CELL IMMOBILIZATION OF RECOMBINANT *KLUYVEROMYCES LACTIS* ON CARBON NANOMATERIAL FOR THE IMPROVEMENT OF XYLANASE PRODUCTION

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DEDICATION

Dedicated to: My late father (Abdul Manaf Hamid), my mother (Che Hasnah Che Indok), my brothers (Khairulizam and Kamaruizani), my lecturers and friends for their endless support and encouragement through the years. Them, who understand me the most and guided me all the way and for all who never tired to hold my back.

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ABSTRACT

Xylanase is a major hydrolysis enzyme that is important for xylan degradation in applications such as paper pulping, food additive production and animal feedstocks. It is typically found in fungi with low productivity and complex processes. As a result, an alternative method for increasing xylanase production that is simple and less time-consuming is desired. The goal of this research is to produce a large-scale xylanase by immobilizing recombinant *Kluyveromyces lactis* with carbon nanomaterial and to apply a direct whole cell biocatalyst method for xylooligosaccharides production. Therefore, four carbon nanomaterials were screened using the pretreatment process that measured xylanase activity and cell growth. Carbon nanotubes (CNT) and graphene oxide (GO) were analyzed and their immobilization and culture condition factors were optimized using Response Surface Methodology (RSM) with different design models, as well as large-scale production process using a bioreactor. Analysis on the carbon nanomaterial was done using a Field Emission Scanning Electron Microscopy with Energy Dispersive X-ray (FESEM-EDX) and Fourier Transform Infrared spectroscopy (FTIR) while Ultra High-Performance Liquid Chromatography (UHPLC) was used to analyze the final sugar product. The most important factors in xylanase production with low cell leakage are cell loading and agar concentration. Following RSM screening and optimization, the xylanase production from free cells (1.39 U/mL) increased tenfold after cell immobilization (10.30 U/mL), and increased to 15 U/mL during the upscale process in the bioreactor. The immobilized cells can be reused for up to 7 fermentation cycles and stored at 4 ℃ for up to 90 days. The end products of lignocellulosic biomass bioconversion are xylobiose and xylotriose. Cell immobilization with carbon nanomaterials has been shown to successfully enhance xylanase production, opening up a new path to improved bioprocessing, particularly for the production of enzymes with reusability and long-term storage.

ABSTRAK

Xilanase adalah enzim hidrolis utama yang penting untuk pendegradan xilan dalam aplikasi seperti mempulpa kertas, bahan tambahan makanan dan makanan ternakan. Xilanase biasanya dijumpai pada spesies kulat dengan penghasilan xilanase yang rendah dan proses yang rumit. Justeru, cara alternatif untuk meningkatkan penghasilan xilanase dengan proses yang senang dan kurang memakan masa diperlukan. Tujuan kajian ini untuk menghasilkan xilanase dalam skala besar dengan pengimobilisasian *Kluyveromyces lactis* dengan bahan nanokarbon dan untuk menggunakan kaedah langsung biokatalis seluruh sel untuk penghasilan xilooligosakarida. Oleh itu, empat jenis bahan nanokarbon disaring menggunakan proses prarawatan yang diukur oleh aktiviti xilanase dan pertumbuhan sel. Nanotiub karbon (NTK) dan grafin oksida (GO) dianalisis dan factor imobilisasi serta keadaan kultur dioptima menggunakan kaedah permukaan respon (RSM) dengan bentuk model yang berbeza, serta proses penghasilan xilanase yang banyak menggunakan bioreaktor. Analisis bahan nanokarbon dijalankan melalui Mikroskop Pengimbasan Elektron Pancaran Medan dengan Serakan Tenaga Sinaran-X (FESEM-EDX) dan spektroskopi Fourier Transform Infra Merah (FTIR) sementara Kromatografi Cecair Berprestasi tinggi ultra (UHPLC) digunakan untuk menganalisa produk gula akhir. Faktor yang paling penting di dalam penghasilan xilanase dengan kebocoran sel yang rendah adalah pembekalan sel dan kepekatan agar-agar. Melalui saringan dan pengoptimunan melalui KSR, peningkatan xilanase daripada sel bebas (1.39 U/mL) adalah sepuluh kali ganda selepas imobilisasi sel (10.30 U/mL) dan meningkat kepada 15 U/mL di dalam bioreaktor. Sel yang diimobilisasi boleh diguna semula untuk 7 kitaran fermentasi dan boleh disimpan di 4 °C sehingga 90 hari. Xilobiosa dan xilotriosa adalah produk akhir biokonversi biojisim lignoselulosa. Imobilisasi sel dengan bahan nanokarbon berjaya meningkatkan penghasilan xilanase, menyediakan laluan baru untuk menambah baik biopemprosesan, khususnya penghasilan enzim dengan kebolehgunaan semula and penyimpanan jangka panjang.

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LIST OF SYMBOLS AND ABBREBRIATIONS

α-MF	-	α-mating factor
μ_{max}	-	Maximum specific growth rate
%	-	Percentage
°C	-	Degree Celcius
ADH	-	Alcohol dehydrogenase
ANOVA	-	Analysis of variance
ATR-FTIR	-	Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy
BBD	-	Box-Behnken design
BSA	-	Bovine serum albumin
$CaCO_3$	-	Calcium carbonate
CCD	-	Central Composite design
cDNA	-	Complementary DNA
CGTase	-	Cyclodextrin glucanotransferase
CL	-	Coconut leaves
CNP	-	Carbon nanoparticles
CNS	-	Carbon nanospheres
CNT	-	Carbon nanotubes
dF	-	Degree of freedom
DM	-	Defined medium
DNA	-	Deoxyribonucleic acid
DNS	-	3,5-dinitrosalicyclic acid
DO	-	Dissolved oxygen
EDX	-	Energy dispersive X-ray
FC	-	Free cells

ELSD	-	Evaporative light scattering detector
FESEM	-	Field emission scanning electron microcopy
GA	-	Glutaraldehyde
GO	-	Graphene oxide
GRAS	-	Generally recognized as safe
HCl	-	Hydrochloric acid
HM	-	Medium obtained from Hun et al. (2013)
IM	-	Medium obtained from Irfan et al. (2016)
kDa	-	Kilodaltons
M	-	Molar
mg	-	Miligram
mL	-	Mililiter
MWCNT	-	Multi walled carbon nanotubes
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
OD	-	Optical density
OFAT	-	One-factor-at-a-time
O-H	-	Hydroxyl group
OPEFB	-	Oil palm empty fruit bunches
OPL	-	Oil palm leaves
PTM	-	Post translational modifications
PVA	-	Polyvinyl alcohol
rpm	-	Revolutions per minute
R^2	-	R-squared
ROS	-	Reactive oxygen species
RSM	-	Response surface methodology
SB	-	Sago bark
SDS	-	Sodium dodecyl sulphate
SDS-PAGE	-	SDS-Polyacrylamide gel electrophoresis
SEM	-	Scanning electron microscopy

SH	-	Sago hampas
U	-	Unit (enzyme activity)
UHPLC	-	Ultra high-performance liquid chromatography
UV	-	Ultraviolet
v/v	-	Volume per volume
w/v	-	Weight per volume
XOS	-	Xylooligosaccharide
XYL	-	Xylan from birchwood
Xyn2	-	Endo 1,4- β xylanase
YM	-	Medium obtained from Yatmaz *et al*. (2016)
YPD	-	Yeast extract peptone dextrose
μ	-	Specific growth rate

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Xylanase is an enzyme that cleaves the *1,4*-glycosidic linkages in the xylan backbone, resulting in the formation of xylooligosaccharides (XOS). The use of xylanase as an important enzyme can be observed in industrial processes, such as bioethanol production, animal feed, food additives, baking industry, xylitol synthesis, and paper and pulp production (Kalim *et al*., 2015). Furthermore, xylanase also acts synergistically with other enzymes to produce commercial sugar through hydrolysis process (Gonçalves *et al*., 2015, Chakdar *et al*., 2016). Recently, the safety of materials used in industrial applications is now given more consideration. For example, traditional chemical food additives such as potassium bromate and azodicarbonamide were used in the baking industry to increase loaf volume, lengthen shelf life, and improve bread taste. Nevertheless, it is now known that these chemical food additives are carcinogenic to humans. It was discovered that xylanase contributes to the search for safe food additives (Zhan *et al*., 2014). Hence, the vast industrial applications have led to a significant investment in research aimed at enhancing xylanase productivity for improved performance of production.

Attempts are made for high productivity of enzymes to meet specific industrial needs and economic viability. Most of the reported xylanases show low yield and incompatibility of the standard fermentation processes that do not meet the demand of industries, which makes the process non-economical. Therefore, recombinant DNA techniques must be employed as excellent tool for the construction of genetically modified strains of microbes with selected characteristics for enzyme production. In this case, isolation and cloning of xylanase gene designate an important step in the engineering of the most efficient microorganisms. Till date, xylanase gene isolated from various microorganisms have been cloned and expressed into suitable hosts with various objectives. To attempt these processes for commercial purposes, cloning of xylanases gene have been reported in both heterologous and homologous protein expression hosts. Heterologous expression is the main tool for the xylanase production at industrial level. Protein engineering by recombinant DNA technology could be beneficial in refining the specific characteristics of present xylanases. Recombinant xylanases have shown better properties than the native enzymes, which can be employed in the fermentation industry (Walia *et al*., 2017).

Historically, a type of yeast known as *Saccharomyces cerevisiae* has predominantly been utilized as a host to produce recombinant proteins. Nevertheless, this yeast is not always the optimal host for the large-scale production of foreign proteins as technical fermentation requires highly sophisticated equipment. Consequently, the development of expression systems using so-called "non-conventional" yeasts, such as *Pichia pastoris, Yarrowia lipoytica*, and *Kluyveromyces lactis* are introduced into the system of heterologous protein production (Gomes *et al.*, 2018). Particularly, *Kluyveromyces lactis* is gaining attention as a credible alternative host for heterologous protein secretion, especially xylanase in a large-scale production (Fuzi *et al*., 2014). In this study, the recombinant *K. lactis* producing xylanase was used. Xylanase gene was originated from *Trichoderma* species of fungus and their limitations of extensive purification of pure enzyme and its low yield led to gene cloning in *K. lactis* (Chakdar *et al*., 2016).

K. lactis is another respiratory Crabtree-negative yeast and also known for producing *β*-galactosidase on an industrial scale. *K. lactis* is primarily used in the food industry for lactose-free products. Bovine chymosin was the first recombinant protein produced using *K. lactis* as a host. To date, recombinant proteins with applications in the food and pharmaceutical industries have been produced more frequently. Unlike some methylotrophic yeasts, *K. lactis* requires methanol-free media for growth, which does not necessitate the need for explosion-proof fermentation equipment and high-cost carbon sources (Fuzi *et al*., 2012). Nonetheless, the traditional technique of producing an enzyme has several drawbacks, which include low productivity, product separation issues, and the inability to be recycled (Ivanova *et al*., 2011; Szymańska *et al*., 2011).

Although the traditional technique of producing an enzyme has several drawbacks, they can be overcome using cell immobilization as a solution to these challenges. Under this process, microbial cells are confined or localised in a defined region where they can be used repeatedly and increase productivity (Willaert, 2011). The immobilization of whole cells for extracellular enzyme production offers various advantages such as improvement in the production of target product, cell separation from bulk liquid for reuse, continuous operation for an extended period, and increased reactor productivity (Sankaralingam *et al*., 2016). Additionally, immobilized cells are noticed to have better operational stability, higher resilience to environmental perturbations such as pH, and adequate cell protection from shear damage (Dogan *et al.*, 2016). Furthermore, both cell immobilization techniques and support types, such as polymers, lignocellulosic material, silica, and hydrogel were found to be well-established. Nonetheless, the use of nanomaterials has not been extensively studied as cell supports in the cell immobilization process. The use of nanomaterials offers promising potential as they are found to be an excellent candidate as matrix/support in cell immobilization due to their unique properties (Manaf *et al*., 2020).

The utilization of nanomaterials, particularly carbon nanomaterials, has emerged in multiple fields, which includes the development of nanocarriers for drugs, proteins, DNA, cell imaging, and also for adsorption and degradation of environmental pollutants (Manaf *et al.*, 2020). In addition to their excellent electronic, optical, thermal, and mechanical properties, carbon nanomaterials are gaining interest because of their high catalytic properties that can improve the production system (Zaytseva & Neumann, 2016). An interesting part of carbon nanomaterials is their high surface area, which can contribute to the higher catalytic activity and reactivity towards biological interactions (Navya & Daima, 2016). Hence, the unique properties of carbon nanomaterials can be utilized as a matrix/support for cell immobilization studies.

Generally, calcium alginate beads have been the focus of multiple studies reporting on the production of xylanase through cell immobilization (Amani *et al*., 2007; Kundu & Majumdar, 2018). In terms of using cell immobilization to improve xylanase productivity, carbon nanomaterials were used as support in this study. The study that was most similar to this research was conducted using graphene oxide as a support for xylanase production from recombinant *E. coli* (Nor Ashikin *et al*., 2017). From the reported work, the improvement achieved by increased xylanase production allows it to be potentially used in this study. Thus, carbon nanomaterials including CNT and GO were chosen as the matrix/support for cell immobilization through recombinant *K. lactis*. To the best of our knowledge, there has been no study on xylanase production through recombinant *K. lactis* using a dual cell immobilization approach. The dual cell immobilization approach usually involves the adsorption of carbon nanomaterials and entrapment in a polymeric gel network.

In immobilized cells, they are applied in the bioconversion of lignocellulosic materials into fermentable sugar. Additionally, the conversion of biomass into fermentable sugar applies to xylanase as a hydrolytic enzyme. Generally, there is an abundance of lignocellulosic waste as biomass in the agriculture sector, which requires proper waste management. Hence, this study applies the waste-to-wealth approach by using a single direct process where the biomass is used as a substrate for xylanase to produce fermentable sugars, such as xylose and XOS. Also, fermentable sugars have a wide range of applications such as for food additives, sweetener, probiotics and for bioethanol production (Abu Bakar *et al*., 2012). Therefore, this study focused on the development of the cell immobilization process through the implementation of carbon-based nanomaterials for improved xylanase production, which includes a large-scale process and its bioconversion to sugar.

1.2 Problem statement

Xylanase is one of the hydrolytic enzymes that are normally found in fungi species. Yet, they always involved complicated large-scale production, which is also an expensive process. Hence, recombinant DNA technology can be applied for better production of proteins (Juturu & Wu, 2012; Zhan *et al*., 2014). In the production of proteins, the yeast system expression has the advantage of a platform for heterologous protein secretion. Yeast is an attractive host for heterologous protein expression with the benefits, such as high cell density growth, and extracellular proteins secretion with a Generally Recognized as Safe (GRAS) status. Nonetheless, there are limitations to the use of common yeasts in the production of proteins. To this extent, *K. lactis* proves itself as a promising host for gene cloning as it can grow on a variety of inexpensive carbon sources while efficiently secreting extracellular proteins, and also has a GRAS status (Fuzi *et al*., 2012; Zhan *et al*., 2014). Based on the advantages of *K. lactis*, the recombinant *K. lactis* was chosen in this study to produce xylanase.

In the production process of enzymes, the traditional approach of the free cell system has a few limitations of low productivity and stability, and a complex product separation process (Beshay *et al*., 2011, Zhuang *et al*., 2017). Additionally, the free cell system cannot be reused, which is normally demanded for certain applications that need a cost-effective process (Szymańska *et al*., 2011). To improve xylanase production and overcome the limitations of the traditional free cell system, the cell immobilization approach can be implemented. The cell immobilization approach comes as an alternative as it may increase cell stability, improves the downstream process, reduce contamination risks, and protect cells from environmental stress.

In this study, carbon-based nanomaterials have been chosen as the cell immobilization support due to the unique properties of high surface area, improved catalytic activity, and high electrical conductivity, which leads to enhanced immobilization efficiency (Abdul Manaf *et al.*, 2021). Nonetheless, carbon-based nanomaterials were found to be only used in a very small number of studies to support the immobilization of cells, particularly in the production of enzymes. Although carbon-based nanomaterials are beneficial for multiple applications such as drug delivery and cell imaging, there is still a need for an in-depth study on the potential of carbon-based nanomaterials as support in cell immobilization. A thorough investigation is required to screen and optimize the factors influencing the cell immobilization process. Additionally, cell immobilization studies using carbon-based nanomaterials for the fermentation aspect should also be investigated.

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APPENDIX F

PUBLICATIONS

Journals

1. Ashikin, N. A. L. N., Fuzi, S. F. Z. M., Abdul Manaf, S. A. ., Manas, N. H. A., Shaarani, S. M., Nawi, M., & Illias, R. M. (2022). Optimization and characterization of immobilized E. coli for engineered thermostable xylanase excretion and cell viability. *Arabian Journal of Chemistry*, *15*(6), 103803.
2. Abdul Manaf, S. A., Mohamad Fuzi, S. F. Z., Low, K. O., Hegde, G., Abdul Manas, N. H., Md Illias, R., & Chia, K. S. (2021). Carbon nanomaterial properties help to enhance xylanase production from recombinant Kluyveromyces lactis through a cell immobilization method. *Applied Microbiology and Biotechnology*, *105*(21), 8531-8544.
3. Abdul Manaf, S. A., Mohamad Fuzi, S. F. Z., Abdul Manas, N. H., Md Illias, R., Low, K. O., Hegde, G., ... & Matias-Peralta, H. M. (2021). Emergence of nanomaterials as potential immobilization supports for whole cell biocatalysts and cell toxicity effects. *Biotechnology and applied biochemistry*, *68*(6), 1128-1138.

VITA

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