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Immobilization of *Pseudomonas stutzeri* Lipase for the Transesterification of Wood Sterols with Fatty Acid Esters

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Abstract Lipase from *Pseudomonas stutzeri* PL-836 was immobilized on hydrophobic supports and evaluated in the transesterification of wood sterols in solvent-free and solvent-containing media. Triton X-100 was used as additive during immobilization in butyl and octadecyl sepabeads increasing enzyme activity yield by 5% and 60%, respectively. Hyperactivation was observed during immobilization in EC octadecyl sepabeads with enzyme activity yield of 200% and protein immobilization yield of 93%. Thermostability of the immobilized enzyme was assessed at 50 °C in different media in the absence and presence of exogenous solvents. The presence of Triton X-100 during immobilization reduced enzyme stability while *tert*-butanol increased it. Transesterification in solvent-free and solvent-containing medium with lipase immobilized in EC octadecyl sepabeads showed that the presence of exogenous solvent increased both conversion yield and productivity. At rather high levels of biocatalyst hydration (40% on wet basis) the presence of *tert*-butanol in the reaction medium more than doubled conversion yield and productivity.

Keywords Lipase · Hydrophobic supports · Enzyme immobilization · Transesterification · Wood sterols · Fatty acid esters

Introduction

Phytosterols (sterols and stanols derived from plants) have proven to be effective in reducing blood serum cholesterol levels when included in the regular diet [1–3]. These products, particularly stanol esters, have conclusively proven to reduce the amount of low-density lipoproteins and are accepted as anticholesterolemic agents [4, 5]. Nowadays, sterols and stanols are obtained mainly from soy [6]. However, woody materials, as those derived from pulp and paper manufacturing, represent interesting alternative raw materials. In fact, wood sterols (actually a mixture of sterols and stanols where β-sitosterol and β-

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sitostanol amount more than 90%) can be obtained as by-products of the Kraft pulping process contributing to pollution abatement, have a narrower sterol and stanol distribution (see Table 1), and derive from nongenetically manipulated biomass [7]. Esters are the preferred form of administrating sterols and stanols because of their increased solubility in the food matrix and increased efficacy [8, 9]. Esterification can be done chemically [10, 11] or enzymatically [12, 13]. Lipase transesterification involves the reaction between wood sterols and esters of fatty acids acting as acyl donors [14]. Enzymatic transesterification, despite its lower productivity when compared to chemical transesterification, is a valuable option in terms of the specificity of the reaction and product quality attainable; however, enzyme cost is a major hurdle so that enzyme immobilization is desirable since efficiency of biocatalyst use can be significantly increased [7, 15]. Hydrophobic matrices are a preferred option for lipase immobilization since they promote the adoption of an active configuration, especially in those lipases bearing a lid (polypeptide chain covering the active site) that moves away from the active site (open configuration) allowing catalysis to take place [16, 17]. Hyperactivation and increase in stability have been obtained by immobilization of lipases on highly hydrophobic supports [18]. Transesterification can be conducted in the presence or absence of added solvents. In the first case, one of the substrates can act as solvent (i.e., the esterification agent). Using this strategy, product (alcohol) inhibition can be alleviated by conducting the reaction under vacuum promoting alcohol removal by stripping [12]. However, a considerable excess of fatty acid ester is required to dissolve the sterol mixture, so the use of exogenous solvent allows maintaining a more proper stoichiometric ratio. Hexane has been extensively used as a suitable exogenous solvent in lipase transesterification reactions [19, 20], but product inhibition cannot be alleviated when using hydrophobic organic solvents. Methanol is an inhibitory by-product that is poorly soluble in the mixture of fatty acid methyl ester (FAME) and *n*-hexane [21], so that the use of moderately polar solvents, like *tert*-butanol may reduce product inhibition by dissolving out the methanol produced in the reactions of transesterification [22].

The transesterification of wood sterols with fatty acid esters has been studied using a previously selected lipase from *Pseudomonas stutzeri* (lipase TL) [14, 23]. The enzyme was immobilized with the purpose of increasing its activity and stability under reaction conditions. The enzyme was immobilized in highly hydrophobic supports (butyl and octadecyl sepabeads) in the presence and absence of Triton X-100 and the biocatalyst selected was characterized in terms of activity and stability in different media containing fatty acid esters, in the absence and presence of hexane and *tert*-butanol. The transesterification of wood sterols with fatty acid esters was then performed with the selected biocatalyst in the selected reaction media.

Table 1 Composition of wood sterols and sterols from soy [7]

Compound	Weight percentage	
	Wood sterols	Soy sterols
β-sitosterol	75	50
Campesterol	7	25
Stigmasterol	<1	20
Brasicasterol	–	3
β-sitostanol	15	2
Campestanol	2	<1

Materials and Methods

Materials

Lipase (triacylglycerol acylhydrolase EC 3.1.1.3) from *P. stutzeri* PL-836 (lipase TL) was kindly donated by Meito Sangyo (Tokyo, Japan). The enzyme was in the form of a fine powder with 17.3% (*w*/*w*) protein and 121 international units (IU)/g. Immobilization supports (EC butyl and EC octadecyl Sepabeads™) were a gift from Resindion Mitsubishi Chemical Corporation, Italy. Wood sterols (WS) were kindly supplied by Arboris (Savannah Georgia, USA) and methyl esters of sunflower fatty acids by Härting S.A. (Santiago, Chile); *p*-nitrophenol (pNP), *p*-nitrophenylpalmitate (pNPP), *n*-hexane (99.9%), Triton X-100, N,O-Bis-(trimethylsilyl)-acetamide were from Sigma (Saint Louis, MO, USA), bovine serum albumin, brilliant blue Coomassie G-250, ethanol (95%), ortophosphoric acid (85%), methanol (99.9%), NaOH, pyridine and *tert*-butanol were from Merck (Darmstadt, Germany).

Analyses

Protein was determined according to Bradford [24]. Activity was determined according to the method proposed by Ten and Xu [25], and adapted by Vega and Villalón [23]. Transesterification activity was determined spectrophotometrically as the initial rate of formation of pNP from pNPP. A reaction mixture was prepared by mixing 1 mL of 8 mM pNPP standard solution, 8.7 mg of wood sterols and 200 μL of anhydrous hexane (99.9%) and then contacted with 10 mg of immobilized lipase (IL). One IU of transesterification activity was defined as the amount of IL producing 1 μmol of pNP per minute from pNPP and WS at 50 °C at the above conditions. A temperature of 50 °C has been previously reported as optimum temperature for transesterification of wood sterols with the same lipase [23]. Reaction was stopped by cooling and the cromophore was extracted with 5–15 mL of 0.1 M NaOH, the suspension was then filtered through a 0.45-μm membrane and the organic phase removed after complete separation; the absorbance of the aqueous phase was determined at 410 nm and converted to pNP concentration through a suitable calibration curve.

Water concentration in IL was determined by the method of Karl–Fischer in a Mettler Toledo DL 38 titrator.

Transesterification reaction was determined by measuring the decrease in (non-esterified) WS. Non-esterified sterols were determined by gas chromatography in an HP 6890 chromatograph using a Hewlett-Packard (Rockville, MD, USA) HP-5 capillary column (5% diphenyl, 95% dimethylpolysiloxane; 30 m×0,32 mm; 0.25-μm film thickness). Sample volumes were 0.5 μL and the carrier gas was helium. A split injector was used with a 50:1 split ratio and temperature of 320 °C. The FID detector (set at 300 °C) was used in combination with internal standard (cholesterol). The final oven temperature was 300 °C and total run time was 15 min.

Enzyme Immobilization

Lipase TL was immobilized on EC butyl sepabeads (TLBS) and EC octadecyl sepabeads (TLOS). Lipase TL (20–100 mg) was dissolved in 10 mL of 0.1 M sodium phosphate buffer pH 7.0 with Triton X-100 (0.05%) and without it. One gram dry weight of support (EC butyl or EC octadecyl sepabeads) was washed and equilibrated in the same buffer and

stored wet at 4 °C. The support was then contacted with the enzyme solution in a roller bottle at room temperature (18±2 °C) until protein concentration in the liquid phase remained constant. The immobilized enzyme was then recovered by filtration in a 40 μm filter crucible, thoroughly washed with hexane and then dried under vacuum for 15 min and left for 12 h under vacuum at room temperature. The immobilized enzyme was then stored dry at 4 °C for further use.

Protein immobilization yield was defined as the fraction of contacted protein retained in the support, the latter being determined as the difference between contacted protein and protein remaining unbound in solution. Enzyme activity yield was determined as the fraction of the contacted activity (in IU of transesterification) that was expressed in the immobilized enzyme.

Immobilization experiments were done in triplicate differences never exceeding 5%.

Thermal Stability of Immobilized Enzyme

Thermal stability of TLOS (17.3 mg of protein/g support) in the presence and absence of Triton X-100 was determined at 50 °C in FAME. Two hundred milligram of 200 of TLOS were suspended in the different media, this amount corresponding to the activity (UI) of the free enzyme when contacted at a ratio of 5% by weight with WS (0.4 g). Exogenous solvent volume for each media (A, B, C, and D) was calculated from this amount of WS. Thermal inactivation of TLOS was conducted in all reaction media described in the next section (see Table 2) but not including WS. Samples were taken at intervals and residual transesterification activity was determined as already described.

Selection of Reaction Media

Reaction media without and with the addition of exogenous solvents were considered. In the first case, the substrate FAME acted as solvent. FAME was used as acyl donor in the reaction of transesterification of WS, which has been previously selected for giving the higher conversions [7]. Composition of WS is in Table 1. Composition of reaction media is presented in Table 2. FAME/WS ratio in medium A was determined to completely dissolve the WS in the FAME. In medium B, the addition of hexane was determined to completely dissolve WS at a stoichiometric ratio with respect to FAME. The relative amount of *tert*-butanol in medium C was determined according to the results reported for other methanolysis reactions by Li et al. [21] and Wang et al. [26]. Medium D was designed considering the *tert*-butanol/hexane ratio employed by Damstrup et al. [27] in reactions of glycerolysis, and the stoichiometric FAME/WS ratio, as in medium B.

Transesterification Reaction with Lipase TL Immobilized in EC Octadecyl Sepabeads

Based on the results on immobilization and thermal inactivation, transesterification was conducted with TLOS without Triton X-100 in a medium with FAME, WS and *tert*-butanol;

Table 2 Composition of reaction media

Medium		Mass ratio
A	FAME/WS	5:1
B	FAME/WS/Hex	0.74:1:10
C	FAME/WS/Tb	5:1:4.32
D	FAME/WS/Tb/Hex	0.74:1:11.8:10

FAME methyl esters of sunflower fatty acids, *WS* wood sterols, *Hex* hexane, *Tb tert*-butanol

transesterification in FAME/WS medium was conducted as control. WS was dissolved in FAME and then *tert*-butanol was added. Reactor temperature was stabilized at 50 °C and operated at 300 rpm at atmospheric pressure when using *tert*-butanol and under vacuum (20 mmHg) in the control medium without *tert*-butanol. Samples were taken at intervals during the reaction and the amount of non-esterified sterols was determined by gas chromatography. Conversion yield (Y) was determined as:

$$Y = \frac{C_{WS0} - C_{WS}}{C_{WS0}} \quad (1)$$

where, C_{WS0} is the initial mass concentration of non-esterified wood sterols and C_{WS} is its mass concentration at a certain reaction time.

Results and Discussion

Enzyme Immobilization

The crude powder lipase TL preparation had 17.3% protein on a dry weight basis and 121 IU/g dry powder. Enzyme immobilization on both supports, in the absence and presence of Triton X-100, was studied at different protein loads (3.46, 8.65, and 17.3 mg protein contacted/g support) and results are presented in Fig. 1 in terms of protein and enzyme activity yield. Protein immobilization yield was about 60% for TLBS and 93% for TLOS for all amounts of contacted protein with and without Triton X-100. Enzyme activity yields were much higher in TLOS than in TLBS both in the absence and presence of Triton X-100; higher hydrophobicity of the latter may be responsible for the higher activity yields obtained [28]. In the case of TLOS, hyperactivation was observed as previously shown for other lid-containing lipases immobilized in hydrophobic sepabeads, where the hydrophobic residues in the matrix strongly interact with the hydrophobic regions close to the active site displacing the structural equilibrium to the open active configuration [16–18]. However, as shown in Fig. 1, the magnitude of hyperactivation is reduced as the support is challenged to higher protein loads, as already reported for an immobilized lipase from *Alcaligenes* sp in similar supports [7]. The above analysis is further supported by the values of specific activities obtained, which were one order of magnitude higher in TLOS than in TLBS, both in the absence and presence of Triton X-100, as shown in Fig. 2, so that TLOS was selected for further studies. These results are in agreement with those reported by Petkar et al. [29], who demonstrate that the specific activity of lipases increased with the length of the hydrophobic group attached to the matrix. As shown in Fig. 2, the use of Triton X-100 during immobilization had a positive effect both in terms of enzyme activity yield and specific activity. It has been claimed that, in general, detergents may prevent the formation of lipase dimers in solution [18, 30–32] and may also have some direct effect on the conformational equilibrium stabilizing the open active configuration prior to immobilization [33].

Thermal Stability of Immobilized Enzyme

Studies were conducted with TLOS under nonreactive conditions, in all reaction media described in Table 2, without WS. Thermal stability of TLOS, at a protein load of 17.3 mg protein/g support, was evaluated at 50 °C both in the absence and presence of Triton X-100. Methanol is produced as a by-product in transesterification reactions and being an inhibitor

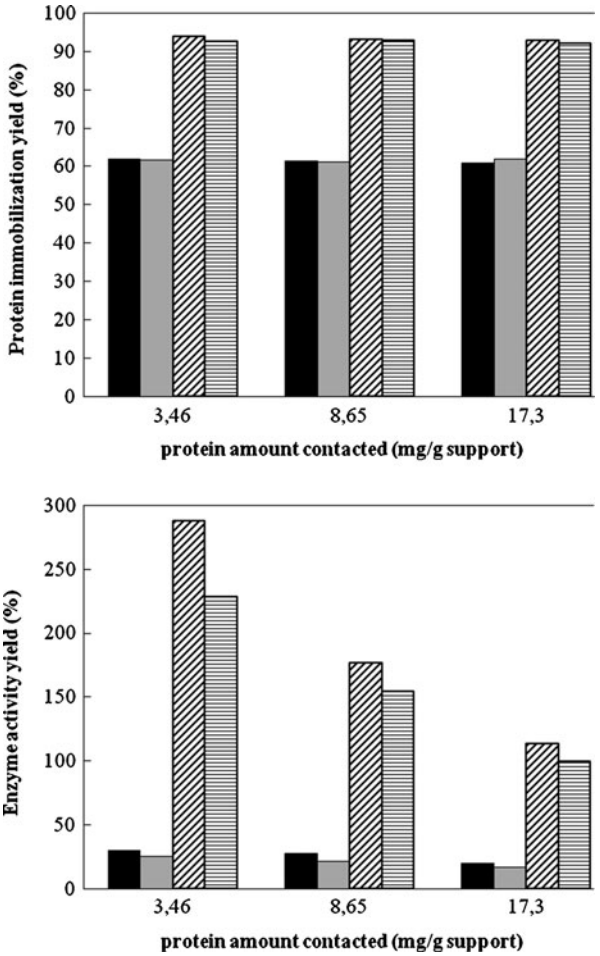

Fig. 1 Protein immobilization and enzyme activity yields in EC butyl sepabeads with Triton X-100 (*black bars*) and without it (*gray bars*) and in EC octadecyl sepabeads with Triton X-100 (*diagonal stippled bars*) and without it (*horizontal stippled bars*)

of lipase activity, its removal is beneficial, which can be done by stripping it under vacuum when FAME acts as the solvent; however, this is no longer possible when hexane or *tert*-butanol are used as solvents because they are volatile enough to be stripped along with methanol. Therefore, to make the stability tests more comparable among these nonreactive media, amounts of methanol equivalent to those produced by transesterification were added stepwise during the course of inactivation in mediums B, C, and D. Results are presented in Fig. 3. Experimental data were well represented by a one-stage inactivation mechanism whose model is:

$$\frac{e}{e_0} = A' + (1 - A')\ \exp(-k_D \cdot t) \tag{2}$$

where, e is the specific activity of biocatalyst at incubation time t (IU/g), e_0 is its initial value (IU/g), k_D is the transition rate constant (h^{-1}) and A′ is the specific activity ratio of the

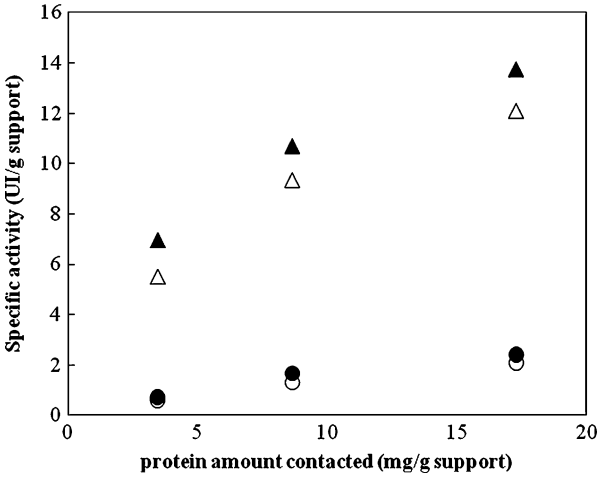

Fig. 2 Specific activity of EC butyl sepabeads (*circles*) and EC octadecyl sepabeads (*triangles*) immobilized lipase TL in the presence (*black figures*) and absence (*white figures*) of triton X-100

final and initial enzyme species. Model parameters were calculated by the method of least squares and results are summarized in Table 3.

Stability was higher for the enzyme immobilized in the absence of Triton X-100. This may be attributed to the fact that in the absence of detergent, the dimeric form of the

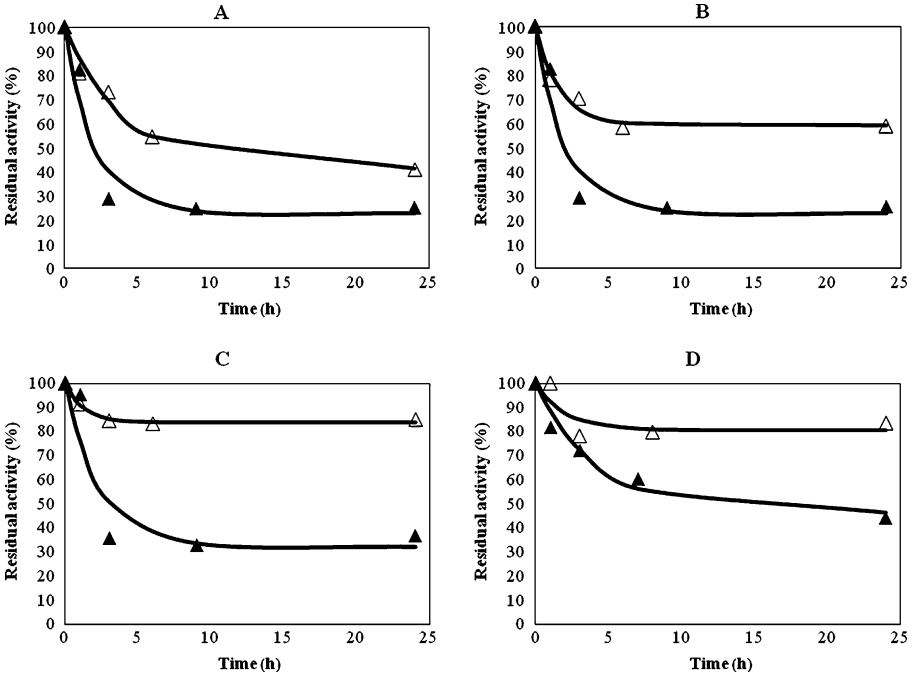

Fig. 3 Thermal stability at 50 °C of EC octadecyl sepabeads immobilized lipase TL with Triton X-100 (*black triangles*) and without it (*white triangles*) in different media: **a** FAME, **b** FAME/hexane, **c** FAME/*tert*-butanol, **d** FAME/*tert*-butanol/hexane. *Symbols* correspond to experimental data, *lines* correspond to the fitted inactivation model

Table 3 Parameters of the model of thermal inactivation of TLOS according to a one-stage inactivation mechanism with activity residual

Medium		A′	k_D (h^{-1})	R
A	With TritonX-100	0.2273	0.4914	0.9712
	Without TritonX-100	0.4114	0.2446	0.9873
B	With TritonX-100	0.3583	0.3012	0.9910
	Without TritonX-100	0.5899	0.6085	0.9845
C	With TritonX-100	0.3208	0.4292	0.9326
	Without TritonX-100	0.8388	0.8229	0.9933
D	With TritonX-100	0.4613	0.2389	0.9812
	Without TritonX-100	0.8055	0.4946	0.8716

A′ Specific activity ratio of the final and initial enzyme species, k_D first-order transition rate constant, R correlation coefficient

enzyme may prevail over the monomeric one, being the stability of both different, as already determined for immobilized lipases from *Bacillus thermocatenulatus* [18] and *Alcalígenes* sp [34]. Stability was significantly increased in media containing exogenous solvents, being higher for the enzyme immobilized in the absence of Triton X-100 in medium containing *tert*-butanol as solvent (medium C without WS). This effect might be due to the ability of *tert*-butanol to dissolve methanol so the negative effect of methanol can be eliminated. [21], improving enzyme performance.

Stability of the free enzyme in media A and B was determined and results are presented in terms of residual activity after 24 h in Table 4. As seen, there is no definite increase in stability as a consequence of immobilization.

Transesterification Reaction with Lipase TL Immobilized in EC Octadecyl Sepabeads

Transesterification of FAME with WS was conducted with TLOS with humidity of 40% on wet basis. Reaction media A and C were used, but the relative amounts of FAME, WS, and *tert*-butanol were modified in medium C since it was possible to reduce them without affecting the homogeneity of the system.

As shown in Fig. 4, both conversion yield and productivity (amount of product per unit volume per unit time) increased considerably by including *tert*-butanol in the reaction medium, more than doubling the values obtained in a reaction medium without added exogenous solvent. This effect is most probably due, as mentioned above, to trans-

Table 4 Residual activity of lipase TL after 24 h at 50 °C

Medium	Residual activity (%)	
	Free enzyme	TLOS without Triton X-100
A	39.6	41.1
B	57.1	59.0

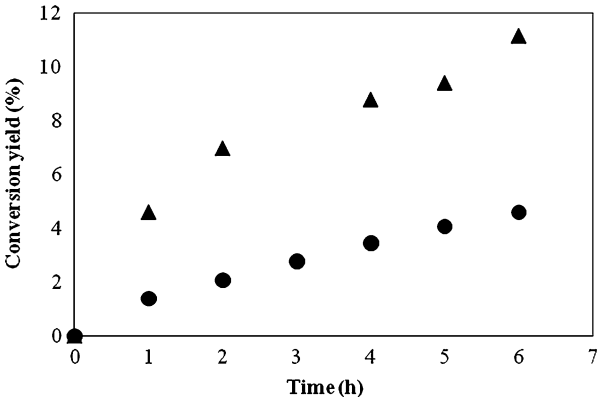

Fig. 4 Time course of the transesterification reaction for the synthesis of wood sterol esters with EC octadecyl sepabeads immobilized lipase (without Triton X-100) in FAME/WS 10:1 medium (*circles*) and FAME/WS/*tert*-butanol 2:1:1.7 medium (*triangles*)

esterification reactions that are favored in moderately polar solvents [35]. However, the reaction in the absence of *tert*-butanol (medium A) was conducted under vacuum, while in its presence (medium C) was conducted at atmospheric pressure thereby increasing the inhibition effect by methanol so that results may be partly due to this fact. The effect of *tert*-butanol on dried LTOS is now being studied. Values of conversion yield in medium C are modest but according to expected for a reaction conducted at atmospheric pressure where methanol inhibition is present [14].

Conclusions

Hydrophobic matrices were excellent supports for immobilizing TL lipase from *P. stutzeri* PL-836. Results were significantly better in terms of enzyme activity yield and specific activity for TL immobilized in the more hydrophobic support (EC octadecyl sepabeads) where hyperactivation was observed being quite significant at low to moderate protein loads. Triton X-100 had a positive effect on enzyme immobilization parameters.

Absence of triton X-100 during immobilization and the presence of exogenous solvents significantly increased TLOS stability. In the presence of *tert*-butanol, the enzyme was very stable at the rather harsh conditions required for transesterification. After an initial decay, enzyme activity remained unaltered for a long period of time, which makes biocatalyst reuse feasible with a considerable increase in biocatalyst efficiency.

Transesterification of WS with FAME was conducted with TLOS in medium without and with exogenous solvents showing a significant positive effect of *tert*-butanol in terms of conversion yield and productivity of the reaction. Conversion yields, though modest, are according to expected for a reaction conducted at atmospheric pressure.

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