyme TLIM), used as biocatalyst, was a generous gift from Novozyme India, Ltd., Bangalore, India. The original moisture content of the enzyme (2%, w/w) was kept intact to initiate the reaction.

All other chemicals used were of analytical grade and procured from SRL, Mumbai, India.

Production of Sterol Esters in the Stirred Tank Batch Reactor

β-Sitosterol and oil were taken in different molar ratios and stirred at 200 rpm at different temperatures for different time periods with varying amounts of lipozyme TLIM enzyme. A minimum amount ($\sim$20 ml) of hexane was added to bring fluidity to the reaction mixture. The total volume of the substrate was 100 ml. The synthesis of esters was first monitored by thin layer chromatography (TLC), and the typical yield of each reaction set was determined separately by column chromatography. The total sterol esters produced were isolated from the reaction product, which comprised excess sterol, excess oil, the product sterol esters and other by-products (Make: SIBATA, Japan, Serial no. N 40394) at 170 °C and 0.15 millibar pressure.

The effects of different reaction parameters for the stirred tank batch reactor, such as temperature, substrate molar ratio and concentration of enzyme, were optimized with a single reaction mixture, and the optimized parameters were followed in the successive reactions.

The molar ratio of the substrates was varied as 3:1, 3:2, 3:3, 3:4, 3:5 and 3:6 of fish oil:β sitosterol to study the effect of the substrate molar ratio on the ester yield, keeping the other reaction parameters such as temperature (60 °C) and enzyme concentration (10% w/w, on the total substrate weight) constant.

The effect of temperature on the reaction rate was studied by conducting the reaction at temperatures ranging from 40, 45, 50, 55, 60 and 65 °C, keeping other reaction parameters such as the substrate ratio (oil:sterol :: 3:6) and enzyme concentration (10% w/w, on the total substrate weight) constant.

The variation in enzyme concentration from 2 to 12% (w/w, on the total substrate weight) was also studied, keeping the other reaction parameters such as temperature (60 °C) and substrate ratio (oil:sterol :: 3:6) constant.

The yield was expressed as grams of ester present in 100 g of the product mixture (%w/w).

Production of Sterol Esters in the Packed Bed Reactor

The reactor consisted of a tubular glass column of 10-mm ID and 50 cm long. It was also provided with a water jacket for temperature control. The immobilized enzyme packed into the reactor was retained in place by means of a sintered plate. The substrates were fed from the top of the bed, and the products were collected at the bottom. The substrates were taken in an optimized substrate ratio, i.e., oil:sterol :: 3:6, and a minimum amount of hexane was added to bring fluidity to the reaction mixture. The substrates previously had been blended and well-mixed at the reaction temperature before conducting the packed bed reaction and were poured into the enzyme bed, maintaining a fixed sample head. Water from a constant temperature bath was circulated through the jacket by a peristaltic pump. A partial suction from a vacuum pump was given to maintain the constant flow rate (0.27 ml/min to get 100 ml product in 6 h); 20 gm of enzyme was closely packed into the column by repeated tapping to avoid any air gaps.

Transesterification reactions were then carried out by passing the substrate through the column. The temperature was maintained at the desired value of 60 °C by passing water through the column jacket. The product mixture was collected at the outlet (after each 100 ml product) and analyzed. The formation of sterol ester was confirmed by TLC, and then the percent yield of ester and fatty acid composition of the ester was determined simultaneously. The yield was expressed as grams of ester present in 100 g of product mixture (%w/w).

Qualitative Analysis of Sterol Esters by Thin Layer Chromatography

The formation of sterol esters was first confirmed by thin layer chromatography (TLC) by spotting the lipid mixture on a Silica Gel G plate (0.2 mm thick) using hexane-diethyl ether-acetic acid (90:10:1) as a developing solvent system [18]. The lipid spots were visualized by iodine absorption identified by measuring R_f values.

Quantitative Analysis of Sterol Ester

The amount of sterol ester produced was determined by column chromatography using silicic acid as an adsorbent and 150 ml of hexane: di-ethyl ether :: 99:1 as eluting solvent [19], which separated sterol ester from the unreacted sterol and oil and other reaction products.

Qualitative Analysis of Sterol Esters

The percent compositions of various sterol esters according to fatty acid compositions were determined by GC. The GC instrument (Agilent, model 6890 N) used was equipped with a FID detector and capillary HP 5 column (30 ml, 0.32 mm I.D, 0.25 μm FT). N_2, H_2 and airflow rates were maintained at 1, 30 and 300 ml/min, respectively. Inlet and detector temperature was kept at 250 and 275 °C, respectively, and the oven temperature was programmed at 65–230–280 °C with a 1-min hold at 65 °C and an increase rate of 20 °C/min and 1 min hold up to 230 and 8 °C/min with 24 min hold up to 280 °C. Sterol esters were fractionated according to the fatty acid composition from which the amount of each fatty acid incorporated in the ester was calculated. The R_t of each sterol ester had been previously standardized in GC by preparing esters of β-sitosterol with different fatty acids.

Fatty acid compositions of the sterol esters produced were further confirmed by hydrolyzing the esters, and the corresponding fatty acids thus obtained were characterized by GC analysis. Methyl esters of the fatty acids were prepared by the method described by Metcalfe [20], and the compositions were determined by GC analysis using an analytical gas chromatography (Agilent 6890 Series Gas chromatograph) equipped with a flame ionization detector (FID) and HP-Wax capillary column (J&W Scientific Columns from Agilent Technologies) of 30 m length with 0.25 mm (i.d.) and 0.25 mm (film thickness). The GC inlet temperature and FID detector temperature were maintained at 250 °C, and the oven temperature was maintained at 250 °C for 2 min, then the temperature was increased at 10 °C/min, up to 280 °C, then there was a 20-min hold at 280 °C. The gas flow was 30, 300 and 29 ml/min for hydrogen, air and nitrogen, respectively.

Results and Discussion

Optimization of Reaction Parameters in STBR

The yield of ester with time at various molar ratios of oil to sterol is shown in Fig. 1. The yield increased as the molar ratio of fish oil to sterol increased from 3:1 to 3:6. Therefore, the molar ratio of 3:6 of oil to sterol could be taken as the optimum substrate ratio. Actually according to functionality 3 mol of oil could provide nine reactive fatty acid molecules, and therefore the effective molar ratio should be counted as 9:6 (3:2).

In the next set of experiments, the substrate concentration was reversed, i.e., substrates were taken in a molar ratio of sterol:oil, starting from 6:1 and increasing up to 6:5. The percent yield of sterol obtained is plotted in Fig. 2, and this figure also demonstrated that the yield of ester also increased as the molar ratio of sterol to oil increased from 6:1 to 6:3, but did not increase much at molar ratios higher

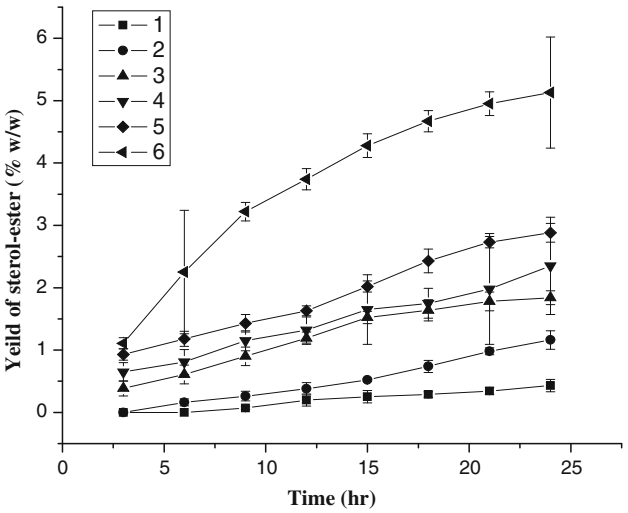

Fig. 1 Yield of sterol ester (% w/w) with time at different molar ratios of oil:sterol (*filled square box*), 3:1; (*filled diamond*), 3:2; (*filled triangle*), 3:3; (*filled down-pointing triangle*), 3:4; (*filled diamond*), 3:5; (*filled left-pointing triangle*), 3:6

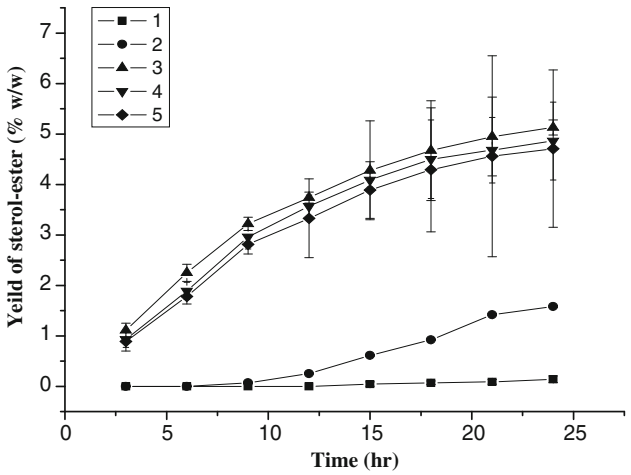

Fig. 2 Yield of sterol ester (% w/w) with time at different molar ratios of sterol:oil (*filled square box*), 6:1; (*filled circle*), 6:2; (*filled triangle*), 6:3; (*filled down-pointing triangle*), 6:4; (*filled diamond*), 6:5

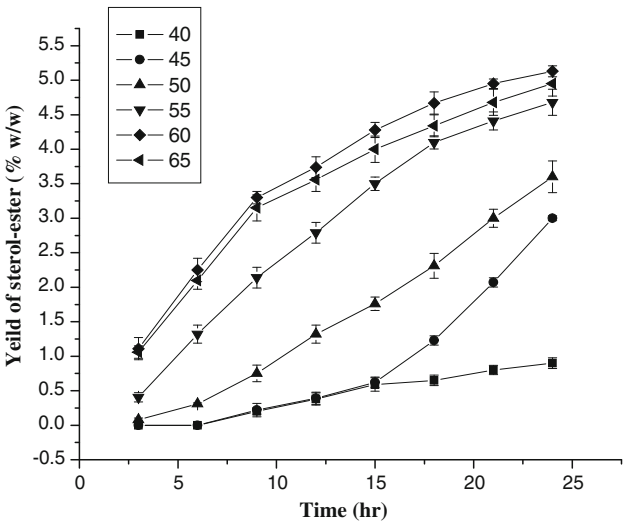

Fig. 3 Yield of sterol ester (% w/w) with time at different temperatures: (*filled squared box*), 40 °C; (*filled circle*), 45 °C; (*filled triangle*), 50 °C; (*filled down-pointing triangle*), 55 °C; (*filled diamond*), 60 °C; (*filled left-pointing triangle*), 65 °C

than 6:3. Therefore, the optimality of the molar ratio of 3:6 of oil to sterol and vise versa was again established.

Reaction temperature always affects the reaction rates, but in case of enzymatic reactions, higher temperature does not always lead to higher rates of reaction as higher temperature sometimes leads to deactivation of the enzyme. The yield of ester increased with temperature starting from 40 °C and increased up to 60 °C, but there was a slight decrease in the yield above 60 °C. When Shimada et al. [12] synthesized sterol esters by an enzymatic esterification process using crude *Pseudomonas sp*. lipases, they obtained the maximum yield at 40 °C. But in our study we observed that the highest yield was obtained at 60 °C (Fig. 3). The difference in optimum temperature may be due to the nature of enzymes used and the substrates used. The enzyme used in this study was immobilized, and the reaction was a transesterification reaction between sterol and triacylglycerol oils. Therefore, a little higher temperature could provide better mixing and compatibility between the substrates and enzymes, which led to a higher yield.

When substrate concentration was much higher than enzyme concentration, other factors such as mass transfer limitation could be ignored. The increase in enzyme concentration would make the reaction faster. The effect of enzyme concentration was evaluated with a fixed substrate ratio (oil:sterol :: 3:6) and reaction temperature (60 °C). The different concentrations of enzyme taken were 2, 4, 6, 8, 10 and 12% on the basis of total substrate weight as this was an immobilized enzyme. Figure 4 is the plot of ester yield with the increase in enzyme concentration. From the figure it could be inferred that the optimum enzyme concentration was 10%. There was a decrease in ester yield

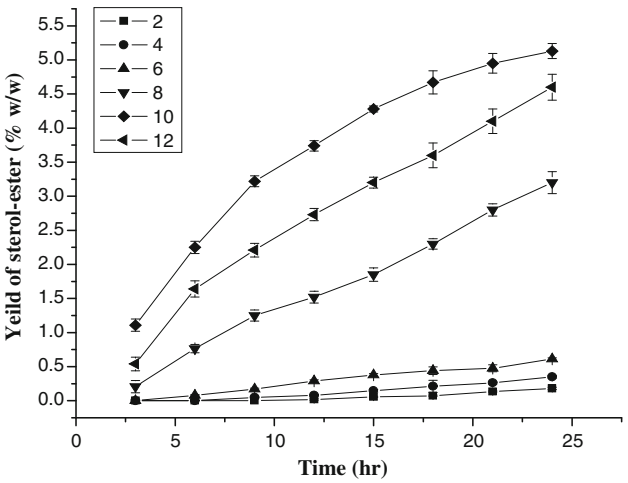

Fig. 4 Yield of sterol ester (% w/w) with different enzyme concentrations at different time intervals: (*filled left-pointing pointer*), 12% enzyme; (*filled diamond*), 10% enzyme; (*filled down-pointing triangle*), 8% enzyme; (*filled triangle*), 6% enzyme; (*filled circle*), 4% enzyme; (*filled squared box*), 2% enzyme

when the amount of enzyme exceeded 10%. This could be attributed to the fact that an increase in enzyme concentration probably initiated the product breakdown because of the simultaneous increase in water content that was present in the enzyme, or because it was a reversible reaction, the backward reaction was initiated because of an increase in enzyme concentration.

Yield of Sterol Ester with Time in STBR

Figure 5 shows that the yield of various sterol esters with time in a STBR and the highest yield of ester were obtained

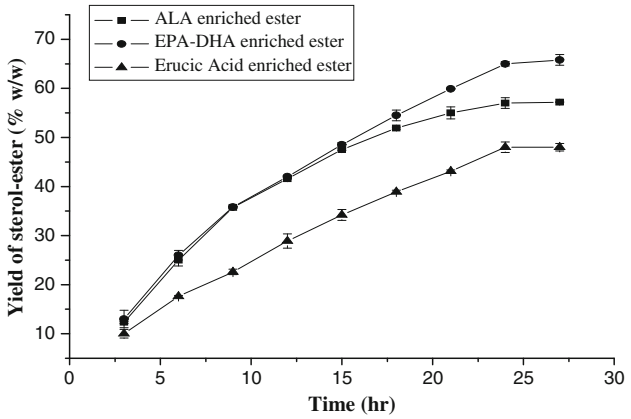

Fig. 5 Yield of different sterol esters (% w/w) with time in a STBR

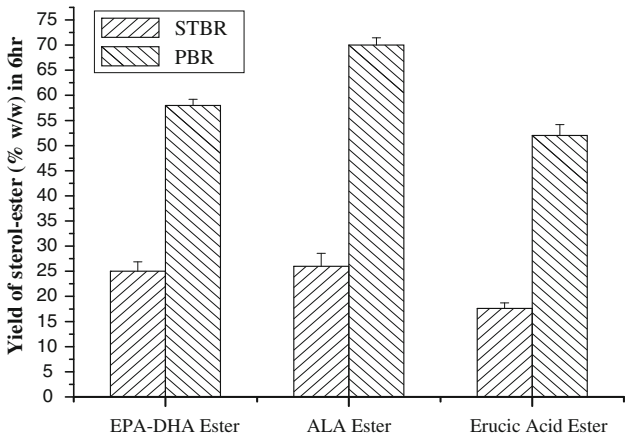

Fig. 6 Comparison of yield of different sterol esters (% w/w) in 6 h by STBR and PBR

at 24 h. From Fig. 5 it is also evident that the ester formation increased with time from 3 to 24 h, and after that there was no significant increase in yield. The yield of ester was highest in case of transesterification with linseed oil, and transesterification with mustard oil gave the least yield among the three.

Comparative Yield of Sterol Ester in STBR and PBR

The yields of sterol ester in the two reactors were compared after a definite time period and plotted in Fig. 6. The flow rate of PBR was so maintained that an equal amount of sample was processed in each reactor in a definite time period. The bar diagrams plotted in Fig. 6 clearly indicate that the production of different esters in a PBR was significantly faster than the STBR. Here also the yield of ALnA-enriched ester was the highest and that of erucic acid was the lowest. The reason for the difference in yield may be the viscosity of the corresponding oil. Though 60 °C temperature and the presence of a small amount of

Table 1 Fatty acid profile of the sterol esters produced by STBR and PBR corresponding to fish oil

Sample	Fatty acid (% w/w)											
	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{20:1}$	$C_{20:5}$	$C_{22:0}$	$C_{22:1}$	$C_{24:0}$	$C_{22:6}$
Fish oil	0.49 ± 0.08	0.98 ± 0.04	4.99 ± 0.76	5.20 ± 0.54	13.70 ± 1.02	5.25 ± 0.93	1.31 ± 0.07	5.04 ± 0.35	**31.36** ± 1.97	3.52 ± 0.20	2.05 ± 0.05	3.87 ± 0.08	**22.24** ± 1.65
n-3 fatty acid ester of sterol(STBR)	–	2.14 ± 0.03	1.30 ± 0.05	0.61 ± 0.23	2.32 ± 0.33	3.15 ± 0.15	2.77 ± 0.13	5.18 ± 0.14	**37.06** ± 1.04	8.86 ± 1.30	2.18 ± 0.06	4.44 ± 0.22	**29.99** ± 0.97
n-3 fatty acid ester of sterol(PBR)	0.10 ± 0.00	2.44 ± 0.04	1.23 ± 0.02	1.0 ± 0.15	1.90 ± 0.27	3.05 ± 0.34	2.75 ± 0.64	5.12 ± 0.83	**39.20** ± 1.10	5.65 ± 0.37	2.19 ± 0.08	3.84 ± 0.19	**31.98** ± 1.63

Values are the average of analysis from triplicate sets. Bold values indicate the amount of significant fatty acids

Table 2 Fatty acid profile of the sterol esters produced by STBR and PBR corresponding to linseed oil

Sample	Fatty acid (% w/w)				
	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Linseed oil	8.62 ± 0.36	3.27 ± 0.09	22.53 ± 2.3	10.97 ± 0.73	**54.61** ± 1.78
ALA ester of sterol (STBR)	12.07 ± 0.45	6.50 ± 0.39	23.91 ± 1.84	15.73 ± 1.07	**36.79** ± 2.08
ALA ester of sterol (PBR)	12.48 ± 0.60	1.89 ± 0.17	28.28 ± 1.09	12.83 ± 1.01	**44.52** ± 1.76

Values are the average of analysis from triplicate sets. Bold values indicate the amount of significant fatty acids

Table 3 Fatty acid profile of the sterol esters produced by STBR and PBR corresponding to mustard oil

Sample	Fatty acid (% w/w)									
	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{20:5}$	$C_{22:0}$	$C_{22:1}$	$C_{24:0}$
Mustard oil	2.24 ± 0.06	1.15 ± 0.03	9.42 ± 0.73	17.94 ± 1.32	10.75 ± 0.95	0.80 ± 0.02	3.20 ± 0.12	5.15 ± 0.26	**48.55** ± 1.4	1.30 ± 0.03
EA ester of sterol (STBR)	8.45 ± 0.87	1.62 ± 0.22	14.17 ± 0.68	15.85 ± 1.01	10.64 ± 1.04	0.90 ± 0.21	7.68 ± 0.49	6.84 ± 0.66	**31.59** ± 1.3	2.26 ± 0.43
EA ester of sterol (PBR)	8.56 ± 0.82	1.98 ± 0.13	12.03 ± 0.72	18.93 ± 0.1.46	11.64 ± 0.94	0.70 ± 0.30	4.8 ± 0.56	3.2 ± 0.20	**38.21** ± 1.1	0.30 ± 0.02

Values are the average of analysis from triplicate sets. Bold values indicate the amount of significant fatty acids

hexane made the reaction mixtures sufficiently fluid, still higher viscosity of the oil, which hinders mobility, consequently led to lesser reactivity of the oil. The partial suction given to the enzyme bed was effective to maintain the mass transfer throughout the bed at a constant rate. The high solubility of sterols in oil and hexane hindered the adhesion of sterols with enzyme.

Characterization of Sterol Esters Corresponding to the Original Oil

The transesterification reaction was carried out in two different reactors, the STBR and PBR. In the first set of reactions, fish oil was used as a source of EPA and DHA fatty acids; in the second set, linseed oil was used as a source of ALnA, and in the third set, mustard oil was used as a source of EA. Approximately 32% EPA and 22% DHA were present in fish oil, 54% ALnA in linseed oil and 49% EA in mustard oil. Analysis of the fatty acid composition of sterol esters by GC showed that almost all the fatty acids present in the different oils (in TAG form) were incorporated in the corresponding esters. Tables 1, 2 and 3 show the fatty acid profile of the original oils and the sterol esters produced in the two reactors. A little variation was observed in the fatty acid profile of the esters produced in the two reactors. The residence time of reactants in the packed bed reactors was much less, and thereby less acyl migration could occur, which minimized the variation in the fatty acid profile.

Conclusion

The present study demonstrates that the enzymatic transesterification reaction can be carried out successfully in a packed bed continuous flow reactor. The study illustrates the inherent advantages of a PBR, such as increased throughput, where a high percentage of ester conversions are obtained more rapidly compared to a batch reactor at lower cost and with better environmental control. As there is no breakdown of enzyme particles in a packed bed reactor due to an absence of stirring, the maximum utility of the enzyme could also be obtained.

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