EXPRESSION AND CHARACTERIZATION OF LIPASE FROM OIL PALM (*ELAEIS GUINEENSIS*)

FATIN FATHIAH BTE SAFIUDIN

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FACULTY OF SCIENCE AND MATHEMATICS
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ABSTRACT

FLL1 gene encodes an endogenous oil palm lipase that influence the innate quality of palm oil. While the genetic information is well-documented, FLL1 characteristics at the protein level remained unexplored. Therefore, this study aims to express and characterize recombinant protein FLL1 to understand its physical and chemical properties. FLL1 was efficiently expressed in Rosetta™ (DE3) pLysS competent cells using pFLL1-pGEX6P2 at 37 °C with 0.4 mM IPTG at A_{660} 0.9. A purification method for producing high-purity FLL1 protein was established through affinity and ion exchange chromatography, suitable for downstream characterization and application. FLL1 enzyme activity peaked at 40 °C, and demonstrated its thermal stability by retaining 50 % of its activity after four hours of incubation at a similar temperature. FLL1 functioned best at pH 7 (PBS buffer) and remained highly stable at pH 8 (PBS and Tris-HCl buffers). Low $LogP_{o/w}$ organic solvents, especially isopropanol, significantly enhanced FLL1 activity, expanding its potential for organic solvent-rich applications. Cofactor analysis showed that Mn^{2+} and Ca^{2+} are good enhancers for FLL1 activity, while Ni^{2+} and Cu^{2+} showed a promising inhibitory effect, thus demonstrating the ability of certain metal ions to regulate FLL1 activity. FLL1 exhibited strong hydrolysis activity towards all substrates tested, especially palm oil, short and long fatty acid chains, highlighting its versatility for lipid processing purposes. In conclusion, FLL1 protein research findings benefit palm oil quality improvement efforts by advancing the research on oil palm lipase. The broad substrate range, metal ion dependency, and stability in organic solvents make this enzyme a promising candidate as a biocatalyst in various industries. Therefore, this study has provided a solid foundation for future FLL1 protein engineering, by contributing valuable insights into the biochemical properties of the enzyme.

PENGKESPRESAN DAN PENCIRIAN LIPASE DARIPADA KELAPA SAWIT (*ELAEIS GUINEENSIS*)

ABSTRAK

Gen *FLL1* mengekod lipase endogen sawit yang mempengaruhi kualiti intrinsik minyak sawit. Walaupun maklumat genetiknya telah didokumentasikan dengan baik, ciri-ciri FLL1 pada peringkat protein masih belum diterokai sepenuhnya. Oleh itu, kajian ini bertujuan untuk mengekspres dan mencirikan protein rekombinan FLL1 bagi memahami sifat fizikal dan kimianya. *FLL1* berjaya diekspres secara efisien dalam sel kompeten *Rosetta™(DE3) pLysS* menggunakan pFLL1-pGEX6P2 pada 37 °C dengan 0.4 mM IPTG pada A_{660} 0.9. Kaedah pemurnian protein FLL1 berketulenan tinggi telah dibangunkan melalui kromatografi keafinan dan pertukaran ion, menjadikannya sesuai untuk pencirian lanjutan dan aplikasi dalam industri. Aktiviti enzim FLL1 paling tinggi pada suhu 40 °C, serta menunjukkan kestabilan suhu yang baik dengan mengekalkan 50 % aktivitinya selepas empat jam dieram pada suhu tersebut. FLL1 menunjukkan paling optimum pada pH 7 dalam larutan penimbal PBS dan kekal sangat stabil pada pH 8 dalam larutan penimbal PBS serta Tris-HCl, menonjolkan keupayaannya dalam pelbagai julat pH. Selain itu, pelarut organik dengan $LogP_{o/w}$ rendah, khususnya isopropanol, meningkatkan aktiviti FLL1 seterusnya memperluaskan potensinya dalam aplikasi persekitaran kaya pelarut organik. Analisis kofaktor menunjukkan bahawa Mn^{2+} dan Ca^{2+} bertindak sebagai penggalak aktiviti FLL1, manakala Ni^{2+} dan Cu^{2+} bertindak sebagai perencat aktiviti yang berkesan lantas menunjukkan kemampuan ion logam tertentu untuk mengawal aktiviti FLL1. Kajian ke atas spesifisiti substrat pula menunjukkan aktiviti hidrolisis yang tinggi terhadap semua substrat yang diuji, terutamanya minyak sawit, serta rantai asid lemak pendek dan panjang lalu menyerlahkan kepelbagaian aplikasi enzim ini dalam pemprosesan lipid. Secara keseluruhannya, hasil kajian protein FLL1 ini dapat menyokong usaha meningkatkan kualiti minyak sawit dengan memajukan penyelidikan mengenai lipase kelapa sawit. Julat substrat yang luas, kemampuan dimanipulasi oleh ion logam, dan kestabilan dalam pelarut organik menjadikan enzim ini calon biokatalis yang berpotensi tinggi dalam pelbagai industri. Justeru itu, kajian ini telah memberikan asas yang kukuh untuk kejuruteraan protein FLL1 pada masa hadapan, dengan menyumbang kepada pemahaman mendalam mengenai sifat biokimia enzim ini.

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LIST OF SYMBOLS AND ABBREVIATIONS

µg	Microgram
µL	Microlitre
°C	Degree Celsius
A	Alanine
AU	Absorbance unit
ANOVA	Analysis of variance
A_{600}	Absorbance at 600 nm
B	Aspartic acid or Asparagine
BSA	Bovine serum albumin
bp	Base pair
C	Cysteine
cDNA	Complimentary deoxyribonucleic acid
C -terminal	Carboxyl terminal
D	Aspartic acid
dh_2O	Distilled water
DMSO	Dimethyl sulfoxside
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
E	Glutamic acid
E. coli	*Escherichia coli*

EDTA	Ethylenediaminetetraacetic acid
F	Phenylalanine
G	Glycine
g	Gram
g/mol	Gram per mole
GSH	Reduced glutathione
GST	Glutathione transferases
H	Histidine
I	Isoleucine
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
J	Leucine or isoleucine
K	Lysine
Kb	Kilobase
L	Leucine
LogP	Logarithm of the partition coefficient
M	Methionine
M	Molar
mAU	Milli absorbance unit
mg/mL	Milligram per millilitre
min	Minutes
mL	Millilitre
mM	Millimolar
MW	Molecular weight
N	Asparagine
NaCl	Sodium chloride

ng	Nanogram
nm	Nanometre
N -terminal	Amino terminus
HCl	Hydrochloric acid
kb	Kilobase
kDa	KiloDalton
O	Pyrrolysine
OD	Optical density
P	Proline
PES	Polyethersulfone
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
*p*NPP	*p*-Nitrophenyl palmitate
Q	Glutamine
R	Arginine
RNA	Ribonucleic acid
rpm	Rotation per minute
S	Serine
s	Second
T	Threonine
TAE	Tris -acetate -EDTA
TEMED	N, N, N′, N′ -Tetramethylethylenediamine
U	Selenocysteine
U	Unit

UV	Ultraviolet
U/mL	Unit per millilitre
w/v	Weight per volume
V	Volt
V	Valine
v/v	Volume per volume
W	Tryptophan
w/w	Weight per weight
X	Any amino acid
Y	Tyrosine
Z	Glutamic acid or Glutamine

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CHAPTER 1

INTRODUCTION

1.1 Research background

Oil palm (*Elaeis guineensis*) is a very common plant in Malaysia. However, the plant is not native but introduced into the country by former British colony as a cash crop (Norhidayu et al., 2017; Murphy *et al.*, 2021). Malaysia is perfect for oil palm to thrive in due to its tropical climate and regular rainfall throughout the year (Murphy *et al*., 2021). Therefore, Malaysia continues to propagate oil palm systemically as an industrial monoculture (Murphy *et al*., 2021; Ferranti & Velotto, 2023). From the 20th century onwards, larger scale plantations were seen in Malaysia as the result of multiple government initiatives due to the promising returns exhibited by the crop (Murphy *et al*., 2021).

In 2023, Malaysia harvested 18.55 million tonnes of crude palm oil which yield a satisfying revenue of RM 94.95 billion (Parveez, *et al*., 2024). Therefore, in 2023, Malaysia has been internationally recognised with the exceptional reputation as the

main exporter of palm oil, by contributing 34.30 % of the global total trade value (Parveez *et al*., 2024). With an estimation of over a million employees in this industry, oil palm is proven to be an extremely significant economic crop for Malaysia and its citizen (Parveez *et al*., 2024).

The most valuable part of an oil palm is the fruit as it can produce oil, namely palm oil all year round. An oil palm fruit bunch weighs roughly 25 kg and contains nearly 3000 individual fruitlets (Raj *et al*., 2021). Instead of sourcing the oil only from the nut to yield crude kernel palm oil, palm oil can also be extracted from the mesocarp to produces crude palm oil that amounted to approximately 90 % of the total crude oil (Maheshwari & Kovalchuk, 2016) Therefore, oil palm is able to generate the highest crude oil yield with minimal land needed in comparison to other oil producing crop like soybean and rapeseed (Murphy *et al*., 2021).

Palm oil quality is threatened by the endogenous lipase present in the fruit (Morcillo *et al*., 2013). Lipase is a functional protein well reported in many hierarchal of life which highlights its importance (Calder, 2015). In microbes, animals and plants, lipase is responsible for lipid metabolism which results in the production of energy for the organisms (Calder, 2016). Unfortunately, the hydrolytic reaction will produce free fatty acid that could acidify the natural oil which affect the overall quality for downstream use (Calder, 2016). Therefore, lipase is abhorred by oil crop breeders to ensure a good oil yield. Along the years, many studies were conducted *via* conventional breeding and various molecular techniques to minimize the detrimental effect of lipase

towards the oil by developing an elite low lipase line (Murphy *et al*., 2021; Weckx *et al.*, 2019).

It was suggested that to properly mitigate the negative effect of endogenous lipase to the oil quality, a proper inspection *via* enzyme characterization must be done (Robinson, 2015). The most recent publication for characterised purified oil palm lipase was by Mohd Din *et al.* (2021) through the characterization of recombinant LIP2 from oil palm mesocarp. Previously, a study on *EgGDSL* lipase gene was conducted via ectopic expression in *Arabidopsis thaliana* ecotype Col-0 which associated the role of *EgGDSL* in altering palm oil quality through fatty acid liberation (Zhang *et al*., 2018). The researchers also managed to deduce the peak *EgGDSL* expression in the tissue which is at the third month (12^{th} weeks after anthesis, WAA). Another gene expression study was conducted with *FLL1* lipase gene isolated from the mesocarp of oil palm fruit (Nurniwalis *et al*., 2015). The researchers discovered that *FLL1* exhibited a huge influence on the oil quality (Nurniwalis *et al*., 2015). Considering the results and the possible impact of the gene towards maintaining good oil quality, FLL1 lipase was chosen as the target protein in this study.

The use of heterologous recombinant protein approach is frequent in the study of target proteins that are affected by challenging natural production and isolation (Contesini *et al*., 2020). The technique involves the insertion of target gene into a vector backbone prior to expression in selected host (Ceccarelli, 2014). The common host is *E. coli* for their simplicity (Gomes *et al.,* 2018). Nowadays, scientists have engineered different strain of *E. coli* to provide the aid needed to maximise target protein

production (Gomes *et al*., 2018). Extensive information on the gene using prediction tools available can detect any arising issues that can be tackled by observing the host used. Therefore, choosing the correct vector backbone and the expression host are important to determine the flow of the experiment (Ceccarelli, 2014). However, optimization of the expression parameter experimentally can be draining and extremely time consuming thus the use of modern technology *via* gene synthesis can be helpful to the researchers. Following the transformation, target protein must be over-expressed *via* the optimization of induction parameters.

The crude of a heterologously expressed protein exists with a myriad of other protein. Therefore, target protein must be isolated *via* protein purification system of choice (Kulkarni & Bose, 2022). The method is crucial to remove the miscellaneous protein prior to target protein characterization (Kulkarni & Bose, 2022). The common method used is protein chromatography technique using the CIpP which means capturing of target protein, intermediate polishing and final polishing (Cytiva Life Sciences, 2018). The purification system can be chosen based on the information gathered through *in-silico* protein profiling (Asenjo & Andrews, 2009; Smith, 2017).

Only purified protein should be used in the characterization of target protein (Scopes, 1995). This is to increase the reliability of data without the interference from the miscellaneous protein and contaminants (Scopes, 1995). There are a lot of different parameters and angle that be observed to understand the target protein and its behaviour. Nevertheless, the basic information on the heat and protein relationship other than buffer and solvent suitability were the cornerstone of proper enzyme

characterization. Additional characterization effort is valued to provide a bigger picture on FLL1 behaviour and preference. However, it is positive that the results gathered from this research will help embark a more specific approach to tackle palm oil quality concern from the enzymatic perspective.

1.2 Problem statements

Lipase exerts a negative effect on the palm oil quality by inducing spontaneous acidification through free fatty acid liberation during its hydrolysis (Calder, 2016) Consequently, the accumulated fatty acids leads to an undesirable taste while decreasing the commercial and nutritional value of palm oil (Sambanthamurthi *et al.,* 2000). Additionally, the affected oil is less stable for storage especially over a long period of time (Sambanthamurthi *et al.,* 2000). This has become one of the major problems facing the palm oil industry, since rancid oil is not suitable for either human consumption or industrial use (Calder, 2016). Therefore, the industry is interested in finding the effective ways of controlling lipase activity to maintain oil quality.

The effort to develop the potential solutions requires an understanding of different species of lipase present in oil palm. Previous gene expression study reveals a gene potentially controlling palm oil quality namely *FLL1* (Nurniwalis *et al*., 2007; Nurniwalis *et al.,* 2015). However, the behaviour of FLL1 as a functional protein is unknown. Targeted study on selected lipase research is expected to help in mitigating the negative effect of lipase towards the oil quality.

Additionally, due to the scarcity information, prevention at the root cause is limited and the potential application for oil palm lipase enzymes remain unexplored. Therefore, oil palm lipase is not applied in any manufacturing industries at all (Chandra *et al*., 2020; Ali *et al*., 2023), while only a few studies have looked into oil palm varieties for their special characteristics and applications (Wong *et al*., 2016; Sambanthamurthi *et al.,* 2000).

This study will bridge the knowledge gap in characterizing the oil palm lipases to formulate specific strategy aimed at the prevention of oil quality deterioration. Eventually, the results may redefine palm oil quality management and broaden potential oil palm lipase uses in other industries.

1.3 Research objectives

The objectives of this study are:

1. To express the lipase gene *FLL1* from oil palm recombinantly *via* the *Escherichia coli* expression system.
2. To characterize the purified recombinant FLL1 lipase.

1.4 Research questions

The research questions of this study are:

1. What are the optimal expression conditions to express the lipase gene *FLL1* from oil palm recombinantly *via* the *Escherichia coli* system?
2. What are the physical and chemical profiles of the purified recombinant FLL1 lipase?

1.5 Research limitations

This thesis focused on the expression and characterization of lipase protein FLL1 by observing the resulting hydrolytic activity exhibited. The research scope following the objectives have been successfully covered in this dissertation. Nevertheless, the results were limited by the designated time frame of the postgraduate study period. A more extensive result can be collected if this research was not bounded by time and money.

From a technical standpoint, the absence of a suitable automated purification system in the facility hindered the progress of this study. To preface, this issue is not a major problem during the initial purification optimization stage, but had become a severe hindrance due to the laborious efforts employed and the time spent to harvest pure protein yield following a certain prerequisite concentration. Nevertheless, this research project was completed successfully by the persevering effort and passion.

1.6 Summary

The expression profile of *FLL1* pointed to the possibility of influencing oil quality thus chosen further studied for functional characterization. In this work, FLL1 lipase is heterologously expressed, purified, and characterized to probe the relation between lipase and oil quality. The findings offer insights to mitigate lipase-caused oil rancidity issue while moulding the future applications of FLL1.