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RECOVERY OF PEPTIDE FROM Nannochloropsis gaditana PROTEIN HYDROLYSATE THROUGH TWO-STAGE ULTRAFILTRATION MEMBRANE

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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 Doctor of Philosophy

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RECOVERY OF PEPTIDE FROM *Nannochloropsis gaditana* PROTEIN HYDROLYSATE THROUGH TWO-STAGE ULTRAFILTRATION MEMBRANE

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March 2021

Chair : Wan Azlina Wan Ab Karim Ghani, PhD
Faculty : Engineering

Microalgae *Nannochloropsis gaditana* (*N.gaditana*) is widely recognized as potential source of biofuel due to its high lipid content. However due to high cost of production, microalgae biorefinery concept was approached. Microalgae's protein which consists up to 50% w/v has a value added. Peptides derived from microalgae's protein offers great potential value for food and pharmaceutical products. In this study, enzymatic hydrolysis of *N.gaditana* using alcalase was optimized using Response surface Methodology (RSM) with different variables; pH, temperature, enzyme concentration and substrate concentration. Optimum conditions were obtained at pH 8.14, temperature 51.4°C, 5.48 g/L substrate concentration and 0.26 g/L enzyme concentration with predicted degree hydrolysis of 55.76%. Microalgae protein hydrolysate (MPH) had 17.9% of total lipid, 55.73 % of total protein, 6.8% of total carbohydrate, 15.42% of ash and 4.15% of moisture with high total amino acid of 2507.297 mg/100g which indicated the high nutritional value in MPH as a potential source of functional food. Since MPH was composed of peptides with different molecular weight, fractionation using ultrafiltration (UF) membrane was conducted with two different membrane configurations; single (10 kDa and 5 kDa) and two-stage (10/5 kDa) to obtain peptide with low molecular weight that could improve the biological activity of *N.gaditana*. Three different parameters (pH, feed flow rate and trans-membrane pressure) were evaluated based on permeate flux and peptide transmission. The best fractionation of MPH was observed using two-stage 10/5 kDa UF membrane at flow rate of 23 ml/min, 1.5 Bar of trans-membrane pressure and pH 2 with permeate flux of 69.85±1.22 L/m^2h and peptide transmission of 79.13±0.50%. High peptide recovery were found using two-stage UF membrane with 58.04%, followed by 10 kDa and 5 kDa UF membrane with 15.70% and 7.26%, respectively. Peptide from two-stage 10/5 kDa UF membrane were mainly consists of peptide with molecular weight below 300 Da with 77.49%. Peptide sequence from fractionated MPH using two-stage 10/5 kDa UF membrane was identified as Leu-Leu-His-Ala-Leu-Leu. The presence of small peptide size, hydrophobic amino acids and histidine were

contributed to high antioxidant activity of the peptide. In summary, MPH fractionation using two-stage 10/5 kDa UF membrane were observed could enrich low molecular weight of peptide and enhance antioxidant activity.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGAMBILAN SEMULA PEPTIDA DARIPADA PROTEIN HIDROLISAT *Nannochloropsis gaditana* MELALUI DUA-PERINGKAT MEMBRAN ULTRAFILTRASI

Oleh

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Microalga *Nannochloropsis gaditana* (*N.gaditana*) dikenali secara meluas sebagai sumber bahan api bio yang berpotensi kerana kandungan lipidnya yang tinggi. Namun disebabkan oleh kos pengeluaran yang tinggi, konsep penapisan bio mikroalga digunakan. Protein Microalga yang terdiri hingga 50% w/v mempunyai nilai tambah. Peptida yang berasal daripada protein mikroalga mempunyai nilai potensi yang besar untuk dijadikan produk makanan dan farmaseutikal. Dalam kajian ini, hidrolisis enzimatik *N.gaditana* menggunakan alcalase dioptimumkan menggunakan Response Surface Methodology (RSM) dengan pemboleh ubah yang berbeza; pH, suhu, kepekatan enzim dan kepekatan substrat. Keadaan optimum diperoleh pada pH 8.14, suhu 51.4 ° C, kepekatan substrat 5.48 g/L dan kepekatan enzim 0.26 g/L dengan tahap darjah hidrolisis yang diramalkan sebanyak 55.76%. Protein mikroalga hidrolisat (MPH) mempunyai 17.9% lipid, 55.73% protein, 6.8% karbohidrat, 15.42% abu dan 4.15% kelembapan dengan jumlah asid amino tinggi 2507.297 mg/100g yang menunjukkan nilai pemakanan yang tinggi MPH sebagai sumber makanan berfungsi yang berpotensi. Oleh kerana MPH terdiri daripada peptida dengan berat molekul yang berbeza, penapisan menggunakan membran ultrafiltrasi (UF) dilakukan dengan dua konfigurasi membran yang berbeza; tunggal (10 kDa dan 5 kDa) dan dua peringkat (10/5 kDa) untuk mendapatkan peptida dengan berat molekul rendah yang dapat meningkatkan aktiviti biologi *N.gaditana*. Tiga parameter yang berbeza (pH, kadar aliran dan tekanan membran trans) dinilai berdasarkan resapan fluks dan transmisi peptida. Penapisan terbaik MPH diperhatikan menggunakan dua peringkat membran UF 10/5 kDa pada kadar aliran 23 ml/min, tekanan membrane trans 1.5 Bar dan pH 2 dengan resapan fluks 69.85 ± 1.22 L/m^2h dan transmisi peptida 79.13 ± 0.50%. Pengambilan semula peptida yang tinggi didapati menggunakan membran UF dua peringkat dengan 58.04%, diikuti oleh membran UF 10 kDa dan 5 kDa masing-masing dengan 15.70% dan 7.26%. Peptida daripada dua peringkat membran UF 10/5 kDa kebanyakkannya terdiri daripada peptida dengan berat molekul di bawah 300 Da dengan 77.49%. Urutan peptida daripada penapisan MPH menggunakan dua peringkat membran UF 10/5 kDa dikenal pasti sebagai Leu-Leu-His-

Ala-Leu-Leu. Kehadiran saiz peptida yang kecil, asid amino hidrofobik dan histidin menyumbang kepada aktiviti antioksidan peptida yang tinggi. Ringkasnya, penapisan MPH menggunakan dua peringkat membran UF 10/5 kDa diperhatikan dapat memperkayakan peptide dengan berat molekul rendah dan meningkatkan aktiviti antioksidan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

MPH	Microalgae protein hydrolysate
DH	Degree of hydrolysis
TNBS	Trinitrobenzenesulfonic acid
OPA	*O*-phthaldialdehyde
SN-TCA	Nitrogen soluble trichloroacetic acid
ROS	Reactive oxygen species
UV	Ultraviolet
UF	Ultrafiltration
NF	Nanofiltration
RO	Reverse osmosis
MF	Microfiltration
RSM	Response surface methodology
WHC	Water holding capacity
OHC	Oil holding capacity
EC	Emulsifying capacity
FPLC	Fast Protein Liquid Chromatography
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
UPLC	Ultra Performance Liquid Chromatography
FTIR	Fourier Transfrom Infrared Spectroscopy
SEC	Size-Exclusion Chromatography
SEM	Scanning Electron Microscopy
BSE	Backscattered electrons
LCMS/MS	Liquid Chromatography Mass Spectroscopy/Mass Spectroscopy
MS/MS	Mass Spectrometry/ Mass Spectrometry
RT	Retention time

MWCO	Molecular weight cut-off
PEF	Pulse electric field
DNA	Deoxyribonucleic acid
E_a	Activation energy
BBD	Box- Behnken design
CCD	Central Composite Design
ACE	Angiotensin I-converting enzyme
R-PE	R-phycoerythrin
E/S	Enzyme-substrate
BHT	Butylated hydroxy toluene
BHA	Butylated hydroxy anisole
TBHQ	Tertiary butyl hydro quinone
PG	Propyl gallate
LEA	Lipid extracted algae
HAT	Hydrogen atom transfer
ET	Electron transfer
DPPH	2,2-diphenyl-1 picrylhydrazyl radical-scavenging capacity
ABTS	2,2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid) radical-scavenging capacity
FAO	Food and Agriculture Organization
AOAC	Association of Official Analytical Chemists
LMBRs	Lipid extreacted microalgae biomass residues
BOD	Biological oxygen demand
COD	Chemical oxygen demand
C_P	Concentration polarization
TMP	Transmembrane pressure
kDa	Kilodalton

Da	Dalton
ANOVA	Analysis of variance
Sp	Species
J_{crit}	Critical flux
J_{lim}	Limiting flux
IEP	Isoelectric point

CHAPTER 1

INTRODUCTION

1.1 Background of study

Approximately 2.4 billion years ago, water is covered more than 70% of algae and is said to be the most dominant group of living organism. At that moment, the process of oxygen accumulation in the atmosphere and water surface had begun. Sufficient amount of carbon dioxide, water, nutrient and light energy make the microalgae capable in producing and storing a variety of bioactive compounds (Chua & Schenk, 2017; Mimouni et al., 2012). Microalgae are photosynthetic marine organisms, also known as alternative raw materials that cannot compete for portable or arable land (Chen et al., 2013; Demirbas, 2010; Shebis et al., 2013). Over the past decade, microalgae and macroalgae have been blasting the worldwide interest (Stengel & Walker., 2015).

Microalgae has been an attractive feedstock to most researchers in the production of biofuels and bioproducts due to its special characteristics; fast growth rate and capable in extracting high concentration of biochemical compounds, for instance, carbohydrates, lipids and proteins (Awaluddin et al., 2016). Since then, researches on secondary metabolites from microalgae have been actively studied. Some species of microalgae showed that excessive amounts of valuable bioactive compounds such as fatty acids, fibers, antioxidants, carotenoids, sterols, proteins, phytocolloids, lectins, oils, amino acids, unsaturated fatty acids, and vitamins, may be commercially utilized (Alassali & Cybulska, 2016). Due to interesting natural bioactive compounds derived from microalgae, various potential applications to humankind of different fields of interest were greatly investigated (Morais et al., 2015).

Since the mid-fifties, intensive efforts have been made in discovering new source of protein for exchange as supplements. Peptide that is derived from protein is one of the major groups in food classes of microalgae and could be obtained during technological processes such as fermentation and storage of foods (Martínez-maqueda et al., 2013). Peptide has been considered for antioxidant, antihypertensive, anticoagulant and antimicrobial activity which could be utilized as a component in pharmaceutical healthcare products (Kim et al., 2001; Suetsuna et al., 2004; Won et al., 2005). These peptides usually contain 3–20 amino acid residues where their activities are depending on amino acids composition and sequences (Pihlanto-leppala, 2001). Bioactive peptide cannot be synthesised by human's body and thus, should be incorporated in daily diet through consumption peptide supplement (Korhonen & Pihlanto, 2006). These peptides are inactive and in intact form of protein in microalgae cells. Prior to peptide production, enzymatic hydrolysis is a preferred method in to break the cells and release the antioxidative peptide from microalgae protein. In the market, there are numerous commercial proteases such as alcalase, pepsin, trypsin, papain, neutrase and many more.

Alcalase has been proven to be one of the most efficient enzymes for the preparation of protein hydrolysate due to its ability in obtaining a high degree of hydrolysis in a relatively short period under mild conditions and can produce protein hydrolysate with high nutrient contents with good functional properties (Adler-Nissen, 1986; See et al., 2011; Shahidi et al., 1995). In order to produce microalgae protein hydrolysate with high degree of hydrolysis (DH) value and desired properties, the performance of enzymatic hydrolysis must be controlled. This includes the enzyme selection as well as parameters affecting the hydrolysis process.

Not only DH, a desired molecular weight of peptide should also be considered to improve antioxidant activities. As the microalgae protein hydrolysate consists of various sizes of peptide, a better understanding of fractionation and purification process is needed to obtain a desired size of bioactive peptide. Chromatographic technique is a common method used to separate this complex matrix containing many hydrolysed protein fractions of similar size due to its high specific separation capacities. However, the high cost of this technology prohibits the production of peptide on a large scale. Therefore, conventional method based on pressure-driven like ultrafiltration membrane can be used to separate the bioactive peptides since the separation is only based in physical mechanism, very simple, cheap and easy to operate (Firdaous et al., 2009; Kumar et al., 2013).

Separation of peptides using ultrafiltration membranes is based on the molecular weight-cut off. Determination of specific peptides might be difficult to identify since different peptides with the same molecular weight might elute at the same retention time. Thus, it is necessary to further purify the microalgae peptide in order to identify the peptide sequence and hence understand the relationship between the sequence and antioxidant activity.

1.2 Problem statement

Nannochloropsis sp. is known for its oleaginous characteristics, with a specific high content of eicosapentaenoic acid (EPA). In Malaysia, *Nannnochloropis* sp is widely used in aquaculture industry as a feed (Abidin et al.,, 2020). Several authors had investigated *Nannochloropsis* sp as potential source of larva feed and biofuel production due to its high triacylglycerol (TAG) content (up to 60% of its dry weight) (Gouveia & Oliveira, 2009; Moazami et al., 2012; Recht et al., 2012; Taylor et al, 2013). Due to high cost of production biofuel, most researchers were approaching microalgal biorefinery concept whereby microalgae biomass is being used as a whole: main product (oil) and by-products (protein, carbohydrates and fiber). Other than lipid, protein is one of the highest content in microalgae, up to 50% w/w which has a value added (Medina et al., 2015). Several studies on protein's *Nannochloropsis* sp have been investigated. The earlier study on peptide derived from protein's *Nannochloropsis oculata* was on the evaluation of Angiotensin I-Converting Enzyme (ACE) activity (Nguyen et al., 2013; Qian et al., 2013; Samarakoon et al., 2013). Eventhough, these studies used enzymatic hydrolysis to obtain the peptide, but no operating parameters were evaluated to determine the degree of hydrolysis. Later on, Medina et al., (2015)

showed that several parameters such as pH, enzyme substrate ratio and time were varied to determine the degree of hydrolysis from microalgae protein hydrolysate (MPH) using papain enzyme. The study demonstrated microalgae *Nannochloropsis gaditana* has potential nutritional value and antioxidant properties. However, some parameters include the effects of different temperature and proteases on the degree hydrolysis were not covered. A better understanding in controlling hydrolysis is required for the optimization process in order to maximize the yield at low cost. Thus, in this research operating parameters; pH, substrate concentration, enzyme concentration and temperature were studied using other commercial protease called, alcalase. Obtaining high DH value with a desired biological activity from MPH requires extra efforts in controlling the hydrolysis parameters during optimization.

MPH obtained from enzymatic hydrolysis is consisted of various sizes of peptides that require separation process. Ultrafiltration (UF) membrane could be used to separate these peptides by obtaining low molecular weight of peptide and hence increase the biological activity. Based on the previous study, separation of peptide using UF membrane has been studied for fish (Chabeaud et al., 2009; Foh et al., 2010; Roslan et al., 2019), milk (Amiot & Bazinet, 2007; Azinet, 2008), sesame (Da et al., 2009) scalloped hammerhead cartilage (Li et al., 2017), yeast (Marson et al., 2021) hydrolysate but similar study on microalgae protein hydrolysate has yet to be found. However, the main drawback of using UF membrane as separation method is membrane fouling, which could reduce permeate flux and peptide transmission. The fouling phenomena could be due to the concentration polarization, formation of a cake layer or membrane pore blocking (Koonani & Amirinejad, 2019). This effect could be reduced by selecting suitable operating parameters, membrane pore size and configuration. To date, there is no study regarding separation of peptide from MPH of *N.gaditana*. Therefore, in this research operating paramaters such as feed flow rate, trans-membrane pressure and pH at different membrane pore size were evaluated. To understand the fouling behaviour, three pore blocking models (cake formation, standard pore blocking and complete pore plugging) were fitted based on the permeate flux.

One of the difficulties that commonly arises when utilising ultrafiltration membranes is the broad pore size distribution of most commercial membranes, which greatly limits the resolving power of the membranes and has been one of the reasons for the low selective transmission during protein fractionation (Yunos & Field, 2008). A better transmission with the enrichment of low molecular weight of peptide is required to improve antioxidant activity. Therefore, a two-stage UF membrane has been introduced as an alternative way of using single UF membrane (Long et al., 2012). A good separation of biomass components were achieved by using two-stage UF membrane as Bottomley, (1991) found that the permeate was enriched with low molecular weight cut-off α-lactalbumin, while Cheang & Zydney, (2003) were able to obtain 100-fold purification and greater than 90% recovery of β-lactoglobulin from a binary mixture with α-lactalbumin. Later, Yap et al, (2014) had successfully separated protein and sugar from microalgae *Tetraselmis suecica* with 50% sugar yield and permeation rate of the sugars had increased approximately up to 90 % at second stage filtration. Nevertheless, no extensive study related to separation of peptide from MPH using two-stage UF membrane has been introduced so far. Thus, in this research, potential of two-

stage UF membrane in improving the transmission with enrichment of low molecular weight of peptide were evaluated and compared with single membrane.

1.3 Research hypothesis

i. Alcalase was efficient protease in preparing protein hydrolysates due to its thermostability (50-70°C) and high optimal pH (pH8-10), which can minimize the growth of microorganisms during hydrolysis process (Salwanee et al., 2013; See et al., 2011). Its capability in hydrolysing proteins with broad specificity for peptide bonds and the hydrolysed short chain peptides containing water soluble hydrophilic peptides have been reported to possess antioxidative properties (Dey & Dora, 2014; Je et al., 2005). Hence, the use of alcalase enzyme in enzymatic hydrolysis of microlagae *N.gaditana* is preferable in producing peptide with high degree of hydrolysis and antioxidant activity.

ii. The main problem in limiting the efficiency of commercial membrane separation was low selective transmission. It was usually related to the distribution of the broad membrane pore size of most commercial membrane (Roslan et al., 2017; Yunos & Field, 2008). Application of two-stage UF membrane had reported to improve the selective transmission during separation and hence increase the purity of hydrolysate (Cheang & Zydney, 2004; Haan et al., 2016; Lin et al., 1997; Vandanjon et al., 2007; Yap et al., 2014; Zuhair et al., 2018). Thus, by introducing two-stage UF membrane in fractionating microalgae protein hydrolysate, the transmission with enrichment of low molecular weight of peptide could be improved and enhance the antioxidant activity.

iii. The molecular weight and peptide sequence of peptide could influence antioxidant activity.

1.4 Research Objectives

i. To optimize enzymatic hydrolysis of microalgae *Nannochloropsis gaditana* using enzyme alcalase.

ii. To assess the fractionation of microalgae protein hydrolysate using single and two-stage ultrafiltration membrane.

iii. To assess the properties of microalgae protein hydrolysate from the UF membrane fractionated product.

1.5 Scopes of study

This study is aimed to produce microalgae protein hydrolysate (MPH) from *Nannochloropsis gaditana sp* with high antioxidant activity through enzymatic hydrolysis, followed by fractionation using cross flow ultrafiltration membrane and purification of low molecular weight of peptide.

Firstly, enzymatic hydrolysis of MPH is performed using alcalase enzyme where the scopes of study is focusing on the screening and optimization of hydrolysis process at different parameters such as pH, temperature (°C), substrate concentration (g/L) and enzyme concentration (g/L). The degree hydrolysis (DH) of the process is monitored using a method called O-phthaldehydde (OPA) method. Based on the screening process, Response Surface Methodology (RSM) is employed to optimize the conditions of enzymatic hydrolysis by obtaining an optimum DH value. Each MPH at optimum conditions is then characterized for its antioxidant activity, chemical properties, physical properties and functional properties. The chemical properties consisting of proximate analysis (moisture, ash, protein, lipid and carbohydrate content) and amino acid analysis. The physical properties include Scanning Electron Microscopy (SEM) of MPH meanwhile the functional properties are characterized for its nitrogen solubility, water holding capacity (WHC), oil holding capacity (OHC) and emulsifying capacity (EC).

The MPH at optimum conditions is then fractionated using cross flow ultrafiltration membrane to obtain bioactive peptide with low molecular weight. In this study, two different cross flow hollow fiber membranes with molecular weight cut-off of 10 and 5 kDa are used. The effects of feed flow rate (23, 29, 35 and 41 ml/min), transmembrane pressure (0.5, 1.0 and 1.5 Bar) and pH (2, 4, 7, 9 and 11) on UF membrane performance are evaluated based on permeate flux and peptide transmission. The UF membranes are performed at two different configurations; single stage (10 and 5 kDa) and two-stage (10/5 kDa).The permeate at optimum conditions from different configuration membranes are then analysed using pore blocking models (cake formation, standard pore blocking, complete pore plugging/blocking). Then, the permeate sample is analysed using Fast Protein Liquid Chromatography (FPLC) for the peptide enrichment. The fraction with highest antioxidant activity obtained from FPLC chromatogram is further purified using Liquid Chromatography Mass Spectroscopy/Mass Spectroscopy (LCMS/MS) to identify the bioactive peptides and peptide sequences.

1.6 Novelty of Research

The novelty of this research is the enhancement recovery of peptide using two-stage cross flow ultrafiltration membrane. High recovery of peptide with enrichment of low molecular weight peptide could improve biological activities. Thus, peptide from MPH *N.gaditana* could be a value added in food and pharmaceutical product.

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LIST OF PUBLICATIONS

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