Chapter 6

Synthesis of 4-chloro-2-methylphenoxy acetic acid (MCPA) esters in non-aqueous media
Chapter 6- Synthesis of 4-chloro-2-methoxyphenoxy acetic acid

6.1. Introduction

Phenoxyacetic acids such as 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), 4-chlorophenoxyacetic acid (4-CPA) are extensively used as herbicides and hormone mimics. MCPA is a selective herbicide widely used in controlling weed growth (Kong, et al., 2011). At 15 mg kg\(^{-1}\) concentration of MCPA adult rats, rabbits and their offspring showed no change in their body weight (Kobal and Budihna, 1999). However, toxicological assays in rats at higher concentration showed teratogenic effect for 4-chloro -2-methylphenoxyacetic acid ethyl ester (MCPEE) (Yasuda and Maeda, 1972) while 2,4-dichlorophenoxyacetic acid (MCPA) induced myotonia in experimental dogs (Beasley, et al., 1991). Four forms of herbicides related to MCPA are MCPA acid, MCPA sodium salt, MCPA dimethylamine salt (DMAS) and MCPA ester. MCPA ester attracts most attention among four forms because of its low water solubility and environmental friendliness (Kong, et al., 2011). 2,4-Dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA) were reported to show bacterial degradation (Pieper, et al., 1988). Esterification reaction of 2-methyl-4-chlorophenoxyacetic acid (MCPA) was reported in the earlier literature using sulphuric acid (Kong, et al., 2011). There are several disadvantages of using homogeneous acid catalysis, which include costly materials of construction, neutralisation of acidic waste leading to pollution and presence of impurities in the final ester. Therefore, the most appropriate process for organic transformations will be the one which avoids homogeneous liquid acids and is eco-friendly and economical. Several general routes of ester preparation have been investigated. Heterogeneous solid acids as catalysts could be used using high temperature (Yadav and Mehta, 1993; Yadav, 2005). In contrast with solid acids, biocatalysts allow synthesis of esters to be performed at moderate temperatures (Yadav and Borkar, 2006; Yadav and Devendran, 2012).

The exploitation of enzymes as catalysts in chemical synthesis has been much in evidence in recent years. Dehydration reaction of polyanhydrides and glycols was reported using *Candida antarctica* lipase with toluene as solvent (Uyama, et al., 2003). An efficient biodiesel production from soybean oil was obtained using a recombinant *Pichia pastoris* displaying *Rhizomucor miehei* Lipase (RML) on the cell surface in an isooctane system (Huang, et al., 2012). Ethyl butyrate was synthesized
using a recombinant *Rhizopus oryzae* lipase immobilized on different supports such as Eupergit®CM, EP100 and octadecyl sephabeads (Guillen, at al., 2012). 4-Ethyl-(2-(1,3-dioxo-1,3-dihydro-2-isooindoylyl))-phenoxy acetic acid (LASSBio 482), an anti-asthma drug, was synthesized by selective hydrolysis of its methyl ester using Lipozyme RM IM (Bevilaqua, et al., 2004). Lipase catalyzed reactions were reported to kinetically proceed via Ping Pong bi–bi mechanism, ternary complex ordered bi–bi mechanism or ternary complex random bi–bi mechanism. In some cases, it involves inhibition by either substrate or product or both. A ping-pong bi–bi mechanism was proposed for *Candida antarctica* lipase B catalyzed resolution of a tertiary alcohol, citalopram intermediate (diol) (Wang, et al., 2009).

Microwave irradiation is established as an efficient heating source for a variety of chemical reactions, where high yields and reaction selectivity can be achieved in short reaction time (Loupy, 2002, Yadav and Dhoot, 2009; Yadav and Borkar, 2006; Yadav and Shinde, 2012b). Microwave irradiation results in an instantaneous localized superheating which is achieved due to dipole rotation or ionic conduction. Hence it was thought desirable to employ microwave irradiation to intensify the reaction rates and conversions. Kinetic modelling for enzymatic esterification of MCPA with different alcohols under microwave irradiation has not been reported so far. Kinetics and mechanism for the lipase catalyzed esterification of MCPA and n-butanol was investigated under microwave irradiation to propose a suitable model. The effect of various parameters on conversion and rate of reaction were studied systematically.

### 6.2. Experimental section

#### 6.2.1. Enzyme and chemicals

The Lipozyme RM IM, Lipozyme TL IM and Novozym 435 were obtained as free gift samples from Novo Nordisk, Denmark. Lipase AYS Amano was a gift sample from Amano Enzyme Inc. Japan. Lipozyme RM IM is *Rhizomucor miehei* lipase immobilized on anionic exchange resin (activity of 30 U g⁻¹, based on tristearin assay) whereas Lipozyme TL IM is *Thermomyces lanuginosus* immobilized on silica. Novozym 435 is *Candida antarctica* lipase B (CALB) immobilized on a macroporous polyacrylic resin beads (bead size 0.3–0.6 mm, bulk density 0.430 g cm⁻³, water content 3%, activity of 7,000 PLU g⁻¹). Lipase AYS Amano is *Candida rugosa* lipase in form of lyophilized powder (activity 30,000 U g⁻¹).
All chemicals used in the study were AR grade, purchased from renowned companies and used with no further purification: 4-Chloro-2-methylphenoxyacetic acid (MCPA) (Sigma Aldrich, India), n-butanol, n-pentanol, n-hexanol, isopropyl alcohol, benzyl alcohol, 2-ethyl-1-hexanol, acetonitrile, diisopropyl ether, diisobutyl ether, tetrahydrofuran and 1,4-dioxan (S.D. Fine Chemicals Pvt. Ltd., Mumbai, India). Solvents used for HPLC analysis were obtained from Thomas Baker (Mumbai, India).

6.2.2. Analytical method

The HPLC analysis was carried out on a Agilent 1260 infinity HPLC-system (pumps G1311C, auto-sampler G1329B, diode-array-detector G1315D) using ZORBAX- RP C-18 column (4.6 × 250 mm; 5 µm, Agilent Industries, USA) under the following conditions: mobile phase, acetonitrile/H₂O (70:30, v/v); flow rate, 1.0 mL min⁻¹; and column temperature, 25 °C.

6.2.3. Experimental set-up

6.2.3.1. Conventional heating

The experimental set up used for conventional heating studies was the same as described in section 3.2.2 of Chapter 3. A typical reaction procedure for lipase catalyzed synthesis of MCPA ester contained 0.1 mmol MCPA and 0.3 mmol n-butanol, diluted to 15 mL with 1,4-dioxan as a solvent. The reaction mixture was agitated at 60 °C for 15 min at a speed of 300 rpm. The reaction was initiated by adding a known amount of lipase. Samples were collected at regular intervals, filtered to eliminate particulate matter, if any, and analyzed by HPLC.

6.2.3.2. Microwave heating

The experimental set up used for microwave heating studies was the same as described in section 4.2.3.2 of Chapter 4. The CEM Discover microwave reactor could be used to conduct experiments up to microwave power of 300 W. The experiments were carried at constant temperature. A constant microwave irradiation was provided (30–40 W). Experimental conditions were maintained same as in conventional heating as described in section 6.2.3.1, unless otherwise stated.
6.2.3.3. Enzyme kinetics

Different process parameters were studied to elucidate the kinetics of lipase catalyzed esterification of MCPA with n-butanol. Concentrations of substrates were systematically varied over a wide range to study their effect on rate of reaction with Novozym 435 loading of 4 mg cm\textsuperscript{-3}. In one set of experiments, n-butanol \((B)\) concentration was varied from 0.1 to 0.4 mmol at a fixed quantity of MCPA \((A)\) (0.05-0.3 mmol) while in another set, different MCPA \((A)\) concentrations were employed from 0.05 to 0.3 mmol at a fixed quantity of n-butanol (ranging from 0.1 mmol to 0.4 mmol). The concentration profiles were used to calculate initial rates of reaction.

6.3. Results and discussion

Scheme 6.1 depicts the reaction.

![Scheme 6.1: Microwave irradiated Novozym 435 catalysed synthesis of MCPA ester](image)

6.3.1. Conventional heating vs. microwave irradiation

Enzymatic esterification of MCPA and n-butanol was studied under both conventional heating and microwave irradiation as a model reaction. A 2-fold increase in initial rate was obtained under microwave irradiation vis-à-vis conventional heating which resulted in reduced time of reaction showing process intensification. This suggested that microwave capturing nature of the reactants (MCPA and n-butanol) was contributing to the higher reaction rate. We have reported similar effect for enzymatic transformation under microwave irradiation for different reactions (Yadav and Shinde, 2012b; Yadav and Pawar, 2012b; Yadav and Devendran, 2012). Enzyme under microwave irradiation may behave differently to some extent. This is due to conformational modification in enzyme under microwave irradiation which assists the substrate to come to the vicinity of active site more easily. Control experiments were also conducted in the absence of enzyme as well as only under microwave irradiation.
without the enzyme. In either case, no conversion was observed. This clearly indicated a synergistic effect between microwave irradiation and enzyme catalysis.

6.3.2. Effect of various biocatalysts

Enzymatic esterification of MCPA with n-butanol was selected as the model reaction to study effect of different immobilized lipases on conversion and rate of reaction (Fig. 6.1). The conversion varied noticeably with the type of lipase. The Lipozyme RMIM and Novozym 435 showed conversion of 9 % and 83 %, respectively whereas very less conversion was obtained with Lipozyme TL IM and Lipase AYS Amano. We chose to study these different enzymes to find out if any significant activation could result owing to microwave irradiation, regardless of their reported applications. Novozym 435 being the most active catalyst amongst those studied was used in further experiments.

Figure 6.1: Effect of various biocatalysts [Reaction Condition: MCPA, 0.1 mmol; n-butanol, 0.3 mmol; solvent, 1,4-dioxan up to 15 ml; temperature, 60°C; speed of agitation, 300 rpm; catalyst, 4 mg cm⁻³; (♦ Novozym 435, ■ Lipozyme RM IM, ▲ Lipozyme TL IM, × Lipase AYS Amano)]
6.3.3. Effect of various solvents

Different solvents such as 1,4-dioxan, acetonitrile, tetrahydrofuran, diisopropyl ether and diisobutyl ether were used to study their effect on process intensification. Maximum conversion of 83% was obtained using 1,4-dioxan as solvent (Fig. 6.2) and hence was used for further studies. Nature of solvent has great impact on activity of enzyme which requires essential water for maintaining the native, catalytically active enzyme conformation in the organic solvent (Wehtje and Adlercreutz, 1997). For non-aqueous enzymatic transformations, hydrophobic solvents are more favoured against hydrophilic solvents. Because hydrophilic solvents cause stripping of the necessary water layer around the enzyme, resulting in reduced enzyme activity (Yadav and Pawar, 2012; Yadav and Shinde, 2012b). Therefore, 1,4-dioxan was used for further studies.

6.3.4. Effect of speed of agitation

The effect of speed of agitation was studied in the range of 100–400 rpm using Novozym 435 and 1,4-dioxan (Fig. 6.3). The conversion profile and initial rate data were obtained from these profiles. An increase in conversion from 32 to 83% was
Figure 6.3: Effect of speed of agitation [Reaction Condition: MCPA, 0.1 mmol; n-butanol, 0.3 mmol; solvent, 1,4-dioxan up to 15 ml; temperature, 60°C; Novozym 435, 4 mg cm⁻³; speed of agitation, (♦ 100 rpm, ■ 200 rpm, ▲ 300 rpm, × 400 rpm)]

found when speed of agitation was increased from 100 to 300 rpm. On the other hand, there was no significant increase in the rate of reaction and conversion at 300 and 400 rpm. At higher speed of agitation, some enzyme particles were found sticking to the reactor wall due to intense agitation which resulted in reduced effective catalyst loading. Thus, further studies were carried out at 300 rpm.

6.3.5. Effect of different alcohols

Esterification of MCPA was investigated using a variety of alcohols such as n-butanol, n-pentanol, n-hexanol, isopropyl alcohol, 2-ethyl-1-hexanol and benzyl alcohol. The conversions obtained with primary alcohols n-butanol, n-pentanol and n-hexanol were 83, 76 and 61 %, respectively (Fig. 6.4). Esterification with branched chain alcohols, isopropyl alcohol and 2-ethyl-1-hexanol resulted in lesser conversions of 46 % and 80 %, respectively whereas aromatic alcohol, benzyl alcohol exhibited conversion of 66 %. The difference in conversion with chain length and nature of alcohol can be related to molecular size of alcohol, solubility in reaction solvent and the affinity of lipase for specific individual alcohol (Verma and Madras, 2010).
Figure 6.4: Effect of different alcohol [Reaction Condition: MCPA, 0.1 mmol; alcohol, 0.3 mmol; solvent, 1,4-dioxan up to 15 ml; temperature, 60°C; speed of agitation, 300 rpm; Novozym 435, 4 mg cm$^{-3}$]

Figure 6.5: Effect of catalyst loading [Reaction Condition: MCPA, 0.1 mmol; n-butanol, 0.3 mmol; solvent, 1,4-dioxan up to 15 ml; temperature, 60°C; speed of agitation, 300 rpm; Novozym 435, ( 2.67 mg cm$^{-3}$, 3.33 mg cm$^{-3}$, 4 mg cm$^{-3}$, × 4.67 mg cm$^{-3}$)]
6.3.6. Effect of catalyst amount

The effect of Novozym 435 amount in esterification reaction of MCPA under microwave irradiation was demonstrated while molar ratios of substrates were maintained constant. Novozym 435 loading in reaction medium was varied from 2.67 to 4.67 mg cm\(^{-3}\) (Fig. 6.5). The reaction rate increased linearly with an increase in enzyme loading up to 4 mg cm\(^{-3}\) beyond which no significant increase in conversion was found and it clearly showed that the biocatalyst loading was much higher than the needed and the rate was limited by the external mass transfer. Hence, loading of 4 mg cm\(^{-3}\) was considered to be the most efficient and optimal under the specified conditions.

6.3.7. Effect of n-butanol concentration

The esterification of MCPA with n-butanol was investigated at different moles of n-butanol, while the moles of MCPA (0.1 mmol) were kept constant using 1,4-dioxan as solvent. Maximum conversion and rate of reaction were achieved at 0.3 mmol of n-butanol (Fig. 6.6). With increase in moles of n-butanol from 0.1 to 0.4 mmol the conversion and reaction rate was increased. The conversion was decreased with

![Figure 6.6: Effect of n-butanol concentration](image-url)
further increase in n-butanol concentration. This could be related to the inhibitory effect of n-butanol at high concentration on Novozym 435 enzyme.

6.3.8. Effect of MCPA concentration

The esterification of MCPA with n-butanol was studied at different moles of MCPA, keeping the moles of n-butanol (0.3 mmol) constant using 1,4-dioxan as solvent. Maximum conversion (83 %) and rate of reaction were obtained at 0.1 mmol of MCPA (Fig. 6.7). By increasing the moles of MCPA from 0.05 to 0.2 mmol, an increase in conversion and reaction rate was obtained, while further increase in MCPA concentration resulted in a decrease in the conversion. The decreased conversion and reaction rate with increased concentration of MCPA could be attributed to the formation of inhibitory dead end complex between MCPA and lipase at higher MCPA concentration.

6.3.9. Effect of temperature

The effect of temperature on conversion and initial rate was investigated under conventional heating and microwave irradiation. As discussed in section 6.3.1, under microwave irradiation overall conversion and the rate of reaction for esterification of MCPA was higher (Fig. 6.8). With an increase in temperature in range of 40–70 °C, an initial rate was found to be increased from $1.4 \times 10^{-3}$ to $2.85 \times 10^{-3}$ mol l$^{-1}$ min$^{-1}$ g$^{-1}$ of enzyme and the conversion increased from 26 % to 83 % (Fig. 6.8). This is due to the momentum provided by microwave energy to surpass energy barrier and thus the reaction is completed more rapidly than conventional heating. Further, under microwave irradiation, high instantaneous heating of the substance(s) above the normal bulk temperature results in greater number of more energetic collisions which is the primary factor for the observed rate enhancements. The activation energy values were obtained by the Arrhenius plot (Fig. 6.9) as 5 and 4.41 kcal mol$^{-1}$ under microwave and conventional heating, respectively. These are not much different thereby suggesting that the pre-exponential factor in the Arrhenius equation is more enhanced due to microwaves resulting into more fruitful collisions.
Figure 6.7: Effect of MCPA concentration [Reaction Condition: MCPA, 0.1 mmol; n-butanol 5-30 mmol; solvent, 1,4-dioxan up to 15 ml; temperature, 60°C; speed of agitation, 300 rpm; Novozym 435, 4 mg cm$^{-3}$; (○ 0.05 mmol; ■ 0.1 mmol, ▲ 0.2 mmol, × 0.3 mmol)]

Figure 6.8: Effect of temperature (conventional vs. microwave heating) [Reaction Condition: MCPA, 0.1 mmol; n-butanol, 0.3 mmol; solvent, 1,4-dioxan up to 15 ml; speed of agitation, 300 rpm; Novozym 435, 4 mg cm$^{-3}$, (● Conversion (%) - Microwave, ■ Conversion (%) - Conventional, Initial rate (mol L$^{-1}$ min$^{-1}$) - Microwave, Initial rate (mol L$^{-1}$ min$^{-1}$) - Conventional)]
6.3.10. Effect of reusability

The reusability of Novozym 435 was studied under optimized conditions to examine the stability and recyclability of enzyme. The enzyme after was filtered, washed with 1,4-dioxan after each use, dried at room temperature and reused for further studies. Only a slight decrease in conversion was observed from 83 % to 79 % after three reuses for esterification of MCPA with n-butanol which may possibly be because of the loss of enzyme during handling (Fig. 6.10). Since no make-up of
enzyme was made for the losses, the studies showed that Novozym 435 was quite stable.

6.3.11. Kinetic model based on initial rate measurements

Based on initial rates ($V$), a kinetic model for esterification of MCPA with n-butanol was proposed. Initial rates for MCPA ester synthesis were determined systematically from the linear section of the conversion plot. Increase in the n-butanol ($B$) concentration, at different fixed MCPA ($A$) concentration, increased the initial rate and reached the maximum at a critical concentration. The reaction rate decreased with further increase in concentration of $B$ and thus the n-butanol inhibition was significant. Thus, it is believed that n-butanol at high concentrations forms dead end inhibitory complex with the lipase. Similar effect was observed for at higher concentration of MCPA ($A$) used. The Lineweaver–Burk plot of 1/initial rate (L min mol$^{-1}$) versus 1/[n-butanol] (L mol$^{-1}$) at different constant MCPA concentration was made (Fig. 6.11). Possibility of sequential mechanism is ruled out because there are no crossings of lines or any common intersection point. In fact, lines are parallel, which suggests the Ping Pong bi–bi mechanism with both substrate inhibitions. It can be seen from Fig. 6.11 that the slope of the lines is not affected at low acid and alcohol concentrations which point towards a mechanism that necessitates the dissociation of one product before the association of the second substrate to the enzyme–substrate complex.

The proposed mechanism is as follows:

\[ E + A \overset{k_1}{\rightleftharpoons} EA \]  \hspace{1cm} (6.1)

\[ EA \rightleftharpoons FP \]  \hspace{1cm} (6.2)

\[ FP \overset{k_2}{\rightleftharpoons} F + P \]  \hspace{1cm} (6.3)

\[ F + B \overset{k_3}{\rightleftharpoons} FB \]  \hspace{1cm} (6.4)

\[ FB \rightleftharpoons EQ \]  \hspace{1cm} (6.5)

\[ EQ \overset{k_4}{\rightleftharpoons} E + Q \]  \hspace{1cm} (6.6)

Inhibition step by MCPA
\[ F + A \xrightarrow{k_1} FA \] \[ \xrightarrow{k_{-1}} \] (6.7)

Inhibition step by n-butanol

\[ E + B \xrightarrow{k_2} EB \] \[ \xrightarrow{k_{-2}} \] (6.8)

From these observations we postulate a ping–pong bi–bi mechanism with inhibition by both substrates. By analogy to the classical mechanism of lipase, it is assumed that MCPA (A) binds first to the free enzyme (E) and forms a noncovalent enzyme acetate complex (EA), which releases the first product, water (P) and (F) modified enzyme. The second substrate alcohol (B) reacts with modified enzyme (F) to give the complex (FB) which gives the product MCPA butyl ester (Q) and free enzyme (E). Along with this, alcohol (B) also forms the dead end complex (E’B) by binding to free enzyme (E) and MCPA (A) also forms the dead end complex with modified enzyme (F) to give complex (FA).

**Figure 6.11:** Lineweaver-Burk plot of 1/initial rate (L min mol\(^{-1}\)) versus 1/[n-butanol] (L mol\(^{-1}\)) at different constant MCPA concentration (\(0.05 \text{ mmol, 0.1 mmol, 0.2 mmol})

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The rate equation obtained with this mechanism is as follows:

\[
v = \frac{V_{\text{max}}[A][B]}{K_m A B + K_m A [A] + K_i A + [A][B]}\]  
(6.9)

Where \([A]\) is MCPA concentration (mol L\(^{-1}\)), \([B]\) is n-butanol concentration (mol L\(^{-1}\)), \(K_{mA}\) is the Michaelis constant for MCPA (mol L\(^{-1}\)), \(K_{mB}\) is the Michaelis constant for n-butanol (mol L\(^{-1}\)), \(K_iA\) is the inhibition constant for MCPA and \(K_iB\) is the inhibition constant due to n-butanol. \(V\) and \(V_{\text{max}}\) are the initial rate and maximum rate (mol L\(^{-1}\) min\(^{-1}\)), respectively.

Kinetic constants were calculated by using Polymath 6.0 software as follows: \(V_{\text{max}}\) (mol L\(^{-1}\)) 0.00413, \(K_{mA}\) (mol L\(^{-1}\)) 0.019, \(K_{mB}\) (mol L\(^{-1}\)) 0.092, \(K_iA\) 0.203 and \(K_iB\) 0.351. A parity plot showed excellent correlation coefficient between experimental versus simulated rates (Fig. 6. 12). This validates proposed Ping Pong bi-bi model with both substrate inhibitions.

6.4. Conclusion

MCPA butyl ester was synthesised using different immobilized lipases among which Novozym 435 was the best. The rate of reaction increased significantly under the influence of microwave irradiation. An optimum enzyme loading of 4 mg cm\(^{-3}\)
and optimum temperature of 60 °C was observed. A kinetic model was postulated based on initial rate data and conversion profiles. The Ping Pong bi–bi mechanism with MCPA as well as n-butanol substrate inhibition was assumed for enzymatic esterification of MCPA with n-butanol. The proposed mechanism was found to fit the data well for microwave assisted Novozym 435 biotransformation reaction. The enzyme was reusable. Esterification of MCPA was also investigated with different alcohols, namely, n-butanol, n-pentanol, n-hexanol, isopropyl alcohol, 2-ethyl-1-hexanol and benzyl alcohol.