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Biocatalytic synthesis of palmitoyl vanillylamide in supercritical carbon dioxide through amidation of vanillylamine hydrochloride and palmitic anhydride by lipase

Kuan-Ju Liu [a,*], Kuan-Miao Liu [b], Hung-Min Chang [c]

[a] Department of Food Science, National Penghu University, Makung, Penghu 88046, Taiwan
[b] Department of Applied Chemistry, Chung Shan Medical University, Taichung 40201, Taiwan
[c] Graduate Institute of Food Science and Technology, National Taiwan University, Taipei 10617, Taiwan

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Abstract

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is the main pungent component in capsicum fruits. Its analog, palmitoyl vanillylamide, with similar properties, was synthesized through amidation by lipase in supercritical carbon dioxide (SC–CO_2) and reaction conditions were optimized. Among five lipases tested, immobilized *Mucor miehei* lipase, Lipozyme IM, was most effective in synthesizing this analog. The reaction conditions for analog synthesis were optimized and were 50 °C, 17 MPa and pH 8 for 23 h using vanillylamine hydrochloride and palmitic anhydride at a molar ratio of 5/15 as substrates catalyzed by Lipozyem IM at a concentration of 0.5% (w/w). The residual enzyme activity was about 40% and 15% after a 46- and 69-h repeated amidation reaction in a batch reaction under optimized conditions, suggesting further modification of conditions was required.

Keywords: Palmitoyl vanillylamide; Lipase; Biocatalytic synthesis; Supercritical carbon dioxide; Amidation

1. Introduction

Red pepper is one of common spices used as a flavoring agent to stimulate appetites. Capsaicin, an amide compound in seeds of red pepper, provides the hot taste, boosts the burning of fat, invigorates the metabolism, and promotes blood circulation (Suzuki & Iwai, 1984; Vass, Brechtelsbauer, Nuttall, & Miller, 1996). Recent reports indicate that capsaicin and its analogs possess some extra bio-functions including anti-bacterial, anti-inflammatory analgesic, anti-nociceptive, and anti-oxidization (Buck & Burks, 1986; Chowdhury, Mukhopadhyay, Bhattacharayay, & De, 1996; Wrigglesworth et al., 1996). The low content of capsaicin in red pepper and the lengthy extraction and purification procedures for this pungent component heats up the development of capsaicin and palmitoyl vanillylamide synthesis.

Chemical synthesis of palmitoyl vanillylamide suffers from some major drawbacks such low efficiency, low quality, unfavorable colour, and high reaction temperature (100–200 °C) (Kaga et al., 1996; Surh & Lee, 1995). Some trials on enzymatic synthesis from vanillylamine and fatty acid derivatives in a two-phase system (water and organic solvent) still meet the problems of low yield (10–40%) and long reaction time (~72 h) (Kobata et al., 1998a; Kobata, Toyoshima, Kawamura, & Watanabe, 1998b). Carbon dioxide has many advantages over conventional organic solvents as a reaction medium such as non-toxicity, non-flammability, low cost, low viscosity, high diffusion, and easy separation from reaction mixture, which makes it the focus of transesterification of triacylglycerols (Erickson, Schyns, & Cooney, 1990; Miller, Blanch, & Prausnitz, 1991), the esterification of various alcohols (Marty, Chulalaksananukul, Willemot, & Condoret, 1992a; Vermue,

* Corresponding author. Tel.: +886 6 926 4115x3803; fax: +886 6 926 0259.
E-mail address: kjliu@npu.edu.tw (K.-J. Liu).

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Tramper, De Jong, & Oostrom, 1992; Rantakyla & Aaltonen, 1994), and intereterification of oils for cocoa butter analog preparation (Liu, Cheng, Chang, & Shaw, 1997).

In the present study, to improve the processes, lipase was used to conduct the biocatalyst, performing the amidation of vanillylamine hydrochloride with palmitic anhydride in supercritical carbon dioxide (SC–CO_2), and parameters affecting enzyme activity such as reaction time, reaction temperature, and reaction pressure were examined. Besides, molar ratio of substrates, acyl donor source, pH memory or water content in reaction mixture versus product formation was also investigated. Finally, residual enzyme activity after repeated operations was assayed to evaluate the practicality of the developed method.

2. Materials and methods

2.1. Materials

Carbon dioxide with a purity of about 99.99% was purchased from a local gas supplier (Yun-Shan-Hang Co., Tainan, Taiwan). Among the five commercial lipases used, *Pseudomonas cepacia* lipase (Amano PS) and *Penicillium camembertii* lipase (Amano G) were purchased from Amano International Enzyme Co. (Nagoya, Japan), *Candida antarctica* lipase (Novozyme 435) and *Mucor miehei* lipase (Lipozyme IM) were products of Novo Nordisk Inc. (Danbury, CT, USA); *Candida cylindracea* lipase was from Sigma Chemical Co. (St. Louis, MO, USA). Vanillylamine hydrochloride, palmitic anhydride, and palmitoyl vanillylamide were the products of Sigma Chemical Co. Novozyme 435 and Lipozyme IM were immobilized enzymes. All chemicals used were of reagent grade.

2.2. Optimization of palmitoyl vanillylamide synthesis in a SC–CO_2 system

Palmitoyl vanillylamide synthesis was carried out in a 40 mL-high pressure reactor (batch type) equipped with a magnetic stirrer, temperature and pressure reading devices (Liu, Chang, & Liu, 2007). First, 40 mL reaction mixture of 5 mM (0.0375 g) vanillylamine hydrochloride and 3 mM (0.0308 g) acyl donor were introduced in the reactor, followed by the dissolution of 200 mg lipase. Then, CO_2 was pumped into the reactor and the amidation reaction was conducted at 17 MPa, 50 °C for 23 h with a fixed agitation speed of 250 rpm.

To optimize palmitoyl vanillylamide formation, experiments were conducted to study factors such as enzyme source, reaction time (3–48 h), reaction temperature (35–60 °C), reaction pressure (10, 17, and 24 MPa), acyl donor source, molar ratio (5/25–25/5) of vanillylamine hydrochloride to palmitic anhydride, enzyme concentration (0.5–1.5%, w/w), water content (0–25%) of the reaction mixture, and pH value (pH 3–10), affecting the palmitoyl vanillylamide synthesis. Effects of lipase source, acyl donor source and molar ratio of vanillylamine hydrochloride to palmitic anhydride on palmitoyl vanillylamide production were studied under the same conditions as described above. Effects of reaction time, temperature and pressure on palmitoyl vanillylamide formation were investigated by varying each factor to the experimental conditions defined above. Factors of enzyme concentration, water content, and pH value of the reaction mixture on palmitoyl vanillylamide formation were conducted using 5 mM (0.0375 g) vanillylamine hydrochloride and 15 mM (0.297 g) palmitic anhydride as substrates under the same conditions. To study the pH effect on analog synthesis, lipase was dissolved in 10 mM mixed Good's buffer solution containing 10 mM each of *N*,*N*–bis (2-hydroxyethyl)-glycine, 3-(cyclohexylamino)-1-propane-sulfonic acid, sodium acetate, and 1,3-bis[tris(hydroxymethyl)-methylamino]propane, followed by pH adjustment to 3–10 by adding concentrated HCl or 2 N NaOH solution and lyophilization by a Savant Speed Vac Concentrator (Liu et al., 1997). Residual enzyme activity was assayed under the same experimental conditions after 1-3 cycles of operations to evaluate the conditions developed. Lipases were previously lyophilized by a Savant Speed Vac Concentrator (Savant Instruments, Inc., Farmingdale, NY, USA) under 50 millitorrs for 24 h before use.

2.3. GC analysis

After reaction over the desired time, depressurization and elution by methanol was conducted to remove lipase by centrifugation (2166*g*, 25 °C, 5 min). The so obtained methanol layer was concentrated using a rotary evaporator (R200A, Büchi, Flawil, Switzerland) (30 °C, 80 rpm) at a reduced pressure of 50 millitorrs to obtain the dried solid. Subsequently, 5 mg of the obtained dried solid were dissolved homogenously in 1.0 mL of methanol, followed by gas chromatographic (GC) analysis as described below.

Methanol solution (1.0 μL) of the reaction mixture was sampled and analyzed using a GC (Hitachi model G-3000; Hitachi, Tokyo, Japan). Experimental conditions were as follows: column, Rtx®-65TG (Restek Corporation, Bellefonte, PA) fused-silica capillary column (length, 30; inner diamete, 0.25 mm); carrier gas, N_2; flow rate, 1.0 mL/min; injection volume, 20 μL; injection port temperature, 310 °C; flame-ionization detector temperature, 330 °C; column temperature, 175 °C/1 min, 20 °C/min to 200 °C, 20 °C/min to 320 °C, 320 °C/9 min. The peaks obtained in the chromatograph were characterized and quantified by injecting known amounts (1.8, 3.5, and 5.3 μg/mL) of palmitoyl vanillylamide mixed with 1 μL of palmitanilide (internal standard). The calibration curve of peak area and the quantity of palmitoyl vanillylamide were linearly related ($r^2 = 0.986$). Triple samples were each analyzed twice.

2.4. Lipase activity assay

Lipase hydrolytic activity was measured according to the method described by R'ua, Maurion, Fernande, Otero,

and Ballesteros (1993) using *p*-nitrophenyl butyzate as substrate. One unit of enzyme was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute. Triple samples were each analyzed twice.

3. Results and discussion

3.1. Enzyme selection

Amidation reaction was carried out with lipases from different sources, in free form or immobilized form to produce palmitoyl vanillylamide. The use of immobilized lipases is becoming important since enzymes in immobilized form have been reported to be more stable against pressure (Calvo, Romero, Alba, Ortiz, & Gutierrez, 2002) and temperature than free ones (Knez & Habulin, 2002), and consequently, is more suitable for continuous flow operation.

Among the five commercial lipases (200 mg) tested in a batch reactor at 17 MPa and 50 °C in a SC–CO_2 system using 40 mL mixture of 5 mM vanillylamine hydrochloride and 3 mM palmitic anhydride as substrate, Lipozyme IM showed most remarkable production (324 μM) of palmitoyl vanillylamide, followed by *C. cylindracea* type VII (250 μM), Amano G (39.6 μM), Novozyme 435 (28.9 μM) and Amano PS (21.1 μM) (Table 1). Difference in yield could be due to the different enzyme intrinsic specificity toward substrates. Therefore, Lipozyme IM was used in the following experiments for optimization of formation of palmitoyl vanillylamide.

3.2. Effects of reaction time, temperature, and pressure on palmitoyl vanillylamide production

Amidation was carried out for 3, 13, 23, 34, and 48 h and the product was quantified to be about 170, 250, 324, 351, and 334 μM, respectively (Table 2). It was obvious that the amount of product increased with increasing reaction time to 34 h and then declined (34–48 h) significantly ($p < 0.05$) due to possible reverse hydrolysis reaction. Since the increase in product amount from 23 to 34 h was only about 27 μM, amidation reaction was terminated at 23 h for the sake of economy of the process and enzyme stability. Mutua and Akoh (1993) reported that the yield of alkyl glycoside fatty acid esters was poor when the reaction catalyzed by *Candida* sp. lipase in a non-aqueous system was lasted for a reaction time longer than 48 h.

Lipozyme IM-catalyzed amidation was conducted between 35 and 60 °C and the level of product was compared. Obviously, increase in reaction temperature resulted in higher product formation, from 237 μM at 35 °C to 341 μM at 60 °C. In SC–CO_2, increase in temperature leads to the increase in substrate solubility (Chrastill, 1982) and decrease in density and viscosity of SC–CO_2 system, and apparently, is favourable for the mass transfer rate of substrates and products in the reaction system. However, increase in reaction temperature adversely affects enzyme stability. Nag (1988) pointed out that high temperature might lead to changes in enzyme conformation and free energy of the reaction system, and thus, might affect enzyme-substrate binding capacity and the yield of product. In addition, temperature also affects the partition of substrates between the SC–CO_2 phase and the enzyme phase (Shishikura, Fujimoto, Suzuki, & Arai, 1994). Based on the above reasons and the fact that only a slight difference (about 17 μM) in product level produced between 50 and 60 °C, the reaction temperature of 50 °C was chosen as the optimal temperature for further reactions to save energy and avoid possible enzyme denaturation during long operation for industrial applications.

To understand the effect of pressure on supercritical fluids, pressure of 10–24 MPa was applied to the reactor and the product was quantified. As shown in Table 2, a signif-

Table 1
Palmitoyl vanillylamide formation by lipases from different sources[a]

Lipase source	Enzyme (trade name or brand)	Palmitoyl vanillylamide (μM)
Candida antarctica	Novozyme 435[b]	28.9 ± 2.2
Mucor miehei	Lipozyme IM[b]	324.2 ± 2.0
Pseudomonas cepacia	Amano PS	21.1 ± 1.5
Penicillium camembertii	Amano G	39.6 ± 1.4
Candida cylindracea type VII	Sigma	250.2 ± 2.1

Triplicate data from separate experiments are expressed as mean ± SEM.
[a] Lipase (200 mg) was dissolved to 40 mL reaction mixture of 5 mM vanillylamine hydrochloride and 3 mM palmitic anhydride to conduct the amidation reaction at 17 MPa and 50 °C for 23 h in SC–CO_2.
[b] Immobilized enzyme.

Table 2
Formation of palmitoyl vanillylamide as affected by reaction time, temperature and pressure[A]

Reaction time (h)	Palmitoyl vanillylamide (μM)
3	169.6 ± 2.1[a]
13	250.3 ± 1.8[b]
23	324.2 ± 1.5[c]
34	350.9 ± 1.6[d]
48	333.8 ± 1.7[e]
Reaction temperature (°C)	
35	237.2 ± 1.6[d]
40	278.8 ± 2.0[c]
50	324.2 ± 1.5[b]
60	341.1 ± 1.2[a]
Reaction pressure (MPa)	
10	150.3 ± 2.2[b]
17	324.2 ± 1.5[a]
24	114.1 ± 0.7[c]

Triplicate data from separate experiments are expressed as mean ± SEM. Mean values in the same column for the same sample with different letters are significantly different ($p < 0.05$).
[A] Lipozyme IM (200 mg) was dissolved to a 40 mL reaction mixture of 5 mM vanillylamine hydrochloride and 3 mM palmitic anhydride to conduct amidation for various reaction periods of time (17 MPa, 50 °C), at various pressures (50 °C, 23 h) or at various temperatures (17 MPa, 23 h) in SC–CO_2.

icant ($p < 0.05$) increase in product amount was detected when pressure increased from 10 MPa (150 μM) to 17 MPa (324 μM), while a sharp decrease in production was detected at a higher pressure of 24 PMa. This could be due to the pressure-induced denaturation of enzyme as a result of the conformational changes (Nakamura, 1990), although increase in pressure increased the substrate solubility at a certain reaction temperature (Chrastill, 1982) and facilitated the reaction rate while decreasing partition of substrates between the immobilized enzyme and supercritical solvent phases. Similarly, Erickson et al. (1990) reported that the rates of acidolysis and esterification decreased with an increase in the reaction pressure at 55 °C in SC–CO_2 or supercritical ethane system due to the decrease in partition of reactants between the supercritical solvent phase and the immobilized enzyme phase. In addition, the increase in pressure also results in a decrease in the diffusion due to the increases in density and viscosity of SC–CO_2 (Perrut, 1992). Apparently, reaction pressure at 17 MPa was optimal for the amidation reaction in the present study (Table 2) conducted at 50 °C for 23 h.

3.3. Acyl donor

Various palmitoyl samples at a level of 3 mM were reacted for 23 h with 5 mM vanillylamine hydrochloride in a SC–CO_2 reactor at 17 MPa and 50 °C, and the product of palmitoyl vanillylamide was quantified (Table 3). Apparently, palmitic anhydride performed superior amidation reaction to any other palmitoyl sample, forming 324 μM product, followed by tripalmitin (168 μM), ethyl palmitate (145 μM), methyl palmitate (145 μM), and palmitic acid (84 μM). Present results were better than that (5%) reported by Kobata et al. (1999) using myristic acid and fatty acid myristic ester as acyl donors catalyzed by Lipase QL in *n*-hexane for capsaicin production. Liu et al. (1997) found similar trends in SC–CO_2 system using different acyl donors for lipase-catalyzed interesterification. Therefore, palmitic anhydride was confirmed to be most suitable for the synthesis of palmitoyl vanillylamide.

Table 3
Effect of acyl donor source on the palmitoyl vanillylamide synthesis[A]

Acyl donor	Palmitoyl anillylamide (μM)
Palmitic acid	83.8 ± 0.6^{e}
Palmitic anhydride	324.2 ± 1.5^{a}
Tripalmitin	167.7 ± 1.8^{b}
Ethyl palmitate	145.1 ± 2.3^{c}
Methyl palmitate	113.1 ± 1.5^{d}

Triplicate data from separate experiments are expressed as mean ± SEM. Mean values in the same column for the same sample with different letters are significantly different ($p < 0.05$).

[A] Lipozyme IM (200 mg) was dissolved to a 40 mL reaction mixture of 5 mM vanillylamine hydrochloride and 3 mM acyl donor to conduct amidation at 17 MPa and 50 °C for 23 h in SC–CO_2.

3.4. Molar ratio of substrate and enzyme concentration

To optimize the effect of molar ratio (5/25–5/1) of vanillylamine hydrochloride to palmitic anhydride on palmitoyl vanillylamide formation, 5 mM vanillylamine hydrochloride and 25-1 mM palmitic anhydride were applied to the reactor and the amidation reaction was carried out at 17 MPa and 50 °C for 23 h (Table 4). The yield of palmitoyl vanillylamide reached 588 and 574 μM when the ratio of vanillylamine hydrochloride/palmitic anhydride was 5/25 and 5/15, respectively. Apparently, at a level of 5 μM vanillylamine hydrochloride, decrease in vanillylamine hydrochloride/palmitic anhydride ratio was favourable for the amidation reaction (Rupley, Gratton, & Carei, 1983). However, in an attempt to save the palmitic anhydride cost and the fact that there is only a slight difference (about 14 μM) in product quantity, molar ratio of vanillylamine hydrochloride to palmitic anhydride was optimized to be 5/15. Therefore, in the following experiments 5 mM vanillylamine hydrochloride and 15 mM palmitic anhydride were used as substrates to perform amidation in SC–CO_2. Of note, only free amine is reacting to lipase during amide synthesis, protection of amine group may be required when reacts under anhydrous organic solvent conditions (Halling, 1994; Klibanov, 1989). In the present study, vanillylamine substrate is in a HCl salt from to protect the amine group.

Concentration of enzyme influences largely the reaction rate and the product quantity. In the present study, 0.5, 1.0, and 1.5% of immobilized lipase was dissolved in the reactor and 574, 602, and 711 μM of product were obtained, respectively, using 5 mM vanillylamine hydrochloride and 15 mM palmitic anhydride as substrates to perform amidation at 50 °C and 17 MPa for 23 h (Table 5). Interestingly, a non-linear dependence of enzyme level on product quantity was observed (Table 5), implying a significant mass-transfer limitation and/or a limited enzyme activity in a SC–CO_2 reactor (Marty, Chulalaksa-

Table 4
Effect of molar ratio of vanillylamine hydrochloride to palmitic anhydride on palmitoyl vanillylamide synthesis[A]

Vanillylamine hydrochloride/ Palmitic anhydride[B]	Palmitoyl anillylamide (μM)
5/25	588.1 ± 1.7^{a}
5/15	574.4 ± 1.6^{b}
5/10	398.5 ± 1.6^{c}
5/5.0	348.4 ± 1.7^{d}
5/2.5	297.6 ± 2.1^{e}
5/1.5	269.3 ± 1.5^{f}
5/1.0	217.8 ± 1.5^{g}

Triplicate data from separate experiments are expressed as mean ± SEM. Mean values in the same column for the same sample with different letters are significantly different ($p < 0.05$).

[A] Lipozyme IM (200 mg) was dissolved to a 40 mL reaction mixture of 5 mM vanillylamine hydrochloride and various levels of palmitic anhydride in SC–CO_2 at 17 MPa and 50 °C for 23 h.

[B] Molar ratio of vanillylamine hydrochloride to palmitic anhydride.

Table 5
Effect of enzyme level to the formation of palmitoyl vanillylamide[A]

Enzyme concentration (%, w/w)	Palmitoyl vanillylamide (μM)
0.5	574.4 ± 1.6^{c}
1.0	602.2 ± 1.8^{b}
1.5	710.9 ± 1.9^{a}

Triplicate data from separate experiments are expressed as mean ± SEM. Mean values in the same column for the same sample with different letters are significantly different ($p < 0.05$).

[A] Various concentrations of enzyme were added to a 40 mL reaction mixture containing 5 mM vanillylamine hydrochloride and 15 mM palmitic anhydride in SC–CO_2 at 17 MPa and 50 °C for 23 h.

nanukul, Willemot, & Condoret, 1992b). For economic reasons, 0.5% (w/w) (200 mg) of enzyme in the reaction mixture was adopted in the following experiments.

3.5. Water content and pH

Water content in the reaction mixture plays an important role in that it influences enzyme conformation (Rupley et al., 1983) and stability (Halling, 1994; Klibanov, 1989). Besides, water also forms a hydrophilic hindrance against hydrophobic substrates and inhibits substrate access to the enzyme (Marty et al., 1992b). In a recent report on the influence of thermodynamic water activity, a_w, on a lipase-catalyzed amidation, Liu, Nag, and Shaw (2001) indicated that the primary effects of a_w included modulation of the equilibrium between hydrolysis and synthetic processes (Dudal & Lortie, 1995; Svensson, Wehtje, Adlercreutz, & Mattiasson, 1994) as well as the hydration state of the enzyme. The latter regulates plasticity and catalytic activity of the enzyme (Klibanov, 1997).

The yield of product versus water content (0–25%) in the reaction mixture was investigated under the experimental conditions of 50 °C and 17 MPa for 23 h. As shown in Fig. 1, palmitoyl vanillylamide formation was reduced with increasing water content in the reaction mixture, implying that the presence of moisture was unfavourable for product formation. This observation is inconsistent with the results reported by Condoret, Vankan, Joullia, and Marty (1997) who indicated that the optimum water content for Lipozyme IM was between 8% and 12%. This could be partly due to the increased mass transfer resistance of reactants in the present study and partly to the observed agglomeration of enzyme and hence reduction of reactive enzyme surface area for the present amidation reaction.

Enzyme shows the same optimum reaction pH value in organic solvent as it does in an aqueous buffer. Lipase in buffer solution at various pH (pH 3–10) was lyophilized (pH memory) (Klibanov, 1989) prior to addition to SC–CO_2 in order to investigate the pH effect on amidation assisted by Lipozyme IM in SC–CO_2. Obviously, yield (570–660 μM) of palmitoyl vanillylamine increased rapidly when amidation reaction was conducted at pH 7, 8, and 9, while it declined to about 370 μM at pH 10 (Fig. 2). Similar results were observed by Liu et al. (1997) who used lipase to synthesize cocoa butter equivalent in SC–CO_2. The "pH memory" affected the enzyme activity in SC–CO_2 by influencing the enzyme-substrate complex formation during amidation.

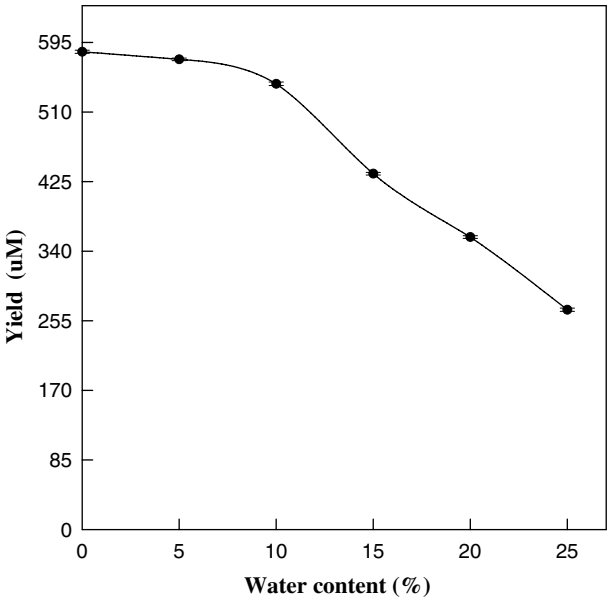

Fig. 1. Effect of water content on the synthesis of palmitoyl vanillylamide by Lipozyme IM. Various amounts of water was added to a 40 mL mixture of immobilized lipase (200 mg)-5 mM vanillylamine hydrochloride-15 mM palmitic anhydride to conduct amidation reaction at 17 MPa and 50 °C for 23 h in SC–CO_2. Each value is the average of three determinations.

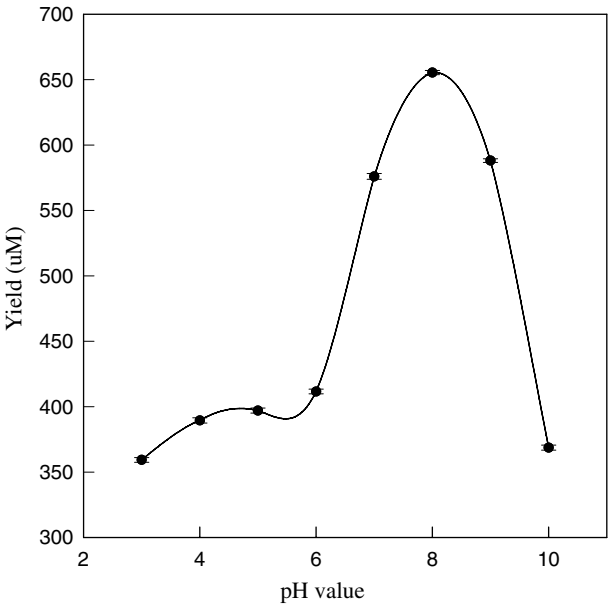

Fig. 2. Dependence of palmitoyl vanillylamide formation on pH value. Lipozyme IM (200 mg) was added to a 40 mL reaction mixture of 5 mM vanillylamine hydrochloride and 15 mM palmitic anhydride to form palmitoyl vanillylamide at 17 MPa and 50 °C for 23 h in SC–CO_2. Each value is the average of three determinations.

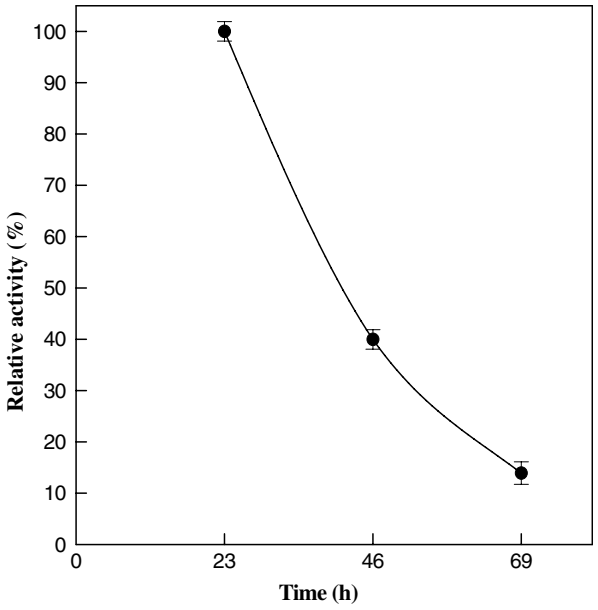

Fig. 3. Change in relative stability of Lipozyme IM in repeated catalysis to produce palmitoyl vanillylamide. Lipozyme IM (200 mg) was added to a 40 mL reaction mixture of 5 mM vanillylamine hydrochloride and 15 mM palmitic anhydride to conduct amidation at 17 MPa and 50 °C for 23 h in SC–CO_2. Reaction time for each reaction cycle was 23 h. Each value is the average of three determinations.

3.6. Stability of Lipozyme IM in repeated operations

For industry, it is important to reduce the running cost especially that for enzyme, thus assay of residual enzyme activity after each operation appears to be essential. It was found that enzyme retained almost the same activity after the first amidation reaction (Fig. 3). However, it lost about 60% and 85% of its activity after the second and third reactions, respectively, at 17 MPa and 50 °C. Loss of about 66% enzyme activity after a 72 h-operation was also reported by Yesiloglu (2004) when using immobilized porcine pancreas lipase in catalyzing ethanolysis of sunflower oil.

4. Conclusions

This work was devoted to explore the feasibility of synthesizing palmitoyl vanillylamide by enzymatic catalysis in SC–CO_2 and the optimized conditions were as follows: 200 mg Lipozyme IM in 40 mL reaction mixture of 5 mM vanillylamine hydrochloride and 15 mM palmitic anhydride at pH 8, 17 MPa, 50 °C for 23 h. The conversion of substrates into product decreased exponentially when water was present in the reaction mixture. Thus, optimized amidation conditions might be too severe for the present reaction. Sacrificing the yield by reducing the reaction temperature and/or pressure might be beneficial for the stability of enzyme for repeated amidation. Thus, improvement of the established experimental conditions is needed.

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