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Profile of Amino Acids Production from Microalgae *Nannochloropsis sp.* Biomass using Subcritical Water Technology

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Abstract. The amino acids from microalgae could be used as a substitute for food and feed supplements in the future. This study investigates the production of amino acids from microalgae *Nannochloropsis sp.* biomass using subcritical water technology approaches. The yield and composition of amino acids produced from subcritical water of microalgae *Nannochloropsis sp.* were evaluated at different temperatures (160-350 °C), time (3-30 min), and biomass loadings (1-15% w/v). Overall results showed that the highest yield of total amino acids (1531.98 mg/100 g algae) was obtained at subcritical water operating conditions of 280 °C, 15 min reaction time, and 1% biomass loading. The studied operating conditions produced a higher yield of non-essential amino acids compared to essential amino acids. The study demonstrated that each of the individual amino acids investigated produced the highest at different ranges of subcritical water conditions. Thus, the obtained profile of the individual amino acid showed that careful management of operating parameters (temperature, time, and biomass loading) is crucial for identifying the amino acids of interest via subcritical water technology.

Keywords: Amino acids, Biomass loading, Microalgae, Subcritical water, Temperature, Time

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INTRODUCTION

The market demand for amino acids increases as they can be used in many industrial applications such as food, animal feed, pharmaceutical, nutraceutical, and cosmetics (D'Este et al. 2018; Ge et al. 2019; Sari 2015). However, the industrial processes to produce amino acids still lack and need to be optimized (D'Este et al. 2018). The lack here means that there are few signs of research progress in amino acid production (Ge et al. 2019). There are still problems such as long reaction time and heavy discharge of wastewater, potentially leading to pollution and environmental problems (Ge et al. 2019). Therefore, finding more cost-effective, environmentally friendly, and sustainable routes for amino acid production is essential.

There are 20 amino acids that are categorized as standard, and these can be classified based on nutrition (essential and non-essential) or R-group. The essential amino acids cannot be synthesized by the body, while the body could synthesize the non-essential amino acids. The R side-chain group of amino acids consisted of hydrophobic non-polar aliphatic, hydrophilic polar aliphatic, hydrophobic and hydrophilic aromatic groups, and positively charged group and negatively charged group (Abdelmoez et al. 2010).

Subcritical water can be used as an alternative method to recover these amino acids (Marcet et al. 2016). However, the production of amino acids using the subcritical water technology is hard to control as the subcritical water hydrolysis process is not specific. The H^+ ion randomly attacked the peptide bond of the protein during the subcritical water process, making it difficult to predict which peptide bond would be cleaved and which amino acids would be produced. Unlike the enzymatic reaction, the enzyme can specifically cleave the desired peptides bonds, which allows us to predict which peptides will be obtained (Marcet et al. 2016).

At an early stage, without a proper investigation of the profiling study, it is difficult to know or predict the composition or type of amino acids that might be produced from microalgae using the subcritical water technique. The temperature, time, and biomass loading might affect the production of these amino acids as the amino acids have different sizes, locations in the protein, and the R side-chain group.

This study deals with the use of the green technology approach, which is subcritical water technology. The yield and profile of amino acids (essential and non-essential) produced from subcritical water of microalgae *Nannochloropsis sp.* biomass are evaluated at different temperatures, times, and biomass loadings.

The microalgae *Nannochloropsis sp*. are selected in this study as only a few studies have been conducted on its protein and amino acid content compared to its lipid and eicosapentaenoic acid (EPA) composition. Hence, our research focuses on amino acids production from microalgae *Nannochloropsis sp.* biomass to improve the utilization of microalgae *Nannochloropsis sp*. as feedstock.

MATERIALS AND METHOD

The list of the chemicals and reagents used in this study are as follows; Hydrochloric acid (HCl), formic acid (R&M Chemicals Ltd.), acetonitrile (J.T. Baker, HPLC Reagent), α-Aminobutyric acid (AABA, MW; 103.12 g/mol), acetonitrile, AccQ Tag Eluent A (Waters), derivatization reagent kit (borate

buffer, acetonitrile, and ACCQ fluor reagent, Waters).

Microalgae *Nannochloropsis sp.* substrate

Microalgae *Nannochloropsis sp.* biomass was obtained from Xi'an Lyphar Biotech (China). The details of the microalgae biomass proximate compositions (crude protein, moisture, ash and fat) and elemental analysis have been discussed by (Zainan et al. 2019a). The highest protein composition (40.5%) showed the suitability of this microalga *Nannochloropsis sp*. biomass to be used as a source of proteins and amino acids (Zainan et al. 2019a).

Apparatus and procedures of subcritical water experiment

A stainless-steel batch reactor with a diameter of 7.5 mm and a length of 150 mm (Swagelok & Co.) was used in this study. The main components of the salt bath include an isolation chamber, inner salt bath, heater (4000 W), temperature sensor, stirrer, stirring motor, and operation panel.

The salt bath used in this study was a mixture of potassium nitrate (R&M Chemicals, Malaysia) and sodium nitrate (R&M Chemicals, Malaysia) at a ratio of 1:1. These salt mixtures become liquid once heated and solidified at ambient temperature. The schematic diagram of the reactor and salt bath (Thomas Kagaku Co. Ltd) used in this study has been provided by (Awaluddin et al., 2016) and (Zainan et al. 2019b)

Initially, the microalgae sample was weighted according to the biomass loading used and placed in a weighing boat. 5 ml of Mili-Q water was then poured into a weighing boat containing the microalgae. The mixture of microalgae and water is then transferred to the reactor and mixed using a vortex mixer.

The reactor was purged with argon gas for a few seconds to release trapped air inside the reactor and was tightly closed with Swagelok caps. Argon is selected as it is a noble gas and completely inert. Purging with Argon is faster due to the higher Argon mass compared to the mass of air. Argon was used in this study to create an inert atmosphere and remove oxygen to avoid corrosion.

Next, the reactor was immersed horizontally into the pre-heated molten salt bath heated to the desired temperature. The stirring speed of the salt bath in this study was set at 20 rpm. The reaction time was recorded after immersion of the reactor in the salt bath. The pressure within the reaction tube was estimated from the steam table under the subcritical conditions.

After the reaction process (based on desired time), the reactor was quenched in a room temperature water bath to stop the reaction. The reactor was cooled in the water bath for at least 2 hours to reduce the high pressure of gas generated inside the reactor.

For experiments involving different temperatures, initially, the salt bath temperature was set at desired low temperature. Once the reaction finished and the reactor was taken out, the salt bath temperature was set again (increased temperature) based on the desired temperature. After the desired set temperature had been reached, another reactor was immersed in the salt bath. The process continues until all the desired temperatures have been investigated.

The generated extract after the subcritical water process was poured into a centrifuge tube. The reactor was washed with 7 ml of Mili-Q water to wash any leftover microalgae residue left inside the reactors. The centrifuge tube was then centrifuged (KUBOTA 2420, Japan) at 4000 rpm for 5 min. The aqueous

phase (supernatant) was decanted and stored at 4 °C for further analysis. The microalgae residue was dried in the oven for 24 h at 60°C.

Production of amino acids at various subcritical water operating conditions

This experiment was performed to determine the effect of temperature, time, and biomass loading on the yield and composition of amino acids. The effect of each parameter was evaluated at varying values, while other parameters were fixed at specific values. For instance, the production of amino acids from microalgae at varying temperatures (160-350 °C) was conducted at a fixed time (10 min) and biomass loading concentration of 5% (w/v).

The selection of the initial temperature (160 °C) was based on the scanning electron microscope (SEM) results obtained by Ho et al. (2018), in which the microalgae *Nannochloropsis* cell wall just began to break at the temperature of 156 °C (Ho et al. 2018).

The selection of fixed time (10 min) is because it takes a while for a batch reactor to reach the set temperature due to the transition in temperature and pressure before reaching the desired reaction condition (Kang et al. 2001).

The selection of biomass loading concentration (5%, w/v) in this study was based on the result obtained by Awaluddin et al. (2016). The best temperature from the screening study was then selected to be evaluated on the effect of time.

The effect of reaction time on the production of amino acids was evaluated from 3-30 min. The temperature used in this experiment was fixed and based on previous screening results on the effect of temperature, while the biomass loading was maintained at 5% (w/v).

Finally, the effect of microalgae loading on amino acids production from subcritical water was conducted using 1% (0.05 g in 5 ml water), 3% (0.15 g in 5 ml water), 5% (0.25 g in 5 ml water), 7% (0.35 g in 5 ml water), 10% (0.5 g in 5 ml water) and 15% (0.75 g in 5 ml water) microalgae loading.

The range of microalgae loading selected in this study was based on the preliminary observation made when microalgae were mixed with water. The temperature and time used in this experiment were fixed and based on the previous screening results on the effect of temperature and time.

The concentration of amino acids in this study was analyzed using Ultra Performance Liquid Chromatography (UPLC).

Production of amino acids using acid hydrolysis method

0.1 g of microalgae *Nannochloropsis sp*. biomass was mixed with 5 ml of 6N HCl acid in a glass-sealed tube (Moore & Stein 1963). The sealed tube was put in the oven for 24 h at 110 °C (Mæhre et al. 2018; Moore & Stein 1963). After 24 h, the sealed tube was cooled, and 50 ml of internal standard (1 µM/ml of α-Aminobutyric acid) was transferred to the hydrolysate.

The solution was mixed with distilled water and made up to 100 ml volume. The mixture was vortexed, and only 10 µl of the solution was needed for the derivatization reaction. After the derivatization reaction, the analysis of amino acids was carried out using UPLC.

Production of amino acids using control experiment

Production of amino acids using the control experiment in this study was conducted at room temperature, 5% (w/v) biomass loading, and 10 min reaction time. The mixture was put in a centrifuge tube and

then vortexed throughout the reaction to ensure a proper mixing had been achieved. The centrifuge tube was then centrifuged (KUBOTA 2420, Japan) at 4000 rpm for 5 min. After that, the aqueous phase (supernatant) was decanted and stored at 4 °C for analysis of amino acids using UPLC. The microalgae residue was dried in the oven for 24 h at 60 °C.

Sample preparation for UPLC analysis

About 0.5 ml of aqueous sample from subcritical water (unknown) was mixed with 0.5 ml of α-Aminobutyric acid (AABA). The mixture was vortex to ensure a homogenous solution. 10 µl of the solution was then transferred to a 1.5 ml Eppendorf tube for derivatization, with 70 µl of borate buffer and 20 µl of AccQ reagent.

The mixture then vortexed at room temperature and was put in the oven for 10 minutes at 55 °C. The mixture was then transferred to a limited volume insert (LVI), and the LVI was placed in the UPLC.

About 2 µl of the content was injected into the UPLC (Waters, Water 2475 model e2695) for amino acid determination. AccQ Tag Ultra Column (2.1 x 100 mm) was used in this analysis, while AccQ Tag Ultra Eluent A and acetonitrile with formic acid were used as mobile phase. The injection mode used in this analysis was a partial loop needle overfill. The flow rate was 0.7 ml/min, while the column and sample temperature were 60°C and 20°C. TUV Detector @ 260 nm was used to detect the presence of the 16 amino acids in the sample.

RESULTS AND DISCUSSION

Amino acids production at various temperatures

The effect of temperature on the amino acid (AA) production from the subcritical water process in this study was conducted at temperatures 160-350 °C, 5% biomass loading, and 10 min time. Figure 1 shows the yield of essential and non-essential amino acids at various temperatures (160-350 °C). The total yield of amino acids represents the combination of essential and non-essential amino acids obtained from subcritical water of microalgae *Nannochloropsis sp.* biomass.

It could be observed that the yield of non-essential amino acids was higher than essential amino acids at most temperatures in this study. The highest yield of essential and non-essential amino acids was observed around 260 °C (345.98 mg AA/100 g algae) and 280 °C (671.94 mg AA/100 g algae), respectively.

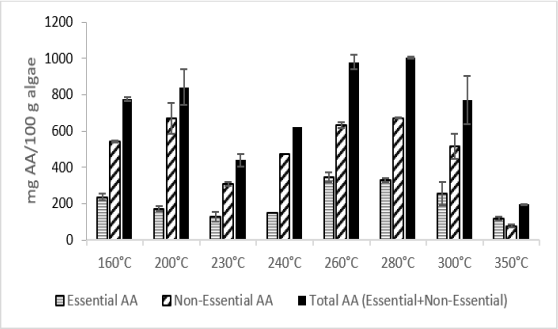

Fig. 1: The yield of essential, non-essential, and total amino acids at different temperatures with time and biomass loading fixed to 10 min and 5% (w/v).

The trend for total amino acid production at different temperatures seems inconsistent. The yield of total amino acids initially increased, starting from 160 °C to 200 °C, then decreased at 230 °C. It was observed that the yield of total amino acids increased again above 230 °C and reduced after 280 °C.

The highest yield of total amino acids (1001.76 mg AA/100 g algae) was obtained at 280°C. The yield then decreased after 280°C

because the amino acids are thermally labile at very high temperatures and decompose to other products such as ammonia, organic acids, carbonic acid, and amines (Abdelmoez et al. 2010).

The decrease of the total amino acids at a temperature of 230 °C might be probably due to the decomposition of some high molecular weight amino acids, which produced around 160-200 °C of subcritical water temperature. These high molecular weight amino acids might be decomposed into low molecular weight amino acids after 200°C temperature (Abdelmoez et al., 2010).

The yield of amino acids then started to increase again after the temperature was raised from 230 °C to 280 °C. At these temperature ranges (230°C to 280°C), the ion product of water (K_w) or also known as the water dissociation constant, is at maximum, and a high K_w value is useful for protein hydrolysis to amino acids (Kang et al. 2001).

Plaza and Turner (2015) reported the K_w increases from 1.0×10^{-14} at 25 °C to 1.2×10^{-12} at 350 °C, with a maximum value of 4.9×10^{-12} at 250 °C. The increases in K_w lead to an increase in hydrogen (H^+) and hydroxide (OH^-) ion concentration (Quitain et al., 2001; Sereewatthanawut et al. 2008). The H^+ ions attack the peptide bond and attach to the peptide bond's nitrogen atom (Zhu et al. 2011a). The attachment leads to splitting the peptide bonds into smaller molecules of amino acids. Hence, the presence of these ions (H^+ and OH^-) in subcritical conditions causes the peptide bond to break down into smaller molecules of peptide and amino acids (Sereewatthanawut et al. 2008).

Figures 2 and 3 show the yield of individual essential and non-essential amino acids at different temperatures. In this study, the essential amino acids being analyzed were Histidine (*His*), Threonine (*Thr*), Lysine (*Lys*), Methionine (*Met*), Valine (*Val*), Isoleucine (*Ile*), Leucine (*Leu*), and Phenylalanine (*Phe*). In contrast, the non-essential amino acids were Serine (*Ser*), Arginine (*Arg*), Glycine (*Gly*), Aspartic acid *(Asp*), Glutamic acid (*Glu*), Alanine (*Ala*), Proline (*Pro*), and Tyrosine (*Tyr*).

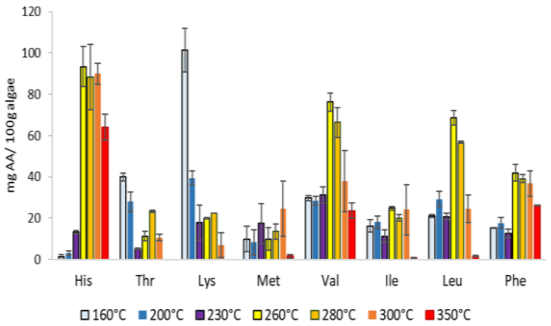

Fig. 2: The yield of essential amino acids at different temperatures with time and biomass loading fixed to 10 min and 5% (w/v)

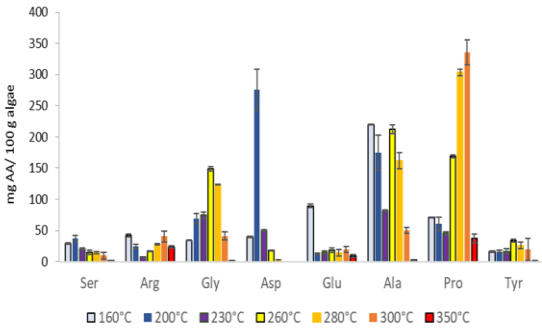

Fig. 3: The yield of non-essential amino acids at different temperatures with time and biomass loading fixed to 10 min and 5% (w/v)

Thr, Met, Ile, Phe, Ser, Arg, and *Tyr* produced less than 50 mg /100 g algae at most temperatures studied. Some of the reasons for the low yield of these amino acids might be due to (1) the subcritical water operating conditions are not suitable to produce these amino acids; (2) the microalgae *Nannochloropsis sp.* in this study might have a low yield of these amino acids; (3) the amino acids analysis method used in

this study might not be suitable to analyze these amino acids; or (4) the amino acids already decomposed at high temperature. To better understand these issues, let us discuss in more detail each of these amino acids.

As mentioned in the introduction part, the amino acids can also be classified into R-group. The R side-chain group consisted of hydrophobic non-polar aliphatic, hydrophilic polar aliphatic, hydrophobic and hydrophilic aromatic groups, and positively charged group and negatively charged group (Abdelmoez et al. 2010).

The negative charge groups consist of *Glu* and *Asp*. The *Glu* and *Asp* (Figure 3) are the non-essential amino acids and can be observed to be produced at low subcritical water temperature (160-200 °C) in this study. *Glu* produced the highest at 160 °C (89.34 mg/100 g algae) and *Asp* at 200 °C (275.60 mg/100 g algae). After the mentioned temperature, the yield of these amino acids decreased at high temperatures.

The reason for the decrease might be due to the size of amino acids. *Asp* (133 Da) and *Glu* (147 Da) are high molecular weight amino acids; hence, decomposed faster at high temperatures (Kang & Chun 2004b). *Asp* decomposes through deamination reactions to organic acids (pyruvic, malic, and fumaric acids), while *Glu* decomposes via lactamization/dehydration processes to produce pyroglutamic acids (Abdelmoez & Yoshida 2013; Yoshida & Tavakoli 2004).

Abdelmoez et al. (2010) also reported that *Asp* and *Glu* are polar amino acids, have carboxyl side chains, and are mostly found on the surface of globular proteins or polypeptides that interact favorably with the solvent molecule. The interaction of *Glu* and *Asp*, which is mostly located at the surface of protein with the subcritical water, might be the reason why *Glu* and *Asp* could be observed to be produced at a low temperature (between 160-200 °C).

The positive charge groups consist of *Lys, Arg,* and *His*. From Figure 2, it can be observed that *Lys* was the highest essential amino acid produced compared to other essential amino acids. The highest yield of *Lys* (101.28 mg/100 g algae) could be observed to be produced at low subcritical water temperature (160 °C). After this temperature, the yield of *Lys* decreased. *Lys* could be observed at low subcritical water temperature (160 °C) because *Lys* is a high molecular weight amino acid (146 Da). At low temperatures, the hydrolysis reaction is not favored compared to high temperatures. Hence, only high molecular weight amino acids such as *Lys* could be produced. As the temperature increases, *Lys* (high molecular weight amino acids) can decompose to *Val* (amino acids with a molecular weight of 117 Da) (Abdelmoez & Yoshida, 2013). This justified the reason for the decrease of *Lys* and increased *Val* as temperature increases.

In contrast to *Lys, His* in this study produced the highest at a temperature between 260-300 °C. The reason behind *His* production at high temperature is still unclear as the molecular weight of *His* is relatively high (155 Da). Esteban et al. (2010) indicated that amino acids with low molecular weights are more suitable to be produced at a high temperature than amino acids with high molecular weights. However, *His* production at a high temperature might probably be due to the structure of microalgae *Nannochloropsis sp*. used in this study. The accessibility of the subcritical water to microalgae is difficult due to the presence of the microalgae cell wall.

The highest yield of protein extracted from microalgae *Nannochloropsis sp.,* according to Zainan et al. (2019a), was

observed at 240 °C. Hence, *His* produced the highest at 260 °C after most of the protein had been extracted.

The *Arg* production in this study behaved very strangely. The production was low at most of the studied subcritical water temperatures, with the peak yield value observed at 160 °C. The yield of *Arg* then dropped to a lower value and then dramatically increased to a high value at 300 °C. Although the molecular weight of *Arg* is high (174 Da), *Arg* can still be observed at high temperatures. This is probably due to the stability of *Arg* in hot pressurized water.

The hydrophobic non-polar aliphatic group in this study consists of two non-essential (*Gly* and *Ala*) and four essential (*Met, Val, Ile,* and *Leu*) amino acids. The yield of *Gly* increased as temperature increased, and the maximum yield of *Gly* was obtained at 260 °C. The yield of *Gly* reached peak value at a temperature of 260 °C because *Gly* was found to be one of the most stable amino acids (Abdelmoez and Yoshida 2013). *Gly* is considered a stable amino acid as *Gly* has a low molecular weight (75 Da) and simple structure (only has an H atom as a side-chain group) (Abdelmoez and Yoshida 2013). Due to this, *Gly* is considered one of the amino acids suitable to be produced using subcritical water technology (Esteban et al. 2008). In the subcritical water process, *Gly* could be produced either by the peptide hydrolysis process or by a thermal transformation product from other amino acids (Abdelmoez et al. 2010).

The yield of *Ala* was higher compared to *Gly*, as *Ala* is stiffer than *Gly*. However, in this study, an inconsistent trend in *Ala* production was observed. The maximum yield of *Ala* was found to be at 160 °C, but it was suddenly reduced and increased again after 230 °C. Such fluctuation in the *Ala* yield is probably due to *Ala's* location in the protein structure and the pathway of *Ala* production in subcritical water. *Ala* is located at both the hydrophilic and hydrophobic regions of the protein structure. *Ala* was observed at low temperature as *Ala* located in a hydrophilic region and could easily interact with the water. As the temperature increased, the protein unfolded and exposed *Ala* which was located in the hydrophobic region. This could explain why *Ala* could be observed at low (160 °C) and high temperatures (260 °C). Similar to *Gly*, the molecular weight of *Ala* is also low (89 Da). Hence, it is not easy to explore the hydrolysis pathway of *Ala* as *Ala* could be produced via hydrolysis of protein and as the thermal transformation of *Ser* and *Cys* (Abdelmoez and Yoshida 2013).

Val and *Leu* were observed the highest at 260°C, and their trend of production was almost similar. However, the yield of *Val* was higher than *Leu*, probably because the molecular weight of *Val* (117 Da) is lower than *Leu* (131 Da). Hence, *Val* is more suitable and stable to be produced than *Leu*. Another reason for the high *Val* production is that *Val* could also be produced via the transformation of other amino acids (*Lys*) (Abdelmoez and Yoshida 2013). The production of *Met* and *Ile* in this study was low (less than 40 mg/ 100 g algae). These might be due to the hydrophobic nature of these amino acids. *Met* and *Ile* are usually located in the interior part of proteins shielded from direct contact with water. Hence, not easy to obtain.

The hydrophilic polar aliphatic group in this study includes *Ser, Thr,* and *Pro*. The yield of *Ser* and *Thr* in this study was lower compared to *Pro*. *Ser* and *Thr* behave similarly as their yield decreases as temperature increases. The reduction in their yield might be related to the location of these amino

acids, as *Ser* and *Thr* are often found buried on protein surfaces; hence, they had access to water (Abdelmoez and Yoshida 2013). Besides, *Ser* (105 Da) and *Thr* (119 Da) can be decomposed to *Ala* (89 Da) and *Gly* (75 Da) as temperature increases, which explains their low yield. In agreement with this study, Abdelmoez and Yoshida (2013) also reported that *Ser* and *Thr* production was generally low. Sato et al. (2004) stated that the decomposition temperature of *Ser* was 228°C, which also described the decrease of *Ser* at high temperatures.

Compared to *Ser* and *Thr,* the yield of *Pro* increases as temperature increases, and *Pro* is observed to be the highest at 300 °C. *Pro* has a molecular weight of 115 Da, and *Pro* is an intermediate product from the decomposition of other amino acids such as *Arg* (174 Da) (Abdelmoez and Yoshida 2013). Hence, this explains its high yield at high temperatures. Abdelmoez and Yoshida (2013) also reported that *Pro* produced the highest compared to *Ser* and *Thr* due to its high stability.

The hydrophilic and hydrophobic aromatic groups include *Tyr* (non-essential) and *Phe* (essential). The yield of *Tyr* and *Phe* increased as temperature increased, and the highest yield of *Tyr* (33.76 mg/ 100 g algae) and *Phe* (41.89 mg/ 100 g algae) were observed at 260 °C. Both amino acids are mainly located to be buried in the hydrophobic interior part of proteins, explaining why they are produced at high temperatures (260 °C). The yield of these amino acids (*Tyr* and *Phe*) is low in this study as the molecular weight of *Tyr* (181 Da), and *Phe* (165 Da) is high and could be decomposed easily. High molecular weight amino acids were prone to degradation and decomposed faster than low molecular weight amino acids (Kang and Chun 2004b).

The above discussion indicates that each amino acid was produced at a specific temperature. Up to this point, it could be assumed most of the high molecular weight amino acids produced at a lower temperature (less than 230 °C). In comparison, the low molecular weight amino acids are mainly produced at a higher temperature (more than 230 °C).

Moreover, the location of these amino acids in the protein also affected the production of these amino acids. Amino acids located at the protein's surface could interact with the water, hence being produced at a lower temperature, while amino acids located at the interior part of the protein are primarily produced at a higher temperature.

The R-groups of the amino acids also affected the production of the amino acids as the hydrophilic amino acids were produced at a lower temperature. In comparison, the hydrophobic amino acids are mainly produced at a higher subcritical water temperature.

Amino acids production at various time

The effect of time on amino acid production was conducted at a temperature of 280 °C and 5% biomass loading. The main guiding factor for selecting process parameters in this work was based on the highest yield of total amino acids obtained at various temperatures. Figure 4 shows the yield of essential, non-essential, and total amino acid production at different times.

The highest yield of essential (353.36 mg AA/100 g algae) and non-essential amino acids (772.44 mg AA/100 g algae) was observed at 20 min reaction time. The yield of total amino acids was increased linearly from 3 min (581.11 mg AA/100 g algae) to 10 min (1001.76 mg AA/100 g algae). At this period (3-10 min), the yield of amino acids increased

to 420.65 mg AA/100 g algae. Above this time (10 to 20 min), the increase in the yield of amino acids was insignificant, as only 124.04 mg AA/100 g algae were increased during that period. The amino acids then slightly decreased at 30 min reaction time. Kang et al. (2001) and Zhu et al. (2011a) reported that under subcritical conditions, the yield of amino acids increases with increasing time until it reaches a point when decomposition is favored over the production of amino acids.

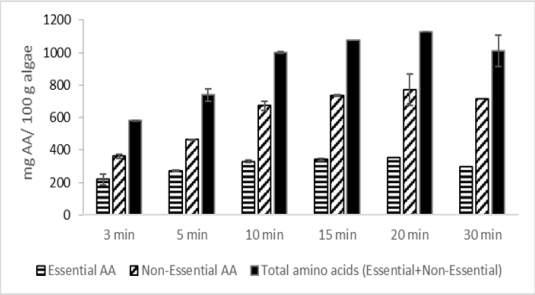

Fig. 4: Amino acids production at 280 °C, 5% (w/v) biomass loading, and various reaction times (3-30 min)

The yield of amino acids in this study increased over time because the hydrolysis process is dominant at a temperature of 280 °C. The ionization constant of water increases at high subcritical water temperature, leading to an increase in hydrogen and hydroxide ions (Sereewatthanawut et al. 2008).

The increase of these ions in subcritical water is suitable for amino acid production as amino acids are mainly produced from hydrolysis reactions. As the time prolonged, the contact time between water containing hydrogen and hydroxide ions to the soluble protein increased, enhancing hydrolysis. Further increase in time allows these ions to have more time to hydrolyze any soluble protein into peptides and amino acids, which explains why the increase of these amino acids as the time increases.

The decreased yield of amino acids at 30 min reaction time might be due to the high temperature causing these amino acids to decompose to organic acids.

The yield of individual essential and non-essential amino acids at different time are shown in Figure 5 and Figure 6. *His* yield in this study decreased as time increased because *His* is a high molecular weight amino acid. As time progressed, the H^+ ions had more time to cleave the high molecular weight amino acids such as *His* to low molecular weight amino acids, which decreased its yield.

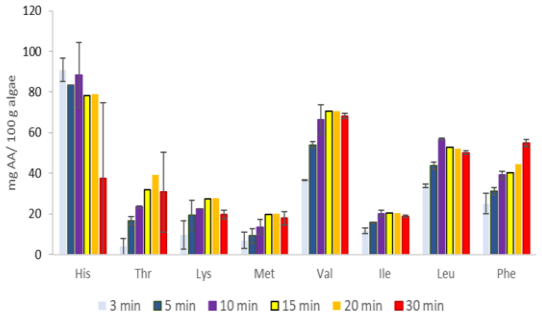

Fig. 5: The yield of essential amino acids at different times, 280 °C and 5% (w/v) biomass loading

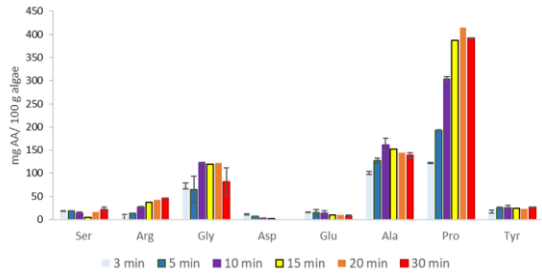

Fig. 6: The yield of non-essential amino acids at different times, 280 °C and 5% (w/v) biomass loading

On the contrary, amino acids such as *Thr, Lys, Met, Val, Ile, Leu, Phe, Arg, Gly, Ala,* and *Pro* increased as time increased in this study. However, the yield of some amino acids in this study remained plateau after 15-20 min reaction time.

Essential amino acids such as *Val, Leu,* and *Phe* produced higher than *Thr, Lys, Met,* and *Ile* as the temperature used in this study (280 °C) was suitable for the production of *Val, Leu,* and *Phe*. There was no substantial difference in the yield of *Lys, Met, Ile, Leu, Thr,* and *Val* after 15 min reaction time.

Pro was among the dominant non-essential amino acids obtained in this study (Figure 6). The yield of *Pro* increased as time increased, and the yield of *Pro* reached a maximum value at 20 min reaction time. A high yield of *Pro* at 20 min reaction time was also observed using the microwave irradiation technique (Chen et al. (2015). However, no further discussion regarding the reason behind the high yield of *Pro* (Chen et al. 2015). We believed the reason might be due to the stability of *Pro* in subcritical water. *Pro* is a low molecular weight (115 Da) amino acid, and *Pro* could be produced from the decomposition of other amino acids such as *Arg* with a molecular weight of 174 Da (Abdelmoez & Yoshida, 2013).

Other non-essential amino acids obtained in this study were *Gly* and *Ala*. It has already been mentioned that *Gly* and *Ala* were dominant as these amino acids resulted from the peptide hydrolysis and decomposition of other high molecular weight amino acids (Chen et al., 2015). Besides, *Gly* and *Ala* are low molecular weight amino acids and simple in structure, making these amino acids suitable to be produced using the subcritical water technique. The yield of *Gly* and *Ala* in this study decreased as the time reached 30 min. However, some studies found that the yield of *Gly* and *Ala* can further increase up to 40 to 50 min reaction time (Chen et al. 2015; Yoshida et al. 1999).

There were no significant differences in the yield of *Ser*, *Asp*, *Glu,* and *Tyr* as time proceeded from 3 to 30 min. The yield of *Ser*, *Asp*, and *Glu* was lower in this study as these amino acids produced the highest at low temperatures (between 160-200 °C), while this study was conducted at 280 °C. The low yield of *Tyr* might probably be due to the composition of *Tyr* in microalgae *Nannochloropsis sp*. in this study was small.

Amino acids production at various biomass loadings

Figure 7 shows the yield of amino acids at different biomass loadings. It could be observed that the yield of non-essential amino acids was higher than the essential amino acids at all biomass loading tests. The total amino acids decreased as the biomass loading increased from 1 to 5% (w/v). The yield of amino acids remained almost constant as the biomass loading increased from 5 to 15% (w/v).

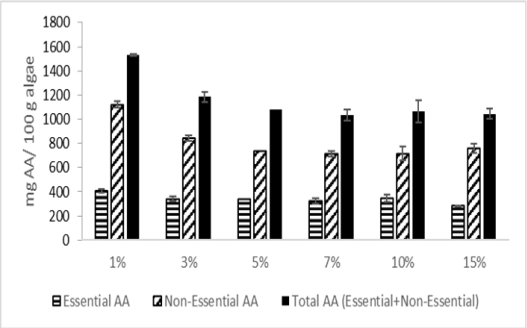

Fig. 7: Amino acids production at 280 °C, 15 min reaction time, and 1-15% (w/v) biomass loading.

In the general case, an increase in the initial substrate concentration favors the production of products. However, this is not the case for amino acid production in this study, as the yield of amino acids decreased when biomass loading increased. This might be due to the excess of an insoluble substrate that could interfere with the cleavage of peptide bonds, affecting the hydrolysis

process and decreasing its yield (Esteban et al. 2010). The excess substrate in the reactor might reduce contact of the peptide bonds with the water (Esteban et al. 2010). The yield of amino acids remained constant after 5% biomass loading as the reaction time (15 min) and the volume of water (5 ml) used for this study were constant.

The yield of amino acids probably could be increased if the time and amount of water increased as the water would have more time and contact to hydrolyze the protein. Ho et al. (2018) reported that a reduction in biomass loading or increased water volume increased the lipid yield of microalgae *Nannochloropsis gaditana*. This clearly showed that the increase in the amount of water might help to disrupt the algal cell wall, solubilize the protein content in the biomass and hydrolyze the protein to the amino acid. However, at this stage, this study focuses on one factor at a time and does not consider any interaction between the parameters tested.

The results indicate that only 1% microalgae loading is needed for high-yield amino acids in this study at the subcritical water temperature of 280 °C and 15 min reaction time. The amino acids obtained from these subcritical water operating conditions (280°C, 15 min, and 1% biomass loading) were the highest (1531.98 mg AA/100 g algae) compared to other subcritical water operating conditions tested at various temperatures and times of this study.

Figures 8 and 9 show the yield of individual essential and non-essential amino acids at different biomass loadings. *Thr, Lys, Val, Ile, Leu, Phe, Ser, Glu, Gly, Ala,* and *Pro*, were the types of amino acids that produced the highest at 1% biomass loading in this study.

Among the amino acids obtained in this study, *Val, Leu, Phe, Gly, Ala* and *Pro* produced higher than *Thr, Lys, Ile, Met, Arg, Asp,* and *Tyr*. Amino acids such as *Val*, *Leu*, *Phe*, *Gly*, *Ala,* and *Pro* produced the highest might be due to the suitability of these amino acids to be produced at this temperature range (260-280 °C). Besides, *Gly*, *Ala*, *Val*, and *Pro* are low molecular weight amino acids, simple in structure, and can be produced via transformation of other amino acids (Abdelmoez et al. 2010; Esteban et al. 2008), which explains their high yield.

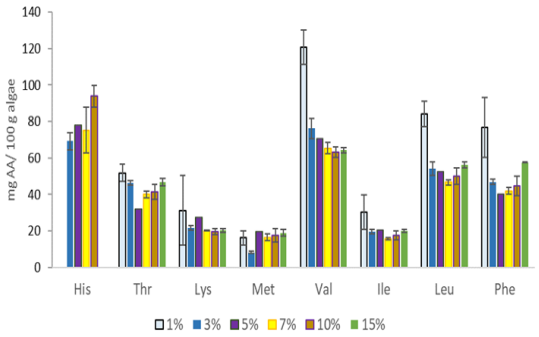

Fig. 8: The yield of essential amino acids at different biomass loadings, 280 °C temperature, and 15 min reaction time.

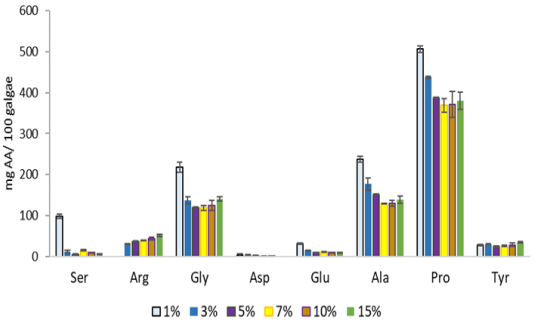

Fig. 9: The yield of non-essential amino acids at different biomass loadings, 280 °C temperature, and 15 min reaction time.

Amino acids such as *Thr*, *Lys*, *Asp*, and *Glu* produced less (from Figures 8 and 9) as these amino acids were observed the highest at 160-200 °C (from Figures 2 and 3), while this study was conducted at 280 °C. Other amino

acids (*Met*, *Ile*, *Arg*, *Ser,* and *Tyr*) also produced less as these amino acids (*Met*, *Ile*, *Arg*, and *Tyr*) showed an insignificant effect on temperature, which then affected the production of these amino acids in this study.

The results indicate the production of individual amino acids at different biomass loading, mainly depending on the temperature selected. Other reasons might be due to the location of some of these amino acids in protein structure or the decomposition of high molecular weight amino acids to low molecular weight amino acids at a high temperature in this study (280 °C) (Abdelmoez et al. 2010). Compositions of amino acids in the original microalgae and selection methods for amino acid analysis might also be other reasons for less amino acid production.

The reason behind *His* production trend in this study is still unknown. The yield of most amino acids (*Thr*, *Lys*, *Val*, *Ile*, *Leu*, *Phe*, *Ser*, *Glu*, *Gly*, *Ala,* and *Pro*) in this study decreased after 1% biomass loading. The decrease might occur because of an excess of the substrate that could interfere with the cleavage of peptide bonds or constant reaction time (15 min) and volume of water (5 ml), leading to an incomplete hydrolysis process.

Comparison of amino acids obtained from the control experiment, acid hydrolysis, and subcritical water process

Figure 10 shows a comparison of the yield of amino acids obtained from the control, subcritical water process, and acid hydrolysis experiment.

The control experiment was conducted as a reference control, and the use of water in this controlled study was not considered a cell disruption technique. The net movement of water molecules through a partially permeable membrane into a region of higher solute concentration is known as osmosis (Safi et al. 2014).

Water travels through the membrane, chloroplast, mitochondria, and vacuole by diffusing across the phospholipid bilayer via water channels (aquaporins). These aquaporins are channel proteins embedded in the cell membrane of microalgae and facilitate water transport between cells.

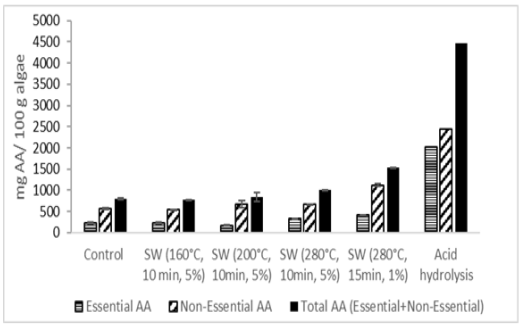

Fig. 10: The yield of amino acids from the control study, acid hydrolysis, and subcritical water (SW) at different operating conditions

Although the cell wall of microalgae is not disrupted, surprisingly, amino acids can still be observed in the control study (Figure 10). This indicates the water can penetrate the cell wall of microalgae *Nannochloropsis sp.* biomass through the osmosis process, release the protein, and hydrolyze this protein to amino acids.

Using vortex in this study might aid in the release of protein as the microalgae collide between microalgae and water molecules. Although the control study was conducted at room temperature, water self-ionization might still help hydrolyze the released protein to amino acids. Safi et al. (2014) also reported that about 6-10% of proteins were released from green microalgae by the osmosis phenomenon. Quitain et al. (2001) also observed the presence of amino acids

when the shrimp was soaked in water for 30 min. According to Quitain et al. (2001), this might be due to the amino acids soluble in water.

It could be observed that there is no significant difference in the yield of amino acids from the control study (796.65 mg AA/100 g algae) and the subcritical water process that operated at 200 °C, 10 min time, and 5% biomass loading (841.15 mg AA/100 g algae). Based on Eq. (1), only a 5.59% increase in the amino acid yield could be observed when subcritical water temperature increased to 200°C.

$$\%AA = \frac{yield\ AA\ (SW) - yield\ AA\ (control)}{yield\ of\ AA\ (control)} x100 \quad (1)$$

The result indicates an only a small amount of amino acids could be obtained from subcritical water of microalgae *Nannonchloropsis sp.* biomass at this temperature (200 °C).

A high yield of amino acids in this study could be observed above 240 °C, as the hydrolysis most likely occurred after 240 °C (Zainan et al. 2019a). This justifies the results, as a 25.75% increase in amino acids could be observed when the subcritical water process operated at a temperature of 280 °C, 10 min, and 5% biomass loading, and a 92.30% increase in the amino acids yield at 280°C, 15 min, and 1% biomass loading.

Zhu et al. (2011b) reported that temperature below 180 °C was considered low enough for a subcritical water hydrolysis reaction to occur. A significant increase in amino acid yield was only observed above 180°C (Zhu et al., 2011b). Sereewatthanawut et al. (2008) mentioned that the hydrolysis process was unlikely to occur at subcritical water conditions of 200 °C and 30 min.

The yield of non-essential amino acids obtained from the control and acid hydrolysis process (Figure 10) was also higher than essential amino acids, which showed that the *Nannochloropsis sp*. biomass in this study has a high content of non-essential amino acids. The yield of total amino acids in this study was the highest via the acid hydrolysis process (4460.98 mg/100 g algae) compared to control and subcritical water technology operated under different conditions.

Based on Eq. (2), only 34% of amino acids could be recovered via subcritical water processes conducted at 280 °C, 15 min and 1% biomass loading (1531.98 mg/100 g algae) compared to the acid hydrolysis process. However, the result is considered good as the time required was 15 min, and only water was used instead of corrosive acid.

$$\%AA = \frac{yield\ of\ AA\ (SW)}{yield\ of\ AA\ (acid\ hydrolysis)} x100 \quad (2)$$

Quitain et al. (2001) and Espinoza et al. (2011) also found that the overall yield of amino acids using subcritical water was relatively low compared to that in acid hydrolysis (D Espinoza et al. 2011). Quitain et al. (2001) found that only 70 mg AA/g shrimp shells could be recovered at subcritical water conditions of 250 °C, 60 min, and 1:125 weight ratio compared to 300 mg AA/g shrimp shells when using acid hydrolysis. However, similar to this study, their goal for carrying out the research was to develop a process that could recover valuable products using a sustainable and environmentally friendly technique (Quitain et al. 2001). The yield of amino acids in this study could be enhanced by various operating conditions, change in the mode of operation (from batch to continuous) (Kang & Chun 2004a), or the addition of acid catalyst (CO_2).

Figures 11 and 12 show the yield of individual essential and non-essential amino acids obtained from the control experiment, acid hydrolysis, and subcritical water operated under different operating conditions.

Based on the acid hydrolysis results, *Lys* and *Leu* were the dominant essential amino acids in microalgae *Nannochloropsis sp.* biomass. The sequence of amino acid composition in this study is similar to microalgae *N. oculata* (Brown, 1991). Brown (1991) reported that *Leu* was the dominant essential amino acid obtained from microalgae *N. oculata* using acid hydrolysis, followed by *Val, Phe, Lys, Thr, Ile, His,* and *Met*. It could also be observed, *Glu* was the highest non-essential amino acid derived from the acid hydrolysis process in this study, followed by *Asp, Ala, Arg, Gly, Pro, Ser*, and *Tyr*. The sequence of non-essential amino acids produced from microalgae *N. oculata* was *Glu* > *Pro* > *Asp* > *Arg* > *Ala* > *Gly* > *Ser* > *Tyr* (Brown, 1991).

Most of the individual amino acids (except *Pro*) produced from the acid hydrolysis process were higher than the individual amino acids produced from the control and subcritical water process. The yield of *Pro* via subcritical water was higher than *Pro* produced via acid hydrolysis, probably due to the combination of both processes (peptide hydrolysis and transformation of other amino acids to *Pro*) during the subcritical water reaction (Abdelmoez & Yoshida 2013).

Abdelmoez et al. (2010) reported that *Arg* could decompose into *Pro* through the aldol cleavage reaction. *Arg* is considered a high molecular weight amino acid (174 Dalton). Thus, at high temperature and reaction time, *Arg* decomposed to *Pro*, whose molecular weight is lower (115 Dalton).

Figures 11 and 12 also show the yield of *His, Thr, Met, Val, Ile, Leu, Phe, Ser, Gly, Asp, Ala, Pro,* and *Tyr* were higher than in the control experiment. A higher yield indicated the suitability of these amino acids to be produced using the selected range of parameters tested in the subcritical water process of this study.

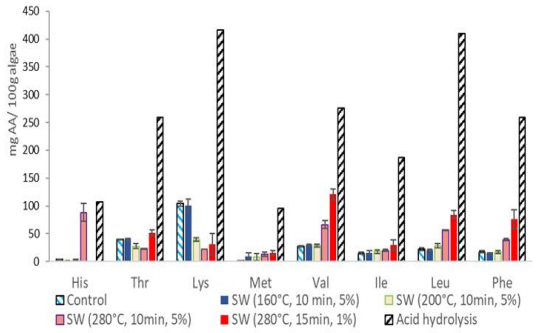

Fig. 11: The yield of essential amino acids from the control study, acid hydrolysis, and subcritical water (SW) under different operating conditions.

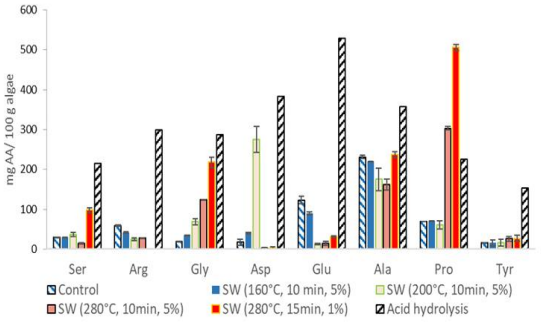

Fig. 12: The yield of non-essential amino acids from control study, acid hydrolysis and subcritical water (SW) under different operating conditions

Lys, Arg, and *Glu* produced a higher yield in the control study than in the subcritical water process tested in this study. The results indicate that the high-temperature condition (above 200 °C) is not suitable for the production of *Lys, Arg*, and *Glu*. The yield of *Lys, Arg,* and *Glu* was also observed the

highest using the acid hydrolysis process (110 °C) and subcritical water process that operated at low temperature (160 °C) of this study. The decrease in *Lys* and *Arg* production at high subcritical water temperature might be due to the transformation of *Lys* to *Val* and *Arg* to *Pro* via the aldol cleavage reaction. It could be observed that the yield of *Val* and *Pro* in this study increased as the temperature increased.

CONCLUSIONS

Overall findings from this study show that proper control of temperature, time, and biomass loading is crucial to acquiring a high yield of amino acids. The study also demonstrated that each of the individual amino acids investigated produced the highest at different ranges of subcritical water conditions. High molecular weight amino acids are mainly produced at a low temperature (160-200 °C), while the low molecular weight amino acids are produced at a high temperature (230 °C and above). Most amino acids produced the highest at the time between 20-30 min and 1% biomass loadings. Amino acids that are located at the protein's surface could interact with the water, hence being produced at a lower temperature, while amino acids located at the interior part of the protein are mostly produced at a higher temperature. The hydrophilic amino acids are produced at a lower temperature, while the hydrophobic amino acids are mainly produced at a higher subcritical water temperature. The studied operating conditions produced a higher yield of non-essential amino acids. Although the yield of amino acids from the subcritical water technique in this study is lower than the conventional method (acid hydrolysis), the subcritical water is still considered more efficient since the goal of the research was to develop a process that could recover valuable products quickly, using a sustainable and environmentally friendly technique.

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NOMENCLATURE

AA	:	Amino acids
His	:	Histidine
Ser	:	Serine
Arg	:	Arginine
Asp	:	Aspartic acid
Glu	:	Glutamic acid
Thr	:	Threonine
Gly	:	Glycine
Pro	:	Proline
Met	:	Methionine
Ala	:	Alanine
Ile	:	Isoleucine
Val	:	Valine
Tyr	:	Tyrosine
Leu	:	Leu
Phe	:	Phenylalanine
Lys	:	Lysine
Da	:	Dalton

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