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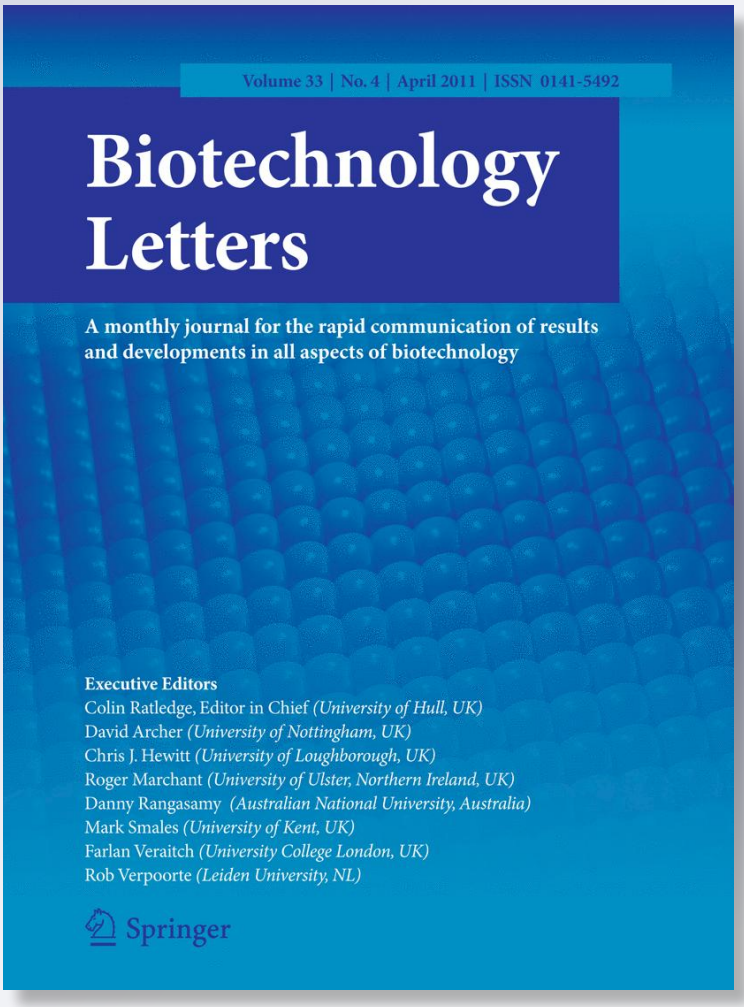

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ORIGINAL RESEARCH PAPER

Improved enzymatic production of phenolated acylglycerols through alkyl phenolate intermediates

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Abstract A novel approach is reported for the synthesis of dihydrocaffoylated glycerols that consists of two steps: enzymatic synthesis of octyl dihydrocaffeate (as a synthetic intermediate) from octanol and dihydrocaffeic acid, and enzymatic interesterification of triacylglycerols with octyl dihydrocaffeate. Due to the good compatibility of the intermediate with triacylglycerols, an improved volumetric productivity [147 mol h^{-1}(kg Novozym 435)$^{-1}$] and high enzyme specific activity [up to 9.6 μmol^{-1} min^{-1}(g Novozym 435)$^{-1}$] have been obtained.

Keywords Alkyl phenolate · Dihydrocaffeic acid · Lipase · Phenolic acids · Interesterification

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Introduction

Phenolic acids are antioxidants that are widely distributed in fruits, vegetables, spices, and aromatic herbs. However, their applications in oil-based food processing and cosmetic industries are limited due to their low solubility in hydrophobic media (Buisman et al. 1998; Figueroa-Espinoza and Villeneuve 2005). To improve the lipophilicity of phenolic acids, an alternative method is incorporation of phenolic acids into triacylglycerols through enzymatic reactions (Compton et al. 2000, 2006; Sabally et al. 2005, 2006, 2007; Laszlo and Compton 2006). However, direct transesterification of phenolic acids with triacylglycerols is generally suffered from long reaction time and low efficiency (Figueroa-Espinoza and Villeneuve 2005; Compton et al. 2000). Thus, many efforts, for example, through medium engineering to improve solubility of phenolic acids (Sabally et al. 2006; Lue et al. 2005), and through chemo-enzymatic approach (Sun et al. 2009), have been made to improve the efficiency of related reactions.

This work reports a two-step approach for enzymatic synthesis of phenolated glycerols with dihydrocaffeic acid (DHCA) as a model penolic acid. An intermediate product,octyl dihydrocaffeate, was first synthesized by lipase-catalyzed esterification of DHCA with octanol. Then, the target products were synthesized through enzymatic interesterification between octyl dihydrocaffeate and triacylglycerol. To develop this approach was from the following considerations: (1) octyl

dihydrocaffeate is supposed to have better compatibility with triacylglycerols than DHCA or short-chain alcohol ester like ethyl dihydrocaffeate; (2) Good compatibility of octyl dihydrocaffeate with triacylglycerols allows a solvent-free reaction to occur; hence a faster reaction and a better productivity could be expected. Response surface methodology (RSM) was employed for reaction optimisation based on a preliminary parameter study. The validation reactions were conducted based on the model optimized reaction conditions. For comparison with the protocol developed in this work, the reactions in other solvent and solvent-free systems were also conducted.

Materials and methods

Materials

Dihydrocaffeic acid, tricaprylin (>98%) and octanol (>99%) were purchased from Sigma-Aldrich Co. Novozym 435 (*Candida antarctica* lipase B), Lipozyme RM IM (*Rhizomucor miehei*) and Lipozyme TL IM (*Thermomyces lanuginosus*) were obtained from Novozyme A/S (Bagsvaerd, Denmark). All other solvents were of analytical or HPLC grades.

Preparing octyl dihydrocaffeate

Octyl dihydrocaffeate was synthesized according to the method of Sabally et al. (2005) with some modifications. The reaction was terminated by removing enzyme through filtration and the unreacted octanol were removed through vacuum evaporation at 70°C. The resulting product was re-dissolved in hexane and washed five times with 0.5 M NaCl to remove the remaining DHCA. The obtained octyl dihydrocaffeate was ~97% pure (containing 3% DHCA) according to HPLC analysis.

Interesterification

Interesterification of octyl dihydrocaffeate with tricaprylin was conducted in a 100 ml thermostat reactor. For a typical reaction, 0.154 g octyl dihydrocaffeate (0.5 mmol) was mixed with 0.705 g tricaprylin (1 mmol). Interesterification was initiated by adding 10% (w/v) immobilized enzyme (on the mass basis of octyl dihydrocaffeate) at 60°C and stirred magnetically (300 rpm). Sample aliquots from reaction mixture were withdrawn and diluted 100 times by methanol, then subjected to HPLC analysis after removing solid impurity by centrifugation (~10000 rpm for 10 min).

For comparison, other two reaction systems: solvent-free direct transesterification of DHCA with tricaprylin and hexane-mediated Interesterification of octyl dihydrocaffeate with tricaprylin were conducted under other identical conditions, respectively. Namely, for the former system 0.5 mmol DHCA was reacted with 1 mmol tricaprylin and for the latter system 0.5 mmol octyl dihydrocaffeate with 1 mmol tricaprylin in 10 ml hexane.

HPLC analysis

HPLC analysis was performed on a RP C18-column (250 × 4.6 mm, 5 μm). 10 μl samples were eluted by 90% solvent A (methanol) and 10% solvent B (water with 0.75% acetic acid) for 16 min at 1 ml/min. Detection was at 284 nm.

The conversion of octyl dihydrocaffeate was calculated based on the deduction of the area percentage of octyl dihydrocaffeate on the basis of all components concerning dihydrocaffoyl moiety (including DHCA) in reaction mixture. The yield of individual dihydrocaffoylated glycerol was calculated as the area percentage of the compound in the total areas of the components concerning dihydrocaffoyl moieties. The enzyme activity was calculated from the initial reaction rate based on the conversion of octyl dihydrocaffeate versus reaction time. The unit of enzymatic activity was defined as μmol octyl dihydrocaffeate consumed per min catalyzed by per g immobilized enzyme preparation.

HPLC–ESI–MS analysis

HPLC–ESI–MS analyses were performed with an electrospray ionisation (ESI) coupled to a quadrupole time-of-flight mass spectrometer (Bruker micrOTOF-Q, Bremen, Germany). The column used and elution conditions for HPLC were the same as for HPLC analysis. Ionisation was performed in the negative mode with 8 l N_2/min at 0.8 bar nebuliser pressure and 190°C. Scan range was from 50 to 1200 m/z.

Experimental design by RSM

Response surface methodology was employed to optimize parameters of lipase-catalyzed interesterification between octyl dihydrocaffeate and tricaprylin. The software Model 8.0, Umetrics (Umeå, Sweden) was used to design the reaction sets and fit the experimental data. Four factors were chosen for investigation with three-level setting: temperature (40, 55 and 60°C), molar ratio of tricaprylin/octyl dihydrocaffeate (1/1, 3/1, 5/1), reaction time (0.5, 24.25 and 48 h) and enzyme load (50, 150 and 250 mg). Central-Composite-Face-centred (CCF) design combined with RSM was used to fit full second-order polynomial model.

Total 27 reactions based on RSM designed parameters were conducted, and the experimental results (conversion of octyl dihydrocaffeate and yield of dihydrocaffoylated glycerols) were then used for model fitting and regression analysis to generate the 2 s polynomial models (the equations not shown). Two sets of optimized parameters could be generated based on the RSM model prediction. Two experimental reactions were thus conducted using the model optimized conditions to validate the model predictions.

All the reactions in this work were conducted in two replicates, and the means ± standard deviations of the data from two replicates (within 95% confidence limitation) were used for evaluation of the results.

Results and discussion

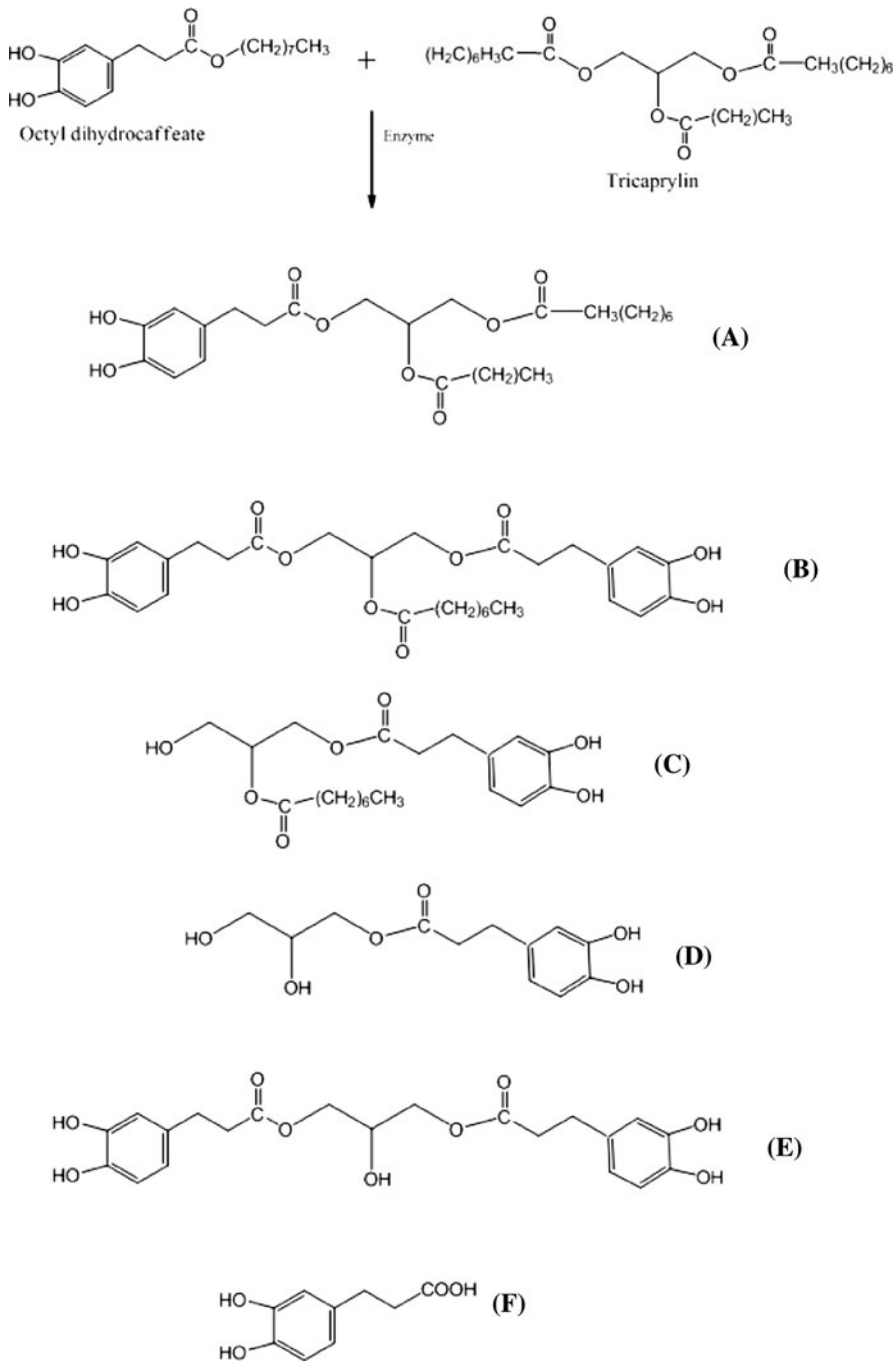
Establishment of reaction protocol

The first step was to synthesize high purity intermediate product—octyl dihydrocaffeate. This was implemented successfully by Novozym 435-catalyzed esterification of DHCA with octanol in 2-butanone/hexane (1:3, v:v) binary solvent system according to the method of Sabally et al. (2006). HPLC analysis showed the purity of the resulting product was 97% with minor unreacted DHCA. The octyl dihydrocaffeate was then used for interesterification with tricaprylin for synthesis of dihydrocaffoylated glycerols in a solvent free system.

Scheme 1 shows that the possible products from interesterification of octyl dihydrocaffeate with tricaprylin. Based on HPLC analysis results, four major peaks were detected in the reaction mixture, and their structures were further indentified by HPLC ESI–MS analysis. The first peak (eluted after 3.14 min) corresponded to products of mono- and di-DHCA glycerol (Compound D and E in Scheme 1) with molecular ion peaks at m/z 255 and 419 ($[M–H]^-$), respectively. The first peak also contained DHCA (181, $[M–H]^-$) (Compound F in Scheme 1), which was in lower abundance than other two products identified. It probably came from the substrate which contains approx. 3% DHCA. The second peak (3.57 min) was identified as di- and mono-DHCA monocaprylin (Compound B and C in Scheme 1) with molecular ion peaks at m/z 545 and 381 ($[M–H]^-$), respectively. Analysis of the ion of the third peak (4.13 min) indicated it was octyl dihydrocaffeate (m/z, 293 ($[M–H]^-$). Similarly, MS analysis of the last peak (5.21 min) showed it corresponded to mono-DHCA dicaprylin with molecular ion peaks at m/z 507 ($[M–H]^-$) (Compound A in Scheme 1). Thus, through HPLC and HPLC–MS analysis, the reaction protocol was established and the structures of desired products were confirmed.

Comparison of different reaction systems

For comparison of the efficiencies of different reaction systems, we also conducted direct transesterification of DHCA with tricaprylin in a solvent free system, and interesterification of octyl dihydrocaffeate with tricaprylin in hexane which the same intermediate was used but performed in solvent instead of solvent-free system (Fig. 1). In terms of conversion, for direct transesterification DHCA with tricaprylin only 3.2% conversion of DHCA could be achieved after 72 h. In contrast, for solvent-free interesterification of octyl dihydrocaffeate with tricaprylin around 60% conversion of octyl dihydrocaffeate could be reached at 24 h and at 72 h the conversion added up to over 70%. However, further dilution of octyl dihydrocaffeate-tricaprylin by hexane did not improve the conversion of octyl dihydrocaffeate; instead it was decreased (14% at 24 h). Poor mixing (or low solubility of DHCA in tricaprylin) will result in a less effective dynamic molecular interaction, which might account for low reaction of DHCA with tricaprylin (Fig. 1). Introducing hexane



Scheme 1 Possible products from interesterification between octyl dihydrocaffeate and tricaprylin. *A* mono-DHCA dicaprylin; *B* di-DHCA monocaprylin; *C* mono-DHCA monocaprylin; *D* mono-DHCA acylglycerol; *E* di-DHCA acylglycerol; *F* dihydrocaffeic acid (DHCA). (The potential isomers not shown.)

to the octyl dihydrocaffeate-tricaprylin system generated a negative effect, contradicting to a possible benefit from solvent introduction—improving mass transfer. The reason for this is not clear; however, the results seem suggest that, compared to solvent-free system, hexane-mediated system showed lower enzyme activity and thus resulted in a lower conversion of octyl dihydrocaffeate (Table 1).

To quantify the efficiency of these three systems further, we calculated the individual enzyme specific

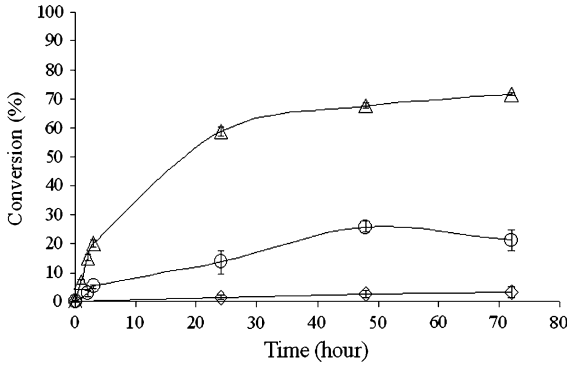


Fig. 1 Conversion of octyl dihydrocaffeate or DHCA in different reaction systems catalyzed by Novozym 435. *Open triangle* solvent-free interesterification of octyl dihydrocaffeate and tricaprylin. *Open diamond* transesterification of DHCA with tricaprylin. *Open circle* hexane-mediated interesterification of octyl dihydrocaffeate with tricaprylin

activity based on an estimation of initial reaction rate (Table 1). The enzyme activity (Novozym 435) of solvent-free octyl dihydrocaffeate-tricaprylin interesterification system is over four times higher than the hexane-mediated system; remarkably, 190 times higher than solvent-free DHCA-tricaprylin system. Sabally et al. (2006) reported lipase-catalyzed transesterification of DHCA with trilinolein and trilinolein in hexane/2-butanone (75:25, v/v), with the specific activity of around 0.5 μmol (g immobilized enzyme)$^{-1}$ min^{-1}. If we neglect the difference between the two systems, in the protocol developed in this work the enzyme activity was >18 times higher than in hexane/2-butanone system. The reason might be ascribed to the significant difference of substrate concentrations between two systems. The general concentration of DHCA in the system of Sabally et al. (2006) was at 10 mM level, while the concentration of octyl dihydrocaffeate in the solvent-free system developed in this work was over 600 mM.

From commercial point of view, a considerable volumetric productivity is an important criterion to evaluate time–space efficiency of a reaction protocol. As indicated in Table 1, the solvent-free octyl dihydrocaffeate-tricaprylin interesterification system was superior to the other two systems in terms of volumetric productivity (15 and 34 times higher, respectively). It also can be estimated that the system developed in this work is also much higher than that of hexane/2-butanone system (Sabally et al. 2006).

Table 1 Effects of enzymes and substrates on conversion of octyl dihydrocaffeate and yield of dihydrocaffoylated glycerides

Substrate	Enzyme	Solvent	Enzyme A activity (μmol g^{-1} min^{-1})	Conversion (%)	Yield (%)		Volumetric productivity [mol h^{-1} (kg enzyme)$^{-1}$][c]
					Product 1[a]	Product 2[b]	
Octyl dihydrocaffeate/tricaprilin (1/2)	Lipozyme RMIM	No	0.44 ± 0.02	14.7 ± 2.4	8.8 ± 2.02	5.3 ± 0.33	21.3 ± 4.12
Octyl dihydrocaffeate/tricaprilin (1/2)	Lipozyme TLIM	No	0.57 ± 0	12.8 ± 0.5	6.9 ± 0.09	5.5 ± 0.12	12.2 ± 3.99
Octyl dihydrocaffeate/tricaprilin (1/2)	Novozym 435	No	9.6 ± 0.06	72.6 ± 0.51	35.6 ± 0.35	28.9 ± 0.21	147 ± 2.14
DHCA/tricaprilin (1/2)	Novozym 435	No	0.05 ± 0	3.22 ± 1.8	1.11 ± 0.97	2.1 ± 0.85	9.1 ± 4.37
Octyl dihydrocaffeate/tricaprilin (1/2)	Novozym 435	Hexane	2.3 ± 0.01	24.3 ± 3.40	9.2 ± 1.2	4.9 ± 0.11	4.5 ± 0.34

[a] Product 1 represents yield of di-DHCA/mono-DHCA monocaprylin

[b] Product 2 represents yield of mono-DHCA dicaprylin

[c] Volumetric productivity was calculated as conversion of octyl dihydrocaffeate or DHCA to dihydrocaffoylated glycerides based on the reaction in 48 h

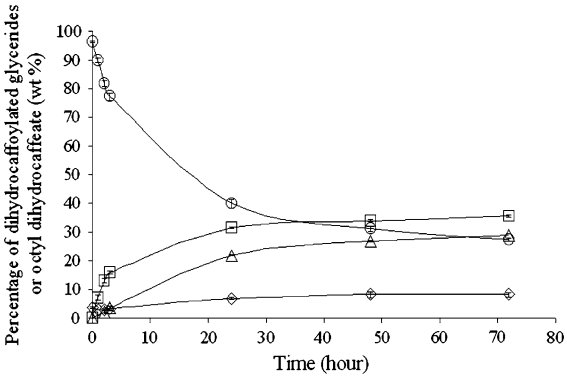


Fig. 2 Time course of Novozym 435 catalyzed interesterification of octyl dihydrocaffeate with tricaprylin. *Open square* yield of di-DHCA/mono-DHCA monocaprylin; *Open triangle* yield of mono-DHCA dicaprylin. *Open diamond* yield of mono-DHCA/di-DHCA acylglycerol. *Open circle* percentage of octyl dihydrocaffeate

Reaction time course and effects of lipase species

Figure 2 displays a typical time course of Novozym 435 catalyzed interesterification of octyl dihydrocaffeate with tricaprylin in a solvent-free system. As depicted, three groups of phenolic derivatives from octyl dihydrocaffeate are generally converted in the concentration di-DHCA/mono-DHCA monocaprylin > mono-DHCA dicaprylin > mono-DHCA/di-DHCA glycerol. This indicated that the reaction simultaneously proceeds in different stages of the interesterification. Less mono-DHCA/di-DHCA glycerol might be a natural result with excessive tricaprylin and little water presence. As can be seen, this solvent-free system was intrinsically fast: within 24 h 60% of octyl dihydrocaffeate converted to products. After another 24 h the reaction conversion reached 70%, thereafter the increases of products levelled off, indicating the reaction came close to the equilibrium of the reaction. Compared with the system previously reported (Sabally et al. 2006; Safari et al. 2006), this system is faster since most of the other systems needed 4–5 days to reach equilibrium. Moreover, this result also presented an important hint for the level setting of reaction time for the following RSM optimization.

In order to determine the appropriate biocatalyst, three commercially available immobilized enzymes were examined for their capacity to catalyze interesterification of octyl dihydrocaffeate with tricaprylin (Table 1). In terms of specific activity, Lipozyme TL IM (0.57 μmol g^{-1} min^{-1}) was not so different from Lipozyme RM IM (0.44 μmol g^{-1} min^{-1}), whereas Novozym 435 was significantly higher (9.57 μmol g^{-1} min^{-1}). This fact again proved that *Candida antartica* lipase B (Novozym 435) was a robust lipase for synthesis application as observed in many different systems (Compton et al. 2000, 2006; Sabally et al. 2005, 2006; Guyot et al. 1997). Therefore, Novozym 435 was chosen as a biocatalyst for RSM optimization.

RSM optimization and experimental validation

RSM was applied to investigate the effects of temperature, reaction time, enzyme load, and substrate molar ratio on the conversion of octyl dihydrocaffeate and yield of dihydrocaffoylated glycerols (the details of statistical analysis not shown). Based on RSM model prediction, two parameter settings could be generated with expected high yield and conversion (Table 2). Two experiments were thus conducted based on the predicted optimum conditions to validate the RSM model predictions (Table 2). As shown in Table 2, the experimental results generally agreed with the predicted values, which confirmed the validity and adequacy of the model prediction. Moreover, high substrate conversion and yield of desired dihydrocaffoylated glycerides in the validation tests were achieved in a relatively shorter reaction time (24–44 h), indicating an intrinsic advantage over the reported systems (Sabally et al. 2006).

Table 2 Optimum conditions predicted by RSM model and the results from validation reactions

	Run 1[a]	Run 2[a]
Temperature (°C)	70	65.2
Reaction time (hour)	24.25	43.6
Enzyme load (%)	14.6	19.9
Molar ratio (tricaprylin/ octyl dihydrocaffeate)	3	4.97
Conversion (%)		
Predicted	81.33	91.91
Experimental	79.8 ± 0.37	86.5 ± 0.21
Yield (%)		
Predicted	76.61	89.86
Experimental	76.5 ± 0.22	78 ± 1.75

[a] Experimental data are means ± standard deviations of two replicates

Conclusions

A novel route for enzymatic synthesis of dihydrocaffoylated glycerides with octyl dihydrocaffeate as a synthetic intermediate has been established. The results demonstrated that the new approach has distinct advantages over reported solvent systems, namely, faster reaction rate and higher volumetric productivity. Optimized reaction conditions were generated through RSM optimization. In two validation reactions based on optimized conditions, further improved conversion of octyl dihydrocaffeate and yield of dihydrocaffoylated glycerols were obtained.

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