Rape Seedling Lipase Catalyzed Synthesis of Flavor Esters Through Transesterification in Hexane

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Summary: Butyl butyrate, a short-chain ester with fruity pineapple odor, is a significant flavor compound that is widely used in the food industry. Enzymatic synthesis of butyl butyrate by crude rape seedlings lipase has been investigated in n-hexane using 50 g/L of enzyme at 40 °C through alcoholysis of ethyl butyrate with butanol. The influence of reaction parameters such aswater content, water activity, substrate concentrations and reaction time were also studied. Ester yield of 60% after 96 h has been obtained with 0.5% (v/v) of added water using reversible reaction or activated ester. A concentration of 0.1M of butanol while 0.6 M of ethyl butyrate was found optimal for butyl butyrate synthesis. The esterification catalyzed by lipase was inhibited by increasing the butanol concentration beyond 0.10 M while no inhibition of enzyme was observed with ethyl butyrate. (Z)-3-hexen-1-ol (*cis*-3-hexen-1-ol) esters posses the odour of freshly cut grass and are used to obtain natural green top notes in food flavours. Two different approaches for rapeseed lipase catalyzed synthesis of these flavour esters were also studied. Acylation of (Z)-3-hexen-1-ol with vinyl acetate (irreversible acyl donor) and butyl caproate (reversible acyl donor) was evaluated. Ester yield of 99 % after 24 h was obtained for (Z)-3-hexen-1-yl acetate with vinyl acetate as acyl donor. Crude rape seedlings lipase has proved to be an efficient catalyst to obtain (Z)-3-hexen-1-ol esters using irreversible acyl donor such as vinyl ester in hexane. Crude lipase also works well at ambient temperature without need of immobilization.

Introduction

Butyl butyrate is in high demand as a component of pineapple flavour in the food, beverage and pharmaceutical industries. Butyrate esters are also applied as nutraceutical agents. Moreover, butyrate esters such as tributyrin as well as synthetic esters have also been reported to have antiproliferative action against a wide variety of cancer cell lines. Anti-tumor activity was also demonstrated in-vivo [1]. Natural flavour esters extracted from plant material are often in short supply and those produced by fermentation are too expensive for commercial use [2]. Therefore, a selective enzyme mediated synthesis can be a good alternative as it is performed under moderate conditions compared to chemical syntheses.

Low molecular flavour esters (LMWE) can be synthesized by organic phase biocatalysis (OPB) to satisfy increasing commercial demands. Esters produced by OPB are thought to comply with the Food and Drug Administration (FDA) definition of natural. This mode of production makes food industry less dependent on seasonal, climatic and geographic variations. The cost for biocatalyst remains an important consideration. Processes that utilize plant enzymes, however, may have advantages because of their lower cost and ready availability [3]. Purified enzymes are more expensive than crude extracts and therefore, commercially not economical. Synthesis of LMWE including butyl butyrate catalyzed by microbial through direct esterification has been extensively studied [4-8]. However, the formation of water in direct esterification reactions poses problems as it favours the reverse (hydrolysis) reaction [9]. Inactivation of the catalyst was also observed due to more polar nature of the substrates [10].

Transesterification is preferable to esterification because, (a) there is no formation of water and (b) inhibition by the acid or alcohol does not occur [11]. Often, increased yields and improved enzyme stability were reported through transesterification (acyl transfer) reactions in organic phase biocatalysis [12, 13]. Microbial lipase catalyzed production of commercially important flavor esters through transesterification has been reported by many authors [14-16]. In lipase catalysed transesterification, the enzyme reacts with acyl donor 1 (AcOR) to give deacylated product 2 (ROH) and acyl-enzyme intermediate (Ac-Enz) and then the acyl-enzyme intermediate reacts with acyl acceptor 3 (R' OH) to give acylated product 4 (AcOR') and free enzyme (scheme1).

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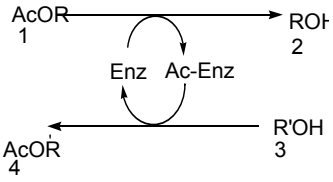
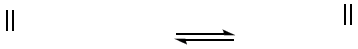


Scheme 1: Lipase catalysed transesterification. Abbreviations are as defined above. The net reaction is also called alcoholysis.

There appear to be no reports describing the use of plant-derived lipases or acetone powder extracted rapeseed lipase for LMWE synthesis through transesterification reactions in organic solvents. Therefore, the ability of acetone powder extracted crude rapeseed lipase to catalyze the synthesis of butyl butyrate through alcoholysis using reversible acyl donor was investigated (as shown in scheme 2). Also in this study, activated or irreversible acyl donor (vinyl acetate) was compared with non-activated or reversible acyl donors (butyl caproate and (Z)-3-hexen-1-yl caproate) to check alcoholysis reaction efficiency using acetone extracted seedlings lipases in organic media.

The influence of reaction parameters such as substrate concentrations, amount of added water and time course of reaction has been studied for efficient ester synthesis using reversible acyl donor. Ethyl butyrate acts as acyl donor1 (AcOR) while butanol has been used as acyl acceptor 2 (ROH) respectively.



Scheme 2: Lipase-catalyzed transesterification of butanol with ethyl butyrate as acyl donor in hexane.

Results and Discussion

In our previous screening and optimization studies [3, 17], highest degree of flavour synthesis was reported with acetone extracted day 4 germinated rapeseed lipase through direct esterification method. Therefore, the acetone extracted rapeseed powder (0.250 g or 50 g/L) lipase was used as source of enzyme at 40 ^{o}C in n-hexane. 40 ^{o}C was used as standard to (a) avoid the excessive evaporation of the organic solvent phase, (b) minimise enzyme denaturation and at the same time (c) increases the rate of reaction.

Effect of Incubation Time on Butyl Butyrate Synthesis Using Non-Activated Ester

The time course for the transesterification of ethyl butyrate with butanol by rape seed lipase is shown in Fig.1 .The reaction reaches equilibrium after 96 h with a final butyl butyrate yield of 60%. Only 1% further increase was noted within the next 24 h. Rate of reaction is not as efficient as in the direct esterification studies where ester yield of 68 % was achieved just after 48 h [18]. These different yields may result from at least one main reason. Different lipase specificity for butyric acid (direct esterification) and ethyl butyrate (Transesterification) is expected to affect the rate of reaction. It is generally believed that transesterification is a more efficient route for synthesis of esters in organic phase [2, 19]. This concept is based on the rational that (a) no additional water is formed during the reaction and (b) enzyme stability is usually high because acidification of the micro aqueous phase does not occur as acid is not present. In this system, lipase stability is more affected by alcohol rather than ester (shown later).

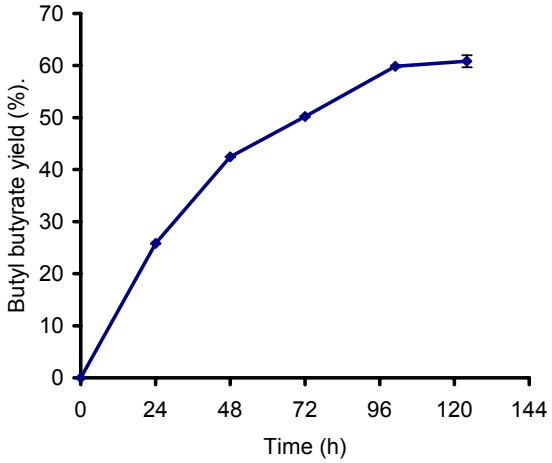


Fig. 1: Time course of butyl butyrate synthesis. The reaction mixture consisted of 0.25 M of butanol and 0.25 M ethyl butyrate in 5 mL of hexane in the presence of 50g/L of rape seedling acetone powder as catalyst at 40 ^{o}C for various times.

Effect of Added Water on Butyl Butyrate Synthesis

The influence of added water on the butyl butyrate yield by transesterification after 48 h is shown in Fig. 2. The optimum water content required for the synthesis of butyl butyrate by transesterification was determined to be 0.5% (v/v). With greater than 0.5% (v/v) water in the reaction

system, a decrease in ester yield was observed. However, both increase and decrease in ester yield was up to 10%, which is not a dramatic change. Normally the enzymatic activity increases with increasing enzyme hydration; this is often explained by water acting like a "lubricant" to increase the internal enzyme flexibility [20]. A decrease in the product yield from 32 to 23% was observed when water contents were increased beyond 20% (v/v). Possible reasons for the decreased activity include mass transfer limitations due to substrate transport through an aqueous phase, aggregation of the catalyst particles, or product hydrolysis. Increased water level, might have acted as competitive inhibitor with the nucleophile alcohol, as expected for the acyl-enzyme mechanism. Higher water levels have been shown to reduce the rate of lipase catalysed esterification or transesterification [21, 22].

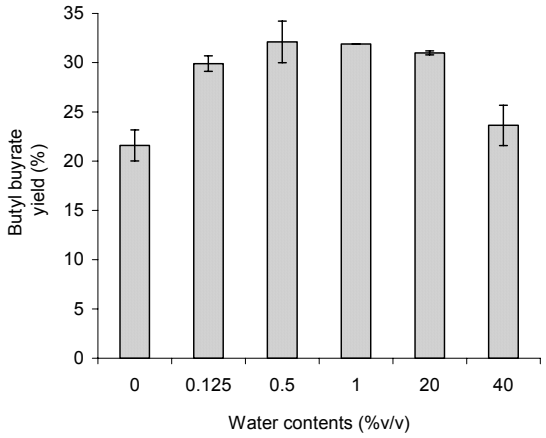


Fig 2: Effects of water content on the synthesis of butyl butyrate through transesterification. The reaction mixture consisted of 0.25 M butanol or ethyl butyrate and 50g/L of rapeseed lipase acetone powder in 5 mL of hexane at 40 °C for 48 h. All experiments were done in duplicate and the values reported are mean of two determinations.

With transesterification reaction using reversible acyl donor or non-activated ester, lower ester yield has been observed compared to direct esterification study where butyl butyrate yield of 89% after 48 h at 40 ^{o}C was observed [18]. Compared to this, *P38* lipase activity was reported optimum at an organic phase water concentration of 0.25% (v/v) to catalyze the synthesis of butyl caprylate through transesterification reactions. At a higher or lower water concentration the yield of ester decreased [20]. Apparently, in present study, the system reaches equilibrium sooner due to competition of acyl enzyme intermediate for eliminated alcohol (ethanol) from ethyl butyrate and substrate alcohol (butanol). There is possibility of further increase in ester yield if ethanol is removed from the reaction mixture by a distillation process. In esterification, the butyric acid has only one alcohol to react with which might explain the higher yields.

Effect of Substrate Concentrations

Effect of Ethyl Butyrate (Acyl Donor)

Synthesis of butyl butyrate increased linearly with increase in acyl donor concentration and a maximum ester formation (88%) was obtained at 0.6 M (Fig. 3) after 48 h at 40 ^{o}C. Moreover, ethyl butyrate, because of its higher polarity, would not disturb the essential water layer at the vicinity of the enzyme, as would butanol. The presence of high concentration of acyl donor (ethyl butyrate) might have enhanced rate of reaction as also described by many other workers to shift reaction towards synthesis [2, 20].

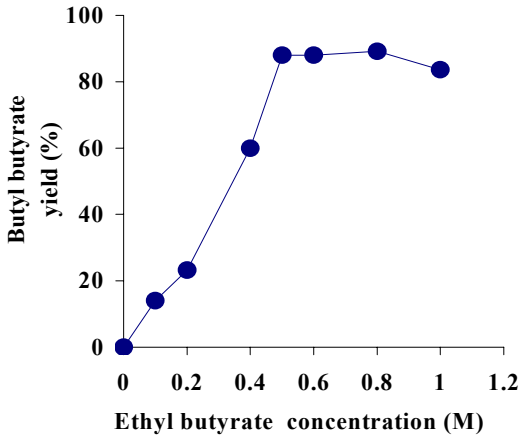


Fig. 3: Effect of ethyl butyrate concentration on the synthesis butyl butyrate. Ethyl butyrate alcohol concentration is changed while the co-substrate was kept constants 0.25 M. System contained 50 g/ L of rape seedling acetone powder in 5 mL of hexane. All reactions were carried out over period of 48 h at 40 °C with no added water.

Effect of Butanol (Acyl Acceptor)

When the acyl donor concentration is fixed of 0.25 M, the optimum concentration of butanol for production of butyl butyrate was found to be 0.10 M (Fig. 4). Butanol concentration above 0.10 M resulted in a steady decline of ester synthesis. It can be deduced that the decrease in ester synthesis is due to

inactivation of rape seeding lipase at high butanol concentrations or substrate inhibition as also reported elsewhere for other enzyme [23].

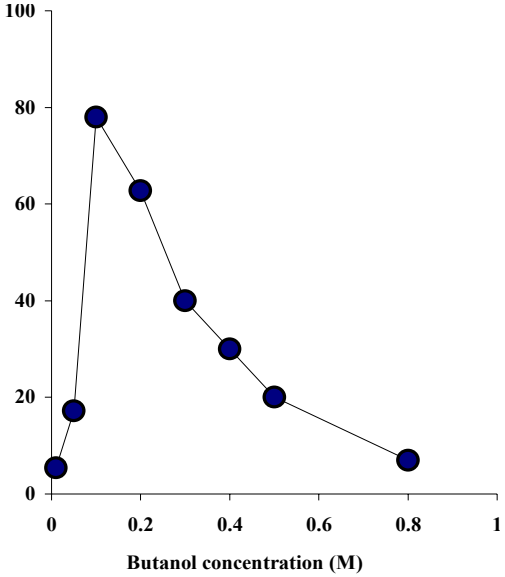


Fig. 4: Effect of butanol concentration on the synthesis butyl butyrate. Butanol concentration is changed while the co-substrate was kept constants 0.25 M. System contained 50 g/ L of rape seedling acetone powder in 5 mL of hexane. All reactions were carried out over period of 48 h at 40 °C with no added water.

In direct esterification reaction, butyl butyrate yield increased with increasing the butanol concentration until 0.25 M while concentration > 0.25 M adversely affected the yield [24]. For alcoholysis, the amount of the alcohol present in the reaction mixture must be in excess to the water present in the system. This serves to avoid the possibility of hydrolysing of the acyl enzyme intermediate during transesterification [25]. This strategy cannot be applied for the systems studied here because excess butanol (> 0.10 M) apparently resulted in the inactivation of rape seedling lipase. Inactivation of *P38* lipase by butanol (> 0.25 M) has also been reported by Tan [20] for the synthesis of butyl caprylate from ethyl caprylate and butanol via transesterification reaction. The difference here could be due to different reactants and enzyme sources used.

Synthesis of Flavor Esters as Function of Acyl Acceptor

Three acyl donors have been tested for alcoholysis reaction in n-hexane. The acylation of (Z)-3-hexen-1-ol (nucleophile) was carried out with (i) vinyl acetate (irreversible acyl donor) and with (ii) butyl caproate (reversible acyl donor*). In third case*, acylation of butanol with (Z)-3-hexen-1-yl caproate (reversible acyl donor) was also checked. Control experiments without added enzyme were also run. The molar conversion yields of alcohols obtained after 24, 48, 72 and 96 h of incubation are shown in Fig. 5. Among the acyl donors investigated, vinyl acetate appeared to be suitable one for the synthesis of (Z)-3-hexen-1-yl acetate and conversions as high as 99% was observed just after 24 h (case 1 irreversible reaction mode). This is followed by synthesis of (Z)-3- hexen-1-yl butyrate with a yield of 50 % and 80 % after 24 and 96 h (case iii reversible reaction mode).

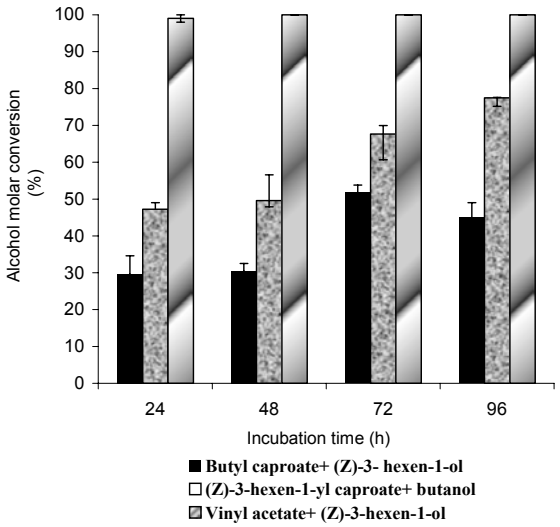


Fig. 5: Effect of different acyl donors on molar conversion yields through transesterification reaction. The reaction mixtures consisted of 0.1 M each of alcohols and 0.25 M acyl donors in 5 mL of hexane in the presence of 50g /L of rape seedling acetone powder at 40 °C.

Comparison of case (ii) and (iii) shows that rape seedling lipase is more specific for ester synthesis with butanol as compared to (Z)-3-hexen-1-ol.This is evident from case (ii) where a maximum of 50% of (Z)-3-hexen-1-ol was converted into ester after 96 h. Apart from the nucleophililic characteristic of alcohol, the differences in ester production can be ascribed to differences in substrate solubility and affinity or specificity of enzyme for the reaction components. In irreversible reaction mode, acyl-enzyme is produced without the chance of back reactions and was described better in terms of reaction rates. Here the unstable liberated (nucleophilic) enol is tautomerised into the

electrophilic carbonyl compound such as acetaldehyde or acetone. In the absence of water, the irreversibly produced acyl enzyme has only the possibility of reacting with the sole nucleophilic present, the alcohol to be acylated.

Irreversible acyl transfer mode of reaction using vinyl or isopropenyl esters has described the method of choice [26, 27]. This study also agrees with many previous investigations where vinyl esters have been reported the most valuable and important acyl donors due to irreversible nature of the reaction and the absence of low volatile side products [28-31]. However, one major drawback associated with use of vinyl esters, is the production of co product "acetaldehyde", which is liberated during the reaction. The by-product acetaldehyde may cause a dramatic loss in activity and selectivity [32, 33]. Acetaldehyde is known to act as alkylating agent on enzymes by forming Schiff's bases in maillard type reactions with lysine thus inactivates enzymes.The extent of the deactivation is dependent on the enzyme's structure, which in turn is governed by its microbial source. Whereas the majority of industrially produced lipases are remarkably stable toward acetaldehyde, the *C. rugosa* and *G. candidum* enzyme is highly sensitive [34]. Acetone powder extracted rapeseed lipase seems to work well and active which could be due to having many co-enzymes and impurities in it. However, it is not clear whether these co-enzymes or impurities are interfering with lipase activity and to what extent. Similarly, it is unknown about protective action of these co-enzymes for lipase against deactivation by acetaldehyde.

The enzymatic synthesis of short-chain acetates has mostly been carried out using acetic acid as acyl donor [4, 35]. However, this direct esterification led to low yields due to the strong acid inhibition on enzyme activity at high concentrations [2, 36]. To avoid acetic acid inhibition of enzyme for the production of short chain flavour esters, synthesis routes such as the transesterification [14, 15, 37, 38] especially alcoholysis reactions [19, 39, 40] have also been investigated successfully.

Experimental

Materials and Methods

Analar grade chemicals such as acids (acetic, butyric, caproic), alcohols (ethanol, butanol, Z-3-hexen-1-ol), esters (vinyl acetate, ethyl and butyl butyrate, Z-3-hexen-1-yl-caproate), 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).

Preparation of Acetone Powder Extracted Rape Seedlings Lipase

Dry w hole rapeseeds were surface sterilized by soaking in 0.1% sodium hypochlorite solution for 30 seconds, 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 [17]. Germinated rapeseeds were 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. Acetone powder extracted rapeseed lipase was washed with 4- volumes of cold acetone and air dried under a hood for 10 h to remove residual acetone. The light greyish powder was kept in sealed bottles at –20 °C until used. Cold acetone (-20 C) has been widely used for powder preparation from seeds such as rapeseed and *Nigella sativa* seeds previously. Primary reason for its use is its miscibility with water or hydrophilic nature. Cold acetone does not affect rapeseed enzyme stability either and it evaporates easily at room temperature.

Transesterification Method

Unless otherwise stated, acetone powder extracted rapeseed lipase (50 g/ L) was suspended in organic solvent (hexane) together with 0.25 M of alcohol (butanol) and 0.25 M of ester (ethyl butyrate). The typical reaction volume was 5 mL. Transesterification 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 at laboratory of food biochemistry, University of Leeds, UK. All experiments were performed in duplicate. Synthesis was also carried out without enzyme.

Ester Analysis

Routine analysis of reactants and products were conducted by a GLC instrument (Model 5160 Carlo Erba) equipped with a BP-20 fused silica capillary column (SGE, UK, 25 m x 0.32 mm ID; film thickness 1 micron), 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 GLC was connected to an integrator (Hewlett Packard 3395 integrator) which recorded the peak areas and retention times in a chromatogram.

Product Identification, *Yield Calculation and Time*

Esters and alcohols were identified according to their retention times on chromatograms and from comparisons with results obtained with standards. Calibration graphs of known ester (ethyl butyrate) and alcohols (butanol and (Z) -3- hexen-1-ol) concentrations versus corresponding peak area were constructed. Various concentrations of ester or alcohols (0.01 M-1 M) were prepared by diluting in n- hexane and 0.2 μL of each was injected in to GC. Injections were repeated twice for each vial. The percentage conversion of alcohols and ester yield was calculated by the following formulae:

Ester yield (%) = (molar ester produced) × 100/ (molar acid added)

Molar conversion (%) = 100 ($[Alcohol]_O$ – $[Alcohol]_F$) / $[Alcohol]_o$,
where subscripts O and F denote initial and final concentrations respectively.

Effect of Incubation Time on Butyl Butyrate Synthesis using non-Activated Ester

For the time course experiment, transesterification reaction was monitored at different time intervals until the reaction reached equilibrium. Samples of the reaction medium were drawn at given timed intervals (24 h) and analyzed for butyl butyrate concentration. Equilibrium was reached when the product concentration remained constant. Substrate concentrations, reaction conditions and procedure remained same as described above for transesterification study.

Effect of Added Water on Butyl Butyrate Synthesis

Varying amounts of distilled water (0-30% v/v) were added to the reaction medium containing acetone powder extracted rapeseed lipase (50 g/ L), 0.25M each of alcohol and acid. Ester synthesis was performed as described above.

Effect of Substrate Concentration

Effect of increasing the concentration of one of the substrates was evaluated, while keeping the other constant. Ethyl butyrate concentrations of 0.06 M, 0.12 M, 0.25 M, 0.4 M, 0.5 M and 1 M were reacted with a fixed 0.25 M concentration of butanol. In the reverse study, ethyl butyrate concentration was fixed at 0.25 M and butanol concentration was varied at 0.06 M, 0.12 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 ^oC. All synthesis experiments were performed in duplicate using separate reaction vials.

Conclusion

The production of low molecular weight esters as flavor compounds has a potential interest for the food industry. The crude acetone powder made from germinating rape seedlings was used for flavour esters synthesis through transesterification of an ester with alcohol in n-hexane. From various acyl donors employed, vinyl ester (activated ester or irreversible acy donor) proved to be most effective for flavour ester synthesis due to irreversible nature of the reaction and the absence of low volatile side products. Rape seedlings catalyzed alcoholysis of fatty acid vinyl ester is efficient way for the synthesis of flavor ester in hexane. Crude rape seedlings acetone powder is easy to prepare, does not need purification and better in terms of production cost, works well at ambient temperature and does not need immobilization.Work is in progress to screen other seedlings lipases and determine optimal reaction conditions for the synthesis of some important flavour esters through alcoholysis using vinyl esters [41-43].

Acknowledgements

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