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Synthesis of Feruloyl Ester Using *Bacillus subtilis* AKL 13 Lipase Immobilized on Celite® 545

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Summary

The lipophilic antioxidants, glyceryl ferulate and feruloyl glyceryl linoleate, were synthesized using lipase from *Bacillus subtilis* AKL 13. The extracellular lipase was produced by cultivation of the strain in modified minimal medium and the enzyme was recovered by fractionation at 80 % ammonium salt saturation. The concentrated enzyme with the specific activity of (4647±66) U/mg was immobilized on Celite® 545 and crosslinked using glutaraldehyde. The prepared enzyme catalyst was used for esterification of ferulic and linoleic acids with glycerol separately in hexane butane solvent system at 50 °C and 3.144×*g* agitation. The maximum ester conversion of 94 % of feruloyl glyceryl linoleate was achieved at 48 h, whereas only 35 % of glyceryl ferulate was synthesized. The reaction products were characterized using RP-HPLC, FTIR, ^{1}H NMR, ^{13}C NMR and fluorescence spectrophotometry. The kinetic parameters of esterification reaction were determined according to ping-pong bi-bi model. The K_m and v_{max} were found to be 69.37 and 3.46 mmol, and 0.387 and 1.02 mmol/(min·g) for glyceryl ferulate and feruloyl glyceryl linoleate, respectively. The kinetic parameters were simulated in MATLAB and the experimental data were in good agreement. Furthermore, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of the blend of feruloyl ester and palm oil was higher than of the plain palm oil and was closer to α-tocopherol.

Key words: Bacillus subtilis lipase, Celite® 545, immobilization, esterification, feruloyl ester

Introduction

The quality of any edible oil is based on its form and composition of fatty acids, which contributes to the functional and textural characteristics. Long chain polyunsaturated fatty acids (PUFA) in the edible oil undergo lipid oxidation during processing and storage, which leads to rancidity and unpleasant odour (*1*). The lipid autoxidation of edible oil occurs in three steps: initiation, propagation and termination, which destroy the essential fatty acids. The primary oxidation products of edible oil are lipid hydroperoxides, which are readily decomposed to alkoxy radicals and yield secondary products such as aldehydes, ketones, acids, esters, alcohols and short chain hydrocarbons. Various factors influence the autoxidation of edible oil. Highly unsaturated oils are readily oxidized. The presence of natural antioxidants in the oil seed prevents the autoxidation. Thus, oxidative stability of refined oil is weaker than that of crude oil. The natural antioxidants are removed during bleaching and refining. The effect of light, especially with a shorter wavelength, has a more detrimental effect on autoxidation (*2*). Many efforts have been taken to improve the oxidative stability of edible oil. One of the recent attempts to achieve oxidative stability is in the addition of phenolic compounds to the refined edible oil. Furthermore, the phenolic compounds exhibit antimicrobial, anti-inflammatory, anti-allergic, antithrombotic, cardioprotective and vasodilatory effects (*3*). Ferulic acid is one of the polyphenols present abundantly in rice, barley, wheat and citrus fruits. It is widely used as natural antioxidant in food and cosmetic industries (*4*). Ferulic acid and

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its derivatives possess several biological activities like anti-inflammatory (*5*), anticardiovascular (*6*) and anticerebral thrombosis (*7*). The amides of ferulic acid are reported as a stimulator of secretion of insulin (*8*). However, the application of ferulic acid in edible oil is limited due to its poor solubility in it (*9,10*). To improve the solubility, it is conjugated with fatty acid or acyl glycerol, so-called designer lipids. The amphipathic nature of designer lipids retains the antioxidant properties of phenolic compounds, together with the enhancement of solubility in the oil (*11*). Recently, many authors have reported the phenolic ester synthesis catalyzed by lipase (*12,13*). According to Kim *et al.* (*14*), the conjugated linoleic acid possesses anticancer activity and reduces the risk of cardiovascular diseases. In general, microbial lipases are used in the synthesis of designer lipids (*15-17*). In enzymatic catalysis, especially in non-aqueous enzymatic reaction, the immobilized enzymes are more preferred than free enzymes due to their high stability (*18*). Among various immobilization processes, adsorption is common, simple to perform and economically acceptable (*19,20*). The Celite® 545 is a highly porous, low-density, diatomaceous earth siliceous material. Furthermore, the conductivity coefficient of Celite® 545 is very low, which makes it a suitable solid support for immobilization (*21*). In the present study, the extracellular lipase was produced using *Bacillus subtilis* AKL 13. The lipase immobilized on Celite® 545 was evaluated for its catalytic activity in glyceryl ferulate and feruloyl glyceryl linoleate synthesis. The obtained feruloyl esters were tested for their antioxidant potential after blending with palm oil at different concentrations.

Materials and Methods

Microorganism and chemicals

Bacillus subtilis AKL 13 was isolated from soil sample of palm oil processing industry located at Virudhunagar, Tamil Nadu, India (latitude 9.575953 °N and longitude 77.961837 °E). The strain was deposited at Microbial Culture Collection, National Centre for Cell Science (NCCS), Pune, India (MCC 3239) after various biochemical and molecular characterizations. The *p*-nitrophenyl palmitate, ferulic acid (4-hydroxy-3-methoxycinnamic acid), linoleic acid, glycerol and Celite® 545 were purchased from Sigma-Aldrich, Bangalore, India. The tributyrin agar and other analytical chemicals were obtained from HiMedia, Bangalore, India. Olive oil and palm oil were kindly donated by Sri Hari oil mill, Virudhunagar, India. The organic solvents used in this study were of HPLC grade (Sigma-Aldrich).

Lipase production and concentration

Submerged batch fermentation was performed to obtain extracellular lipase from *B. subtilis* AKL 13. According to Kanwar *et al.* (*22*), the minimal medium containing (in g/L): $NaNO_3$ 3.0, K_2HPO_4 0.1, $MgSO_4{\cdot}7H_2O$ 0.5, KCl 0.5, $FeSO_4{\cdot}7H_2O$ 0.01, glucose 0.25 %, by volume, and olive oil 0.25 %, by volume, was prepared, inoculated with 1 % (by volume) of mid-log culture (9 h, $A_{600\ nm}$=0.85). The culture broth was kept at 37 °C under agitation (95×*g*) for 14 h. The cell-free culture broth was obtained by centrifugation at 9503×*g* and 4 °C for 20 min. Then, the crude enzyme was subjected to stepwise ammonium sulphate fractionation (20 to 95 % saturation). The required amount of ammonium salt was added to one litre of crude enzyme to achieve 10 % of saturation and the content was kept at 4 °C overnight. The formed precipitate was separated by centrifugation at 9503×*g* and 4 °C for 20 min. The precipitate was reconstituted in sodium phosphate buffer (50 mM, pH=7.5). Further saturation of the remaining crude enzyme was extended until 100 % with an increase of 10 % saturation to precipitate any residual protein. The precipitate formed during salt saturation was subjected to membrane dialysis to completely remove any remaining ammonium salts. The dialysis was carried out in sodium phosphate buffer (50 mM, pH=7.5) for 12 h at 4 °C. Dialysis buffer solution was replaced with freshly prepared buffer every 3 h. The dialyzed lipase was reconstituted in the same buffer for protein and lipase activity measurements (*23*). About 69 % of product recovery with the purification fold of 2.88 was freeze dried and turned into powder (Promega, Chennai, India) for immobilization studies.

Immobilization of lipase on Celite® 545

Initially, the Celite® 545 was thoroughly washed with sodium phosphate buffer (pH=7.5) to remove any impurities adhered to its surface. The washed Celite® 545 was dried in hot air oven (Promega) for 6 h at 110 °C. The lipase powder (20 mg) was suspended in 10 mL of buffer in a 50-mL glass vial. The dried Celite® 545 (2 g) was added and kept under low stirring (0.349-0.786×*g*) at 37 °C. At regular time intervals, an aliquot was taken for the residual protein and lipase activity assay. The immobilization was continued for 2 h and the resulting immobilized enzyme was treated with 10 mL of 1 % (by volume) glutaraldehyde as crosslinking agent for 30 min at 37 °C. The crosslinked enzyme immobilized on Celite® 545 was washed three times in buffer and stored at 4 °C (*24*). The amount of lipase adsorbed on Celite® 545 (q_t, mg/g of support) was calculated according to the following equation:

$$q_t = \left(\frac{\gamma_0 - \gamma_t}{m} \right) V \qquad /1/$$

where q_t is the amount of protein adsorbed onto mass unit of solid matrix (mg/g), γ_0 is the concentration of initial protein (mg/mL), γ_t is the concentration of protein (mg/mL) at time t in the aqueous phase of immobilization medium, V is the volume of the immobilization medium (mL) and m is the mass of solid matrix (g).

Lipase assay

The hydrolytic activity of lipase was assayed using UV-Vis spectrophotometer UV-1700 (Shimadzu, Kyoto, Japan). The assay reaction mixture contained 6 μL of 30 mM *p*-nitrophenyl palmitate (*p*NPP) prepared in isopropanol, 24 μL of 50 mM sodium phosphate buffer (pH=7.6), 25 μL of enzyme solution and 445 μL of distilled water. The reaction was incubated at 37 °C and the release of *p*-nitrophenol was monitored for 2 min at λ=410 nm (*25*). The amount of lipase required to liberate 1 μmol of *p*-nitrophenol per minute per mL of enzyme solution under the assay conditions was considered as one unit of lipase activity.

The amount of protein was determined by micro-Bradford assay using bovine serum albumin (BSA) as standard.

Characterization of B. subtilis AKL 13 lipase immobilized on Celite® 545

The surface morphology of the Celite® 545 and the bound enzyme was recorded (Carl Zeiss EVO 18; Jena, Germany) at 19 kV with 3000× resolution. To determine the thermal stability of the immobilized enzyme, 100 mg of the lipase immobilized on Celite® 545 were taken in 10 mL of phosphate buffer (50 mM, pH=7.5) and exposed to temperatures from 35 to 75 °C for 4 h. Later, the hydrolytic activity towards *p*NPP was assayed at 37 °C. The effect of various organic solvents on the stability of immobilized enzyme was determined. A mass of 100 mg of *B. subtilis* AKL 13 lipase immobilized on Celite® 545 was taken in 10 mL of organic solvents and kept under agitation at 3.14×*g* for 4 h. After incubation the immobilized enzyme was separated, freeze-dried and assayed for hydrolytic activity on *p*NPP under standard conditions.

Solubility of ferulic acid in glycerol

Substrate solvation in the reaction medium is the major factor in considering enzyme-catalyzed activity. Hence, the solubility of ferulic acid (50 mM) in glycerol (10 mL) at various temperatures was tested. The samples were withdrawn at regular intervals and analyzed in reversed phase high-performance liquid chromatograph (RP-HPLC binary gradient system, model LC-2030; Shimadzu Prominence, Kyoto, Japan).

Synthesis of feruloylated acylglycerol

As shown in the reaction scheme (Fig. 1), the esterification activity of the *B. subtilis* AKL 13 lipase immobilized on Celite® 545 was estimated in two-step reaction conditions, synthesis of glyceryl ferulate (step 1) followed by feruloyl glyceryl linoleate (step 2). Reaction mixture A contained 30 mM of ferulic acid and 10 mM of glycerol in a stoppered glass vial. A mass of 100 mg of *B. subtilis* AKL 13 lipase immobilized on Celite® 545 was added as biocatalyst to the reaction mixture to carry out the esterification reaction at 50 °C under shaking at 1.39×*g* for 96 h. In the second step, 10 mM of glyceryl ferulate and 10 mM of linoleic acid in a stoppered glass vial were taken and the reaction was initiated by adding 100 mg of immobilized enzyme under the conditions mentioned in step 1. The reaction progress was monitored with RP-HPLC using free ferulic acid as standard. The reaction mixture without the enzyme was used as control.

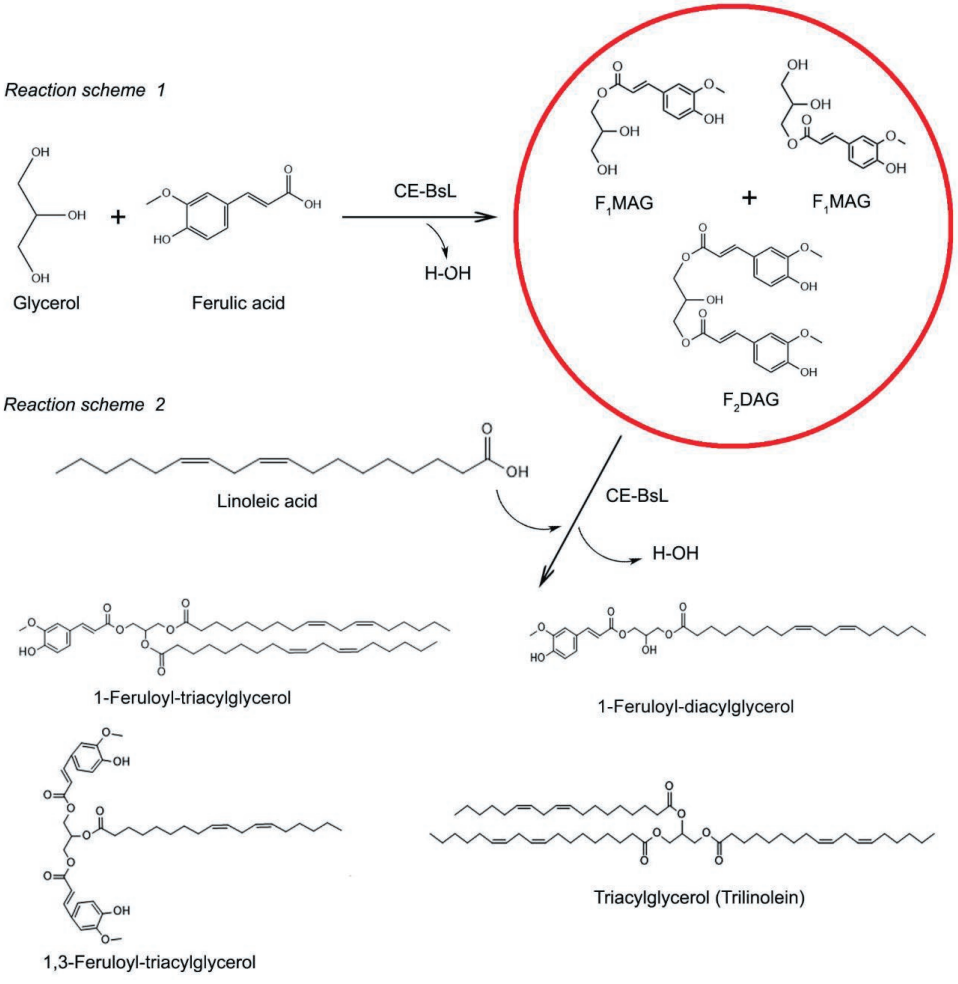


Fig. 1. Esterification reaction scheme of glyceryl ferulate and feruloyl glyceryl linoleate synthesis using *B. subtilis* AKL 13 lipase immobilized on Celite® 545. CE-BsL=*B. subtilis* AKL 13 lipase immobilized on Celite® 545, F_1MAG=monoferuloylated acyl glycerols, F_2DAG=diferuloylated acyl glycerols

Characterization of esterification reaction products

Chromatographic analysis

An aliquot of sample and blank reactions was analyzed by thin layer chromatography (TLC) using a solvent mixture of benzene/ether/dichloromethane/hexane (3:5:2:2, by volume) (*26*). The TLC plates were sprayed with 20 % (by volume) sulfuric acid and visualized (*14*). In RP-HPLC, the reaction product was analyzed in C18 column at 37 °C as described by Zheng *et al.* (*27*). The mobile phase was a mixture of solution A (100 % methanol) and solution B (water containing 0.1 % trifluoracetic acid). The mobile phase was degassed by sonication at 75 % amplitude, 30-second on-off cycles for 10 min (VCX 130, Sonics and Materials, Inc., Newtown, CT, USA). The gradient elution was set as solvent A from 50 to 100 % for 10 min. Then, it was maintained at 100 % for further 10 min. Later, solvent A concentration was gradually brought down to 50 % in 10 min. A volume of 20 μL of the 100-fold diluted samples (50 μg/mL) was injected into the column and feruloylated species were monitored at λ=325 nm with UV-Vis detector (model SPD-20A; Shimadzu). The product conversion was calculated based on the free ferulic acid peak area during the reaction time (*14,26*).

Spectroscopic analysis

The Fourier transform infrared (FTIR) spectrum of the esterification reaction products was recorded in the range from 400 to 4000 cm^{-1} with Shimadzu IRAffinity-1S spectrophotometer. Catalyzed esterification reaction products of *B. subtilis* AKL 13 lipase immobilized on Celite® 545 were dissolved in deuterated chloroform ($CDCl_3$; Sigma-Aldrich). The ^1H and ^{13}C NMR spectra were recorded at 300 and 75 MHz respectively with tetramethylsilane (Cortec, Paris, France) as an internal standard using Bruker Avance III 500 MHz NMR spectrometer (Bruker Corporation, Billerica, MA, USA). The acquisition parameters were: spectral width 6172.8 Hz, relaxation delay 2 s, and number of scans 16. The chemical shifts (δ) and coupling constants (J) were reported in ppm and Hz, respectively. To distinguish the emission property of reactants and products, the emission spectrum (λ_{ex}=325 nm) of the samples before and after esterification was recorded with Shimadzu FL2000 fluorescence spectrophotometer.

Antioxidant activity of palm oil blended with feruloyl acyl glycerols

The free radical scavenging activity of the blend of glyceryl ferulate, feruloyl glyceryl linoleate and palm oil was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method as described by Compton *et al.* (*9*). Fresh palm oil was blended with 1 % of ferulic acid, α-tocopherol, glyceryl ferulate and feruloyl glyceryl linoleate separately. A volume of 100 μL of each sample was suspended in 1.9 mL of 0.1 mM of the ethanolic DPPH solution separately. The mixture was incubated for 20 min and the DPPH reduction was measured at λ=517 nm with a UV-Vis spectrophotometer 1700 (Shimadzu) at 37 °C. Palm oil without any additional antioxidants was used as control. The α-tocopherol was used as standard to calculate the percentage of DPPH free radical scavenging activity.

Kinetic modelling studies

The kinetic parameters such as maximum reaction rate (v_{max}) and Michealis-Menten constant (K_m) of glyceryl ferulate and feruloyl glyceryl linoleate synthesis catalyzed by *B. subtilis* AKL 13 lipase immobilized on Celite® 545 were determined. The determined parameters were used to simulate the esterification reaction products in MATLAB v. 2015a (*28*). The model equations from a set of ordinary differential equations and algebraic equation tools were used. The model assumptions were that the concentration of reacting substrates in each reaction (ferulic acid, glycerol, glyceryl ferulate and linoleic acid) at the external surface of immobilized catalyst is at equilibrium. Similarly, the rheological property of the bulk reaction medium is considered to be constant in all the performed experiments. The predicted values by the model were validated by conducting a separate set of experiments and good correlation was seen.

Statistical analysis

Statistical analysis was performed with SPSS v. 18.0 (*29*) and all the data were expressed as mean value±standard deviation. A value of $p<0.05$ was considered statistically significant.

Results and Discussion

Immobilization of B. subtilis AKL 13 lipase

In the present study, the free lipase with the *p*NPP hydrolytic activity of 4647 U/mg was immobilized on Celite® 545. The immobilized enzyme was further crosslinked using glutaraldehyde. The free aldehyde groups of glutaraldehyde link the ε amino group of Lys residues of adsorbed proteins on solid surface. Furthermore, the crosslinking by glutaraldehyde enhances the thermostability by creating more direct covalent bonds among enzyme molecules (*30*). Initially, the immobilization time and amount of initial protein for immobilization were studied. The maximum protein loading on solid support matrix depends on the enzyme structure, type of matrix and used buffer. De Castro *et al.* (*31*) reported that under conditions of low enzyme loading (0.5 mg/g of support) and optimum time, the enzyme spread itself over the matrix and retained its active conformation.

As indicated in Fig. 2, the immobilization yield and adsorbed amount of protein (q_t) increased with an increase in time and subsequently reached an asymptomatic value at around t=72 min.

The maximum q_t (10 mg/g) was obtained in 75 min. The residual enzyme activity in the aqueous solution gradually decreased and no lipase activity was found after 75 min of incubation with Celite® 545. This result indicated that 100 % of lipase is adsorbed on Celite® 545. Verma *et al.* (*32*) reported 77 % of immobilization of lipase from *Bacillus cereus* MTCC 8372 on Celite® 545. Roby *et al.* (*12*) achieved 94.5 % of adsorption of *Bacillus coagulans* BTS-3 lipase on Celite® 545 after overnight incubation. In contrast to free enzyme, the specific activity of lipase immobilized on Celite® 545 was six times lower, 745 U/mg of immobilized protein. Upon

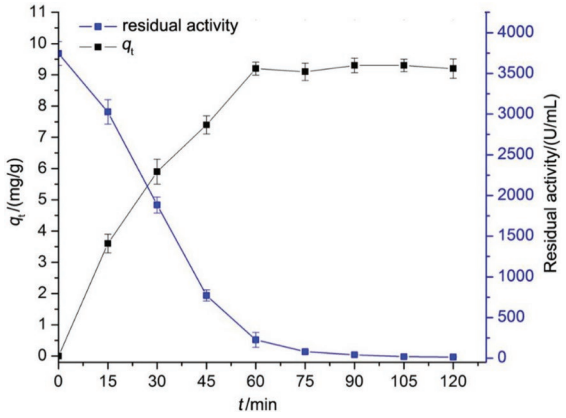


Fig. 2. Time course adsorption profile of *Bacillus subtilis* AKL 13 lipase on Celite® 545 in 50 mM sodium phosphate buffer (pH=7.5) at 37 °C

immobilization, the high molecular enzyme substance attached to the surface of solid matrix and blocked the available catalytic site. Furthermore, at high initial enzyme loading, the proteins were adsorbed on solid matrix at close proximity to each other. This strong attachment could cause structural changes in the protein.

Scanning electron micrograph analysis

The scanning electron micrograph (SEM) analysis of Celite® 545 before and after immobilization was carried out to study the changes in the surface morphology of the matrix (Fig. 3). The SEM image of the Celite® 545 showed the honeycomb structure of the matrix and seemed to be an ideal support for adsorption due to its large surface area. The SEM image of the Celite® 545 after immobilization indicated that the cavities of the honeycomb structure of the matrix were occupied by protein molecules. The results are in agreement with the study of Meunier and Legge (*33*). The SEM image shows the irregular adsorption of protein on the surface of solid matrix and some free cavities.

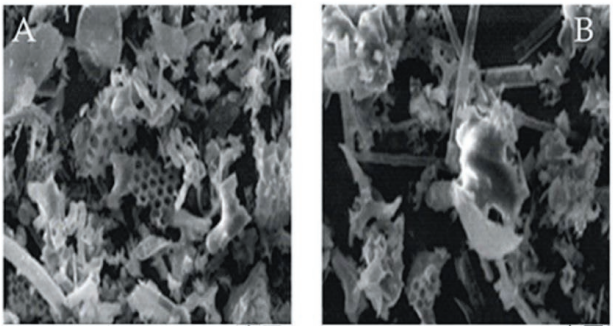


Fig. 3. Scanning electron micrographs of Celite® 545 before (A) and after (B) immobilization of *Bacillus subtilis* AKL 13 showing the honeycomb structure and irregular attachment of proteins on the surface

Thermal stability of B. subtilis AKL 13 lipase immobilized on Celite® 545

The thermal stability of the free lipase and that immobilized on Celite® 545 is shown in Fig. 4, which shows that the immobilized lipase lost 23 % activity at 50 °C, whereas

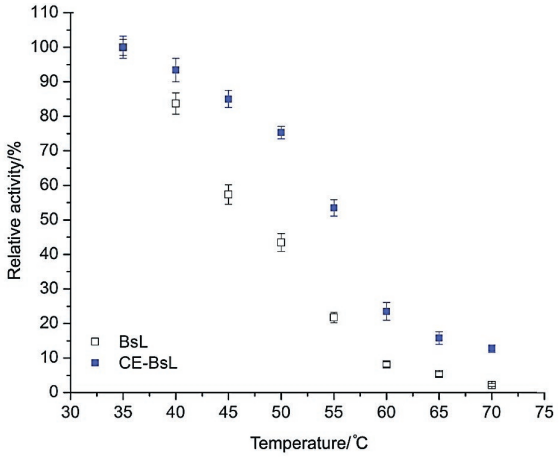


Fig. 4. Effect of temperature on the stability of free (BsL) and immobilized *Bacillus subtilis* AKL 13 (CE-BsL) on Celite® 545 in 50 mM sodium phosphate buffer (pH=7.5) for 4 h

free enzyme showed a loss of 62 %. The high thermal stability of immobilized enzyme is possible due to limitation in conformational changes and strong interaction between the enzyme and solid support.

Substrate solvation studies

In an enzymatic reaction, the solubility of the substrates and products is the most important factor affecting the reaction rate. The stereochemistry and solvent activity coefficient of a molecule determine its solubility in various solvents. In this study, ferulic acid and glycerol were used as the substrates for the synthesis of glyceryl ferulate, hence the solubility of ferulic acid in glycerol was determined at various temperatures (Fig. 5).

At 35 °C, the solubility of the ferulic acid in glycerol was found to be 81.6 %, whereas at 50 °C, it was 100 %. The high viscosity of the glycerol at room temperature may limit the solubility of ferulic acid. Roby *et al.* (*12*) reported that the temperature and liquefaction of the reaction mixture influence the diffusivity of the substrate. Galanakis *et al.* (*34*) reported that the natural phenolic compounds solubilize in moderate polar solvents like alcohols and acetone

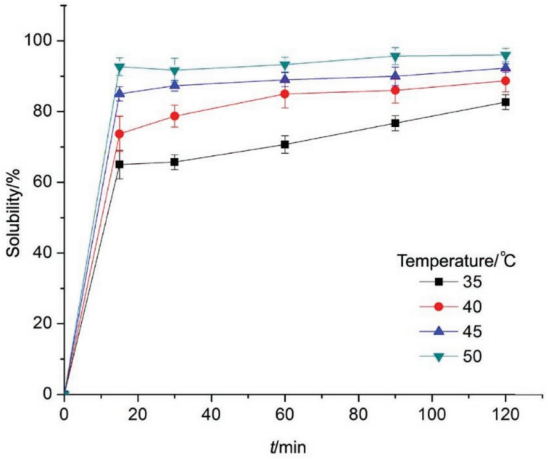


Fig. 5. Effect of temperature on the solubility of ferulic acid in glycerol

rather than in highly polar (water) or less polar solvents like diethyl ether and dichloromethane. From UNIFAC (UNIQUAC Functional Group Activity Coefficients) model analysis (*35*), the ferulic acid exhibited higher solvent activity coefficient in water (13.0) followed by diethyl ether (8.2). However, increasing the temperature reduced the activity coefficient and improved the solubility of phenol in polar solvents. Hence, in the present study the ferulic acid was completely solubilized in glycerol and maintained at 50 °C until the completion of the reaction.

Synthesis of feruloylated lipid

In the present study, feruloylated lipid was synthesized by a two-step esterification using *B. subtilis* AKL 13 lipase immobilized on Celite® 545. Initially, ferulic acid was esterified with glycerol to synthesise glyceryl ferulate, followed by transesterification with linoleic acid to form feruloyl glyceryl linoleate. Similarly, Sun *et al.* (*36*) attempted to synthesise feruloylated lipid using *Candida antarctica* lipase and reported that the conversion was considerably higher in the two-step reaction of ethyl ferulate, oleic acid and glycerol than putting all together in a single step. The presence of a long-chain fatty acid creates the steric hindrance and electrostatic interactions between the phenyl group of ferulic acid and the active site of the enzyme (*36*). Fig. 6 shows the production of glyceryl ferulate with time.

There was no significant increase in the glyceryl ferulate formation observed during the reaction. The maximum glyceryl ferulate conversion, 35 %, was achieved with the maximum reaction rate of 0.010 (mmol/(min·g)) at 48 h of the reaction using immobilized enzyme (Fig. 7).

The slow and poor product conversion may occur due to changes in the water content during the reaction, which diminishes the enzyme activity (*37*). Conversely, the reaction proceeds rapidly in the second step where feruloyl glyceryl linoleate was synthesized. The maximum achieved feruloyl glyceryl linoleate conversion was 95 % (Fig. 6). The maximum reaction rate was found to be 0.0386 (mmol/

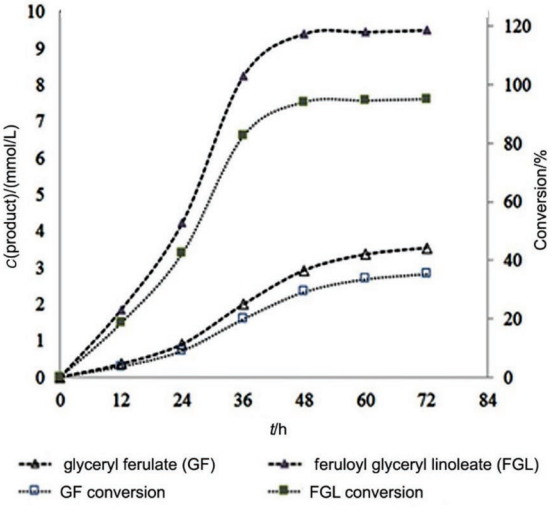


Fig. 6. The production of glyceryl ferulate and feruloyl glyceryl linoleate with time. The esterification of ferulic acid/linoleic acid with glycerol (3:1 molar ratio) was catalyzed by *Bacillus subtilis* AKL 13 lipase immobilized on Celite® 545

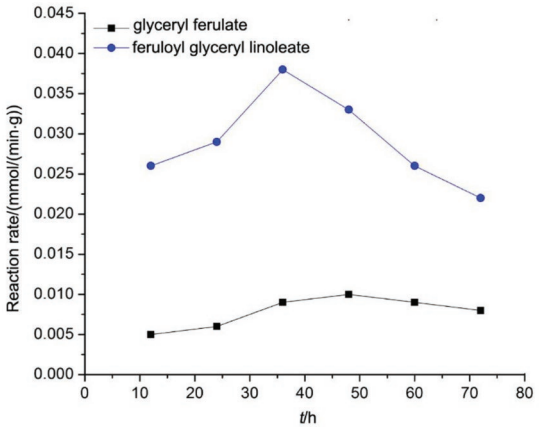


Fig. 7. Reaction rate over time in glyceryl ferulate and feruloyl glyceryl linoleate synthesis

(min·g)) at 36 h (Fig. 7). The reports on the lipase-catalyzed synthesis of feruloyl glyceryl linoleate are very limited. Reddy *et al.* (*38*) studied the synthesis of phenolic lipoconjugate containing ferulic acid, where they achieved 49-58 % yield in 48 h. Similarly, Sorour *et al.* (*39*) reported 65 % conversion for the transesterification of flaxseed oil with 3,4-dihydroxyphenylacetic acid using Novozyme 435. Sabally *et al.* (*40*) reported maximum bioconversion of 66 % in the transesterification of dihydrocaffeic acid with trilinolein using Novozyme lipase after 5 days of reaction.

Kinetic modelling of feruloylated lipid synthesis

According to ping-pong random model (Fig. 8), glycerol (G) and ferulic acid (FA) are bound to the immobilized lipase (E) to form a ternary complex (E.FA), which results in the formation of glyceryl ferulate (GF) and water. In another reaction, glyceryl ferulate (GF) and linoleic acid (LA) bind to enzyme (E) and form ternary complex (E.GFL), which results in the formation of feruloyl glyceryl linoleate (FGL).

The reaction velocity was obtained from the linear regression analysis of time-concentration profile. The initial rate equation and other kinetic parameters were calculated according to the following equations (*41*):

$$v_0 = \frac{v_{max}[G][FA]}{K_m^{FA}[G] + K_m^{G}[FA] + [G][FA] + K_s^{G}K_m^{FA}} \quad /2/$$

$$v_0 = \frac{v_{max}[GF][LA]}{K_m^{LA}[GF] + K_m^{GF}[LA] + [GF][LA] + K_s^{GF}K_m^{LA}} \quad /3/$$

where K_m is Michaelis-Menten constant (mmol), K_s is dissociation constant and v_{max} is maximum velocity (mmol/(min·g)).

The kinetic parameters were found to be K_m^{GF}=69.37 mM, K_m^{GFL}=3.46 mM, v_{max}^{GF}=0.387 mmol/(min·g) and v_{max}^{GFL}= 1.02 mmol/(min·g). In ping-pong reaction with two substrates, the liberation of one substrate from the enzyme in the initial period is irreversible due to negligible concentration (*41*). Hence in the present study K_s^{G}=K_s^{GF}=0. The

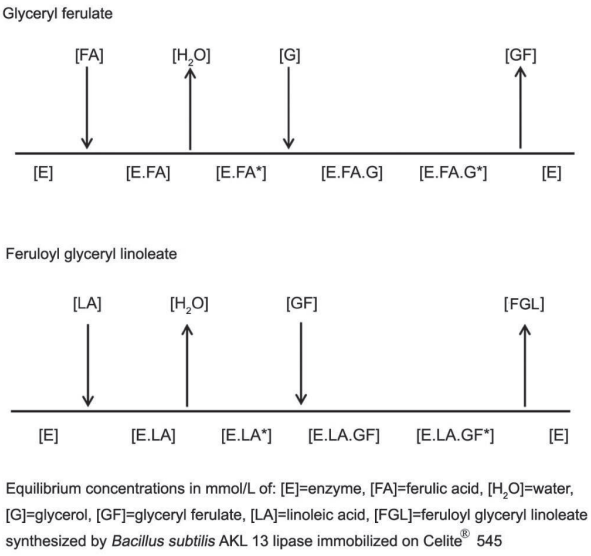


Fig. 8. Scheme of enzymatic synthesis of glyceryl ferulate and glyceryl feruloyl linoleate synthesized by *Bacillus subtilis* AKL 13 lipase immobilized on Celite® 545

results indicate that the affinity between the enzyme and linoleic acid was higher than that of the enzyme and ferulic acid. These kinetic parameters were used to simulate the esterification reaction model in MATLAB v. 2015 (*28*). The experimental data obtained in the present work were compared with a simulated model (Fig. 9). The sum of squared errors (SSE) error function test was carried out to find the significance of the model and experimental values. SSE was 2.879 while adjusted R^2 value was 0.997.

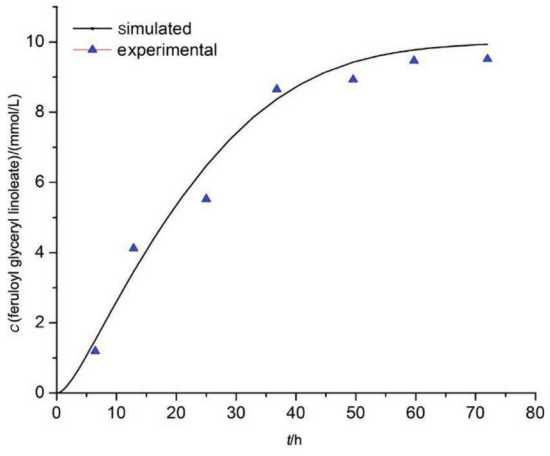


Fig. 9. Product distribution curve for the esterification of glyceryl ferulate with linoleic acid at 1:1 molar ratio using *Bacillus subtilis* AKL 13 lipase immobilized on Celite® 545. The experimental data are shown by the symbol, whereas the line is simulated curve based on the kinetic parameters

Chromatographic analysis of feruloylated acylglycerols

The esterification reaction products of ferulic acid and glycerol were analyzed by thin layer chromatography (TLC) according to the method described by Sabally *et al.* (*15*). The bands of ferulic acid and ferulic acid esters were detected by charring with 20 % sulphuric acid. The glyceryl ferulate migrated more slowly than ferulic acid due to its high non-polar nature when compared to free ferulic acid and glycerol. The retention factor (R_f) of free ferulic acid, monoferuloylated acyl glycerols (F_1MAG) and diferuloylated acyl glycerols (F_2DAG) were 0.68, 0.52 and 0.31, respectively. The bands corresponding to glyceryl ferulate were scraped separately and analyzed further by reversed phase high-performance liquid chromatography (RP-HPLC). The reaction products were identified according to Yang *et al.* (*37*). As expected, highly polar compound (F_1MAG) followed by less polar compound (F_2DAG) were eluted in the C18 column. The identification of the position of a feruloyl group in the glycerol backbone was ambiguous (*42*). Many authors have reported that lipase from *Bacillus* sp. possesses 1-3 specificity (*43-45*).

Spectroscopic analysis of feruloyl acyl glycerol

FTIR spectroscopy studies

The Fourier-transform infrared (FTIR) spectrum of synthesized feruloyl ester (Fig. 10) showed the transmittance band at 1713 cm^{-1}, which confirmed the formation of C=O stretching of the ester group in feruloylated acyl glycerol. The transmittance at 2939 cm^{-1} corresponds to the aliphatic C-H group. The peaks between 699 and 711 cm^{-1} correspond to out-of-plane bending of the phenolic O-H group (*46*).

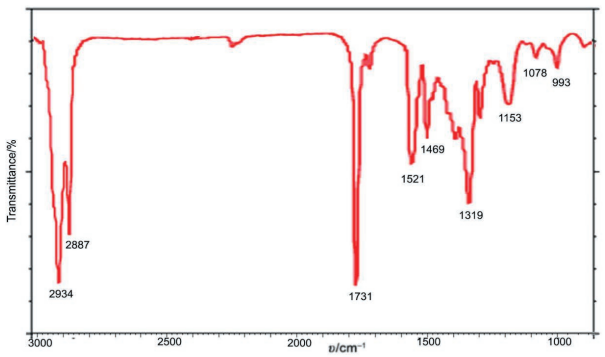


Fig. 10. FTIR spectrum of esterification of glyceryl ferulate and linoleic acid catalyzed by *Bacillus subtilis* AKL 13 lipase immobilized on Celite® 545 to obtain feruloyl glyceryl linoleate

Nuclear magnetic resonance spectroscopy studies

Esterification reaction products of glyceryl ferulate and linoleic acid catalyzed by the *B. subtilis* AKL 13 lipase immobilized on Celite® 545 were subjected to ^{1}H NMR (Fig. 11) and ^{13}C NMR (Fig. 12) analyses.

According to Gholivand *et al.* (*13*) and Nieva-Echevarría *et al.* (*47*), the assignment of the signals corresponding to protons of the glycerol backbone, acyl chains and phenolic moiety are given in Table 1. The multiplet proton signals between δH=0.88 and 1.610 ppm indicated the protons of aliphatic groups. The strong signals at δH=4.071 and 4.223 ppm verified that the reaction product is 1,3-diacylglycerol. The strong signals in the range of δH=6.0-7.6 ppm confirmed the presence of a phenolic compound attached to the glycerol backbone. The proton doublet peaks at δH=6.32 and 7.59 ppm confirmed the

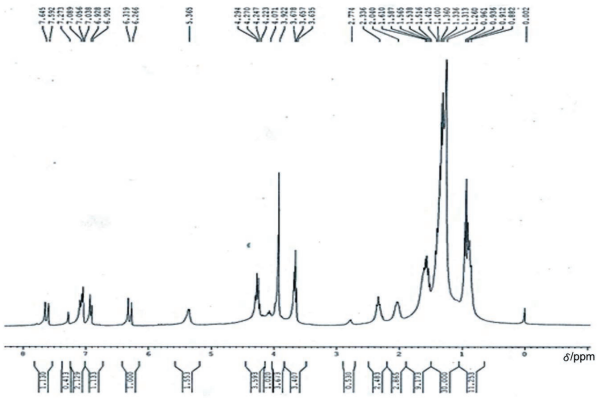


Fig. 11. 1H NMR spectrum analysis of feruloyl acyl glycerol produced during the esterification of ferulic acid and glycerol (3:1 molar ratio) by *Bacillus subtilis* AKL 13 lipase immobilized on Celite® 545

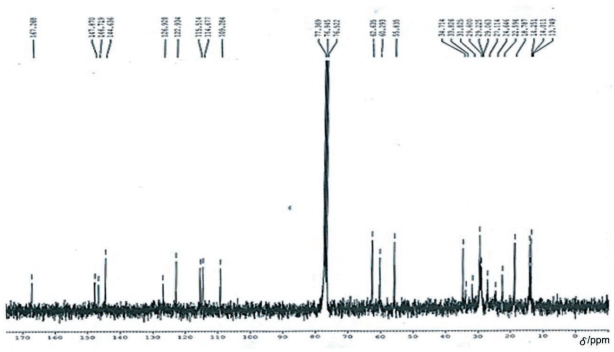
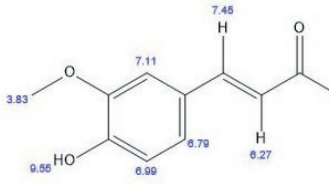


Fig. 12. 13C NMR spectrum analysis of feruloyl acyl glycerol produced during the esterification of ferulic acid and glycerol (3:1 molar ratio) by *Bacillus subtilis* AKL 13 lipase immobilized on Celite® 545

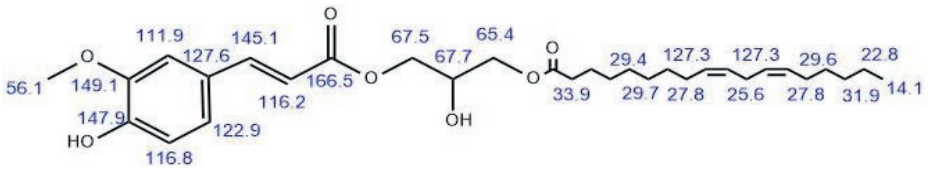
presence of methoxyl group and three aromatic protons, a typical ABX system of ferulic acid. Similar spectrum has been reported by Nieva-Echevarría *et al*. (*47*). In contrast to ^{1}H NMR, the ^{13}C NMR provides the carbon skeleton directly. The number of carbon peaks indicates the different carbon groups in the structure. The peaks from δC=122 to 149 ppm (corresponding to aromatic carbon) confirmed the presence of ferulic acid in the structure. Furthermore, the characteristic peak at δC=167.2 ppm (corresponding to C=O) confirmed the formation of ferulic acid ester. The signals at low ppm (14-34) indicated the presence of linoleic acid in the structure.

Table 1. Chemical shift assignments and multiplicities of the 1H NMR and 13C NMR signals in $CDCl_3$ of the esterification reaction product catalyzed by *Bacillus subtilis* AKL 13 lipase immobilized on Celite® 545

Chemical shift/ppm	Multiplicity	Type of protons	Compound
0.882	t	-C<u>H</u>$_3$	Unsaturated ω-6 acyl groups and fatty acids
1.26-1.425	m	-(C<u>H</u>$_2$)$_n$-	Acyl groups and linoleic acid
1.516-1.610	m	-OCO-CH$_2$-C<u>H</u>$_2$-	Acyl groups in diacylglycerol (1,2-DG or 1,3-DG), except for DHA, EPA and ARA acyl groups
2.336	m	-OCO-C<u>H</u>$_2$-	Acyl groups in 1,3-DG, 1-MG and fatty acid except DHA acyl groups
2.774	s	=HC-C<u>H</u>$_2$-CH=	Polyunsaturated ω-6 and ω-3 acyl groups
3.63-3.67	t	-ROCH$_2$-CHOH-C<u>H</u>$_2$OH-	Glyceryl group in 1-MG
4.071-4.294	t	-ROC<u>H</u>$_2$-C<u>H</u>OH-C<u>H</u>$_2$OR′-	Glyceryl group in 1,3-DG
5.36	s	-C<u>H</u>=C<u>H</u>-	Acyl groups and fatty acids
6.26-7.59	d		Aromatic hydrogen – ferulic acid



Chemical shift (in ppm) in ^{13}C NMR for 1,3-glyceryl feruloyl linoleate



Glyceryl ferulate and linoleic acid were esterified at equimolar concentration (10 mmol/L) and 50 °C for 72 h. s=singlet, d=doublet, t=triplet, m=multiplet, 1-MG=1-monoglyceride, 1,2-DG=1,2-diglyceride, 1,3-DG=1,3-diglyceride, DHA=docosahexaenoate, EPA=eicosapentaenoate, ARA=arachidonate

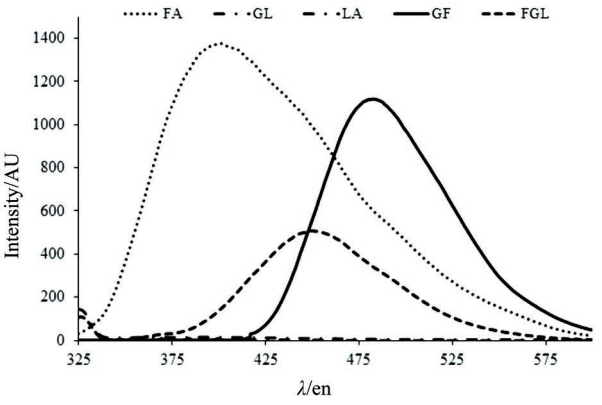


Fig. 13. Fluorescence emission spectra of feruloylated species formed during the esterification of ferulic acid (FA)/linoleic acid (LA) and glycerol (G) in 3:1 molar ratio by *Bacillus subtilis* AKL 13 lipase immobilized on Celite® 545. The excitation wavelength is λ=325 nm. GF=glyceryl ferulate, FGL=feruloyl glyceryl linoleate

Fluorescence spectroscopy studies

The ferulic acid exhibits fluorescence property with emission peaks between 410 and 420 nm (*48*), whereas glycerol and fatty acids do not possess fluorescence properties. Hence, in the present work, the difference in the emission profile of ferulic acid and its ester was analyzed to monitor the progress of the esterification reaction. Fig. 13 shows the fluorescence spectra of the ferulic acid and its ester in methanol at λ_{ex}=325 nm. A strong emission peak at λ_{em}=410 nm was observed for ferulic acid, whereas glyceryl ferulate and feruloyl glyceryl linoleate showed a shift in the emission peak at λ_{em}=484 nm and 448 nm respectively. The fluorescence emission intensity of the glyceryl ferulate was higher than that of feruloyl glyceryl linoleate (Fig. 13).

Radical scavenging activity of feruloylated acylglycerols

Since palm oil is widely used in India, it was selected for this study and blended with feruloyl ester at various mass fractions. The radical scavenging activity of synthesised feruloylated lipid was investigated using DPPH and its antioxidant potential was collated with the activity of α-tocopherol and ferulic acid. The plain palm oil had very little scavenging activity, whereas palm oil with ferulic acid esters manifested augmented scavenging activity (Fig. 14). The highest scavenging activity ((84.5±4.7) %) was noted of the palm oil blend with feruloyl glyceryl linoleate followed by α-tocopherol ((82.2±3.2) %). The radical scavenging activities of palm oil blend with free ferulic acid or glyceryl ferulate were comparable. When increasing the percentage of feruloyl glyceryl linoleate in the oil, the activity was rapidly increased and saturated at 0.5 %. The α-tocopherol required double the amount to exhibit the same activity. Hence, the radical scavenging activity of feruloylated compounds makes it a promising antioxidant in various applications especially in food and cosmetic industries (*49*).

Conclusions

Lipase from *Bacillus subtilis* AKL 13 was produced, purified and immobilized on Celite® 545 under optimum

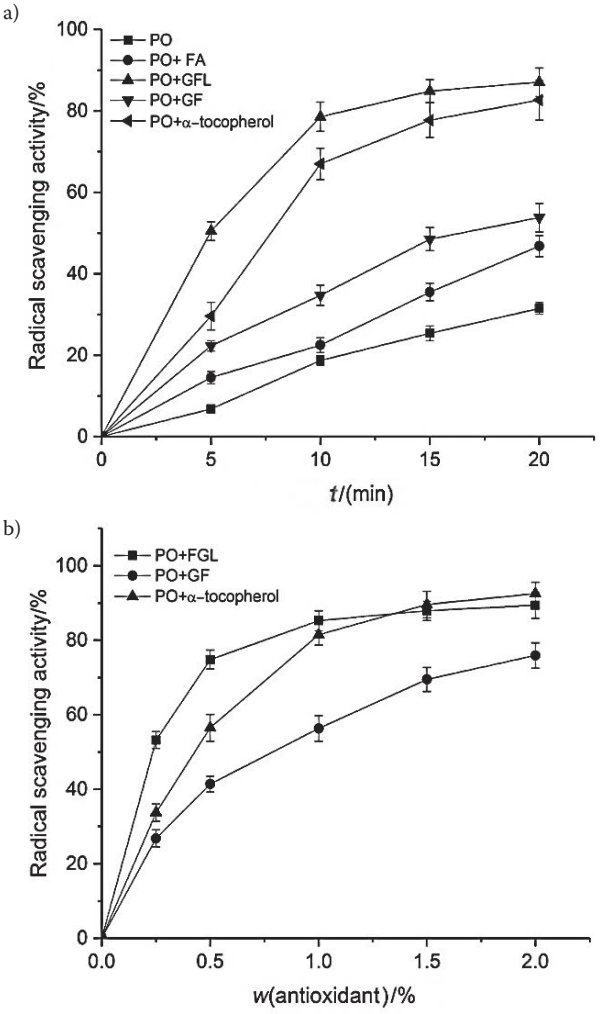


Fig. 14. The effect of the blend of ferulic acid (FA) and its esters with palm oil (PO) on radical scavenging activity with time (a) and the mass fraction of antioxidant in the blend of ferulic acid esters with palm oil (b) in comparison with α-tocopherol. GF=glyceryl ferulate, FGL=feruloyl glyceryl linoleate

immobilization conditions. The scanning electron micrograph verified the attachment of proteins on the surface of the solid support. Maximum q_t (amount of protein attached per unit surface area of solid support) of 10 mg/g was achieved in 75 min of immobilization. The prepared immobilized catalyst showed a good thermal stability by retaining 67 % of activity at 50 °C for 3 h of exposure time. The immobilized lipase showed a preeminent esterification activity (95 % of ester yield) in synthesis of glyceryl feruloyl linoleate. However, deprived activity (35 % yield) was noted in glyceryl ferulate synthesis. The NMR spectra of reaction product confirm the formation of 1,3-diferuloylacylglycerols. Various esterification reaction kinetic parameters were determined and used to simulate the yield in MATLAB. A good agreement of the predicted and experimental results was observed. Furthermore, the blend of feruloyl esters with palm oil improved its antioxidant stability.

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