UNIVERSITI PUTRA MALAYSIA

BIOCHEMICAL AND STRUCTURAL CHARACTERIZATION OF CROSS-LINKED ENZYME AGGREGATES IMMOBILIZED ELASTASE STRAIN K

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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June 2020

Chair : Mohd Shukuri Mohamad Ali, PhD
Faculty : Biotechnology and Biomolecular Sciences

Immobilization of enzyme is a great modification technique that enhances the stability and reusability of an enzyme. Nevertheless, some immobilization techniques have low productivity and require the enzyme to undergo purification process; a process which is laborious and time consuming. CLEA immobilization technique offer better alternative since crude enzyme can be used directly during the preparation of CLEA. In this study, CLEA immobilization technique was tailored and developed to retain and enhance the activity of elastase strain K, while facilitating its recovery after completion of an enzymatic reaction. Another area that has been elusive regarding CLEA is the structural analysis. Organic solvent tolerant protease, elastase strain K was immobilized using CLEA technique and the biochemical as well as the biophysical profiles of CLEA-elastase was analyzed. This valuable enzyme exhibit remarkable tolerance against wide range of organic solvents including methanol, ethanol, 1-propanol and dimethyl sulfoxide (DMSO). Aggregates of elastase strain K was prepared by adding 60% (w/v) ammonium sulfate and treated for 3 h prior to cross-linking with 0.2% (v/v) glutaraldehyde for 2 h. Maximum recovered activity of CLEA-elastase was recorded at 61.4% while CLEA-elastase-SB; derivatives of CLEA-elastase with addition of BSA and starch as co-aggregants, recorded a recovered activity of 81.6%. Immobilized elastase strain K exhibit enhanced thermostability and exhibit increment of optimum temperature at 50°C. In addition to that, CLEA-elastase exhibit broad pH stability between pH 5-10 and high proteolytic activity was recorded at pH 8. The organic solvent tolerant characteristic of elastase strain K was retained even after immobilization. Enhancement of organic solvent tolerance was also detected in CLEA-elastase treated with methanol, acetonitrile, ethanol, 1-propanol, benzene and xylene with 111.4%, 164.6%, 172.7%, 111.4%, 152.7% and 133.2% of recovered activity, respectively. The biophysical analysis conducted using scanning electron microscopy (SEM), dynamic light scattering (DLS), Brunauer-Emmett-Teller (BET) surface area and Fourier-transform

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infrared (FTIR) spectra revealed that CLEA-elastase exhibit a type 2 aggregate morphology; appearance of aggregates are random and less defined, with an average diameter of 1497 nm. In addition, co-aggregation with BSA and starch increase the surface area and porosity of CLEA-elastase. Stretching and vibration of bonding associated with the presence of successful cross-linkages was detected especially within the 1600 – 1700 cm^{-1} of FTIR spectra. In general, organic solvent tolerant elastase strain K has been successfully immobilized using CLEA method. The technique has successfully being tailored and developed to retain and enhance the proteolytic activity of elastase strain K.

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PENCIRIAN BIOKIMIA DAN STRUKTUR PENYEKAT GERAKAN PAUTAN SILANG AGREGAT ENZIM ELASTASE STRAIN K

Oleh

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Penyekat gerakan enzim merupakan kaedah pengubahsuaian yang mampu meningkatkan kestabilan dan guna semula enzim. Walaubagaimanapun, beberapa kaedah penyekat gerakan enzim menghadapi masalah produktiviti rendah selain memerlukan enzim untuk melalui proses penulenan; satu proses yang sukar dan memakan masa. Kaedah penyekat gerakan CLEA menawarkan alternatif yang lebih baik memandangkan enzim mentah boleh digunakan secara terus semasa proses penyediaan CLEA. Dalam kajian ini, kaedah penyekat gerakan CLEA telah dihasilkan dan diubahsuai untuk mengekalkan serta meningkatkan activiti elastase strain K, di samping memudahkan pemulihan selepas proses tindak balas enzim tamat. Selain itu, bidang lain yang masih kurang difahami adalah berkenaan analisis struktur CLEA. Profil biokimia and biofizik elastase strain K; sejenis protease yang mampu bertahan dalam pelarut organik, disekat gerakan menggunakan kaedah CLEA telah dianalisis. Enzim yang berharga ini mempamerkan ketahanan yang baik terhadap pelbagai jenis pelarut organik seperti metanol, etanol, 1-propanol dan dimetil sulfoksida (DMSO). Agregat elastase strain K telah disediakan dengan menambah 60% (w/v) ammonium sulfat dan dirawat selama 3 jam sebelum proses pautan silang dijalankan dengan menambah 0.2% (v/v) glutaraldehyde dan dirawat selama 2 jam. Pengekalan aktiviti maksimum CLEA-elastase direkod setinggi 61.4% manakala pengekalan aktiviti maksimum CLEA-elastase-SB; terbitan CLEA-elastase yang dirawat bersama BSA dan kanji sebagai bahan pengagregatan bersama, direkod setinggi 81.6%. Penyekat gerakan elastase strain K telah meningkatkan kestabilan suhu dan suhu optimum meningkat kepada 50°C. Selain itu CLEA-elastase mempamerkan kestabilan pada pH yang luas diantara pH 5-10 dan aktiviti tertingi direkod pada pH 8. Ciri ketahanan elastase strain K terhadap pelarut organik dikekalkan selepas proses pentakmobilan. Peningkatan ketahanan terhadap pelarut organik juga dikesan pada CLEA-elastase yang dirawat bersama metanol, asetonitril, etanol, 1-propanol, benzena dan xilena dengan aktiviti sebanyak 111.4 %, 164.6 %, 172.7 %, 111.4 %, 152.7 % and

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133.2 %. Analisis biofizik telah dijalankan mengunakan pengimbasan mikroskop electron (SEM), penghamburan cahaya dinamik (DLS), kawasan permukaan Brunauer-Emmett-Teller (BET) dan spektra inframerah transformasi Fourier (FTIR) mendedahkan bahawa CLEA-elastase mempamerkan morfologi agregat jenis 2; penampilan agregat bersifat rawak dan kurang ditakrifkan, serta purata diameter direkot sebesar 1497 nm. Selain itu, penambahan bahan agregat bersama iaitu BSA dan kanji meningkatkan kawasan permukaan dan keporosan CLEA-elastase. Peregangan dan getaran pengikat yang dikaitkan dengan keberhasilan pautan silang telah dikesan pada spektra FTIR 1600–1700 cm^{-1}. Secara umumnya, elastase strain K; enzim mampu tahan pelarut organik telah berjaya disekat gerakan menggunakan kaedah CLEA. Kaedah ini telah berjaya dihasilkan dan diubahsuai untuk mengekalkan dan menigkatkan aktiviti protease elastase strain K.

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LIST OF ABBREVIATIONS

α	Alpha
BJH	Barrett, Joyner, and Halenda
bp	Base pair
β	Beta
BET	Brunauer-Emmett-Teller
BSA	Bovine serum albumin
CLE	Cross-linked enzyme
CLEA	Cross-linked enzyme aggregates
CLEC	Cross-linked enzyme crystals
°C	Degree celsius
DNA	Deoxyribonucleic acid
DMSO	Dimethylsulfoxide
DLS	Dynamic light scattering
FTIR	Fourier-transform infrared
g	Gram
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
L	Litre
LB	Luria bertani
Lys	Lysine
µL	Microliter
µm	Micrometer
µmoles	Micromoles

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mg/mL	Milligram per mililiter
M	Molar
nm	Nanometer
Log $P_{o/w}$	Partition coefficient
rpm	Rotation per minute
SEM	Scanning electron microscopy
NaOH	Sodium hydroxide
TCA	Trichloroacetic acid
v/v	Volume per volume
w/v	Weight per volume

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CHAPTER 1

INTRODUCTION

Nowadays, the demand of hydrolytic enzymes such as proteases has significantly increased since the use of hydrolytic enzymes has been proven to be beneficial for industrial purposes. The application of hydrolytic enzymes for industrial purposes covers wide industrial areas such as detergent industries, leather tanning, food industries and pharmaceutical (Mahmod et. al., 2016). Proteases for instance, are incorporated in detergent formula to remove protein-based stains and are used in leather industries to aid dehairing processes. The use of protease in the industries occupies 60% of the hydrolases in the industrial market. Over the years, proteases have been extracted from different sources and were tailored for commercial uses. Studies on proteases mainly focused on microbial proteases due to its variety and its unique characteristics including thermophilic, alkalophilic and organic solvent tolerant traits (Asgher et. al., 2018). In addition to that, uses of enzyme in general for industrial purposes are inexpensive and sustainable to the environment. Although the use of enzyme as a biocatalyst are undeniably profitable and beneficial, they are limited to single usage as enzyme undergoes denaturation after catalysing a reaction (Sheldon, 2011). Enzymes are also susceptible to denaturation under extreme conditions such as high temperature, extreme pH and presence of organic solvents in the reaction media. These conditions; which are deemed extreme towards enzyme, are frequently utilized in industrial applications to shift the reaction equilibrium towards completion of synthesis (Li et. al., 2013).

Notwithstanding with the limitations, enzymes with high durability and tolerance against the extreme conditions are constantly being discovered and studied. Enzymes that are thermophilic; able to endure high temperature, alkalophiles; able to tolerate alkaline conditions, and solvent tolerant enzymes has a promising feature which are applicable for industrial purposes. Enzymes with these features are abundant in nature and can be isolated from microorganism living in appropriate niches. In this study, elastase strain K is a solvent tolerant metalloprotease isolated from *Pseudomonas aeruginosa* strain K. This valuable enzyme exhibit remarkable tolerance against wide range of polar and non-polar organic solvents such as methanol, ethanol, 1-propanol and dimethyl sulfoxide (DMSO). Natural enzymes with organic solvent-tolerance are useful for applications employing organic solvents as reaction media because they can be used for these applications without any modifications to stabilize the enzymes. In order to expand the practicality of this enzyme for industrial applications, some modifications should be made to enhance the durability of the enzyme. One enzyme modification that can be applied is by immobilization techniques. Whilst individual enzyme molecules are susceptible to denaturation, physical carrier or chemical cross-linker can be incorporated to form a stable immobilized enzyme structure. Example of enzyme immobilization techniques are adsorption, encapsulation and cross-linking.

While each of the enzyme immobilization techniques offers great solution in improving the operational stability of enzyme, there are some limitations of using certain immobilization techniques. Enzyme immobilization by adsorption often experience loss of enzyme activity due to enzyme leaching during recovery processes (Sheldon, 2011). In addition to that, the use of non-catalytic carrier in adsorption reduces the volumetric yield and cause enzyme dilution. Likewise, enzyme immobilization by encapsulation also experience loss of activity due to enzyme leaching and low productivity since most of the volume are taken up by the non-catalytic matrices (Velasco-Lozano et. al., 2015). Above all one major drawback of these immobilization techniques is the need to use a highly purified enzyme. In view of the drawbacks seen in the enzyme immobilization techniques by adsorption, encapsulation and cross-linking, this study therefore proposed the preparation of cross-linked enzyme aggregates (CLEA) of elastase strain K. Cross-linked enzyme aggregates (CLEA) is a versatile, carrier-free enzyme immobilization technique which has a notable reputation due to the advantages it provides. In contrast with adsorption and encapsulation, CLEA immobilization does not require a purified enzyme to begin with. In addition to that, the use of small sized cross-linker ensure that the immobilized enzyme to be comprised of the enzyme mostly (Talekar et. al., 2013). The processes of forming CLEA are also relatively simple; by incorporation of aggregants to precipitate the enzyme thus increasing the selectivity and addition of bifunctional reagent such as glutaraldehyde to cross-link the enzyme. Processes of forming CLEA also varied in term of duration of the treatment and concentration of cross-linking reagent used, depending on the enzyme being immobilized.

To date, only few studies were conducted on immobilization of organic solvent tolerant protease using CLEA immobilization technique. It is noteworthy to study the extent of immobilizing elastase strain K using CLEA method in improving the activity and maintaining the pre-existing organic solvent tolerant characteristic of the enzyme. Apart from that, studies regarding the structural conformation of CLEA remain elusive.
It is hypothesized that understanding of its structural conformational would facilitate the construction of CLEA. This research was conducted to achieve the following objectives:

1. To optimize the preparation of CLEA of the organic solvent tolerant elastase strain K.

2. To characterize the biochemical features of CLEA of organic solvent tolerant elastase strain K.

3. To determine the biophysical profile of CLEA of organic solvent tolerant elastase strain K.

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REFERENCES

Adlercreutz, P. (2013). Immobilisation and application of lipases in organic media. *Chemical Society Reviews*, 42(15), 6406--6436.

Agyei, D., & He, L. (2015). Evaluation of cross-linked enzyme aggregates of Lactobacillus cell-envelope proteinases, for protein degradation. *Food and Bioproducts Processing*, *94*, 59-69.

Ahmad, M. M., & Hassan, Z. (2019). Detection of Milk Clotting Enzyme Produced by Lactic Acid Bacteria Isolated from Fermented Food. *Alexandria Science Exchange Journal*, *40*(July-September), 415-418.

Andler, R., & Steinbüchel, A. (2017). A simple, rapid and cost-effective process for production of latex clearing protein to produce oligopolyisoprene molecules. *Journal of biotechnology*, *241*, 184-192.

Asgher, M., Bashir, F., & Iqbal, H. M. (2018). Protease-based cross-linked enzyme aggregates with improved catalytic stability, silver removal, and dehairing potentials. *International Journal of Biological Macromolecules* , 118, 1247-1256.

Baker, A. H., Edwards, D. R., & Murphy, G. (2002). Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *Journal of Cell Science*, 115(19), 3719-3727.

Baker, S. L., Munasinghe, A., Kaupbayeva, B., Kang, N. R., Certiat, M., Murata, H., Matyjaszewsk, K., Lin, P., Colina, C. M., & Russell, A. J. (2019). Transforming protein-polymer conjugate purification by tuning protein solubility. *Nature communications*, *10*(1), 1-12.

Barbosa, O., Ortiz, C., Berenguer-Murcia, Á., Torres, R., Rodrigues, R. C., & Fernandez-Lafuente, R. (2014). Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization. *RSC Advances*, 4(4), 1583-1600.

Bashir, F., Asgher, M., Hussain, F., & Randhawa, M. A. (2018). Development and characterization of cross-linked enzyme aggregates of thermotolerant alkaline protease from *Bacillus licheniformis. International Journal of Biological Macromolecules*, 113, 944-951.

Bian, H., Cao, M., Wen, H., Tan, Z., Jia, S., & Cui, J. (2019). Biodegradation of polyvinyl alcohol using cross-linked enzyme aggregates of degrading enzymes from Bacillus niacini. *International journal of biological macromolecules*, *124*, 10-16.

Brix, K., & Stöcker, W. (Eds.). (2013). *Proteases: structure and function* (pp. 1-564). Springer Vienna.

© COPYRIGHT UPM

Cao, L. (2005). Immobilised enzymes: science or art? *Current Opinion in Chemical Biology*, 9(2), 217-226.

Cerda-Costa, N., & Xavier Gomis-Ruth, F. (2014). Architecture and function of metallopeptidase catalytic domains. *Protein Science*, 23(2), 123-144.

Chen, Y., Jiang, Q., Sun, L., Li, Q., Zhou, L., Chen, Q., ... & Li, W. (2018). Magnetic combined cross-linked enzyme aggregates of ketoreductase and alcohol dehydrogenase: an efficient and stable biocatalyst for asymmetric synthesis of (R)-3-quinuclidinol with regeneration of coenzymes in situ. *Catalysts*, *8*(8), 334.

Cui, J. D., & Jia, S. R. (2015). Optimization protocols and improved strategies of cross-linked enzyme aggregates technology: current development and future challenges. *Critical Reviews in Biotechnology*, 35(1), 15-28.

Dal Magro, L., Kornecki, J. F., Klein, M. P., Rodrigues, R. C., & Fernandez-Lafuente, R. (2020). Pectin lyase immobilization using the glutaraldehyde chemistry increases the enzyme operation range. *Enzyme and microbial technology*, *132*, 109397.

Di Cera, E. (2009). Serine proteases. *IUBMB life*, *61*(5), 510-515..

Doraiswamy, N., Sarathi, M., & Pennathur, G. (2019). Cross-linked esterase aggregates (CLEAs) using nanoparticles as immobilization matrix. *Preparative Biochemistry and Biotechnology*, *49*(3), 270-278.

Doukyu, N., & Ogino, H. (2010). Organic solvent-tolerant enzymes. *Biochemical Engineering Journal*, 48(3), 270–282.

Duong-Ly, K. C., & Gabelli, S. B. (2014). Salting out of proteins using ammonium sulfate precipitation. *Methods in Enzymology*, 541, 85-94.

Eissa, A. S., Puhl, C., Kadla, J. F., & Khan, S. A. (2006). Enzymatic cross-linking of β-lactoglobulin: Conformational properties using FTIR spectroscopy. *Biomacromolecules*, *7*(6), 1707-1713.

Gaber, Y., & Ismail, M. (2017). Cross-linked enzyme aggregates of pig liver esterase evaluated . *Biotechnology*, 16(3), 123-129.

Galvis, M., Barbosa, O., Ruiz, M., Cruz, J., Ortiz, C., Torres, R., & Fernandez-Lafuente, R. (2012). Chemical amination of lipase B from Candida antarctica is an efficient solution for the preparation of crosslinked enzyme aggregates. *Process Biochemistry*, 47(12), 2373–2378.

Gilani, S. L., Najafpour, G. D., Moghadamnia, A., & Kamaruddin, A. H. (2016). Stability of immobilized porcine pancreas lipase on mesoporous chitosan beads: a comparative study. *Journal of Molecular Catalysis B: Enzymatic* , 133, 144-153.

© COPYRIGHT UPM

Goldstein, J. I., Newbury, D. E., Michael, J. R., Ritchie, N. W. M., Scott, J. H. J., & Joy, D. C. (2017). *Scanning electron microscopy and X-ray microanalysis*. Springer.

Grzonka, Z., Kasprzykowski, F., & Wiczk, W. (2007). Cysteine proteases. In *Industrial enzymes* (pp. 181-195). Springer, Dordrecht.

Gupta, A., & Khare, S. K. (2006). A protease stable in organic solvents from solvent tolerant strain of *Pseudomonas aeruginosa*. *Bioresource Technology*, 97(15), 1788-1793.

Gurumallesh, P., Alagu, K., Ramakrishnan, B., & Muthusamy, S. (2019). A systematic reconsideration on proteases. *International Journal of Biological Macromolecules*, 128, 254-267.

Häring, D., & Schreier, P. (1999). Cross-linked enzyme crystals. *Current Opinion in Chemical Biology*, 3(1), 35-38.

Hwang, K. S., Park, H. Y., Kim, J. H., & Lee, J. Y. (2018). Fully organic CO_2 absorbent obtained by a Schiff base reaction between branched poly (ethyleneimine) and glutaraldehyde. *Korean Journal of Chemical Engineering*, *35*(3), 798-804.

Jegan Roy, J., & Emilia Abraham, T. (2004). Strategies in making cross-linked enzyme crystals. *Chemical Reviews*, 104(9), 3705-3722.

Jian, S., Wenyi, T., & Wuyong, C. (2011). Kinetics of enzymatic unhairing by protease in leather industry. *Journal of Cleaner Production*, 19(4), 325-331.

Jeon, J. G., Kim, H. C., Palem, R. R., Kim, J., & Kang, T. J. (2019). Cross-linking of cellulose nanofiber films with glutaraldehyde for improved mechanical properties. *Materials Letters*, *250*, 99-102.

Kazarian, S. G., & Chan, K. L. A. (2013). ATR-FTIR spectroscopic imaging: recent advances and applications to biological systems. *Analyst*, 138(7), 1940-1951.

Kirk, O., Borchert, T. V., & Fuglsang, C. C. (2002). Industrial enzyme applications. *Current Opinion in Biotechnology* , 13(4), 345-351.

Lauinger, L., Li, J., Shostak, A., Cemel, I. A., Ha, N., Zhang, Y., Merl, P. E., Obermeyer, S., Stankovic-Valentin, N., Schafmeier, T., Wever, W. J., Bowers, A. A., Carter, K. P., Palmer, A. E., Tschochner, H., Melchior, F., Deshaies, R. J., Brunner, M., & Diernfellner, A. (2017). Thiolutin is a zinc chelator that inhibits the Rpn11 and other JAMM metalloproteases. *Nature chemical biology*, *13*(7), 709.

Li, Q., Marek, P., & Iverson, B. L. (2013). Commercial proteases: Present and future. *FEBS Letters*, 587(8), 1155-1163.

© COPYRIGHT UPM

Mahmod, S., Yusof, F., Jami, M. S., & Khanahmadi, S. (2016). Optimizing the preparation conditions and characterization of a stable and recyclable cross-linked enzyme aggregate (CLEA)-protease. *Bioresources and Bioprocessings*, 3(1), 1-11.

Mahmod, S. S., Yusof, F., Jami, M. S., & Khanahmadi, S. (2015). Development of an immobilized biocatalyst with lipase and protease activities as a multipurpose cross-linked enzyme aggregate (multi-CLEA). *Process Biochemistry*, 50(12), 2144–2157.

Mandujano-González, V., Villa-Tanaca, L., Anducho-Reyes, M. A., & Mercado-Flores , Y. (2016). Secreted fungal aspartic proteases: A review. *Revista Iberoamericana de Micología*, 33(2), 76-82.

Mansur, H. S., Sadahira, C. M., Souza, A. N., & Mansur, A. A. (2008). FTIR spectroscopy characterization of poly (vinyl alcohol) hydrogel with different hydrolysis degree and chemically crosslinked with glutaraldehyde. *Materials Science and Engineering C* , 28(4), 539–548.

Mat Said, Z. S., Arifi, F. A. M., Salleh, A., Rahman, R. N. Z. R. A., Leow, A. T. C., Latip, W., & Ali, M. S. M. (2019). Unravelling protein-organic solvent interaction of organic solvent tolerant elastase from *Pseudomonas aeruginosa* strain K crystal structure. *International Journal of Biological Macromolecules*, 127, 575-584.

Mateo, C., Palomo, J. M., Van Langen, L. M., Van Rantwijk, F., & Sheldon, R. A. (2004). A new, mild cross-linking methodology to prepare cross-linked enzyme aggregates. *Biotechnology and Bioengineering*, 86(3), 273-276.

Matijošytė, I., Arends, I. W., de Vries, S., & Sheldon, R. A. (2010). Preparation and use of cross-linked enzyme aggregates (CLEAs) of laccases. *Journal of Molecular Catalysis B: Enzymatic*, *62*(2), 142-148.

Mehde, A. A., Mehdi, W. A., Özacar, M., & Özacar, Z. Z. (2018). Evaluation of different saccharides and chitin as eco-friendly additive to improve the magnetic cross-linked enzyme aggregates (CLEAs) activities. *International journal of biological macromolecules*, *118*, 2040-2050.

Migneault, I., Dartiguenave, C., Bertrand, M. J., & Waldron, K. C. (2004). Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. *BioTechniques*, 37(5), 790-802.

Nadar, S. S., Muley, A. B., Ladole, M. R., & Joshi, P. U. (2016). Macromolecular cross-linked enzyme aggregates (M-CLEAs) of α-amylase. *International Journal of Biological Macromolecules*, 84, 69-78.

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Nagase, H. (2001). Metalloproteases. *Current protocols in protein science*, *24*(1), 21-4.

Nair, A. R., & Chellapan, G. (2020). Improving operational stability of thermostable Pythium myriotylum secretory serine protease by preparation of cross-linked enzyme aggregates (CLEAs). *Preparative Biochemistry & Biotechnology*, *50*(2), 107-115.

Nguyen, L., Seow, N., & Yang, K.-L. (2017). Hollow cross-linked enzyme aggregates (h-CLEA) of laccase with high uniformity and activity. *Colloids Surface B Biointerfaces*, 151, 88-94.

Nuijens , T., Cusan , C., van Dooren, T. J., Moody, H. M., Merkx, R., Kruijtzer, J. A., et al. (2010). Fully Enzymatic Peptide Synthesis using C-Terminal tert-Butyl Ester Interconversion. *Advanced Synthesis & Catalysis*, 2399-2404.

Özacar, M., Mehde, A. A., Mehdi, W. A., Özacar, Z. Z., & Severgün, O. (2019). The novel multi cross-linked enzyme aggregates of protease, lipase, and catalase production from the sunflower seeds, characterization and application. *Colloids and Surfaces B: Biointerfaces*, *173*, 58-68.

Park, S. H., Kim, S. J., Park, S., & Kim, H. K. (2019). Characterization of organic solvent-tolerant lipolytic enzyme from Marinobacter lipolyticus isolated from the Antarctic Ocean. *Applied biochemistry and biotechnology*, *187*(3), 1046-1060.

Park, S. H., Soetyono, F., & Kim, H. K. (2017). Cadaverine production by using cross-linked enzyme aggregate of *Escherichia coli* lysine decarboxylase . *J. Microbiol. Biotechnol*, 27(2), 289–296.

Pecora, R. (Ed.). (2013). *Dynamic light scattering: applications of photon correlation spectroscopy*. Springer Science & Business Media..

Polgar, L. (2005). The catalytic triad of serine peptidases. *Cellular and Molecular Life Sciences*, 62(19-20), 2161-2172.

Rahman, R. N. Z. R. A., Lee, P. G., Basri, M., & Salleh, A. B. (2005). An organic solvent-tolerant protease fromPseudomonas aeruginosa strain K, Nutritional factors affecting protease production. *Enzyme and Microbial Technology*, 36(5-6), 749–757.

Rahman, R. N. Z. R. A., Salleh, A. B., Basri, M., & Wong, C. F. (2011). Role of α-Helical structure in organic solvent-activated homodimer of elastase strain K. *International Journal of Molecular Sciences*, 12(9), 5797-5814.

Rathankumar, A. K., SaiLavanyaa, S., Saikia, K., Gururajan, A., Sivanesan, S., Gosselin, M., Vaidyanathan, V. K., & Cabana, H. (2019). Systemic Concocting of Cross-Linked Enzyme Aggregates of Candida antarctica

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Lipase B (Novozyme 435) for the Biomanufacturing of Rhamnolipids. *Journal of Surfactants and Detergents*, *22*(3), 477-490.

Rawlings, N. D. (2013). Protease families, evolution and mechanism of action. In *Proteases: structure and function* (pp. 1-36). Springer, Vienna.

Rehman, S., Bhatti, H. N., Bilal, M., & Asgher, M. (2016). Cross-linked enzyme aggregates (CLEAs) of *Pencilluim notatum* lipase enzyme with improved activity, stability and reusability characteristics. *International Journal of Biological Macromolecules*, 91, 1161-1169.

Reimer, L. (2013). *Transmission electron microscopy: physics of image formation and microanalysis* (Vol. 36). Springer.

Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, Á., Torres, R., & Fernández-Lafuente, R. (2013). Modifying enzyme activity and selectivity by immobilization. *Chemical Society Reviews*, 42(15), 6290-6307.

Rzychon, M., Chmiel , D., & Stec-Niemczyk, J. (2004). Modes of inhibition of cysteine proteases. *Acta Biochimica Polonica*, 51(4), 861–873.

Sahin, S., & Ozmen, I. (2020). Covalent immobilization of trypsin on polyvinyl alcohol-coated magnetic nanoparticles activated with glutaraldehyde. *Journal of Pharmaceutical and Biomedical Analysis*, *184*, 113195.

Sajid, M., & McKerrow, J. H. (2002). Cysteine proteases of parasitic organisms. *Molecular and Biochemical Parasitology*, 120(1), 1-21.

Salleh, A. B., Razak, C. N., Rahman, R. N. Z. R. A., & Basri, M. (2006). Protease: Introduction. In A. Salleh, R. Rahman, & M. Basri , *New Lipases and Proteases* (pp. 23-39). New York: Nova Science Publishers.

Salvesen, G. S., Hempel, A., & Coll, N. S. (2016). Protease signaling in animal and plant regulated cell death. *The FEBS journal*, 283(14), 2577-2598.

Sangeetha, K., & Abraham, T. E. (2008). Preparation and characterization of cross-linked enzyme aggregates (CLEA) of subtilisin for controlled release applications. *International Journal of Biological Macromolecules*, *43*(3), 314-319.

Santhalembi, L., Periyasamy, K., Pennathur, G., Seileshkumar, T., Gangmei, L., & Ningombam, A. (2020). Preparation and characterization of cross-linked enzyme aggregates (cleas) of *Aeromonas caviae* AU04 protease. *Journal of Advanced Scientific Research*, *11*(1), 35-43.

Saran, S., Mahajan, R. V., Kaushik, R., Isar, J., & Saxena, R. K. (2013). Enzyme mediated beam house operations of leather industry: a needed step towards greener technology. *Journal of Cleaner Production*, 54, 315-322.

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Satar, R., Jafri, M. A., Rasool, M., & Ansari, S. A. (2017). Role of glutaraldehyde in imparting stability to immobilized β-galactosidase systems. *Brazilian Archives of Biology and Technology*, *60*.

Sawant, R., & Nagendran, S. (2014). Protease: an enzyme with multiple industrial applications. *World Journal of Pharmacy and Pharmaceutical Sciences*, 3(6), 568-579.

Schoevaart, R., Wolbers, M. W., Golubovic, M., Ottens, M., Kieboom, A. P. G., Van Rantwijk, F., Van Der Wielen, L. A. M., & Sheldon, R. A. (2004). Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs). *Biotechnology and Bioengineering*, 87(6), 754-762.

Shah, S., Sharma, A., & Gupta, M. N. (2006). Preparation of cross-linked enzyme aggregates by using bovine serum albumin as a proteic feeder. *Analytical Biochemistry*, 351(2), 207–213.

Sheldon, R. A. (2007). Enzyme Immobilization: The Quest for Optimum Performance. *Advanced Synthesis & Catalysis*, 349(8-9), 1289 – 1307.

Sheldon, R. A. (2011). Characteristic features and biotechnological applications of cross-linked enzyme aggregates (CLEAs). *Appl Microbiol Biotechnol*, 92(3), 467-477.

Sheldon, R. A. (2011). Cross-linked enzyme aggregates as industrial biocatalysts. *Organic Process Research & Development*, 15(1), 213-223.

Stetefeld, J., McKenna, S. A., & Patel, T. R. (2016). Dynamic light scattering: a practical guide and applications in biomedical sciences. *Biophysical Reviews*, 8(4), 409-427.

Sun, S., Li, H., & Xu, Z. J. (2018). Impact of SurfaceArea in Evaluationof Catalyst Activity. *Joule*, 2(6), 1024-1027.

Talekar, S., Joshi, A., Joshi, G., Kamat, P., Haripurkar , R., & Kambale, S. (2013). Parameters in preparation and characterization of cross linked enzyme aggregates (CLEAs). *RSC Advances*, 3(31) 12485–12511.

Talekar, S., Nadar, S., Joshi, A., & Joshi, G. (2014). Pectin cross-linked enzyme aggregates (pectin-CLEAs) of glucoamylase. *RSC Advances*, 4(103), 59444–59453.

Tang, J. (2012). Gastricsin. In A. J. Barrett, J. Woessner, & N. D. Rawling, *Handbook of proteolytic enzymes* (pp. 38-43). London: Elsevier science.

Tudorache, M., Gheorghe, A., Viana, A. S., & Parvulescu, V. I. (2016). Biocatalytic epoxidation of α-pinene to oxy-derivatives over cross-linked lipase aggregates B Enzymatic.

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Van der Hoorn , R. A. (2008). Plant proteases: from phenotypes to molecular mechanisms. *Annual Review of Plant Biology*, 59, 191-223.

Velasco-Lozano, S., López-Gallego, F., Mateos-Díaz, J. C., & Favela-Torres, E. (2015). Cross-linked enzyme aggregates (CLEA) in enzyme improvement - a review. *Biocatalysis*, 1, 166–177.

Wahab, M. K. H. A., El-Enshasy, H. A., Bakar, F. D. A., Murad, A. M. A., Jahim, J. M., & Illias, R. M. (2019). Improvement of cross-linking and stability on cross-linked enzyme aggregate (CLEA)-xylanase by protein surface engineering. *Process Biochemistry*, *86*, 40-49.

Wang, S., Zheng, D., Yin, L., & Wang, F. (2017). Preparation, activity and structure of cross-linked enzyme aggregates (CLEAs) with nanoparticle. *Enzyme and microbial technology*, *107*, 22-31.

Wingfield, P. T. (2016). Protein precipitation using ammonium sulfate. *Current Protocols in Protein Science*, 84(1), A.3F.1–A.3F.9.

Wong, C. F., Salleh, A. B., Basri, M., & Rahman, R. N. Z. R. A. (2010). Organic-solvent stability of elastase strain K overexpressed in an Escherichia–Pseudomonas expression system. *Biotechnology and Applied Biochemistry*, 57(1), 1-7.

Xu, D. Y., & Yang, Z. (2013). Cross-linked tyrosinase aggregates for elimination of phenolic compounds from wastewater. *Chemosphere*, 92(4), 391-398.

Yang, Y. F., Zhang, L. Z., Du, X. P., Zhang, S. F., Li, L. J., Jiang, Z. D., Wu, L. M., Ni, H., & Chen, F. (2017). Recovery and purification of limonin from pummelo [*Citrus grandis*] peel using water extraction, ammonium sulfate precipitation and resin adsorption. *Journal of Chromatography B*, *1060*, 150-157.

You, Y., Sun, X., Cui, Q., Wang, B., & Ma, J. (2016). The retention and drainage behavior of cross-linked gelatin with glutaraldehyde in a papermaking system. *BioResources*, *11*(3), 6162-6173.

Yu, P., Huang, X., Ren, Q., & Wang, X. (2019). Purification and characterization of a H2O2-tolerant alkaline protease from *Bacillus* sp. ZJ1502, a newly isolated strain from fermented bean curd. *Food Chemistry*, 274, 510-517.

Zdarta , J., Meyer, A. S., Jesionowski , T., & Pinelo, M. (2018). A general overview of support materials for enzyme immobilization: characteristics, properties, practical utility. *Catalysts*, 8(2), 92.

Zerva, A., Antonopoulou, I., Enman, J., Iancu, L., Rova, U., & Christakopoulos, P. (2018). Cross–Linked Enzyme Aggregates of Feruloyl Esterase Preparations from *Thermothelomyces thermophilia* and *Talaromyces wortmannii*. *Catalyst*, 8(5), 208.

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