

**ENHANCEMENT OF *CANDIDA RUGOSA* LIPASE VIA  
IMMOBILIZATION ONTO BENZYLTRIEETHYL AMMONIUM  
CHLORIDE-MODIFIED SUPPORTS FOR THE SYNTHESIS OF  
NONYL-HEXANOATE**

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## AUTHOR DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged.

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## ABSTRAK

Kajian menunjukkan bahawa tanah liat kaolin mempunyai potensi besar untuk digunakan sebagai penyerap dan penyokong yang baik untuk biopemangkin kerana ianya menepati keperluan dan kehendak mesra alam industri moden masakini. Tambahan pula, ianya berpatutan, tahan mikrob, stabil secara terma dan mempunyai kekuatan mekanikal yang baik. Walau bagaimanapun, penggunaannya sebagai penyokong sekatgerak biopemangkin adalah terhad disebabkan sifat hidrofiliknya. Bagi mengatasi masalah tersebut, kation yang boleh ditukarganti ( $\text{Na}^+$ ) dalam struktur telah digantikan dengan kation ammonium kuartenari melalui proses penukargantian ion. Dalam kajian ini, keupayaan penukargantian kation (CEC) kaolin asli dan kaolin yang telah dirawat pada  $650\text{ }^\circ\text{C}$  (metakaolin) telah ditentukan melalui kaedah methilin biru dan didapati berada pada nilai  $2.5\text{ meq}/100\text{g}$  tanah liat and  $1.5\text{ meq}/100\text{g}$  tanah liat, masing-masing. Klorida benziltriethylamonium (BTEA-Cl) pada kepekatan berbeza ( $0.5 - 2.0\times$  CEC kaolin asli dan metakaolin) telah digunakan dalam penyediaan kaolin termodifikasi-organo dan metakaolin termodifikasi-organo. Pencirian kaolin termodifikasi-organo dan metakaolin termodifikasi-organo telah dilakukan menggunakan difraktometer sinar-X (XRD), spectrometer Fourier transform inframerah (FTIR), mikroskop pengimbas elektron (SEM) dan pengalisa luas permukaan dan keporosan. Kejayaan modifikasi-organo terhadap kaolin asli telah dipamerkan melalui peningkatan dalam nilai  $d_{001}$  dari  $7.12\text{ \AA}$  kepada di antara  $7.20 - 7.34\text{ \AA}$  dalam corak belauan XRD. Begitu juga dengan perubahan kaolin kepada metakaolin sebagai hasil rawatan haba telah disahkan melalui kehilangan puncak kaolinit pada  $12.46^\circ$ . Corak belauan XRD terhadap kaolin dan metakaolin asli dan termodifikasi didapati serupa menunjukkan struktur tanah liat yang tidak terubahsuai setelah modifikasi-organo dilakukan menggunakan BTEA-Cl. Kejayaan modifikasi terhadap kaolin and metakaolin juga dapat dilihat melalui kemunculan puncak utama pada spektra FTIR di  $2800\text{--}3000\text{ cm}^{-1}$  and  $1474\text{ cm}^{-1}$  yang mewakili CH stretching and aromatic C=C dalam BTEA<sup>+</sup>. Mikrograf SEM bagi kaolin and metakaolin termodifikasi-organo menunjukkan peningkatan kepingan dan ampaian berbanding kaolin dan metakaolin yang tidak termodifikasi. Bukti modifikasi-organo ke atas kaolin asli and metakaolin juga dapat diperhatikan melalui pengurangan drastik luas permukaan tanah liat dari  $25.34\text{ m}^2/\text{g}$  tanah liat asli kepada di antara  $5.90 - 13.11\text{ m}^2/\text{g}$  tanah liat termodifikasi-organo, dan dari  $19.91\text{ m}^2/\text{g}$  untuk metakaolin kepada di antara  $9.04\text{--}18.14\text{ m}^2/\text{g}$  untuk metakaolin termodifikasi-organo. Kaolin asli, metakaolin dan tanah liat yang termodifikasi-organo kemudiannya telah digunakan sebagai penyokong dalam proses sekatgerak lipase dari *Candida rugosa* (CRL) melalui kaedah penjerapan fizikal. Metakaolin termodifikasi-organo sebanyak  $2.0\times$  CEC menunjukkan penjerapan protein tertinggi sehingga  $14.83 \pm 1.37\text{ mg protein/g}$  tanah liat dan hampir  $70\%$  sekatgerak. Pencirian lipase tersekatgerak menggunakan XRD menunjukkan peningkatan jarak  $d_{001}$  dari  $7.12$  hingga  $7.22\text{ \AA}$  untuk kaolin, sementara jarak  $d_{001}$  kaolin yang diubah suai organo menurun sekitar  $2\%$ . Imej SEM menunjukkan dengan jelas, permukaan luar yang tebal dan kasar sebagai bukti kewujudan lipase pada permukaan

kaolin and metakaolin. Puncak-puncak pada  $1656\text{ cm}^{-1}$ ,  $1540\text{ cm}^{-1}$  adalah berkaitan dengan kewujudan CRL apabila ditentukan menggunakan FTIR. Kejayaan sekatgerak CRL ke atas kaolin and metakaolin asli dan termodifikasi-organo juga dapat dilihat di mana luas permukaan, isipadu pori-pori juga dapat dilihat di mana luas permukaan, isipadu pori-pori menurun sekitar 46% -72% dan 1% -52%, sementara saiz liang liat meningkat sekitar 42% -53% setelah sekatgerak terhadap CRL dilakukan. CRL tersekatgerak, bersama-sama dengan CRL asli kemudiannya telah diuji untuk aktiviti esterifikasi dalam tindakbalas di antara nonanol and asid heksanoik. CRL tersekatgerak ke atas kaolin asli termodifikasi-organo 2.0x CEC (CRL-2.0 NK) dan CRL tersekatgerak ke atas metakaolin termodifikasi-organo 2.0x CEC (CRL-2.0 MK) menunjukkan peningkatan aktiviti sebanyak 1.5 kali ganda berbanding CRL asli. FTIR dan kromatografi gas-spektrometer jisim (GC-MS) telah digunakan untuk mengesahkan kejayaan sintesis nonil heksanoat menggunakan CRL tersekatgerak ke atas tanah liat termodifikasi-organo. CRL-2.0 NK dan CRL-2.0 MK juga turut menunjukkan kestabilan terma yang tinggi di antara 57.67% - 51.39% walaupun setelah inkubasi pada suhu  $70\text{ }^{\circ}\text{C}$ . CRL-2.0 MK mempamerkan kebolehan guna semula tertinggi di mana ia mengekalkan 65.15 % daripada aktiviti asal ( $5.24 \times 10^{-3}$  mmol ester/min/mg protein) walaupun setelah 10 ulangan penggunaan. Keupayaan lipase tersekatgerak yang terpilih (CRL-2.0 NK dan CRL-2.0 MK) untuk mensintesis nonil heksanoat telah dikaji dari sudut kinetik, di mana parameter kinetik,  $K_m$  and  $V_{max}$ , telah ditentukan menggunakan model kinetik Michaelis-Menten dan dapat diterangkan dengan tepat melalui mekanisma Ping-Pong Bi-Bi. Nilai  $K_{m(\text{Hex})}$  yang rendah mencadangkan bahawa CRL, CRL-2.0 NK dan CRL-2.0 MK mempamerkan kecenderungan yang lebih tinggi terhadap asid heksanoik (Hex) berbanding terhadap nonanol (Non),  $K_{m(\text{Non})} > K_{m(\text{Hex})}$ . Lipase tersekatgerak yang terpilih juga menunjukkan nilai  $V_{max}$  yang lebih tinggi (2.1786-2.3504 mmol/L/min) berbanding CRL (1.5810 mmol/L/min) dalam tindakbalas berenzim. Kajian ini telah mengupas secara terperinci ciri fiziko-kimia pelbagai jenis terbitan kaolin untuk manfaat penggunaannya sebagai penyokong biopemangkin. Aktiviti dan kestabilan lipase yang telah disekatgerak ke atas tanah liat termodifikasi yang terpilih didapati meningkat, mencadangkan bahawa penyediaan biopemangkin tersekatgerak yang berfungsi tersebut adalah lebih sesuai dan praktikal bagi menggantikan pemangkin asid berbahaya, yang lazim digunakan dalam penghasilan nonil heksanoat.

## ABSTRACT

Studies have shown that kaolin clay has huge potentials to be utilized as excellent adsorbent and support for biocatalysts because they meet the requirement of today's increasing eco-friendly demands of modern industries. Furthermore, they are inexpensive, microbial resistant, thermally stable and possesses appreciable mechanical strength. However, their use as support for biocatalysts immobilization are limited due to their hydrophilic nature. To overcome this, exchangeable cations ( $\text{Na}^+$ ) in their structures are replaced with quaternary ammonium cations (QACs) through ion exchange process. In this study, cation exchange capacity (CEC) by methylene blue method of natural kaolin and kaolin treated at  $650\text{ }^\circ\text{C}$  (metakaolin) were determined and found to be  $2.5\text{ meq}/100\text{g}$  clay and  $1.5\text{ meq}/100\text{g}$  clay, respectively. Benzyltriethylammonium chloride (BTEA-Cl) at varying concentrations ( $0.5 - 2.0\times$  CEC of the natural kaolin and metakaolin) were used in the preparation of the organo-modified kaolin and organo-modified metakaolin. The organo-modified kaolin and metakaolin were characterized using X-ray diffractometer (XRD), Fourier transform infrared (FTIR) spectrometer, scanning electron microscope (SEM), and surface area and porosity analyzer. Successful organo-modifications of the natural kaolin were exhibited by the increase in  $d_{001}$  spacing from  $7.12\text{ \AA}$  to between  $7.20 - 7.34\text{ \AA}$  in XRD patterns. Similarly, successful conversion of kaolin to metakaolin as a result of thermal treatment was confirmed through the disappearance of kaolinite peak at  $12.46^\circ$ . XRD patterns of the modified and unmodified kaolin and metakaolin were found to be similar indicating the unchanged clay structures upon organo-modification with BTEA-Cl. Results of the successful modifications of kaolin and metakaolin were also exhibited by the appearance of major peaks in FTIR spectra at  $2800-3000\text{ cm}^{-1}$  and  $1474\text{ cm}^{-1}$  which represented the existence of CH stretching and aromatic C=C in BTEA<sup>+</sup>. The SEM micrographs of the organo-modified kaolin and metakaolin exhibited increasing individualized platelets and agglomeration as compared to the unmodified kaolin and metakaolin. Evidence of the organo-modification on natural kaolin and metakaolin was also noticed as surface areas of the clays showed drastic decrease from  $25.34\text{ m}^2/\text{g}$  natural clay to between  $5.90 - 13.11\text{ m}^2/\text{g}$  organo-modified clay, and from  $19.91\text{ m}^2/\text{g}$  for metakaolin to between  $9.04 - 18.14\text{ m}^2/\text{g}$  for organo-modified metakaolin clays. The natural kaolin, metakaolin and their organo-modified counterparts were then used as supports in the immobilization of *Candida rugosa* lipase (CRL) via physical adsorption method. Organo-modified metakaolin with  $2.0\times$  CEC showed highest protein loading of up to  $14.83 \pm 1.37\text{ mg protein/g}$  clay and almost 70% of immobilization. Characterization of the immobilized lipases using XRD showed increased  $d_{001}$  spacing from  $7.12$  to  $7.22\text{ \AA}$  for kaolin, while  $d_{001}$  spacing of organo-modified kaolin decreased about 2%. SEM images exhibited substantial, thick and rough external surface as evidence of the existence of lipase on the surface of the kaolin and metakaolin clay. The absorption bands at  $1656\text{ cm}^{-1}$ ,  $1540\text{ cm}^{-1}$  were linked to the existence of CRL when identified using FTIR. Successful immobilization of CRL onto natural and organo-modified kaolin and metakaolin clay were also observed where surface areas and pore

volumes decreased about 46%-72% and 1%-52%, respectively, while pore sizes of the clay increased about 42%-53% upon immobilization of CRL. The immobilized CRL, together with free CRL were then screened for esterification activities in the reactions which consisted of nonanol and hexanoic acid. CRL immobilized onto 2.0x CEC organo-modified natural kaolin (CRL-2.0 NK) and CRL immobilized onto 2.0x CEC organo-modified metakaolin (CRL-2.0 MK) showed 1.5 folds increased in activities as compared to free CRL. FTIR and gas chromatography-mass spectrometer (GC-MS) were used to confirm the successful synthesis of nonyl hexanoate using CRL immobilized onto the organo-modified clay. CRL-2.0 NK and CRL-2.0 MK also showed high thermal stabilities 57.67% and 51.39%, respectively even after incubation at 70 °C. CRL-2.0 MK exhibited highest reusability where it maintained 65.15% of the initial activity ( $5.24 \times 10^{-3}$  mmol ester/min/ $\mu$ g protein) even after 10 cycles of continuous uses. The abilities of the selected immobilized lipases (CRL-2.0 NK and CRL-2.0 MK) to synthesis the nonyl hexanoate were kinetically studied where kinetic parameters,  $K_m$  and  $V_{max}$ , were determined by means of Michaelis-Menten kinetic model and were best explained by the Ping-Pong Bi-Bi mechanism. The lower  $K_{m(Hex)}$  values suggested that the CRL, CRL-2.0 NK and CRL-2.0 MK displayed higher affinity towards hexanoic acid (Hex) than towards nonanol (Non),  $K_{m(Non)} > K_{m(Hex)}$ . The selected immobilized lipases also demonstrated considerably higher  $V_{max}$  values (2.1786-2.3504 mmol/L/min) compared to free CRL (1.5810 mmol/L/min) in the enzymatic reaction. The present study had extensively explored the structure and physico characteristics of the different types of kaolin derivatives for their beneficial use as support for biocatalyst. Activities and stabilities of lipase immobilized onto the selected organo-modified clay were improved, suggesting that the preparation of functional immobilized biocatalyst are more adapted and practical substitute to the hazardous acid catalyst commonly used for nonyl hexanoate production.

## الملخص

أظهرت الدراسات أن طين الكاولين لديه إمكانات هائلة لاستخدامه كمتماز ممتاز ودعم للمحفزات الحيوية لأنها تلبى متطلبات المتطلبات الصديقة للبيئة المتزايدة اليوم للصناعات الحديثة. علاوة على ذلك، فهي غير مكلفة، ومقاومة للميكروبات، ومستقرة حرارياً وتمتلك قوة ميكانيكية ملحوظة. ومع ذلك، فإن استخدامها كدعم لتثبيت المحفزات الحيوية محدود بسبب طبيعتها المحبة للماء. للتغلب على هذا، يتم استبدال الكاتيونات القابلة للتبديل ( $\text{Na}^+$ ) في هياكلها مع كاتيونات الأمونيوم الرباعية (QACs) من خلال عملية التبادل الأيوني. في هذه الدراسة، تم تحديد سعة التبادل الكاتيوني (CEC) بطريقة الميثيلين الأزرق للكاولين الطبيعي والكاولين المعالجين عند 650 درجة مئوية (ميتاكولين) ووجد أنهما 2.5 ميغا / 100 جرام من الطين و 1.5 ميغا / 100 جرام من الطين، على التوالي. تم استخدام كلوريد بنزول تراي إيثيل الأمونيوم (BTEA-Cl) بتركيزات مختلفة (0.5-2.0 CEC x من الكاولين الطبيعي والميتاكولين) في تحضير الكاولين المعدل عضوياً والميتاكولين المعدل عضوياً. تم تمييز الكاولين والميتاكولين المعدلين عضوياً باستخدام مقياس حيود الأشعة السينية (XRD) ومطياف فورييه للأشعة تحت الحمراء (FTIR) وميكروسكوب المسح الإلكتروني (SEM) ومساحة السطح ومحلل المسامية. تم عرض التعديلات العضوية الناجحة للكاولين الطبيعي من خلال زيادة تباعد  $d_{001}$  من 7.12 Å إلى ما بين 7.20-7.34 Å في أنماط XRD. وبالمثل، تم تأكيد التحويل الناجح للكاولين إلى ميتاكولين نتيجة للمعالجة الحرارية من خلال اختفاء ذروة الكاولين عند 12.46 درجة. تم العثور على أنماط XRD للكاولين المعدلة وغير المعدلة والميتاكولين لتكون متشابهة مما يشير إلى أن هياكل الطين لم تتغير عند التعديل العضوي باستخدام BTEA-Cl. تم عرض نتائج التعديلات الناجحة للكاولين والميتاكولين أيضاً من خلال ظهور القمم الرئيسية في أطياف FTIR عند 2800-3000 سم<sup>-1</sup> و 1474 سم<sup>-1</sup> مما يمثل وجود تمدد CH والعطرية C=C في BTEA<sup>+</sup>. أظهرت الصور المجهرية SEM للكاولين والميتاكولين المعدلين عضوياً زيادة الصفائح الدموية الفردية والنكتل مقارنةً بالكاولين والميتاكولين غير المعدل. ولوحظ أيضاً دليل على التعديل العضوي على الكاولين الطبيعي والميتاكولين حيث أظهرت المساحات السطحية للطين انخفاضاً حاداً من 25.34 م<sup>2</sup> / جم من الطين الطبيعي إلى ما بين 5.90-13.11 م<sup>2</sup> / جم من الطين المعدل عضوياً، ومن 19.91 م<sup>2</sup> / جم للطين ميتاكولين إلى ما بين 9.04-18.14 م<sup>2</sup> / جم لطين الميتاكولين المعدل عضوياً. تم استخدام الكاولين الطبيعي والميتاكولين ونظرائهم المعدلة عضوياً كدعم في تثبيت المبيضات الليباز (CRL) عبر طريقة

الامتزاز الفيزيائي. أظهر الميناكولين المعدل عضوياً مع  $2.0 \times \text{CEC}$  أعلى تحميل بروتين يصل إلى  $14.83 \pm 1.37$  مجم بروتين / جم طين وما يقرب من 70 % من التثبيت. أظهر توصيف الليباز الثابت باستخدام XRD زيادة تباعد  $d_{001}$  من 7.12 إلى  $7.22 \text{ \AA}$  للكولين، بينما انخفض تباعد  $d_{001}$  للكولين المعدل عضوياً بنحو 2%. أظهرت صور SEM سطحاً خارجياً سميماً وخشناً كدليل على وجود الليباز على سطح طين الكولين والميناكولين. تم ربط نطاقات الامتصاص عند 1656 سم<sup>-1</sup>، 1540 سم<sup>-1</sup> بوجود CRL عند تحديدها باستخدام FTIR. كما لوحظ التثبيت الناجح لـ CRL على الكولين الطبيعي والمعدل عضوياً وطين ميناكولين حيث انخفضت مساحات السطح وحجم المسام بحوالي 46% - 72% و 1% - 52%، بينما زادت أحجام مسام الطين بحوالي 42% - 53% عند تجميد CRL. تم بعد ذلك فحص CRL المجمد، جنباً إلى جنب مع CRL الحر من أجل أنشطة الأسترة في التفاعلات التي تتكون من حمض النونانول وحمض هيكسانويك. تم تثبيت CRL على الكولين الطبيعي المعدل عضوياً  $2.0 \times \text{CEC}$  (CRL-2.0 NK) و CRL المثبت في  $2.0 \times \text{CEC}$  ميناكولين المعدل عضوياً (CRL-2.0 MK) وأظهر زيادة 1.5 أضعاف في الأنشطة مقارنة بـ CRL المجاني. تم استخدام FTIR ومقياس طيف الكتلة اللوني للغاز (GC-MS) لتأكيد التوليف الناجح لهكسانوات النونيل باستخدام CRL المثبت على الطين المعدل عضوياً. أظهر CRL-2.0 NK و CRL-2.0 MK أيضاً ثباتاً حرارياً عالياً حوالي 57.67% و 51.39%، على التوالي حتى بعد الحضانة عند 70 درجة مئوية. أظهر CRL-2.0 MK أعلى قابلية لإعادة الاستخدام حيث حافظ على 65.15% من النشاط الأولي ( $5.24 \times 10^{-3}$  ملي مول استر / دقيقة / مجم بروتين) حتى بعد 10 دورات من الاستخدامات المستمرة. تمت دراسة قدرات الليباز المحدد المعطل (CRL-2.0 NK و CRL-2.0 MK) لتخليق هيكسانوات النونيل بشكل حركي حيث تم تحديد المعلمات الحركية،  $K_m$  و  $V_{max}$ ، عن طريق النموذج الحركي ميكاليس-مينتن وتم شرحها بشكل أفضل بواسطة آلية ثنائية بي البنق بونق. تشير قيم  $K_m$  (Hex) المنخفضة إلى أن CRL و CRL-2.0 NK و CRL-2.0 MK أظهروا تقارباً أعلى تجاه حمض هيكسانويك (Hex) من تجاه نونانول (Non)،  $K_m(\text{Non}) > K_m(\text{Hex})$ . أظهرت الليباز المختارة أيضاً قيم  $V_{max}$  أعلى بكثير (2.1786-2.3504 ملي مول / لتر / دقيقة) مقارنة بـ CRL (1.5810 ملي مول / لتر / دقيقة) في التفاعل الأنزيمي. استكشفت الدراسة الحالية على نطاق واسع التركيب والخصائص الفيزيائية لأنواع مختلفة من مشتقات الكولين لاستخدامها المفيد كدعم للمحفز الحيوي. تم تحسين أنشطة واستقرار الليباز المثبت على الطين المختار المعدل عضوياً، مما يشير إلى أن تحضير المحفز الحيوي

المثبت وظيفياً يكون أكثر تكيفاً وبديلاً عملياً لمحفز الحمض الخطير المستخدم بشكل شائع لإنتاج الهكسانويات نونيل.

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## LIST OF ABBREVIATIONS

<b>CRL</b>	<i>Candida rugosa</i> Lipase
<b>EC</b>	Enzyme Commission
<b>QACs</b>	Quaternary Ammonium Cations
<b>BTEA-Cl</b>	Benzyltriethylammonium-chloride
<b>MB</b>	Methylene-Blue
<b>CEC</b>	Cation Exchange Capacity
<b>BSA</b>	Bovine Serum Albumin
<b>NK</b>	Natural Kaolin
<b>MK</b>	Metakaolin
<b>Rpm</b>	Revolutions per minute
<b>XRD</b>	X-Ray Diffraction Analysis
<b>FTIR</b>	Fourier Transform Infrared Spectroscopy
<b>SEM</b>	Scanning Electron Microscopy
<b>BET</b>	Brunauer, Emmett and Teller
<b>GC-MS</b>	Gas Chromatography-Mass Spectrometry
<b>V</b>	Velocity
<b>V<sub>max</sub></b>	Maximum Velocity
<b>K<sub>m</sub></b>	Michealis-Menten Contant
<b>K<sub>i</sub></b>	Inhibition Constant
<b>Å</b>	Angstrom
<b>m/z</b>	mass-to-charge ratio