UNIVERSITI PUTRA MALAYSIA

PRODUCTION OF AMINO ACIDS FROM MICROALGAE *Nannochloropsis sp*. BIOMASS USING SUBCRITICAL WATER TECHNOLOGY

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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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Chairman : Assoc. Prof. Mohd Razif Bin Harun, PhD
Faculty : Engineering

The increase in the world population resulted in high demand for food, particularly proteins. Proteins are made up of long chains of amino acids. At present, animals and plants-based amino acids constitute the majority of the food that humans and livestock consume. However, due to the increase in demand, these amino acid resources will not be sufficient. Therefore, sources such as microalgae could be an alternative. Chemical synthesize and biological (enzyme and fermentation) methods are current methods to produce these amino acids. These methods used harmful chemicals, long processing steps, and high operating costs. Hence, finding a suitable amino acid production technique could significantly protect the environment, save time, and cost. This study deals with the use of the green technology approach, which is subcritical water technology. The yield and composition of amino acids produced from subcritical water of microalgae *Nannochloropsis sp*. were evaluated at different temperatures, time, and biomass loadings. The response surface methodology (RSM) was employed to generate empirical equations that correlate the subcritical water process parameters and the response variables, which are amino acids. The empirical equation generated was then evaluated to ensure the model adequately fit to describe and predict the production of amino acids. This study was also conducted to evaluate the kinetic and thermodynamic parameters of amino acid production from the microalgae using subcritical water technology. The results revealed the total and individual amino acids investigated produced the highest yield at different subcritical water conditions. Hence, careful selection of operating parameters (i.e., temperature, time, and biomass loading) is crucial to identify the selected amino acids when using subcritical water technology. The empirical equations obtained from RSM are inaccurate for predicting the yield of amino acids from the subcritical water of microalgae *Nannochloropsis sp*. biomass. However, the RSM study provides some ideas about the range of the optimum subcritical water conditions that might lead to a high yield of amino acid production. A high yield of total amino acids (1959 mg/100 g algae), leucine (134 mg/100 g algae), glycine (323 mg/100 g algae) and alanine (495 mg/100 g algae) was obtained in the kinetic study when using the optimum operating conditions, as suggested by the RSM approach. A single consecutive model used in the kinetic study is adequate to predict the production of the total amino acids, glycine, leucine and alanine at the studied temperature. The thermodynamic analysis results showed the subcritical water process as endothermic and constant energy supply needed to produce these amino acids. Overall, the findings from this study are useful to understand the production of the amino acid from microalgae *Nannochloropsis sp*. via the subcritical water process. Thus, the research will benefit food and pharmaceutical industries, where microalgae can be used as an alternative feedstock to meet the population's need for a more sustainable food supply, specifically concerning protein and amino acid demand.

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

PENGHASILAN AMINO ASID DARIPADA BIOJISIM MIKROALGA *Nannochloropsis sp.* MENGGUNAKAN TEKNOLOGI AIR SUBGENTING

Oleh

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Peningkatan populasi dunia mengakibatkan permintaan tinggi terhadap makanan, terutama protein. Protein adalah molekul yang terdiri daripada rantaian panjang amino asid. Pada masa ini, protein daripada haiwan dan tumbuhan yang berasaskan amino asid merupakan sebahagian besar makanan yang dimakan oleh manusia dan haiwan ternakan. Walau bagaimanapun, pada masa depan, sumber amino asid ini tidak akan mencukupi. Oleh itu sumber mampan seperti mikroalgae boleh menjadi alternatif. Kaedah kimia dan biologi (enzim dan fermentasi) adalah kaedah yang digunakan buat masa ini untuk menghasilkan amino asid. Kaedah ini menggunakan bahan kimia berbahaya, langkah pemprosesan yang panjang, dan kos operasi yang tinggi. Oleh itu, teknik yang sesuai dalam penghasilan amino asid perlu dicari supaya dapat melindungi alam sekitar, menjimatkan masa, dan kos. Kajian ini melibatkan penggunaan teknologi hijau iaitu teknologi air subgenting. Jumlah dan komposisi amino asid yang dihasilkan dari teknologi air subgenting menggunakan mikroalga *Nannochloropsis sp*. di nilai pada suhu, masa dan jumlah biojisim yang berbeza. Metodologi permukaan tindak balas (RSM) digunakan untuk menghasilkan persamaan empirikal yang boleh menghubungkan parameter teknologi air subgenting dan pemboleh ubah tindak balas, iaitu amino asid. Persamaan empirikal yang dihasilkan kemudian di nilai untuk memastikan modelnya sesuai untuk menerangkan dan meramalkan penghasilan amino asid ini. Selain itu, kajian ini juga dilakukan untuk menilai parameter kinetik dan termodinamik pengeluaran amino asid dari mikroalga *Nannochloropsis sp*. menggunakan teknologi air subgenting. Hasil kajian menunjukkan jumlah tertinggi penghasilan keseluruhan dan setiap satu amino asid yang disiasat dihasilkan pada julat keadaan air subgenting yang berbeza. Oleh itu, pemilihan operasi parameter seperti suhu, masa dan jumlah biojisim yang teliti sangat penting untuk mengenal pasti amino asid yang dikaji menggunakan teknologi air subgenting. Persamaan empirikal yang diperoleh dari RSM tidak tepat untuk digunakan dalam meramalkan hasil amino asid dari teknologi air subgenting

menggunakan mikroalga *Nannochloropsis sp.* biojisim. Walau bagaimanapun, kajian RSM masih memberikan idea mengenai julat keadaan yang optimum yang boleh meningkatkan pengeluaran amino asid. Penghasilan amino asid keseluruhan yang tinggi (1959 mg AA/100 g alga), leucine (134 mg/100 g algae), glycine (323 mg/100 g algae) dan alanine (495 mg/100 g algae) dapat diperoleh dalam kajian kinetik dengan menggunakan keadaan optimum yang disarankan oleh pendekatan RSM. Model tunggal berturut yang digunakan dalam kajian kinetik boleh digunakan untuk meramalkan pengeluaran amino asid keseluruhan, glycine, leucine dan alanine pada suhu yang dikaji. Analisis termodinamik menunjukkan proses air subgenting ini sebagai endotermik dan tenaga luaran yang berterusan diperlukan untuk penghasilan amino asid. Secara keseluruhan, penemuan dari kajian ini sangat berguna untuk memahami penghasilan amino asid daripada mikroalgae *Nannochloropsis sp.* melalui teknologi air subgenting. Oleh itu, penemuan penyelidikan ini akan memberi manfaat kepada industri makanan dan farmaseutikal di mana mikroalga berpotensi menjadi sumber alternatif untuk memenuhi keperluan populasi bagi bekalan makanan yang lebih mampan, khususnya berhubung dengan permintaan yang tinggi terhadap protein dan amino asid.

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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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AA	Amino acids
His	Histidine
Arg	Arginine
Ser	Serine
Gly	Glycine
Asp	Aspartic acid
Glu	Glutamic acid
Thr	Threonine
Ala	Alanine
Pro	Proline
Cys	Cystine
Lys	Lysine
Tyr	Tyrosine
Met	Methionine
Val	Valine
Ile	Isoleucine
Leu	Leucine
Phe	Phenylalanine
UPLC	Ultra-performance liquid chromatography
min	minute
h	hour
AABA	A-aminobutryic acid
HCl	Hydrochloric acid
BSA	Bovine Serum Albumin
EPA	Eicosapentaenoic acid
IEC	Ion-exchange chromatography
GC	Gas chromatography
HPLC	High pressure liquid chromatography
FID	Flame ionization detector
RP-HPLC	Reverse-phase-high pressure liquid chromatography
CCD	Central composite design
FCCD	Face centred central composite design
TUV	Tuneable detector
DAD	Photodiode array detector
FLD	Fluorescence detector
ANOVA	Analysis of variance
OFAT	One-factor-at-time
BBD	Box-Benhken Design
SW	Subcritical water
MW	Molecular weight
n.d.	No date
Da	Dalton

CHAPTER 1

INTRODUCTION

This chapter briefly introduces the background and context of this thesis. The purpose of conducting this research is discussed in the problem statement. Also, the objectives and scope of this study are further outlined in this chapter. The significance of this research and the organization of this thesis are also included in this chapter.

1.1 Research Background

The expansion in the world population resulted in an increase of food needed and hence lead to increased demand for protein (Sari, 2015). Protein is important as they play a critical role in the growth and maintenance of human and animal (livestock) body (Delimaris, 2013). Proteins are made up of different combinations of amino acids, and the nutritional quality of protein depends on the amino acid composition (Wielen et al., 2017). Nowadays, about 0.6 million tonnes of amino acids are used in Europe as supplements for food and feed (Sari, 2015). It is predicted that about 9 million tonnes of protein in the feed sector could be reduced if there are 38 million tonnes of amino acids could be used for feed supplements in the future (Sari, 2015). The use of amino acids as a supplement could alleviate the protein demand due to the increase in the world population.

At present, animals (meat, poultry, milk, and egg), fish, shellfish, plants, and crops-based amino acids constitute most of the food that humans and livestock consume (Delimaris, 2013; Henchion et al., 2017). Foods derived from animal sources are considered a complete protein because they contain all the amino acids that a human body required (Bleakley & Hayes, 2017). However, these animal sources consist of high levels of saturated fats and cholesterol, which could be linked to the development of cardiovascular disease and diabetes (Bleakley & Hayes, 2017). Source of amino acids from plants such as soy, vegetables, cereals, grains, nuts, and seeds are also useful for human and animal diet (Ejike et al., 2017; Lonnie et al., 2018). However, a million hectares of land is required to produce these plants and crops (Henchion et al., 2017). In contrast, fish sources (wild fish) could be linked to chemical contamination by heavy metals such as mercury (Sidhu, 2003). In the future, the sources of amino acids discussed above might not be sufficient due to the increasing demand. Therefore, another alternative sustainable protein and amino acid source should be discovered.

Microalgae are considered a promising and sustainable source of protein. The protein content of microalgae is around 30-65% (Becker, 2007). The amino acid compositions of some microalgae are also comparable to other food proteins (Becker, 2007). Microalgae are considered sustainable sources as they can be produced all-year-round, and they can be grown in seawater, freshwater, and wastewater without the requirement of land (Dragone et al., 2010; Mobin & Alam, 2014). However, the amount of protein and amino acid composition in microalgae usually varies between microalgae species (Becker, 2007; Mobin and Alam, 2017). This variation is due to the microalgae cell wall and cultivation conditions (Safi et al., 2014; Surendhiran & Vijay, 2014). Figure 1.1 shows the schematic presentation of marine microalgae with the cell wall. These cell walls make the protein, amino acids, and other intracellular components (carbohydrate and lipid) of microalgae not easily accessible. Therefore, greater effort has been placed on developing methods to disrupt the cell wall and extract the protein and other intracellular components of microalgae.

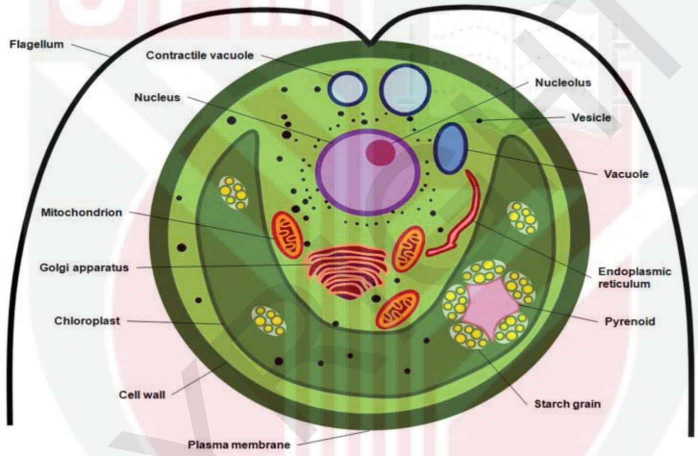

Figure 1.1: Schematic presentation of unicellular microalgae with cell wall
(Source: Poojary et al., 2016)

An emerging green technology known as subcritical water has been shown to extract the protein in microalgae (Moscoso et al., 2013; Awaluddin et al., 2016). The protein that has been extracted could further hydrolyze to produce amino acids (Sunphorka et al., 2012b). The microalgae *Nannochloropsis sp*. used in this study is widely used for aquaculture, pharmaceutical, and biofuel applications due to its lipid and eicosapentaenoic acid (EPA) content (Ma et al., 2016; Sukarni et al., 2014). However, limited study has been conducted on the protein and amino acid content, which limits its commercialization. Furthermore, the amino acid composition of the microalgae *Nannochloropsis sp.* shown in the past was obtained using the acid hydrolysis process rather than using the subcritical water technique (Safi et al., 2013; Brown, 1991). Hence, identifying, understanding the factor, mechanism, and suitable operating conditions for amino acid production via subcritical water technology is vitally important in establishing the nutritional value of microalgae *Nannochloropsis sp*. in this study.

1.2 Problem statement

The market demand for amino acids is increasing as they have potential to be used in many industrial applications such as food, animal feed, pharmaceutical, nutraceutical, cosmetics, polymer materials, and agricultural chemicals sectors (Sari, 2015; D'Este et al., 2018; Ge et al., 2019). However, the study reported that the industrial processes to produce amino acids still lack and need to be optimized (D'Este et al., 2018). The lack here means that they are not many signs of progress of research in amino acids production for decades (Ge et al., 2019). There are still problems such as long reaction time and heavily discharge of wastewater which could potentially lead to pollution and environmental problem (Ge et al., 2019). Nowadays, the demand for greener development and technology related to chemical industries are arising due to the stringent policies for environmental protection (Ge et al., 2019). Therefore, the research in finding more cost-effective, environmentally friendly and sustainable routes for amino acids production is essential.

The existing methods to produce amino acids can be categorized into three types; chemical synthesis, microbial method (enzymatic synthesis and fermentation), and extraction from protein hydrolysate (D'Éste et al., 2018; Sari, 2015; Ikeda, 2003; Breuer et al., 2004). Among these methods, fermentation is the most common industrial process to produce amino acids. However, some major problems related to fermentation including contamination, high energy consumption (for mixing and oxygen transfer), variability in the quality of the substrate, infection by bacteriophages, and the requirement for a bigger reactor (D'Este et al., 2018). The chemical synthesis methods for the industrial process of amino acid production are known as Chloroacetic acid, Strecker synthesis, and Bucherer-Bergs route (Breuer et al., 2004; Karau & Grayson, 2014). However, the main downsides of chemical synthesis are related to the use of hazardous cyanide sources, the high price of catalysts, and the production of a mixture of D and L-amino acids (D'Éste et al., 2018; Breuer et al., 2004).

The production of D and L-amino acids from chemical synthesis can be avoided using the enzymatic route (D'Este et al., 2018). However, the enzymatic route for the production of amino acids is usually associated with high cost due to the use of several enzymes and instability of enzymes (Ikeda, 2003). The extraction method has been reported as a suitable method to produce amino acids using biomass sources, waste, and industrial by-products (D'Este et al., 2018). These methods are well established utilizing the available chemicals (acid, alkali, ethanol, acetonitrile, methanol). However, the process is lengthy (18-72 h) (Mustatea et al., 2019) and generates waste liquid (acidic waste) (Xiu et al., 2019). An additional process such as lyophilization, rotary evaporation, drying/washing with sodium hydroxide is required to remove or neutralize the acidic waste from the products, which increases the operational cost (Xiu et al., 2019; Sereewathanawut et al., 2008; "Amino acid analysis-Biochrome", 2020).

An alternative method known as subcritical water can be used to produce amino acids (Zhu et al., 2011a; Marcet et al., 2016). The use of water as a solvent has made this method sustainable, economical, and environmentally friendly to be used for food, pharmaceutical, and animal feed applications (feed pellet, granulated solids or liquid concentrate, e.g., MetAMINO from Evonik and Rhodimet AT88 from Adisseo) (Karau & Grayson, 2014). The subcritical water technology is considered economical and sustainable as water is readily available and has a positive life cycle assessment, ensuring a safe and superior technology (Gbashi et al., 2017). Also, subcritical water adopts a relatively less sophisticated technology, thus requiring much lower engineering costs for process-scale apparatus/equipment (Gbashi et al., 2017; Kubatova et al., 2001; Bart, 2005). Furthermore, the dielectric constant and ionic properties of water changes at subcritical conditions (101-374°C, 0.1-22.1 MPa), making water act as an organic solvent and acid catalyst (Thiruvenkadam et al., 2015; Espinoza et al., 2011; Shitu et al., 2015). However, at present, an additional process (pre-treatment with alkaline and acid addition) is employed either before or after the subcritical water process to produce or analyze these amino acids (Du et al., 2020; Moscoso et al., 2013). This extra step makes the process a two-step process. The acid addition will make the analysis results inaccurate as this acid will further hydrolyze the protein that presents in the aqueous phase after the subcritical water process. Hence, this study proposes a one-step process of amino acid production from microalgae biomass using the subcritical water technique.

The information on free amino acids that could be obtained from the subcritical water of microalgae is currently limited compared to other components of microalgae such as lipid (Ho et al., 2018) and carbohydrate (Awaluddin et al., 2016). Up to now, only microalgae *Chlorella* and *Scenedesmus sp*. have been used for extraction of protein and production of amino acids using the subcritical water technology (Awalludin et al., 2016; Moscoso et al., 2015). The limitation is probably due to the temperature (100-374°C) of subcritical water that could lead to protein and amino acid degradation. Most of the studies reported thermal degradation of protein and amino acids were observed when subcritical water hydrolysis was carried out at high temperature for an extended time (Empikul et al., 2012; Uddin et al., 2010; Kang & Chun, 2004a). The degradation conditions (temperature, time, pressure) of these proteins and amino acids are depending on the source of the substrate. Besides, individual amino acids were also susceptible to degradation under different conditions (Sato et al., 2004). Therefore, proper control of subcritical water operating conditions is required to enhance the process and obtain the desired product of interest, which is free amino acids.

1.3 Research Objectives

The overall aim of this study is to determine the feasibility of subcritical water technique as a one-step process and green technology approach for free amino acids production from microalgae *Nannochloropsis sp.* biomass. The objectives of this study are;

1. To investigate the factors (temperature, time, and microalgae loading) that affect the composition of amino acids production from microalgae *Nannochloropsis sp*. biomass
2. To evaluate the empirical equation that generated from the response surface methodology (RSM) for predicting the production of amino acids from microalgae *Nannochloropsis sp*. biomass
3. To evaluate the kinetic and thermodynamic parameters of amino acids production from microalgae *Nannochloropsis sp.* biomass using subcritical water technology

1.4 Research Scopes

1.4.1 Identification of the factors that affect the composition of amino acids production

The main scope of this study is to identify the yield of protein and free amino acids composition obtained via subcritical water technology at different ranges of temperatures, biomass loadings and time. The effect of various parameters in the production of amino acids from microalgae *Nannochloropsis sp.* were evaluated, and the range of values that results in the highest production of amino acids could be obtained. The results obtained provide a basis or reason for the range of parameters and type of amino acids chosen in the kinetic and RSM study. The amino acids production via subcritical water in this study was also compared with amino acids obtained from the acid hydrolysis process and control study.

1.4.2 Evaluation of empirical equations generated from RSM for amino acids production from microalgae *Nannochloropsis sp*. biomass

Response surface methodology (RSM) under central composite design (CCD) was employed to develop the empirical equations. The empirical equations can be used to predict the yield of amino acids production from microalgae *Nannochloropsis sp*. biomass using subcritical water technology. The adequacy of the empirical model developed was then tested and verified. Significant factors or parameters that could affect the production of amino acids were identified by analysis of variance (ANOVA) method, and the interaction of each factor on amino acids production were evaluated by 3D-graphical representation.

1.4.3 Evaluation on the kinetic and thermodynamic parameters of amino acids production from microalgae *Nannochloropsis sp*. biomass

A kinetic model is required to describe the production of amino acid from microalgae *Nannochloropsis sp*. biomass. The range of experimental conditions selected in this study was based on the suggested operating conditions from RSM. The data obtained from the experimental study was used to determine the rate of amino acids production and decomposition via subcritical water. Computer software MATLAB was used in kinetic modelling to determine the rate constant of amino acids production and decomposition. The activation energy (Ea), as well as the pre-exponential factor (A), were calculated using this so-called Arrhenius plot. The thermodynamic analysis was evaluated using the transition-state theory and focus on determining the enthalpy, entropy and Gibbs free energy of activation of the reaction.

1.5 Significance of study

This study contributed to the development of an efficient method for the recovery of amino acids through safe and green technique, especially for food, and pharmaceutical application. A better insight on microalgae protein extraction and hydrolysis via subcritical water technology can contribute to a higher protein and amino acids. The amino acids obtained could alleviate the shortage of protein in the future, thus proven the capability of microalgae as one of the novel amino acids source and subcritical water as a feasible and environmentally friendly technique. The identification of high concentration amino acids from profiling study provides an idea for product development using microalgae *Nannochloropsis sp.* biomass as feedstock and contribute to current existing amino acids.

1.6 Thesis organization

The thesis first gives a general overview of the research. The literature review in Chapter 2 discusses the structure, type, classification, and function of amino acids. The use of microalgae as a source of protein is included in Chapter 2. Chapter 2 also discussed different techniques or methods used for amino acids production and analysis. The review then focused on the technique selected in this study, which is subcritical water. Explanation on theory of subcritical water, previous and current study that have been conducted are included in this chapter. Introduction on kinetic and different models that have been used in the past are also presented in Chapter 2. The description of the theory selected for thermodynamic study and response surface methodology (RSM) approach are explained in brief in chapter 2. Chapter 3 describes the materials and methodology used in obtaining and analyzing the amino acids. The results and discussion section are shown in Chapter 4. Finally, Chapter 5 discusses the conclusion, together with recommendations on the work that could be conducted in the future.

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The student was born in Malacca on 26th July 1985. She received her bachelor's degree in Biochemical-Biotechnology Engineering at International Islamic University Malaysia and graduated in 2009. She then continues her master studies in Biotechnology Engineering at International Islamic University Malaysia and received her master's degree in 2012. In 2017, she started her PhD in Chemical and Environmental Engineering at Universiti Putra Malaysia under the supervision of Associate Prof. Dr. Razif Harun. Her research focuses on protein extraction and amino acids production from microalgae *Nannochloropsis sp.* biomass using subcritical water technology. Her area of interest includes extraction of biomasses using chemical, biochemical and thermochemical conversion techniques.

LIST OF PUBLICATIONS

Journal

Zainan, N.H., Thiruvenkadam, S., Danquah, M.K., Harun, R. (2019) Biochemical analysis and potential applications of aqueous and solid products generated from subcritical water extraction of microalgae *Chlorella pyrenoidosa* biomass. *Journal of Applied Phycology, 32: 111–126.*

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