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Immobilized Lipases—A Versatile Industrial Tool for Catalyzing Transesterification of Phytosterols Solubilized in Plant Oils to Produce their Fatty Acid Esters

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Abstract: The conjugation of phytosterols (PSs) with fatty acids results in producing phytosterol esters (PSEs) characterized with enhanced lipophilicity and improved functional properties of major interest in food and nutraceutical applications. The use of immobilized lipases to catalyze direct transesterification reactions between PSs and plant oils to form PSEs as a green alternative to conventional chemical production methods has attracted interest during the last two decades. The low solubility of PSs in common plant oil triglycerides, typically below 3% at ambient temperatures, remains the main challenge for bringing lipase-catalyzed direct transesterification reactions of PSs and oil triglycerides to commercial scales. This study focuses on the enzymatic synthesis of PSEs starting from solubilized PSs at concentrations of up to 30% wt./wt. of oil mixtures comprising fatty acid ethyl esters (FAEEs), monoglycerides (MGs), diglycerides (DGs) and triglycerides (TGs) as a homogeneous medium for the direct transesterification reaction. The results of this study show for the first time that the addition of FAEEs into the reaction medium results in altering of the substrate preference of the enzyme making MGs as the favorite fatty acyl group donors for PSs amongst all other fatty acyl donors present in the reaction system. The proposed new enzymatic route allows starting with high concentrations of solubilized PSs making the direct transesterification of oil glycerides attractive for production of PSEs at industrials scales.

Keywords: Immobilized lipases; phytosterols; phytosterol esters; fatty acyl group donors; esterification; and direct transesterification reactions

1. Introduction

PSs are naturally occurring in plants, typically found in seeds, vegetable oils, and nuts. Structurally, they belong to the same family of cholesterol derived from animal sources [1]. During the last two decades, the use of PSs in oils and fats has gained attention due to their potential health benefits. Once incorporated in oils and fats PSs have been shown to reduce the absorption of dietary cholesterol in the intestine, which results in lowering blood cholesterol levels, maintaining a healthier lipid profile therefore, contributing to the reduction of the risk of heart diseases, such as atherosclerosis, heart attacks, strokes, and other related body functional disorders [2–6]. Furthermore, many research studies have shown that PSs in their pure form or mixed in oils/fats offer a natural alternative to synthetic cholesterol-lowering drugs [5,7,8]. Despite the health benefits of the various formulations of PSs approved in many research studies, their use remains subject to regulatory guidelines and limitations, and these guidelines may vary in different countries [4,9,10].

PSs have limited solubility in fats and oils, typically 1-3% at ambient temperatures depending on the fatty acid profile of the oil medium, which means that high concentrations cannot be easily incorporated into functional food products [11]. Furthermore, PSs are susceptible to oxidation, especially under certain processing conditions, responsible for negatively altering the taste, odor, and nutritional quality of oils and fats [12,13]. To overcome limited solubility of PSs as well as their low oxidative stability, many research studies have shown that esterified PSs offer an attractive solution for facilitating their applications in functional foods, cosmeceuticals, and pharmaceuticals [2,5,7,14,15]. One of the most common methods for synthesizing PSEs involves esterification of PSs with free fatty acids. This process typically employs strong acids as catalysts, such as sulfuric and p-toluene sulfonic acids [2,16–18], or use of native and immobilized lipases as biocatalysts [7,19,20]. The reaction can be carried out under various conditions, including solvent-based or solvent-free microaqueous systems. Solvent-free lipase-catalyzed esterification methods have gained popularity due to their eco-friendly nature and ease of product recovery [20]. Common fatty acids used for esterification of PSs include palmitic, stearic, and oleic acids, typically of vegetable oil sources [21–23]. Transesterification is another possible method for synthesizing PSEs. This reaction involves direct donation of fatty acyl groups to PSs by using oil TGs or fatty acid short-chain alkyl esters, such as FAEEs, as fatty acyl group donors, in solvent as well as solvent-free reaction medium. This reaction can be catalyzed by strong alkaline catalysts such as sodium methoxide/ethoxide, or by using lipases in their free or immobilized forms [15,20,24]. On the contrary to strong alkaline and acid catalysts the major advantages of lipases are their capability to catalyze esterification and transesterification reactions simultaneously [25], operate under mild reaction conditions therefore are low in energy consumption and less concurrent byproducts, as well as their tolerance to micro-amounts of water present in the reaction medium [15]. Free as well as lipases immobilized on an organic or inorganic polymer support matrix, have been used for the esterification/transesterification of PSs and fatty acyl group donors [22,23]. In many cases immobilization allows lipases to be more stable, reusable, and easily separated from the reaction mixture. It also improves their operational efficiency in industrial applications and allows the use of conventional industrial reactor configurations, including batch- and continuous-wise stirred-tank, fluidized- and fixed-bed reactors [17,19].

Due to the higher solubility of PSs in free fatty acids medium as compared to using oil TGs as fatty acyl group donors, most studies in the field have used native or immobilized lipases to catalyze esterification reactions between both substrates in solvent and solvent-free systems. Most of these studies have found that lipases derived from *Candida rugosa*, *Candida antarctica*, *Alcaligenes sp*., and *Rhizomucor miehei*, are the best performing enzymes to catalyze the production of PSEs in stirred batch and continuous stirred-tank reactors [20,21,23,26]. After the desired esterification reaction conversion is achieved, the product PSEs characterized with its high solubility in oil medium can be separated from the reaction mixture. Common separation methods include solvent extraction, distillation, crystallization, or chromatographic techniques, can be applied to obtain high purity PSEs. Lipase-catalyzed direct transesterification reactions between PSs with either oil TGs or FAEEs have been less documented due to the low solubility of PSs in hydrophobic oil-based medium [17,20]. To increase PSs concentrations above 3%, the transesterification reaction medium containing PSs at a maximum concentration of 10% wt./wt. of oil, has been heated up to 90-130°C which led to severe losses in enzyme activity, staining of the final product as well as technical complexity in removal of the free enzyme residues from the reaction medium [11,20]. Although direct enzymatic transesterification exhibits advantages such as one-step reaction under milder reaction conditions, its use to produce PSEs remains unresolved at industrial scales.

The present work reveals a versatile production process based on use of free or immobilized lipases for catalyzing direct transesterification reactions between oil glycerides as fatty acyl group donors, and solubilized PSs with initial concentrations of up to 30% wt./wt. of oil at temperature ranges tolerated by most enzymes. The proposed process demonstrates for the first time the use of a homogeneous direct transesterification reaction medium where MGs serve as the most favored fatty acyl group donor for PSs to produce PSEs in the presence of a lipase as a catalyst. Such a system would offer a versatile tool for paving the way for employing immobilized lipases to produce PSEs at commercial scales starting from oil glycerides with no limitations for the low solubility of PSs in the reaction medium as well as with no restrictions for choice of reactor type and configuration.

2. Materials and Methods

2.1. Materials

Different lipases derived from various microbial species have been kindly contributed by different enzyme manufacturers as detailed in Table 1. Edible oils derived from different sources have been purchased from local groceries (Israel). Distilled MGs (Verol IG-90, 95% as MGs) and a mixture of mono- and di-glycerides (Verol IG, 38-42% wt. as MGs) both were a generous gift from Lasenor (Spain). Oleic acid and ethyl oleate of 96% purity were purchased from Sigma (Israel). A mixture of PSs with a purity of 95%, composed of 40% β-sitosterol, 30% stigmasterol, and 32% campesterol, was kindly contributed by NuSci Institute & Corp. (CA, USA). Ethanol, n-hexane, sodium hydroxide, sodium phosphate salts and other reagents of analytical grades, were purchased from Sigma (Israel).

Table 1. Commercial lipases, their sources, form, and manufacturers, which have been tested in this study.

Commercial name	Species of microorganism	Form lipase	Manufacturer
Lipase F-AP	*Rhizopus oryzae*	Powder	Amano Enzymes, Japan
Lipase G	*Penicillium camemberti*	Powder	
Lipase A	*Aspergillus niger*	Powder	
Lipase PS	*Pseudomonas cepacia*	Powder	
Lipase PF	*Pseudomonas fluorescens*	Powder	
Lipase R	*Penicillium roqueforti*	Powder	
Lipase F	*Rhizopus niveus*	Powder	
Lipase AY	*Candida (C.) rugosa*	Powder	
Lipase CR	*C. rugosa*	Powder	Enzyme Development Corporation, USA
Lipase AN	*Aspergillus niger*	Powder	
Lipase QLM	*Burkholderia (B.) ubonensis*	Powder	Meito Sangio, Japan
Lipase OF	*C. rugosa*	Powder	
Lipase TL	*Pseudomonas stutzeri*	Powder	
Lipase SL	*Pseudomonas (B.) cepacia*	Powder	
Novocor AD L	*C. antarctica A*	Liquid	Novozymes, Denmark
Eversa Transform 2.0	*Thermomyces lanuginosus*	Liquid	
Lipozyme CALB L	*C. antarctica B*	Liquid	
Novozym 435 (Immobilized)	*C. antarctica B*	Immobilized	
Lipozyme RM IM (Immobilized)	*Rhizomucor miehei*	immobilized	

2.2. Analysis Methods

The concentrations of all reaction components including FFAs, FAEEs, MGs, DGs, TGs, PSs and PSEs were determined by gas chromatography. A Hewlett-Packard gas chromatograph (Agilent 6890; USA) equipped with a flame-ionization detector, and a capillary column Agilent DB-1HT, 0.25 mm i.d. x 5m length, and 0.1 µm film thickness (USA), was used under the following separation conditions: Injector and detector temperatures were maintained at 360°C; initial column temperature was 120°C, for 1 min, thereafter, the oven temperature was raised at a rate of 20°C/min to reach 360°C for 5mins. FFAs content (%) in the reaction system was also measured by Karl Fischer titrator 684 KF (Metrohm, Herisau, Switzerland).

2.3. Preparation of Immobilized Lipases

Lipases were immobilized following standard procedures where a lipase derived from a certain microorganism was solubilized in phosphate buffer solution of 0.1M at a certain pH value, typically 7.5 [23]. A macroporous hydrophobic organic polymer resin, such as polypropylene beads (Accurel MP1000, beads size 300-700 µm, and mean pore diameter 25 nm, Product of EVONIK, Germany), was first washed twice with three bed volumes of distilled water and then the wet beads were washed with three bed volumes of ethanol. Ethanol was removed by filtration and then the beads were added into the lipase solution. The mixture was shaken at room temperature for 8 hours. Cold acetone was added into the mixture to increase the enzyme precipitation on the polymer resin. The immobilized enzyme beads were obtained after removal of the aqueous solution by filtration followed by drying of the wet polymer beads by lyophilization down to water content of less than 3% wt./wt.

2.4. Enzymatic Transesterification/Esterification Between PSs and Various Fatty Acyl Donors

A mixture of PSs (0.3g) and fatty acyl donors (3g) comprised of any combination of the following two compositions: either a mixture of plant oil TGs, ethyl oleate (as FAEEs), oleic acid (as FFAs), MGs and DGs at different weight ratios, or partly transesterified plant oil with ethanol comprised mainly of FAEEs, MGs, DGs, TGs and a minor amount of FFAs (less than 3%), with the weight ratios as described below. The oil mixtures before reactions were degassed by bubbling nitrogen gas, then shaken for 1 hour at 60°C. Reaction between the oil mixture components was initiated by the addition of a commercial or an immobilized lipase preparation (150 and 300mg, respectively) into the mixtures. The reaction mixtures were incubated at 60°C and shaken at 160rpm for 24 hours. The water concentration in the reaction medium was in the range of 200-300ppm based on Karl Fischer titrator analysis. Samples of 60 microliters were withdrawn periodically from the reaction mixtures for GC analysis for determining the concentrations (presented as GC peak area ratios %) of the reaction medium components including FFAs, MGs, PSs, DGs, PSEs, and TGs. The relative activity of enzymes was calculated as the peak area ratios for the product PSEs divided by the sum of peak area ratios for PSs and PSEs after one hour of reaction. Under the above-described reaction conditions PSs were not completely soluble in reaction mediums comprised of predominantly oil TGs as fatty acyl group donors, while were completely soluble in reaction mediums comprising any combination of fatty acid acyl group donors however in the presence of FAEEs prepared as detailed below. Each analysis value is the average for three repetitions.

2.5. Preparation of Partially Transesterified Plant Oils via Lipase-catalyzed Transesterification of Oil TGs and Ethanol

A mixture of canola oil (100g) containing 10% wt. ethanol and 0.1% wt. water, was incubated at 60°C. *Thermomyces lanuginosus* lipase immobilized on Accurel MP 1000 beads (5g), was added into the mixture. The reaction mixture was then incubated at 40°C and shaken at 160 rpm for 6 hours. Samples of 60 microliters were withdrawn periodically from the reaction mixture for GC analysis for determining the concentrations (as GC peak area ratios %) of FAEEs, FFAs, MGs, DGs and TGs. The reaction mixture after 6 hours of reaction was filtered off for the removal of the biocatalyst to obtain a partially transesterified canola oil, residual water, and nonreacted ethanol. Residual ethanol and water were flash evaporated, and the remaining reaction mixture with the composition of 67.8% FAEEs, 1% FFAs, 12.4% MGs, 9.7% DGs, and 9.1% TGs (as GC peak area ratios), was used as a medium for solubilization of PSs at concentrations of up to 30% wt./wt. of oil medium, and as a fatty acyl group donor in transesterification/esterification reactions of PSs. Each analysis value is the average for three repetitions.

3. Results and Discussion

3.1. Screening of Lipases of Transesterification Activity to Produce PSEs

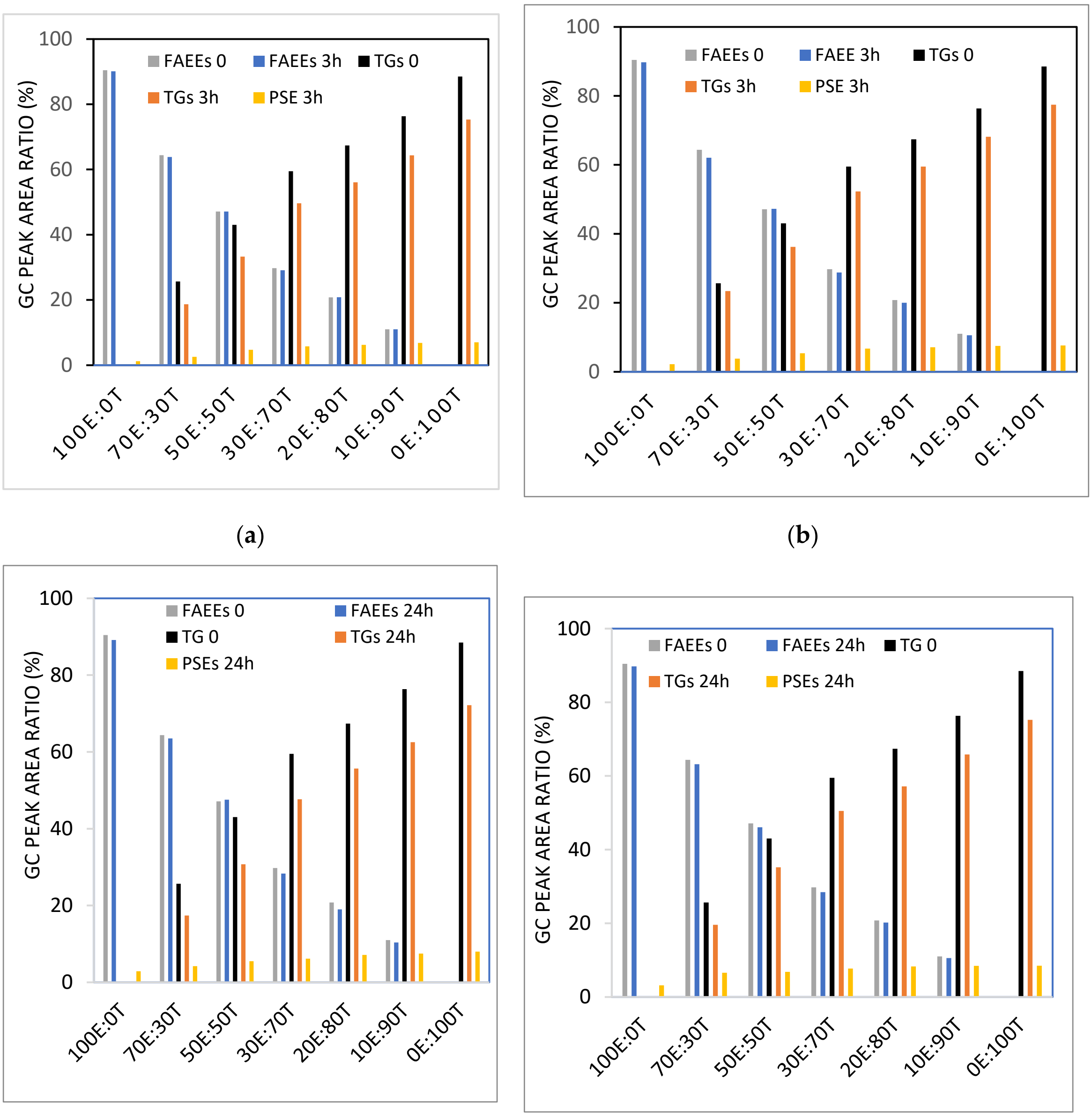
Table 2 shows the concentrations of the transesterification reaction components FFAs, MGs, PSs, DGs, PSEs, and TGs after 24 hours of reaction between PSs and canola oil TGs. In agreement with other research studies, the results presented in Table 2 show that lipases derived from *C. rugosa*, *B. ubonensis*, *C. antarctica A*, and *Pseudomonas sp.*, are the most active enzymes for transesterification of oil TGs and PSs to produce PSEs, MGs and DGs [17,20,23]. Since the maximum solubility of PSs in oil TGs is limited to 3%, the reaction medium was heterogeneous at the beginning and with the progress of reaction became homogenous once adequate amounts of PSEs and MGs are produced which are responsible for solubilization of PSs in the reaction medium. Because of the low solubility of PSs in oil TGs at ambient temperatures the direct transesterification between the reaction components is limited to initial concentrations of PSs lower than 3% to ensure homogeneous reaction medium as a precondition for use of packed-bed column reactors. Furthermore, the results presented in Table 2 show that the concentrations of FFAs, MGs and DGs, have increased in the reaction medium after 24 hours of reaction. This result is attributed to the competing hydrolysis side reaction catalyzed by most lipases tested in this study in the presence of micro-amounts of water in the reaction medium. A substantial increase in the hydrolytic products was obtained when a commercial liquid lipase containing water was used which resulted in increasing the levels of water in the reaction medium and therefore enhancing of the hydrolysis side reaction.

Table 2. The concentrations of the reaction components after 24 hours of reaction using different types of lipases in their free form. Reaction conditions: See Materials and Methods Section 2.4.

		GC Peak area ratio (%)					
Name of lipase	**Source of lipase**	**FFAs**	**MGs**	**PSs**	DGs	PSEs	TGs
Control	-	1.4	0.3	9	5.1	0	84.2
Lipase F-AP	*Rhizopus oryzae*	4.8	3	6.8	7	2.2	76.2
Lipase G	*Penicillium camemberti*	3.3	1.3	7	2.3	2	84.1
Lipase A	*Aspergillus niger*	2.5	1.2	7.5	3.2	1.5	84.1
Lipase PS	*Pseudomonas cepacia*	7.9	3.6	4	11.9	5	67.6
Lipase PF	*Pseudomonas fluorescens*	6.8	2.6	3	12.5	6	69.1
Lipase R	*Penicillium roqueforti*	8.4	3.9	7	11.4	2	67.3
Lipase F	*Rhizopus niveus*	3.7	0.9	7.1	2.4	1.9	84
Lipase AY	*C. rugosa*	7.4	3.7	0.3	13.4	8.7	66.5
Lipase CR	*C. rugosa*	7.6	3.9	0.1	15	8.9	64.5
Lipase AN	*Aspergillus niger*	8.6	3.9	2.8	9.5	6.2	69
Lipase QLM	*B. ubonensis*	9.5	3.7	0.1	14.2	8.7	63.8
Lipase OF	*C. rugosa*	8.4	3.5	0.3	13.9	8.7	65.2
Lipase TL	*Pseudomonas stutzeri*	8.2	4.3	0.2	12.7	8.6	66
Lipase SL	*B. cepacia*	11.3	3.2	0.8	13.5	8.2	63
Novocor AD L	*C. antarctica A*	17.5	6.5	6	18.4	3	48.6
Eversa Transform 2.0	*Thermomyces lanuginosus*	21.6	9.9	7.5	16.6	1.5	42.9
Lipozyme CALB L	*C. antarctica B*	3.3	2	7.5	1.7	1.5	84
Novozym 435 (Immobilized)	*C. antarctica B*	7.5	3.8	6.7	12.2	2.3	67.5
Lipozyme RM IM (Immobilized)	*Rhizomucor miehei*	9.7	3.3	7	10.9	2	67.1

3.2. Enzymatic Transesterification of PSs and Mixtures of Different Weight Ratios of Canola Oil TGs and FAEEs

In an attempt to increase the solubility of PSs in the reaction medium as well as determine the favored fatty acyl group donors, different combination of oil TGs and ethyl oleate (as FAEEs) was prepared and reacted with PSs (10%wt./wt.) in the presence of two different lipases immobilized on a hydrophobic macroporous support, separately. Figure 1A-D show the concentrations of the transesterification reaction components between PSs and mixtures of oil TGs and ethyl oleate at different weight ratios after 3 and 24 hours of reaction using immobilized lipases derived from *C. rugosa,* and *B. ubonensis,* separately. The results show that the concentrations of TGs were significantly reduced with time till reaching equilibria after 24 hours of reaction (equilibria is not shown), while the concentrations of ethyl oleate remained approximately with no significant changes in both cases. These results indicate that both different lipases prefer TGs rather than FAEEs as fatty acyl group donors in the direct transesterification reaction process for PS.



(**a**) (**b**)

(c) (d)

Figure 1. The concentrations of the reaction components for the transesterification reaction between PSs and mixtures of ethyl oleate (E) and canola oil TGs (T) at different weight ratios (E:T) after 3 and

24 hours of reaction using either *B. ubonensis* lipase (a and c) or *C. rugosa* lipase (b and d), both immobilized on Accurel MP 1000 beads. Reaction conditions: See Materials and Methods Section 2.4.

3.3. Transesterification/Esterification of PSs with Mixtures Comprised of Different Weight Ratios of Canola oil TGs, MGs and FFAs as Fatty acyl donors

Table 3 depicts the results for determining the favorite fatty acyl group donor for both lipases derived from *B. ubonensis* and *C. rugosa*, separately, to produce PSEs through transesterification/esterification reactions starting from PSs and a mixture of fatty acyl group donors comprised of FFAs, MGs, DGs and TGs at different weight ratios. The presence of FFAs, MGs and DGs in the reaction medium led to completely solubilizing PSs producing a homogeneous reaction medium. The results presented in Table 3 show explicitly that TGs are better fatty acyl group donors than MGs, DGs and FFAs for PSs to produce PSEs under the described reaction conditions. The results show also that there was an increase in the concentrations of FFAs, typically in the range of 4-8%, as well as for MGs and DGs after 3 and 24 hours of reaction as compared to their initial concentrations. This increase is attributed to the competing hydrolysis side reaction catalyzed by both lipases in the presence of micro-amounts of water in the reaction medium.

Table 3. The concentrations of the reaction components comprised of PSs and a mixture of different weight ratios comprising canola oil (TGs), DGs, MGs, and FFAs after 3 and 24 hours of reaction using an immobilized lipase derived either from *B. ubonensis* or *C. rugosa*. Reaction conditions: As described in the Materials and Methods Section 2.4.

Time (h)	**Lipase source**	**GC Peak area ratio (%)**					
		FFAs	**MGs**	**PSs**	**DGs**	**PSEs**	**TGs**
0	Control	24.25	38.27	25.14	11.92	0	0.42
	Control	14.01	22.74	20.29	10.94	0	32.02
	Control	9.36	14.83	16.75	8.73	0	50.33
	Control	5.1	8.01	15.04	5.8	0	66.05
	Control	4.5	4.11	13.67	4.5	0	73.22
	Control	2	2.67	10.59	3.02	0	81.72
	Control	0.86	0.02	10.14	2.77	0	86.21
3	*B. ubonensis*	29.55	34.88	23.38	10.22	1.76	0.21
	B. ubonensis	20.22	22.47	17.59	22.55	2.7	14.47
	B. ubonensis	12.66	20.77	14.11	26.57	2.64	23.25
	B. ubonensis	8.55	15.46	11.41	28.66	3.63	32.29
	B. ubonensis	7.2	11.47	9.34	27.82	4.33	39.84
	B. ubonensis	5.8	6.6	5.39	23.94	5.2	53.07
	B. ubonensis	4.6	1.45	3.74	12.12	6.4	71.69
	C. rugosa	28.89	34.66	23.03	11.12	2.11	0.19
	C. rugosa	19.88	17.47	16.48	18.64	3.81	23.72
	C. rugosa	16.25	12.36	12.42	18.51	4.33	36.13
	C. rugosa	12.48	7.55	10.27	19.5	4.77	45.43
	C. rugosa	9.55	6.32	8.86	19.96	4.81	50.5
	C. rugosa	6.3	4.11	5.5	17.02	5.09	61.98
	C. rugosa	4.6	1.32	3.93	9.94	6.21	74
24	*B. ubonensis*	31.22	33.54	19.19	9.99	5.95	0.11
	B. ubonensis	21.44	20.69	10.03	22.52	10.26	15.06
	B. ubonensis	17.1	19.39	7.42	26.04	9.33	20.72
	B. ubonensis	13	14.72	2.44	28.17	12.6	29.07
	B. ubonensis	11.3	11.98	4.55	26.5	9.12	36.55
	B. ubonensis	9.8	8.1	2.04	24.68	8.55	46.83
	B. ubonensis	8.5	2.74	0.59	13.3	9.55	65.32

	C. rugosa	31.92	31.89	22.79	10.83	2.35	0.22
	C. rugosa	21.01	18.47	14.97	18.88	5.32	21.35
	C. rugosa	15.77	12.65	10.81	18.27	5.94	36.56
	C. rugosa	12.91	8.83	6.84	21.14	8.2	42.08
	C. rugosa	12	6.76	6.62	21.39	7.05	46.18
	C. rugosa	9.9	5.35	2.71	18.24	7.88	55.92
	C. rugosa	5.31	1.72	1.38	11.46	8.76	71.37

3.4. Screening of Lipases of Transesterification Activity to Produce PSEs Using Partially Transesterified Plant Oils with Ethanol as a Reaction Medium

The results presented in Table 4 are in harmony with the results depicted in Table 2 where lipases derived from *C. rugosa*, *B. ubonensis*, C. *antarctica A*, *Pseudomonas sp.*, and *C. cylindracea* exhibited the highest activity for catalyzing the direct transesterification of PSs and a mixture comprised of FAEEs and oil glycerides (mono-, di- and tri-glycerides). The results show also that when liquid lipase preparations containing water were used the concentrations of FFAs were accordingly high due to the competing hydrolysis side reactions with preference to MGs as a substrate. The results clearly show that the concentrations of FAEEs and DGs remained approximately unchanged before and after the transesterification reaction. Furthermore, the results show that the concentrations of TGs increased after 24 of reaction which can be attributed to the synthetic capability of most of the used immobilized enzymes to esterify MGs and DGs to TGs. The results show explicitly that the concentrations of MGs have dropped significantly after reaction due to two main competing reactions, the first is its conversion to TGs by most lipase preparations while the second because of its transesterification with PSs which results in producing PSEs. These results show surprisingly that MGs are predominantly the most favored fatty acyl group donors amongst all other reaction medium components under the described reaction conditions. Based on the results presented in Tables 2 and 3, the preference of both lipases for MGs as fatty acyl group donors compared to TGs, DGs and FFAs, is ascribed to the addition of FAEEs into the reaction medium.

Table 4. The concentrations of the reaction components comprised of PSs and partially transesterified canola oil with ethanol with the composition as described in the Materials and Methods Section 2.5, after 24 hours of reaction using lipases from different sources immobilized on Accurel MP 1000 beads. Reaction conditions: As described in the Materials and Methods Section 2.4.

		GC Peak area ratio (%)						
Name of lipase	**Immobilized lipase**	**FFAs**	**FAEEs**	**MGs**	**PSs**	**DGs**	PSEs	TGs
Control	-	1.2	56.5	13.4	10.5	10.4	0	8
Lipase F-AP	*Rhizopus oryzae*	8.3	56.2	6.9	8.5	10.3	2	7.8
Lipase G	*Penicillium camemberti*	4	57.2	8	9.6	12.3	0.9	8
Lipase A	*Aspergillus niger*	6.2	56.8	7.1	9.5	11.8	1	7.6
Lipase PS	*Pseudomonas cepacia*	8.6	56.8	6.2	6.3	10.1	4.2	7.8
Lipase PF	*Pseudomonas fluorescens*	9.5	55.5	6.3	8.4	10.2	2.1	8
Lipase R	*Penicillium roqueforti*	7.3	56.3	8.1	9.6	10.1	0.9	7.7
Lipase F	*Rhizopus niveus*	3.6	57.5	7.8	9.7	12.8	0.8	7.8
Lipase AY	*C. rugosa*	14	55.2	5.2	0.4	8.2	10.1	6.9
Lipase CR	*C. rugosa*	10.47	55.18	5.64	0.7	10.51	9.8	7.7
Lipase AN	*Aspergillus niger*	6.57	56.2	7.74	0.4	11.19	10.1	7.8
Lipase QLM	*B. ubonensis*	12.8	55.6	4.8	0.5	8.4	10	7.9
Lipase OF	*C. rugosa*	13.9	53.3	5.7	0.8	8.6	9.7	8
Lipase TL	*Pseudomonas stutzeri*	14.9	55.2	6.2	0.2	6.5	10.3	6.7
Lipase SL	*Pseudomonas (B.) cepacia*	12.7	56	5.6	0.4	7.4	10.1	7.8
Novocor AD L	*C. antarctica A*	9.7	55.8	5.8	9.4	10.6	1.1	7.6
Eversa Transform 2.0	*Thermomyces lanuginosus*	10.68	55.32	7.4	9.6	8.1	0.9	8

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Lipozyme CALB L	*C. antarctica B*	9.9	56	7.4	9.6	8.4	0.9	7.8
Novozym 435 (Immobilized)	*C. antarctica B*	7.1	55.2	8.3	9.6	11	0.9	7.9
Lipozyme RM IM (Immobilized)	*Rhizomucor miehei*	9.2	56.1	6.9	9.5	10.5	1	6.8

3.5. Enzymatic Transesterification of PSs and Partially Transesterified Oils with Ethanol Containing Low Levels of FAEEs

Table 5 shows the concentrations of the transesterification reaction components at different time intervals using PSs dissolved in partially transesterified canola oil with ethanol however containing 20.8% instead of 57.7% of FAEEs, using lipases from different sources immobilized on macroporous hydrophobic polymer beads. Contrary to the results reported in former research studies [17,20], the results presented in Table 5 show explicitly that MGs in the presence of FAEEs in the reaction medium, serve as the favored fatty acyl group donor for PSs as compared to TGs, DGs and FFAs. The results also show that the addition of water into the reaction system would lead to increasing the transesterification activity of both enzymes as well as reaching higher concentrations of PSEs after 24 hours of reaction. The increase in the concentrations of FFAs in all experiments where water was added is attributed to occurrence of the competing hydrolysis side reactions to form FFAs as a hydrolytic byproduct for the partial hydrolysis of MGs, mainly. Furthermore, there was an increase in the concentrations of TGs which can be ascribed to the esterification activity of both lipases to partly convert MGs and FFAs to TGs.

Table 5. The concentrations of the reaction components comprised of PSs and partially transesterified canola oil containing low concentrations of FAEEs (prepared as described in the Materials and Methods Section 2.5) at different time intervals using lipases from different sources immobilized on Accurel MP 1000 beads. Reactions were performed without and with the addition of water at weight ratios of 0.5% of the reaction medium after bringing the oil mixture to a neutral pH with sodium hydroxide solution of 3M. Reaction conditions: As described in the Materials and Methods Section 2.4.

			GC Peak area ratio (%)						
Time (h)	**Immobilized lipase**	**Water (wt./wt.%) of oil**	**FAEEs**	**FFAs**	**MGs**	**PSs**	**DGs**	**PSEs**	**TGs**
0	Control	0	20.8	1.2	27.3	14.0	20.8	0.0	15.9
3	*B. ubonensis*	0	20.2	2.3	17.2	10.6	19.5	5.3	24.8
	B. ubonensis	0.5	21.1	6.5	16.2	11.2	20.8	4.9	19.3
	C. rugosa	0	20.2	4.0	20.4	8.6	23.1	8.7	15.0
	C. rugosa	0.5	22.0	7.3	12.5	3.5	19.2	16.9	18.6
6	*B. ubonensis*	0	20.5	1.5	14.5	8.1	25.0	9.0	21.3
	B. ubonensis	0.5	20.8	6.8	14.5	9.3	19.6	7.9	21.1
	C. rugosa	0	20.7	3.2	18.9	9.1	23.7	8.9	15.5
	C. rugosa	0.5	21.0	7.7	10.8	3.1	20.3	17.4	19.7
24	*B. ubonensis*	0	20.1	1.6	11.9	4.2	25.0	15.7	21.5
	B. ubonensis	0.5	20.3	6.8	12.2	4.9	21.4	14.4	19.9
	C. rugosa	0	21.0	3.5	18.8	9.1	22.9	8.8	15.9
	C. rugosa	0.5	21.3	8.3	11.0	3.0	18.5	17.1	20.7

3.6. Enzymatic Transesterification of PSs with Partially Enzymatically Transesterified Oil Comprised of FAEEs, MGs, and TGs/or with Oil Mixtures Prepared by Mixing Appropriate Weight Ratios of FAEEs, MGs, DGs and TGs

The following is the composition of two oil mixtures A and B with approximately a similar composition.

Mixture A: Partially enzymatically transesterified canola oil with ethanol with the following composition which was prepared as described in the Materials and Methods Section 2.5:

FAEEs %	MGs %	DGs %	TGs %
67.8	12.4	9.7	9.1

Mixture B: An oil mixture prepared by mixing appropriate weight ratios of ethyl oleate (as FAEEs), MGs, DGs and canola oil (as TGs).

FAEEs %	MGs %	DGs %	TGs %
68.4	12.7	9.6	9.3

Both oil mixtures have been enzymatically transesterified with 10% wt./wt. PSs, separately, under the same reaction conditions. Table 6 shows the concentration profiles of the reaction components after 3, 6 and 24 hours of reaction using lipases from two different sources immobilized on Accurel MP 1000, separately. The results presented in Table 6 show clearly that MGs were primarily the favored donor of fatty acyl groups to PSs, rather than TGs or FAEEs under the described direct transesterification reaction conditions. To the best of our knowledge, this is the first time to show that the addition of FAEEs into lipase-catalyzed transesterification reaction medium for PSs and fatty acyl donors including FFAs, MGs, DGs and TGs, MGs would serve predominantly as the favored fatty acyl group donor for PSs amongst the other possible fatty acyl group donors.

Table 6. The concentrations of the reaction components at different time intervals for the transesterification of PSs and oil mixtures A and B, separately, using different immobilized lipases. Reaction conditions: As described in the Materials and Methods Section 2.4.

			GC Peak area ratio (%)						
Time (h)	**Reaction mixture**	**Immobilized lipase**	**FAEEs**	**FFAs**	**MGs**	**PSs**	**DGs**	**PSEs**	**TGs**
0	Mixture A	T=0	62	1.4	11	10.6	7.9	0	7.1
	Mixture B	T=0	61.1	1.7	11.2	10.6	7.8	0	7.6
3	Mixture A	*B. ubonensis*	60.9	5.1	8.9	4.7	7.7	5.9	6.8
	Mixture B	*B. ubonensis*	59.8	5.5	9.5	5.6	7.5	5	7.1
	Mixture A	*C. rugosa*	58.9	7.1	9.9	1.8	7.5	8.8	6
	Mixture B	*C. rugosa*	59.2	7.9	8.7	3.5	6.8	7.1	6.8
6	Mixture A	*B. ubonensis*	58.8	5.3	10.5	2.8	7.8	7.8	7
	Mixture B	*B. ubonensis*	58.4	6.8	10.1	4.4	7.5	6.2	6.6
	Mixture A	*C. rugosa*	59	7.1	9.7	0.6	7.2	10	6.4
	Mixture B	*C. rugosa*	58.3	7.7	8.7	1.8	7.5	8.8	7.2
24	Mixture A	*B. ubonensis*	59	6.6	9.8	1.1	7.6	9	6.9
	Mixture B	*B. ubonensis*	59.3	6.2	10.5	2.2	7	8.4	6.4
	Mixture A	*C. rugosa*	58.2	6.8	9.5	0.1	7.8	10.5	7.1
	Mixture B	*C. rugosa*	59.2	7.9	9.2	1.6	7.6	9.5	5

3.7. The Effect of Phytosterol Concentration

The results presented in Figure 2 illustrate the effect of PSs concentration on the relative direct transesterification activity of *B. ubonensis* and *C. rugosa* lipases, separately, immobilized on Accurel MP 1000. Phytosterols at concentrations of up to 30% wt./wt. of oil glycerides (mono-, di- and tri-glycerides) mixture containing 20% FAEEs, could be completely dissolved at the reaction temperature 60° making a homogeneous reaction medium. Unlike to the results reported in other research studies [17,20], the results illustrated in Figure 2 show that the enzyme transesterification activity did not decrease either due to increasing the PSs concentrations or due to an increase in the rate of formation of the byproduct DGs when the initial concentrations of PSs were raised in the range of 5-30%. These results show for the first time the applicability of lipase-catalyzed direct transesterification of phytosterols in homogeneous oil media with no restrictions for its initial concentrations as well as with no enzyme activity inhibition caused by the byproducts MGs and DGs. Furthermore, the GC analysis results in these tests show explicitly that MGs present in the oil medium are the favorite fatty acyl group donors amongst all other possible used fatty acyl group donors, for PSs to produce their fatty acid esters.

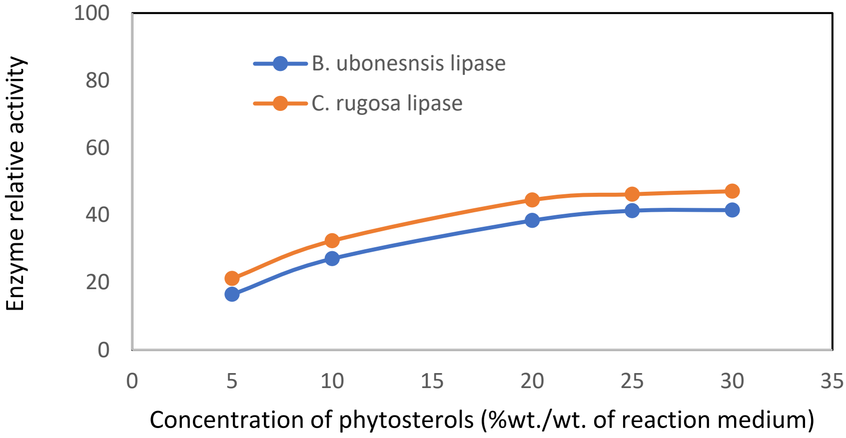

Figure 2. The effect of the initial concentration of PSs dissolved in partially transesterified oil (with the composition as described in Table 5) on the relative transesterification activity of *B. ubonensis* and *C. rugosa* lipases both immobilized on Accurel MP 1000. Reaction conditions: See Materials and Methods Section 2.4. .

4. Conclusions

The present work demonstrates an industrially feasible solvent-free direct transesterification process for oils/fats and PSs using lipases immobilized onto a support matrix as an alternative method to conventional chemical processes to produce PSEs for different industrial applications. The findings of this study show for the first time that under specific conditions in the presence of FAEEs in lipase-catalyzed direct transesterification reaction medium comprised of PSs and mixtures of different ratios of FFAs, MGs, DGs and TGs, MGs would predominately serve as the favored fatty acyl group donors amongst all other reaction medium components for PSs. Furthermore, this method offers several advantages over traditional methods using chemical catalysts or free enzymes, such as avoid the limited solubility of PSs in oil TGs, ease of separation and reuse of the immobilized enzymes, allow enzyme recyclability, and improved efficiency in PSEs production processes. Another major advantage of these findings is the allowance for use of homogeneous reaction medium comprised of various combinations of FAEEs, MGs, DGs, TGs and solubilized PSs up to 30% wt., a prerequisite for utilizing recyclable immobilized enzymes in batch and continuous packed-bed reactors to avoid mass transfer limitations in the vicinity of the immobilized enzyme as well as avoid blockage of the column reactors.

5. Patents

A US patent has been filed based on the results reported in this manuscript.

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