CHAPTER III

SCREENING ON THE PERFORMANCE OF DUAL LIPASES SYSTEM IN FERULATE ESTERS SYNTHESIS

3.1 Introduction

The versatility of lipase (triacylglycerol acylhydrolase; EC 3.1.1.3) has paved the way for its wide applications especially in fats and oils segment. Besides being biodegradable non-toxic compound, lipase displays varied selectivity, regiospecificity and mild operating conditions. Nowadays, the used of lipases in modifying fats and oils to form structured lipids are a mature topic due to their improvable characteristics, like certain physical (Palla & Carrin, 2014) and chemical properties (Ciftci & Saldaña, 2012) as well as nutritional benefits (Ma et al., 2014; Palla & Carrin, 2014).

Ferulic acid is a phenolic acid naturally found in plant kingdom possessing functional and nutritional benefits (Barone et al., 2009). Due to the hydrophilicity, however, ferulic acid exhibits low solubility and stability in various solvent systems and thus restricts its application in diverse fields. Therefore, the strategy to transesterify of the ferulic acid or its ethyl ester (ethyl ferulate) with lipophilic media was a trendy idea to enhance the solubility of the compounds (Compton et al., 2000; Yang et al., 2012; Sun et al., 2013; Sun & Bi, 2014). The new amphiphilic molecule possesses surface activity because it

contains both hydrophobic head (fatty acid moiety) and hydrophilic tail (phenolic moiety) (Yang et al., 2012). Together, its original function properties such as antioxidant, anti-inflammatory, antiviral and UV absorbing also showed an improvement (Murakami et al., 2002; Lin et al., 2005).

Non-specific lipase of Novozym 435 from Antartic yeast *Candida antarctica* commonly reported as an efficient biocatalyst for the synthesis of ferulate esters (Xin et al., 2011; Yang et al., 2012; Sun et al., 2013). However its application for industrial purpose are limited due to the relative high production cost of this lipase which has a direct impact on the overall process cost (Fjerbaek et al., 2009; Reyes-Duarte et al., 2011; Chattopadhyay & Sen, 2013). Combination of Novozym 435 with any specific lipase simultaneously seems to be an alternative way to overcome such shortcoming and reveal their combined interactions.

Consequently, the principal objective of this part was to scrutinize the possible interaction between two immobilized lipases of non-specific Novozym 435 and 1, 3-specific Lipozyme RM IM (from fungi *Rhizomucor miehei*) in a dual lipases system, for the high yield production of ferulate esters. Olive oil was selected as a model substrate due to its beneficial dermatologic effects (Badiu et al., 2010) containing unique set of triacylglycerols mainly triolein and other fatty acids such as palmitic, palmitoleic, stearic, linoleic and linolenic (Baccouri et al., 2008). Detection and identification of reaction products were facilitated by Fourier Transform Infrared (FTIR).

3.2 Materials and Methods

3.2.1 Materials

Substrates (ethyl ferulate and olive oil) were obtained from Sigma-Aldrich (St. Louis, USA), solvents and chemicals (toluene, ethanol, acetone and potassium hydroxide) were purchased from Merck, Germany. Commercial lipases of Novozym 435 (immobilized lipase B from *Candida antarctica*) and Lipozyme RM IM (immobilized lipase from *Rhizomucor miehei*) were purchased from Sigma-Aldrich (St. Louis, USA). All chemicals were commercially available and of analytical grade unless otherwise specified.

3.2.2 Enzymatic Synthesis of Ferulate Esters: Screening of Dual Lipases System

The transesterification method was modified from Compton et al. (2000). 100 mg of various ratio of immobilized lipases of Novozym 435 to Lipozyme RM IM (1: 0, 9: 1, 8: 2, 7: 3, 6: 4, 5: 4, 4: 4, 4: 5, 4: 6, 3: 7, 2: 8, 1: 9 and 0: 1) were added into 25 mL of screw capped vials containing a mixture of 1 g of ethyl ferulate and 4 g of olive oil in 5 mL of toluene. The vials were placed in a controlled water-bath shaker at 60 °C and shaken at 200 rpm. The mixture was continuously reacted for 12 hrs. All batch reactions were conducted under the above conditions unless stated elsewhere.

3.2.3 Percentage Conversion of Ferulate Esters

After completion of each assigned period, the reaction was terminated with 7 mL of ethanol: acetone (1: 1 v/v) and the lipases were filtered. The percentage conversion (%) of ferulate esters was measured by determining the remaining unreacted fatty acids in the reaction mixture by titration with 0.3 M KOH in an automatic titrator (Metrohm, Switzerland). All the samples were assayed in triplicate and the experiment was repeated twice. The percentage conversion of ferulate esters was calculated based on the Equation 3.1.

Conversion of ferulate esters (%) =

$$\frac{\text{Volume of KOH (without lipases)} - \text{Volume of KOH (with lipases)}}{\text{Volume of KOH (without lipases)}} \times 100$$

(Equation 3.1)

3.2.4 Verification of Reaction Components Using FTIR Analysis

All the samples before and after incubation period were analyzed using Fourier Transform infrared spectrophotometer (Perkin Elmer, Varian 3100). The samples were placed in horizontal attenuated total reflectance (HATR) consisting of germanium crystal at a controlled temperature (20 °C). All spectra were measured and subtracted with background spectrum of air, at each scanning of the sample. The spectrum was recorded as transmittance value at each frequency point data conducted in three replicates.

3.3 Results and Discussion

3.3.1 Enzymatic Synthesis of Ferulate Esters

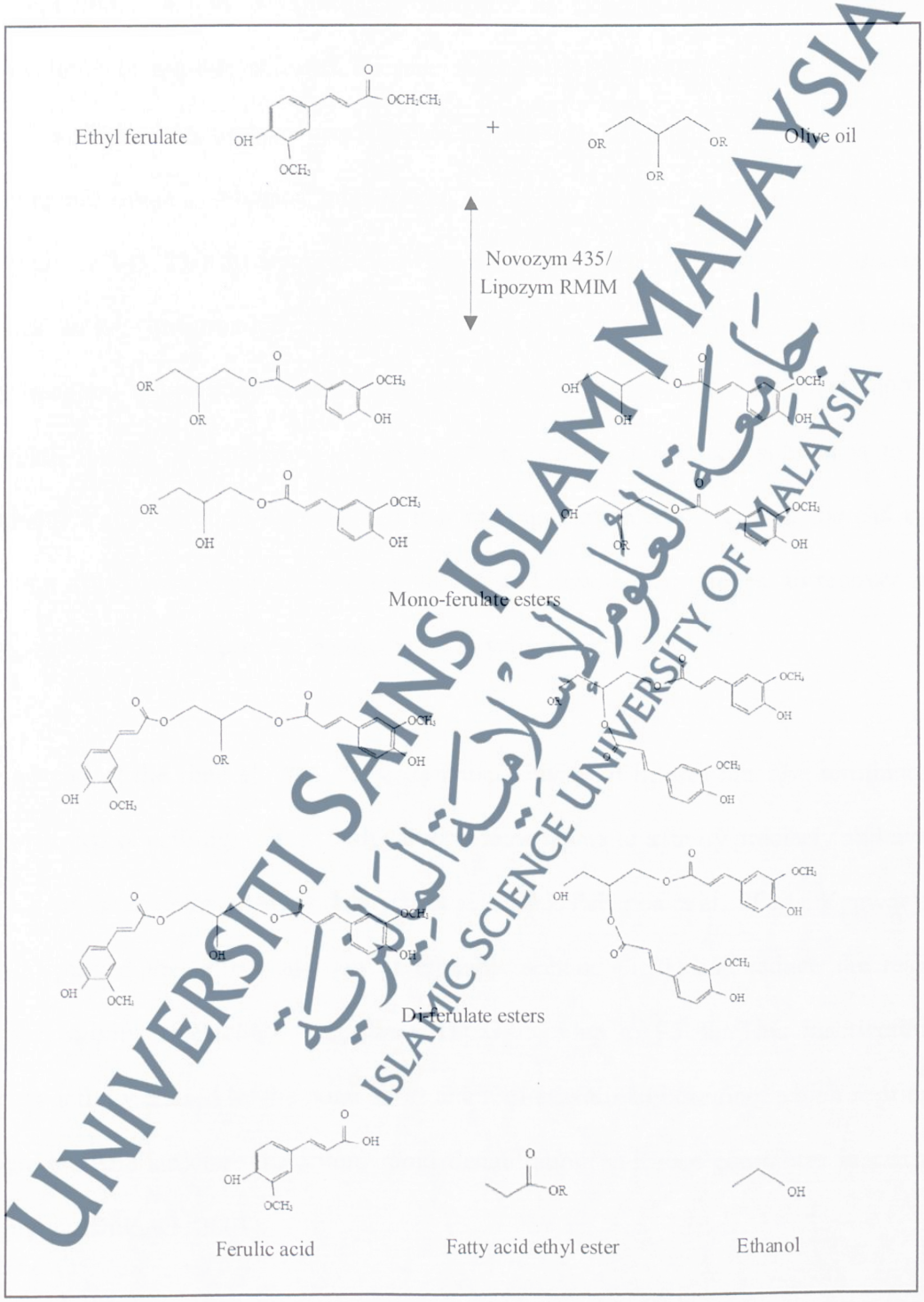
Enzymatic synthesis of ferulate esters was successfully carried out by transesterification between ethyl ferulate and olive oil using dual lipases system of Novozym 435-Lipozyme RM IM as biocatalyst. Transesterification refers to a process that converts one ester to another ester. Due to the difference type of acyl donor (ethyl ferulate) and acyl acceptor (olive oil) involved, this enzymatic synthesis also known as interesterification reaction. The reaction was performed in the presence of toluene as medium to dissolve the reaction components as well as shift the equilibrium to the synthetic process.

The order by which the components migrated in the reaction system was deeply discussed by several authors (Burham et al., 2009; Xin et al., 2009; Xin et al., 2011) in the synthesis of ferulate esters, which can mirror the sequence of our reaction process even under different developing conditions. The acyl donor of ethyl ferulate binds first to the lipase forming acyl-enzyme complex with the release of ethanol. At the same time, acyl acceptor of triacylglycerol undergoes hydrolysis to generate a diacylglycerol containing a free hydroxyl group (Laszlo & Compton, 2006). Once this initial hydrolysis achieved, transesterification proceeds smoothly because the pool of available water molecules has attained equilibrium (Balcaõ et al., 1998). Thermodynamically unstable structure of the acyl-enzyme complex is further reacted with diacylglycerol to form ferulate esters

(Laszlo & Compton, 2006). The mechanism action of dual lipases system in enhancing the process is still under discovery, yet, it may contributed by the difference specificities of these two lipases towards different fatty acids present in natural oil (Rodrigues & Ayub, 2011).

Due to the multiple fatty acid compositions attached to the glycerol backbone of olive oil, transesterification with ethyl ferulate may result in a mixture of ferulate esters species. Referring to the previous studies which have used different sources of acyl acceptor such as castor oil (Sun & Bi, 2014), monostearin (Sun et al., 2013), fish oil (Yang et al., 2012), triolein (Xin et al., 2009; Xin et al., 2011) and soybean oil (Laszlo & Compton, 2006), we can summarize that there were two types of ferulate esters formed: mono-ferulate esters and di-ferulate esters. Mono-ferulate esters refer to one feruloyl moiety on glycerol backbone while di-ferulate esters contain two feruloyl moieties on glycerol backbone. Possible products of transesterification of ethyl ferulate with olive oil are depicted in Figure 3.1.

FIGURE 3.1: Possible Products of Transesterification of Ethyl Ferulate with Olive Oil



The application of toluene as solvent in this study was useful to liquefy the substrates for efficient lipases action. According to Yahya et al. (1998), lipases are well suited for applications in organic solvents because their catalytic feature involves a lipid-water interface. This characteristic gives lipases an inherent affinity for hydrophobic media. Among the organic solvents, toluene can be easily removed because of its volatility (Clough, 2014). This hydrophobic solvent also dissolves less in water, so distort the lipases' active conformation only weakly (Laane et al., 1987). In the absence of solvents, high reaction temperature is needed to reduce the viscosity of the substrates and long incubation time is required to improve effective contact between substrates to form products. Yahya et al. (1998) claimed that the elimination of solvents at the end of the reaction offers significant cost savings than that of downstream process to recover fewer components would be present in solvent-free systems.

At the end of the time allotted, the enzymatic activity of lipases must be terminated to maintain reproducibility of the results, determine enzymatic activity precisely and avoid a false increase in color intensity (Kanwar et al., 2005; Palacios et al., 2014). Kanwar et al. (2005) proved that direct addition of ethanol: acetone (1: 1) can reduce the residual lipases activity of *Bacillus coagulans* MTCC-6375 up to 95 %. This inactivation of lipases activity caused by the polar short chain of ethanol and acetone, which resulted in severe enzyme structure distortion, rapid denaturation and even completes inactivation (Ogino & Ishikawa, 2001).

3.3.2 Screening of Dual Lipases System in the Synthesis of Ferulate Esters

In this study, Novozym 435 and Lipozyme RM IM were screened individually and in combinations (dual lipases system) for their potential combined interactions. The performance of different ratios of Novozym 435 to Lipozyme RM IM in the synthesis of ferulate esters is shown in Figure 3.2. The results are clearly shown that the mixtures of Novozym 435-Lipozyme RM IM at any ratios gave a significant effect to the degree of transesterification at more than 80 %, with the highest conversions were obtained using 1: 9 ratio (86.47 %), as compared to the single system of Novozym 435 (69.71 %) and Lipozyme RM IM (70.06 %). These observations agree well with Kuo et al. (2012) and Banerjee et al. (2013) whom used the same dual lipases system of Novozym 435-Lipozyme RM IM for higher conversion of esters.

In addition, the work of Lee et al. (2006) on the synthesis of biodiesel has also shown high conversion of esters up to 99 % using a combination of 1, 3-specific immobilized *Rhizopus oryzae* with non-specific immobilized *Candida rugosa* as compared to the single lipase system. Later, Guan et al. (2010) reported that *Rhizomucor miehei* catalyzed 68.5 % of methanolysis of soybean oil for biodiesel production. However, by combining the lipase with *Penicillium cyclopium* lipase, the production yield was increased up to 95 %. This demonstrated a dual lipases system has high potential to be employed in various bioorganic syntheses.

Banerjee et al. (2013) suggested that interactive effects of a dual lipases system were depended on the mixing ratio. They found that the application of 1:1 ratio of non-specific lipase to specific lipase led to the highest production of biodiesel from coffee ground oil. The work of Ibrahim et al. (2008) also showed the preference for equivalent amount mixture of non-specific lipase and specific lipase in the interesterification palm stearin with coconut oil. As shown in Figure 3.2, the percentage conversion obtained for various ratios of dual lipases system were fluctuated. The real reason accounting for above observations are not clear. However, one possible explanation is due to the small gap employed between the ratios. Our results seem to suggest that the ratio of 1 : 9 Novozym 435-Lipozyme RM IM contributed to the highest percentage conversion (86.47 %) of ferulate esters.

The result obtained was quite unexpected, as Lipozyme RM IM exhibited better conversion than Novozym 435. In fact, lipases screening were conducted by previous researchers in the synthesis of ferulate esters and revealed Novozym 435 exhibited greater conversion over Lipozyme RM IM (Compton et al., 2000; Sun et al., 2013; Sun & Bi, 2014). They found that by using Novozym 435, more than 98 % conversions of ferulate esters were achieved in solvent-free system exceptional for the work done by Compton et al. (2000), where only 44 % conversion was produced in toluene as medium. On the contrary, Salis et al. (2003) proved that Lipozyme RM IM was more active than Novozym 435 in n-hexane medium towards wax esters production. Besides, Lipozyme RM IM has reported to lose its activity after being used in solvent-free medium (Oguntimein et al., 1995). Obviously, the presence of solvent plays a crucial role which

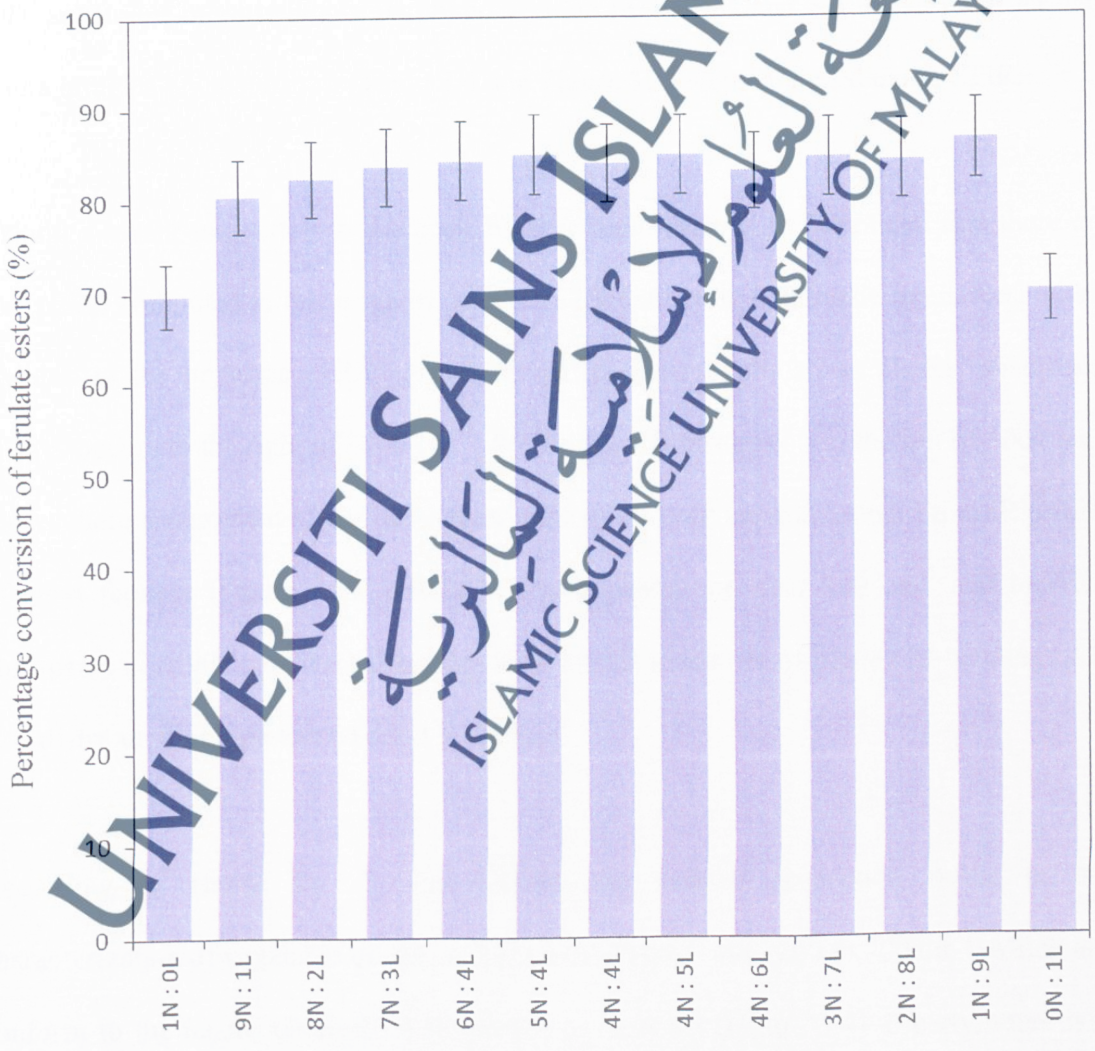
reflects our finding. In a study by Stamatis et al. (2001) proposed that electronic and stearic effect of hydroxylated dericatives of cinnamic acid caused lipase from *R. miehei* showed higher reaction rate and yield compared to the lipase from *C. antarctica*.

Although Novozym 435 and Lipozyme RMIM were employed simultaneously in the same medium environment, the microenvironments for both lipases are different. Novozym 435 is an immobilized lipase on macroporous acrylic resin containing 1-2 % (w/w) of water. This explains why Novozym 435 consistently showed high yield of ferulate esters in solvent free-system (Yang et al., 2012; Sun et al., 2013). A small amount of water is essential to retain structural integrity, active site polarity and protein stability. Novozym 435 also exhibits a very small lid and a funnel-like catalytic site, appropriately for short acyl chains (Martinsa et al., 2014). On the other hand, the support of Lipozyme RM IM is Duolite ES 562, a weak anion–exchange resin. An anion-exchange resin would tend to attract carboxylic acid due to its positive charges. Moreover, due to the crevice-like binding site located near the protein surface, Lipozyme RM IM is suitable to accommodate long acyl chains (Yahya et al., 1998; Guti'errez-Ayesta, 2007).

Referring to our finding, the application of large portion of 1, 3-specific Lipozyme RM IM lipase would trigger hydrolyzation of triacylglycerol of olive oil to form diacylglycerol for rapid acyl-enzyme actions. Further addition of small quantity of nonspecific Novozym 435 would create an environment containing various specificity of lipases for the diverse fatty acids presented in the system, thus result in an enhanced percentage conversion. Considering the degree of percentage conversion and economical

point of view, 1: 9 Novozym 435-Lipozyme RM IM was used as an optimum ratio for further study.

FIGURE 3.2: Screening of Dual Lipases System in Transesterification of Ethyl Ferulate with Olive Oil. The Reaction Was Carried Out At 60 °C, 1: 4 g Ethyl Ferulate/ g Olive Oil, 5 mL of Toluene and 100 mg of Immobilized Lipases



3.3.3 Identification of Reaction Components Using FTIR Analysis

Since every different type of bond in a compound or same type of bond in two different compounds have a different natural frequency of vibration, thus, Fourier transform infrared spectrophotometer (FTIR) can be used as a fingerprint to give structural information about the compounds (Hsu, 1997). Data position, peak shape and peak intensity will often give a clue to its identity as well (Appendix C). In this study, the characteristic of ethyl ferulate (Table 3.1 and Figure 3.3), olive oil (Table 3.2 and Figure 3.4), substrates mixture before incubation period (Table 3.3 and Figure 3.5) and ferulate esters after 12 hrs incubation (Table 3.4 and Figure 3.6) were analyzed using FTIR.

Taking a closer inspection to the each IR spectrum obtained, it is noticed that there are two peaks computed in the range of 3300 to 2750 cm^{-1} with difference intensities, which are due to the presence of CH-stretching. In aliphatic hydrocarbon chains, absorption always occurs to the right of 3000 cm^{-1}. While, the CH absorption to the left of 3000 cm^{-1} shows there are existent of aromatic compound in the each sample tested. From IR profile of ethyl ferulate (Figure 3.3), visibly, a strong absorption near 800 cm^{-1} and medium absorption near 900 cm^{-1} were detected. It is reflects to the out-of-plane CH-bending of 1, 2, 4-distributed ring pattern of ethyl ferulate.

According to Henna Lu & Tan (2009), the interest absorption peaks in the characterization of vegetable oil are at 3600 to 2800 cm^{-1} and 1800 to 700 cm^{-1}, which are conform to the results obtained in IR profile of olive oil (Figure 3.4). Clearly, there is a

visible peak observed near 3008 cm^{-1}. The band was associated to the stretching vibration of *cis* double bonds CH groups while the latter denoted the stretching of C-C double bonds. The *cis* double bonds CH groups and C-C double bonds are found widely in unsaturated fatty acids such as oleic acids, linoleic acids and linolenic acids as presented in the chemical structure of olive oil.

The two most characteristic features in the spectrum of an ester are the strong C=O and C-O stretching absorptions. The C=O group gives rise to strong absorption in the region 1820 to 1660 cm^{-1} while C-O group shows strong intensity absorption near 1300 to 1000 cm^{-1}. Almost all the sample tested (Figure 3.3, 3.4, 3.5 and 3.6) display these types of absorptions with difference intensities. The formation of ferulate esters can be figured out from the comparison result of IR spectra before (Figure 3.5) and after (Figure 3.6) incubation time. The comparison between these two results shows an increment in intensity of C=O group at peak 1743 cm^{-1} and C-O group at peak 1157 cm^{-1} after being incubated for 12 hrs. This shows that both ethyl ferulate and olive oil have reacted with each other to form ferulate esters. Interestingly, a broad peak of OH-bond was detected at peak 3414 cm^{-1} after 12 hrs of incubation time (Figure 3.6). This may be due to the formation of ethanol, the side product of the reaction synthesis.

TABLE 3.1: Summary of IR Absorption Peak of Ethyl Ferulate

Type of vibration	Frequency (cm^{-1})	% Transmittance	Intensity
-CH-aromatic	3178	74.66	w
-CH-aliphatic	2916 & 2854	73.46 & 81.91	w
C=O stretching	1730	45.06	m
C=C aromatic	1512	29.78	m
$-CH_2$ bend	1465	51.98	m
$-CH_3$ bend	1373	44.19	m
-CO-stretching	1157	2.91	s
-CH-aromatic OOP	972 & 810	34.38 & 20.39	m & s

Weak (w); medium (m); strong (s)

FIGURE 3.3: IR Spectrum of Ethyl Ferulate

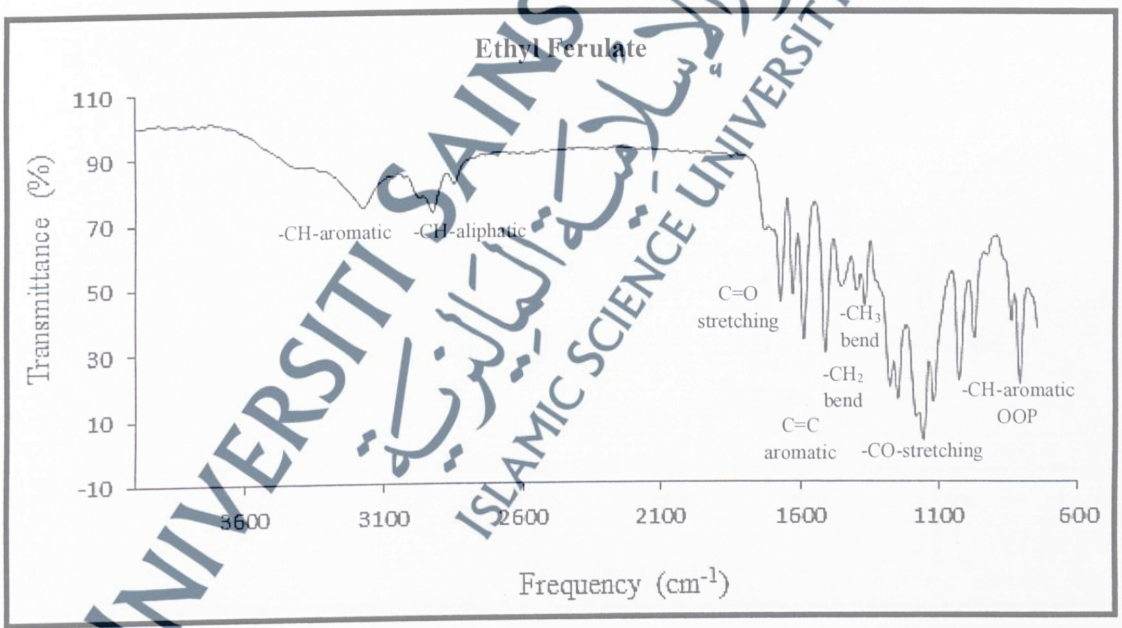


TABLE 3.2: Summary of IR Absorption Peak of Olive Oil

Type of vibration	Frequency (cm^{-1})	% Transmittance	Intensity
=CH-stretching (*cis*)	3008	92.97	w
-CH-aliphatic	2924 & 2854	60.20 & 73.25	m & w
C=O stretching	1743	58.31	m
$-CH_2$ bend	1458	77.58	w
$-CH_3$ bend	1373	84.46	w
-CO-stretching	1157	61.85	m

Weak (w); medium (m); strong (s)

FIGURE 3.4: IR Spectrum of Olive Oil

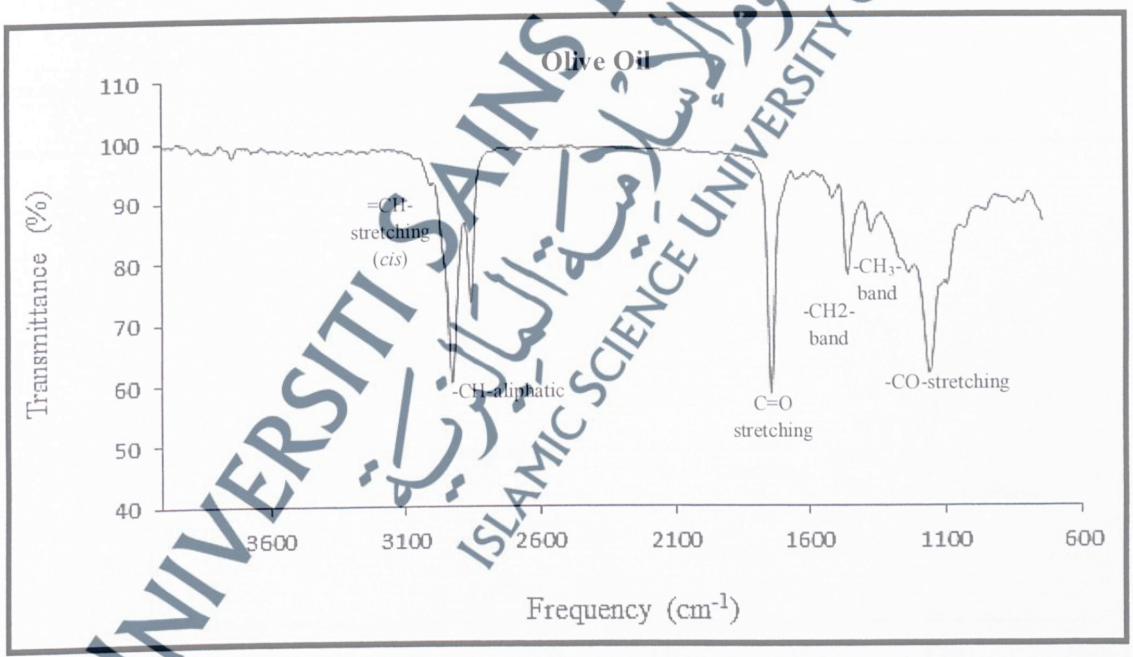


TABLE 3.3: Summary of IR Absorption Peak of Substrates Mixture before Incubation

Type of vibration	Frequency (cm^{-1})	% Transmittance	Intensity
=CH-stretching (*cis*)	3016	91.86	w
-CH-aliphatic	2924 & 2854	70.73 & 81.53	w
C=O stretching	1743	71.23	w
C=C aromatic	1512	83.57	w
$-CH_2$ bend	1458	77.38	w
$-CH_3$ bend	1373	85.10	w
-CO-stretching	1157	63.82	w

Weak (w); medium (m); strong (s)

FIGURE 3.5: IR Spectrum of Substrates Mixture before Incubation

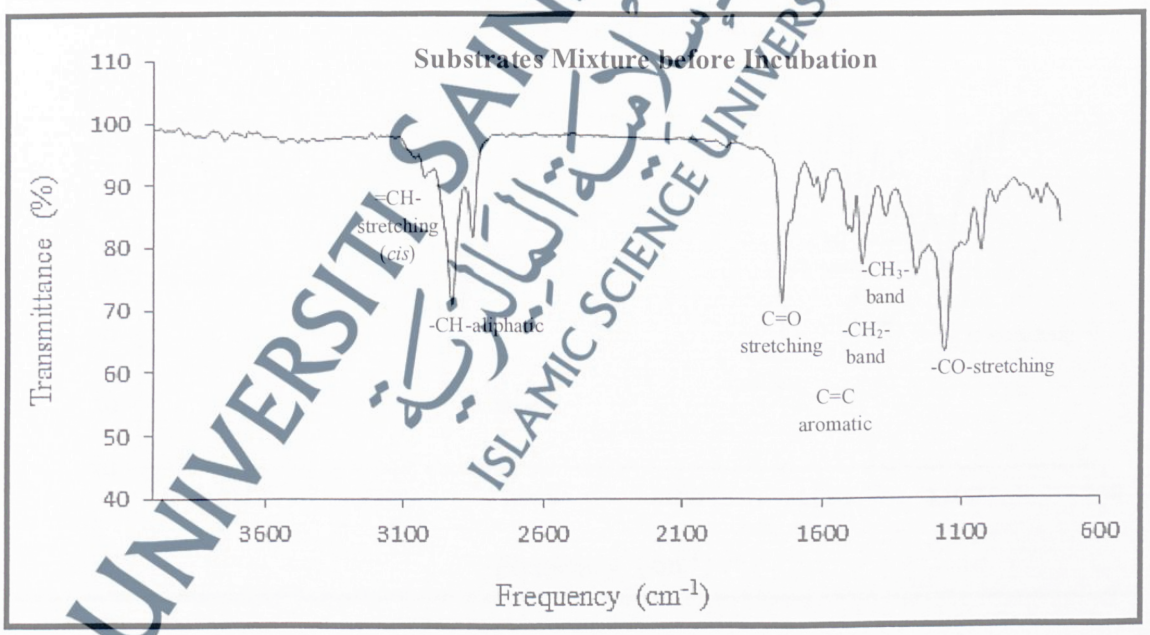
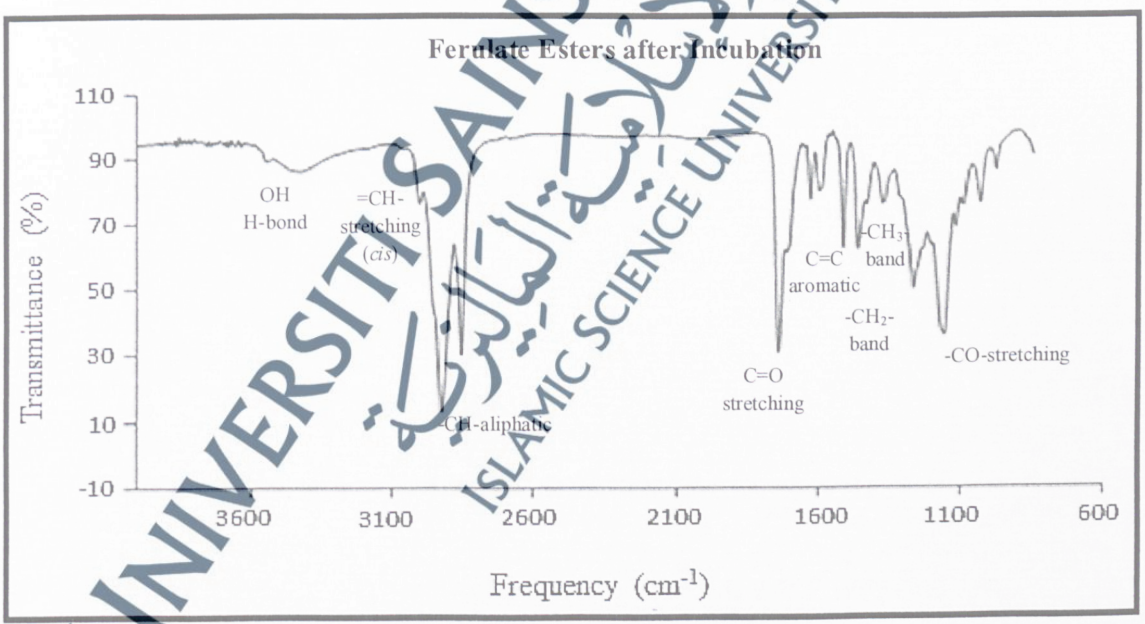


TABLE 3.4: Summary of IR Absorption Peak Of Ferulate Esters after Incubation

Type of vibration	Frequency (cm^{-1})	% Transmittance	Intensity
OH H-bond	3414	88.32	w
=CH-stretching (*cis*)	3016	79.23	w
-CH-aliphatic	2924 & 2854	11.58 & 29.10	s
C=O stretching	1743	28.13	s
C=C aromatic	1512	58.02	w
$-CH_2$ bend	1458	62.38	w
$-CH_3$ bend	1373	77.42	w
-CO-stretching	1157	38.82	m

Weak (w); medium (m); strong (s)

FIGURE 3.6: IR Spectrum of Ferulate Esters after 12 hrs Incubation Period



3.4 Conclusion

In this work, ferulate esters were successfully synthesized and the samples were identified using FTIR. A dual lipases system of 1: 9 Novozym 435-Lipozyme RM IM was developed and found to be a promising biocatalyst offering myriad possibilities in enhancing the ferulate esters synthesis.