Parameters Affecting the Synthesis of (Z)-3-hexen-1-yl acetate by Transesterifacation in Organic Solvent

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Summary: (Z)-3-hexen-1-yl esters are important green top-note components of food flavors and fragrances. Crude acetone powders extracted lipases from five plant seedlings of rapeseed, wheat, barley, linseed and maize were investigated for their use in the synthesis of flavor esters with vinyl acetate by transesterification in organic solvents. Rape seedlings showed the highest degree of (Z)-3-hexen-1-yl acetate synthesis with a yield of 76 % in 72 h. Rape seedling was chosen as promising biocatalyst to evaluate the effects of some of reaction parameters on (Z)-3-hexen-1-yl acetate synthesis using vinyl acetate and (Z)-3-hexen-1-ol at 40 °C in n-hexane with 50 g/L enzyme as catalyst. Acetonitrile proved distinctly superior solvent. The percent remaining activity relative to fresh seedlings powders was highest in wheat and barley. Highest ester yield of 80% was obtained with 0.8 M of substrate concentrations within 48 h. Crude rapeseed lipase afforded a conversion 93% of ethyl alcohol. Higher ester yield was achieved within first 24 h with added molecular. The crude rape seedlings lipase is low cost yet effective, showed potential for the production of green note esters industrially.

Key words: Seedlings lipase, Transesterification, Flavor ester, (Z)-3-hexen-1-yl acetate

Introduction

(Z)-3-hexen-1-yl acetate is of special interest since it represents a model of flavor esters [1]. Chemical synthesis of food flavours has many disadvantages and nowadays under question, such as poor reaction selectivity leading to undesirable side reactions, low yields, pollution, high manufacturing costs, and impossibility of labelling the resulting products as natural. Production of esters industrially from plant and through fermentation has also many disadvantages, as it is time consuming, expensive and dependent on supply of natural material. The use of enzyme catalysed reactions circumvents many of these drawbacks, due to the substrate specificity, regio- and enantioselectivity of these biocatalysts, which can be utilized at mild reaction conditions. Therefore, the use of lipases for a variety of biotechnological applications is rapidly and steadily increasing. Flavor esters 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. Biocatalyst cost also remains an important factor. Processes that utilize plant enzymes, however, may have advantages because of their lower cost and ready availability [2]. Lipase catalysed synthesis of flavor esters by direct esterification method has been reported by many authors [3-11]. However, the formation of water in direct esterification reactions has been described a major problem which favours the reverse (hydrolysis) reaction [12]. Moreover, inactivation of the catalyst was also observed due to more polar nature of the substrates [13].

Transesterification reaction for the flavor synthesis is thought more efficient to esterification because (a) there is no formation of water and (b) inhibition by the acid or alcohol does not occur [14]. Moreover, often, increased yields and improved enzyme stability were reported through transesterification reactions in OPB [15-18]. Transesterification reactions have also been proposed to circumvent the inhibitory effects of polar substrate (acetic acid) on lipases and have been investigated successfully [19-22]. Transesterification includes reversible acyl-transfer and irreversible acyl-transfer reactions [23]. However, low ester yields (less than 60%) have been reported with lipase mediated reversible acyl-transfer reactions when alkyl or non-activated esters were used as acyl donors [24-26]. Ester interchange involving alkyl esters have been described rather slow and required a large excess of acylating agent to shift the equilibrium towards formation of the desired compound [27]. Irreversible acyl-transfer mode of reaction using vinyl or isopropenyl esters (acetates) has become the method

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of choice for flavor synthesis. Vinyl esters especially vinyl acetate and butyrate have been described the most valuable and widely used acyl donors [28, 29] due to irreversible nature of the reaction and the absence of low volatile side products [30, 31]. Moreover, vinyl esters have been demonstrated to give better reaction rate than the isopropenyl esters [28]. 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 (scheme-1). 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. Fang and Wong [32] reported that by using this reaction mode reaction rates increased 10 to 1000 times when compared to reversible acyl transfer.

Current paper describes the ability of various crude seedlings lipases for the preparation of green top note esters (Z)-3-hexen-yl acetate through transesterification (irreversible acyl-transfer) of vinyl acetate with (Z)-3-hexen-ol at 40 ^{o}C in hexane. Vinyl alcohol formed after transesterification of vinyl acetate tautomerises to acetaldehyde, thus making the process irreversible as shown in Scheme1. Crude rape seedling lipase will be further investigated to study effects of parameters such as specificity of vinyl acetate for ethanol, butanol and (Z)-3-hexen-1-ol; solvent choice; effect of substrate concentrations, molecular sieves and residual activity in seedlings powder used for transesterification reactions. Vinyl ester was used as the acylating agent for transesterification because it offers an effective solution to overcome equilibrium. Previously (Z)-3-hexen-yl acetate was synthesized with purified microbial *Candida Antarctica* (Novozym 435) lipase through direct esterification reaction using different reactants and under different conditions [1].

Experimental

Plant Material and Chemicals

Analytical grade chemicals such as, alcohols, acids, organic solvents (HPLC grade), were purchased from Sigma-Aldrich Co. Ltd. (England, UK).While, hexane and heptane were bought from Fisher (Loughborough, UK). Molecular sieves (3A, 8-12 mesh were used to dry hexane and both were obtained from Sigma-Aldrich Co. Ltd.) for at least 24 h before use. Nickerson Seeds Ltd., (Lincoln, UK) provided seed material.

Seed Germination and Preparation of Acetone Powder from Rape Seedlings

Crude acetone powder was prepared from rape seedlings as described earlier [26].

Preparation of Crude Lipase Extract from Acetone Powder

Lipase was extracted from acetone powder by stirring acetone powder (1g) with 32 ml of MacIlvaine buffer for 1h at room temperature. The slurry obtained was centrifuged at 12,000 X g for 30 min. at 4°C. The supernatant was removed and this lipase preparation was used lipase activity determination and protein contents experiments.

Protein Determination in Crude Lipase Extract

The protein contents of plant seedlings were determined by the modified Bradford method [57]. The Bradford reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 ml of 99.7% ethanol and adding 100 ml of 85% (w/v) orthophosphoric acid. The final volume of the solution was made up to 1liter. The final concentration in the reagent was Coomassie Brilliant Blue G-250 0.01% (w/v) ethanol 4.7% (w/v) and phosphoric acid 8.5% (w/v). 0.2 ml aliquots of seed extract was placed in a reaction tube and 5 ml of Bradford's reagent was added with mixing. Absorbance was read at 595 nm approximately after 5 min of reaction against a blank reagent. The blank was prepared by adding 0.2 ml of buffer solution instead of sample extract. Bovine Serum Albumin (1mg/ml buffer) was used in preparing a calibration graph.

Determination of Residual Lipase Activity in Powder used for Transesterification

Lipase activity was determined with 4-methylumbelliferyl heptanoate as substrate using fluorimetric method following procedure described earlier by Liaquat [2].

Transesterification Reactions

Rape seedlings acetone powder (50 g/ l) together with 0.25 M of vinyl ester and 0.25 M of alcohol (s)) were suspended in organic solvents. The total reaction volume made was 5 ml. Transesterification was carried out with shaking at 100 rpm at 40 °C temperature for 48 h. 0.1 ml of sample was taken at fixed intervals then centrifuged (1300 X g for 5 min), and samples were stored at -10

°C or until analyzed. Experiments were done in duplicate using separate reaction vials.

Product Yield

Procedure for identifying acid, alcohol and esters has been described by Liaquat *et al*. [26]. Percent ester yields or molar conversion were calculated by following formulae:

Molar conversion (%) = 100 ([Acid] ^{O} – [Acid] ^{F}) / [Acid]^{o}

where subscripts O and F denote initial and final concentrations respectively, while

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

Gas Chromatographic and Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Reactants and products were routinely analysed by GLC. Esters identity was established through GC-MS following method described earlier by Liaquat *et al*. [26].

Screening of Plant Seedlings for the Synthesis of (Z)-3-hexen-1-yl acetate via irreversible acyl transfer reaction

Five crude seedlings lipases were investigated to transesterification of vinyl ester and (Z)-3-hexen-1-ol. 0.25 M of each (Z)-3-hexen-1-ol (148 µl) and 0.25 M of vinyl acetate (115 µl) were mixed and total volume of reaction mixture were brought to 5 ml with hexane, to which 250 mg of corresponding seedling lipase preparation were added. Samples were prepared and analyzed for product by GC at given time intervals.

Residual Lipase Activity Determination in Used Powder

To examine the effect of reactant substrate / or products on stability of enzyme, residual activity of lipase was evaluated. After a 72 h for transesterification period, lipase from rapeseedings was recovered through centrifugation and remaining catalytic activity was determined spectrofluorimetrically.

Factors Effecting Reaction Conditions for Vinyl Ester Synthesis

Effect of Solvents

The reaction between vinyl acetate and (Z)-3-hexen-1-ol was investigated with eight organic solvents of different hydrophobicity; hexane, dioxane, tetrahydrofuran, acetonitrile heptane diethyl ether, or toluene under similar reaction conditions.

Substrate Concentrations

In this experiment, the concentration of one of the substrates increased, while the other kept constant. The effects were evaluated. A fixed 0.25M concentration of vinyl acetate was reacted with varying concentration of 0.06 M to 1M of (Z)-3-hexen-1-ol. In reverse study, vinyl concentration was varied from 0.06 to 1M while (Z)-3-hexen-1-ol concentration was fixed at 0.25M. Reaction was carried in hexane at 40 ºC.

Effect of Molecular Sieves

The reaction mixtures contained 0.25 M vinyl acetate, 0.25 M alcohol in 5 ml of hexane. 250 mg of rape seedling acetone powder was used as catalyst at 40 C. Samples were taken at 100 rpm at various time intervals.

Vinyl Acetate Specificity for Alcohols During Transesterification

0.25 M of ethanol (74.4 µl), butanol (115 µl), and (Z)-3-hexen-1-ol (148 µl) were mixed with 115 µl of vinyl acetate in three different vials. Final volume of each vial was brought up to 5 ml with hexane to which 250 mg of rape seedling were added and conversion was followed by GC.

Results and Discussion

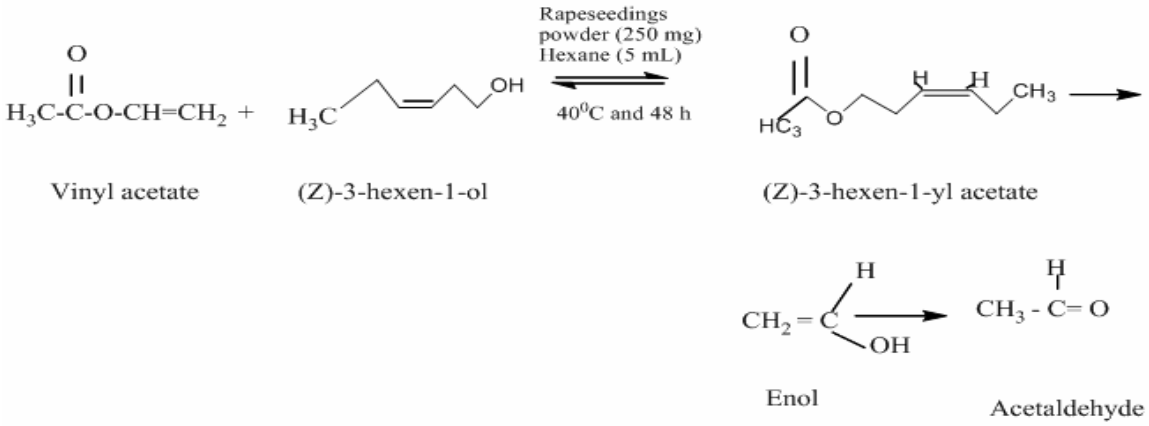
Screening of Plant Enzymes for (Z)-3-hexen-1-yl acetate Synthesis as Function of Time

Screening of plant seedlings was performed to select the most appropriate plant enzyme for transesterification involving vinyl acetate with (Z)-3-hexen-1-ol. The reaction was carried out using acetone powders from germinated plant seedlings of rapeseed, wheat, barley, linseed and maize using standard conditions with no added water in hexane.

Fig. 1 illustrates the biocatalytic ability of five seedlings acetone powders for the synthesis of (Z)-3-hexen-1-yl acetate as function of time. Generally, synthesis increased with time. Of the five plant lipases screened, the highest transesterification activity was observed with rape seedlings, with yield of 37, 45, 54 and 64 % within 12, 24, 48 and 72 h. This was followed by linola with 23-48% yield within same time course. Briefly, acetone powders from seedlings of wheat, maize and barley gave a yield < 48% after a reaction period of 72 h. Acetone powder from maize seedlings performed poorly as compared to other powders and gave a final yield of 24% within 72 h. In this study, ester production was not as efficient as direct esterification study [2]. Through direct esterification, two green notes esters (Z)-3-hexen-1-yl butyrate and (Z)-3-hexen-1-yl

caproate were produced with yields about 96% using rape seedling powder after 72 h. However, with rape seedlings lipase catalysed transesterification reaction (reversible acyl transfer mode), comparatively lower yield (60 %) was obtained for butyl butyrate within 96 h using non- activated ester (ethyl butyrate) [25]. There are various reasons for different synthesis yields. Firstly, as crude lipase was used, the specific activity of lipase in the different powders is different (Table-1) which affected the yields. Secondly, lipase specificity is expected to affect the conversion yields as different substrates were used in direct esterification and transesterification studies.

Protein contents of seedling acetone powders were also determined and are given in Table-1. It is evident that enzymes with lower specific activity (rapeseed and linola) gave highest synthesis through transeterification reaction (Fig. 1). However, these enzymes exhibited least residual activity (Fig. 4).The hydrolytic activity of crude acetone powder extracted lipase preparations were also determined using 4-methylumbelliferyl heptanoate (4-MUH) through spectrofluorimetric assay. Highest hydrolytic activity was observed for crude lipase reparations from wheat and maize which were not as efficient for synthetic purposes. Therefore hydrolysis of 4-MUH is not an indicator for synthetic activity for these lipase preparations. Based on results from previous direct esterification [2] and current transesterification studies, rape seedlings were selected for further study.



Scheme-1: Lipase-catalysed transesterification of (Z)-3-hexen-1-ol with vinyl acetate to produce (Z)-hexen-1-yl acetate in hexane.

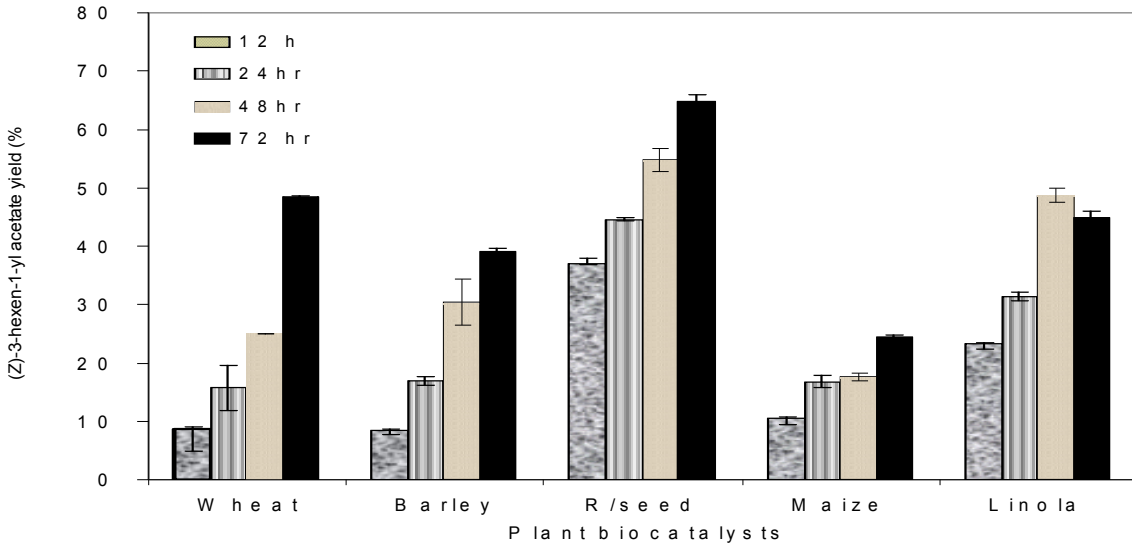


Fig. 1: Time course showing the (Z)-3-hexen-1-yl acetate yields catalysed by acetone powder from various plant seedlings. Reaction conditions: 0.25 M of each vinyl acetate and (Z)-3-hexen-1-ol in 5ml of hexane, 250 mg (5% w/v) enzyme. Samples were taken at 100 rpm, at 40 ^{o}C at various time intervals.

Table-1: Protein contents, hydrolytic and specific activities of acetone powders from day-4-old germinating plant seedlings used for transesterification study. Activity was determined through fluorimetric method using 4-MUH as substrate for 30 min at 30 °C. Results are average of three independent determinations with standard deviations less than 1%.

Seedlings (cultivar name)	Protein(mg/ml)	Hydrolytic activity(nmol 4-MU/ ml/min)	Specific activity (nmol 4MU/mg protein /ml)
Wheat (cultivar IPM)	8.38	14.17	1.69
Barley (Decanter)	4.81	12.04	2.81
Maize (River)	48.15	7.94	0.16
Linola (Windermere)	7.25	11.56	2.49
Rape seed (Boston)	10.43	10.33	0.46

For lipase catalysed transesterification of (Z)-3-hexene-1-ol with vinyl acetate under conditions of irreversible acyl transfer produces a chromatogram of product mixtures along with their retention times is shown in Fig. 2. The identification of ester peak was carried out by comparing their retention times with authentic standard ester and by matching its mass spectra with those of standard ester as well as with the NBS library of flavor and fragrances in the data base system (Fig. 3).

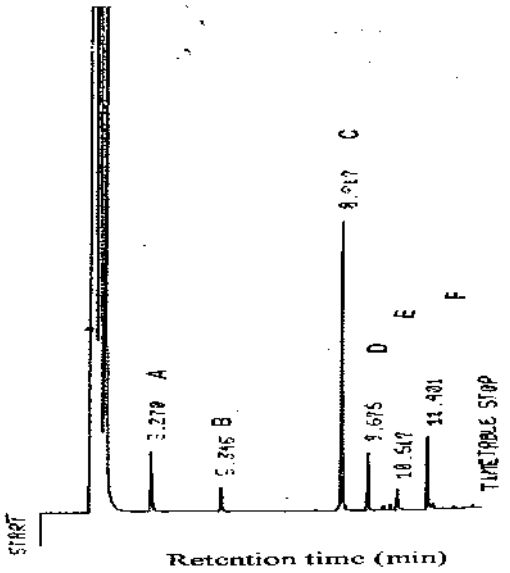


Fig. 2: GC profile of samples from the transesterification experiment between vinyl acetate and (Z)-3-hexen-1ol at 40 °C after 48 h: Vinyl acetate (A), Unknown (B), (Z)-3-hexen-1- yl acetate (C), (Z)-3-hexen-1ol (D), acetic acid (E), Internal standard (F).

Residual lipase activity in seeding powders used for transesterification

Five seedling powders, used for the synthesis of ester in hexane were also evaluated for their hydrolytic activity before and after transesterification reaction using 4-MUH by fluorimetric assay. Crude lipase preparations from wheat and maize exhibited highest hydrolytic activity (Table-1). The percent remaining activity relative to fresh seedlings acetone powders after a reaction time of 72 h at 40 C is given in Fig. 4. As observed in previous section, wheat contained highest residual hydrolytic activity (52%) followed by barley (46.6%) while lowest activity was shown by linola powder. This proves that despite having highest activity, crude lipase preparation from wheat was not better than rape seedlings for synthesis of esters. It can be concluded that hydrolysis does not correlate with synthesis. Furthermore, synthesis or hydrolysis are not only enzyme dependent but also depend on substrate used, type of organic solvent and reaction mode.

Vinyl acetate in many ways is an acyl donor of first choice in lipase catalysed transesterification reactions [33] But 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 [34, 35]. Acetaldehyde is known to act as alkylating agent on enzymes by forming Schiff's bases in Maillard type reactions with lysine thus inactivates enzymes [34].The extent of the deactivation is dependent on the enzyme's structure, which in turn is governed by its source. Whereas the majority of industrially produced microbial lipases are remarkably stable toward acetaldehyde, the *Candida rugosa* and *Geotricum candidum* enzyme have been described as highly sensitive [33, 36]. However, as reported by Weber *et al*. [35] total numbers of lysine residues of lipase are apparently not the criterion for lipase stability. The deactivation extent is determined by the relative reactivity (*i.e*., nucleophilicity) of particular lysine residues within the enzyme. As a consequence, those lipases that posses highly exposed lysine residues (e.g., *C. rogusa* and *G. candidum)* are likely to be more readily deactivated by the acetaldehyde than others whose lysine groups are buried and exhibit only moderate basicity. It is anticipated that lipase from rape seedlings, which exhibited least residual activity and highest yield, could have moderate basicity since lysine the contents are buried within enzyme. This needs to be proved with further studies.

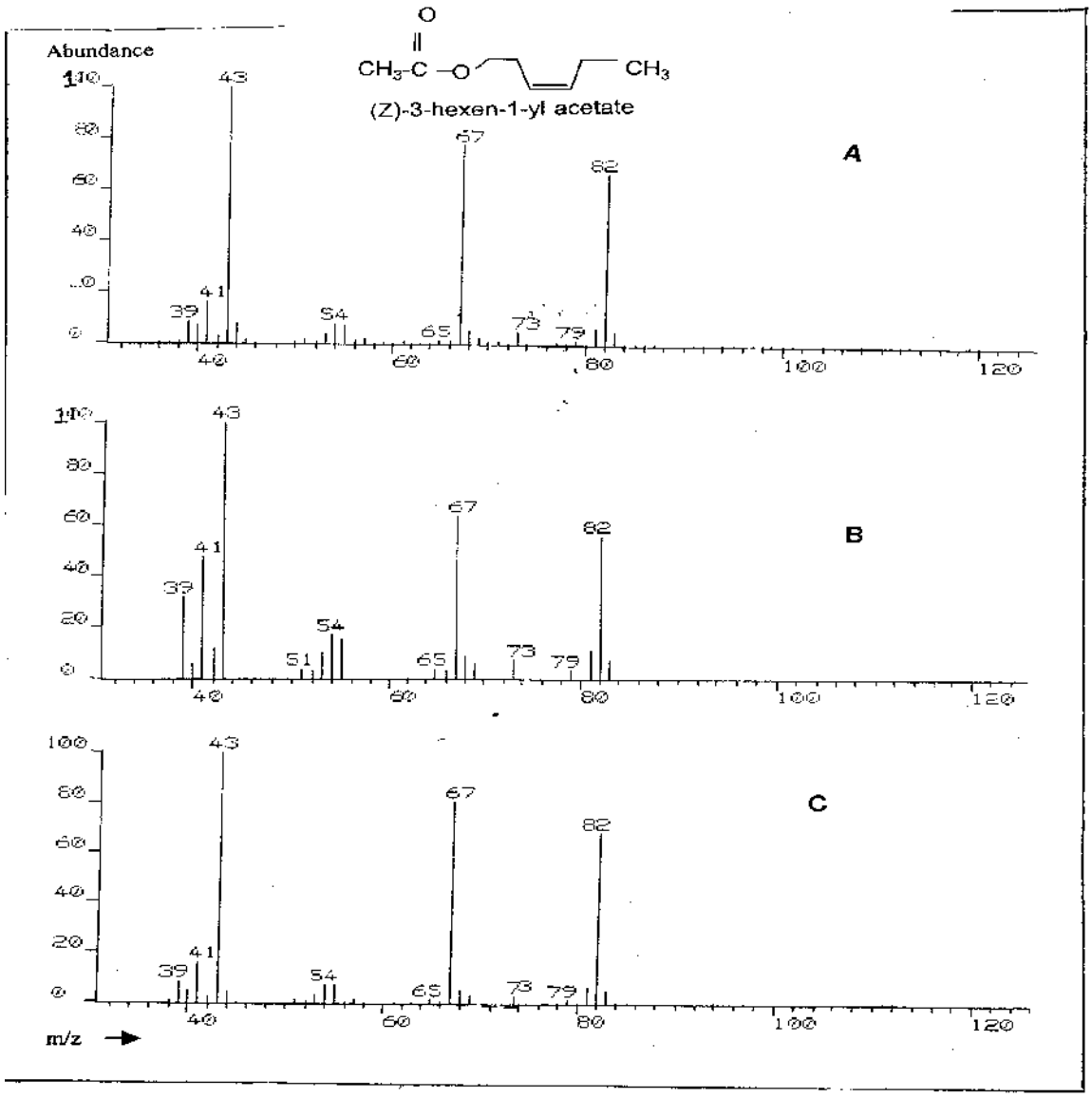


Fig. 3: GC-Mass spectra of peak (C), which was identified as a (Z)-3-hexen-1- yl acetate, and its comparison with standard (A) library (B), synthesised (C).

Effects of Parameters on (Z)-3-hexen-1-yl acetate ester

Effects of Solvents

Organic solvents are known to affect the activity and specificity of lipases. Use of organic solvents can have drastic effects on equilibrium conversions as has been demonstrated for direct esterification reaction [37] and has also been reported previously [38, 39]. The choice of solvent can therefore be crucial for the success of the desired transformation. In view of these effects of solvents, eight solvents (*i.e.*, dioxane, acetonitrile, toluene, diethyl ether, pentane, hexane, and heptane) were assessed for rapeseed lipase-catalysed production of (Z)-3-hexen-1-yl acetate. As shown in Table-2**,** the transesterification reaction did occur in all solvents, but acetonitrile proved distinctly better solvent. Log P pattern has not proved to be correct for this particular solvent. Apart from acetonitrile, a progressive increase in yield has been observed with solvents with Log P values higher than 2.5.High transformation by acetonitrile means that synthesis in organic solvents depends also on the nature of organic solvents. Acetonitrile might have caused some useful changes in the enzyme active site to fit the substrates or in the reaction media to make it more suitable for vinyl acetate reaction. It is well

known that polar organic solvents such as ethanol and acetornitrile compete with water. This higher ester synthesis in polar acetronitrile solvent is opposite to the findings of Zaks and Klibanov [40] who reported that the activity of enzymes was higher in hydrophobic solvents as compared to hydrophilic ones. Present results of acetonitrile study is also in disagreement with some other studies [40] who first pointed out that log P, as a solvent parameter, correlates best with enzyme activity. Higher ester yield in acetonitrile through transesterification (irreversible acyl-transfer) is also surprisingly different than esterification study [36] where hexane and heptane were the most effective organic solvents for maximum ester yield.

Table-2: Rape seedling lipase catalysed synthesis of (Z)-3-hexen-1-yl acetate through transesterification in various organic solvents.

Solvent	Log P value	Alcohol molar Conversion (%)	Ester yield (%)
1,4-Dioxane	-1.1	7 ± 0.3	18 ± 0.4
Acetonitrile	-0.3	74 ±1.9	69±0.4
THF	0.49	20 ±1.2	8±0.0
Diethyl ether	0.85	8 ±1.7	19±0.2
Toluene	2.5	28 ± 2.3	21±0.0
Pentane	3.0	34 ± 5.3	29±1.8
Hexane	3.5	54 ± 3.8	48±0.0
Heptane	4.0	56 ±2.3	43±0.4

Generally, solvents of low polarity and low water solubility do not strongly affect enzyme stability. Although the Log P value of a solvent has been useful to predict the behaviour of enzymes in systems containing low amount of water [40]. This approach may be less useful for monophasic systems containing water miscible solvents. Enzymes are known to be denatured in monophasic systems containing polar solvents, mainly because the hydration state of the enzyme molecules is distorted to the extent that the catalytically active conformation is lost [41, 42]. The solvent effects on the transesterification of hexanol with ethyl acetate catalysed by the *C.antarctica* (Novozyme 435) lipase have been investigated [43]. Their results indicated that solvent effect on conversion was very small as compared to that for the direct esterification reaction. The differences in two modes of production were ascribed to differences in reactants and water involved.

Effect of Substrate Concentration

The effect of substrate concentrations on deacetylation of vinyl acetate by (Z)-3-hexen-1-ol catalysed by crude rape seedling powder was also investigated. Various vinyl acetate (0.1-1 M) were added to the reaction mixture while (Z)-3-hexen-1-ol concentration remained constant (0.25 M) and vice versa.

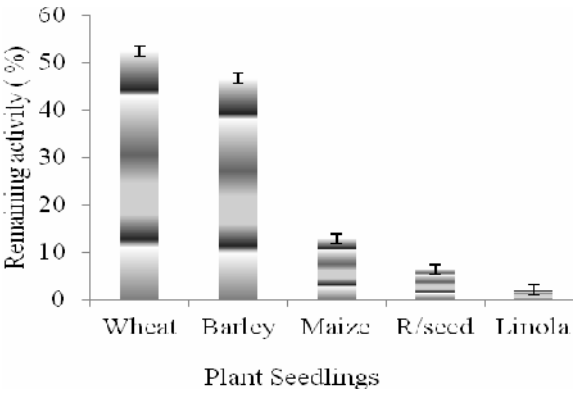


Fig. 4: Residual lipase activities in seedlings powders. The activity was measured after using these powders for transesterification for 72 h. The powders were washed with hexane and evaporated with nitrogen. The hydrolytic activity was measured using 4-MUH as substrate. Results are average of three independent determinations with standard deviations less than 1%.

As depicted in Fig. 5a and 5b, increasing substrate concentrations led to an increase in transesterification. An optimal ester yield of 80% and 54% was obtained with 0.8 M of (Z)-3-hexen-1-ol and 0.8 M of (Z)-3-hexen-1-yl acetate respectively. Acyl donor concentration greater than 0.25 M did not result in a significant increase in ester yield. It could be because the enzyme was inhibited by substrates above these concentrations. This was evident from large alcoholic peak found in the organic phase which could have resulted in inhibition of enzyme. Other reasons could either be saturation of enzyme active site by either substrate or the action of product acetaldehyde when high vinyl acetate concentrations were used.

Lipases of a newly isolated *Pseduomonas aeruginosa* MTCC 5113 were assessed for transesterification of benzyl alcohol and vinyl acetate to produce the flavoring agent benzyl acetate. Crude lipase preparations achieved benzyl alcohol conversion of 89% within 3 h at 30 ºC [44]. Evidence suggests that *P. aeruginosa* lipase experienced possible substrate inhibition by benzyl alcohol. No clear evidence was seen for inhibition of the enzyme by vinyl acetate. Few other researchers also investigated enzymatic synthesis of terpenyl esters by transesterifcation catalysed by microbial lipases with fatty acid vinyl esters as acyl donors [45, 46]. In this case, no inhibition by substrates such as geraniol and vinyl acetate was observed. Similar results were obtained by Kumari *et al*. [47] reported synthesis of

isoamyl acetate by transesterification of isoamyl alcohol with vinyl acetate using immobilized *Rhizopus oryzae* NRRL 3562 lipase. The substrates had no inhibitory effect on immobilized lipase.

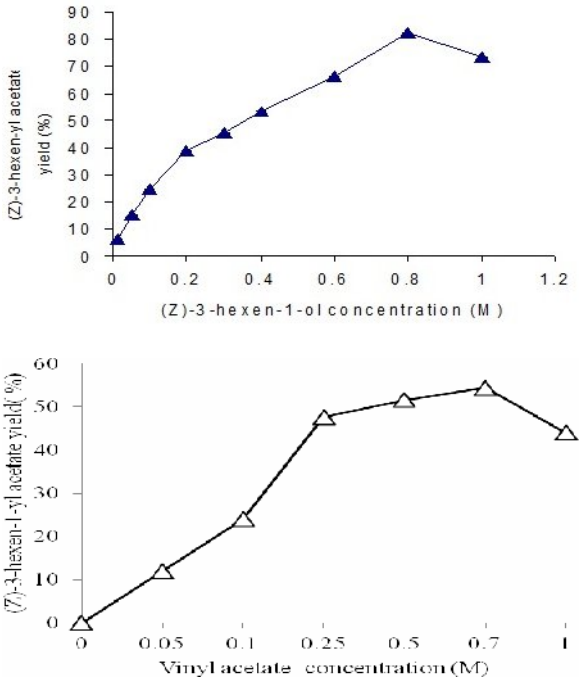


Fig. 5: Effect of (Z)-3-hexen-1-ol (a) vinyl acetate (b) concentrations on the production of (Z)-3-hexen-1-yl acetate while the (Z)-3-hexen-1-ol or vinyl acetate concentration of the respective systems remain constant at 0.25 M. 250 mg of rape seedling powder was suspended in 5 ml of hexane containing the substrates. All reactions were carried out for 48 h at 40 °C.

Transacetylation of Alcohols with Vinyl Ester

Ethanol, butanol and (Z)-3-hexen-1-ol were tested. Ester interchange of vinyl acetate with various alcohols proved the efficiency of this reaction as shown in Fig. 6. Molar conversion yield of 93, 71 and 64% were obtained with ethanol, butanol and (Z)-3-hexen-1-ol after 24 h. Conversion yield obtained is double as compare to our previous esterification study [48] where less than 40% conversion yield was obtained in 72 h with ethanol, butanol, and (Z)-3-hexen-ol. During esterification study, enzyme was more specific to (Z)-3-hexen-1-ol where in transacylation study, greater yield was observed with ethanol. This is not surprising as the acyl donors used are different in both cases. Using immobilized lipase from *Mucor miehei* (Lipozyme 1M) or *Candida antarctica* (Novozym 435) in the absence of water trapping, (Z)-3-hexen-1-yl butyrate, isovalerate, or caproate were produced with yields about 95% in direct esterification study [48, 49]. However, the yield for (Z)-3-hexen-1-yl acetate production using Lipozyme 1M was less than 2%. Novozym 435 afforded (Z)-3-hexen-1-yl acetate with yields greater than 90% [1, 50]. The nature of the substrates also exerts a great deal of influence on both the activity and selectivity of the enzyme [51]. Bearing in mind that lipase-catalysed reactions involve an acyl enzyme intermediate, it might be expected that the reactivity of a specific vinyl ester would depend on the affinity between the vinyl ester and the enzyme itself [52].

The enzymatic synthesis of favor esters by transesterification from vinyl esters and alcohols catalysed by free *Candida antarctica* lipase B in ionic liquids has been studied by de los Ríos *et al*. [51] Not only does the nature of the vinyl acyl donor affect the efficiency of enzyme in ester synthesis but also the chain length of the alcohol played an important role in overall enzyme activity. It was observed that when the alkyl chain length of the alcohols was increased, the synthetic activity of enzyme showed a bell curve, the highest activity being obtained for 1-butanol. When the number of carbon atoms of the vinyl ester was increased, the optimum activity was found for six carbon atoms. The influence of the alkyl chain length of the alcohol was tested, the optimal length being four carbon atoms.

Additions of Molecular Sieves

To remove the water from an organic solvent, addition of molecular sieves is an efficient method. There are reports that the activity of /or stability of enzymes is affected by the addition of molecular sieves [53-56]. Two different reasons have been described for this phenomenon. One is the low water activity maintained by the molecular sieves and another is the actual presence of the sieves. Besides, the addition of molecular sieves (3 A) to the medium in order to trap acetaldehyde seems to be having beneficial, but further studies needs to be carried out.

In a set of experiments, the reaction medium was dehydrated using the molecular sieves. Enzymatic reaction was performed using this dehydrated medium with or without molecular sieves (Table-3).

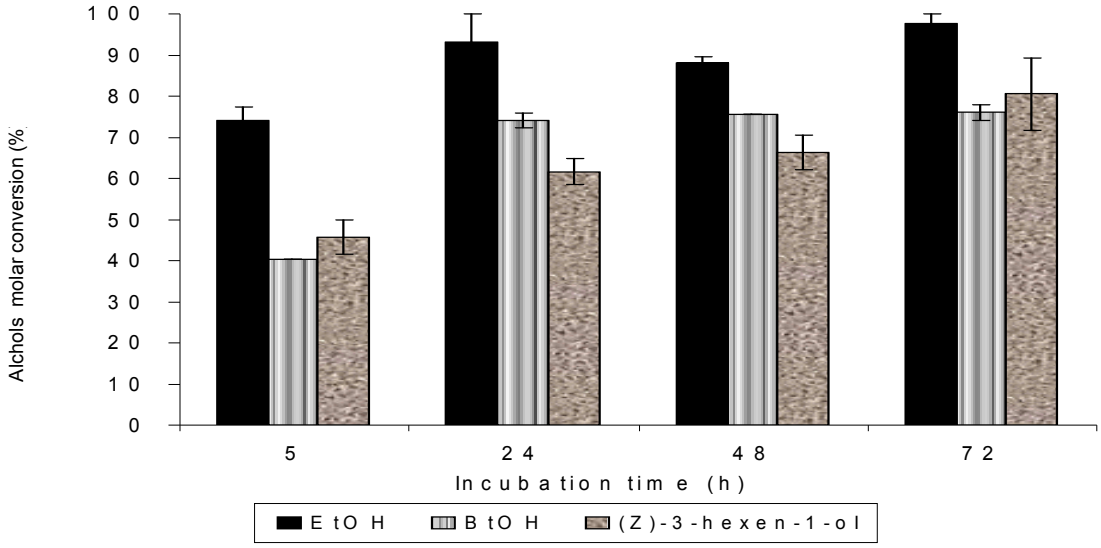


Fig. 6: Transesterification of alcohols with vinyl acetate. The reaction mixtures consisted of 0.25 M of each alcohol and 0.25 M vinyl acetate in 5 ml of hexane. 250 mg of rape seedling acetone powder was used as catalyst at 40 °C.

Table-3: Synthesis of vinyl acetate with and without molecular sieves. The reaction mixtures consisted of 0.25 M alcohol and 0.25 M vinyl acetate in 5 ml of hexane. 250 mg of rape seedling acetone powder was used as catalyst at 40 °C. Samples were taken at 100 rpm at various time intervals.

Time (hr)	(Z)-3-hexen-1-yl acetate yield (%)	
	*with MS	without MS
5	45.7±2.2	46.45±4.3
24	80.1±1.4	61.6±3.1
48	70.3±4.7	66.3±4.2
72	42.9±0.85	80.5±3.9

* MS – Molecular sieves

Conversion yield was higher with molecular sieves during first 24 h of reaction. A yield of 80% was achieved within first 24 h and then dropped to 42% after 72 h. It is assumed that the molecular sieves first adsorb the acetaldehyde in the medium which gave higher yield in the first 24 h. After this molecular sieves were fully saturated with acetaldehyde and could not hold any more acetaldehyde. It is also possible that the presence of molecular sieves for long time lowered and maintained a low water activity, which would have caused decreased enzyme activity. There is no evidence that product ester was absorbed by molecular sieves. However this might be possible and needs to be examined in future. Results also show that yield of ester without molecular sieves increased with time. This result is in close agreement with the findings of Wehtje *et al*. [56] who reported higher activity if the molecular sieves were omitted.

Conclusion

(Z)-3-hexen-1-yl acetate is an extremely aromatic compound with 'green notes' flavor. It is wildly used in the food industry and is a short-chain ester with fruity odor. Lipases extracted from crude acetone powders of five plant seedlings were tested to catalyse transesterification of vinyl acetate with (Z)-3-hexen-1-ol to produce (Z)-3-hexen-1-yl acetate. A maximum of 64% ester yield was obtained with rape seedlings lipase after 72 h. However, highest hydrolytic and remaining lipase activity was noted for wheat seedling. Transesterification of vinyl acetate was highest with ethyl alcohol. The optimum transesterification conditions to achieve highest yields were: substrate concentration 0.8 M for duration of 48 h, and acetonitrile as the organic solvent in the presence of 50 g/L (5% w/v) of crude rape seedling lipase at 40 °C.

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