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Optimized synthesis of (Z)-3-hexen-1-yl caproate using germinated rapeseed lipase in organic solvent

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ABSTRACT

(Z)-3-hexen-1-yl esters are important green top-note components of food flavors and fragrances. Effects of various process conditions on (Z)-3-hexen-1-yl caproate synthesis employing germinated rapeseed lipase acetone powder in organic solvent were investigated. Rapeseed lipase catalyzed ester formation more efficiently with non-polar compared to polar solvents despite high enzyme stability in both types of solvents. Maximum ester yield (90%) was obtained when 0.125 M (Z)-3-hexen-1-ol and caproic acid were reacted at 25 °C for 48 h in the presence of 50 g/L enzyme in heptane. Enzyme showed little sensitivity towards a_w with optimum yield at 0.45, while added water did not affect ester yield. Esterification reduced by increasing molecular sieves (>0.0125%, w/v). The highest yields of caproic acid were obtained with isoamyl alcohol (93%) followed by butanol and (Z)-3-hexen-1-o1 (88%) respectively reflecting the enzyme specificity for straight and branched chain alcohols. Secondary alcohols showed low reactivity, while tertiary alcohol had either very low reactivity or not esterified at all. A good relationship has been found between ester synthesis and the solvent polarity (log *P* value); while no correlation for the effect of solvents on residual enzyme activity was observed. It may be concluded that germinated rapeseed lipase is a promising biocatalyst for the synthesis of valuable green flavor note compound. The enzyme also showed a wide range of temperature stability (5–50 °C).

1. Introduction

Derivatives of (Z)-3-hexen-1-ol including C6 aldehydes and acyl esters impart green-flavor notes and a character of freshness to foods and fragrances. Within higher plants, (Z)-3-hexen-1-ol is formed by the lipoxygenase catalyzed oxidation of linoleic acid to form a lipid hydroperoxide. This intermediate is cleaved by hydroperoxide lyase forming (Z)-3-hexen-1-al, (E)-2-hexenal and hexanal. The aldehydes can be reduced using NADH-linked alcohol dehydrogenase to form (Z)-3-hexen-1-ol, (E)-2-hexen-1-ol and hexan-1-ol. These compounds possess the aroma of freshly cut grass. The production of "leaf alcohol" green notes using the lipoxygenase reaction has been achieved on an industrial scale [1,2]. Currently 5–10 metric tons of green flavor notes are produced annually at price of 2500–6000 $US kg^{-1} [3,4].

Conversion of (Z)-3-hexen-1-ol to the acyl ester provides a means to lower its odor threshold and increase its chemical stability [5,6]. Short chain flavor esters such as (Z)-3-hexen-1-yl acetate, butyrate, and caproate can be synthesized using microbial lipases suspended in non-aqueous solvents [7–22]. Fungal lipases are preferred for organic phase biocatalysis (OPB) owing to their ready availability and low cost [23–25]. Lipases from higher vegetative plants including peanut [24], wheat germ [27], papaya [28–30], rapeseed [31–34] and *nigella sativa* seeds [35] have also been used for OPB. The cost for biocatalyst remains an important consideration in OPB. In our previous study [36], various plant seedlings were evaluated for their ability to catalyze synthesis of flavor esters in organic solvents. Of the systems examined, germinated rapeseed showed the highest degree of flavor synthesis.

Enzyme activity, stability, and selectivity are generally influenced by the reaction media and temperature. And also one of the criteria for choosing best biocatalyst is its ability to remain stable in solvent over wide range of temperature. In this study, the influence of process variables (reaction time, temperature, moisture contents, water activity, molecular sieves, enzyme load, its reuse, acid alcohol concentration, organic solvents and various alcohol chain lengths) on (Z)-3-hexen-1-yl caproate synthesis is described under the following headings: solvent characteristics, biocatalyst related factors and substrate. The aim of study is to perform a comprehensive evaluation of (Z)-3-hexen-1-yl caproate synthesis using crude rapeseed lipase acetone powder in accordance with the scheme

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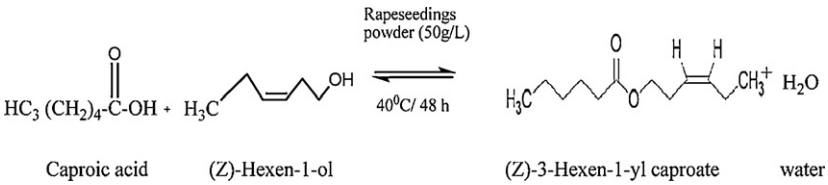

Scheme 1. Synthesis of (Z)-3-hexen-1-yl caproate catalyzed by rape seedlings lipase in organic solvent via esterification.

below and to determine the optimal conditions for (Z)-3-hexen-1-yl caproate synthesis (Scheme 1).

2. Materials and methods

2.1. Materials and chemicals

Analar grade chemicals, acids, alcohols, organic solvents (HPLC grade), were obtained from Sigma–Aldrich Co., Ltd. (Poole, England). Hexane and heptane were obtained from Fisher (Loughborough, UK). Hexane was dried over molecular sieves (3 A, 8–12 mesh; both from Sigma–Aldrich Co., Ltd.) for at least 24 h prior to use. Seeds were supplied by Nickerson Seeds Ltd., Lincoln (UK).

2.2. Acetone powder preparation from rape seedlings

Dry whole rapeseeds were surface sterilized by soaking in 0.1% sodium hypochlorite solution for 30 s, rinsed thoroughly with running tap water and soaked for 24 h at 26 °C (designated as day 1st) in a dark incubator. Germination was achieved by placing rapeseed on moist filter paper towels, on top of moist perlite (Silvaperl graded horticultural) in shallow plastics trays, and then covering with perforated aluminium foil. Samples of seedlings were withdrawn on day 4 after germinating for further processing. In preliminary studies, lipase activity reached to a maximum at 4–6 days after germination [19]. Germinated rapeseed was washed with distilled water three times, equilibrated in a refrigerator at 4 °C for 10 min, cut into small pieces, and then homogenized with 5 volumes of cold acetone (−18 °C or less) for 1 min. The resulting solid was recovered by vacuum filtration using a Buchner funnel, fitted with a Whatman No. I filter paper. Rapeseed lipase acetone powder was washed with 4-volumes of cold acetone and air dried under a hood for 10 h. The light greyish powder was kept in sealed bottles at −20 °C until used.

2.3. Esterification method

Rapeseed acetone powder (50 g/L) was suspended in organic solvents together with 0.25 M of acid and 0.25 M of alcohol. The typical reaction volume was 5 mL. Esterification was performed with shaking at 100 rpm at a temperature of 40 °C for 48 h. Thereafter, 0.1 mL of reaction mixture was withdrawn at known intervals, centrifuged (1300 × *g* for 5 min) to remove suspended matter, and stored at −10 °C until analyzed (usually within 24 h) as described below. All experiments were performed in duplicate. Synthesis was also carried out without enzyme.

2.4. Ester analysis

Routine analysis of reactants and products was conducted by a GLC instrument (Model 5160 Carlo Erba) equipped with a BP-20 fused silica capillary column (SGE, UK, 25 m × 0.32 mm ID; film thickness 1 μm), and a flame ionization detector. The carrier gas was helium (2 mL/min, split ratio 1:15). The GLC oven temperature was maintained at 50 °C for 2 min and then increased to 210 °C at a rate of 15 °C/min and held for 4 min. The injector temperature was fixed at 250 °C and detector temperature at 240 °C. The

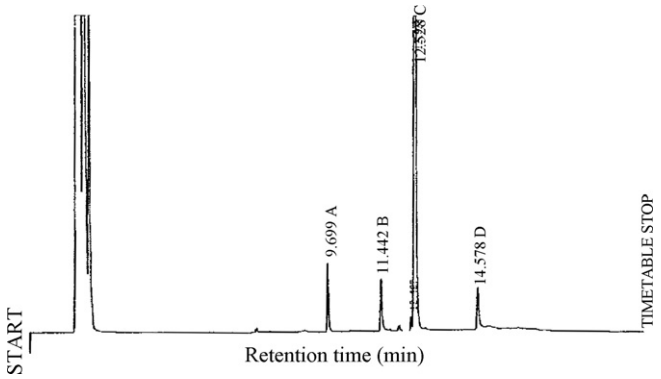

Fig. 1. Gas chromatogram for the analysis of samples obtained from the esterification of caproic acid with (Z)-3-hexen-1-ol after 48 h at 40 °C: (Z)-3-hexen-1ol (A), internal standard (B), (Z)-3-hexen-1-yl caproate (C), caproic acid (D). For gas chromatographic conditions see Section 2.

GLC was connected to an integrator (Hewlett Packard 3395 integrator) which recorded the peak areas and retention times in a chromatogram.

2.4.1. Product identification, yield and reaction time

The identity of ester products is possible to achieve either (a) using the peak retention times obtained with standard compounds (Fig. 1) or (b) interpretation of fragmentation patterns in a GC–MS analysis or comparing these with a library of standard esters (Fig. 2). Ester yield was determined by quantitative analysis using a calibration graph of peak area versus concentration. Standard concentrations of acids, alcohol or ester (0.0125–0.25 M) were diluted with n-hexane and 0.2 μL was subjected to GLC analysis. Injections were repeated twice for each vial. Reaction time-course was determined by monitoring the formation of ester at different time intervals. Percent ester yield was defined as molar ester

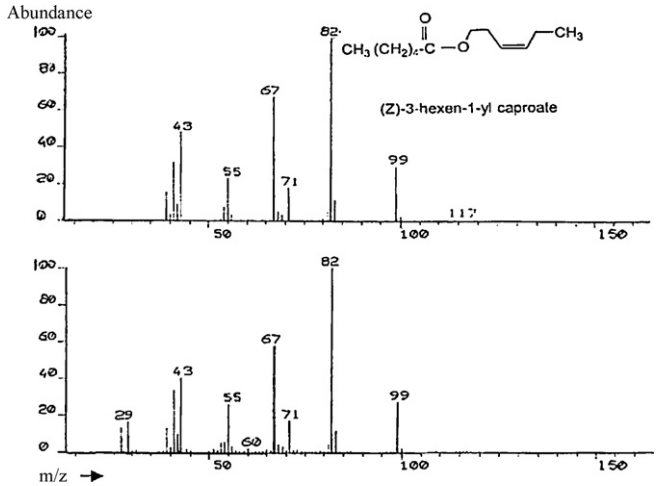

Fig. 2. GC–MS spectra of peak C identified as (Z)-3-hexen-1-yl caproate and its confirmation in this study. (A) Synthesized and (B) library.

formed × 100/molar acid added. The identity of different esters was established by GC–MS analysis (Carlo Erba GC Model 4200, Kratos MS 80 RFA). The GS–MS instrument was equipped with a 38 m × 0.32 μm × 0.5 μm film thickness BP-20 column (SGE, UK); elution was performed as described above for conventional GLC analysis. Injections (0.2 μL) were made on column. Mass spectra were recorded with the ion source energy of 70 eV.

2.5. Effect of solvents

The reaction of (Z)-3-hexen-1-ol with caproic acid was examined using seven common organic solvents of varying hydrophobicity; dioxane, acetonitrile, tetrahydrofuran (THF), diethyl ether, toluene, hexane or heptane. All other reaction conditions were as described above.

2.6. Effect of added moisture and water activity (a_w)

Varying amounts of distilled water (0–30%, v/v) was added to the reaction medium containing rapeseed lipase acetone powder (50 g/L), alcohol and acid. Ester synthesis was performed as above. To examine the influence of a_w on ester yield, first reactants, enzyme, and organic solvents were equilibrated with standard saturated salt solutions at room temperature (21 °C) in separate desiccators for 7 days as reported by Chamouleau et al. [53]. The salt standards were $MgCl_2$ (a_w = 0.33), Mg $(NO_3)_2$ (a_w = 0.55), NaCl (a_w = 0.75), KCl (a_w = 0.86), $ZnSO_4 \cdot 7H_2O$ (a_w = 0.90), and molecular sieves (a_w = 0.04). Reaction was started by mixing the two separately equilibrated phases as described above.

2.7. Effect of reaction temperature

Ester synthesis was performed at 0–80 °C. For temperature below 40 °C a thermo controller (refrigeration unit) was used. Reaction temperatures above 40 °C were maintained using an oil bath filled with Dow Corning Silicon Oil.

2.8. Effect of substrate concentration

Effect of increasing the concentration of one of the substrates was evaluated, while keeping the other constant. (Z)-3-hexen-1-ol concentrations of 0.625 M, 0.125 M, 0.25 M, 0.4 M, 0.5 M and 1 M were reacted with a fixed 0.25 M concentration of caproic acid. In the reverse study, (Z)-3-hexen-1-ol concentration was fixed at 0.25 M and caproic acid concentration was varied at 0.0625 M, 0.125 M, 0.25 M, 0.4 M, 0.5 M and 1 M. The organic solvent phase was hexane at a reaction temperature of 40 °C.

2.9. Enzyme selectivity for alcohols

Caproic acid was reacted with 13 common alcohols in addition to (Z)-3-hexen-1-ol; ethanol, propanol, 2-propanol, butanol, *tert*-butanol, pentanol, isopentanol, hexanol, *tert*-hexanol, heptanol, 3-heptanol, octanol, and geraniol. The reactions were performed with hexane as solvent as described above.

2.10. Lipase stability and reuse

Enzyme stability was determined by incubation with various organic solvents for 24 h followed by drying under nitrogen. The recovered powders were re-suspended with a known volume of distilled water, centrifuged and assayed for remaining lipase activity (see below). To examine the effect of co-substrates on enzyme stability, lipase residual activity was determined following 48 h ester synthesis reaction. Enzyme stability was also evaluated in terms of the degree of re-use. After a 48 h ester synthesis episode, rapeseed lipase was recovered by centrifugation, washed with excess organic solvent and dried under nitrogen. The dried enzyme powder was then added to a fresh mixture of reactants and the yield of (Z)-3-hexen-1-yl caproate analyzed by GLC.

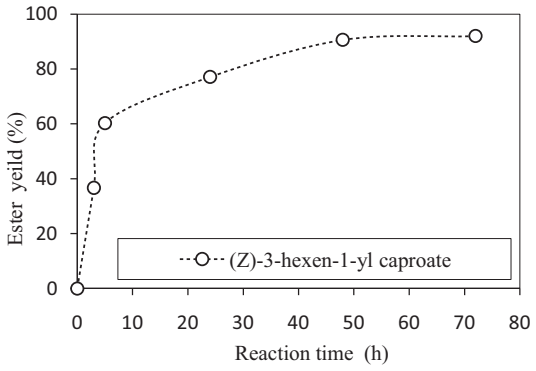

Fig. 3. Time course of (Z)-3-hexen-1-yl caproate synthesis. The reaction mixtures consisted of 0.25 M caproic acid, and 0.25 M butanol or (Z)-3-hexen-1-ol and 50 g/L of rapeseed lipase acetone powder was at 40 °C. All experiments were done in duplicate and the values reported are mean of two determinations.

2.11. Determination of lipase residual activity

Lipase was assayed using the 4-methylumbelliferyl heptanoate (4-MUH) fluorimetric assay. The assay mixture (3 mL) comprised Tris–HCl buffer (0.1 M, pH 8), 25 μL of the 4-MUH (0.01 M in 99–100% ethanol), and 200 μL of lipase solution. The reaction was stopped with the addition of 1 M HCl (0.5 mL) after 30 min and fluorescence was recorded with fluorescence spectrophotometer (Perkin-Elmer, LS-203) at λ_{ex} = 330 and λ_{em} = 450 nm. Blank assays were performed by adding HCl to the reaction mixture before enzyme. A calibration graph for 4-MU determination was constructed from fluorescence measurements recorded with 3–70 μL (or 7.5–175 nmol) of 4-MU stock per 3 mL of assay mixture. A unit of lipase activity was expressed as mole 4-MU produced/min/mL of enzyme solution.

3. Results and discussion

The time scale for (Z)-3-hexen-1-yl caproate synthesis is presented in Fig. 3. With increasing reaction time, an increase in ester yield was observed. (Z)-3-hexen-1-yl caproate synthesis reached completion in 48 h at 40 °C. For the baseline study, product yield was 90% with no further increase beyond 48 h. Non availability of the either of substrates to the enzyme could be the one major reasons, because after 48 h, as 90% of the caproic acid was converted to ester (results not shown). This high conversion was quite surprising since no attempts were made to prevent water accumulation in the reaction mixture. This is a considerably faster reaction compared to reaction of butyl carpylate catalyzed by the psychrotrophic *Pseudomonas fluorescens* P38 lipase which reached an equilibrium after 96 h at 20 °C with a final molar conversion of 75% [54]. In our case, organic phase biocatalysis at 40 °C is expected to be associated with a higher rate of reaction and low organic solvent phase viscosity. Reaction time and product yield are two important process endpoints in this study. A short reaction time reduces overall process cost, decreases substrate inventory, and reduces the requirement for energy. The time of reaction is dependent on kinetic factors such as, enzyme specific activity, amount of biocatalyst used, concentrations of co-substrates, reaction temperature, choice of organic solvent, and the degree of stirring, shaking or sonication that affects mass transfer limitations also affects the reaction rate [38,39].

Table 1
Effect of solvent choice on the yield of (Z)-3-hexen-1-yl caproate.

Organic solvent	Log *P*	Ester yield (%)
1,4-Dioxane	−1.1	0.1 ± 1.7
Acetonitrile	−0.33	1.0 ± 0.0
Tetrahydrofuran	0.49	2.0 ± 0.0
Diethyl ether	0.85	7.9 ± 0.0
Toluene	2.5	36.6 ± 0.7
Hexane	3.5	48.3%[a]
Heptane	4.0	86.5 ± 0.6

[a] See text for details.

3.1. Solvent factors

3.1.1. Effect of solvent on ester synthesis

The effect of different organic solvents, with log *P* values ranging from −1.1 to 4.0, on (Z)-3-hexen-1-yl caproate yield is shown in Table 1; *P* is the partition coefficient for *solvent molecules* between water and octanol [37,38]. Clearly, (Z)-3-hexen-1-yl caproate yield was high for non-polar solvents with log *P* > 2.5. A yield of 86% was obtained in n-heptane followed by hexane (48.3%) after 48 h at 40 °C. In agreement with earlier reports [39,40] hydrophilic solvents (log *P* < 2.0) were associated with a low yield of ester. Hydrophilic solvents such as dioxane (log *P* = −1.1), acetonitrile (log *P* = −0.33), and tetrahydrofuran (log *P* = 0.48) remove a layer of adsorbed water from enzyme particles leading to inactivation [41]. By contrast, non-polar solvents such as hexane (log *P* = 3.5), and n-heptane (log *P* = 4.0) preserve the micro aqueous layer surrounding biocatalyst particles and so help to retain enzymatic activity [41,42] leading to high product yields.

3.1.2. Effect of added water and water activity (a_w)

A minimum amount of water may be essential to produce a degree of enzyme flexibility necessary for catalysis [56]. The yield of (Z)-3-hexen-1-yl caproate (94–97%) was not affected by the addition of water to the organic solvent phase over the range 0.125–30% (w/w) (biocatalyst) (Fig. 4A). Analysis of lipase powders used in this study showed that these contained 9–9.5% (w/w) water measured by oven drying to a constant weight at 105 °C overnight. The total amount of water present in the current reaction system was 0.48–1.95% (v/v) or 0.27–1.08 M expressed with respect to the net solvent volume.

Compared to this, P38 lipase activity was optimum at an organic phase water concentration of 0.25% (v/v) to catalyze the synthesis of butyl caprylate. At a higher or lower water concentration the yield of ester decreased [54]. The optimum amount of water required for organic phase biocatalysis may depend on factors such as the type of organic phase and choice of enzyme [37,56]. Compared to the nominal moisture content of 0.48% (w/v) used for the present baseline studies, previous investigators found 0.1–0.6% (v/v) of added water to be compatible with ester synthesis [43,44]. However, >0.6% (v/v) added water encouraged ester hydrolysis and decreased yield. Inappropriately high moisture levels also reduce the efficiency of OPB due to increased diffusion limitations, interfacial inactivation of enzymes and decreased enzyme stability. In this study, adding 20% (w/w) of water led to agglomeration of lipase powder though this did not seem to affect ester synthesis adversely. As well as the initial moisture content, a_w also rises during OPB due to water produced during esterification [38].

In further investigations of water-relations, all reaction components were pre-equilibrated to known a_w values using standardized saturated salt solution. Under such circumstances ester synthesis was maximum at $a_w = 0.45$ declining slightly at $a_w > 0.53$ (Fig. 4B). Overall, rapeseed acetone powder lipase preparations showed little sensitivity to a_w. Data in Fig. 4B suggests that rape seedling lipase belongs to a group of lipase that functions

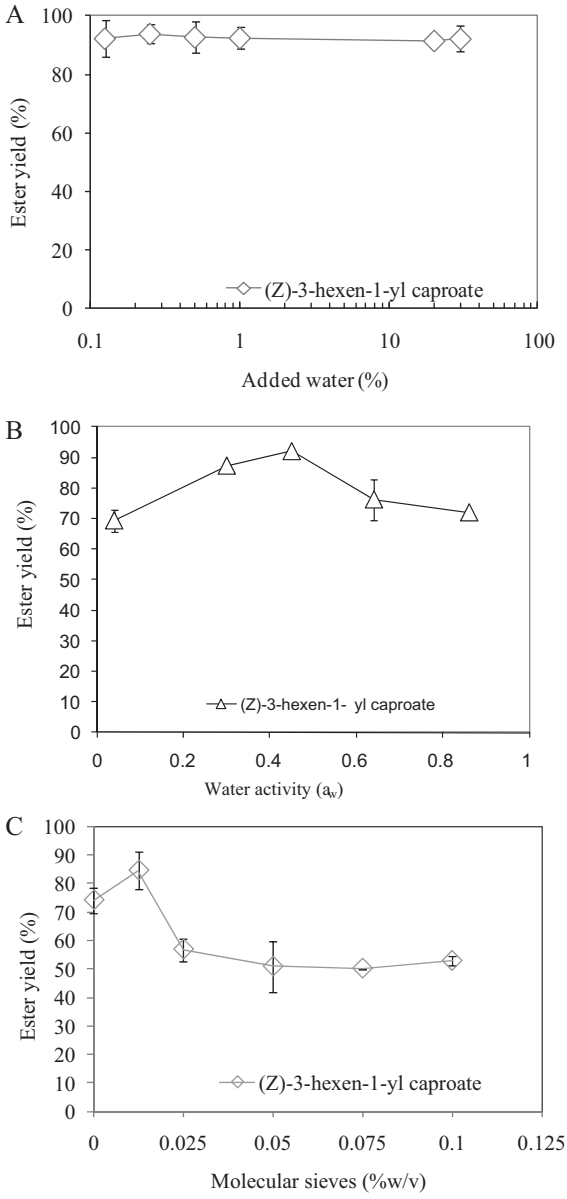

Fig. 4. Moisture–activity relations for ester synthesis: effect of added water (A), water activity a_w (B) or addition of molecular sieves (C) on the synthesis of (Z)-3-hexen-1-yl caproate. The reaction mixture consisted of 0.25 M of butyric or caproic acid with 0.25 M butanol or (Z)-3-hexen-1-ol in 5 mL of hexane at 40 °C in the presence of rape seedling acetone powder (50 g/L) for 48 h.

better at a medium a_w. Most lipases fall into to second category [45]. Lipases from different sources vary widely in their dependence on a_w in non-aqueous solvents [43,44]. Low, medium and high water activity dependent lipases work best at a_w 0.19, 0.60 and 0.75 respectively. Wheat germ belongs to last type with optimum water activity of 0.90.

Moisture effect was also examined by adding 0–0.125% (w/v) of desiccant (molecular sieves 3A) directly to the reaction system. At low levels of addition (<0.0125%, w/v), the yield of (Z)-3-hexen-1-yl caproate ranged from 74 to 84%. Further addition of molecular sieves (>0.0125%, w/v) decreased product yield by about 20% (Fig. 4C). The effect of added desiccants is two fold. At low levels of addition, molecular sieves increase product yield by sequestering water thereby shifting K_{EQ} (reaction equilibrium) towards increase synthesis. However, high amounts of molecular sieves reduce the yield of ester by absorption. Torres and Otero [46] and also Omar

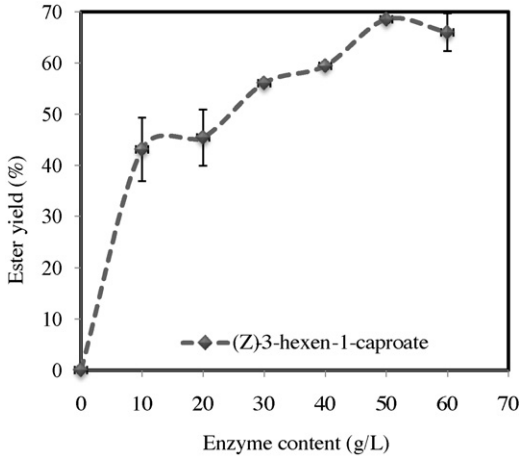

Fig. 5. Effect of enzyme amount on the yield of (Z)-3-hexen-1-yl caproate. The reaction mixture consists of 0.25 M of (Z)-3-hexen-1-ol and 0.25 M caproic acid in 5 mL of hexane containing different amounts of rape seedling acetone powder at 40 °C for 48 h.

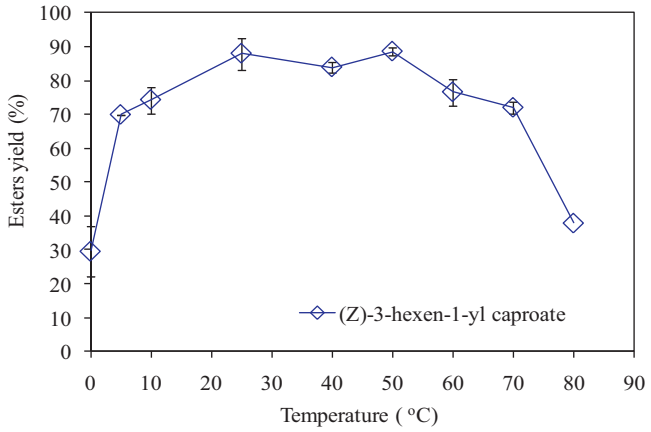

Fig. 6. Effect of temperature on the synthesis of (Z)-3-473 hexen-1-yl caproate. The reaction mixture consisted on 0.25 M of caproic acids and (Z)-3-hexen-1-ol in 5 mL of hexane at 40 °C in the presence of 50 g/L of acetone powder from day 4 of germinating rape seedling.

et al. [42] reported a reduction in the yield of esters in the presence of desiccant due to the absorption of co-substrates particularly acids. Controlling the amount of desiccant employed is necessary to achieve a balance between the opposing effects of the molecular sieves.

3.2. Biocatalyst factors

3.2.1. Effect of enzyme concentration

The amount of lipase is a crucial economic factor for any bioconversion process. The yield of (Z)-3-hexen-1-yl caproate increased with increasing amount of biocatalyst (Fig. 5). However, no significant increase in yield was observed with lipase loadings of more than 50–60 g/L (Fig. 5). At low additions of biocatalyst, ester synthesis did not reach equilibrium within the 48 h reaction time. The concentrations of lipase reported are often too high to meet industrial requirements [57]. For P38 lipase, a concentration of 20 g/L has been reported [54].

3.2.2. Rape seedlings lipase stability in organic solvents

The stability of rapeseed lipase acetone powder was influenced by exposure to solvent choice (Table 2). No significant relation was observed between log *P* values and activity retention. When suspended with pure solvents, high residual lipase activities were observed with THF (log *P* = 0.47), diethyl ether (log *P* = 0.85), hexane (log *P* = 3.5) and heptane (log *P* = 4.0) but toluene (log *P* = 2.5) was deactivating. It has been reported previously that log *P* values is not always a good parameter to predict the toxic effects of solvent in enzyme reactions at high temperature [47,48]. Residual activity of P38 lipase samples after heating at 10–60 °C for 48 h was also determined [54]. Lipase dissolved in water was found to be least stable. In comparison, the dry P38 lipase was stable until 40 °C but became increasingly inactivated at higher temperatures.

Table 2
Effect of organic solvent on rapeseed lipase stability.

Solvent	Log *P*	Residual activity (%)[a]
1,4-Dioxane	−1.1	5 ± 0.00
Acetonitrile	−0.3	63 ± 0.00
THF	0.49	89 ± 0.05
Diethyl ether	0.85	85 ± 0.08
Toluene	2.5	40 ± 0.02
Pentane	3.0	77 ± 0.00
Hexane	3.5	72 ± 0.06
Heptane	4.0	81 ± 0.04

Untreated control has 100% activity. THF, tetrahydrofuran.

[a] Rape seedling acetone powder (50 g/L) was exposed to different solvents for 24 h at 25 °C, dried under nitrogen gas and residual activity was determined by fluorimetric assay.

The effect of different organic solvents on the stability of rapeseed acetone powder lipase is likely to be different from observations using purified proteins suspended in pure organic solvents [38–42]. In such idealized systems, biocatalyst stability is generally higher for high log *P* solvents accounting for the high product yield normally associated with non-polar solvents. Crude acetone powder preparations also contain considerable amounts of starch which is likely to affect the outcome of these studies. Finally, regardless of the destabilizing effect of co-substrates, rapeseed acetone powder lipase could be reused. The yield of (Z)-3-hexen-1-yl caproate was 87%, 62% and 48% for three successive uses.

3.2.3. Effect of reaction temperature

The temperature dependence of ester synthesis is shown in Fig. 6. (Z)-3-hexen-1-yl caproate formed over a broad temperature range of 5–50 °C, with the maximum yield at 25–50 °C. A low temperature sensitivity is observed in this study which suggests that (Z)-3-hexen-1-yl caproate could be produced at ambient temperatures (25–30 °C), without stringent temperature control; this feature can be expected to lead to considerable savings in energy cost for ester production. Compared to the present results, the optimum temperature for ester synthesis using papaya (*Carica papaya*) lipase was 63 °C [30]. Temperature optimum for butyl caprylate synthesis, using *Pseudomonas* P38 lipase was 20 °C [54]. The decrease in ester synthesis above 20 °C was associated with lipase inactivation at higher temperature. In general, thermostability within an organic solvent is achieved if the enzyme is intrinsically rigid, or if the environment (e.g. low water activity) prohibits enzyme flexibility. Enzymes that are stable/rigid within organic solvent phases are also likely to exhibit low specific activity at low temperatures. On the other hand, high specific activity within solvents may be associated with a highly flexible and relatively heat-labile enzymes [55].

3.3. Substrate factors

3.3.1. Effect of alcohol structure

The effect of alcohol structure on ester synthesis was examined by considering chain length, position of the hydroxyl groups, degree of branching, presence of unsaturated bonds and geometric isomerism (Table 3). Rapeseed lipase showed selectivity for C3:0–C6:0

Table 3
Effect of alcohol structure on the synthesis of caproic acid esters catalyzed by rapeseed lipase.

Alcohol	Ester yield (%)
Methanol	43
1-Propanol	82
2-Propanol (isopropanol)	35
1-Butanol	88
2-Methyl-2-propanol (*tert*-butanol)	0
1-Pentanol (amyl alcohol)	71
3-Methyl-1-butanol (isoamyl alcohol)	93
Hexanol	60
2-Methyl-2-pentanol (*tert*-hexyl alcohol)	2
(Z)-3-hexen-1-ol	88
(E)-2-hexen-1-ol	36
1-Heptanol	53
3-Heptanol	29
1-Octanol	49

Reaction was carried out at 40 °C for 48 h in 5 mL of hexane containing 0.25 M of various alcohols and 0.25 M caproic acid, and 50 g/L of rape seeding lipase. Results are average of two independent determinations with highest errors on mean was less than 10%.

alcohols whilst the yield of ester decreased for C7:0–C8:0 alcohols. Primary alcohols (1-propanol and 1-butanol) were more effectively esterified compared with secondary or tertiary alcohols (2-propanol, t-butanol or t-hexanol). Yields were higher for (Z)-3-hexen-1-o1 compared to either hexan-1-ol or (E-) 3-hexen-1-ol. The cis/trans selectivity may be due to shape differences between cis- and trans-isomers. Previous studies show that lipases from *Mucor miehei*, *Aspergillus*, *Candida rugosa*, and *Rhizopus arrhizus* [25] and also goat pregastric lipase [49] have selectivity for C4:0–C6:0 alcohols. The purified lipase from oilseed rape (*Brassica napus* L. cv Ceres) did not esterify secondary alcohols [50] and papaya lipase was equally reactive with isoamyl alcohol and pentanol [30].

3.3.2. Effect of substrate concentration

The goal is to increase the concentration of reactants in hexane without causing adverse effects on the reaction rate or the yield. The concentration of acid or alcohol used in this study affected the yield of (Z)-3-hexen-1-yl caproate (Fig. 7). When the concentration of caproic acid or (Z)-3-hexen-1-ol was increased (0.06–1.00 M) whilst the other co-substrate was fixed at 0.25 M, product yield increased until the caproic acid and alcohol concentration reached 0.125 M. Further increasing the concentration resulted in a decrease synthesis. The loss of effectiveness resulting from an increase of substrate concentration may be due either to a substrate inhibition or, more likely, to a modification of the polarity of the medium. Effect was more pronounced for alcohol. Loss of synthetic activity at high alcohol concentrations might be due to its dehydrating effects to rape seed lipase stability.

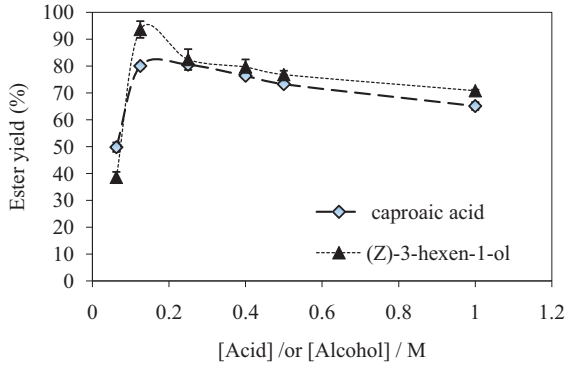

Fig. 7. Effect of caproic acid (acid) and (Z)-3-hexen-1-ol (alcohol) concentration on the synthesis (Z)-3-hexen-1-yl caproate. Acid or alcohol concentration is changed while the co-substrate was kept constants 0.25 M. System contained 50 g/L of rape seedling acetone powder. All reactions were carried out over period of 48 h at 40 °C with no added water.

Alcohols have been shown to inhibit lipases from *Bacillus licheniformis* [26], *M. miehei* [40], *Candida antarctica* [51], *Candida cylinracea* [52], and goat pregastric lipase [49]. The inactivating effect of different alcohol declines with their log *P* value which increases with chain length. Methanol is more inactivating compared to long chain alcohols such as butanol and octanol. The inactivating effects of acid co-substrates are related to their pK_a value which increases (acidity decreases) with chain length. During OPB using lipases, high concentrations of butyric acid were tolerated [23,40,34,52] but not acetic acid [5,6,25].

4. Conclusions

This study shows that (Z)-hexen-1-yl caproate can be produced in quantitative yields using rapeseed lipase (50 g/L) suspended in hexane or heptane as organic solvent under mild reaction conditions (40 °C, 48 h). Ester synthesis is dependent on organic solvent characteristics, biocatalyst factors and substrate factors. The cost of biocatalyst remains an important consideration in OPB as purified enzymes are expensive. Crude seedling powder is potentially inexpensive alternative form of biocatalyst for OPB. Procedures for preparing acetone powder are simple, making it quite suitable for synthesis of green note flavor ester. Moreover, the impact of the amount of enzyme is crucial and rape seedlings acetone powder can be reused without stringent control of moisture at a broad range of temperature (5–50 °C) in non-polar solvents. This work illustrates the possibility of using acetone powder lipase from germinated rapeseeds for low temperature biocatalysis. This is particularly important because, commercially, use of ambient temperature (25–30 °C) is economical. Low temperature OPB might in future have applications in the preparation of heat-sensitive, high value, products. The present data is highly encouraging suggest that optimization studies employing pilot scale studies are warranted. Finally, certain limitations of the current discussion should be highlighted chief amongst which is the realization that rapeseed acetone powder could conceivably contain more than one lipases species. The substrate selectivity demonstrated here, applies to caproic acid as the co-substrate which may change when different acid substrates are considered.

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