Chapter 3

Lipase catalysed transesterification of cinnamyl alcohol with vinyl acetate in organic media
3.1 INTRODUCTION

The phenylpropanoids are a diverse family of organic compounds that are synthesized by plants from the amino acid phenylalanine. Their name is derived from the six-carbon, aromatic phenyl group and the three-carbon propene tail of compounds (Christine et al., 1984). Phenylpropanoids are found throughout the plant kingdom, where they serve as essential components of a number of structural polymers, provide protection from ultraviolet light, defend against herbivores and pathogens, and mediate plant-pollinator interactions as floral pigments and scent compounds (Alexander and Choudhary, 1996; Kumar et al., 2005). Acid derivatives are widely used as precursor or intermediates in the synthesis of range of active pharmaceutical ingredients/drugs molecules. Alcohol derivatives are widely used in fine chemicals, flavor and fragrance synthesis (Jakubas et al., 1992; Sharma, 2011).

Cinnamyl acetate, a phenyl propanoid class of compound, reportedly found in the essential oil of mimosa (black wattle), Larus azorica and Cinnamomum zeylancium flowers (Jayaprakasha et al., 2000; Pedro et al., 2001). Cinnamyl acetate is widely employed in perfumery because of their excellent sensory and fixative properties. Apart from flavor and fragrance, cinnamyl acetate also used as starting material/precursor in a variety of applications which include the production of photosensitive polymers, the creation of inks for multicolor printing, the formulation of animal repellent compositions, and the development of effective insect attractants. Worldwide use of cinnamyl acetate is about 100 metric ton per annum (Bhatia et al., 2007). Traditionally, cinnamyl acetate is produced via several chemical catalytic routes by varying different acyl donors such as acid, ester or acyl halides. Chuanfa et al, 2008 reported the direct esterification of cinnamyl alcohol with acetic anhydride in presence of 85-98% p-toluenesulfonic acid. Another process is the reaction between cinnamyl bromide and sodium acetate in the presence of tetra-butyl ammonium bromide as a phase transfer catalyst at high temperature (Devulapelli and Weng, 2009). Wolfson et al., 2012 reported the insitu one-pot synthesis of cinnamyl acetate by transesterification of cinnamyl alcohol which was previous obtained by chemical/bioreduction of cinnamaldehyde, with triacetin in presence of Amberlyst -36 (sulfonic acid ion-exchange resin). All these processes are require hazardous
chemicals, more sophisticated instruments for controlling reaction temperature and pressure.

Lipase catalysed esterification and transesterification reactions are among the most promising alternatives to traditional chemical methods. Lipases are inherently superior to chemical catalysis, due to their broad substrate specificity; they have good ability to recognize regio and stereo selectivity; are inexpensive; need no co-factors; work under mild reaction conditions; and require low energy input (Krishna et al., 2001a; Priya and Chadha, 2003). Lipase catalysed synthesis of flavour and fragrance esters are well adopted method at industries level. There is dearth of information on the synthesis of cinnamyl acetate using lipase as catalyst. Thus it was useful to study the lipase catalyzed transesterification of cinnamyl alcohol with vinyl acetate for the synthesis of cinnamyl acetate in non-aqueous medium. The present work highlights the effects of various parameters such as nature of lipase, speed of agitation, mole ratio, catalyst loading, temperature, and reusability of catalyst. Reaction mechanism and kinetics are also studied.

### 3.2 MATERIALS AND METHODS

All enzymes and chemicals were received from reputed companies. Novozym 435 (Lipase B from *Candida antarctica*, supported on a macroporous acrylic resin and enzyme activity 7,000 PLU/g. PLU is propyl laurate unit, based on reaction between propyl alcohol and lauric acid.). Lipozyme RM-IM (Lipase from *Rhizomucor miehei*, supported on a macroporous anion exchange resin and enzyme activity 30 U/g, based on tristearin assay), Lipozyme TL IM (Lipase from *Thermomyces lanuginosus*, supported on porous silica granulates and enzyme activity 175 IU/g, IU is international unit, based on tributyrin assay) were received as gift samples from Novo Nordisk, Denmark. Amano AYS (*Candida rugosa* lipase and enzyme activity 240 U/g, based on tributyrin assay) was received from Amano Pharmacueticals, Japan. Vinyl acetate, toluene, 1,4-dioxane, tetrahydrofuran, t-butanol and other analytical reagents were purchased from S.D. Fine Chemicals Pvt. Ltd., Mumbai, India. Cinnamyl alcohol was purchased from Sisco Chemicals Pvt. Ltd., Mumbai. All chemicals and enzymes were used without any further modification.
3.2.1 Experimental setup

The experimental set-up consisted of a 3 cm i.d. mechanically agitated glass reactor of 50 cm$^3$ capacity, equipped with four baffles and a six-bladed turbine impeller. The entire reactor assembly was immersed in a thermostatic water bath, which was maintained at the desired temperature with an accuracy of ±1 °C. A typical reaction mixture consisted of 0.01 mol cinnamyl alcohol and 0.02 mol vinyl acetate diluted to 15 cm$^3$ with toluene as a solvent. The reaction mixture was agitated at 40 °C for 15 min at a speed of 200 rpm and then 10 mg of enzyme was added to initiate the reaction. Clear liquid samples free from the catalyst particles were withdrawn periodically from the reaction mass and analysed by gas chromatography.

3.2.2 Analysis

Analysis of liquid samples was carried out by GC (Chemito 8610) equipped with flame ionization detector using 4 m×3.8 mm stainless steel column packed with 10% SE-30 stationary phase. Nitrogen was used as carrier gas at a flow rate of 1 cm$^3$.min$^{-1}$. The temperature program was as follow: 50°C for 1 min; 5 °C/min up to 160°C; 20°C/min up to 275 °C; then steady temperature for 5 min. The injector and detector temperatures were both kept at 290 °C. Undecane was used as internal standard and percentage conversion was calculated based on area under curve of limiting reactant as follows: Conversion (%) = [(A$_0$/I$_0$) – (A/I)] *100 / (A$_0$/I$_0$), Whereas A$_0$, A = Area under curve of limiting reactant at time t = 0 and t = t min, I$_0$, I = Area under curve of internal standard at time t = 0 and t = t min. Formation of cinnamyl acetate was confirmed by GC–MS (Clarus 500 GC/MS, PerkinElmer, USA).

3.3 RESULTS AND DISCUSSION

In enzymatic reactions, several factors have significant influence on enzyme activity and rate of reaction. The effect of all these parameters was studied systematically by varying one parameter at a time approach. Scheme 1 represents the reaction.
Lipase catalysed transesterification of cinnamyl alcohol with vinyl acetate in organic media

Scheme 3.1: Lipase catalyzed transesterification of cinnamyl alcohol with vinyl acetate.

3.3.1 Effect of catalysts

Lipases from different microbial origin such as lipase B from Candida antarctica, Rhizomucor miehei lipase, Thermomyces lanuginosus lipase and Candida rugosa lipase were used under similar reaction condition (Fig. 3.1).

Figure 3.1: Effect of catalysts in transesterification of cinnamyl alcohol with vinyl acetate.

cinnamyl alcohol – 0.01 mol; vinyl acetate – 0.02 mol; solvent up to 15 cm³; speed of agitation – 200 rpm; catalyst loading – 4.67 U/ml; temperature – 40 °C, Cal-B (Lipase B from Candida antarctica), RMIM (Rhizomucor miehei lipase), TLIM (Thermomyces lanuginosus lipase), AMANO AYS (Candida rugosa lipase).
Lipase B from *Candida antarctica* (Novozym 435) gave a conversion of 96% in 1 h, which was the maximum as compared to other lipases. *Rhizomucor miehei* lipase and *Thermomyces lanuginosus* lipase gave only 6 and 4% conversion, respectively. These two lipases are suitable for esterification/interesterification of high molecular weight fatty acids and their ester derivatives. *Candida rugosa* lipase gave less than 2% conversion; it shows that this enzyme may be deactivated by acetaldehyde, a co-product of reaction which forms the Schiff base with lysine residue of enzyme (weber et al., 1995). Thus, *Candida antarctica* lipase B was chosen as the best catalyst for this system and used in further experiments.

3.3.2 Effect of solvents

The effect of solvents on the enzymatic reactions is significantly important in the non-aqueous medium. Solvent affects the catalytic power of enzyme by changing the three-dimensional conformation of protein, and therefore significantly alters conversion and rate. It has been reported that biocatalysts are more stable in non-polar solvents than polar solvents or in other words, solvents which have high logP value showed good compatibility with enzymes molecules that leads to improved activity (Arcos et al., 2001). Therefore, the effect of different organic solvents with varying log P values, such as toluene [logP = 2.5], tertrahydrofuran [logP = 0.53], t-butanol [logP = 0.35] and 1, 4 dioxane [logP = -0.42] were studied under similar conditions (Fig. 3.2). Maximum conversion of 96% was obtained by employing toluene as solvent which has high logP value among those studied. 1,4-Dioxane and tertrahydrofuran led to a conversion of 70 and 44%, respectively. The conversion was very low (28%) with t-butanol. It clearly indicated that enzymes in hydrophobic solvent with high logP value show good activity compared to organic solvents with low logP value. Therefore, further experiments were carried out by using toluene as a solvent.
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Figure 3.2: Effect of solvents for transesterification of cinnamyl alcohol with vinyl acetate

cinnamyl alcohol – 0.01 mol; vinyl acetate – 0.02 mol; solvent up to 15 cm³;
speed of agitation – 200 rpm; catalyst loading – 10 mg; temperature – 40 °C,
Toluene, ▲ 1, 4 Dioxane, ■ Tetra-hydrofuran × t-Butanol

3.3.3 Effect of speed of agitation

The influence of external mass transfer resistances and intra-particle diffusion limitation of reactants, from bulk phase to active site of enzyme in immobilized beads, must be overcome to study intrinsic kinetics of reaction (Yadav and Lathi, 2003). To select the appropriate speed of agitation for transesterification reaction, a number of experiments were performed in the range of 100 to 250 rpm under similar conditions (Fig. 3.3). It was observed that the conversion increased with an increase in speed of agitation up to 200 rpm. Above this value, there was no significant change in the conversion. This indicated that there was no external mass transfer limitation above 200 rpm. This was further confirmed by comparing the time constants for reaction ($t_r$) and diffusion ($t_d$) using theoretical calculations. These are defined as follows: $t_r = C_0/r (C_0)$ and $t_d = D_S/ (k_{SL})^2$, where $D_S$ is the diffusivity of limiting reactant $(cm^2. s^{-1})$ and $k_{SL}$ is the liquid side mass transfer coefficient $(m. s^{-1})$. If $t_r >>$
t_d means that the reaction was not mass transfer controlled. Both initial concentration of cinnamyl alcohol (C_0) and rate of reaction (r(C_0)) were determined experimentally and their values were as 0.66 mol.dm^-3 and 3.83×10^{-4} mol.dm^{-3}.s. Diffusivity of cinnamyl alcohol at 40 °C was calculated by using the Siebel equation as 2.29×10^{-5} cm^2. s^{-1}. The average diameter (d_p) of the support particle was taken as 0.06 cm since the particle size ranged between 0.03 and 0.09 cm. The value of mass transfer coefficient of cinnamyl alcohol was calculated by using Sherwood number (k_{SL} = 2D/d_p) to be 7.66×10^{-4} cm.s^{-1}. From these values, the calculated time constants for reaction and diffusion were 1.72×10^{3} s and 39.15 s. The time constant for the reaction was much higher as compared to that for diffusion indicating that there was no external mass transfer resistance.

Figure 3.3: Effect of speed of agitation for transesterification of cinnamyl alcohol with vinyl acetate

- cinnamyl alcohol – 0.01 mol; vinyl acetate – 0.02 mol; solvent up to 15 cm^3;
- speed of agitation 100 rpm – 250 rpm; catalyst loading – 10 mg; temperature – 40 °C,

- *250 RPM,*  *200 RPM,*  *150 RPM*  *× 100 RPM*

Further, it is necessary to rule out the intra-particle diffusion resistance. It could be done by comparing the rate of substrate diffusion per unit interfacial area (k_{SL}C_0)
with the reaction rate per unit area \((\varphi r_0/a)\), where \(\varphi\) is the phase volume ratio and ‘\(a\)’ is the interfacial area per volume of organic phase. As the particles were spherical, \(\varphi/a = R_p/3\), where \(R_p\) is the average radius of the particles. It was found that the value of \(k_{SL}C_0 = 5.056 \times 10^{-4}\) mol.cm\(^{-2}\).s\(^{-1}\) and \(\varphi r_0/a = 3.83 \times 10^{-6}\) mol.cm\(^{-2}\).s\(^{-1}\). It indicated that the rate of substrate diffusion per unit interfacial area was higher than the reaction rate per unit area. Therefore, intra-particle diffusion limitation did not have any effect on the reaction and the reaction was controlled by intrinsic enzyme kinetics (Perry and Green, 1984). Therefore, further experiments were performed at a speed of 200 rpm.

### 3.3.4 Effect of temperature

Temperature plays a significant role in catalytic reactions. Increasing temperature may reduce mixture viscosity, enhance mutual solubility, improve diffusion process of substrates, and enhance the interactions between catalytic particles and substrates. In the case of biocatalytic process, high temperature may disrupt the active conformation of enzyme which leads to loss of activity and selectivity. So it is important to find the desired temperature for optimum enzyme activity. The effect of temperature was studied in the range of 30 to 50°C by maintaining other parameters constant (Fig. 3.4). It was observed that the conversion and initial rate of reaction increased with increase in temperature up to 40°C. Above this temperature, there was relatively smaller increase in conversion and initial rate of reaction; it also showed that the enzyme remained active at 50°C. However, repeated use at this temperature may denature the enzyme. Therefore, 40°C was chosen as the reaction temperature to carry out further reactions. Similar temperature was employed in synthesis of isoamyl acetate in non-aqueous medium as mild temperature to reduce the operational flexibility and possible enzyme denaturation in a large scale process (Romero et al., 2005). Arrhenius plot was made in the range of 303 to 323 K to find the activation energy as 6.51 kcal/mol (Fig. 3.5). This value lies within the typical value for enzyme catalytic reaction (Segel, 1975).
Figure 3.4: Effect of temperature for transesterification of cinnamyl alcohol with vinyl acetate

cinnamyl alcohol – 0.01 mol; vinyl acetate – 0.02 mol; solvent up to 15 cm³; speed of agitation - 200 rpm; catalyst loading – 10 mg; temperature – 30 °C - 50 °C, 30 °C
35 °C, 40 °C, 45 °C, 50 °C
3.3.5 Effect of enzyme loading

To select the appropriate amount of enzyme, a number of experiments were performed in the range of 5-12.5 mg enzyme loading under similar conditions. The conversion profiles obtained with various enzyme loadings are shown in Fig. 3.6. The conversion and rate of reaction increased with increase in enzyme loading up to 10 mg; beyond which there was a marginal increase in conversion. Initially, the increase of enzyme loading will accelerate the reaction rate and enhance the conversion. However, at high enzyme loading, the excess enzyme particles have a tendency to attract each other and form the enzyme aggregate which may reduce the accessibility of enzyme particles to reactants. The enzyme particle found on external surface of such aggregate are easily exposed to substrate but the mass transfer could drastically diminish the substrate availability to enzyme particles present inside the aggregates (Yang et al., 2005). Thus further experiments were carried out at 10 mg enzyme loading.

Figure 3.5: Arrhenius plot for transesterification of cinnamyl alcohol with vinyl acetate

cinnamyl alcohol – 0.01 mol; vinyl acetate – 0.02 mol; solvent up to 15 cm$^3$; speed of agitation - 200 rpm; catalyst loading – 10 mg

$$y = -3280x + 2.4199$$
$$R^2 = 0.9759$$
Figure 3.6: Effect of enzyme loading for transesterification of cinnamyl alcohol with vinyl acetate

cinnamyl alcohol – 0.01 mol; vinyl acetate – 0.02 mol; solvent up to 15 cm³; speed of agitation – 200 rpm; catalyst loading – 5 - 12.5 mg; temperature – 40 °C, 5 mg 7.5 mg 10 mg 12.5 mg
Figure 3.7: Initial rate versus enzyme loading for transesterification of cinnamyl alcohol with vinyl acetate

cinnamyl alcohol – 0.01 mol; vinyl acetate – 0.02 mol; solvent up to 15 cm³; speed of agitation – 200 rpm; catalyst loading – 5 - 12.5 mg; temperature – 40 °C

The plot of initial rate of reaction against enzyme loading shows that the initial rate increases linearly with enzyme loading (Fig. 3.7). It clearly indicated that the reaction was kinetically controlled.

3.3.6 Effect of mole ratio

A number experiments were carried out to analyse the effect of substrate concentration on the rate and conversion in transesterification of cinnamyl alcohol with vinyl acetate at 40 °C temperature using 10 mg of enzyme loading in toluene as solvent. The concentration of cinnamyl alcohol was kept constant (0.010 mol), whereas vinyl acetate concentration was varied from 0.010 to 0.030 mol; the mixture volume was kept constant at 15 cm³ by adjusting the amount of toluene. It was found that the conversion and rate of reaction had positive relationship with concentration of vinyl acetate (Fig. 3.8). The overall conversion increased with an increase in
concentration of vinyl acetate suggesting that there was no inhibition of enzyme by
vinyl acetate.

Figure 3.8: Effect of concentration of vinyl acetate for transesterification of
cinnamyl alcohol with vinyl acetate

<table>
<thead>
<tr>
<th>C:V Ratio</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>0</td>
</tr>
<tr>
<td>1:1.5</td>
<td>10</td>
</tr>
<tr>
<td>1:2</td>
<td>30</td>
</tr>
<tr>
<td>1:3</td>
<td>50</td>
</tr>
</tbody>
</table>

In second set of experiments, the concentration of vinyl acetate was kept constant
(0.010 mol) and cinnamyl alcohol concentration was varied from 0.005 to 0.020 mol
under similar condition (Fig. 3.9). The results indicated that the conversion and rate of
reaction had negative relationship with cinnamyl alcohol concentration. The overall
conversion decreased with an increase cinnamyl alcohol concentration. The possible
reason for this behaviour is that at high concentration, the alcohol is bound to the
active site of enzyme molecules by reversible competition and prevents the binding of
ester molecules. The driving force for alcohol binding might be high polarity of the
region around active serine site of the enzyme (Puskas et al., 2011). Similar kinds of
alcohol inhibition were observed in synthesis of octyl acetate and citronellyl acetate
(Yadav and Trivedi, 2003; Yadav and Borkar, 2009a).
3.3.7 Catalyst reusability

The reusability study was conducted at 40 °C in toluene as solvent. After completion of each run, the catalyst was filtered by using membrane filter then washed with the toluene, dried at room temperature and reused as such under similar condition. Typically a loss of about 5% catalyst was observed. It was found that there was a marginal decrease in conversion from 94% to 85% after three reuses, which was due to loss of enzyme during filtration and drying. There was no loss of activity but the material was lost during filtration (Fig. 3.10).
Catalyst reusability test for transesterification of cinnamyl alcohol with vinyl acetate

- Cinnamyl alcohol – 0.01 mol; vinyl acetate – 0.02 mol; solvent up to 15 cm$^3$;
- Speed of agitation – 200 rpm; catalyst loading – 10 mg; temperature – 40 °C

**3.4 KINETIC MODEL**

The kinetic model for transesterification of cinnamyl alcohol was determined by using the initial rate data under similar condition. The initial rate of reaction was calculated systematically by changing reactant concentrations over a wide range. There was no inhibition of enzyme observed with increasing concentration of vinyl acetate. On other hand, the rate of reaction decreased with increasing concentration of cinnamyl alcohol. It suggests that alcohol acts as reversible inhibitor at high concentration and forms the dead end complex with enzyme.
The Lineweaver-Burk plot was constructed by plotting reciprocal of both initial rates and vinyl acetate concentrations for different concentrations of cinnamyl alcohol at 40 °C and 200 rpm using 10 mg enzyme (Fig. 3.11). The plot showed no parallel lines and therefore the possibility of ping-pong bi-bi mechanism was ruled out. The slope and intercept changed linearly with increased concentration of cinnamyl alcohol which confirmed again that the enzyme was inhibited via formation of dead-end complex with alcohol at high concentration. The lines were intersecting at a certain point suggesting the ternary complex mechanism. The synthesis of phenylethyl ester derivatives by the same enzyme (Novozym 435) also followed the similar mechanism (Borkar, 2008). According to that mechanism, the lipase (E) first binds with vinyl acetate (A) to form the enzyme – vinyl acetate complex (AE). Then this complex (AE) combines with cinnamyl alcohol (B) to form ternary complex ABE. This ternary complex then isomerizes to another ternary complex EPQ, which releases the product.
cinnamyl acetate (P) and vinyl group (Q) and frees the enzyme E. The formation of ternary complex is sequenced as follows:

\[
E + A \xrightleftharpoons[k_2]{k_1} EA
\]  
(3.1)

\[
EA + B \xrightleftharpoons[k_{3a}]{k_{3b}} EAB
\]  
(3.2)

\[
EPQ \xrightleftharpoons[k_1]{k_2} P + EQ
\]  
(3.3)

\[
EQ \xrightleftharpoons[k_{4a}]{k_{4b}} E + Q
\]  
(3.4)

Dead end complex formation step is:

\[
E + B \xrightleftharpoons[k_{5a}]{k_{5b}} EB
\]  
(3.5)

The equation obtained with the above mechanism is:

\[
v = \frac{V_{\text{max}} [A][B]}{K_{iA} K_{mB} \left(1 + \frac{[B]}{K_{iB}}\right) + K_{mB}[A] + K_{mB}[B]\left(1 + \frac{[B]}{K_{iB}}\right) + [A][B]}
\]  
(3.6)

whereas \(v\) is the rate of reaction, \(V_{\text{max}}\) the maximum rate of reaction, \([A]\) the initial concentration of vinyl acetate, \([B]\) the initial concentration of cinnamyl alcohol, \(K_{mA}\) the Michaelis constant for vinyl acetate, \(K_{mB}\) the Michaelis constant for cinnamyl alcohol, \(K_{iA}\) the inhibition constant for vinyl acetate, and \(K_{iB}\) the inhibition constant for cinnamyl alcohol. The initial rate data were used to determine the kinetic parameters of the above mechanism by using non-linear regression analysis with the software package Polymath 5.1 (Table 3.1).
Table 3.1: Values of kinetic parameters for transesterification of cinnamyl alcohol with vinyl acetate

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{max}}$ (mol.dm$^{-3}$.s$^{-1}$.g$^{-1}$ of enzyme)</td>
<td>0.091</td>
</tr>
<tr>
<td>$K_{mA}$ (mol.dm$^{-3}$)</td>
<td>0.0252</td>
</tr>
<tr>
<td>$K_{mB}$ (mol.dm$^{-3}$)</td>
<td>0.0842</td>
</tr>
<tr>
<td>$K_{iA}$ (mol.dm$^{-3}$)</td>
<td>0.0889</td>
</tr>
<tr>
<td>$K_{iB}$ (mol.dm$^{-3}$)</td>
<td>0.0167</td>
</tr>
</tbody>
</table>

Figure 3.12: Parity plot of reaction versus simulated rates for transesterification of cinnamyl alcohol with vinyl acetate

The rates of reaction for different reactant concentrations were stimulated using the above equation to verify the proposed kinetic model. The parity plot between
stimulated rate and experimental rate suggested that the proposed mechanism was valid (Fig. 3.12).

3.5 CONCLUSION

In this study, the effects of various parameters on the conversion and rate of lipase catalysed transesterification of cinnamyl alcohol with vinyl acetate in non-aqueous medium were studied. Among the different lipases, Novozym 435 was found to be the most active catalyst in toluene as solvent. Overall conversion of 96% was achieved in 1 h with a mole ratio of cinnamyl alcohol to vinyl acetate of 1:2 using 10 mg of catalyst at 40°C. From the progress curve analysis, it was established that the reaction followed the ternary complex mechanism with inhibition by cinnamyl alcohol. The kinetic parameters were refined by using non-linear regression. There was an excellent agreement between the experimental data and stimulated data. Compared to traditional methods, this enzymatic process is better.