CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Previous literatures have proven that there are strong justifications to use kaolin clay minerals as adsorbents for enzymes due to their high availability, low cost and unique structural properties that make them susceptible for further modifications.

In this study, natural kaolin obtained from Perak, Malaysia was transformed into metakaolin, an amorphous, through calcination at 650 ^{o}C. Surface properties of the natural and metakaolin were enhanced and successfully converted from hydrophilic to hydrophobic, for suitable enzyme immobilization. This was done by replacing exchangeable cation, Na^{+}, with organic surfactant containing quaternary ammonium cation, benzyltriethylammonium ($BTEA^{+}$), through ion exchange process.

All of these clays have been extensively studied for their use to immobilize lipase from *Candida rugosa* (CRL) and applied in the synthesis of nonyl hexanoate. The conclusions drawn from the experimental results are as outlined below, which clearly describes the outcomes in relation to the objectives of the research.

Evidence of the successful conversion of kaolin to metakaolin is the absence of kaolinite peaks (12.46°, 25°, 35.14°, 38.56° and 62.44°) observed in XRD patterns of metakaolin. Changes observed denote successful formation of metakaolin due to thermal treatment, which had caused elimination of H_2O^{+} and/or OH^{-} groups, causing the kaolinite layered structure to collapse and becomes amorphous.

As for the basal spacing of the samples, modified kaolin showed significantly higher basal spacings of between 7.20 - 7.34 Å, as compared to the basal spacing of

natural kaolin (7.12 Å). Basal spacing was not recorded for non-crystalline amorphous of metakaolin.

In addition, conversion of kaolin into metakaolin observed through FTIR spectrum showed disappearance of bands corresponding to internal and external hydroxyl groups, which commonly occurs after calcination. Furthermore, after calcination, all peaks that corresponded to Si-O-Si group at 1111, 1032, and 1007 cm^{-1} wavenumbers were found to group together forming single broad band at 1051 cm^{-1}. This is due to the induced deformation of the silica tetrahedral structure in metakaolin.

When modification takes place in natural kaolin and metakaolin, major peaks showing the identity of kaolinite clays remain unchanged. However, as the amount of surfactant was increased, changes in the O-H stretching vibrations and H-O-H bending vibrations of hydrogen linked water molecules occurred in kaolin and metakaolin, revealing alterations in surface affinity from hydrophilic to hydrophobic. Intensity of C-H stretching at wavenumbers between 2800 - 3000 cm^{-1} and aromatic C=C vibrations at 1474 cm^{-1} present in modified clays were originated from $BTEA^{+}$ added as modifier.

SEM images of metakaolin also showed existence of platelets with irregular shapes, disordered morphology and individualized platelets, as a result of thermal treatment, although both natural kaolin and metakaolin clays possesses similar major plate-like structures. These major plate-like structures observed in SEM images of both clays were further confirmed through the EDX analysis, which revealed the presence of silica and alumina as major constituents along with traces of potassium and iron oxides for both natural kaolin and metakaolin clays. The percentage of Al_2O_3 and SiO_2 are 36.12% and 36.04% respectively, for kaolin, and 44.83% and 50.79%, respectively for metakaolin. When observed using SEM, modified clays showed existence of small

particles on the surface of the clay after modification, which could be associated with the presence of the organic surfactant.

BET surface area analysis showed decrease in surface area of metakaolin (19.91 m^2/g) as compared to the BET surface area of natural kaolin (25.34 m^2/g). Upon modification with $BTEA^+$, both clays exhibited further decrease in BET surface areas, where the average surface area for modified natural kaolin is 8.98 m^2/g while average surface area for modified metakaolin is 13.01 m^2/g. Interestingly, pore volume of natural kaolin (0.113 cm^3/g) and metakaolin (0.121 cm^3/g) showed gradual decrease with increasing amount of organic surfactant. The organo-modified clays also formed type IV isotherm behaviour, with narrow hysteresis loops (H3 type).

This study continued with the immobilization of *Candida rugosa* lipase (CRL) onto natural kaolin, metakaolin and the organo-modified clays through physical adsorption method. With regards to the immobilization percentage, 2.0 MK showed highest immobilization (70.14% ± 2.41) and protein loading (14.83% ± 1.37), while 0.5 MK showed lowest immobilization (42.14% ± 1.77) and protein loading (5.30% ± 0.52).

After CRL adsorption onto the kaolin, metakaolin, and modified clays, a shift in XRD peak intensity was detected, which was accountable for the reduced surface crystallinity caused by CRL loading. The XRD result showed the d_{001} value did not change significantly after CRL immobilization. There was a slight shifting of the peak to a higher 2θ angle, suggesting that few of the layers are intercalated by the enzymes. In conclusion, CRL were assumed to be attached on the surface area of the supports. This is expected due to the globular size of the protein in CRL molecule (50Å × 42Å × 33Å, molecular weight of 60 kDa) as compared to the d-spacing of natural and modified kaolin (7.12 Å - 7.34 Å).

FTIR analysis of kaolin loaded with CRL showed existence of amide I, C=O stretching at 1656 cm^{-1}, corresponding to the existence of amino acids in CRL. This peak however showed sign of interference with existing C=O from kaolin and metakaolin at 1636 cm^{-1}, when combined resulted in further increase in intensity of amide I, C=O stretching in all immobilized CRL. The existence of CRL was also confirmed by the appearance of amide II, CN stretching and NH bending at 1540 cm^{-1}, which confirmed the success of immobilization process.

Therefore, an increase in particle size and appearance of spherical structures on the surface of clays after immobilization as shown in the SEM micrographs were observed. Furthermore, upon immobilization, the external surface of the supports became substantially thick, with rough surface of irregular domains. This may be due to agglomeration of lipase molecules on the support surface after immobilization.

Adsorption of CRL onto the supports also led in a reduction in pore volume and surface area, and the increase of pore size. This were attributed to the accommodation of CRL molecules in the pores and between layers of the supports, which further confirmed successful immobilization of the enzyme onto the supports.

Considerably high percentage of conversion (52.99% to 68.03%) was achieved using immobilized CRL, as compared to free CRL (50.71%). Similarly, enzyme specific activity was higher in immobilized CRL (3.63×10^{-3} µmol/min/mg protein - 5.24×10^{-3} µmol/min/mg protein) as compared to the specific activity of free CRL (3.27×10^{-3} µmol/min/mg protein). Highest conversion of nonyl hexanoate (68.03%) was achieved when CRL-2.0 MK, with specific activity of 5.24×10^{-3}µmol/min/mg protein was used as catalyst. On the other hand, lowest conversion of nonyl hexanoate (52.99%) was achieved when CRL-NK with specific activity of 3.63×10^{-3} µmol/min/mg protein was used as catalyst, as compared to other immobilized lipases.

In the lipase thermostability assay, CRL-2.0 NK showed the highest relative activity of 80.47% followed by CRL-2.0 MK with considerably high relative activity of 70.81%, even after 1 hour of exposure to high temperature (70 °C). Operational stability assay of the immobilized lipases also revealed that CRL-1.0 MK and CRL-2.0 MK were able to retain 52.65% - 62.56% of relative activity and 33.74% - 42.56% ester conversion, while CRL-1.0 NK and CRL-2.0 NK still maintained 36.11% - 38.53% of relative activity with 23.23% -26.16% ester conversion, even after 10 cycles of continuous subsequent uses. These results proved that modifications with $BTEA^+$ of between 1.0 - 2.0 time of the CEC of natural kaolin and metakaolin, were beneficial to improve hydrophobicity of the supports, thus enhanced the catalytic activity of CRL loaded onto the supports, when used in the synthesis of nonyl hexanoate.

From the immobilized lipase activity assay conducted, CRL-2.0 NK and CRL-2.0 MK have shown high stabilities and activities in the synthesis of nonyl hexanoate. These enzymes were selected for further studies of their kinetics behaviours. Initial reaction rates obtained from the experiments and SigmaPlot® version 12.5 software simulation showed that the esterification of nonyl hexanoate catalyzed by free CRL and selected immobilized enzymes (CRL-2.0 NK and CRL-2.0 MK) followed Michaelis Menten kinetics model.

In general, all enzymes showed higher affinities toward hexanoic acid (Hex) as compared to nonanol (Non). These were based on the values of Michaelis Menten constant, K_m, $K_{m(Hex)} < K_{m(Non)}$. In addition, the V_{max} of immobilized lipases were found to be higher than that of free CRL. These data were supported by the Ping Pong Bi Bi mechanism, which suggested that the immobilized lipases followed the one substrate competitive inhibition mechanism, while free CRL followed the two substrates competitive inhibition mechanism.

The work presented in this study had contributed to the overall knowledge needed to potentially apply the use of the $BTEA^+$ modified kaolin/metakaolin immobilized lipases in industries. To meet the demands for industrial applications, cost is one of the major factors for consideration which may be achieved with the use of the immobilized enzymes prepared in this study. Their applications can be further extended to meet the demands in flavour industry especially when natural sources and extracts are hindered by limited supply and high production costs.

Thus, suitable technologies for value added together with large-scale applications of the immobilized lipases prepared herein, may serve as a substitute for those expensive catalysts and supports commercially available. In this study, the nonyl hexanoate synthesized were verified where its presence was confirmed through FTIR spectrum, showing a prominent signal at 1717 cm^{-1}, corresponding to the presence of the conjugated carbonyl (C=O) group in esters A signal at 1246 cm^{-1}, which matched to the presence of the carboxyl (C-O) group, also indicated the presence of the ester. Presence of the product was also confirmed in the GC-MS chromatogram which showed its existence at 30.43 min retention time, with molecular mass of 242 m/z.

5.2 Recommendations for Further Studies

This research provides insights into the structure and the properties of organo-modified kaolin and metakaolin, and their possible applications as effective adsorbents for lipase immobilization. Although the present work has unveiled several research breakthroughs in enzyme technology, the following are recommendations which can be considered to further improve the catalytic performance of enzyme and provide new perspectives of enzyme catalysis for efficient industrial applications.

1. Optimizing the present enzymatic esterification performance using response surface methodology (RSM) as a substitute to the conventional one-at-a-time optimization approach.

2. Conducting comparative study on the use of other types of microbial lipases from various sources, such as *Rhizopus* and *Pseudomonas*.

3. Enhancing the support physico-chemical properties through physical and/or chemical means, such as sylilation and heat treatment, including the use of other modification reagents.

4. Combining two or more enzymes with unique catalytic properties in order to maximize yields of products.