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Rapeseed Lipase Catalyzed Synthesis of Butyl Butyrate for Flavour and Nutraceutical Applications in Organic Media

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Abstract. Butyl butyrate, a short chain ester with fine fruity pineapple odour, is a significant flavour compound. Recent investigations show that butyrate esters also have anticancer activity. Factors influencing the synthesis of butyl butyrate by organic phase biocatalysis were investigated. Maximum ester yield of 89% was obtained when 0.25 M butanol and butyric acid were reacted at 25 °C for 48 h in the presence of 250 mg rape seed lipase acetone powder in hexane. Addition of water did not affect synthesis, while a water activity of 0.45 was found optimum. Of 15 different alcohols evaluated, isoamyl and (*Z*)-3-hexen-1-ol were esterified most effectively with molar conversion yields of 92.2 and 80.2%. Short chain primary alcohols such as methanol and medium-long chain alcohols, such as heptanol and octanol were esterified more slowly. The results show that rape seed lipase is versatile catalyst for ester synthesis with temperature stability range 5-50 °C.

Keywords: flavour, butyl butyrate, rape seedling, biocatalysis, esterification, anticancer agent

Introduction

Esters of butyric acid are important as flavour compounds (Leblanc *et al*., 1998). Ethyl butyrate and isoamyl butyrate are found in the aroma of strawberry and banana. The butyrate ester of isoamyl alcohol is a valuable, high demand flavour and fragrance compound widely used in the food, beverage and pharmaceutical industries. The world market for flavours is thought to account for a quarter of the total food additive market. An emerging area of application of butyrate esters is as nutraceutical agents. Naturally occurring butyrate esters such as tributyrin as well as synthetic esters have been shown to possess antiproliferative action against a wide variety of cancer cell lines. Anti-tumour activity was also demonstrated *in-vivo* (Kuefer *et al*., 2004).

Direct synthesis of esters from fatty acids and alcohols by enzymatic methods has been suggested as a good alternative route to industrial catalysis. Butyrate esters and other short chain flavour esters can be synthesized by organic phase biocatalysis (OPB) to satisfy commercial demands (de Baros *et al*., 2009; Pires-Cabral *et al*., 2009; Torres *et al*., 2009; Ben Salah *et al*., 2007; Romero *et al*., 2005). Fungal lipases are preferred for organic phase biocatalysis (OPB) owing to their ready availability and low cost (Abbas and Comeau, 2003; Krishna *et al*., 2000; Langrand *et al*., 1999). Lipases from higher vegetative plants including wheat germ (Xia *et al*., 2009), papaya (Miyazawa *et al*., 2008; Caro *et al*., 2000) and rapeseed lipase (Mukherjee and Jachmanián, 1996; Ncube *et al*., 1993; Hills *et al*., 1990) have also been used for various purposes in OPB. 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 technical use (El *et al*., 1998). Earlier, we had evaluated various plant seedlings in OPB and results showed that acetone powder obtained from day 4 germinated rape seed was potentially useful biocatalyst for the synthesis of low molecular weight flavour esters (Liaquat and Apenten, 2000). Butyl butyrate was amongst the esters formed in a good yield.

In the present study, the impact of several parameters on synthesis of butyl butyrate catalyzed by crude rape seedlings powder was carefully investigated first time, which included effect of added water, water activity, substrate concentration, temperature and incubation time. The ability of enzyme to catalyze the synthesis of

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organic esters commonly used in the flavour industry by esterification of butyric acid as acylating agent with various alcohols in hexane was also studied.

Materials and Methods

Reagent grade chemicals, acids, alcohols, organic solvents (HPLC grade), salts and esters were obtained from Sigma-Aldrich Co. Ltd., Poole, England. Hexane and heptane were obtained from Fisher Scientific Ltd. (Loughborough, UK). Hexane was dried over molecular sieves (3A, 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.

Acetone powder preparation from rape seedlings. Dry whole rapeseeds were surface sterilized by soaking in 0.1% sodium hypochlorite solution for 30 sec., 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. 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.

Direct esterification conditions. Unless otherwise stated, 0.25 g of seedlings powder was added to 5 mL of hexane containing 0.25 M of acid and 0.25 M of alcohol. Synthesis was performed by shaking reaction vessels at about 100 rpm at a constant temperature of 37 °C. The concentrations of ester formed were determined by withdrawing samples (1 mL). These were then centrifuged (1300 × g for 5 min at room temperature) to remove the residual lipase. Aliquots of 0.5 mL reaction mixture were taken from the supernatant and stored at -10 °C until analysed (usually within 24 h). The frozen samples were allowed to warm to room temperature and then analyzed by gas chromatography to determine the concentration of ester, alcohol and acids. Esters synthesis is expressed as percentage molar conversion of acids. All synthesis experiments were performed in duplicate using separate reaction vials. A control experiment was also carried out to check spontaneous esterification. Products of synthesis were analyzed by GC.

Gas chromatographic analysis. The gas chromatography system consisted of Carlo Erba apparatus (Model 5160) equipped with a flame ionization detector. Separation involved a BP-20 fused silica capillary column (SGE, UK, 25 m × 0.32 mm ID; film thickness 1 micron) operated with helium gas as carrier (2 mL/min, split ratio 1:15). The 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 GC was connected to an integrator (Hewlett Packard 3395 integrator) which recorded the peak areas and retention times in a chromatogram.

Esters identification and quantification. Esters, alcohol and acids were identified according to their retention times on chromatograms and from comparisons with results obtained with standards. A calibration graph of known acid concentration *vs* corresponding peak area was constructed. Various concentrations of acid (0.0125 M-1 M) were prepared by diluting in *n*- hexane and 0.2 μL of each was injected into GC. Injection was repeated twice for each vial. The percentage conversion of acids and alcohols were calculated by the following formulae:

$$\text{Ester yield (\%)} = \frac{\text{(molar ester produced)} \times 100}{\text{(molar acid added)}}$$

$$\text{Molar conversion (\%)} = \frac{100\,([\text{Acid}]_0 - [\text{Acid}]_F)}{[\text{Acid}]_0}$$

where subscripts 0 and F denote initial and final concentrations, respectively.

Effect of added moisture and water activity (a_w). Varying amounts of distilled water (0-30% v/v) were added to the reaction medium containing rapeseed lipase acetone powder 25 mg (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 described by Goderis *et al.* (1987). The salt standards were $MgCl_2$ (a_w = 0.113), Mg $(NO_3)_2$ (a_w = 0.45), NaCl (a_w = 0.74), KCl (a_w = 0.86), $ZnSO_4.7H_2O$ (a_w = 0.90), and molecular sieves (a_w = 0.04). Ester synthesis was initiated by mixing the three reaction components followed by incubation at 40 °C with shaking (100 rpm) for 48 h. Synthesis was also carried out without enzyme (controls).

Reaction temperature. The effect of temperature on ester synthesis was studied by incubating reaction mixtures at various temperatures (0-80 °C). For temperatures below 20 °C, a thermo controller (cooled refrigerator) was used. For temperatures above 40 °C, an oil bath filled with Dow Corning silicon oil was used for incubation.

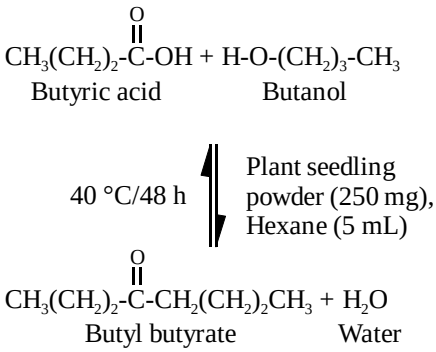
Effect of substrate concentration. The effect of increasing the concentration of one of the substrates was evaluated, while keeping the other constant. The concentration of butyric acid added was 0.0625, 0.125, 0.25, 0.4, 0.5, 1 M while keeping the alcohol concentration and other variables constant. Similarly concentration of alcohol (butanol) was varied while keeping the acid concentration constant (0.25 M). Studies were carried out in hexane at 40 °C (optimum temperature for ester synthesis as determined above).

Time course studies. For the time course experiment, the esterification reaction was monitored at different intervals until the reaction reached equilibrium. Samples of the reaction medium were drawn at given timed intervals and analyzed for butyl butyrate (percent acid molar conversion) concentration. Equilibrium was reached when the product concentration remained constant.

Alcohol specificity for ester synthesis. The specificity of lipase from rape seedlings for different alcohols was checked using butyric acid as acyl donor. The alcohols used were ethanol, propanol, 2-propanol, butanol, ter-butanol, pentanol, isopentanol, hexanol, (*Z*)-3-hexen-1-ol, ter-hexanol, heptanol, 3-heptanol, octanol and geraniol.

Results and Discussion

Optimization of butyl butyrate synthesis in *n*-hexane. Butyl butyrate was formed by direct esterification of butanol with butyric acid in hexane as shown in the given scheme. Esters were identified by comparing their retention times with those of authentic standard esters and by matching their mass spectra with those of standard esters as well as with the NBS library of flavours and fragrances as described previously (Liaquat and Apenten, 2000).



Time course of butyl butyrate synthesis. The reaction catalyzed by the rape seed lipase acetone powder reached equilibrium after 48 h at 40 °C with a final ester yield of 68% (Fig. 1). Both ester yield and substrate molar percentage yield are shown. Compared to reaction for butyl caprylate using cold-adapted lipase from psychrotrophic *Pseudomonas* P38 lipase in *n*-heptane at 20 °C (Tan *et al.*, 1996) which reached equilibrium after 96 h, this is a considerably faster reaction. High temperature organic phase biocatalysis is expected to be associated with a faster rate of reaction and lower 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

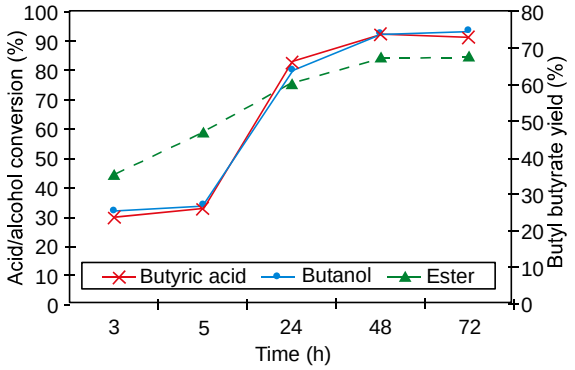


Fig. 1. Time course of the synthesis of butyl butyrate. The reaction mixtures consisted of 0.25 M of butyric acid and 0.25 M butanol in 5 mL of hexane. Reaction medium was incubated at 40 °C in the presence of 250 mg rape seed acetone powder.

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 and also the reaction rate (Halling, 1994; Takahashi *et al*., 1985).

Effect of added water on butyl butyrate synthesis. Ester yield of 96% was obtained with up to 30% (v/v) added water (Fig. 2) and the yield was not affected by the amount of water in the reaction mixture. The acetone powder used in the present study contained 8.7 to 9.5% water measured by oven drying the sample to a constant weight at 105 °C overnight. Water contents greater than 20% appeared to produce agglomeration of lipase powder but did not have any effect on the synthesis.

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 (Tan *et al*., 1996). The optimum amount of water required for organic phase biocatalysis may depend on factors such as the type of organic phase and the choice of enzyme (Zaks and Russell, 1988). An organic phase water content of 0.1-0.6% (v/v) has commonly been adopted (Shaw and Lo, 1994) and 1% for goat pregastric lipase (Lai and O'Connor, 1999).

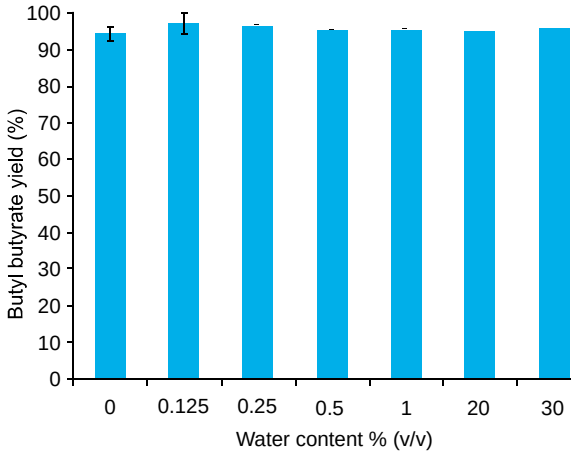


Fig. 2. Effect of added water on butyl butyrate synthesis. The reaction mixture consisted of 0.25 M of butyric acid with 0.25 M butanol in 5 mL of hexane. Reaction medium was incubated at 40 °C for 48 h in the presence of 250 mg rape seed acetone powder.

Higher water content levels have been shown to reduce the product yield of lipase catalyzed reactions in organic phase (Valivety *et al*., 1993).

Effect of water activity (a_w) on the synthesis. The effect of water activity (a_w) on the synthesis of butyl butyrate is shown in Fig. 3. Crude rape seedlings lipase showed maximum ester synthesis activity at a_w = 0.45. Lipases from different sources vary widely in dependence of catalytic activity on a_w (Valivety *et al*., 1992). Wheat germ lipase had a high activity and enantioselectivity in *n*-hexane with a high initial water activity 0.97 (Xia *et al*., 2009). Rape seedling lipase is thought of belonging to a group of lipases that function medium a_w. This feature is useful for synthetic applications in order to suppress hydrolytic side reactions.

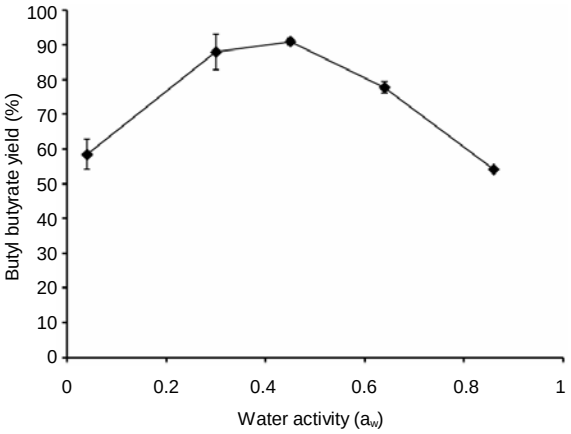


Fig. 3. Effect of water activity ($a_{w)}$ on the butyl butyrate synthesis. The reaction mixture consisted on 0.25 M of butyric and 0.25 M butanol in 5 mL hexane. Reaction medium was incubated at 40 °C for 48 h in the presence of 250 mg rape seed acetone powder.

Effect of reaction temperature on butyl butyrate synthesis. The reaction was optimal at 30-50 °C (Fig. 4). However, rape seedling powder can also be used to synthesize esters in good yield even at low temperatures (5-25 °C) leading to a yield of 61-78% for butyl butyrate. The decrease in ester yield at temperature above 50 °C is probably a result of the catalyst instability. Optimum temperature for a given enzymatic reaction depends on the enzyme source, type of immobilization (if any) and the pH of the reaction mixture (Dordick, 1989). Esterification yield of 90% was reported for the same ester in hexane with

immobilized porcine pancreatic lipase (deCastro *et al*., 1999). However, temperature optimum for butyl caprylate synthesis, using *Pseudomonas* P38 lipase was 20 °C (Tan *et al*., 1996). 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.

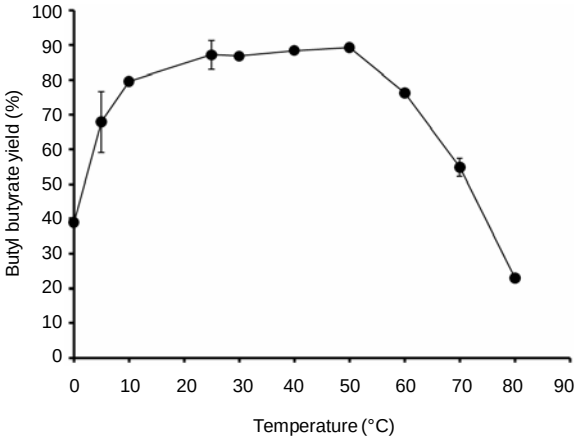


Fig. 4. Effect of reaction temperature on butyl butyrate synthesis. The reaction mixture consisted of 0.25 M of butyric acid and 0.25 M butanol in 5 mL of hexane. Reaction medium was incubated at 40 °C for 48 h in the presence of 250 mg rape seed acetone powder.

Effect of alcohol and acid on ester synthesis. With a system containing a fixed concentration of 0.25 M of alcohol, increasing the acid concentration from 0.0625-0.25 M increased ester yield up to 86.46%. Further increase did not lead to improvement (Fig. 5). The lower conversion obtained below 0.25 M of butyric acid is probably a simple consequence of the lower concentration of acid substrate. Likewise the ester yield increased with increasing the butanol concentration until 0.25 M (Fig. 6). Increasing butanol concentration above 0.25 M adversely affected the yield. Loss of synthetic activity at high alcohol concentrations might be due to its dehydrating effects on rape seed lipase stability. Such results indicate that butanol may act as a competitive inhibitor to the reaction, or a high concentration of the alcohol might be detrimental to rape seed lipase stability. Alcohols are reported to be terminal inhibitors of lipases (Alvarez-Macarie and Baratti, 2000; Chowdary *et al*., 2000; Chulalaksananukul *et al*., 1993) and acids may cause acidification of the microaqueous interface leading to enzyme inactivation. However, the acidification of the microaqueous interphase may be less pronounced in the present case, since butyric is a comparatively weak acid and more hydrophobic than the other low molecular weight acids such as acetic and propionic acids.

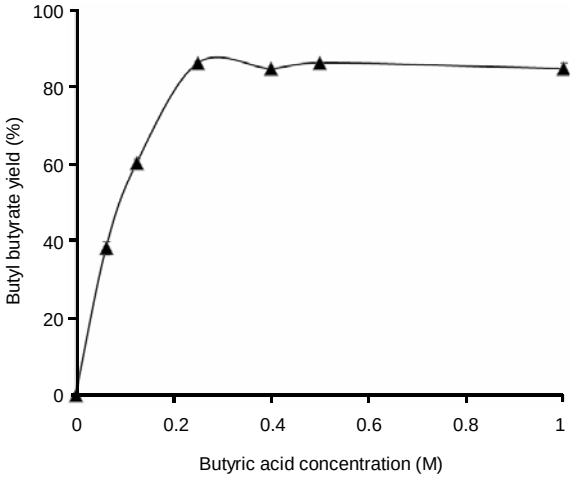


Fig. 5. Effect of butyric acid concentration on ester butyl butyrate synthesis while butanol concentration of the respective system remains constant at 0.25 M. 250 mg of rape seedling acetone powder was suspended in 5 mL of hexane containing the substrates. All reactions were carried out over period of 48 h at 40 °C with no added water.

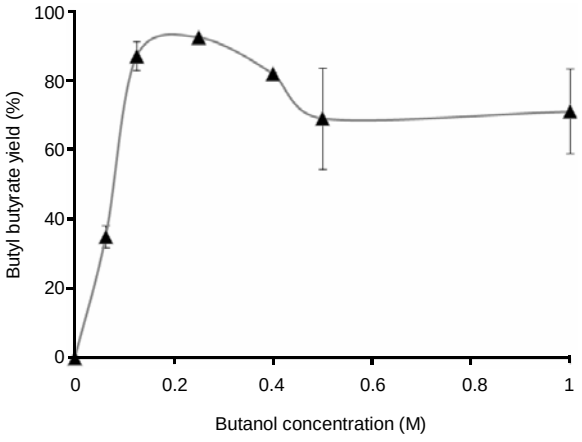


Fig. 6. Effect of butanol concentration on the butyl butyrate synthesis, while butyric acid concentration of the respective systems remain constants at 0.25 M. 250 mg of rape seed acetone powder was suspended in 5 mL of hexane containing the substrates. All reactions were carried out over period of 48 h at 40 °C with no added water.

Effect of alcohol structure on the esters synthesis. Short chain primary saturated alcohols (1- propanol and 1- butanol) were esterified by rape seedling powder in higher yields than *sec*-alcohol (2-propanol). The molar conversion yield of butyric acid with branched chain isopentanol and (*Z*)-3- hexen-1-o1 was 92.2 and 80.5%, respectively, which are the highest yields obtained in the present study (Table 1). These results demonstrate that rapeseed lipase has good specificity for both isoamyl alcohol and (*Z*)-3-hexen-1-ol. Tertiary alcohols were either not esterified by the rapeseed lipase or showed little reactivity. There was a decrease in reactivity as the chain length of the alcohol increased. The greater affinity of crude rape seedling lipase for isoamyl and (*Z*)-3-hexen-1-ol alcohols as compared to their reaction with 1-pentanol and hexyl alcohol, indicates that these are recognized by the rape seedling lipase as different and not like simple alcohols. Broad specificity of seedling powder in the present study might be due to the crude nature of acetone powder. The crude rape seedling lipase behaves similar to the crude papaya lipase (Gandhi and Mukherjee, 2000) and to microbial lipases (Karra-Chaabouni *et al*., 1998; Rotticci *et al*., 1998) which esterify primary alcohols but not tertiary alcohols. Literature reports also demonstrate that branched substrates (either alcohol or acid) are poor substrates for lipase catalysis (Chowdary *et al*., 2000; Rangheard *et al*., 1992).

Table 1. Effect of alcohol structures on the synthesis of butyric acid esters catalyzed by rape seed lipase. Reaction was carried out at 40 °C for 48 h in 5 mL of hexane containing 0.25 M of alcohols and 0.25 M butyric acid, and 50 g/L of rape seed lipase. Results are average of two independent determinations; highest errors on means were less than 10%

Alcohols	Butyric acid molar conversion (%)
Methanol	35.6
1-Propanol	48.8
2-Propanol/(*iso*-propanol)	20.1
1-Butanol	54.5
Tert-butyl alcohol/(2-methyl-2-propanol)	0
1-Pentanol/(amyl alcohol)	53.6
Iso-amyl alcohol/(3-methyl-1-butanol)	92.2
Hexyl alcohol	27.5
Tert-hexyl alcoho/(2-methyl-2-pentanol)	8.1
(*Z*)-3-hexen-1-ol/*cis*-3-hexen-1-ol	80.5
Trans-2-hexen-1-ol	0
1-Heptanol	26.4
3-Heptanol	25.0
1-Octanol	23.4
Geraniol	49.0

Geranyl esters are essential fragrance compounds used in food, cosmetic and pharmaceutical industries. Rape seedlings powder has also catalyzed the reaction with geraniol and a yield of 49% in 48 h was obtained. This yield is 20% lower than that obtained by Kim *et al*. (1998) after 72 h. Further studies are needed to maximize synthesis of gerenyl esters with this enzyme.

Conclusion

Production of low molecular weight esters as flavour compounds by biotechnological processes has a potential interest for the food industry. Crude acetone powder made from germinating rape seedlings was used for butyl butyrate ester synthesis by direct esterification of butyric acid with butanol in *n*-hexane which was rarely investigated. Different reaction parameters for enhancing ester formation were investigated. Conversion yield of 89% was obtained at 25 °C after 48 h at water activity of 0.45 in *n*-hexane without added water. The esterification reduced by increasing alcohol concentration beyond 0.25 M and by raising reaction temperature above 50 °C. The highest molar conversion yields of butyric acid were obtained with isopentanol and (*Z*)-3-hexen-1-o1 reflecting the enzyme specificity for branched chain alcohols. Alcohol chain length higher than (C_6) reduced butyric acid ester formation. This work illustrates the possibility of using rape seed lipase acetone powder 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. 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.

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