ENTRAPMENT OF MALTOGENIC AMYLASE AND CYCLODEXTRIN GLUCANOTRANSFERASE FOR MALTO-OLIGOSACCHARIDES SYNTHESIS

NURHIDAYAH KUMAR BINTI MUHAMMAD FIRDAUS KUMAR

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Faculty of Engineering
Universiti Teknologi Malaysia

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DEDICATION

To my dearest Ibu and Abah,
For all their love and prayers that have always nourished and sustained me.

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ABSTRACT

Starches in various forms are extensively utilized industrially for a variety of purposes. Malto-oligosaccharides (MOS) can be synthesized by maltogenic amylase (MAG1) from starch. However, using maltogenic amylase alone has significant drawbacks, including inadequate specificity for starch and low MOS yield. Cyclodextrin glucanotransferase (CGTase) can convert starch to cyclodextrins, the preferred substrates for MAG1. Employing the substrate diversity of MAG1 and CGTase, the use of both enzymes could provide a new synthesis pathway for MOS from a low-cost and widely available starch. Although free enzymes can be used to produce MOS, this method is hindered by low enzyme recovery and lack of enzyme stability, making it unsuitable for industrial applications. Enzyme immobilization strategy was used in this study to alleviate these disadvantages. Entrapment immobilization technique that uses calcium alginate (CA) beads were developed to improve the stability and reusability of free MAG1 and CGTase. Factors affecting the formation of the beads for both CA-MAG1 and CA-CGTase were investigated. These include sodium alginate concentration, calcium chloride concentration and enzyme loading. The highest activity recovery of CA-MAG1 was obtained with 75 U MAG1 loading, 2 % (w/v) sodium alginate, and 0.8 % calcium chloride that yielded 88.06 % of activity recovery. Meanwhile, the optimum conditions for CA-CGTase were achieved at 1000 U CGTase loading with 2.5 % (w/v) sodium alginate and 0.8 % calcium chloride exhibited 89.45 % activity recovery. CA-MAG1 retained 41 % of the initial activity after 60 min incubation at 45 °C compared to free MAG1 in which the activity dropped after 10 min of incubation. After incubation for an hour at 70 °C, CA-CGTase retained 44 % of its initial activity compared to 11 % of free CGTase. A kinetic study discovered that CA-MAG1 and CA-CGTase have shown a relatively high affinity towards starch, with K_m value of 6.86 mM. Moreover, the developed beads revealed low enzyme leaching and were able to be reused up to 8 cycles with more than 50% of activity retention. Starch hydrolysis reactions were carried out by two-steps and one-pot methods. For the two-steps method, MOS yield of 183.82 mg/g was obtained when 3 U MAG1 loading, 10 U CGTase loading, 2 % (w/v) of starch, and a total reaction of 5 h. With a 2 h reaction time, MAG1 loading to CGTase loading in a 3 U: 7 U ratio, and 1.5 % (w/v) starch loading, the one-pot technique provided 190.48 mg/g of MOS, which was 0.97-fold more than the two-steps method. The entrapment method used to produce CA-MAG1 and CA-CGTase have proven to be a viable strategy for developing stable and reusable enzymes that are potentially useful biocatalysts for starch conversion in MOS production.

ABSTRAK

Kanji dalam pelbagai bentuk digunakan secara meluas secara industri untuk pelbagai tujuan. Malto-oligosakarida (MOS) boleh dihasilkan oleh amilase maltogenik (MAG1) daripada kanji. Walaubagaimanapun, penggunaan MAG1 sahaja mempunyai kelemahan yang ketara, iaitu kekhususan yang tidak mencukupi untuk kanji dan penghasilan MOS yang rendah. Glukanotransferase siklodekstrin (CGTase) pula boleh menukarkan kanji kepada sikoldekstrin, substrat pilihan bagi MAG1. Menggunakan kepelbagaian substrat MAG1 dan CGTase, penggunaan kedua-dua enzim boleh menyediakan laluan sintesis baharu bagi penghasilan MOS daripada kanji dengan kos yang rendah. Walaupun enzim bebas boleh digunakan untuk menghasilkan MOS, kaedah ini tidak realistik kerana perolehan enzim yang rendah dan kekurangan kestabilan enzim, menjadikannya tidak sesuai untuk aplikasi perindustrian. Strategi imobilisasi enzim digunakan dalam kajian ini untuk mengurangkan kelemahan ini. Teknik imobilisasi pengepungan yang menggunakan manik kalsium alginat (CA) telah dihasilkan untuk meningkatkan kestabilan dan kebolehgunaan semula MAG1 dan CGTase bebas. Faktor-faktor yang mempengaruhi pembentukan manik bagi CA-MAG1 dan CA-CGTase telah disiasat. Faktor tersebut ialah kepekatan natrium alginat, kepekatan kalsium klorida dan pemuatan enzim. Perolehan aktiviti tertinggi CA-MAG1 diperoleh dengan 75 U MAG1, 2 % (w/v) natrium alginat, dan 0.8 % kalsium klorida yang menghasilkan 88.06 % perolehan aktiviti. Sementara itu, keadaan optimum untuk CA-CGTase dicapai pada 1000 U CGTase dengan 2.5 % (w/v) natrium alginat dan 0.8 % kalsium klorida mempamerkan 89.45 % perolehan aktiviti. CA-MAG1 mengekalkan 41 % daripada aktiviti sebenar selepas 60 min inkubasi pada 45 °C berbanding MAG1 bebas di mana aktiviti itu menurun selepas 10 minit inkubasi. Selepas inkubasi selama sejam pada 70 °C, CA-CGTase mengekalkan 44% daripada aktiviti sebenarnya berbanding 11 % daripada CGTase bebas. Kajian kinetik mendapati bahawa CA-MAG1 dan CA-CGTase telah menunjukkan pertalian yang agak tinggi terhadap kanji, Km = 6.86 mM. Selain itu, CA-MAG1 dan CA-CGTase dapat digunakan semula sehingga 8 kitaran dengan lebih daripada 50 % pengekalan aktiviti. Tindakan hidrolisis kanji dilakukan oleh kaedah dua langkah serentak dan serentak. Untuk kaedah dua langkah, hasil MOS sebanyak 183.82 mg/g diperoleh apabila 3 U MAG1, 10 U CGTase, 2 % (w/v) kanji, dan jumlah tindakan 5 jam. Dengan masa tindak balas 2 jam, MAG1: CGTase dalam nisbah 3 U: 7 U, dan kanji 1.5 % (w/v), teknik serentak menghasilkan 190.48 mg/g MOS, iaitu 0.97 kali ganda lebih banyak daripada kaedah dua langkah. Kaedah pengepungan yang digunakan untuk menghasilkan CA-MAG1 dan CA-CGTase telah terbukti sebagai strategi yang baik untuk menghasilkan enzim yang stabil dan boleh diguna semula yang berpotensi sebagai biomangkin untuk penghasilan MOS daripada kanji.

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LIST OF ABBREVIATIONS

A600	-	Absorbance at optical density of 600 nm
BSA	-	Bovine serum albumin
β-CD	-	Beta-cyclodextrin
CA	-	Calcium alginate
CCD	-	Central composite design
CGTase	-	Cyclodextrin glucanotransferase
DNS	-	Dinitosalicylic acid
FTIR	-	Fourier transform infrared spectroscopy
IPTG	-	Isopropyl β-D-1-thiogalactopyranoside
kDa	-	Kilodalton
LB	-	Luria Bertani
MAG1	-	Maltogenic amylase
ml	-	Mililiter
MOS	-	Malto-oligosaccharide
OFAT	-	One-factor-at-a-time
rpm	-	Revolution per minute
RSM	-	Response Surface Methodology
SEM	-	Scanning electron microscopy
U	-	Unit
U/ml	-	Unit per mililiter
V	-	Volt
w/v	-	Weight per volume

LIST OF SYMBOLS

β	-	Beta
°C	-	Degree Celsius
h	-	Hour
µl	-	Microliter
µmol	-	Micromole
min	-	Minutes
ml	-	Mililiter
%	-	Percentage
±	-	Plus minus

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Starch is found naturally, the cheapest carbohydrate commercially available, and can be used to produce oligosaccharides. Due to the high interest in functional oligosaccharides for health benefits and other characteristics, the enzymatic process has been recognized in producing functional oligosaccharides with high yields and purity at a lower cost. The enzymatic process of oligosaccharides production involves more straightforward steps compared to the complicated procedures of a chemical process. Besides, it offers stereochemical specificity and is more environmentally friendly, without harmful chemicals usage. Malto-oligosaccharides are an essential kind of functional oligosaccharides with good adaptability to food processing as well as beneficial to human digestion and absorption performance. Therefore, they have been used widely in food industries. Malto-oligosaccharides (MOS) are low sweeteners with properties like antistaling effect on bread, high water holding capacity, and preventing sucrose crystallization.

The enzymatic reaction requires more than one enzyme to improve the production yield. The enzymatic process of MOS can be produced by adding malto-oligosaccharide forming amylase to starch. Ibrahim (2018) added pullulanase (EC 3.2.1.9) to the starch as a second enzyme to break down α-(1→6) glycosidic bonds in the branched amylopectin starch. Maltogenic amylase (EC 3.2.1.133) is one of the glycoside hydrolase (GH13) family that exhibits high affinity of substrate specificity toward cyclodextrin (CD) than starch and pullulan (Abdul Manas, Pachelles, Mahadi, and Md Illias, 2014). On the other hand, cyclodextrin glucanotransferase (EC2.4.1.19) an enzyme from the GH13 family undergone a cyclization process to split the starch and formed CD. Owing to the substrate diversity of both enzymes, the

use of these enzymes could provide a potential synthesis strategy for MOS production.

The process of converting raw materials into MOS must be inexpensive and geared toward improving enzyme productivity and stability. Currently, several enzyme-based immobilization processes have proven economically and have already been implemented on a larger scale, mostly in the food industry in which the free enzyme-catalyzed process is replaced with the immobilized enzyme. For standard industrial practices, immobilization is a requirement to assist the enzyme recovery and reusable ability provided that they are stable enough. Nonetheless, these days, the objective of immobilization does not revolve around the reusability of the enzyme but to improve the enzyme features such as enzyme stability and activity that may be achieved by an appropriate immobilization approach.

Research on various materials and techniques of enzyme immobilization has been established and is still ongoing. Different materials have been used as immobilization carriers such as ceramics (Gao, Feng, Li, Jiang, and Zhou, 2015), chitosan (Gür, İdil, and Aksöz, 2018), and carbon nanotubes (Tavares et al., 2015). Previously, Jaafar et al. (2021) employed synergistic action of free maltogenic amylase and cyclodextrin glucanotransferase using starch as the substrate for MOS production. This study improves the stability and reusability of both enzymes by entrapping them individually into calcium alginate beads to prevent substrate transfer limitation, allowing efficient substrate channeling.

1.2 Problem Background

This study was motivated to solve three main issues. First, a complete hydrolysis of polysaccharides complex structure (starch) into MOS is limited due to the low efficiency of a single maltogenic amylase enzyme for starch hydrolysis. Second, the use of a free enzyme to completely hydrolyze the starch is inadequate due to shorter enzyme lifetime, low enzyme operational stability, and impossible to reutilizing for continuous operation which will eventually increase the production

cost. Third, poor operation stability, substrate diffusion limitation and the enzyme can be easily detached from the support and lead to enzyme leakage by adsorption and cross-linking method.

Forth, using enzymes mixture co-immobilization may lead to mass transfer limitations of the substrates to the active site of the enzymes. By designing an individual entrapped maltogenic amylase and cyclodextrin glucanotransferase, the activity, stability, and reusability of both enzymes can be enhanced for dual-enzyme starch hydrolysis system in a one-pot reaction for continuous production of MOS at a lower cost. Immobilizing enzymes independently can be accomplished using a different protocol for each enzyme that can be optimized. A partial inactive enzyme can also be restored by adding fresh biocatalyst of just that enzyme, without having to discard or deal with other enzymes. Bulk diffusion associated with the substrate channeling can be overcome by entrapping enzymes individually. It would also minimize competing reactions, minimize leaching and improve catalysis. It is also essential to study the kinetic of bioconversion and mechanical stability using this engineered biocatalyst to understand the new mechanism produced.

1.3 Research Objectives

The study aims to immobilize maltogenic amylase (MAG1) and cyclodextrin glucanotransferase (CGTase) for conversion of starch into MOS. To achieve the aim of the study, some objectives have been set. The objectives of this project are as follows:

1. To optimize the entrapment conditions for MAG1 and CGTase in calcium alginate beads.
2. To characterize the entrapped maltogenic amylase (CA-MAG1) and cyclodextrin glucanotransferase (CA-CGTase).
3. To evaluate the performance of CA-MAG1 and CA-CGTase in the starch hydrolysis reaction for MOS synthesis.

1.4 Scope of Study

This study focuses on the entrapment method to improve MAG1 and CGTase activity and recyclability. In order to achieve the objectives, the following scopes are outlined:

(a) Expression and purification of recombinant MAG1 and CGTase from *Bacillus lehensis* G1.

(b) Screening and optimization for entrapment conditions of MAG1 and CGTase on several parameters; (1) sodium alginate concentration (1 – 4 % (w/v)), (2) calcium chloride concentration (0.2 – 1.0 % (w/v)), and (3) enzyme loading (MAG1; 12.5 – 100 U; CGTase; 500 – 1500 U). Optimization of MAG1 and CGTase entrapment by central composite design (CCD) to obtain the highest activity recovery of entrapped enzymes.

(c) Characterization of CA-MAG1 and CA-CGTase in terms of thermostability, optimum temperature and pH, reusability and kinetic. Mechanical study of leaching analysis of CA-MAG1 and CA-CGTase.

(d) Physical characterization of the morphology and chemical composition of CA-MAG1 and CA-CGTase.

(e) Production of MOS by CA-MAG1 and CA-CGTase using optimum conditions for two-steps (3 U MAG1 loading, 10 U CGTase loading, 2 % (w/v) of starch, and a total reaction of 5 h) and one-pot (MAG1 loading to CGTase loading in a 3 U: 7 U ratio, 1.5 % (w/v) starch loading, and 2 h reaction time).

1.5 Significance of Study

The potential of MAG1 and CGTase for the synthesis of MOS has been developed before. The production of MOS by employing both enzymes could provide a new route to fulfill the market demand for oligosaccharides. Enzymatic synthesis is highly preferable in meeting the rising demand for sustainable and greener production. To provide a robust enzyme for industrial practice, a promising method of enzyme immobilization need to be established to improve enzyme activity and offer a simpler process for enzyme to be reutilize. Enzymes are attached to the support in beads form that allows them to be easily separated from the reaction. By immobilizing both enzymes individually into their respective support, any unfavorable kinetics such as diffusional resistance can be avoided. A partial inactive enzyme can also be restored by adding fresh biocatalyst of just that enzyme, without having to discard or deal with other enzymes. Alginate is utilized as the immobilization support that gives a milder and non-toxic condition for enzyme immobilization. Over the years, glutaraldehyde has been utilized for crosslinking immobilization support, the harmful effects on humans and enzyme deactivation have prompted researchers to look for another crosslinker that is safe, biodegradable, and environmentally friendly. In this study, the alginate beads formed demonstrate minimal enzyme leaching and do not require an extra crosslinker to hold enzymes into the support, and be easily detachable for the production of MOS at a lower cost. The biocatalyst produced from this study could be a potential for industrial application with high activity and reusability for continuous operation.

REFERENCES

Abd Rahim, S. N., Sulaiman, A., and Edama, N. A. (2014). Kinetics of Tapioca Slurry Saccharification Process Using Immobilized Multi-Enzyme System Enhanced with Sg. Sayong Clay. Journal of Mechanical Engineering, 11(2), 67-77.

Abdul Manas, N. H., Pachelles, S., Mahadi, N. M., and Md Illias, R. (2014). The Characterisation of an Alkali-Stable Maltogenic Amylase from Bacillus lehensis G1 and Improved Malto-Oligosaccharide Production by Hydrolysis Suppression. PLOS ONE, 9(9), e106481. doi:10.1371/journal.pone.0106481

Ahmad, R. A., Jaafar, N. R., Abdul Manas, N. H., Wan Azelee, N. I., Md Illias, R., and Nawawi, N. N. (2020). Bioconversion of Starch to Maltooligosaccharides (Mos) by the Reaction of Maltogenic Amylase. Jurnal Teknologi (Sciences & Engineering), 82(1), 99-101. doi:https://doi.org/10.11113/jt.v82.14136|

Andler, S., M., and Goddard, J., M. (2018). Transforming food waste: how immobilized enzymes can valorize waste streams into revenue streams. npj Science of Food, 2(1), 19. doi:10.1038/s41538-018-0028-2

Ariffin, M. F. K., Idris, A., and Ngadiman, N. H. A. (2019). OPTIMIZATION OF LIPASE IMMOBILIZATION ON MAGHEMITE AND ITS PHYSICO-CHEMICAL PROPERTIES. Brazilian Journal of Chemical Engineering, 36, 171-179. Retrieved from http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0104-66322019000100171&nrm=iso

Athalye, A. (2020). Enzymatic desizing for effective processing. Technology of Fibres and Textile Processing.

Ayarza, J., Coello, Y., and Nakamatsu, J. (2016). SEM–EDS study of ionically cross-linked alginate and alginic acid bead formation. International Journal of Polymer Analysis and Characterization. doi:http://dx.doi.org/10.1080/1023666X.2016.1219834

Bai, Y., and Wu, W. (2021). The Neutral Protease Immobilization: Physical Characterization of Sodium Alginate-chitosan Gel Beads. Research Square. doi:https://doi.org/10.21203/rs.3.rs-680678/v1

Beatriz Brena , P. G.-P., and Francisco Batista-Viera. (2013). Immobilization of Enzymes and Cells. In J. M. Guisan (Ed.), Methods in Molecular Biology (3 ed., Vol. 1051). New York: Springer Science+Business Media

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., and Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta, 76(5), 965-977.

Bibi, Z., Shahid, F., Qader, S. A. U., and Aman, A. (2015). Agar–agar entrapment increases the stability of endo-β-1,4-xylanase for repeated biodegradation of xylan. International Journal of Biological Macromolecules, 75, 121-127. doi:https://doi.org/10.1016/j.ijbiomac.2014.12.051

Blanco, K. C., dos Santos, F. J., Bernardi, N. S., Júnior, M. J., Monti, R., and Contiero, J. (2013). Reuse of Cyclodextrin Glycosyltransferase through Immobilization on Magnetic Carriers. Enzyme Engineering, 2(111). doi:10.4172/2329-6674.1000111

Božić, N., Lončar, N., Slavić, M., and Vujčić, Z. (2017). Raw starch degrading α-amylases: an unsolved riddle. Amylase, 1(1), 12-25. doi:https://doi.org/10.1515/amylase-2017-0002

Bradford, M. M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry, 72(1 - 2), 248 - 254.

Brena, B., Gonzalez-Pombo, P., and Batista-Viera, F. (2013). Immobilization of enzymes: a literature survey. (Vol. 1051): Humana Press, Totowa, NJ.

Burghardt, J. P., Baas, M., Gerlach, D., and Czermak, P. (2019). Two-Step Production of Neofructo-Oligosaccharides Using Immobilized Heterologous Aspergillus terreus 1F-Fructosyltransferase Expressed in Kluyveromyces lactis and Native Xanthophyllomyces dendrorhous G6-Fructosyltransferase. Catalysts, 9(8), 673. doi:https://doi.org/10.3390/catal9080673

Cabana, H., Jones, J. P., and Agathos, S. N. (2007). Preparation and characterization of cross-linked laccase aggregates and their application to the elimination of endocrine disrupting chemicals. Journal of Biotechnology, 132(1), 23 - 31.

Calvo, T. A., and Santagapita, P. (2016). Physicochemical Characterization of Alginate Beads Containing Sugars and Biopolymers. Journal of Quality and Reliability Engineering, 2016. doi:https://doi.org/10.1155/2016/9184039

Cedrone, F., Ménez, A., and Quéméneur, E. (2000). Tailoring new enzyme functions by rational redesign. Current Opinion Structural Biology, 10, 405-410.

Chen, Z., Wang, Y., Liu, W., Wang, J., and Chen, H. (2017). A novel cross-linked enzyme aggregates (CLEAs) of papain and neutrase-production, partial characterization and application. International journal of biological macromolecules, 95, 650 - 657.

Daemi, H., and Barikani, M. (2012). Synthesis and characterization of calcium alginate nanoparticles, sodium homopolymannuronate salt and its calcium nanoparticles. Scientia Iranica, 19(6), 2023-2028. doi:https://doi.org/10.1016/j.scient.2012.10.005

Dai, X. Y., Kong, L. M., Wang, X. L., Zhu, Q., Chen, K., and Zhou, T. (2018). Preparation, characterization and catalytic behavior of pectinase covalently immobilized onto sodium alginate/graphene oxide composite beads. Food Chemistry, 253, 185-193. doi:https://doi.org/10.1016/j.foodchem.2018.01.157

Danial, E. N., Hamza, A. H., and Mahmoud, R. H. (2015). Characteristics of Immobilized Urease on Grafted Alginate Bead Systems. Brazilian Archives of Biology and Technology, 58, 147-153. Retrieved from http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1516-89132015000200147&nrm=iso

Datta, S., Christena, L. R., and Sriramulu Rajaram, Y. R. (2013). Enzyme immobilization: an overview on techniques and support materials. Biotech, 3, 1-9. doi:10.1007/s13205-012-0071-7

de Souza, M. F., Rodriguesa, M. A., Freitas, S. P., and da Silva Bon, E. P. (2020). Effect of milling and enzymatic hydrolysis in the production of glucose from starch-rich Chlorella sorokiniana biomass. Algal Research, 50. doi:https://doi.org/10.1016/j.algal.2020.101961

Devi, B., Unni, B. G., Wann, S. B., and Samanta, R. (2012). Immobilization of partial purified alpha-amylase enzyme produced by soil born Bacillus species. Advances in applied science Research, 3, 2739-2744.

Diem, M. (2018). Comments on recent reports on infrared spectral detection of disease markers in blood components. Journal of Biophotonics, 11(7). doi:10.1002/jbio.201800064

Egharevba, H. O. (2019). Chemical Properties of Starch and Its Application in the Food Industry. In M. Emeje (Ed.), Chemical Properties of Starch

Fortes, C. C. S., Daniel-da-Silva, A. L., Xavier, A. M. R. B., and Tavares, A. P. M. (2017). Optimization of enzyme immobilization on functionalized magnetic nanoparticles for laccase biocatalytic reactions. Chemical Engineering & Processing: Process Intensification, 117, 1-8. doi:http://dx.doi.org/10.1016/j.cep.2017.03.009

Gajdhane, S. B., Bhagwat, P. K., and Dandge, P. B. (2016). Response surface methodology-based optimization of production media and purification of α-galactosidase in solid-state fermentation by Fusarium moniliforme NCIM 1099. 3 Biotech, 6(2), 260. doi:10.1007/s13205-016-0575-7

Ganonyan, N., Benmelech, N., Bar, G., and Gvishi, R. (2020). Entrapment of enzymes in silica aerogels. Materials today, 33, 24-35. doi:https://doi.org/10.1016/j.mattod.2019.09.021

Gao, J., Feng, K., Li, H., Jiang, Y., and Zhou, L. (2015). Immobilized lipase on porous ceramic monoliths for the production of sugar-derived oil gelling agent. RSC Advances, 5(84), 68601-68609. doi:10.1039/C5RA10570F

Gorecka, E., and Jastrzebska, M. (2011). Immobilization techniques and biopolymer carriers. Biotechnol Food Sci, 75, 65-86.

Graebin, N. G., Schöffer, J. D. N., Andrades, D. D., Hertz, P. F., Ayub, M. A. Z., and Rodrigues, R. C. (2016). Immobilization of Glycoside Hydrolase Families GH1, GH13, and GH70: State of the Art and Perspectives. Molecules, 21(8), 1074. doi:https://doi.org/10.3390/molecules21081074

Gür, S. D., İdil, N., and Aksöz, N. (2018). Optimization of Enzyme Co-Immobilization with Sodium Alginate and Glutaraldehyde-Activated Chitosan Beads. Applied Biochemistry and Biotechnology, 184(2), 538-552. doi:10.1007/s12010-017-2566-5

Homaei, A. A., Sariri, R., Vianello, F., and Stevanato, R. (2013). Enzyme immobilization: an update. Journal of Chemical Biology, 6(4), 185-205. doi:10.1007/s12154-013-0102-9

Ibrahim, O. O. (2018). Functional Oligosaccharides: Chemicals Structure, Manufacturing, Health Benefits, Applications and Regulations. J Food Chem Nanotechnol 4(4), 65-76.

Inaba, S., Kamiya, N., Bekker, G. J., Kawai, F., and Oda, M. (2019). Folding thermodynamics of PET-hydrolyzing enzyme Cut190 depending on Ca2+ concentration. Journal of Thermal Analysis and Calorimetry, 135, 2655-2663. doi:https://doi.org/10.1007/s10973-018-7447-9

Ivanova, V. (2010). Immobilization of Cyclodextrin Glucanotransferase from Paenibacillus Macerans ATCC 8244 on Magnetic Carriers and Production of Cyclodextrins. Biotechnology & Biotechnological Equipment, 24, 516-528. doi:10.1080/13102818.2010.10817893

Jaafar, N. R., Ahmad, R. a. A., Nawawi, N. N., Abd Rahman, N. H., Shamsul Annuar, N. A., Rahman, R. A., and Md Illias, R. (2021). Synergistic action of cyclodextrin glucanotransferase and maltogenic amylase improves the bioconversion of starch to malto-oligosaccharides. Process Biochemistry, 103, 9-17. doi:https://doi.org/10.1016/j.procbio.2021.02.002

Jadhav, S. B., and Singhal, R. S. (2014). Pullulan-complexed α-amylase and glucosidase in alginate beads: Enhanced entrapment and stability. Carbohydrate Polymers, 105, 49-56. doi:https://doi.org/10.1016/j.carbpol.2014.01.066

Jamil, N., Man, R. C., Suhaimi, S., Shaarani, S. M., Arshad, Z. I. M., Mudalip, S. K. A., and Sulaiman, S. Z. (2019). Immobilization of cyclodextrin glucanotransferase on hollow fiber membrane: optimization of the immobilization parameters by response surface methodology. IOP Conference Series: Earth and Environmental Science, 268, 012008. doi:10.1088/1755-1315/268/1/012008

Janecek, S., Svensson, B., and MacGregor, E. A. (2014). α-Amylase: an enzyme specificity found in various families of glycoside hydrolases. Cell Mol Life Sci., 71(7), 1149-1170. doi:10.1007/s00018-013-1388-z

Kaneko, T., Kato, T., Nakamura, N., and Horikoshi, K. (1987). Spectrophotometric Determination of Cyclization Activity of β-Cyclodextrin-Forming Cyclomaltodextrin Glucanotransferase. Journal of the Japanese Society of Starch Science, 34(1), 45-48. doi:10.5458/jag1972.34.45

Kanpariya, H. H., and Parekh, S. N. (2019). Immobilization of α-Amylase by Entrapment Method and its Comparative Study with Free α-Amylase. International Journal for Research in Applied Science & Engineering Technology, 7(IV). doi:https://doi.org/10.22214/ijraset.2019.4495

Kaur, N., Singh, B., and Sharma, S. (2018). Hydrogels for potential food application: Effect of sodium alginate and calcium chloride on physical and morphological properties. The Pharma Innovation Journal, 7(7), 142-148.

Keerti, Gupta, A., Kumar, V., Dubey, A., and Verma, A. K. (2014). Kinetic Characterization and Effect of Immobilized Thermostable Glucosidase in Alginate Gel Beads on Sugarcane Juice. ISRN Biochemistry, 2014, 178498. doi:10.1155/2014/178498

Kelley, T. R. (2010). Optimization, an important stage of engineering design. Technology Teacher, 69(5), 18-23.

Klein, M. P., Scheeren, C. W., Lorenzoni, A. S. G., Dupont, J., Frazzon, and Hertz, P. F. (2011). Ionic liquid-cellulose film for enzyme immobilization. Process Biochemistry, 46, 1375-1379. doi:https://doi.org/10.1016/j.procbio.2011.02.021

Kobayashi, S., Shoda, S.-I., Kashiwa, K. M. D. O.-R., and Shimada, J. (2022). Japan Patent No.: E. P. APPLICATION.

Kokkonen, P., Beier, A., Mazurenko, S., Damborsky, J., Bednar, D., and Prokop, Z. (2021). Substrate inhibition by the blockage of product release and its control by tunnel engineering. RSC Chemical Biology(2).

Kolcuoğlu, Y., Colak, A., Faiz, O., and Belduz, A. O. (2010). Cloning, expression and characterization of highly thermo- and pH-stable maltogenic amylase from a thermophilic bacterium Geobacillus caldoxylosilyticus TK4. Process Biochemistry, 45(6), 821-828. doi:https://doi.org/10.1016/j.procbio.2010.02.001

Kumar, K., and Loos, K. (2019). Deciphering Structures of Inclusion Complexes of Amylose with Natural Phenolic Amphiphiles. ACS Omega, 4, 17807-17813. doi:0.1021/acsomega.9b02388

Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature, 227, 680 - 685. doi:10.1038/227680A0

Larosa, C., Salerno, M., de Lima, J. S., Merijs Meri, R., da Silva, M. F., de Carvalho, L. B., and Converti, A. (2018). Characterisation of bare and tannase-loaded calcium alginate beads by microscopic, thermogravimetric, FTIR and XRD analyses. Int J Biol Macromol, 900-906. doi:10.1016/j.ijbiomac.2018.04.138

Li, Z., Ji, K., Jie, Z., Ye, X., Wang, T., Luo, X., . . . Kong, Y. (2017). A debranching enzyme IsoM of Corallococcus sp. strain EGB with potential in starch

processing. International Journal of Biological Macromolecules, 105, 1300-1309. doi:https://doi.org/10.1016/j.ijbiomac.2017.07.153

Ling, H. L., Rahmat, Z., Abdul Murad, A. M., Mahadi, N. M., and Md Illias, R. (2017). Proteome-based identification of signal peptides for improved secretion of recombinant cyclomaltodextrin glucanotransferase in Escherichia coli. Process Biochemistry, 61, 47-55. doi:https://doi.org/10.1016/j.procbio.2017.06.018

Lombard, V., Ramulu, H. G., Drula, E., Coutinho, P. M., and Henrissat, B. (2014). The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res, 42, 490-495.

Londoño-Restrepo, S. M., Rincón-Londoño, N., Contreras-Padilla, M., Millan-Malo, B. M., and Rodriguez-Garcia, M. E. (2018). Morphological, structural, thermal, compositional, vibrational, and pasting characterization of white, yellow, and purple Arracacha Lego-like starches and flours (Arracacia xanthorrhiza). Int J Biol Macromol 113, 1188-1197.

Lumdubwong, N. (2019). Applications of Starch-Based Films in Food Packaging. Reference Module in Food Science. doi:https://doi.org/10.1016/B978-0-08-100596-5.22481-5

Lv, J.-S., Liu, X.-Y., Xu, J.-X., Deng, Y.-F., Wu, Z., Wang, Y.-M., . . . Xu, H. (2013). Preparation and properties of adsorption material from corn stalks core when used for enzyme immobilization and the subsequent activities of the adsorbed enzymes. Industrial Crops and Products, 50, 787-796. doi:http://dx.doi.org/10.1016/j.indcrop.2013.08.068

Mahmod, S. S., Yusof, F., Jami, M. S., and Khanahmadi, S. (2016). Optimizing the preparation conditions and characterization of a stable and recyclable cross-linked enzyme aggregate (CLEA)-protease. Bioresour. Bioprocess, 3(3). doi:10.1186/s40643-015-0081-5

Malhotra, I., and Basir, S. F. (2020). Immobilization of invertase in calcium alginate and calcium alginate-kappa-carrageenan beads and its application in bioethanol production. Preparative Biochemistry & Biotechnology, 1-10. doi:10.1080/10826068.2019.1709979

Marshall, J. (2012). Mechanisms of saccharide polymerization and depolymerization. Elsevier.

Martín, M. C., López, O. V., Ciolino, A. E., Morata, V. I., Villar, M. A., and Ninag, M. D. (2019). Immobilization of enological pectinase in calcium alginate hydrogels: A potential biocatalyst for winemaking. Biocatalysis and Agricultural Biotechnology, 18. doi:https://doi.org/10.1016/j.bcab.2019.101091

Miao, M., Jiang, B., Jin, Z., and BeMiller, J. (2018). Microbial Starch-Converting Enzymes: Recent Insights and Perspectives: Microbial starch-converting enzymes…. Comprehensive Reviews in Food Science and Food Safety, 17. doi:10.1111/1541-4337.12381

Míguez, N., Gimeno-Pérez, M., Fernández-Polo, D., Cervantes, F. V., Ballesteros, A. O., Fernández-Lobato, M., . . . Plou, F. J. (2018). Immobilization of the β-fructofuranosidase from Xanthophyllomyces dendrorhous by Entrapment in Polyvinyl Alcohol and Its Application to Neo-Fructooligosaccharides Production. Catalysts, 8(5). doi:10.3390/catal8050201

Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Analytical Chemistry, 31(3), 426-428. doi:10.1021/ac60147a030

Minteer, S. D. (2016). Cell-Free Biotechnologies. Biotechnology for Biofuel Production and Optimization, 433 - 448. doi:10.1016/b978-0-444-63475-7.00016-9

Mohamad, N. R., Marzuki, N. H. C., Buang, N. A., Huyop, F., and Wahab, R. A. (2015). An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. Biotechnology & Biotechnological Equipment, 29(2), 205-220. doi:10.1080/13102818.2015.1008192

Mohd Sukri, S. S., and Mimi, S. A. M. (2017). Effects of Support Matrix for Xylanase Immobilisation on Alginate Hydrogel Beads. International Journal of Engineering Technology and Sciences, 7(1). doi:http://dx.doi.org/10.15282/ijets.7.2017.1.4.1066

Møller, M. S., and Svensson, B. (2016). Structural biology of starch-degrading enzymes and their regulation. Current Opinion in Structural Biology, 40, 33-42. doi:http://dx.doi.org/10.1016/j.sbi.2016.07.006

Montgomery, D. C. (2010). Design and Analysis of Experiments (Seven ed ed.). New Delhi: Wiley India Pvt. Ltd.

Muller, S., Concha, D., Vasquez, P., Rodriguez-Nunez, K., Martinez, R., and Bernal, C. (2022). Effect of the immobilization of pectinase on the molecular weight distribution of pectin oligosaccharides obtained from citrus pectin. Biocatalysis and Agricultural Biotechnology, 43. doi:https://doi.org/10.1016/j.bcab.2022.102389

Narayanan, T. S. N. S., Park, I.-S., and Lee, M.-H. (2015). Surface modification of magnesium and its alloys for biomedical applications: Biological interactions, mechanical properties and testing. Woodhead Publishing: Elsevier.

Nawawi, N. N., Hashim, Z., Rahman, R. A., Murad, A. M. A., Bakar, F. D. A., and Illias, R. M. (2020). Entrapment of porous cross-linked enzyme aggregates of maltogenic amylase from Bacillus lehensis G1 into calcium alginate for maltooligosaccharides synthesis. International Journal of Biological Macromolecules, 150, 80-89. doi:https://doi.org/10.1016/j.ijbiomac.2020.02.032

Nawaz, M. A., Rehman, H. U., Bibi, Z., Aman, A., and Ul Qader, S. A. (2015). Continuous degradation of maltose by enzyme entrapment technology using calcium alginate beads as a matrix. Biochemistry and Biophysics Reports, 4, 250-256. doi:https://doi.org/10.1016/j.bbrep.2015.09.025

Nguyen, L. T., Lau, Y. S., and Yang, K. L. (2016). Entrapment of cross-linked cellulase colloids in alginate beads for hydrolysis of cellulose. Colloids and Surfaces B: Biointerfaces, 145, 862 - 869.

Niu, D., Qiao, J., Li, P., Tian, K., Liu, X., Singh, S., and Lu, F. (2017). Highly efficient enzymatic preparation of isomalto-oligosaccharides from starch using an enzyme cocktail. Electronic Journal of Biotechnology, 26, 46-51. doi:https://doi.org/10.1016/j.ejbt.2016.12.002

Osbon, Y., and Kumar, M. (2019). Biocatalysis and Strategies for Enzyme Improvement. In Biophysical Chemistry - Advance Applications: IntechOpen.

Osho, M., Popoola, T., Adeleye, T., and Adetunji, C. (2016). Response surface methodology for optimal immobilization of Aspergillus niger ATCC 1015 lipase by adsorption method. International Journal of Biological Research, 4, 56. doi:10.14419/ijbr.v4i1.6008

Parreidt, T. S., Müller, K., and Schmid, M. (2018). Alginate-Based Edible Films and Coatings for Food Packaging Applications. Foods, 7(170). doi:10.3390/foods7100170

Parthasarathy, M., and Sethuraman, S. (2014). Chapter 2 - Hierarchical Characterization of Biomedical Polymers. In S. G. Kumbar, C. T. Laurencin, & M. Deng (Eds.), Natural and Synthetic Biomedical Polymers (pp. 33-42). Oxford: Elsevier.

Pereira, A. d. S., Diniz, M. M., De Jong, G., Gama Filho, H. S., dos Anjos, M. J., Finotelli, P. V., . . . Amaral, P. F. F. (2019). Chitosan-alginate beads as encapsulating agents for Yarrowia lipolytica lipase: Morphological, physico-chemical and kinetic characteristics. International Journal of Biological Macromolecules, 139, 621-630. doi:https://doi.org/10.1016/j.ijbiomac.2019.08.009

Priyanka, P., Kinsella, G. K., Henehan, G. T., and Ryan, B. J. (2019). The Effect of Calcium Alginate Entrapment on the Stability of Novel Lipases from P. Reinekei and P. brenneri. Trends in Peptide and Protein Sciences, 4(2019). doi:http://dx.doi.org/10.22037/tpps.v4i0.26682

Raghu, H. S., and Achur, R. (2015). Immobilization of α- amylase (1, 4-α-D-glucanglucanohydralase) by calcium alginate encapsulation. International Food Research Journal, 22, 869-871.

Rahim, S. N. A., Sulaiman, A., Hamid, K. H. K., Edama, N. A., and Baharuddin, A. S. (2015). Effect of agitation speed for enzymatic hydrolysis of tapioca slurry using encapsulated enzymes in an enzyme bioreactor. International Journal of Chemical Engineering and Applications, 6(1).

Rahmani, N., Rohana, R., Sukarno, S., Andriani, A. D. E., and Yopi, Y. (2013). Production of Maltooligosaccharides from Black Potato (Coleus tuberosus) Starch by α-amylase from a Marine Bacterium (Brevibacterium sp.). Microbiology Indonesia, 7, 129-136. doi:10.5454/mi.4.3.6

Rajput, K. N., Patel, K. C., and Trivedi, U. B. (2016). b-cyclodextrin Production by Cyclodextrin Glucanotransferase from an Alkaliphile Microbacterium terrae KNR 9 Using Different Starch Substrates. Biotechnology Research International. doi:http://dx.doi.org/10.1155/2016/2034359

Rakmai, J., Cheirsilp, B., & Prasertsan, P. (2015). Enhanced thermal stability of cyclodextrin glycosyltransferase in alginate–gelatin mixed gel beads and the

application for β-cyclodextrin production. Biocatalysis and Agricultural Biotechnology, 4(4), 717-726. doi:10.1016/j.bcab.2015.10.002

Rehman, H. U., Aman, A., Silipo, A., Qader, S. A. U., Molinaro, A., and Ansari, A. (2013). Degradation of complex carbohydrate: Immobilization of pectinase from Bacillus licheniformis KIBGE-IB21 using calcium alginate as a support. Food Chemistry, 139, 1081-1086. doi:10.1016/j.foodchem.2013.01.069

Reis, C. L. B., Sousa, Emerson Yvay Almeida de, Serpa, Juliana de França, Oliveira, Ravena Casemiro, & Santos, José Cleiton Sousa dos. (2019). DESIGN OF IMMOBILIZED ENZYME BIOCATALYSTS: DRAWBACKS AND OPPORTUNITIES. Química Nova, 42(7), 768-783. doi:https://dx.doi.org/10.21577/0100-4042.20170381

Rios, N. S. A.-P., S.; Mendez-Sanchez, C.; Lokha, Y.; Cortes-Corberan, V.; Gonçalves, L.R.B.; Fernandez-Lafuente, R. (2019). Increasing the Enzyme Loading Capacity of Porous Supports by a Layer-by-Layer Immobilization Strategy Using PEI as Glue. Catalysts, 9(576). doi: https://doi.org/10.3390/catal9070576

Rodríguez Gastón, J. A., Costa, H., Rossi, A. L., Krymkiewicz, N., & Ferrarotti, S. A. . (2012). Maltooligosaccharides production catalysed by cyclodextrin glycosyltransferase from Bacillus circulans DF 9R in batch and continuous operation. Process Biochemistry, 47(12), 2562-2565. doi:doi:10.1016/j.procbio.2012.08.008

Rueda, N., dos Santos, J. C. S., Ortiz, C., Torres, R., Barbosa, O., Rodrigues, R. C., . . . Fernandez-Lafuente, R. (2016). Chemical Modification in the Design of Immobilized Enzyme Biocatalysts: Drawbacks and Opportunities. The Chemical Record, 16(3), 1436-1455. doi:https://doi.org/10.1002/tcr.201600007

Salari, D., Niaei, A., Aghazadeh, F., Hosseini, S. A., and Seyednajafi, F. (2012). J. Environ. Sci. Health A: Toxic/Hazard. Subst. Environ. Eng., 47(3), 423.

Sambrook, J., and Russell, D. W. (2001). Molecular Cloning : A Laboratory Manual. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory.

Samsudin, N., Arifin, M. A., and Nordin, N. (2017). Statistical Optimization of Gelatin Immobilisation on Modified Surface PCL Microcarrier to Improve PCL Microcarrier Compatibility. Jurnal Teknologi (Sciences & Engineering), 79(6), 167-175. doi:https://doi.org/10.11113/jt.v79.10417

Sathali, A. A. H., and Varun, J. (2012). Formulation, development and in vitro evaluation of candesartan cilexetil mucoadhesive microbeads. International Journal of Current Pharmaceutical Research, 4(3), 109-118.

Sethuramiah, A., and Rajesh, K. (2016). Chapter 6 - Statistics and Experimental Design in Perspective. In Modeling of Chemical Wear (pp. 129-159).

Shahid, F., Aman, A., and Qader, S. A. U. (2019). Immobilization of Dextranase Using Anionic Natural Polymer Alginate as a Matrix for the Degradation of a Long-Chain Biopolymer (Dextran). International Journal of Polymer Science, 2019, 1354872. doi:10.1155/2019/1354872

Sharma, M., Sharma, V., and Majumdar, D., K. . (2014). Entrapment of a-Amylase in Agar Beads for Biocatalysis of Macromolecular Substrate. International Scholarly Research Notices 2014. doi:https://doi.org/10.1155/2014/936129

Sheldon, R. A., and van Pelt, S. (2013). Enzyme immobilisation in biocatalysis: why, what and how. Chemical Society Reviews, 42(15), 6223-6235. doi:10.1039/C3CS60075K

Shen, Q., Yang, R., Hua, X., Ye, F., Zhang, W., Zhao, W. (2011). Gelatin-templated biomimetic calcification for b-galactosidase immobilization. Process Biochemistry, 46, 1565-1571.

Shofiyah, S. S., Yuliani, D., Widya, N., Sarian, F. D., Puspasari, F., Radjasa, O. K., . . . Natalia, D. (2020). Isolation, expression, and characterization of raw starch degrading α-amylase from a marine lake Bacillus megaterium NL3. Heliyon, 6(12), e05796. doi:https://doi.org/10.1016/j.heliyon.2020.e05796

Shruti Pathania, N. S. a. S. H. (2017). Optimization of culture conditions using response surface methodology for synergism production of cellulase, xylanase and pectinase by Rhizopus delemar F2 under solid state fermentation. Journal of Pharmacognosy and Phytochemistry, 6(6), 1872-1878.

Sirisha, V. L., Jain, A., and Jain, A. (2016). Chapter Nine - Enzyme Immobilization: An Overview on Methods, Support Material, and Applications of Immobilized Enzymes. In S.-K. Kim & F. Toldrá (Eds.), Advances in Food and Nutrition Research (Vol. 79, pp. 179-211): Academic Press.

Smeets, V., Baaziz, W., Ersen, O., Gaigneaux, E. M., Boissière, C., Sanchez, C., and Debeckera, D. P. (2019). Hollow zeolite microspheres as a nest for enzymes:

a new route to hybrid heterogeneous catalysts for chemo-enzymatic cascade reactions. Chemical Science, 11(4). doi:10.26434/chemrxiv.7796648.v2

Sojitra, U. V., Nadar, S. S., and Rathod, V. K. (2017). Immobilization of pectinase onto chitosan magnetic nanoparticles by macromolecular cross-linker. Carbohydrate Polymers, 157, 677-685. doi:https://doi.org/10.1016/j.carbpol.2016.10.018

Straksys, A., Kochane, T., and Budriene, S. (2016). Catalytic properties of maltogenic α-amylase from Bacillus stearothermophilus immobilized onto poly(urethane urea) microparticles. Food Chemistry, 211, 294-299. doi:https://doi.org/10.1016/j.foodchem.2016.05.071

Suhaimi, S., Che Man, R., Jamil, N., Md Shaarani, S., Abdul Mudalip, S. K., Sulaiman, S. Z., and Ramli, A. N. M. (2018). Stability and reusability of cyclodextrin glucanotransferase immobilized on hollow fiber membrane. IOP Conference Series: Earth and Environmental Science, 185. doi:10.1088/1755-1315/185/1/012002

Sukri, S. S. M., and Mimi Sakinah, A. M. (2018). Production of high commercial value xylooligosaccharides from meranti wood sawdust using immobilised xylanase. Appl. Biochem. Biotechnol, 184, 278 - 290.

Sulaiman, S., Cieh, N. L., Mokhtar, M. N., Naim, M. N., and Kamal, S. M. M. (2017). Covalent immobilization of cyclodextrin glucanotransferase on kenaf cellulose nanofiber and its application in ultrafiltration membrane system. Process Biochemistry, 55, 85-95. doi:https://doi.org/10.1016/j.procbio.2017.01.025

Talekar, S., and Chavare, S. (2012). Optimization of immobilization of a-amylase in alginate gel and its comparative biochemical studies with free a-amylase. Recent Research in Science and Technology, 4(2), 1-5.

Tavares, A. P. M., Silva, C. G., Drazˇic´, G., Silva, A. M. T., Loureiro, J. M., and L., F. J. (2015). Laccase immobilization over multi-walled carbon nanotubes: Kinetic, thermodynamic and stability studies. Journal of Colloid and Interface Science, 454, 52-60. doi:http://dx.doi.org/10.1016/j.jcis.2015.04.054

TheStar Online. (2009). Dietary fibre science. TheStar Online.

Thirugnanasambandham, K., Sivakumar, V., and Maran, J. P. (2014). Optimization of electrocoagulation process to treat biologically pretreated bagasse effluent.

Journal of the Serbian Chemical Society, 79(5), 613-626. doi:10.2298/JSC130408074T

Thomas, M., Priest, F. G., and Starck, J. R. (1980). Characterization of an extracellular a-amylase from Bacillus megaterium sensu stricto. J Gen Microbiol, 118(1), 67-72. doi:https://doi.org/10.1099/00221287-118-1-67

Torabizadeh, H. (2020). Nano co-immobilization of α-amylase and maltogenic amylase by nanomagnetic combi-cross-linked enzyme aggregates method for maltose production from corn starch. Carbohydrate Research, 488, 107904. doi:https://doi.org/10.1016/j.carres.2019.107904

Vetrano, A., Gabriele, F., Germani, R., and Spreti, N. (2022). Characterization of lipase from Candida rugosa entrapped in alginate beads to enhance its thermal stability and recyclability. New Journal of Chemistry, 46(21), 10037 - 10047. doi:https://doi.org/10.1039/D2NJ01160C

Viswanathan, S., and Nallamuthu, T. (2014). Extraction of sodium alginate from selected seaweeds and their physiochemical and biochemical properties. International Journal of Innovative Research Science, Engineering and Technology, 3. doi:10998-11003

Wan Azelee, N. I. (2015). Optimisation of Kenaf Pretreatment and Enzymatic Reaction Conditions of Kenaf Hemicellulose for xylooligosaccharides Production. (PhD Thesis). Universiti Teknologi Malaysia, Skudai, Johor.

Wang, L., Wu, D., Chen, J., and Wu, J. (2013). Enhanced production of γ-cyclodextrin by optimization of reaction of γ-cyclodextrin glycosyltransferase as well as synchronous use of isoamylase. Food Chemistry, 141(3), 3072. doi:10.1016/j.foodchem.2013.05.149

Xiang, G., Buwalda, P. L., van der Maarel, M. J. E. C., and Leemhuis, H. (2021). The thermostable 4,6-α-glucanotransferase of Bacillus coagulans DSM 1 synthesizes isomalto-oligosaccharides. Amylase, 5, 13-22. doi:https://doi.org/10.1515/amylase-2021-0002

Xiao, A., Xiao, Q., Lin, Y., Ni, H., Zhu, Y., and Cai, H. (2017). Efficient immobilization of agarase using carboxyl-functionalized magnetic nanoparticles as support. Electronic Journal of Biotechnology, 25, 13-20. doi:https://doi.org/10.1016/j.ejbt.2016.10.007

Yagar, H., and Balkan, U. (2017). Entrapment of laurel lipase in chitosan hydrogel beads. Artificial Cells, Nanomedicine, and Biotechnology, 45(5), 864-870. doi:10.1080/21691401.2016.1182920

Zhang, Mao, H., Li, M., and Su, E. (2019). Cyclodextrin glucosyltransferase immobilization on polydopamine-coated Fe3O4 nanoparticles in the presence of polyethyleneimine for efficient βcyclodextrin production. Biochemical Engineering, 150. doi:https://doi.org/10.1016/j.bej.2019.107264

Zhang, Yuwen, L. X., and Peng, L. J. (2013). Parameters Affecting the Performance of Immobilized Enzyme Journal of Chemistry. doi:doi:10.1155/2013/946248

Zhang, Zhang, R., Chen, L., and McClements, D. J. (2016). Encapsulation of lactase (β-galactosidase) into κ-carrageenan-based hydrogel beads: Impact of environmental conditions on enzyme activity. Food Chemistry, 200, 69-75. doi:10.1016/j.foodchem.2016.01.014

Zhao, X., Qi, F., Yuan, C., Du, W., and Liu, D. (2015). Lipase-catalyzed process for biodiesel production: Enzyme immobilization, process simulation and optimization. Renew. Sustain. Energy Rev., 44, 182-197. doi:https://doi.org/10.1016/j.rser.2014.12.021

Zhou, Li, Z., Zhang, H., Wu, J., Ye, X., Dong, W., . . . Schottel, J. L. (2018). Novel Maltogenic Amylase CoMA from Corallococcus sp. Strain EGB Catalyzes the Conversion of Maltooligosaccharides and Soluble Starch to Maltose. Applied and Environmental Microbiology, 84(14), e00152-00118. doi:doi:10.1128/AEM.00152-18

Zhou, Pan, S., Wei, X., Wang, L., and Liu, Y. (2013). Immobilization of β-glucosidase onto magnetic nanoparticles and evaluation of the enzymatic properties. BioResources, 8(2), 2605-2619.

Zhu, X., Tian, Y., Xu, W., Guang, C., Zhang, W., Zhang, T., and Mu, W. (2018). Bioconversion of sucrose to maltooligosaccharides by the synergistic action of amylosucrase and α-amylase. Process Biochemistry, 74, 74-76. doi:https://doi.org/10.1016/j.procbio.2018.08.026

Zusfahair, Ningsih, D. R., Kartika, D., Kurniasih, M., Nofiani, R., and Fatoni, A. (2020). Improved reuse and affinity of enzyme using immobilized amylase on alginate matrix. Journal of Physics: Conference Series, 1494(2020). doi:10.1088/1742-6596/1494/1/012028

LIST OF PUBLICATIONS

1. Kumar, N. K. M. F., Chew, C. M., Manas, N. H. A., Ahmad, R. A., Fuzi, S. F. Z. M., Rahman, R. A., Illias, R. M. (2022). 'Immobilization of Maltogenic amylase in Alginate-chitosan for Improved Enzyme Retention and Stability', *Malaysian Journal of Fundamental and Applied Sciences*, 18, pp. 25-33.

2. Kumar, N. K. M. F. K., Manas, N. H. A., Mahmud, H., Dailin, D. J. (2022). Enzyme Entrapment Techniques and Strategies. Penerbit UTM Press. Book Chapter. (Submitted)