Journal of Molecular Catalysis B: Enzymatic 58 (2009) 181–186

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Lipase-catalyzed synthesis and antibacterial activity of N-vanillylnonanamide

Kuan-Ju Liu*

Department of Food Science, National Penghu University, Makung, Penghu 88046, Taiwan

ARTICLE INFO

Article history:
Received 9 June 2008
Received in revised form 18 December 2008
Accepted 18 December 2008
Available online 27 December 2008

Keywords:
N-vanillylnonanamide
Lipase
Antibacterial activity
Supercritical carbon dioxide
Amidation

ABSTRACT

N-vanillylnonanamide (VAN) was successfully synthesized from vanillylamine hydrochloride by enzymatic catalysis in supercritical carbon dioxide (SC–CO_2). Five commercial lipases, Novozyme 435, Lipozyme IM, Amano PS, Amano G and Sigma *Candida cylindracea* type VII, as biocatalysts for VAN synthesis were compared. Lipozyme IM exhibited best yields of tested lipases. Various parameters such as time, temperature, pressure and vanillylamine hydrochloride/nonanoic anhydride ratio that influenced the reaction were investigated. Nonanoic anhydride showed the best acyl donor of the employed substrates. An amidation yield of 40% was obtained when nonanoic anhydride and Lipozyme IM were used at 170 bar and 50 °C for 23 h in SC–CO_2. Besides, addition of 2 mM divalent salts ($CuCl_2$ and $ZnCl_2$) significantly increased 11–23% yield of the VAN. The enzyme operational stability suggested that Lipozyme IM maintained over 50 °C of the initial activity for the synthesis of VAN after reuse for 69 h. Furthermore, in vitro, VAN behaved as a potential antibacterial against *Escherichia coli*.

1. Introduction

Capsaicin is the major pungent component in a variety of capsicum fruits such as hot green and red peppers, and thus, represents an important ingredient in the majority of spicy foods [1]. It is widely consumed as a food additive throughout the world, particularly in Mexico, China, and Japan. Capsaicin is also typically used to treat various diseases, such as rheumatoid arthritis, osteoarthritis, diabetic neuropathy, and postherpetic neuralgia [2]. N-vanillylnonanamide (VAN), or so-called synthetic capsaicin, is a capsaicin substitute which has a similar chemical structure and similar pharmacological effects as capsaicin [3]. In recent years, specialists have found that capsaicin, VAN, and capsaicin analogs have many concurrent functions, such as antibacterial, anti-inflammatory, analgesic, anti-nociceptive and anti-oxidization functions [4–6]; therefore, they are widely applied to food and pharmaceutical industries. Various capsaicin analogs have been discovered in the Capsicum family of plants [7] and more analogs have been synthesized chemically in order to research their capacity for various physiological and biological activities [8]. In industry, capsaicin, VAN, and capsaicin analogs are produced from fatty acid chlorides and amines at temperatures of 140–170 °C under moderate pressure [8]. Enzyme-catalyzed synthesis has many advantages compared to chemical synthesis because it is done under mild conditions, without toxic reagents, and with specificity to the substrate. Although some analogs have been enzymatically synthesized from vanillylamine and fatty acid derivatives in a two-phase system, only low yields were obtained; the amide yield was 10–40% at 70 °C for 72 h [9]. Thus, in order to substantially improve the yield and conform to the contemporary requirements for environmental sensation and protection, different approaches, using enzyme as biocatalyst and changes of reaction environment, for unique and desired chemicals have been initiated. Supercritical carbon dioxide with many advantages over conventional organic solvents as reaction media due to non-toxic, non-flammable, low cost, easy separation and purification, low viscosity and high diffusivity, is reasonably selected as another alternative solvents for yield enhancement [10,11].

To date, enzymatic catalysis in SC–CO_2 has been restricted to a few applications mainly involving hydrophobic substrates, while lipases are used almost exclusively either for the transesterification of triglycerides [10,11] or the esterification of various alcohols [12]. The resolution of racemic compounds has also been described [13]. Besides, it has been reported that VAN can inhibit bacterial growth [4,14], which makes it was regarded as an antibacterial agent. In the present study, the amidation of vanillylamine hydrochloride with nonanoic anhydride was investigated in supercritical carbon dioxide (SC–CO_2) using Lipozyme IM as the biocatalyst. To examine the effects on the production of VAN, various parameters affecting enzyme activity, such as temperature, pressure, and metal salt, along with the influence of the amine/acid anhydride molar ratio, reaction time, and stability of the lipase and acyl donor have been

* Tel.: +886 6 926 4115x3803; fax: +886 6 926 0259.
E-mail address: kjliu@npu.edu.tw.

1381-1177/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2008.12.015

investigated. Moreover, the antibacterial activity of VAN is investigated using *Escherichia coli* as an index organism.

2. Experimental

2.1. Materials

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). Novozyme 435 and Lipozyme IM were immobilized enzymes. Vanillylamine hydrochloride, nonanoic anhydride, nonanoic acid, trinonanoin, and 8-methyl-N-vanillyl-6-nonenamide were the products of Sigma Chemical Co. All chemicals used were reagent grade. Carbon dioxide with a purity of approximately 99.99% was purchased from a local gas supplier (Yun-Shan-Hang Co., Tainan, Taiwan).

2.2. VAN synthesis in SC–CO_2 system

VAN synthesis was carried out in a 40-mL high pressure reactor (batch type) equipped with a magnetic stirrer, and temperature and pressure reading devices [15]. First, the 40 μL reaction mixture of 5 mM (0.0375 g) vanillylamine hydrochloride and 3 mM (0.0358 g, d = 0.893 g/mL) 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 170 bar, 50 °C for 23 h with a fixed agitation speed of 250 rpm. The effects of the enzyme concentration of the reaction mixture on VAN formation were investigated using 5 mM (0.0375 g) vanillylamine hydrochloride and 20 mM (0.239 g) nonanoic anhydride as substrates under the same conditions. To study the effect of the metal ions on analog synthesis, lipase was dissolved in 2 mM metal ion solution and lyophilization was performed by a Savant Speed Vac Concentrator (Savant Instruments Inc., Farmingdale, NY, USA) under 50 mTorr for 24 h before use [16]. Residual enzyme activity was assayed under the same experimental conditions after 1–4 cycles of operations to evaluate the conditions developed.

2.3. Bacterial cultures

E. coli CCRC 10675 obtained from the Food Industry Research and Development Institute, Department of Bioresources Collection and Research Center, Hsinchu, Taiwan, was employed as the test organism. *E. coli* was grown aerobically in 15 mL conical glass flasks containing 2 mL of nutrient broth (NB, Difco, Detroit, MI, USA) at 37 °C for 12 h. Aliquots (40 μL) of this culture were aseptically transferred to 4 mL of fresh medium and incubated at 37 °C to the mid logarithmic phase (absorbance ~0.6 at 660 nm). After two successive transfers of the test organism in nutrient broth at 37 °C for 12 h, the activated culture was again inoculated into 100 mL NB at 37 °C for 12 h. Cells were then harvested by centrifugation (8000 × g for 10 min), suspended in 10 mL of 0.85% NaCl to ca. 10^{10} cfu/mL, and used as the inoculum.

2.4. Determination of antibacterial activity of VAN

Antibacterial tests were carried out using the disc diffusion method [17]; 10 mL of suspension containing 10^{10} cfu/mL of bacteria was poured onto the LB agar plate. Broth cultures of bacteria were freshly prepared for each assay overnight (24 h, 37 °C). Agar plates (20 mL) were prepared, allowed to set, and then surface dried (37 °C, 30 min). Broth cultures were vortexed for 30 s and 500 μL was immediately removed and spread over the surface of the agar and till surface dried (37 °C, 15 min). Ten microlitres (2.69 mM) of VAN was pipetted onto a 6-mm sterile disc (Oxoid) and the disc was placed onto the surface of the prepared agar plate. Following 24 h incubation at 37 °C, the diameters of inhibition zone for each dilution were then recorded in mm (including the disc). For control plates, 10 μL of nanopure water was pipetted onto the disc. Assays were completed in triplicate and repeated independently three times. Assays were also repeated using a commercial antibiotic (ampicillin (10 μg)) for the positive controls.

2.5. GC analysis

After the reaction over the desired time, depressurization and elution by methanol were conducted to remove lipase by centrifugation (2166 × g, 25 °C, 5 min). The obtained methanol layer was concentrated using a rotary evaporator (R200A, Büchi, Flawil, Switzerland) (30 °C, 80 rpm) at a reduced pressure of 50 mTorr to obtain the dried solid. Subsequently, 5 mg of the obtained dried solid was dissolved homogenously in 1.0 mL of methanol, which was 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, USA); fused-silica capillary column (length, 30 m; inner diameter, 0.25 mm); carrier gas, N_2; flow rate, 1.0 mL/min; injection volume, 20 μL; injection port temperature, 280 °C; flame-ionization detector temperature, 300 °C; column temperature gradient, 175 °C/1 min, 20 °C/min to 200 °C, 20 °C/min to 310 °C, 310 °C/5 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 VAN mixed with 1 μL of palmitanilide (internal standard). The calibration curve of the peak area and the quantity of VAN were linearly related (r^2 = 0.986). Triple samples were each analyzed twice.

2.6. Lipase activity assay

Lipase hydrolytic activity was measured according to the method described by R'ua et al. [18] using *p*-nitrophenyl butyzate as the 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.

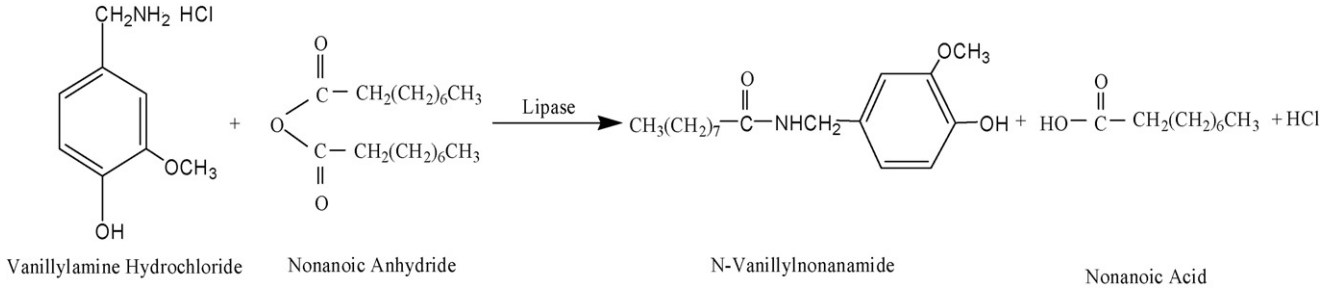
2.7. Statistical analysis

A variance analysis of results was carried out using the General Linear Model Procedure of SAS Statistical Software, Version 6.11 [19]. Lipase source, acyl donor, reaction time, temperature, pressure or vanillylamine hydrochloride/nonanoic anhydride ratio, and metal ions were each tested in triplicate.

3. Results and discussion

3.1. Selection of the enzyme

The amidation reaction was carried out with lipases from different sources, in free form or immobilized form to produce VAN (Scheme 1). The use of immobilized lipases is becoming more important since enzymes in immobilized form have been reported to be more stable against pressure and temperature than intact ones [20,21], and consequently, is more suitable for continuous flow operations. Among the five commercial lipases (200 mg) tested in a batch reactor at 170 bar and 50 °C in a SC–CO_2 system using 40 mL of a mixture of 5 mM vanillylamine hydrochloride and 3 mM



Scheme 1. Synthesis of N-vanillylnonanamide catalyzed by lipase in SC–CO_2.

nonanoic anhydride as substrates, Lipozyme IM had the best production (11.8%) of VAN, followed by *P. cepacia* (7.0%), Amano G (2.1%), Novozyme 435 (0.7%), and *C. cylindracea* type VII (0.2%) (Table 1). Differences in yield could be due to the intrinsic specificity of the different enzymes toward the substrates. Lipozyme IM was used in the following experiments for optimization of the formation of VAN.

3.2. Effect of type of acyl donor

Various nonanoyl samples at a level of 3 mM were reacted for 23 h with 5 mM vanillylamine hydrochloride in an SC–CO_2 reactor at 170 bar and 50 °C, and the product of VAN was quantified (Table 2). Nonanoic anhydride performed better in the amidation reaction than any other nonanoyl sample, forming 11.8% of product, followed by trinonanoin (1.1%), ethyl nonanoate (0.5%), nonanoic acid (0.4%), and methyl nonanoate (0.3%). These results were better than those (20%) reported by Kobata et al. [22] using myristic acid and fatty acid myristic ester as acyl donors, catalyzed by Lipase OF in n-hexane for capsaicin production. Liu et al. [16] found similar trends in the SC–CO_2 system using different acyl donors for lipase-catalyzed interesterification. Romero et al. [23] reported that acid anhydride performed a better yield than those of others, yielding 93% esterification in SC–CO_2 at 15 MPa and 313 K (1.5-fold increase with respect to acetic acid). Nonanoic anhydride was confirmed to be the most suitable for the synthesis of VAN.

Table 1
N-vanillylnonanamide (VAN) synthesized by lipases from various sources[a].

Lipase source	Trade name or brand	VAN yield (%)	Specific activity (U/mg protein)[b]
Candida antarctica	Novozyme 435	0.7 ± 0.03	36.3
Mucor miehei	Lipozyme IM	11.8 ± 0.07	34.0
Pseudomonas cepacia	Amano PS	7.0 ± 0.05	111
Penicillium camembertii	Amano G	2.1 ± 0.03	109
Candida cylindracea type VII	Sigma	0.2 ± 0.03	82.0

Triplicate data from separate experiments are expressed as mean ± SEM.

[a] Lipase (200 mg) was added to a 40 mL reaction mixture of 5 mM vanillylamine hydrochloride and 3 mM nonanoic anhydride in SC–CO_2 at 170 bar and 50 °C for 23 h.

[b] Specific activity was measured by hydrolyzing *p*-nitrophenyl butyrate as substrate. One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol per minute.

Table 2
Effect of acyl donor source on the N-vanillylnonanamide synthesis[a].

Acyl donor	VAN yield (%)
Nonanoic anhydride	11.8 ± 0.07
Nonanoic acid	0.4 ± 0.07
Trinonanoin	1.1 ± 0.05
Methyl nonaonate	0.3 ± 0.06
Ethyl nonaonate	0.5 ± 0.07

[a] The reaction conditions are exactly the same as Table 1 except acyl donor was substituted. Triplicate data from separate experiments are expressed as mean ± SEM.

3.3. Effects of reaction time, temperature, and pressure on VAN production

Amidation was carried out for 3, 23, 34, and 48 h and the product was quantified to be approximately 7.7, 11.8, 21.6, and 3.4%, respectively (Fig. 1). 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 a possible reverse hydrolysis reaction [24]. Since the increase in product amount from 23 to 34 h was approximately 9.8%, the amidation reaction was terminated at 34 h for the economy of the process and enzyme stability. Mutua and Akoh [24] 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 had a reaction time longer than 48 h. Lipozyme IM-catalyzed amidation was conducted between 35 and 60 °C and the amount of product was compared (Fig. 2). Obviously, increase in reaction temperature resulted in higher product formation, from 1.9% at 35 °C to 11.8% at 50 °C. However, our results showed that temperatures greater than 50 °C resulted in lower yield formation. This may be due to enzyme inactivation [25,26].

Knez et al. [25] has already reported that the immobilized *M. miehei* lipase activity increases between 33 and 50 °C for the esterification of oleic acid with oleyl alcohol in SC–CO_2; however, with further increases in temperature, thermal deactivation occurs. Additionally, Oliveira and Oliveira [27] reported that the Lipozyme IM optimum condition was found to be 51 °C, 146 bar, $R = 1{:}10$ (oil/ethanol molar ratio), and $W = 0$ (water concentration) with a predicted reaction conversion of 31.0% for the ethanolysis of palm

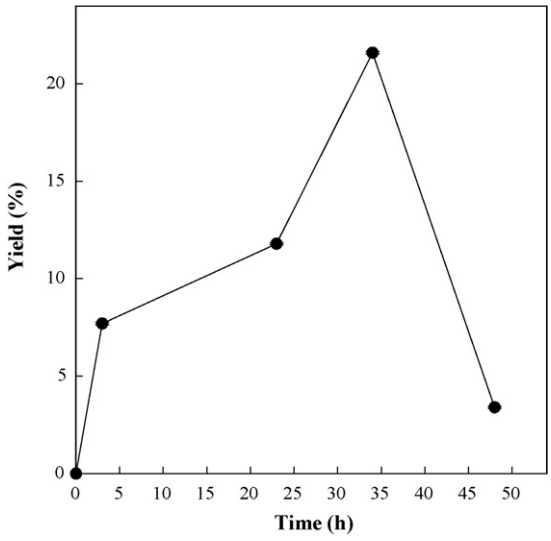


Fig. 1. Time course of the amidation between vanillylamine hydrochloride and nonanoic anhydride in SC–CO_2. Lipozyme IM (200 mg) was added to a reaction mixture (40 mL) containing 5 mM vanillylamine hydrochloride and 3 mM nonanoic anhydride at 170 bar and 50 °C for 48 h in SC–CO_2.

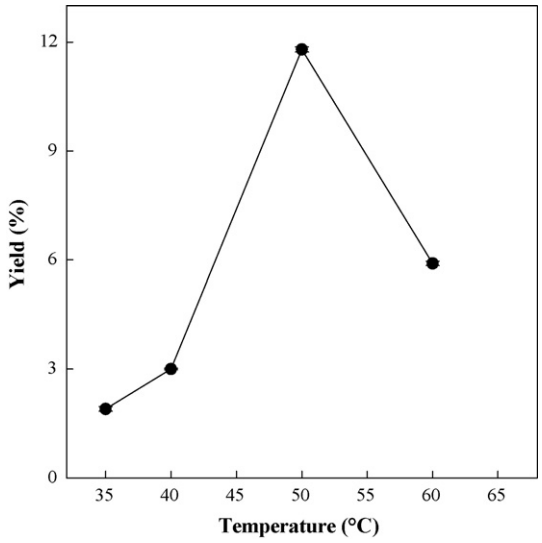


Fig. 2. Effect of temperature on the synthesis of VAN. Lipozyme IM (200 mg) was added to a reaction mixture (40 mL) containing 5 mM vanillylamine hydrochloride and 3 mM nonanoic anhydride in SC–CO_2 at various temperatures and 170 bar for 23 h.

kernel oil in SC–CO_2. Similar results were suggested by Liu et al. [26] for the enzymatic synthesis of palmitoyl vanillylamide at 170 bar in SC–CO_2. Optimal temperature was 50 °C.

In SC–CO_2, an increase in temperature leads to an increase in substrate solubility [28] and a decrease in density and viscosity of the SC–CO_2 system, and apparently, is favourable for the mass-transfer rate of substrates and products in the reaction system. However, an increase in reaction temperature adversely affects enzyme stability. Nag [29] 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 [30]. The reaction temperature of 50 °C was employed as the optimal temperature for further reactions studies.

To understand the effect of pressure on supercritical fluids, pressure of 100–240 bar was applied to the reactor and the product was quantified. As shown in Table 3, a significant ($p < 0.05$) increase in product amount was detected when pressure increased from 100 bar (8.2%) to 170 bar (11.8%), whereas a sharp decrease in production was detected at the higher pressure of 24 MPa. This result could be due to the pressure-induced denaturation of the enzyme due to conformational changes [31], although an increase in pressure increased the substrate solubility at a certain reaction temperature [24], and facilitated the reaction rate while decreasing the partition of substrates between the immobilized enzyme and supercritical solvent phases. Similarly, Erickson et al. [10] reported that the rates of acidolysis and esterification decreased with an increase in the reaction pressure at 55 °C in SC–CO_2 or the 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 [32].

Table 3
Formation of N-vanillylnonamide as affected by reaction pressure[a].

Reaction pressure (bar)	VAN yield (%)
100	8.2 ± 0.07
170	11.8 ± 0.07
240	7.1 ± 0.03

Triplicate data from separate experiments are expressed as mean ± SEM.
[a] The reaction conditions are exactly the same as Table 1.

Table 4
Formation of N-vanillylnonamide as affected by molar ratio of vanillylamine hydrochloride to nonanoic anhydride[a].

Vanillylamine hydrochloride/nonanoic anhydride[b]	VAN yield (%)
0.2	15.6 ± 0.07
0.25	40.0 ± 0.08
0.3	13.2 ± 0.07
0.5	13.1 ± 0.07
1.0	12.2 ± 0.08
2.0	21.3 ± 0.07
3.0	29.9 ± 0.07
5.0	10.2 ± 0.06

Triplicate data from separate experiments are expressed as mean ± SEM.
[a] The reaction conditions are exactly the same as Table 1.
[b] Molar ratio of vanillylamine hydrochloride to nonanoic anhydride.

Ikushima et al. [33] reported that SC–CO_2 can promote the activation of the enzyme and provokes drastic conformational changes of the enzyme, causing the movement of the surface groups and creating an active site producing stereoselective machinery. Furthermore, accounting for the low viscosity and high diffusivity of SC–CO_2, the substrates are easier to be transferred into pores of the immobilized carrier to contact with enzyme. The yield of VAN decreased while system pressures are elevated over 170 bar. The reason could attribute to increasing activation energy of the amidation reaction under higher system pressures. Hence, 170 bar was selected as the optimal operating pressure for the amidation reaction in the present study (Table 3) conducted at 50 °C for 23 h. Similar results were reported for lipase from *Rhizomucor miehei* (Lipozyme RM IM) [34]. Besides Knez et al. [25] also demonstrated that the immobilized *M. miehei* lipase (Lipozyme IM) was unstable at 200 bar and 70 °C in SC–CO_2. With higher pressure the conversion decreases slightly. Initial reaction rates increase at temperatures from 40 to 60 °C when the pressure is increased from 80 to 300 bar. However, they decrease with higher pressure, over 300 bar.

3.4. Effect of substrate ratio and enzyme concentration

To optimize the effect of the molar ratio (0.2–5.0) of vanillylamine hydrochloride to nonanoic anhydride on VAN formation, 5 mM of vanillylamine hydrochloride and 25∼1 mM nonanoic anhydride were introduced into the reactor and the amidation reaction was carried out at 170 bar and 50 °C for 23 h (Table 4). The yield of VAN reached 40 and 29.9% when the ratio of vanillylamine hydrochloride/nonanoic anhydride was 0.25 and 3.0, respectively. Apparently, at a level of 5 μM vanillylamine hydrochloride, the decrease in vanillylamine hydrochloride/palmitic anhydride ratio was favourable for the amidation reaction. It may be inferred that the activity of enzyme could be inhibited by higher concentration of the enriched vanillylamine hydrochloride substrate [26]. The excessive vanillylamine hydrochloride might form a layer of membrane-like substance on the surface of enzyme to prohibit interaction between substrate and active site of enzyme, and hence inhibit the amidation. Another rationale might attribute to steric effect of the enriched vanillylamine hydrochloride. Since the vanillylamine hydrochloride–enzyme complex hinders the other free nonanoic anhydride to form a covalent bond with enzyme. This hindering mechanism is the so-called substrate inhibition in the kinetics of enzyme. The inhibitory effect of vanillylamine hydrochloride on enzymatic amidation has been reported [26].

However, in an attempt to save on the cost of vanillylamine hydrochloride, the molar ratio of vanillylamine hydrochloride to nonanoic anhydride was maintained to be 0.25. Therefore, in the following experiments, 5 mM vanillylamine hydrochloride and

20 mM nonanoic anhydride were used as substrates to perform amidation in SC–CO_2. It is noteworthy that only free amine is reacting to lipase during amide synthesis; protection of the amine group may be required when reacting under anhydrous organic solvent conditions [35,36]. In the present study, vanillylamine substrate is in an HCl salt form to protect the amine group. The enzyme concentration greatly influences the reaction rate and the product quantity. In the present study, 0.5, 1.0, and 1.5% immobilized lipase was dissolved in the reactor and 40, 7.2, and 4.9% of yields were obtained, respectively, using 5 mM vanillylamine hydrochloride and 20 mM nonanoic anhydride as substrates to perform amidation at 50 °C and 170 bar for 23 h (data not shown). It seems that a decrease in enzyme concentration was favourable for the amidation reaction, implying a significant mass-transfer limitation and/or limited enzyme activity in an SC–CO_2 reactor [12]. Furthermore, the condition of high enzyme concentration, all the available substrate is forming the enzyme/substrate complex, and so the extra enzyme molecules (active sites) cannot further improve reaction rate. Based on good yield consideration, 0.5% (w/w) (200 mg) of enzyme in the reaction mixture was used in the following experiments. Similar results have been reported for the esterification of citronellol with lauric acid in SC–CO_2 using Novozym 435 [37] and for myristyl glucose synthesis by *C. antarctica* lipase [38].

3.5. Effect of metal salt

It is known that metal ions play a role in the maintenance of stable and active structures of enzymes by binding on the specific sites, which are generally formed by negatively charged amino acid side chains. The effect of metal ions on the rate of amidation was studied. $CuCl_2$ and $ZnCl_2$ significantly increased the yield, whereas $CaCl_2$ and $MgCl_2$ decreased the yield of VAN by the catalysis of Lipozyme IM in SC–CO_2 (Table 5). The production of different amides requires different metal ions to improve the yield. Namely, $CuCl_2$ and $ZnCl_2$ greatly increased the yield of VAN to 49.2 and 44.3%, respectively. Similar results were observed by Liu et al. [16] who used lipase to synthesize a cocoa butter equivalent in SC–CO_2. Yamamoto and Fugiwara [39] reported the inhibiting effect of Ca^{2+} and Mg^{2+} on lipase activity for the hydrolysis of castor oil. Different salts of metals (Fe^{2+}, Hg^{2+}, Ca^{2+} and Mg^{2+}) inhibited the lipase, suggesting that they were able to alter the enzyme conformation [40]. The effect of various metal ions, as a cofactor for catalytic activity, on *Staphylococcus epidermidis* lipase activity was also reported by Simons et al. [41]. In addition, thermostability is influenced by environmental factors such as pH and the presence of metal ions. Lo'pez-Lo'pez et al. [42] reported that *Cherax quadricarinatus* lipase was inactivated by Ca^{2+}. This inactivation could be caused by adding $CaCl_2$, suggesting that they were able to alter the enzyme conformation.

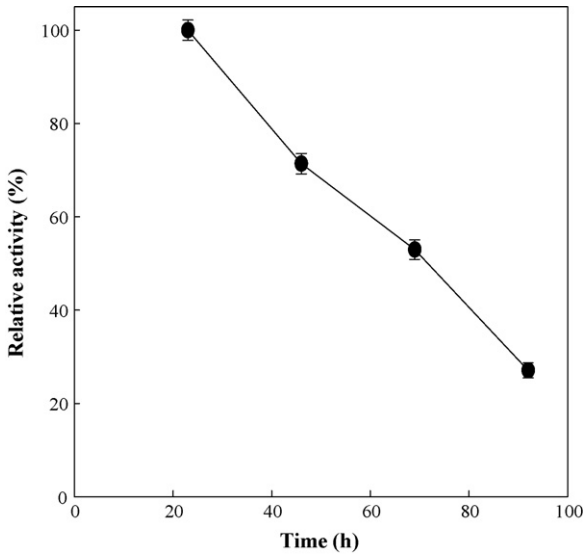


Fig. 3. Operational stability of Lipozyme IM. The reaction mixture was refreshed every 23 h, and relative activity was assayed immediately after each change. The lipase (200 mg) was added to a reaction mixture (40 mL) containing 5 mM vanillylamine hydrochloride and 20 mM nonanoic anhydride at 170 bar and 50 °C for 23 h in SC–CO_2. Each value is the average of three determinations.

Table 5
Formation of N-vanillylnonamide as affected by metal salts[a].

Metal salt	VAN yield (%)
Control	40.0 ± 0.08
$CaCl_2$	35.7 ± 0.06
$CuCl_2$	49.2 ± 0.06
$ZnCl_2$	44.3 ± 0.06
$MgCl_2$	37.6 ± 0.07

Triplicate data from separate experiments are expressed as mean ± SEM.
[a] The reaction conditions are exactly the same as Table 1.

Table 6
Antibacterial activity of N-vanillylnonamide (VAN) against *Escherichia coli*.

Compound	Diameter of inhibition zone (mm)
Ampicillin	9.0 ± 0.8
VAN[a]	10.6 ± 0.1

Triplicate data from separate experiments are expressed as mean ± SEM.
[a] The reaction conditions are exactly the same as Table 1.

3.6. Stability of Lipozyme IM

It has been demonstrated that the cost of lipase accounts for a large part of the total cost of VAN production, and one of the main advantages of an immobilized lipase is that it can be used repeatedly over an extended period of time. To investigate lipase stability, the amidation was repeated every 23 h. After completion of the reaction in 23 h of each cycle, the lipase was transferred into the same system for a new cycle. The results demonstrated that almost 100% enzyme activity was maintained after the first amidation reaction and is shown in Fig. 3. However, it lost about 29 and 47% of its activity after the second and third amidation, respectively, at 170 bar and 50 °C. Loss of about 73% of the enzyme activity after a 96-h operation was also reported by Yesiloglu [43] when using immobilized porcine pancreas lipase in catalyzing ethanolysis of sunflower oil.

3.7. Antibacterial activity of N-vanillylnonanamide

In the present study, the antibacterial activities of VAN were determined against Gram-negative bacteria (*E. coli*) in vitro by disc diffusion methods. Ampicillin was employed as a positive control against bacteria. The result of the antibacterial study of the synthesized compound is shown in Table 6. VAN indicated a better antibacterial activity (zone of inhibition, 10.6 mm) against *E. coli* and its sensitivity is similar to that of the reference ampicillin (zone of inhibition 9.0 mm). This is probably due to the lipophilic alkyl chain that avails the molecule to penetrate through the lipid cell membrane of Gram-negative bacteria.

4. Conclusions

The results of current study indicate that using SC–CO_2 as a safe and potential reaction medium for the lipase-catalyzed synthesis of VAN can be decently considered. Due to non-toxicity and nonflammability, it represents as a suitable solvent in the preparation of capsaicin analogs. The synthesis of VAN by enzymatic catalysis in SC–CO_2 and the maintained conditions were as follows:

6800 U Lipozyme IM in 40 mL reaction mixture of 5 mM vanillylamine hydrochloride and 20 mM nonanoic anhydride in SC–CO_2 at 170 bar and 50 °C for 23 h. Thus, maintained amidation conditions might be too strict for the present reaction. Compromise of the VAN yield by reducing the reaction temperature and/or pressure might be beneficial for the stability of enzyme for the aim of continual amidation. Thus, improvement of the confirmed experimental conditions is demanded. Furthermore, another novel function of VAN against *E. coli* suggested that VAN could be considered as a potential antibacterial agent for trials in controlling of food protection when more safety issues have been conducted.

Acknowledgement

Financial support for this study from the National Science Council of The Republic of China under grant (NSC 91-2313-B-346-001) is greatly appreciated.

References

[1] V.S. Govindarajan, M.N. Sathyanarayana, CRC Crit. Rev. Food Sci. Nutr. 29 (1991) 435–474.
[2] M.M. Fusco, M. Giacovazzo, Drugs 53 (1997) 909–914.
[3] I.J. Chen, J.M. Yang, J.L. Yeh, B.N. Wu, Y.C. Lo, S.J. Chen, Eur. J. Med. Chem. 27 (1992) 187–192.
[4] S.H. Buck, T.F. Burks, Pharmacol. Rev. 38 (1986) 179–226.
[5] B. Chowdhury, S. Mukhopadhyay, D. Bhattacharayay, A.K. De, Med. Sci. Res. 24 (1996) 669–670.
[6] R. Wrigglesworth, C.S.J. Walpole, S. Bevan, E.A. Campbell, A. Dray, G.A. Hughes, I. James, K.J. Masdin, J. Winter, Eur. J. Med. Chem. 39 (1996) 4942–4951.
[7] T. Suzuki, K. Iwai, in: A. Brossi (Ed.), The Alkaloids: Chemistry and Pharmacology, 1984, pp. 227–299.
[8] H. Kaga, K. Goto, T. Takahashi, M. Hino, T. Tokuhashi, K. Orito, Tetrahedron 52 (1996) 8451–8470.
[9] K. Kobata, M. Kawamura, M. Toyoshima, Y. Tamura, S. Ogawa, T. Watanabe, Biotechnol. Lett. 20 (1998) 451–454.
[10] J.C. Erickson, P. Schyns, C.L. Cooney, AICHE J. 36 (1990) 299–301.
[11] A.D. Miller, W.H. Blanch, M.J. Prausnitz, Ind. Eng. Chem. Res. 30 (1991) 939–946.
[12] A. Marty, W. Chulalaksananukul, R.M. Willemot, J.S. Condoret, Biotechnol. Bioeng. 39 (1992) 1–8.
[13] H. Michor, R. Marr, T. Gamse, T. Schilling, E. Klingsbichel, H. Schwab, Biotechnol. Lett. 18 (1996) 79–84.
[14] H. Tsuchiya, J. Ethnopharmacol. 75 (2001) 295–299.
[15] K.J. Liu, H.M. Chang, K.M. Liu, Food Chem. 100 (2007) 1303–1311.
[16] K.J. Liu, H.M. Cheng, R.C. Chang, J.F. Shaw, J. Am. Oil Chem. Soc. 74 (1997) 1477–1482.
[17] P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Tenover, R.H. Yolke, Manual of Clinical Microbiology, 6th ed., ASM, Washington, DC, 1995.
[18] M.L. R'ua, T.D. Maurion, V.M. Fernande, C. Otero, A. Ballesteros, Biochim. Biophys. Acta 1156 (1993) 181–189.
[19] SAS Institute Inc. SAS user's guide. Statistics, version 6.11, SAS Institute Inc., Cary, NC, 1995.
[20] L. Calvo, M.D. Romero, C. Alba, F. Ortiz, M. Gutierrez, Chem. Eng. Trans. 2 (2002) 181–186.
[21] Z. Knez, M. Habulin, J. Supercrit. Fluids 23 (2002) 29–39.
[22] K. Kobata, M. Kobayashi, Y. Tamura, S. Miyoshi, S. Ogawa, T. Watanabe, Biotechnol. Lett. 21 (1999) 547–550.
[23] M.D. Romero, L. Calvo, C. Alba, M. Habulin, M. Primozic, Z. Knez, J. Supercrit. Fluids 33 (2005) 77–84.
[24] L.N. Mutua, C.C. Akoh, J. Am. Oil Chem. Soc. 70 (1993) 43–46.
[25] Z. Knez, M. Habulin, V. Krmelj, J. Supercrit. Fluids 14 (1998) 17–29.
[26] K.J. Liu, K.M. Liu, H.M. Chang, Food Chem. 102 (2007) 1020–1026.
[27] J.V. Oliveira, D. Oliveira, Ind. Eng. Chem. Res. 39 (2000) 4450–4454.
[28] J. Chrastill, J. Phys. Chem. 86 (1982) 3016–3021.
[29] A. Nag, J. Surf. Sci. Technol. 4 (1988) 91–96.
[30] A. Shishikura, K. Fujimoto, T. Suzuki, K. Arai, J. Am. Oil Chem. Soc. 71 (1994) 961–967.
[31] K. Nakamura, Trends Biotechnol. 8 (1990) 228–292.
[32] M. Perrut, in: C. Balny, R. Hayashi, K. Heremans, P. Massor (Eds.), High Pressure and Biotechnology, 1992, pp. 401–410.
[33] Y. Ikushima, N. Saito, K. Hatakeda, O. Sato, Chem. Eng. Sci. 51 (1996) 2817–2822.
[34] C.G. Laudani, M. Habulin, Z. Knez, G.D. Porta, E. Reverchon, J. Supercrit. Fluids 41 (2007) 92–101.
[35] M. Habulin, S. Šabeder, M. Paljevac, Ž. Knez, J. Supercrit. Fluids 43 (2007) 199–203.
[36] P. Degn, W. Zimmermann, Biotechnol. Bioeng. 74 (2001) 483–491.
[37] A.M. Klibanov, Trends Biochem. Sci. 14 (1989) 141–144.
[38] P.J. Halling, Enzyme Microb. Technol. 16 (1994) 178–206.
[39] K. Yamamoto, N. Fugiwara, Agric. Biol. Chem. 52 (1988) 3015–3021.
[40] C. Sharon, S. Furugoh, T. Yamakido, H. Ogawa, Y. Kato, J. Ind. Microbiol. Biotechnol. 20 (1998) 304–307.
[41] J.W. Simons, M.D. van Kampen, S. Riel, F. Gotz, M.R. Egmond, H.M. Verhey, Eur. J. Biochem. 253 (1998) 675–683.
[42] S. López-López, H. Nolasco, F. Vega-Villasante, Comp. Biochem. Physiol. B 135 (2003) 337–347.
[43] Y. Yesiloglu, J. Am. Oil Chem. Soc. 81 (2004) 157–160.