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ORIGINAL ARTICLE

Lipase catalysed synthesis of N-trans-feruloyltyramine and a quantitative HPLC-UV method for analysis

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Abstract

N-trans feruloyltyramine amide was successfully synthesized from 4-hydroxy-3-methoxycinnamic acid and tyramine hydrochloride in a one-step lipase catalysed reaction. The use of immobilized lipase, lipozyme TL IM as the catalyst in the reaction allowed simple isolation of the enzyme from the products and other components in the reaction mixture. N-feruloyltyramine amide was characterized using Fourier Transform Infrared (FTIR) Spectroscopy, Proton Nuclear Magnetic Resonance (1H NMR) and elemental analysis. Under optimized conditions 93.5% yield was obtained when the process was carried out for 48 h using a molar ratio of cinnamic acid:tyramine HCl, 6:1 at 40°C. In addition, a rapid simple and sensitive HPLC-UV method was developed for the determination of N-feruloyltyramine using an ®Rp-8 end-capped column. The optimum mobile phase used was acetonitrile:disodium hydrogen phosphate, 30:70(v/v.). N-feruloyltyramine amide was detected at a retention time of 12 min. The calibration curve was linear over the range of $5.27–12.30 \times 10^{-4}$ M with correlation factor r = 0.9958. Consequently, the method was considered valid for quantitative analysis samples of N-trans-feruloyltyramine amide.

Keywords: *N-trans-feruloyltyramine, lipase, tyramine HCl, cinnamic acid, HPLC*

Introduction

Amides are biologically interesting compounds and play a very important role in modern drug chemistry (Fana et al. 2010; Ahmad et al. 2010). N-trans-feruloyltyramine amide is classified within the cinnamic acid amide group, with IUPAC name: (E)-3-(4-hydroxy-3-methoxyphenyl)-N-[2-(4-hydroxyphenyl) ethyl] prop-2-enamide, CAS no, 65646-26-6, molecular weight, 313.34 g/mol and molecular formula, $C_{18}H_{19}NO_4$ (King & Calhoun 2010). It had higher activity than the reference medicine acarbose in treating α-glucosidase inhibition in the glucosidase bioassay (Fana et al. 2010). In addition, feruloyltyramine amide is able to suppress P-selectin expression on platelets by 31% (Park 2009) at a concentration of 0.05 μM. COX enzymes are involved in the regulation of P-selectin expression on platelets and N-feruloyltyramine amide was subsequently shown to be a COX enzyme inhibitor. N-trans-feruloyltyramine amide has been isolated from many plants such as litsea *(Lauraceae)*, *Actinodaphne Longifolia*, *Aristolochia gehrtii*, *Porcelia macrocarpa* and *Potato common scab* lesions (Tanaka et al. 2009; Hosana & Lucia 2001; Chaves & Roque 1997; Kinga & Calhounb 2005; Cutilloa et al. 2003). However, the yield of the extracted feruloyltyramine is very low, meaning that large amounts of plant material are needed for extraction and thus the process is not economic. N-trans-feruloyltyramine amide can be synthesized by reacting amino ethyl phenol with chloro carbonyl vinyl methoxy ester (Nomuraa et al. 2003). Although this method gave a yield of 94.0%, toxic hydrazine was needed as a reagent. Park and Schoene (2003) have

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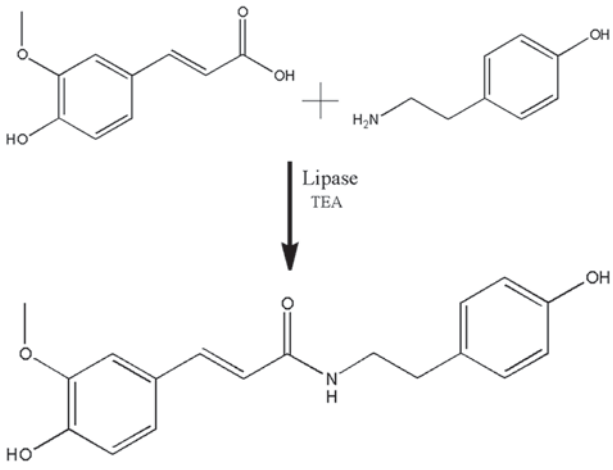
also synthesized feruloyltyramine amide with a lower yield of 55.0%. In addition, feruloyltyramine amide was synthesized by coupling of ferulic acid with tyramine in t-BuOH using dicyclohexylcarbodiimide (Yamazaki et al. 2008). Many catalysts are used to synthesize amides; however, enzymes such as lipases are considered to be the best catalysts due to their high stability in organic media and their ability to accept a variety of substrates (Fernández-Pérez & Otero 2001). In amide synthesis, protection of the reactive amine groups is needed which then must be released after the reaction. The protection is used to avoid carbonation of the amine with carbon dioxide. In this work, the amine group was protected as the HCl derivative. Tyramine was to be released by the addition of a stronger non-reactive tertiary amine base, such as N, N-diisopropylethylamine or triethylamine (Reyes-Duarte et al. 2002). To date, no reports on the synthesis of feruloyltyramine amide from 4-hydroxy-3-methoxycinnamic acid with tyramine hydrochloride in the presence of lipase have been published. In addition, a new simple and sensitive analytical HPLC method for quantitative analysis of feruloyltyramine was developed.



Scheme 1. Lipase catalysed synthesis of N-trans-feruloyltyramine amide from 4-hydroxy-3-methoxycinnamic acid and tyramine hydrochloride in the presence of Lipozyme TL IM in acetonitrile.

Methods

Materials

4-hydroxy-3-methoxycinnamic acid, tyramine hydrochloride having a purity of 99% (w/w), methanol and acetonitrile (HPLC grade) were obtained from Acros Organics (Germany). Tert-butanol, triethylamine, tetrahydrofuran, dimethylformamide, hexane and disodium hydrogen phosphate were purchased from Merck (Germany). The enzymes; Novozyme 435, Lipozyme RM IM, Lipozyme TL IM, Flavourzyme 500MG, Lipase from *Candida rugosa* and Lipase Acidic were obtained from Novozymes (Denmark).

Synthesis

Scheme 1 shows the reaction of amine and acid substrates catalysed by lipase. The reaction was carried out in 25-ml screw-capped tubes at 40°C for 48 h with stirring at 400 rpm. Tyramine HCl (0.29 mmol) was first treated with triethylamine (5 ml) and stirred at 400 rpm for 30 min in order to release the free amine from its salt. Then, 4-hydroxy-3-methoxycinnamic acid (0.26 mmol) was added to the mixture in the presence of enzyme (50 mg) (0.3%, w/w) and molecular sieves (150 mg). Finally, acetonitrile (15 ml) was added to the reaction mixture and stirred for 48 h at 40°C (Reyes-Duarte et al. 2002). The reaction mixture was evaporated under reduced pressure at room temperature and then diluted with water (200 ml). The resulting precipitate was extracted with ethyl acetate and the procedure was repeated three times (each 100 ml). The organic layer was washed successively with 5% HCl (100 ml × 2), 5% $NaHCO_3$ (100 ml × 2), and dried over $MgSO_4$.

Spectroscopic analysis

Identification of feruloyltyramine amide was performed using FT-IR (Perkin Elmer - model 1650, CA, USA), UV (Shimadzu model 1650, Tokyo, Japan) and NMR (500 MHz, JEOL, Tokyo, Japan). Elemental analysis was carried out using CHNS (Leo model 932, CA, USA).

HPLC analysis

Chromatographic analysis was carried out using HPLC (ACQUITY UPLC Waters, Milford, USA). The mobile phase was prepared by mixing acetonitrile with disodium hydrogen phosphate buffer at pH 2.50. The pH of the buffer was adjusted to 2.50 using phosphoric acid and triethylamine. The best mobile phase used was acetonitrile:disodium hydrogen phosphate (30:70, v/v.). The mixture was filtered through a 0.45-mm membrane and degassed by ultrasonic mixing prior to use. Solvent delivery was at a flow rate of 0.5 mL/min. The stationary phase used was chromolith ®Rp-8 endcapped (100–4.5 mm) with a particle size of 2 µm. Detection of the analytes was carried out at 220 nm. The injection volume was 20 µL.

Calibration curve

Stock solution was prepared by dissolving 55.0 mg feruloyltyramine amide in a 100 mL volumetric flask with methanol. The final stock standard solution concentration was 1.8 mM. Diluted standards were prepared by diluting 3, 4, 5, 6 and 7 mL of stock standard to 10 mL with methanol to five different concentrations (Shurbaji et al. 2010).

Optimization of the synthesis reaction

Effect of organic solvents. The effect of various organic solvents on the synthesis reaction was studied. The solvents used were tert-butanol (log kow = 0.35), methanol (log kow = −0.77), dimethylformamide (log kow = −1.01), acetonitrile (log kow = −0.34), hexane (log kow = 3.9), triethylamine (log kow = 2.38) and tetrahydrofuran (log kow = 0.46). The reaction was carried out at a mole ratio of 1:1, 50 mg Novozyme 435, temperature 30°C and reaction time 96 h. Novozyme 435 was selected as the testing enzyme and the optimized enzyme in this section. The yield was determined using the standard calibration curve equation of feruloyltyramine amide.

Effect of enzyme type. Novozyme 435, Lipozyme RM IM, Lipozyme TL IM, Flavourzyme 500MG, *Candida rugosa* lipase and Lipase Acidic were screened for the best activity. The reaction was at a mole ratio of 1:1, 50 mg lipase enzymes, temperature 40°C and reaction time of 48 h. The yield was determined based on the standard calibration curve of feruloyltyramine amide.

Effect of reaction time and temperature. The reaction mixture was incubated at various temperatures, 30, 40 and 50°C and various times 24–96 h using mole ratio, 1:1 and 50 mg Lipozyme TL IM. The yield was determined based on the standard calibration curve equation of feruloyltyramine amide.

Effect of enzyme amount. The reaction mixture consisted of the different amounts of Lipozyme TL IM (50–500 mg) at mole ratio, 1:1 and temperature, 40°C and reaction time, 48 h. The yield was determined based on the standard calibration curve equation of feruloyltyramine amide.

Effect of molar ratio. Tyramine HCl was reacted with different quantities of cinnamic acid, with mole ratios (cinnamic acid:tyramine HCL) of 1:1, 2:1, 3:1, 4:1, 5:1, 6:1 and 8:1 at 40°C, 48 h reaction time and 250 mg of lipozyme TL IM. The yield was determined based on the standard calibration curve equation of feruloyltyramine amide.

Results and discussion

Synthesis

No reaction was detected when the tyramine-HCl derivative (protected amine) was used, suggesting the requirement for a free amino group in the reaction due to the inability of the amino group –NH to form the corresponding amide. When equimolar concentrations of tyramine-HCl and 4-hydroxy-3-methoxycinnamic acid were used, low production rates of feruloyltyramine amide were obtained. However, when an excess of the tertiary amine was used in the reaction with acetonitrile as solvent, the rate increased considerably. The dried ethyl acetate layer was evaporated under reduced pressure to give an oily yellowish product.

Spectroscopic analysis

Analysis (in MeOH) of the yellow oil product obtained showed UV max at 220,288, 319 nm. On silica gel 60 F254 TLC plates (solvent $CHCl_3$-Acetone 5:1, v/v.) only one spot of the product was evident, with a higher Rf than the substrates, indicating the purity of the compound. FTIR analysis showed absorption bands at 3414, 1700 and 1600 cm^{-1} attributed to N-H group stretching, C = O stretching and N-H bending of amide, respectively. These data confirm the formation of N-feruloyltyramine amide.

H1-NMR (500 MHz, CD3OD): 3.14 (2H, t, J = 11 Hz), 3.30 (2H, t, J = 1.65 Hz), 3.80 (2H, s, phenolic OH), 3.83 (3H, s, −OCH3), 5.5 (1H, d, J = 15.6 Hz), 6.57 (1H,m) 6.70 (2H,m), 6.80–6.87, (1H, m), 6.98 (4H, m),7.90 (1H, s). Elemental analysis CHNS results were 69% C, 6% H, 4.5% N and 20.5% O. The spectral data obtained indicated that N-feruloyltyramine amide was formed with good purity.

HPLC analysis

A standard containing N-feruloyltyramine amide (12.8×10^{-4}M) was prepared and injected under the optimum chromatographic conditions. A completely separated peak for N-feruloyltyramine with high purity exceeded (98.0%) was obtained at a retention time Rt = 12 min (Figure 1).

Calibration curves (Figure 2) for N-feruloyltyramine amide were obtained in the concentration range of ($5–12 \times 10^{-4}$) M, each point represents the mean value ± standard deviation (SD) from three independent experiments. The linearity of this method was evaluated by linear regression analysis calculated by the least-squares method. The representative linear equation of N-feruloyltyramine

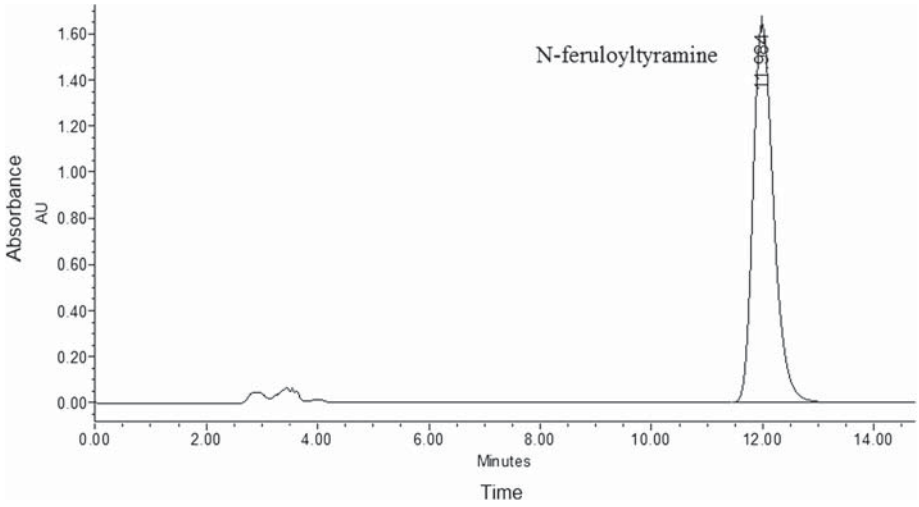


Figure 1. Typical chromatogram for N-trans-feruloyltyramine amide standard (12.8×10^{-4}M).

amide standard was: $y = 138.65x + 94.61$, with $r^2 = 0.9916$ and correlation factor $r = 0.9958$.

Where y = (Area of the peak $\times 10^4$) and x = (concentration of sample $\times 10^{-4}$ M).

Optimization of the synthesis reaction

Effect of organic solvents. Figure 3 shows the effect of organic solvents on amide formation. It was found that the highest yield was obtained in acetonitrile (log kow = −0.34), (yield = 39.8%). Acetonitrile is a polar aprotic solvent which can dissolve moderately polar compounds such as N-*trans*- feruloyltyramine amide. The yield was relatively low with solvents having a log kow more than 3, such as hexane (yield = 2.5%). Therefore, acetonitrile was used as the optimum solvent in the subsequent experiments.

Effect of enzyme type. Six commercial enzymes were screened to select the most efficient enzyme for amide formation (Figure 4). The results indicated that the reaction using Lipozyme TL IM gave the highest yield 69.9% followed by Lipozyme RM IM at 69.2%. Novozyme 435 was the least effective enzyme with a yield of 63.1%. Therefore, Lipozyme TL IM was used as the lipase in the subsequent experiments. The effect of organic solvent was re-evaluated using Lipozyme TL IM instead of Novozyme 435, confirming that acetonitrile remained

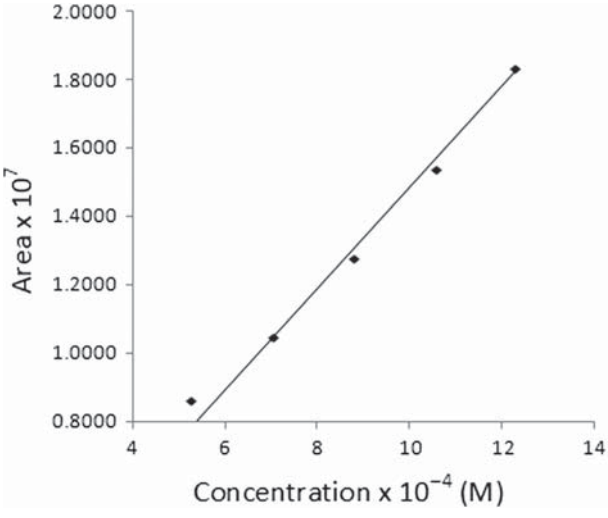


Figure 2. Calibration curve of N-transe-feruloyltyramine amide at five different concentrations, each point represents the mean value ± standard deviation (SD) from three independent experiments, Relative SD (RSD) was less than 2.0%.

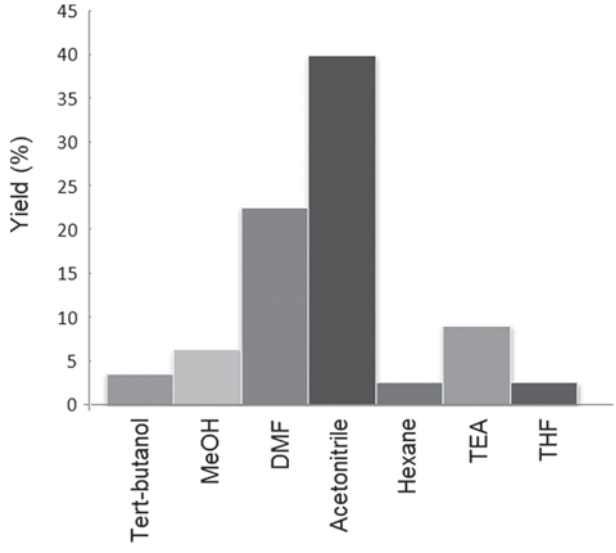


Figure 3. Effect of various organic solvents using mole ratio 1:1 and 50 mg Novozyme 435, at temperature 30°C and reaction time 96 h. Each point represents the mean value ± SD from three independent experiments, RSD was less than (2.0)%.

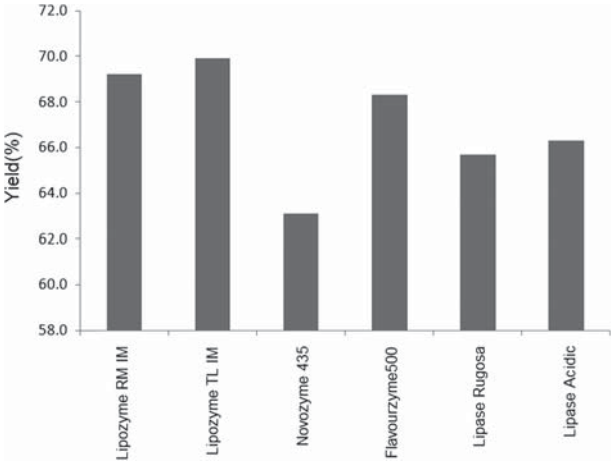


Figure 4. Effect of different types of lipase enzymes using mole ratio 1:1 and 50 mg of enzyme, at temperature 40°C and reaction time 48 h, each point represents the mean value ± SD from three independent experiments, RSD was less than 2.0%.

the optimum (Figure 5). Lipozyme TL IM is considered a good catalyst in organic reactions due to its high stability in organic media and ability to accept a great variety of substrates (Abd Rahman et al. 2011; Adnani et al. 2010).

Effect of reaction time and temperature. The effect of temperature and time on amide formation at 30, 40 and 50°C, and 24–96 h is shown in Figure 6. At 30°C, the lowest yield (26%) was obtained after 96 h. The yield obtained at 40°C was lower than that obtained at 50°C at 24 h (51.5%, 55.1, respectively).

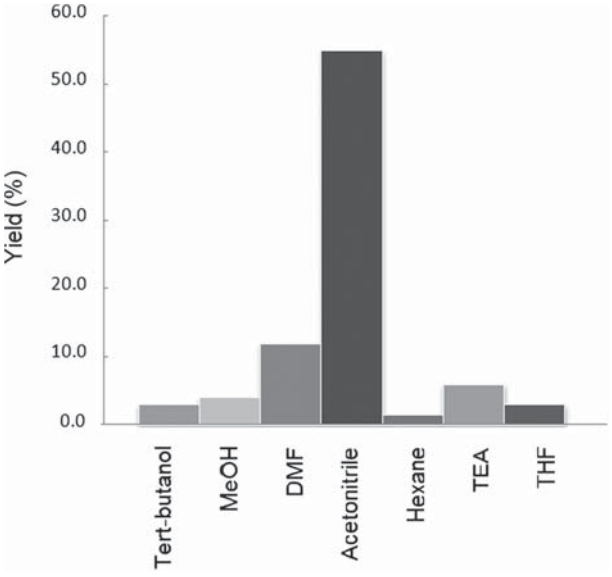


Figure 5. Effect of various organic solvents using a mole ratio 1:1 and 50 mg Lipozyme TL IM, at 40°C and reaction time 96 h; each point represents the mean value ± SD from three independent experiments, RSD was less than 2.0%.

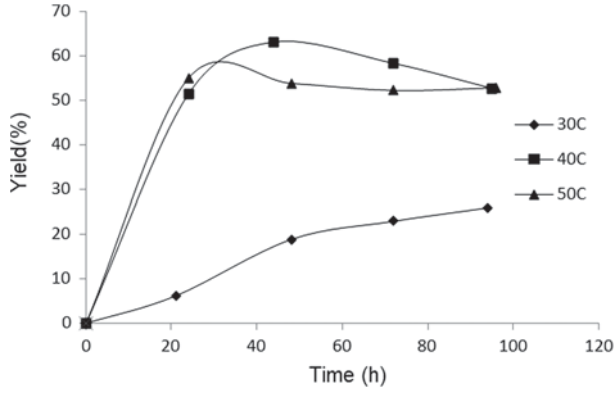


Figure 6. Effect of various temperatures (30, 40 and 50°C) and times (24–96 h) using a mole ratio 1:1 and 50 mg Lipozyme TL IM, each point represents the mean value ± SD from three independent experiments, RSD was less than 2.0%.

However, after extending the reaction time to 48 h, the yield at 40°C was higher than 50°C, (63.1%, 53.9% respectively). After that time the relative yield decreased slightly, which may be due to denaturation of the lipase. Therefore, the highest yield was obtained when the process was carried out for 48 h at 40°C

Effect of enzyme amount. Figure 7 illustrates the results of using different amounts of Lipozyme TL IM from 50 to 500 mg. The yield increased marginally with an increase in enzyme amount. The highest yield of amide (73.1%) was obtained when 250 mg of Lipozyme TL IM was used. This limit may due to substrate and mass transfer limitation (Reyes-Duarte et al. 2002).

Effect of molar ratio. The effect of mole ratio of substrates on amide formation reaction is shown in Figure 8. Increasing the amount of cinnamic acid

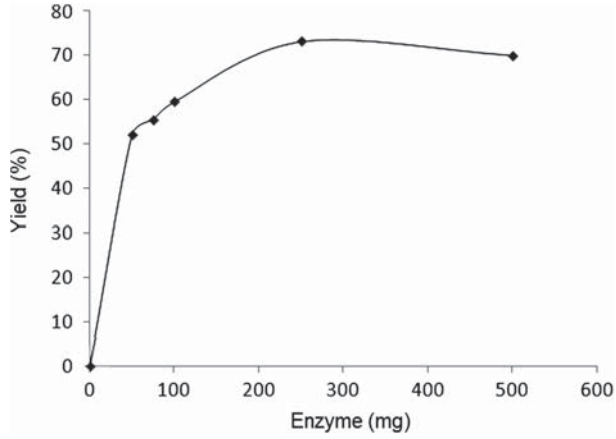


Figure 7. Effect of different quantities of Lipozyme TL IM from 50 to 500 mg using a mole ratio of 1:1 at 40°C and reaction time 48 h; each point represents the mean value ± SD from three independent experiments, RSD was less than 2.0%.

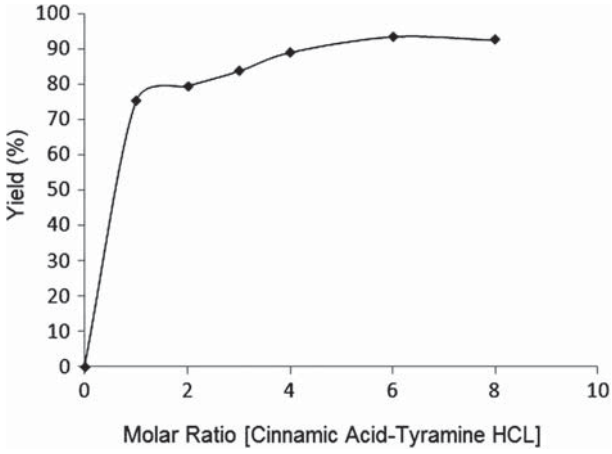


Figure 8. Effect of molar ratio of cinnamic acid:tyramine HCl (1:1, 2:1, 3:1, 4:1, 5:1, 6:1 and 8:1) at 40°C, reaction time 48 h and 250 mg of lipozyme TL IM, each point represents the mean value ± SD from three independent experiments, RSD was less than 2.0%.

had the greatest effect, with a mole ratio of 6:1 (cinnamic acid:tyramine HCl) resulting in a higher yield of 93.5% when 250 mg of Lipozyme TL IM was used. However, increasing the mole ratio to >6:1 did not increase the yield.

Conclusion

Lipase catalysed synthesis of N-feruloyltyramine amide was successfully achieved in a one-step reaction. The optimum reaction conditions were as follows: temperature 40°C, 250 mg of Lipozyme TL IM, molar ratio of 6:1 for cinnamic acid:tyramine HCl, and reaction time 48 h. The optimized percentage yield obtained was 93.5%. A precise and sensitive HPLC method has been developed and applied; it involved simple sample preparation and provides short analyte peak retention times, which make it suitable for the quantitative determination of N-feruloyltyramine amide. Design of products and processes for this reaction minimizes the use of hazardous substances which complies with green chemistry in seeking to reduce pollution.

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