

Bioproduction of Natural Isoamyl Esters from Coconut Cream as Catalysed by Lipases

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Received: March 4, 2013 Accepted: March 25, 2013 Online Published: March 28, 2013

doi:10.5539/jfr.v2n2p157

URL: <http://dx.doi.org/10.5539/jfr.v2n2p157>

Abstract

This study investigated the bioproduction of isoamyl esters in coconut cream by lipases. Five lipases (palatase 20000 L, lipase AYS “Amano”, lipase A “Amano” 12, piccantase A and piccantase AN) were used to biosynthesize isoamyl esters in coconut cream supplemented with isoamyl alcohol. The lipases have different abilities to synthesize isoamyl esters with lipase AYS “Amano”, palatase 20000 L and piccantase A showing the highest potential. Bioproduction of isoamyl octanoate by palatase 20000 L was further examined under different conditions of temperature, pH, isoamyl alcohol concentration and lipase amount. Biosynthesis of isoamyl octanoate by palatase was not significantly affected at 30-50°C or pH of 4 to 7 and its maximum bioproduction was obtained at isoamyl alcohol concentration of 4% (v/v) and lipase amount of 6 mg 100 mL⁻¹ reaction mixture. The lipase-treated coconut cream may serve as a bioflavouring ingredient for food applications or for extraction of pure aroma chemicals.

Keywords: esters, lipases, alcoholysis, esterification, flavour, coconut cream

1. Introduction

Esters of short-chain fatty acids, aromatic and acetate esters are arguably the most important aroma chemicals in food and beverages such as wine, imparting various fruity flavour notes (Cristiani & Monnet, 2001; Ugliano & Henschke, 2009). Among the esters, isoamyl esters are flavour-active esters with wide applications in the flavour and fragrance industry. Isoamyl esters of short-chain fatty acids of C₂-C₈ are commonly used in flavour compositions for foods and beverages (Arctander, 1994). Examples of such flavour compositions include banana, apricot, apple, cherry, peach, strawberry, raspberry, pineapple, chocolate, candy, chewing gum, honey, butter, butterscotch, coffee, rum, liqueur, and brandy etc.

Isoamyl esters are traditionally produced through chemical synthesis. However, the increasing consumer demand has led to a rapid shift toward the production and use of natural flavour compounds. Extraction from natural sources such as plant materials is one but expensive option. More commercially acceptable alternatives are biocatalysis and fermentation using enzymes and microorganisms such as yeasts (Cristiani & Monnet, 2001; Longo & Sanroman, 2006; Ugliano & Henschke, 2009; Tan et al., 2011).

The bioproduction of natural isoamyl acetate, a key aroma chemical, has been the subject of several studies involving microbial fermentation and biocatalysis. Yeasts such as a *Saccharomyces cerevisiae* mutant and *Williopsis saturnus* var. *saturnus* are found to produce isoamyl acetate of up to 354 mg L⁻¹ (Quilter et al., 2003; Yilmaztekin et al., 2009). On the other hand, it is reported that immobilized lipase can catalyse the esterification of isoamyl alcohol and acetic acid in organic solvent with a conversion of over 95% (Krishna et al., 2001). Another study shows that an immobilized esterase from *Bacillus licheniformis* can synthesize isoamyl acetate from isoamyl alcohol and *p*-nitrophenylacetate in organic solvent with a yield of 43% (Torres et al., 2009).

Despite the studies on isoamyl acetate production mentioned above, few reports have been published on the production of natural isoamyl esters of other fatty acids. There are two reports on the production of isoamyl esters from fusel oil (> 50% *i*-amyl alcohol (= active amyl and isoamyl alcohols)) and fatty acids. One report

describes the uncatalyzed esterification of fusel oil with acetic, propionic and butyric acids to produce *i*-amyl acetate, *i*-amyl propionate and *i*-amyl butyrate under non-aqueous and solvent-free conditions (Küçük & Ceylan, 1998). The other study describes a lipase-catalyzed esterification of *i*-amyl alcohol with oleic acid in organic solvent to form *i*-amyl oleate as a biolubricant (Nemestothy et al., 2008).

Besides hydrolysis and esterification, lipases can catalyze ester synthetic reactions between esters and alcohols (alcoholysis or transesterification), acids (acidolysis or transesterification) or other esters (interesterification) under certain conditions (Gunstone, 1999). Indeed, several studies have shown the production of fatty acid esters of various carbon-chain length via lipase-catalyzed alcoholysis of milk fat and vegetable oils in solvent-free media or in organic solvent (Soumanou & Bornscheuer, 2003; Liu et al., 2004; Lubary et al., 2009).

Coconut fat is rich in short-chain fatty acid of C₈ (octanoic or caprylic acid) (7.5% w/w). Coconut cream is produced from coconut milk after centrifugation and contains about 25% (w/v) of fat. This fatty material is cheap and readily available in many parts of the world, especially Southeast Asia. Coconut cream may be an attractive substrate for the production of natural esters through alcoholysis.

The bioproduction of isoamyl esters from coconut cream via lipase-catalyzed alcoholysis has received little research attention. This study aimed to investigate the bioproduction of isoamyl esters from coconut cream and isoamyl alcohol with a focus on aroma-active isoamyl octanoate.

2. Method

2.1 Materials

Coconut cream with a fat content of 25.4% (w/v) (Kara brand, Fairtech Holdings Pte Ltd, Singapore) was used as starting lipid material for the synthesis of esters. Natural octanoic acid and isoamyl alcohol were supplied by Firmenich Asia Pte Ltd (Singapore). Trioctanoin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Palatase 20000 L (Novozymes, Bagsvaerd, Denmark), lipase AYS "Amano" and lipase A "Amano" 12 (Amano Enzymes Inc., Nagoya, Japan), piccantase A and piccantase AN (DSM Food Specialties, Village-Neuf, France) were biocatalysts used in this study. Palatase 20000 L and piccantase A were isolated from *Rhizomucor miehei*, lipase AYS "Amano" was obtained from *Candida rugosa*, and lipase A "Amano" 12 and piccantase AN were produced from *Aspergillus niger*. Lipases obtained from different microorganisms have distinct properties with respect to positional specificity, substrate selectivity, operation temperature and pH. Therefore, in this study the five lipases originated from three microorganisms were tested for their ability to produce desirable flavor esters.

2.2 Experimental Procedures and Reaction Conditions

The reactions were carried out under aseptic conditions to investigate the synthetic activities of different lipases. Reagents including 100 mL of coconut cream and 1% (v/v) of isoamyl alcohol were added in 250 mL capped bottles together with 0.5 mg of protein. Two types of control test were conducted, one control contained isoamyl alcohol without lipase and the other one contained only coconut cream. The reactions were performed at 30°C in a shaking water bath with a shaking speed of 100 rpm for 24 h.

The effects of isoamyl alcohol concentration, enzyme amount, reaction temperature and pH on the synthesis of isoamyl octanoate by lipase were studied. Coconut cream (80 mL), certain amounts of isoamyl alcohol, deionised water and lipase were added in 250 mL capped bottles with a resulted volume of 100 mL to get desired concentration of reactants. The reaction conditions applied were illustrated in the figure legends. The reaction was carried out in a shaking water bath (100 rpm) for 11 h.

To have a better understanding of biosynthesis of isoamyl octanoate by lipase, transesterification and esterification assays were carried out in buffer system. The reagents including 460 mM isoamyl alcohol, 30 mM trioctanoin (transesterification) or 95 mM octanoic acid (esterification) were added in a 250 mL capped bottle containing potassium phosphate (80 mM, pH 7.0), lipase (0.5 mg of protein) and proper amount of deionised water. The total volume of the reaction mixture was 100 mL. Control tests without adding lipase were also conducted. The reaction was carried out for 90 min in a shaking water bath (100 rpm) at 30°C. Reactions were stopped by adjusting pH to 2.0 with 2 M sulphuric acid.

2.3 Sample Analysis

The protein content of the lipase preparations was determined with a protein assay kit from Bio-Rad Laboratories (CA, USA) by using bovine serum albumin (Sigma-Aldrich) as standard.

Volatile compounds in samples were extracted by headspace solid-phase microextraction (HS-SPME) method. Extracted volatile compounds were analyzed by gas chromatography-mass spectrometry/flame ionization detector (GC-MS/FID). Two millilitres of sample was added into a 20 mL capped SPME vial and pre-incubated

at 85°C for 15 min with agitation (250 rpm). Then an 85 µm Carboxen/PDMS fibre (Supelco, Bellefonte, PA, USA) was exposed to the sample headspace for 40 min to extract volatile compounds. After that, the fibre was desorbed for 5 min in the injector port. The HS-SPME sampling process was performed by the autosampler (Gerstel, Germany).

The analysis was carried out in an Agilent 6890N GC equipped with FID and a 5975 inert mass selective detector (MSD) (Agilent, Palo Alto, CA, USA). Separation of the volatiles was performed on a 50 m × 0.25 mm × 0.25 µm FFAP capillary column from Quadrex (Woodbridge, USA). The injector temperature was 250°C. Splitless mode was applied with helium as carrier gas (flow rate was 1.2 mL min⁻¹). The initial oven temperature was set at 50°C for 4 min, then was raised to 230°C at 5°C min⁻¹ and held for 30 min. MSD was operated in the electron impact (EI) mode at 70 eV. The flavour compounds were identified by comparing mass spectra information with WILEY database.

The reproducibility of the method was obtained from analyses of 10 identical samples and analytical error was approximately 5%. For comparative purposes, the FID peak areas of target compounds were used as the responses. The FID peak areas demonstrated in this study were the mean of two values obtained from duplicate analyses.

3. Results and Discussion

The main objective of this research was to investigate the lipase-catalysed bioproduction of isoamyl octanoate from coconut cream and isoamyl alcohol under aqueous conditions, as this was quantitatively the major aroma-active isoamyl ester of short-chain fatty acid expected to be produced in coconut cream in relation to the fatty acid composition of coconut fat (see Introduction). However, isoamyl esters of other fatty acids such as isoamyl hexanoate and isoamyl laurate were also produced but not presented here, because these esters were either quantitatively minor (e.g. isoamyl hexanoate) or were not particularly potent (e.g. isoamyl laurate) due to high odour thresholds. Further, as mentioned in Introduction and demonstrated elsewhere (Liu et al., 2003), besides alcoholysis reaction lipases can also hydrolyze triglycerides in coconut cream and form free fatty acids.

3.1 Bioproduction of Isoamyl Octanoate by Different Lipases in Coconut Cream

The bioproduction of isoamyl octanoate by five commercial lipases is presented in Figure 1, which also shows formation of octanoic acid and consumption of isoamyl alcohol. The blank control (coconut cream only) had no detectable isoamyl octanoate; neither did the control with added isoamyl alcohol without added lipase.

The synthesis of 2-phenylethyl octanoate was investigated in a previous study (Tan et al., 2011). Since different lipases from varied sources and even the same lipase from the same source have distinct substrate selectivity and specificity, it is of value to understand their ability to produce different esters. Thus, in this study, the ability of five lipases for the synthesis of other esters was investigated. The varied ability of the five lipases to produce isoamyl octanoate is shown in Figure 1a. The lipase AYS produced most isoamyl octanoate, followed by lipases palatase and Picc A. The three lipases also produced different amounts of octanoic acid in the same order as that of isoamyl octanoate (Figure 1b), indicating simultaneous alcoholysis and hydrolysis. The lipases A12 and Picc AN produced only trace amounts of isoamyl octanoate and octanoic acid (Figure 1a and 1b). The lack of octanoic acid production by lipases A12 and Picc AN was unexpected, suggesting that the two lipases were not active on coconut fat.

As shown in Figure 1c, the consumption of isoamyl alcohol (residual amount indicated in Figure 1c) correlated with the bioproduction of isoamyl octanoate. Relative to the controls (coconut cream and isoamyl alcohol), the lipase AYS consumed most isoamyl alcohol, followed by lipases palatase and Picc A, whereas the lipases A12 and Picc AN consumed little isoamyl alcohol.

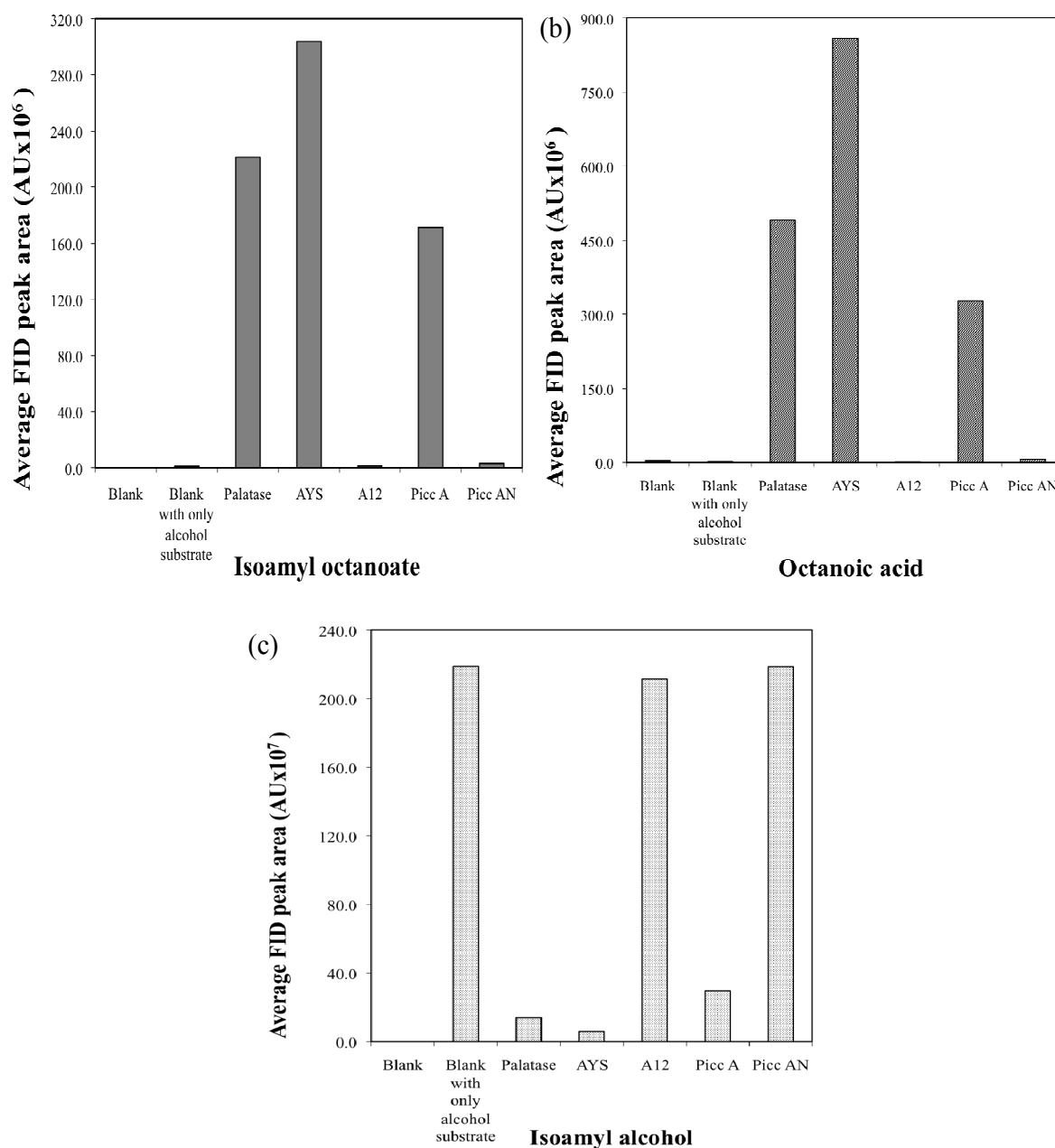


Figure 1. Bioproduction of isoamyl octanoate (a), formation of octanoic acid (b) and consumption of isoamyl alcohol (c) in coconut cream, as catalysed by lipases (palatase, AYS, A12, Picc A and Picc AN)

Reaction conditions: 100 mL of coconut cream, 1% v/v of isoamyl alcohol, 0.5 mg of protein, 30°C, pH 6.0, 24 h of incubation. The ability of synthesizing isoamyl octanoate was evaluated by observing the formation of isoamyl octanoate and consumption of isoamyl alcohol. Octanoic acid was also generated via the lipase-catalyzed hydrolysis of triglycerides.

To gain further insight into the characteristics of three lipases of palatase, AYS and Picc A, their kinetic data of isoamyl octanoate production, octanoic acid formation and isoamyl alcohol consumption were compared (Figure 2). AYS was the most active lipase, followed by palatase and then Picc A. Moreover, AYS produced the highest yield of isoamyl octanoate with the fastest reaction rate (Figure 2a). Palatase and Picc A, while generating lower ester yields with slower reaction rates, did not show ester hydrolysis. A good correlation between the kinetics and concentrations of isoamyl octanoate, octanoic acid and isoamyl alcohol was demonstrated, showing simultaneous ester production, octanoic acid release and alcohol utilisation.

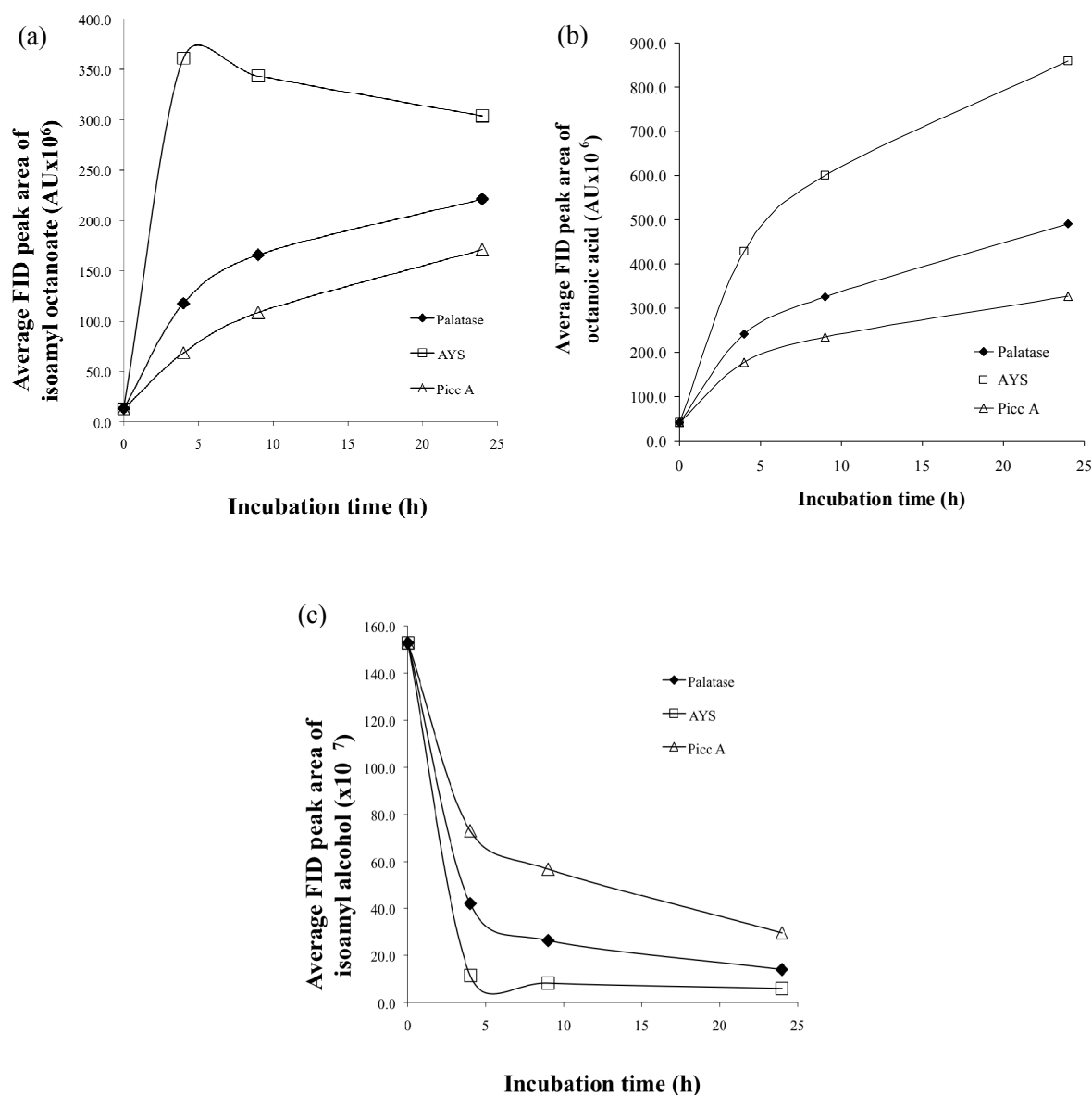


Figure 2. Time-course changes of isoamyl octanoate (a), octanoic acid (b) and isoamyl alcohol (c) in coconut cream treated with lipases palatase, AYS and Picc A

Reaction conditions: 100 mL of coconut cream, 1% v/v of isoamyl alcohol, 0.5 mg of protein, 30°C, pH 6.0, 24 h of incubation. Base on the screening test results, three lipases including palatase, AYS and Picc A were selected as catalysts for the synthesis of isoamyl octanoate. The time-course study was conducted to obtain the reaction rates and kinetic reaction curves.

The findings in this study are in agreement with those reported elsewhere that not all lipases can synthesise esters in aqueous media (Liu et al., 2003). The cited study reports that mammalian lipases generally lack the ability to produce esters in aqueous media, whereas fungal lipases tend to do so.

The differences in the ester-synthesising activity among the five lipases could be related to their biological origins. The most effective lipase AYS was produced by yeast *C. rugosa*. The less efficient lipases palatase and Picc A were derived from mould *R. miehei* and the least active lipases Picc AN and A12 were prepared from mould *A. niger*. Lipases are known to possess various specificities for fatty acid substrates and the positions on the triglycerides (Lotti & Alberghina, 2007), which may result in different catalytic abilities.

Langrand et al. (1990) evaluated various commercial lipases (including lipases from *C. rugosa*, *R. miehei* and *A. niger*) for their ability to synthesise flavour-active esters from alcohols (including isoamyl alcohol) and free fatty acids in organic media. They found extremely low activity of *A. niger* lipase to generate significant yields of esters relative to other lipases. These findings are in agreement with our observation of the absence of or extremely weak synthesising and hydrolytic activities of lipases A12 and Picc AN derived from the same mould.

C. rugosa lipases are well known for their broad range of specificity with a lack of preferences for specific ester bonds in the triglycerides and accept different esters with varying carbon chain-length fatty acid and alcohol moieties (Benjamin & Pandey, 1998). According to the manufacturer, the lipase AYS is able to attack short-, medium- and long-chain fatty acids at 1, 2, 3 positions of tri-, di- and monoglycerides, which might explain the high activities of the lipase AYS observed here. For the *R. miehei* lipases, they are 1,3-specific lipases (*i.e.* attack only bonds 1 and 3 of triglycerides) and this could explain the lower productivity and ester yield obtained by using the lipases palatase and Picc A, relative to the lipase AYS.

The aim of this study was to synthesize isoamyl esters of short- to medium-chain fatty acids from coconut fat and isoamyl alcohol. The lipase AYS, which has a broad specificity for various carbon chain-length of fatty acids, might complicate the flavour profile by producing undesirable esters of longer-chain fatty acids in spite of its high yield. The lipase palatase has a preference for short-chain fatty acids according to the manufacturer. This would likely result in a desirable flavour profile enriched with the desired esters. Further, comprehensive studies have been conducted on *R. miehei* lipases (*i.e.* fungal source of palatase) regarding its structure-function properties (Alcantara et al., 1998), indicating that its value is recognized. Thus, the lipase palatase was selected for subsequent investigation.

3.2 Effects of Physicochemical Parameters on Bioproduction of Isoamyl Octanoate by Lipase Palatase in Coconut Cream

The effects of physicochemical parameters including isoamyl alcohol concentration, lipase amount, temperature and pH on the lipase palatase-catalyzed bioproduction of isoamyl octanoate from coconut cream and isoamyl alcohol were investigated. Figure 3 (a, b, c, d) shows the lipase-catalyzed formation of isoamyl octanoate under different conditions.

As the isoamyl alcohol concentration increased from 2 to 4% (v/v), the rate and yield of isoamyl octanoate bioproduction increased markedly (Figure 3a). Correspondingly, the rate and yield of octanoic acid release decreased dramatically (data not presented). Further increment of isoamyl alcohol concentration from 4 to 8% (v/v) did not cause any significant changes in the reaction rate and yield of isoamyl octanoate. This result indicates that 4% (v/v) of isoamyl alcohol was sufficient for the biotransformation of octanoic acid in coconut cream into isoamyl octanoate.

The effect of lipase amount on isoamyl octanoate bioproduction is shown in Figure 3b. Our preliminary study showed that the rate and yield of isoamyl octanoate bioproduction increased almost proportionally as the lipase amount increased from 0.25 to 2.00 mg (data not shown). Further studies demonstrated that the rate and yield of isoamyl octanoate bioproduction further increased as the lipase amount increased from 2.00 to 8.00 mg, especially from 2.00 to 4.00 mg of protein (Figure 3 b).

The effects of temperature and pH on isoamyl octanoate bioproduction are shown in Figure 3 (c, d). Temperatures in the range of 30-50°C did not show any significant effect on the bioproduction of isoamyl octanoate and reaction rate. The production of isoamyl octanoate was well correlated with the consumption of isoamyl alcohol. This suggests that palatase are able to catalyze the synthetic reactions under a wide temperature range. Similarly, pH in the range of 4.4 to 7.1 had no marked effects on the rate and yield of isoamyl octanoate bioproduction, corresponding well with the consumption of isoamyl alcohol. This result indicates that palatase can be operated under a wide pH range. Our findings are similar with those reported by Oliveira and Rosa (2006), and they also found that the lipase palatase was able to perform the transesterification reaction under broad temperature and pH optimum range in an aqueous-oil biphasic system. The exception is that the latter study found significant inhibition of transesterification from 40 to 50°C.

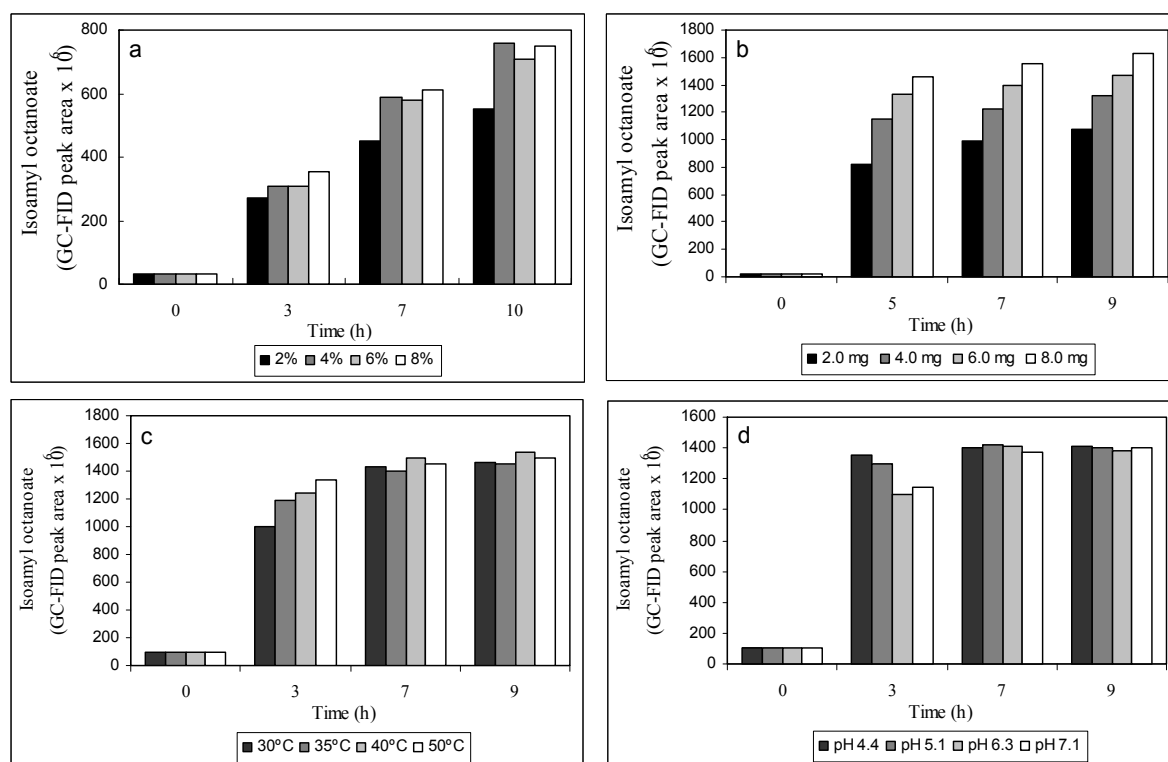


Figure 3. Effects of physicochemical parameters on the bioproduction of isoamyl octanoate in coconut cream (total volume = 100 mL), as catalysed by lipase palatase

(a) isoamyl alcohol concentration (v/v), reaction conditions: 0.5 mg of protein, pH 6.0, 30°C; (b) lipase amount (mg), reaction conditions: 4% v/v of isoamyl alcohol, pH 6.0, 30°C; (c) temperature (°C), reaction conditions: 4% v/v of isoamyl alcohol, pH 6.0, 6.0 mg of protein; (d) pH, reaction conditions: 4% v/v of isoamyl alcohol, 30°C, 6.0 mg of protein. The palatase was used for the synthesis of isoamyl octanoate. Effects of four different physicochemical parameters on the lipase activity were investigated through observing the time-course production of isoamyl octanoate.

3.3 Bioproduction of Isoamyl Octanoate by Lipase Palatase in Buffer-Esterification vs. Alcoholysis

This experiment was performed to gain further understanding of bioproduction of isoamyl octanoate by the lipase palatase by conducting reactions with isoamyl alcohol and octanoic acid (esterification) or trioctanoin (alcoholysis) as reactants in aqueous buffer media. The findings are illustrated in Figures 4 (a, b).

In the esterification reaction (Figure 4a), isoamyl octanoate was surprisingly synthesised and correspondingly octanoic acid was consumed, indicating that esterification occurred in the aqueous media. In the alcoholysis reaction (Figure 4b), as expected, isoamyl octanoate was produced and octanoic acid was released, suggesting simultaneous alcoholysis and hydrolysis. These findings imply that the lipase palatase catalysed the bioproduction of isoamyl octanoate via both alcoholysis and esterification in coconut cream. However, the time-course data on the release of octanoic acid in coconut cream showed no visible consumption of octanoic acid in alcoholysis, which would have been expected if esterification occurred. This could be due to very fast and dominant esterification upon release of octanoic acid to such an extent that hydrolysis and esterification steps occur simultaneously (Cheirsilp et al., 2008). It is also possible that the lipase palatase behaved differently in the aqueous buffer media and coconut cream because of the microaqueous milieu of the latter.

These findings are in contrast with a previous report that the same lipase can catalyse alcoholysis between tributyrin and ethanol, but not esterification between butyric acid and ethanol (Liu et al., 2003), which may be associated with the harmful effect of polar butyric acid on the activity of the enzyme (Bezbradica et al., 2007), relative to the less harmful, more non-polar octanoic acid used in this study. This discrepancy could also be due to the difference in the hydrophobicity (polarity) of the alcohol and acid substrates used. Butyric acid (Log P = 0.79) and ethanol (Log P = -0.235) used in the study cited above are hydrophilic (polar), which makes

esterification difficult to proceed in an aqueous environment. Isoamyl alcohol ($\text{Log } P = 1.36$) and octanoic acid ($\text{Log } P = 3.05$) are more hydrophobic (non-polar), which may facilitate esterification in aqueous media.

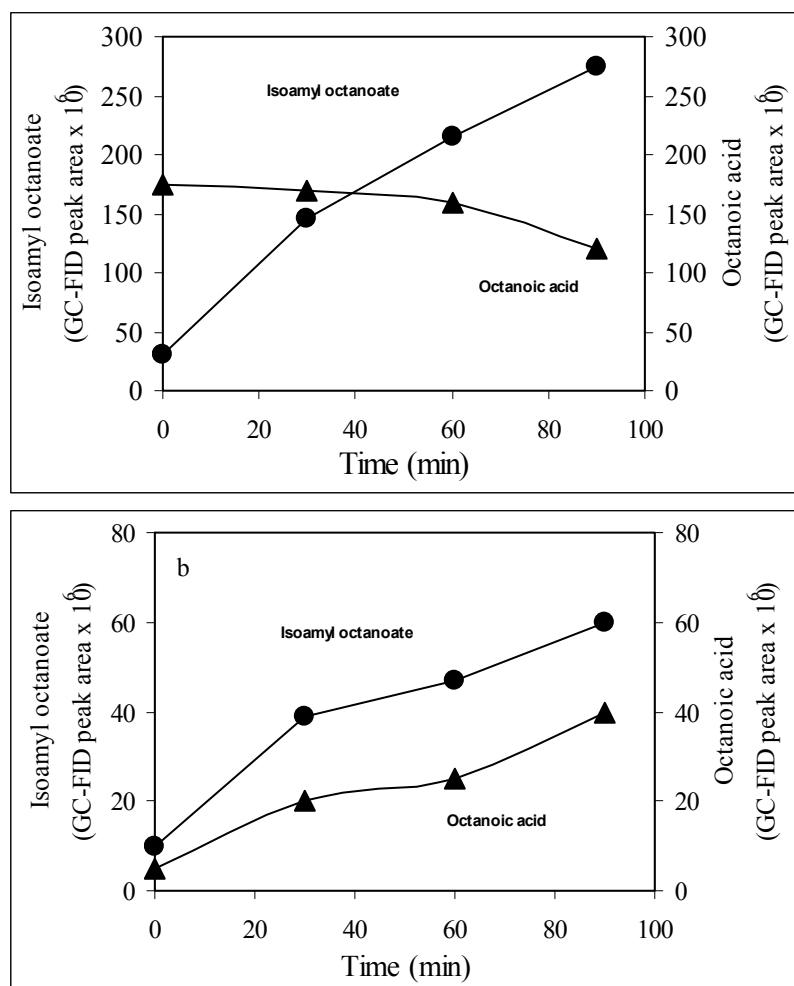


Figure 4. Changes in isoamyl octanoate (●) and octanoic acid (▲) during esterification (a) and transesterification (alcoholysis) (b), as catalysed by lipase palatase

Reaction conditions: 460 mM isoamyl alcohol, 0.5 mg of protein, 95 mM octanoic acid (esterification) or 30 mM trioctanoin (transesterification), 30°C, pH 7.0, 80 mM phosphate buffer (total volume = 100 mL). The catalytic behaviour of lipase during the alcoholysis and esterification reactions in an aqueous buffer system was investigated for the comparison with that in coconut cream system. This result shows the effect of reaction system polarity on the lipase catalytic behaviour.

There are a few studies on lipase-catalysed esterifications in aqueous media, all involving hydrophobic long-chain fatty acids (C_{16} and above), polar and non-polar C_{1-10} alcohols (Ishii et al., 1990; Boutur et al., 1995; Lecointe et al., 1996). Nonetheless, there is still a lack of understanding about esterification and alcoholysis in aqueous systems. By comparison, in solvent-free and organic media systems, the relative polarity of substrates (and products), water activity, water molecules and/or solvent are known to affect biocatalytic activities of the enzyme and thus, esterification and transesterification reactions (Goldberg et al., 1990; Yang et al., 1994; Bezbradica et al., 2007). More hydrophobic fatty acids and alcohols tend to accumulate at the oil-water interface, allowing lipase the access to act on these substrates for direct esterification in both aqueous and non-aqueous environments (Boutur et al., 1995; Marangoni, 2002).

4. Conclusions

This study demonstrated the bioproduction of isoamyl esters by lipases in coconut cream supplemented with isoamyl alcohol. The effects of several physicochemical parameters on the lipase palatase-catalyzed bioproduction of isoamyl octanoate were investigated. It was found that the lipase palatase was able to perform the reactions under a wide range of temperature and pH. Besides physicochemical parameters, the polarity of reaction systems could also affect the catalytic behaviour of the lipase palatase. After reaction, the coconut cream may be of use as flavouring in food applications. This biotechnology may also be applied to *in-situ* generation of fruity flavour in some food products such as cheese.

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