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Short chain flavour ester synthesis by a new esterase from *Bacillus licheniformis*

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Abstract

The newly characterized esterase from *Bacillus licheniformis* was used for ethyl ester synthesis in *n*-heptane by direct esterification with fatty acids of different chain lengths. The highest reaction rates and yields were obtained with caproic (C6) and caprylic (C8) acids reflecting the enzyme specificity for mid-chain length fatty acids. The rate of ethyl caproate formation increased linearly with the enzyme concentration in the range 0–40 mg/ml. The enzyme displayed maximum activity with 0.1% (v/v) addition of water. The initial reaction rate was maximal at 67°C but the highest yield (95%) was obtained at 45°C. Kinetic parameters were determined for caproic acid: K_M of 38.4 mM and V_M of 8.3 μmol/min/ml (at 0.25 M ethanol), and for ethanol K_M of 12.3 mM and V_M of 1.3 μmol/min/ml (at 0.25 M caproic acid). The highest activities and yields were observed for solvents having log $P \geq 3.2$. The enzyme was used three times without activity loss.

Keywords: *Bacillus licheniformis*; Esterase; Ester synthesis; Flavors; Non-conventional media

1. Introduction

Short chain fatty acids esters are commonly used in the manufacturing of flavors and fragrances because of their fruity odor. They can be obtained either by extraction from their natural sources or by chemical synthesis. The first method is expensive and the second resulted in products without the ''natural'' label [1]. Therefore, growing interest has been devoted to the enzymatic synthesis, which allows the use of ''natural'' label. Usually, hydrolases retain their activity and specificity in organic solvents as long as sufficient water is present to maintain optimal enzyme conformation for catalysis [2–4]. There are several advantages of using enzymatic synthesis in organic media: (i) higher solubility of hydrophobic substrates in organic solvents compared to water; (ii) enzymes are more stable in organic solvents than in water [5]; (iii) easy product and enzyme recovery; (iv) elimination of microbial contamination risks; and (v) yield increased by reduction of substrate and product inhibitions either by maintaining a lower concentration in the microenvironment of

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the enzyme [6,7] or by changing the interactions between the inhibitor and the active site of the enzyme [8].

In our laboratory, several thermotolerant bacterial strains have been isolated from soil [9,10]. Among them, a *Bacillus licheniformis* strain has been identified and an esterase gene has been cloned in *Escherichia coli* and characterized [11]. This thermostable esterase activity showed and interesting non-typical specificity for *p*-nitrophenyl esters of mid-chain length fatty acids (C4–C8). Although it had no activity on *p*-nitrophenyl esters of high chain length (C16) or olive oil, it shared several structural and biochemical similarities with lipases [12]. Therefore, we suspected that this esterase might be useful for esters synthesis using fatty acids of intermediate chain length such as caproic acid (C6).

In this work, we report the use of the cloned *B. licheniformis* esterase for the synthesis of ethyl caproate from ethanol and caproic acid in *n*-heptane.

2. Experimental

2.1. Enzyme preparation

A lyophilized enzymatic powder was prepared from an *E. coli* recombinant strain carrying the esterase gene as previously described [12]. The activity of this powder was 0.31 U/mg measured by the rate of hydrolysis *p*-nitrophenyl caproate in water. From 2 l of culture, 500 mg of powder and 775 total units were obtained. The enzyme powder was dried over NaOH for at least 48 h before use.

2.2. Esterification method

In the standard protocol, ester synthesis was carried out in screw-capped test tubes as bioreactors. Unless otherwise specified, 25 mg of enzymatic powder were added to 2.5 ml of *n*-heptane containing 0.25 M ethanol, 0.25 M caproic acid, and 0.1% (v/v) water. All reagents were purchased from Sigma (St. Louis, MO, USA). They were previously dried by adding 3 Å molecular sieve (Fluka Chemie, Switzerland) and by desiccation for 48 h under solid NaOH. The tubes were incubated in an orbital shaking water bath at 37°C. All experiments were run in triplicate.

2.3. Extraction and analysis

Samples from reaction mixtures (100 μl) were diluted in 900 μl of *n*-heptane containing 10 mM of an appropriate internal standard (decane, dodecane, tetradecane or octadecane depending on the esters tested). The remaining acids were extracted with 100 μl of 0.05 M Tris–HCl, pH 9. Samples were taken every 30 min during 2 h to determine the ester concentration. The initial reaction rate was calculated from the slope of the curve of the formed ester vs. time. For yield determination the incubation time was 16 days. Esters were analyzed by gas–liquid chromatography. A 1-μl aliquot was injected in a Shimadsu GC-14A chromatograph equipped with a flame ionization detector and a Perkin-Elmer Nelson integrator. A BP10 capillary column (SGE, Austin, TX) was used. The carrier gas was helium.

2.4. Repeated uses

The enzyme was incubated in standard ester synthesis conditions for several 24-h runs. After each run, the reaction media was centrifuged ($3000 \times g$, 5 min) and the enzyme was washed with 2.5 ml of *n*-heptane. Then, fresh reaction medium was added. The initial reaction rate was determined over a 2-h period of incubation.

3. Results and discussion

3.1. Effect of enzyme concentration

The system used for ester synthesis was biphasic with the solid enzyme powder in suspension in the liquid phase of *n*-heptane. In such heterogeneous catalysis, the powder showed tendency to adsorb water and to aggregate. For

this reason, all the liquids used in the reaction mixture (*n*-heptane, caproic acid and ethanol) were dried before use by using both a molecular sieve and NaOH drying (see Section 2). The enzyme powder was lyophilized and dried over NaOH for 48 h before use. The initial rate of ethyl caproate synthesis was proportional to the amount of enzyme added to the reaction mixture. This result demonstrated that the reaction was kinetically limited in the range tested.

3.2. Effect of added water

In organic systems the amount of water present in the reaction medium is very important. Water is necessary to maintain the active tertiary structure of the enzyme [13] and it controls the thermodynamic equilibrium of the enzymatic reactions [4]. Generally, a low water content favors ester synthesis over hydrolysis [14]. To study the effect of water addition on the reaction rate, 0–0.6% (v/v) were added to the reaction media (Fig. 1). Activity first increased to 0.96 mmol/l/h and then went down sharply. This result agrees with the fact that a minimal quantity of water is necessary to maintain the active conformation of the enzyme. Above this quantity (1 μl/ml) the enzyme started to aggregate and to bind to the tube glass-wall. All further experiments were run with 1 μl/ml added water.

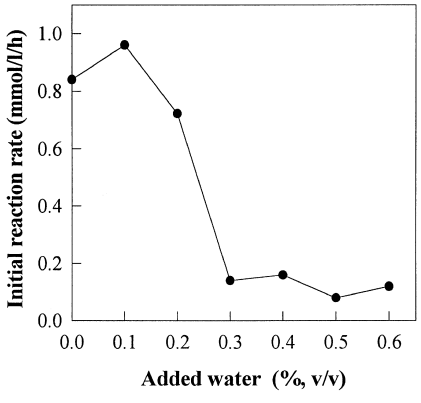

Fig. 1. Effect of water addition on esterification velocity. Standard conditions for ester syntheses were used and the amount of added water was varied. Initial rates were assayed in less than 2 h. Results are average of three independent determinations with standard deviations less than 10%.

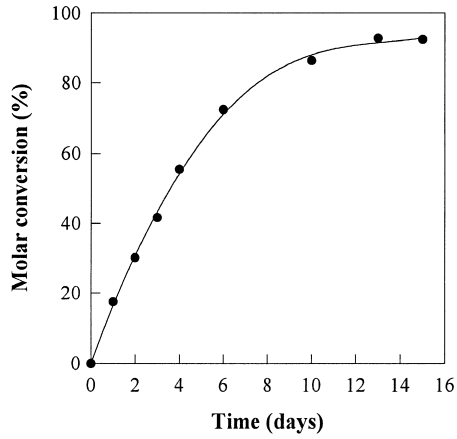

Fig. 2. Kinetic of ethyl caproate synthesis. Standard conditions for ester syntheses were used. Results are average of three independent determinations with standard deviations less than 10%.

3.3. Reaction kinetics

The reaction kinetics was studied using 0.25 M concentrations of each substrates (Fig. 2). The reaction rates went down after 2 days but a high conversion (95%) was obtained after 13 days. This high conversion was quite surprising since no attempts were made to prevent water accumulation in the reaction mixture. In fact, one mole of water was formed per mole of ethyl caproate formed. This water may affect both the initial reaction rate (see Fig. 1) and the reaction equilibrium. Nevertheless, a high degree of substrate conversion was achieved in these conditions, further improvement in yield is expected by removing the water in continuous from the reaction mixture.

3.4. Effect of chain length of acids on ethyl ester synthesis

Interest in the esterase from *B. licheniformis* is linked to its unusual specificity for hydrolysis of *p*-nitrophenyl ester of mid chain length fatty acids [12]. On the other hand, short-chain fatty

acids ethyl esters are the most commonly used in the food industry [1]. Therefore, we have investigated the fatty acid specificity for ester synthesis in organic media. This specificity could not be determined from the experiments in aqueous medium since it may change in organic media due to increased structure rigidity [4]. Thus, the initial rates of ester synthesis and final reaction yields (after 16 days of reaction at 37°C) were determined using ethyl alcohol and different fatty acids with chain lengths from C2 to C18 (Fig. 3). The reaction rates increased from acetic acid (C2) to caprylic acid (C8) and it went down rapidly for fatty acids with higher chain lengths. A low activity was detected with palmitic (C16) and stearic (C18) acids but none with oleic acid (C18:1). These results are similar, but not identical, to those obtained in aqueous media [12]. In water, the highest activity was observed for *p*-nitrophenyl caproate (C6) hydrolysis and no activity at all was detected for *p*-nitrophenyl palmitate and stearate. The conversion yield followed the same general tendency (Fig. 3) as the initial rate with a maximum for caproic acid (C6). On the whole, the *B. licheniformis* esterase catalyzed efficiently the synthesis of esters of caproic and caprylic acids (C6 and C8) and to a lesser extent of butyric (C4) acid.

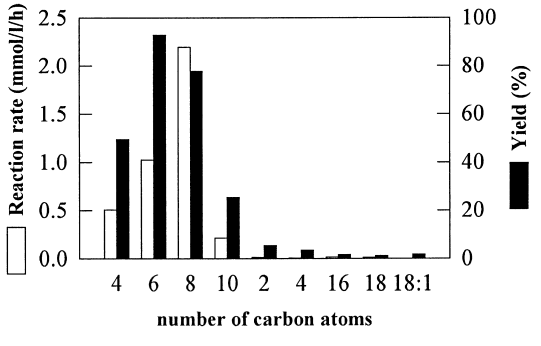

Fig. 3. Effect of chain length of fatty acid on esterification rate and yield of ethyl caproate. Standard conditions for ester syntheses were used but the nature of the acid was varied. Results are average of three independent determinations with standard deviations less than 10%.

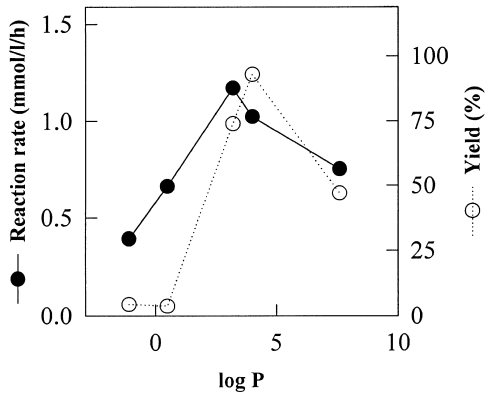

Fig. 4. Effect of solvents on esterification velocity and ethyl caproate synthesis. Standard conditions for ester syntheses were used but the nature of the solvent was changed. All solvents were dried as described for *n*-heptane before use. Solvents were dioxane (log $P = -1.1$), tetrahydrofuran (log $P = 0.49$), cyclohexane (log $P = 3.2$) heptane (log $P = 4$) and tetradecane (log $P = 7.6$). Results are average of three independent determinations with standard deviations less than 10%.

3.5. Effect of solvent

In the reaction conditions tested, i.e., without control of water activity, it is well known that the nature of the organic solvents greatly influence the activity of biocatalysts. Solvents can strip the essential water that maintains the active conformation of enzymes [3]. Log P (the partition coefficient between water and 1-octanol) is generally used to describes the solvent hydrophobicity [15]. Hydrophilic organic solvents (low log P values) are expected to dissolved more water than hydrophobic ones and thus to reduce the catalytic activity of enzymes [3]. Solvents can also act directly on enzyme and inhibit activity by altering hydrogen bonds and hydrophobic interactions [4]. Solvents can also compete with the substrate for binding into the active site [16,17]. Therefore, the esterification rate and yield of ethyl caproate formation were tested with six solvents of Log P varying from −1.1 to 7.6 (Fig. 4).

The initial reaction rate first increased from dioxane to cyclohexane and it went further down for tetradecane. The reaction yield followed the

same tendency. Similar results have been published for the *Candida cylindracea* lipase [18]. The initial velocity for butyl butyrate formation was maximal with hexane and decreased with more hydrophobic solvents like octane or decane. Therefore, solvents with intermediate Log *P* (around 4) are suitable for ethyl caproate synthesis in the conditions used.

3.6. Effect of substrate concentration

In organic media, changes in substrate concentration not only affect the reaction rates (Michaelis–Menten kinetics) but also the enzyme activity by affecting the water solubility in the solvent [3]. The effect of varying the caproic acid (Fig. 5) or ethanol (Fig. 6) concentrations on the initial rates of ester ethyl caproate synthesis was studied in the range 0–1 M. Both curves showed similar trends with apparent Michaelis–Menten kinetics for the lowest concentrations tested (up to 0.25 M). Both Lineweaver–Burk representations were linear (inserts in Figs. 5 and 6) which allowed the calculations of kinetics parameters using nonlinear regression analysis. The estimated values of K_M and V_M were 38.4 ± 3.9 mM and 8.3 ± 0.6 μmol/min/ml. for caproic acid. This K_M value is relatively low compared to that for the *Pseudomonas fluorescens* lipase (0.22 M) [19], for oleic acid (0.3 M) and *C. cylindracea* lipase [20], for butyric acid and *Mucor miehei* lipase (0.14 M) [1], and for acetic acid and *C. cylindracea* lipase (0.146 M) [18]. It is similar to that of *P. fluorescens* lipase for butyric acid (55 mM) [1] and to that of *Rhizopus arrhizus* lipase for oleic acid (15.3 mM) [21].

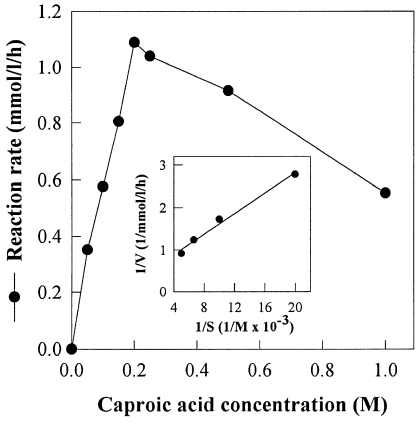

Fig. 5. Effect of caproic acid concentration on esterification velocity. Standard conditions for ester syntheses were used but the concentration of caproic acid was varied. Initial rates were assayed in less than 2 h. A Lineweaver–Burk plot is shown in the insert. Results are average of three independent determinations with standard deviations less than 10%.

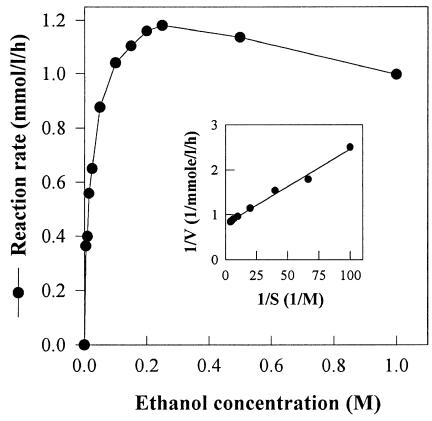

Fig. 6. Effect of ethanol concentration on esterification velocity. Standard conditions for ester syntheses were used but the concentration of ethanol was varied. Initial rates were assayed in less than 2 h. A Lineweaver–Burk plot is shown in the insert. Results are average of three independent determinations with standard deviations less than 10%.

A clear inhibition was observed at caproic acid concentrations higher than 0.25 M. Several studies have also reported inhibitory effects of acids on esterification catalyzed by lipases [20–28]. This inhibition may result from (i) substrate inhibition, (ii) pH reduction in the aqueous microenvironment of the enzyme, (iii) enzyme dehydration. This phenomenon is described with lipases with the exception of the esterification rate of *C. cylindracea* lipase [26] which was not inhibited even at butyric acid concentration as high as 1 M. Other enzymes are inhibited by low acid concentrations. For instance, the *Mucor miehei* lipase was inhibited by 0.05 M acetic acid [18].

The variations of ethyl alcohol concentrations showed similar trends on reaction rate (Fig. 6).

The K_M value was 12.3 ± 0.5 mM, which is similar to that of *Candida antarctica* for methanol (16.4 mM) [29] and the V_M was 1.3 ± 0.1 μmol/min/ml. Alcohol concentrations higher than 0.25 M were inhibitory but with an intensity much less than that observed for caproic acid. Alcohol inhibitions have been reported for several lipases, like those of *C. cylindracea* [26], *M. miehei* [30] and *C. antarctica* [29].

3.7. Effect of temperature on initial rate

The initial rate of ethyl caproate synthesis was studied in standard conditions with incubation temperatures ranging from 27°C to 77°C (Fig. 7). The rate increased from 27°C to 67–77°C with no apparent real decrease. This curve is typical of enzyme with high thermostability and which thermal denaturation, during the time of the assay, is negligible. The Arrhenius representation (Fig. 7) was linear and activation energy of 15.5 kJ/mol was deduced. The activity curve profile is similar to that obtained for *M. miehei* lipase (60°C) [31].

The effect of temperature on the esterification yield was studied at three selected temperatures of 37°C, 45°C and 52°C (Table 1). After a reaction time of seven days the highest yield (95%) was observed for 45°C while it was lower for the two other temperatures tested. However, the yield for 37°C also reached 95% but only after ten days of incubation.

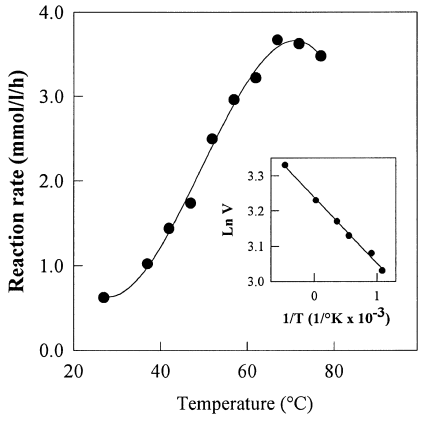

Fig. 7. Effect of temperature on reaction rate. Standard conditions for ester syntheses were used but the incubation temperature wad varied. Initial rates were assayed in less than 2 h. An Arrhenius plot is shown in the insert. Results are average of three independent determinations with standard deviations less than 10%.

Table 1
Effect of temperature on ethyl caproate yield
Standard conditions for ester syntheses were used and the incubation temperature was varied. Results are average of three independent determinations with standard deviations less than 10%.

Temperature (°C) (°C)	Yield (%)	
	7 days	10 days
37	95	95
45	90	95
52	62	69

3.8. Operational stability

The enzyme stability was checked after several 24-h runs in standard conditions (Table 2). Initial (during 2 h) reaction rates were determined. Results showed full retention of activity for the first three runs and almost complete losses (95%) after eight runs. This inactivation most probably resulted from the water accumulation within the catalyst since it was visually observed that the enzymatic powder stuck to the glass tubes more and more during the different runs. Others have also reported similar results [18,32].

Table 2
Effect of enzyme reuse on activity
0.25 M solutions of ethyl alcohol and caproic acid were incubated at 37°C during 24 h per run. Initial reaction rates were determined after enzyme recovery and incubation into fresh reaction medium.

Number of runs	Remaining activity (%)
1	100
2	100
3	100
4	89
5	76
6	41
7	17
8	6

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