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Screening of novel feruloyl esterases from *Talaromyces wortmannii* for the development of efficient and sustainable syntheses of feruloyl derivatives

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ABSTRACT

The feruloyl esterases Fae125, Fae7262 and Fae68 from *Talaromyces wortmannii* were screened in 10 different solvent: buffer systems in terms of residual hydrolytic activity and of the ability for the transesterification of vinyl ferulate with prenol or L-arabinose. Among the tested enzymes, the acetyl xylan-related Fae125 belonging to the phylogenetic subfamily 5 showed highest yield and selectivity for both products in alkane: buffer systems (*n*-hexane or *n*-octane). Response surface methodology, based on a 5-level and 6-factor central composite design, revealed that the substrate molar ratio and the water content were the most significant variables for the bioconversion yield and selectivity. The effect of agitation, the possibility of DMSO addition and the increase of donor concentration were investigated. After optimization, competitive transesterification yields were obtained for prenyl ferulate (87.5–92.6%) and L-arabinose ferulate (56.2–61.7%) at reduced reaction times (≤ 24 h) resulting in good productivities (> 1 g/L/h, > 300 kg product/kg FAE). The enzyme could be recycled for six consecutive cycles retaining 66.6% of the synthetic activity and 100% of the selectivity.

1. Introduction

Feruloyl esterases (FAEs, EC 3.1.1.73) are a subclass of carbohydrate esterases belonging to the CE1 family of the CAZy database (www.cazy.org). They are generally known for their role in the enzymatic hydrolysis of lignocellulosic biomass as they are able to catalyze the hydrolysis of the ester bond between hydroxycinnamic acids such as ferulic acid (FA) and sugars acting synergistically with other lignocellulolytic enzymes such as xylanases [1]. FAEs are a very diverse class of enzymes that have been initially categorized to type A–D based on their specificity on methyl hydroxycinnamates and diferulates [2] and more recently have been assigned to 13 different phylogenetic subfamilies (SF1-13) [3,4].

During the last decades, FAEs have been employed as a biosynthetic tool for the modification of hydroxycinnamic acids and their esters via esterification or transesterification reactions, respectively, with alcohols or sugars altering their lipophilicity and maintaining their bioactive properties, among which is the potent antioxidant activity [5]. As hydroxycinnamic acids are partially soluble in water or oil media, the modified lipophilicity of resulting esters could facilitate their application in the food, pharmaceutical and cosmetics industries [6]. Reports on FAE-catalyzed modifications include the (trans)esterification of hydroxycinnamates with alcohols (1-propanol, 1-butanol or glycerol) resulting in varying yields (1–97%) [7–11] while the synthesis of sugar esters has offered significantly lower yields (1–52%) due to the solubility limitations of sugars in the low water content media that are essential for transesterification [12–17]. The vast majority of FAE-based bioconversions has been performed in ternary solvent systems forming detergentless microemulsions, protecting the enzyme from inactivation in aqueous microdroplets, as this class of enzymes is less stable in organic solvent media than lipases, while little attention has been paid on the stability of this class of enzymes in different solvent systems.Moreover, optimization studies have been focused on maximizing the transesterification yield having little interest in the minimization of the side-hydrolytic reactions that occur naturally due to the presence of water, while only methyl or ethyl hydroxycinnamates have been employed as donors for transesterification.

Abbreviations: AFA, L-arabinose ferulate; FA, ferulic acid; FAE, feruloyl esterase; PFA, prenyl ferulate; RSM, response surface methodology; SF, subfamily; VFA, vinyl ferulate

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The advantage of employing FAEs as biocatalysts is the high specificity towards hydroxycinnamic acids that constitutes them a superior tool for the synthesis of feruloyl esters. On the other hand, the widely studied lipases might be more stable in pure organic solvents but can only catalyze the esterification of phenolic acids only if the aromatic moiety is not para-hydroxylated and the lateral chain is saturated [5,9]. Therefore, there is a need for the selection of novel FAEs with increased synthetic potential and the assessment of their stability in organic solvents for the development of competitive synthetic processes. Moreover, the use of engineered substrates, such as vinyl esters, can result in high yields and reduced reaction times, as the transesterification by-product, vinyl alcohol, tautomerizes to acetaldehyde shifting the reaction towards synthesis instead of hydrolysis, that occurs as side-reaction.

In this work, three novel FAEs from *Talaromyces wortmannii* expressed in the C1 platform were screened in a variety of solvents in terms of enzyme activity and synthetic ability. Two targeted compounds of different lipophilicity, prenyl ferulate (PFA) and L-arabinose ferulate (AFA), were synthesized via FAE-catalyzed transesterification of vinyl ferulate (VFA) with the respective acceptors (prenol or L-arabinose), as presented in Fig. 1. The relationship between reaction parameters (water content, substrate ratio, time, enzyme concentration, pH and temperature) and the bioconversion yield and selectivity was investigated in the best solvent using the best enzyme. The optimal conditions were determined using response surface methodology (RSM) offering an insight into the potential of the industrial applications of FAEs as biocatalytic tools for antioxidant modification.

2. Materials and methods

2.1. Materials and enzymes

MFA was purchased from Alfa Aesar (Karlsruhe, Germany). VFA was provided by Taros Chemicals GmbH & Co. KG (Dortmund, Germany). Ionic liquids 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIm][BF_4]) and 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIm][PF_6]) and other solvents were purchased from Sigma-Aldrich (Saint-Louis, USA). All solvents were of analytical grade (> 99% purity). Genomic DNA sequences encoding the three putative feruloyl esterases, Fae125 (type A, SF5, Genbank ID: MF362595.1), Fae7262 (type B, SF6; Genbank ID: MF362597.1) and Fae68 (type B, SF1, Genbank ID: MF362596.1), were synthesized (GeneArt, Germany) and cloned in a transformation vector as previously described [18]. In each case, the resulting expression cassette containing the gene of interest under the control of a strong promoter was co-transformed with a selection marker in a C1 production strain. The production strains were grown aerobically in a fed-batch system in minimal medium supplemented with trace elements [19]. After fermentation, the broth was centrifuged (15,000 x g for 1 h, 4 °C) and the cell-free broth was concentrated and dialyzed to 10 mM phosphate buffer pH 6.5. Samples were subsequently freeze-dried for storage.

2.2. Protein assay and determination of FAE content

The protein concentration of each enzymatic sample was determined using the Pierce™ BCA Protein Assay (Thermofisher Scientific, Waltham, USA). The FAE content (g FAE/g protein) of the enzymatic preparations was determined by a densitometric method, including SDS-PAGE using a Novex Sharp pre-stained protein standard (Thermofisher Scientific, Waltham, USA) and subsequent quantification of bands using JustTLC software (Sweday, Lund, Sweden). It was determined that the FAE content of the enzymatic preparations was 0.10–0.15 g FAE/g protein for Fae68, 0.15–0.25 g FAE/g protein for Fae7262 and 0.10 g FAE/g protein for Fae125.

2.3. Effect of reaction media on the enzyme activity

The stability of FAEs was tested by incubating 0.5 mg/mL in 96.8:

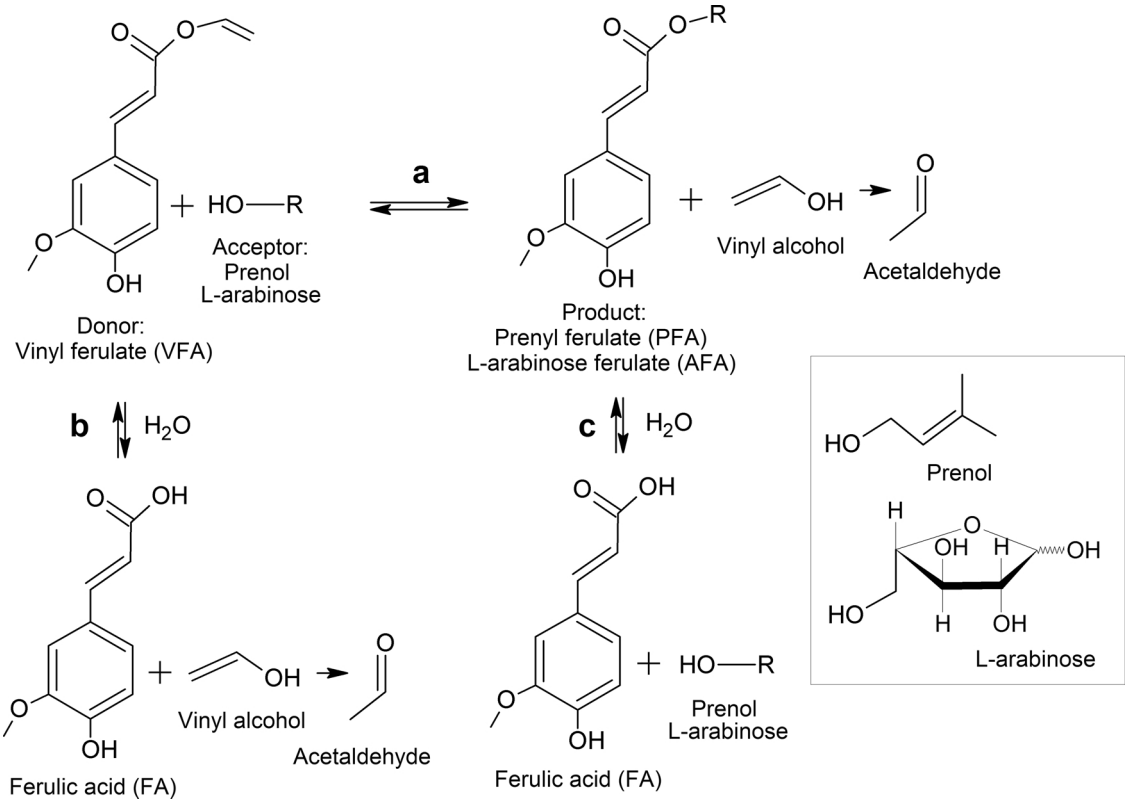

Fig. 1. Schematic representation of enzymatic a) transesterification of VFA (donor) with prenol or L-arabinose (acceptor) b) hydrolysis of VFA (competitive side-reaction) c) hydrolysis of product (competitive side-reaction). Under normal conditions vinyl alcohol tautomerizes to acetaldehyde.

3.2 v/v solvent: 100 mM MOPS-NaOH pH 6.0 for 8 h at 25 °C and 1000 rpm agitation in an Eppendorf thermomixer (Eppendorf, Hamburg, Germany). Different solvents were used including alkanes (*n*-hexane, *n*-octane), alcohols (*t*-amyl alcohol, prenol, *t*-butanol), an ester (ethyl acetate), a ketone (methyl ethyl ketone) and ionic liquids ([BMIm][BF_4], [BMIm][PF_6]). The enzyme was introduced in the solvent as stock solution in buffer. At the end of 8 h, samples were agitated to homogeneity following withdrawal of 10 μL and subsequent dilution of the aliquot in 970 μL 100 mM MOPS-NaOH pH 6.0. In the case of water immiscible solvents (*n*-octane, *n*-hexane and [BMIm][PF_6]) where the enzyme was enclosed in the water phase forming a biphasic system, the solvent was removed and the water phase was re-diluted in buffer achieving the desired concentration for determining enzymatic activity.

The activity was assayed by introducing 1 mM MFA (20 μL of 50 mM stock solution in dimethyl sulfoxide, DMSO) at a total volume of 1 mL. Samples were incubated at 45 °C for 10 min while reactions were ended by subsequent incubation at 100 °C for 5–10 min. Subsequently, they were 5-fold diluted in acetonitrile. All reactions were carried out in duplicate accompanied by appropriate blank samples that were prepared for each reaction containing buffer instead of enzyme. One unit (U) is defined as the amount of enzyme (mg) releasing 1 μmol FA per minute under the defined conditions. No substrate consumption was observed in the absence of esterase. The initial enzyme activity was assessed for each enzyme after incubation in buffer for 8 h at 25 °C and 1000 rpm agitation.

2.4. Effect of reaction media on the transesterification yield and selectivity

Transesterification reactions were performed at 98.6: 3.2 v/v solvent: 100 mM MOPS-NaOH pH 6.0 using 50 mM VFA as donor and 200 mM prenol or 30 mM L-arabinose, aiming to the synthesis of prenyl ferulate (PFA) and L-arabinose ferulate (AFA), respectively. Reactions were performed in an Eppendorf thermomixer (Eppendorf, Hamburg, Germany) for 8 h at 40 °C and 1000 rpm agitation, using fixed enzyme load for each enzyme. In accordance with paragraph 2.3, a variety of solvents was screened (*n*-hexane, *n*-octane, *t*-amyl alcohol, prenol, *t*-butanol, ethyl acetate, methyl ethyl ketone, [BMIm][BF_4] and [BMIm][PF_6]). Additionally, a ternary system (*n*-hexane: *t*-butanol: buffer 53.4: 43.4: 3.2 v/v/v) forming detergentless microemulsions was used as a reference based on previous reports [11,16]. At the end of incubation, reactions were ended by addition of acetonitrile. Each reaction was carried out in duplicate and was accompanied by a blank sample, containing buffer instead of enzyme.

2.5. Experimental design for optimization in best solvent

The synthesis of the two targeted compounds (PFA and AFA) was optimized in the best solvent using the most promising biocatalyst by response surface methodology (RSM) provided by Design-Expert Software 9.0 (Stat-Ease Inc., Minneapolis, USA). A five-level ($-\alpha$, -1, 0, $+1$, $+\alpha$), six-variable central composite design (CCD) was applied for studying the effect of transesterification variables. The variables selected for optimization were: X_1 water content (% v/v), X_2 substrate molar ratio (mM acceptor/mM donor), X_3 time (h), X_4 enzyme concentration (g FAE/L), X_5 pH and X_6 temperature (°C). Table 1 lists the levels of the six independent variables for the synthesis of each targeted compound. The independent variables are coded to two levels, low (-1) and high ($+1$), whereas the axial points are coded as minimum ($-\alpha$) and maximum ($+\alpha$). The $\pm\ \alpha$ levels for variables X_1, X_2, X_4 were selected based on limitations during the preparation of reaction mixtures. The chosen responses were Y_1 transesterification yield (% mM targeted compound/mM VFA) and Y_2 selectivity (mM targeted compound/mM FA). The full factorial design consisted of 64 factorial points, 12 axial points and 10 center points, leading to 86 sets of experiments. Each reaction condition was carried out in duplicate at 1000 rpm agitation and fixed donor concentration (60 mM VFA for PFA and 80 mM VFA for AFA). The following buffers were used at 100 mM concentration: sodium acetate (pH 4–6) and MOPS-NaOH (pH 6–8). In the case of AFA synthesis, 5% v/v of DMSO was used in order to aid the solubilization of substrates in the solvent. Each reaction was ended by addition of acetonitrile.

2.6. Statistical analysis

Regression analysis was performed based on the acquired experimental data and was fitted into the following empirical polynomial equation using the software Design-Expert 9.0 (Stat-Ease Inc., Minneapolis, USA): $Y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j$, where Y is the predicted response, b_0, b_i and b_{ij} are coefficients and x_i,xj are the non- coded values of the transesterification variables. The variability of the fit of the equation was expressed by the coefficient of determination (R^2) and its statistical significance was evaluated using Fisher's test (*F*-test).

2.7. Analysis of products

Quantitative analysis was made by HPLC on a 100-5 C18 Nucleosil column (250 mm × 4.6 mm) (Macherey Nagel, Düren, Germany) and detection of feruloyl compounds by a PerkinElmer Flexar UV/VIS detector (Waltham, USA) at 300 nm. Elution was done with 70:30 v/v acetonitrile: water for 10 min at 0.6 mL/min flow rate and room temperature. Retention times for FA, AFA, MFA, VFA and PFA were 4.1, 4.4, 6.1, 7.4 and 8.7 min, respectively. Calibration curves were prepared using standard solutions of feruloyl compounds in acetonitrile (0.1–2 mM). The transesterification yield was calculated as the molar amounts of generated transesterification product (PFA or AFA) compared to the initial molar amount of limiting reactant, expressed as a percentage. The selectivity was defined by the molar concentration of produced transesterification product (PFA or AFA) divided by the molar concentration of produced FA.

3. Results and discussion

3.1. Effect of reaction media on enzyme activity

Although the use of organic solvents is explicit for the enzymatic synthesis of esters by hydrolases, a major disadvantage is the inactivation of enzymes by organic solvents resulting in significant limitations of the enzymatic reaction processes. The FAE activity was assessed after 8 h of incubation in 96.8: 3.2 v/v solvent: buffer at 1000 rpm and 25 °C using three feruloyl esterases from *T. wortmanni* as biocatalysts belonging to different phylogenetic subfamilies: Fae68 (SF1), Fae7262 (SF6) and Fae125 (SF5). Fae125 retained its activity in water immiscible solvent- water systems (*n*-octane, *n*-hexane and [BMIm][PF_6]) indicating that interfacial inactivation events were negligible in these biphasic systems. More than 50% of the initial activity was maintained in the case of the *t*-amyl alcohol, *t*-butanol, ethyl acetate and [BMIm][BF_4] (Fig. 2A). The same trend was observed for Fae7262 however the enzyme's activity was more than halved by the aforementioned solvents maintaining only 20–40% of the initial activity (Fig. 2B). Lastly, Fae68 was stable only in *n*-octane, while it maintained more than > 60% of its activity for most solvents (Fig. 2C). Interestingly, all tested FAEs underwent severe inactivation in prenol and methyl ethyl ketone resulting in detrimental effects on the residual activity. Fae125 maintained only 20–30%, Fae7262 5–15% and Fae68 < 3% of its initial activity in these solvents.

Several factors influence the loss of activity during incubation in organic solvents such as the conformational changes and in particular the decreased conformational flexibility of the enzymes, the loss of crucial water (in the case of water miscible solvent systems), the thermodynamic

Table 1
Levels of transesterification variables.

Independent variable	Factor		PFA synthesis				AFA synthesis				
		Minimum (−α)	Low (−1)	Central (0)	High (+1)	Maximum (+α)	Minimum (−α)	Low (−1)	Central (0)	High (+1)	Maximum (+α)
Water content (% v/v)	X_1	0	1.8	5.0	8.2	10	0	4.5	12.5	20.5	25
Substrate molar ratio (mM acceptor/mM donor)	X_2	5	18.5	42.5	66.5	80	0.5	1.0	2.0	3.0	3.5
Time (h)	X_3	1	9.5	24.5	39.5	48	1	3.5	8	12.5	15
Enzyme concentration (g FAE/L)	X_4	0.004	0.04	0.10	0.16	0.20	0.002	0.02	0.05	0.08	0.10
pH	X_5	4	4.7	6	7.2	8	4	4.7	6	7.2	8
Temperature (°C)	X_6	20	24.5	32.5	40.5	45	20	24.5	32.5	40.5	45

stabilization of substrate and the interfacial inactivation (in the case of water- water immiscible solvent systems) [20,21]. Several attempts have been made on the correlation between enzyme activity and the nature of organic solvents of which representative is often the logarithm of partition coefficient (logP), a measure of hydrophobicity, as it is generally accepted that the enzyme activity is higher in hydrophobic (logP > 4) than hydrophilic solvents (logP < 1). Indeed, in our work a linear decrease of enzymatic activity was observed by increasing solvent hydrophobicity for the acetyl xylan-related FAEs (R^2 = 0.8488 for Fae125 and 0.8382 for Fae7262) (Fig. 2D). Moreover, a similar trend between the residual activity of the three FAEs from *T. wortmannii* was observed in different solvents (correlation equal to 0.774 for Fae7262 and Fae68, 0.853 for Fae125 and Fae68 and 0.885 for Fae125 and Fae7262).

3.2. Effect of reaction media on the transesterification yield and selectivity

Synthesis of two targeted compounds of different lipophilicity (PFA and AFA) was performed in 96.8: 3.2 v/v solvent: buffer for 8 h at 1000 rpm and 40 °C. Highest PFA yield was observed in water immiscible- water systems comprising of alkanes. Fae125 showed highest yield in *n*-hexane (52.2%) and in *n*-octane (50.0%) with similar selectivity (3.6–3.8). PFA yield reached 16.5 and 15.4% in *t*-butanol and [BMIm][PF_6], respectively, while it was very low (< 7%) in other solvents. There was no observed synthesis in prenol and [BMIm][BF_4] (Fig. 3A). Fae7262 and Fae68 had similar trend with Fae125 during PFA synthesis. However, they showed lower yields than Fae125 in *n*-octane (25–29%) with lower selectivities (equal to 1.095 and 0.419

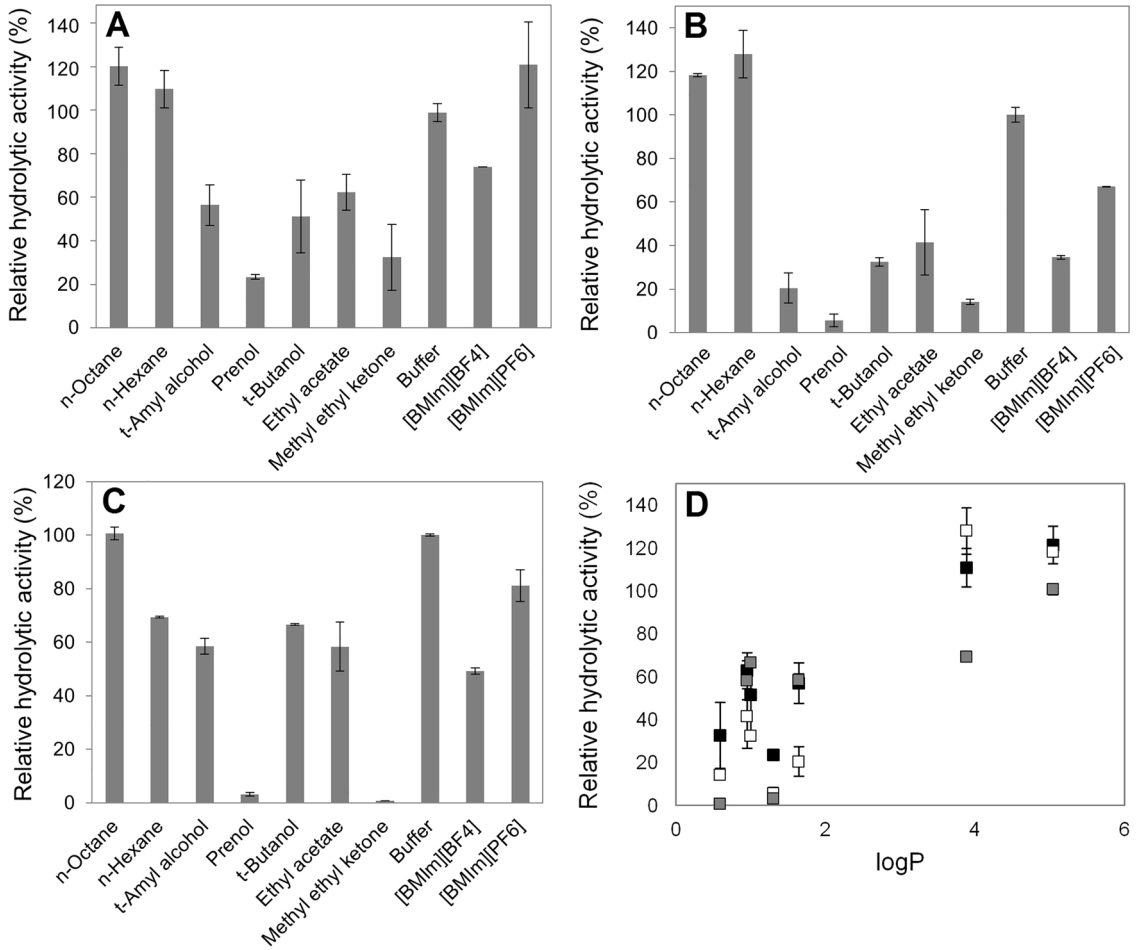

Fig. 2. Residual activity of (A) Fae125 (B) Fae7262 (C) Fae68 after 8 h incubation at 98.6: 3.2 v/v solvent: buffer at 25 °C and 1000 rpm. (D) Residual activity versus the logarithm of partition coefficient (logP). Black: Fae125, White: Fae7262, Grey: Fae68.

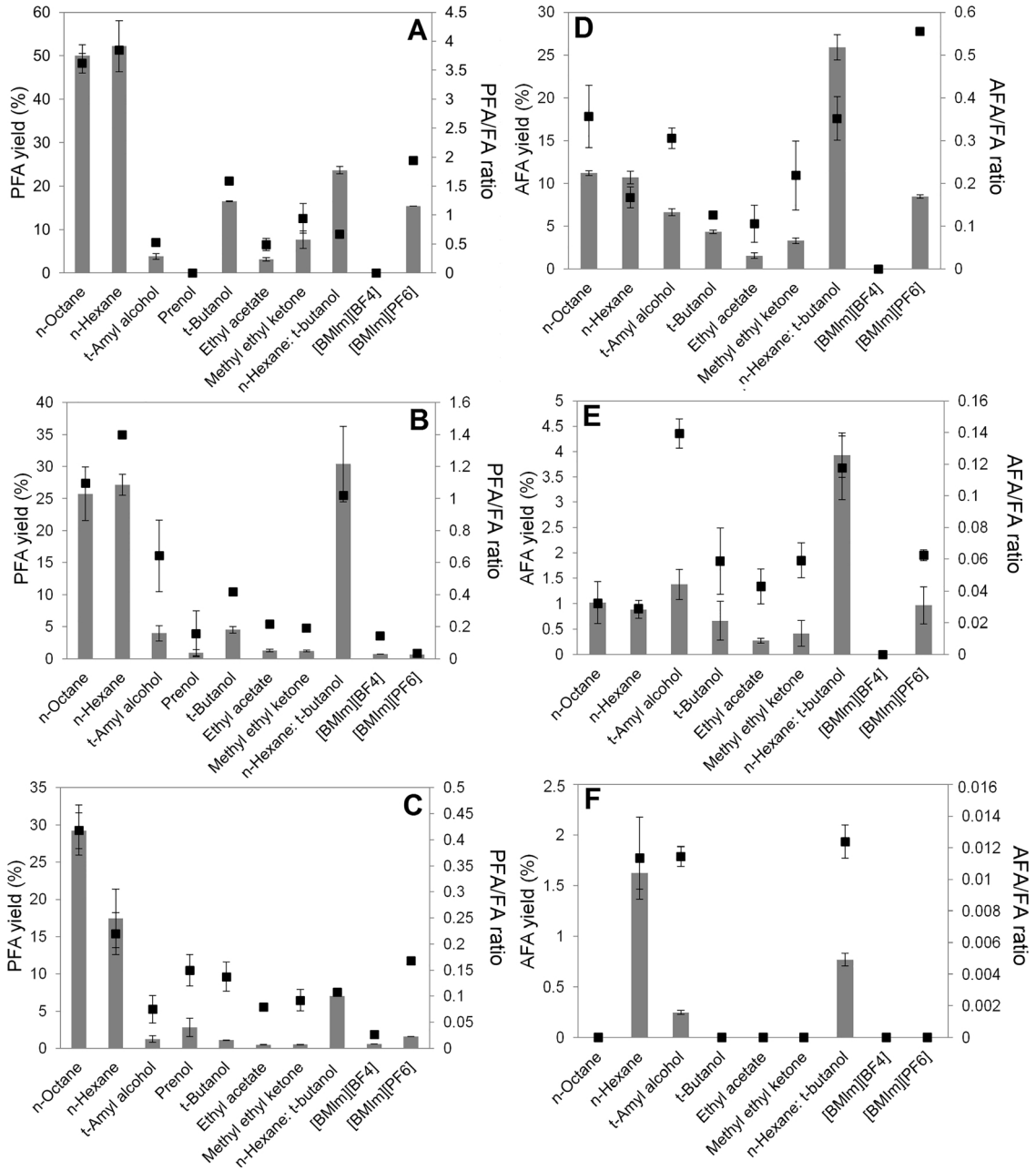

Fig. 3. Transesterification yield (grey column) and selectivity (black squares) of (A), (D) Fae125 (B), (E) Fae7262 (C), (F) Fae68. Transesterification reactions were carried out at 50 mM VFA, 200 mM prenol or 30 mM L-arabinose, 96:8: 3.2 v/v solvent: buffer, 1000 rpm and 40 °C for 8 h.

Fae7262 and Fae68, respectively) and the use of other water miscible solvents resulted in limited yields (< 5%) (Fig. 3B and C). PFA synthesis in a ternary system of *n*-hexane: *t*-butanol: buffer forming detergentless microemulsions resulted in good yields for all tested FAEs.

Highest AFA yield was observed in detergentless microemulsions followed by water immiscible- water systems for Fae125 and Fae7262. In all cases, selectivity was considerably lower than PFA synthesis and < 1, showing that hydrolysis was a predominant reaction. Fae125 showed highest yield in microemulsions (25%) followed by *n*-hexane or octane (10.7 or 11.2%) while selectivity was highest in *n*-octane and in microemulsions (~0.35) (Fig. 3D). Fae7262 showed lower yields (up to 4%) but had similar trend with Fae125 (Fig. 3E). Fae68 offered negligible yields (< 2%) in all tested solvents (Fig. 3F). There was no observed synthesis in [BMIm][BF_4] for any tested FAE.

Transesterification in organic solvents and ionic liquids has been widely performed with lipases where application of hydrophobic solvents usually results in improved synthetic yields [5,22,23]. In our work, there has been a linear relation between the yield or selectivity and solvent hydrophobicity where transesterification is more favored in alkanes than polar water-miscible solvents (R^2 = 0.8346–0.9659). Although use of detergentless microemulsions resulted in higher transesterification yields than all tested water miscible solvents in this work, as they offer protection from inactivation by enclosing the enzyme in aqueous microdroplets stabilized by *t*-butanol, the even higher yields in pure alkane:buffer systems could be attributed to possible interfacial activation of the enzymes. Furthermore, there has been a good correlation between the residual hydrolytic activity and the transesterification yield in different tested organic solvents (0.9187, 0.9742, 0.6617 for Fae125, Fae7262, Fae68 during PFA synthesis, 0.8457 for Fae125 during AFA synthesis) (Figs. 2 and 3).

3.3. Optimization of reaction conditions via RSM and model verification

Based on the above, Fae125 was selected as best biocatalyst for the synthesis of both products (PFA and AFA) as it can transesterify VFA with acceptors of different size and lipophicility (prenol and L-arabinose) with good yields. *n*-Hexane was used as most adequate solvent since it offered highest yields and selectivity for both products. Furthermore, it has a significantly lower boiling point (69 °C) than octane (125 °C) facilitating future downstream processing approaches. Optimization of reaction conditions for each product was performed via RSM.

To determine the best fitting model for each bioconversion, multiple regression analysis was investigated using Design-Expert. The models were compared and evaluated for significance (*F* values, *P* values, lack of fit and R^2 values) (Tables 2 and 3). Among the proposed models, the quadratic and the two-factor interaction (2FI) model were statistically more suitable for the description of the FAE-catalyzed transesterification of VFA with prenol and L-arabinose, resulting in similar *F* values. Comparing the statistical significance of each variable for both models, it was concluded that the exported quadratic model could be simplified into a 2FI model, since only a single quadratic factor (X_5^2) was significant ($p < 0.05$) (data not shown). Thus, a 2FI model was used for the prediction of responses of both bioconversions.

3.3.1. PFA synthesis

The analysis of variance (ANOVA) for the 2FI models for the PFA yield and selectivity is presented in Tables 2 and 3, respectively. The high *F* value for the two responses, 20.83 and 14.55, implies that the models were significant ($p < 0.001$). The regression analysis resulted in a coefficient of determination (R^2) of 0.8741 and 0.8291 (Fig. 4A and B), respectively, indicating that the models have high significance for describing the relationship of the responses (yield and selectivity) and variables. According to the established models, the substrate molar ratio (X_2, 192.9), the water content (X_1, 78.64), the time (X_3, 68.36) and the enzyme load (X_4, 24.94) were significant linear terms while the synergistic effects of water content-substrate molar ratio (X_1X_2, 29.58) and substrate molar ratio-time (X_2X_3, 15.63) were most significant interactive terms having an impact on the PFA yield (Table 2). Similarly, the substrate molar ratio (X_2, 150.02), the water content (X_1, 37.54), the time (X_3, 28.56) and the enzyme load (X_4, 19.60) were most significant linear terms while the synergistic effects of substrate ratio-time (X_2X_3, 14.13) and substrate molar ratio-temperature (X_2X_6, 12.05) were most significant interactive terms having an impact on the selectivity (Table 3). The significance of synergistic action of substrate molar ratio and time on the selectivity reflects the almost exclusive conversion of VFA to PFA over time at high acceptor concentrations. On the other hand, the significance of the interaction between the substrate molar ratio and the temperature underlines the adverse impact of very high prenol concentration in combination with increased temperature on the stability of Fae125. The effect of substrate molar ratio and time on the selectivity is presented in Fig. 4C. The full predictive equations for PFA synthesis are shown inthe Supplementary material (File S1).

Neglecting the insignificant terms ($p > 0.05$), the final predictive equations are presented below:

$$Y_1 \text{ (PFA yield (\% mM PFA/mM VFA}_{initial})) = 102.09 + 3.42X_1 - 2.13X_2 + 0.60X_3 + 215.45X_4 + 0.078X_1X_2 - 27.66X_1X_4 + 0.012X_2X_3 + 3.96X_2X_4 + 0.014X_2X_6$$

$$Y_2 \text{ (Selectivity (mM PFA/mM FA))} = 7.27 + 0.597X_1 - 0.143X_2 + 0.034X_3 + 15.7X_4 - 0.081X_6 + 0.002X_1X_2 - 0.005X_1X_3 - 2.31X_1X_4 + 0.0009\ X_2X_3 + 0.273\ X_2X_4 + 0.002\ X_2X_6$$

The optimal conditions for PFA synthesis catalyzed by Fae125 in *n*-hexane: buffer, determined as the ones maximizing both responses were predicted via the numerical optimization of Design Expert software. The optimal conditions, presented in Table 4, were determined as: 8.2% v/v water, 18.56 mM prenol/mM VFA, 24.5 h, 0.04 g FAE/L, 4.7 pH, 24.5 °C. Under the optimal conditions, the predicted PFA yield was 86.5% and the predicted selectivity 7.202. The model was validated

Table 2
Analysis of variance (ANOVA) for response surface 2FI model for yield.

Source	Yield									
	PFA synthesis					AFA synthesis				
	Sum of squares	df	Mean square	*F* value	*P* value	Sum of squares	df	Mean square	*F* value	*P* value
Model	33885.63	21	1613.602	20.83258	8.24E-21	15682.8	21	746.7999	7.320045	4.96E-10
X_1	6090.919	1	6090.919	78.63747	1.1E-12	2652.621	1	2652.621	26.00068	3.56E-06
X_2	14938.83	1	14938.83	192.8694	7.7E-21	5013.393	1	5013.393	49.14069	2.23E-09
X_3	5294.844	1	5294.844	68.35966	1.21E-11	122.6805	1	122.6805	1.2025	0.277133
X_4	1932.022	1	1932.022	24.94358	4.96E-06	0.004427	1	0.004427	4.34E-05	0.994766
X_5	93.94015	1	93.94015	1.212824	0.274963	4.530597	1	4.530597	0.044408	0.833798
X_6	70.05461	1	70.05461	0.904448	0.345226	26.25051	1	26.25051	0.257304	0.613807
X_1X_2	2291.034	1	2291.034	29.57864	9.32E-07	2303.69	1	2303.69	22.5805	1.27E-05
X_1X_3	80.94565	1	80.94565	1.045058	0.310558	1794.902	1	1794.902	17.59341	9E-05
X_1X_4	489.6721	1	489.6721	6.321965	0.014491	417.0017	1	417.0017	4.087401	0.047597
X_1X_5	99.74773	1	99.74773	1.287804	0.260752	27.73543	1	27.73543	0.271859	0.603975
X_1X_6	0.248497	1	0.248497	0.003208	0.95501	191.6921	1	191.6921	1.878944	0.175477
X_2X_3	1210.316	1	1210.316	15.62592	0.000198	162.5396	1	162.5396	1.593194	0.211675
X_2X_4	563.5109	1	563.5109	7.275268	0.008959	7.949389	1	7.949389	0.077919	0.781082
X_2X_5	59.77225	1	59.77225	0.771696	0.383031	0.309784	1	0.309784	0.003036	0.956236
X_2X_6	433.531	1	433.531	5.597149	0.021079	6.521683	1	6.521683	0.063925	0.801247
X_3X_4	0.024966	1	0.024966	0.000322	0.985733	1428.277	1	1428.277	13.9998	0.000406
X_3X_5	8.338336	1	8.338336	0.107653	0.74392	3.396448	1	3.396448	0.033292	0.855827
X_3X_6	79.53092	1	79.53092	1.026793	0.314789	31.35409	1	31.35409	0.307329	0.581351
X_4X_5	0.173694	1	0.173694	0.002243	0.96238	11.77602	1	11.77602	0.115427	0.735215
X_4X_6	17.1637	1	17.1637	0.221594	0.639454	621.8869	1	621.8869	6.095662	0.016368
X_5X_6	131.0159	1	131.0159	1.691494	0.198142	0.627491	1	0.627491	0.006151	0.937746
Residual	4879.708	63	77.45568			6223.294	61	102.0212		
Lack of Fit	4879.708	54	90.36496			6223.294	52	119.6787		
Pure error	0	9	0			0	9	0		
Cor total	38765.34	84				21906.09	82			

Table 3
Analysis of variance (ANOVA) for response surface 2FI model for selectivity.

Source	Selectivity									
	PFA synthesis					AFA synthesis				
	Sum of squares	df	Mean square	*F* value	*P* value	Sum of squares	df	Mean square	*F* value	*P* value
Model	135.1276	21	6.434649	14.55301	7.76E-17	12.30857	21	0.586123	12.27704	8.71E-15
X_1	16.59717	1	16.59717	37.53721	6.45E-08	3.465622	1	3.465622	72.59163	5.63E-12
X_2	66.33225	1	66.33225	150.0212	2.56E-18	3.022151	1	3.022151	63.30259	5.23E-11
X_3	12.62849	1	12.62849	28.5614	1.33E-06	0.479154	1	0.479154	10.03645	0.002398
X_4	8.666881	1	8.666881	19.60157	3.87E-05	0.14794	1	0.14794	3.098776	0.083364
X_5	0.270831	1	0.270831	0.612528	0.43677	0.043991	1	0.043991	0.921447	0.340885
X_6	4.040274	1	4.040274	9.13774	0.003618	0.321546	1	0.321546	6.735172	0.011825
X_1X_2	1.821311	1	1.821311	4.119193	0.046628	2.535082	1	2.535082	53.10036	7.44E-10
X_1X_3	4.264338	1	4.264338	9.644498	0.002846	0.16698	1	0.16698	3.497606	0.066258
X_1X_4	3.410038	1	3.410038	7.712358	0.007215	0.612307	1	0.612307	12.82551	0.000679
X_1X_5	0.442761	1	0.442761	1.001377	0.320806	0.019334	1	0.019334	0.404981	0.526909
X_1X_6	1.040228	1	1.040228	2.352646	0.130077	0.002516	1	0.002516	0.052703	0.819193
X_2X_3	6.249887	1	6.249887	14.13514	0.000375	0.01704	1	0.01704	0.356916	0.552435
X_2X_4	2.6873	1	2.6873	6.077769	0.016426	0.034417	1	0.034417	0.720908	0.399166
X_2X_5	0.076701	1	0.076701	0.173472	0.67846	0.0212	1	0.0212	0.444052	0.507686
X_2X_6	5.329859	1	5.329859	12.05435	0.000939	0.020706	1	0.020706	0.433718	0.512648
X_3X_4	0.347677	1	0.347677	0.786327	0.378586	0.346109	1	0.346109	7.249679	0.009142
X_3X_5	0.00704	1	0.00704	0.015923	0.899987	0.004961	1	0.004961	0.10392	0.748277
X_3X_6	0.338887	1	0.338887	0.766448	0.384644	5.37E-07	1	5.37E-07	1.12E-05	0.997336
X_4X_5	0.00021	1	0.00021	0.000476	0.982663	0.005487	1	0.005487	0.114928	0.735767
X_4X_6	0.278933	1	0.278933	0.630853	0.430024	0.363799	1	0.363799	7.620199	0.007612
X_5X_6	0.296569	1	0.296569	0.67074	0.415881	0.025393	1	0.025393	0.531879	0.468609
Residual	27.8556	63	0.442152			2.912222	61	0.047741		
Lack of Fit	27.8556	54	0.515844			2.912222	52	0.056004		
Pure Error	0	9	0			0	9	0		
Cor Total	162.9832	84				15.2208	82			

experimentally, giving 87.5% yield and 7.616 selectivity. The presence of water during transesterification was essential as at 0% v/v water, the enzyme was not active.

3.3.2. AFA synthesis

Accordingly, the ANOVA for the AFA yield and selectivity showed that the exported 2FI models were significant ($p < 0.001$), with high *F* values (7.32 and 12.28, respectively) (Tables 2 and 3). The regression analysis resulted in a coefficient of determination (R^2) of 0.716 and 0.809, respectively (Fig. 4D and E). Regarding the variables affecting the AFA yield, the substrate molar ratio (X_2, 49.14) and the water content (X_1, 26.00) were the most significant linear terms while the water content-time (X_1X_3, 17.59) and the enzyme load-time (X_3X_4, 14.00) were the most important interactive terms (Table 2). Regarding the variables affecting the selectivity, the water content (X_1, 72.59), the substrate molar ratio (X_2, 63.30) and the time (X_3, 10.03) were most important among the linear terms while the interaction of water content-substrate molar ratio (X_1X_2, 53.10), water content-enzyme load (X_1X_4, 12.82), enzyme load-temperature (X_4X_6, 7.62) and enzyme load-time (X_3X_4, 7.25) were important interactive factors (Table 3). The effect of water content and substrate ratio on the selectivity is presented in Fig. 4F. The synergistic effect of water content-time and enzyme load-time reflects the reaction limitations due to the side hydrolysis of AFA by Fae125, after a critical ratio of AFA and VFA has been attained. On the other hand, it is an indication for the increased specificity of FAEs towards natural-like substrates, such as AFA, as no-side hydrolysis of transesterification product was observed during PFA synthesis over time. The full predictive equations for AFA synthesis are shown in the Supplementary material (File S2).

Neglecting the insignificant terms ($p > 0.05$), the final predictive equations are presented below:

$$Y_1 \text{ (AFA yield (\% mM AFA/mM } VFA_{initial})) = -63.9 + 2.78X_1 + 14.51X_2 - 0.807X_1X_2 - 0.153 \quad X_1X_3 + 21.02X_1X_4 - 69.48 X_3X_4 - 25.67X_4X_6$$

$$Y_2 \text{ (Selectivity (mM AFA/mM FA))} = -0.120 + 0.008X_1 + 0.738X_2 + 0.028X_3 + 0.001X_6 - 0.027X_1X_2 + 0.806X_1X_4 - 1.08 X_3X_4 - 0.621 X_4X_6$$

The optimal conditions for AFA synthesis catalyzed by Fae125 in *n*-hexane: DMSO: buffer, defined as the ones maximizing both responses, were estimated via the numerical optimization of Design Expert software. The optimal conditions, presented in Table 4, were determined as: 4.5% v/v water, 2.96 mM L-arabinose/mM VFA, 12 h, 0.02 g FAE/L, 4.7 pH, 38.9 °C. The increased optimal temperature, comparing to PFA synthesis, underlines the need for increased solubilization of L-arabinose for efficient transesterification. Furthermore, it appears that time and enzyme load are crucial during AFA synthesis, as at long incubations times or increased enzyme load, hydrolysis of AFA occurs after a crucial ratio between VFA and AFA has been achieved. This phenomenon was also observed in previous work based on the optimization of AFA synthesis in detergentless microemulsions by FAEs from *Myceliophthora thermophila* [16]. The lower optimal value of water content (4.5%) underlines the need for reduced water activity, in order to reduce the extent of side-hydrolytic reactions. Under the optimum conditions, the predicted AFA yield was 60.3% and the predicted selectivity was 1.442. The model was validated experimentally, resulting in 56.2% yield and 1.284 selectivity. The presence of water during transesterification was essential as at 0% v/v water, the enzyme was not active.

3.4. Effect of DMSO concentration and agitation on the yield and selectivity

As a next step, the effect of DMSO addition (0–20% v/v) and agitation (0, 1000 rpm) were investigated at optimal conditions using Fae125 versus time. During PFA synthesis and at 0% v/v DMSO (after 24 h of incubation), reactions performed without agitation resulted in significantly lower yield and selectivity (61.5%, 5.570) comparing to reactions where agitation was applied (87.5%, 7.616), indicating that mass transfer phenomena are significant (Fig. 5A and B). The same trend was observed at 2% v/v DMSO. On the other hand, at 5–10% v/v DMSO, mass transfer phenomena appeared to be less significant as the

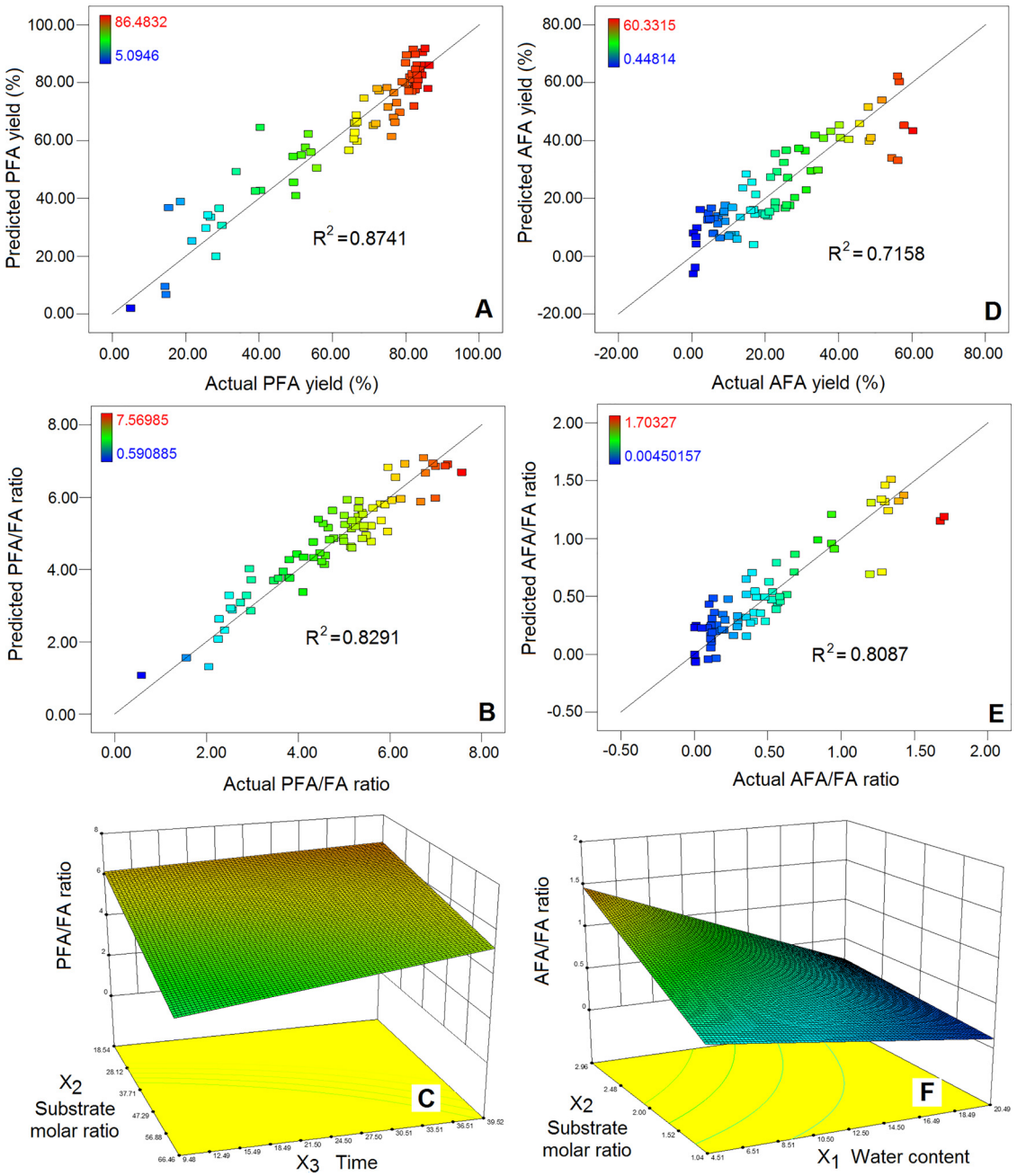

Fig. 4. Model diagnostic plots of predicted vs actual (A) PFA yield (B) PFA/FA ratio (C) Response surface plot showing the effect of substrate molar ratio (X_2, mM acceptor/mM VFA) and time (X_3, h) on the selectivity during PFA synthesis (PFA/FA ratio). Model diagnostic plots of predicted vs actual (D) AFA yield (E) AFA/FA ratio. (F) Response surface plot showing the effect of water content (X_1, % v/v) and substrate molar ratio (X_2, mM acceptor/mM VFA) on the selectivity during AFA synthesis (AFA/FA ratio).

resulting yield was > 90% after only 5 h of incubation and selectivity was 2-fold increased independently of agitation after 24 h. At 20% v/v DMSO, Fae125 appeared to be inactivated (< 2% yield) (data not shown). This is in accordance with findings on the effect of DMSO on the hydrolytic activity of feruloyl esterases where AnFaeA from *Aspergillus niger* and NcFaeB from *Neurospora crassa* were inactivated over 30% v/v DMSO whereas the commercial Ultraflo and TsFaeC from *Talaromyces stipitatus* were inactivated over 40% and 50% v/v DMSO, respectively [24].

Overall, at 5% v/v DMSO and 1000 rpm, the obtained yield and selectivity was 92.8% and 14.090 at 24 h. Thus, an addition of DMSO could increase significantly the selectivity of the synthesis of aliphatic ferulates in water-water immiscible solvent systems. At these conditions, the yield was 1.85-fold increased and the selectivity was 4-fold higher comparing to the initial conditions tested in paragraph 3.2. Furthermore, the selectivity was approximately 2-fold increased comparing to reactions at optimal conditions and 0% v/v DMSO. The achieved yield (92.8%) is among the highest reported for aliphatic esters synthesis based on FAEs and significantly improved comparing to the PFA synthesis by FAEs from *M. thermophila* in microemulsions (72.2%) [11]. More specifically, a > 90% transesterification yield has been achieved by the immobilized commercial enzymatic preparation Ultraflo L during synthesis of butyl ferulate [11,25]. Esterification of FA with glycerol (81%) by FAE-PL (purified from the multienzymatic preparation Pectinase PL "Amano" produced by Aspergillus *niger*) [26], esterification of sinapic acid or transesterification of methyl sinapate

Table 4
Summary of optimal values of variables maximizing both responses (yield and selectivity) and green chemistry/process metrics.

Independent variable	PFA synthesis		AFA synthesis	
Optimal parameters				
Water content (% v/v)	8.2		4.5	
Solvent (hexane: DMSO v/v)	91.8: 0		90.5: 5.0	
Substrate molar ratio (mM acceptor/mM donor)	18.56		2.96	
Time (h)	24.5		12.0	
Enzyme concentration (g FAE/L)	0.04		0.02	
pH	4.7		4.7	
Temperature (°C)	24.5		38.9	
Model validation				
Predicted yield (% mM product/mM $VFA_{initial}$)	86.5		60.3	
Actual yield (% mM product/mM $VFA_{initial}$)	87.5 ± 0.9		56.2 ± 1.2	
Predicted selectivity (mM product/mM FA)	7.202		1.442	
Actual selectivity (mM product/mM FA)	7.616 ± 0.623		1.284 ± 0.184	
Process metrics				
DMSO addition (% v/v)	0	5	0	5
Yield (% mM product/mM $donor_{initial}$)	87.5 (24 h)	92.5 (24 h)	32.3 (12 h)	56.2 (12 h)
Catalyst productivity[a] (g product/g FAE)	345–908	362–1178	410–1045	735–1795
Productivity[a] (g product/L/h)	0.57–1.51	0.60–1.96	0.68–1.74	1.23–2.99
Product concentration[a] (g/L)	13.8–36.3	14.6–47.1	8.2–20.9	14.7–35.9
Green chemistry metrics				
Atom efficiency (%)	85.6	85.6	88.1	88.1
Carbon efficiency (%)	88.2	88.2	88.2	88.2
Reaction mass efficiency[a] (%)	10.0–12.6	12.9–13.4	15.4–15.7	11.0–27.0
Solvent intensity[a,b] (% kg solvent/kg product)	166–436	132–427	299–762	180–441
Water intensity[a,b] (% kg water/kg product)	23–59	17–56	22–55	13–31
E-factor[a,b] (kg waste/kg product)	27.8–56.6	22.2–55	37.3–87.2	22.0–49.8

[a] Calculated for a range of yields; 60–200 mM VFA for PFA synthesis; 80–200 mM VFA for AFA synthesis.
[b] Assuming no recycling of enzyme, solvent or substrate.

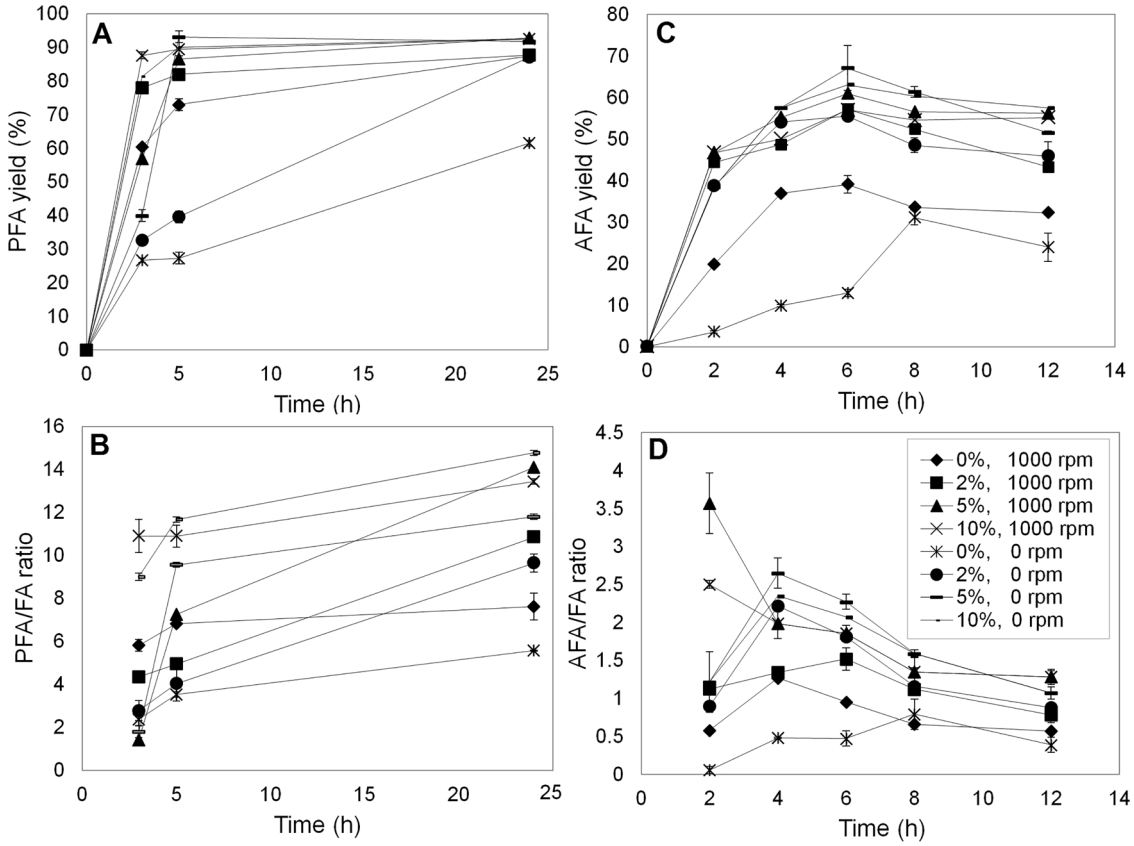

Fig. 5. (A) Effect of DMSO (% v/v) and agitation (0, 1000 rpm) on the (A) PFA yield (B) PFA/FA ratio (C) AFA yield (D) AFA/FA ratio. Reactions were performed at optimal conditions as determined by the response surface model for each bioconversion using Fae125 as biocatalyst and were monitored over time.

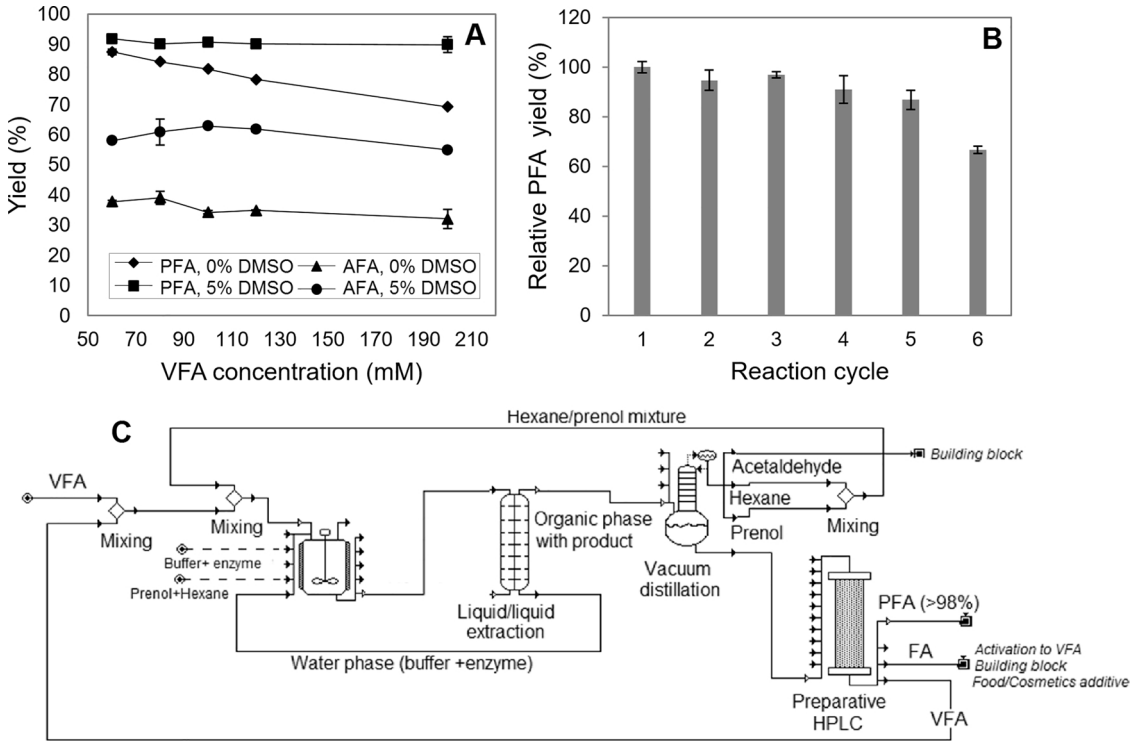

Fig. 6. (A) Effect of donor concentration at optimal conditions and 1000 rpm. PFA synthesis: 24 h, AFA synthesis: 6 h (B) Reusability of enzyme (water phase) during PFA synthesis as performed in hexane: water at optimal conditions. (C) Scheme of PFA synthetic process in *n*-hexane: buffer with solvent and enzyme recycling.

with glycerol (70–78%) by AnFaeA from *A. niger* [27,28] and transesterification of methyl ferulate with 1-butanol (78%) by immobilized AnFaeA [28] are indicative reports of increased yields after optimization of reaction conditions in FAE-catalyzed reactions.

During AFA synthesis, lack of agitation and DMSO resulted in very low yield and selectivity. At 0 rpm and 0% v/v DMSO, the yield was 31.1% and selectivity was 0.792 at 8 h of incubation while they stabilized at 24.0% and 0.386 until 12 h. At 1000 rpm and 0% v/v, the yield and selectivity were improved and stabilized at 32.3% and 0.569 after 12 h. The addition of DMSO is essential in this reaction as a 2-fold increase in yield and 4-fold increase in selectivity was observed at 2–10% v/v (Fig. 5C and D). This expected increase is owed to the effective solubilization of L-arabinose in the formed polar phase, enabling the transesterification over hydrolysis. Mass transfer phenomena were less significant over 5% v/v of DMSO. More specifically, at 5% v/v and 0 rpm agitation, a 67.1% yield and 2.270 selectivity were achieved at only 6 h of incubation that subsequently decreased and stabilized to 51.5% and 1.068 at 12 h. At 1000 rpm, a 60.9% yield and 1.857 selectivity were observed at 6 h, while a decrease to 56.2% and 1.284 was observed up to 8 h remaining constant until 12 h. At 20% v/v, the enzyme was inactivated (0% yield) (data not shown). Interestingly, an increase of selectivity is observed up to 4 h of incubation in all cases, except for 5–10% v/v DMSO and 1000 rpm, where the selectivity is quite high at the first 2 h (> 2.5) and then decreases linearly. This could be explained by the improved contact of enzyme with the substrates. The observed decrease in the selectivity and yield up to 12 h indicates that the enzyme is hydrolyzing AFA after a critical ratio of AFA and VFA is achieved, a phenomenon that is not observed during PFA synthesis. Nevertheless, after optimization via RSM with DMSO addition, the obtained yield and selectivity at 6 h was increased 6-fold and 14-fold, respectively, comparing to the initial screening conditions tested in paragraph 3.2. To the authors' best knowledge, a > 60% yield is the highest reported in literature regarding FAE-catalyzed AFA synthesis. Works reporting high AFA yields include the transesterification of methyl ferulate by StFaeC from *Sporotrichum thermophile* (syn *M. thermophila*; 40–50%) [29] and the transesterification of VFA by FaeA1 from *M. thermophila* C1 (52.2%) in microemulsions [16].

3.5. Distribution of feruloyl compounds in hexane: DMSO: water systems

The addition of DMSO imposed changes in the composition and formation of phases of the reaction system. Thus, the organic and water phase at 0% and 5% v/v DMSO was analyzed by HPLC at 0 h and at the end of the reaction for both bioconversions. When prenol was used as acceptor at 0% v/v DMSO, two distinct transparent phases were formed: a water-rich phase containing the enzyme and buffer and a hexane-rich phase containing the acceptor (prenol). When 5% v/v DMSO was used, the polar nature of this reagent formed a macroscopically defined "polar phase" containing water and DMSO in which the enzyme was enclosed. At both cases, > 90% of solubilized VFA in the organic phase at 0 h. At the end of 24 h at 0% v/v DMSO > 90% of the produced PFA and of the remaining VFA and 65% of the produced FA were encountered in the organic phase. At 5% v/v DMSO, slightly more FA (89% of the produced FA) was found in the organic phase.

During AFA synthesis at 0% v/v DMSO, both substrates were practically insoluble in the reaction mixture, hence the very low observed transesterification yields (Fig. 5C). In particular, there was a formation of three phases: a water-rich phase containing the enzyme and solubilized L-arabinose, an organic phase containing 5% of the total amount of VFA and a solid phase comprised by insoluble VFA and L-arabinose. On the contrary, at 5% v/v DMSO a two phase system was formed: an a-polar hexane rich phase comprising of hexane and solubilized VFA and a polar phase containing water and DMSO comprising of 52% solubilized VFA, L-arabinose and enzyme. At the end of 24 h, AFA was partially solubilized in both phases.

3.6. Effect of donor concentration on the yield and selectivity

Although enzymes operate optimally at low substrate concentrations, the possibility of increasing the VFA concentration was

investigated at optimal conditions and fixed substrate molar ratio, since it could benefit further the productivity of the process. It was observed that the PFA yield was decreased by 15% and the respective selectivity by 23%, when the donor concentration increased from 60 mM to 200 mM in reactions without DMSO. At 5% v/v DMSO, the yield and selectivity remained constant up to 200 mM, increasing the PFA concentration 2.5 times (up to 180 mM). On the other hand, the AFA yield decreased only by 7–8% by increasing the VFA concentration to 200 mM, in reactions with 0 or 5% v/v DMSO (Fig. 6A).

3.7. Enzyme reusability

Use of water immiscible-water systems for enzymatic bio-catalysis offers various advantages such as the reusability of water phase containing the free enzyme. Transesterification of VFA with prenol in *n*-hexane: water (0% v/v DMSO) was carried out for 6 consecutive reaction cycles at optimal conditions as determined by RSM. At the end of each reaction, the upper organic phase was isolated and analyzed by HPLC while a newly prepare organic solvent mixture containing hexane and substrates (VFA and prenol) was introduced. As presented in Fig. 6B, the achieved yield was decreased only by 10% compared to the initial PFA yield by the end of fourth cycle while at the end of sixth cycle the PFA yield was 66.6% of the initial one. Selectivity was retained at 100% of the initial value throughout the six consecutive reaction cycles. The obtained results are comparable with the reusability of the commercial multienzymatic preparation Depol 740 L immobilized on mesoporous silica MPS-5D or MPS-9D that was relatively stable throughout 6 runs, maintaining 70–96% of its initial activity during the transesterification of MFA with 1-butanol in a solvent-free system [10]. C1FaeB1 from *M. thermophila* C1 immobilized on SBA-15 maintained its initial yield and selectivity for the same reaction after 9 consecutive cycles [30].

3.8. Metrics and conceptual process design

The bioactive activities of feruloyl derivatives constitute them an attractive candidate for use in the food, pharmaceutical and cosmetic industries. Although enzymatic transesterification offers milder operational conditions (T, pH) and increased selectivity comparing to chemical counterpart processes, there are various economic and environmental challenges that limit industrial application. For instance, nature has evolved enzymes to operate at low concentration of substrates (i.e. donor) and hence products, limiting productivity and placing a particular burden on downstream processing. At the same time, the large amount of required solvent and acceptor increase the cost and impose the need of recycling along with the increased price of enzymes. In our work, optimization offered exceptional biocatalyst productivities (up to 1795 kg product/ kg free FAE) (Table 4) that are required and adequate for pharmaceutical processes (100–250 kg product/kg free enzyme) or even fine chemical processes (670–1700 kg product/kg free enzyme) [31] while the possibility of Fae125 reuse for 6 consecutive cycles could offer more than a 5-fold increase in biocatalyst productivity for PFA synthesis. Moreover, productivity > 1 g product/L/h was achieved for both products (PFA and AFA), fitting well with the needs of early stage analysis for process development [33]. The increased yields (i.e. > 80% for PFA) and productivities can be attributed to the identification of potent biocatalysts such as Fae125 and the use the activated donor VFA. The use of vinyl activated donors allows the shift of equilibrium towards transesterification, as the produced vinyl alcohol tautomerizes to acetaldehyde (Fig. 1). However, the need of vinyl-activated donor and the resulting by-product (acetaldehyde) should be addressed in terms of cost, product safety and downstream processing.

Focusing on the biocatalytic process itself, assuming at a theoretical level that the production of substrates, biocatalyst and downstream processing results in zero waste and that no materials are recycled, the calculated E-factor is within the range of pharmaceutical processes (25–100 kg waste/kg product) [32,33]. A 2.5-fold increase in donor concentration, enabled in general a 2-fold decrease of E-factor and a 4-fold decrease of solvent intensity (Table 4). Nevertheless, solvent recycling is essential as solvent intensity is about 130–180% for most of the synthetic conditions described in this work. Furthermore, the water and enzyme used in this synthetic process, could be easily reused by liquid-liquid extraction as *n*-hexane is a water immiscible solvent. The resulting byproducts, FA and acetaldehyde, could be sold or used as *building-block chemicals in a prospect that the particular process could be integrated as part of a future biorefinery*. A conceptual process design for PFA synthesis in *n*-hexane: buffer is presented in Fig. 6C.

4. Conclusions

In this work, we evaluated the synthetic potential and assessed the stability of three FAEs from *T. wortmannii* in different solvents. After optimization of reaction conditions, competitive yields were obtained for the synthesis of two feruloyl derivatives of different lipophilicity (PFA and AFA) using the type A Fae125 from SF5 as biocatalyst. The developed syntheses were evaluated based on green chemistry and process metrics resulting in exceptional productivities. The investigation of the potential of FAEs for the modification of feruloyl compounds could enable the application of greener and milder processes with exceptional selectivity for use in the food, pharmaceutical and cosmetics industry. Moreover, the modeling and early stage process analysis sets the basis for scale up and enables the performance of a preliminary and/or techno-economic assessment for future development of cost-effective FAE-based bioconversions.

Author contributions

IA (Luleå University of Technology, Sweden) designed and performed the experiments, analyzed the data and wrote the manuscript; UR and PC designed the experiments and analyzed the data; LI (DuPont Industrial Biosciences, the Netherlands) provided the enzymatic preparations Fae68, Fae7262 and Fae125; PJ and AP (Taros Chemicals, Germany) provided VFA as donor for enzymatic transesterification; All authors reviewed and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.enzmictec.2018.08.007.

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