Towards the use of microalgae as a source of alternative protein : using plant extract as a growth enhancer, protein quality quantification, and a method of biochemical content estimation

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TOWARDS THE USE OF MICROALGAE AS A SOURCE OF
ALTERNATIVE PROTEIN: USING PLANT EXTRACT AS A
GROWTH ENHANCER, PROTEIN QUALITY
QUANTIFICATION, AND A METHOD OF BIOCHEMICAL
CONTENT ESTIMATION

DARYL LEE

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2021

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PROTEIN: USING PLANT EXTRACT AS A GROWTH ENHANCER, PROTEIN
QUALITY QUANTIFICATION, AND A METHOD OF BIOCHEMICAL
CONTENT ESTIMATION

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School of Chemical and Biomedical Engineering

A thesis submitted to the Nanyang Technological University

in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

2021

Statement of Originality

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I cherish every moment throughout the entire period and without a doubt, Prof.

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Daryl Lee, 2020

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Summary

Food security, increase in global population, ecological impacts of climate change, and greater health awareness have led to the recent rise in alternative protein products. One such product is microalgae. Low technology readiness level and high cost of production, however, have greatly hampered its commercial viability. A simpler and more direct way to improve the economics is to increase the biomass productivity or output of the process.

Hence, the first objective of this thesis is to investigate the use of herbs and spices to determine if its use could improve the growth of microalgae. Herbs and spices of different concentrations and forms were screened in the study. It was found that onion extract was the most effective and managed to double the growth rate and increase the cell density to about 6 times that of control.

The second objective is to determine the microalgal protein digestibility, specifically the PDCAAS method to determine the protein quality of microalgae. This is important if the microalgae (whether whole or extracted proteins) are to be used as food to supply humans with essential amino acids (and nutrients). The PDCAAS value was found to be relatively high, 0.88, comparable to beef and soy. The digestibility was found to have a maximum value of 1 but dropped to 0.88 due to the lack a certain amino acid (methionine).

It is said that the biorefinery approach is the way to go for a microalgae production process that is viable and economical. High value products are produced and sold alongside cheaper and lower value commodity products. However, the technologies available for microalgae are still at an infancy and a lack of understanding of the processes is hindering its further development. The third objective of this thesis is thus

to develop a method to estimate the biochemical composition of the biomass that avoids the laborious and time-consuming disruption/extraction/measurement processes. Data available from literature were used to evaluate the performance of the prediction model and it was found to be highly predictive with low root mean squared errors.

1 Introduction

1.1 Background

With the rise of global population and climate effects, alternative and more sustainable food/protein sources are needed. And with the recent emergence of deadly pandemic like COVID-19 exacerbating the food security issues, coupled with recent inclination towards healthier and more sustainable non-meat-based proteins, various alternative protein sources have emerged. Some examples are insect proteins, cultured meat, legumes/soybean-based proteins, and microalgae-based proteins [1]. Microalgae is said to be the mother of all plants on Earth and said to account for more than half of all the oxygen produced. Microalgae have been researched for probably almost a century. Its applications however are currently still towards the high-value products such as nutraceuticals, pigments, and pharmaceuticals [2]. Its use for lower value commodity products such as biofuel and food have not achieved much success due to the high cost of processing (downstream) [3].

1.2 Hypotheses

Economic viability of the production process is highly dependent on the biomass productivities achievable. Hence, one of the aims of this work is to improve the biomass productivity of the microalgae. A higher productivity or biomass output of the production process can significantly impact the economics of the process. Secondly, even though microalgae have been around for quite some time, their digestibility is still unclear. Methods available are often unstandardized whereas digestibility results reported vary widely, partly because of the methods used and also partly because of the cultivation/processing that the biomass has undergone (freeze-dried vs. sun-dried

or different methods of cell disruption or different stage of harvest). In this work, it is one of the aims to perform and report a more standardized method of determining the protein digestibility of microalgae, or any protein-rich products for that matter. Lastly, because of the vast number of species available with different characteristics, different growth conditions and modes carried out, and various processing technologies available and their different combinations, determining the biochemical composition of the biomass easily, quickly, and accurately is a challenge. This is even more apparent considering the complexity of the extraction process and the various non-standardized methods available that are not tailored to a certain species or a once-size-fits-all. Hence, it is the final aim of this work to develop a method that is easy, accurate, and fast while at the same time not relying on completely new technologies or requiring huge technical expertise.

1.3 Objectives

Therefore, the first objective of this thesis is to improve the productivity of microalgae through the use of herbs and spices. This has been done elsewhere, such as in aquaculture and agriculture to improve productivity and reduce the harmful bacteria contamination. Different herbs are screened, in different forms and different concentrations, to identify potential natural chemical promoters of growth/productivity (Chapter 3). The different biochemical contents are also quantified to investigate the effect of these herbs/spices' additions.

In chapter 4, the objective is to determine the protein-digestibility corrected-amino acid score (PDCAAS) of *Chlorella* biomass. This method was recommended by FAO/WHO as the preferred method. The method involves two parts. First, the

digestibility of the proteins is determined by enzyme hydrolysing the sample to simulate the gastric digestion in the body and then measuring the released amino acids. It is also important to determine, even if the proteins are digested, the amino acid profile/composition of the proteins digested. The amino acids available after digestion are needed to supply the essential amino acids needed by humans. The lack of essential amino acids makes the protein a poor quality one, even though it's completely digestible.

The last objective of this thesis is to develop a method that is able to predict the biochemical composition of a biomass through elemental analysis. This quick and easy method can potentially speed up the development process of producing microalgae biomass in a viable and commercial manner. The prediction model is then validated using results from literature and it's accuracy (or error) is reported (Chapter 5)

2 Literature review

2.1 Background

Growing at a rate of 1.07% annually, the current global population of about 7.7 billion is expected to reach close to 10 billion by mid-century [1]. The projected rise in consumption of meat and dairy products, 173% and 158%, respectively, clearly will lead to further ecological problems and climate changes [4]. It is therefore necessary to reassess our food sources (animal vs. plant-based) and to adopt a more sustainable and secure approach as we navigate into the future.

Microalgae are one potential source of proteins [5]. When considering the environmental impact of a product (food/protein in this case), there are three primary indicators that are often used. On a basis of per kg product, they are the greenhouse emissions (GHGs), water consumption, and land use efficiency. The GHG emission for whole algae protein is shown in Figure 2-1 below.

Figure 2-1 Carbon footprint of whole algae protein compared with other sources of proteins. Taken from [6].

One observation from Figure 2-1 is that the carbon footprint (in terms of kg CO₂ equivalent released per kg protein produced) for beef production is close to 40 times that of whole algae protein. And although whey has a much lesser carbon footprint, it is still about 10 times that of microalgae. Microalgae in general, is on par or lesser in terms of carbon footprint when compared with vegan sources of protein [6].

The water consumed in the production of proteins is shown in Figure 2-2 below for whole algae proteins, as well as other conventional sources of proteins.

Figure 2-2 Water consumption per weight protein produced for whole algae proteins and conventional protein sources. Taken from [6].

Again, beef and whey account for much higher consumption of water per kg protein produced, about 40 and 7 times more than that needed for microalgae. The low

consumption of water for microalgae, even when compared with crops, is due to the fact that much of the water used in the production process can be recycled back after the harvesting process.

Lastly, the land use efficiency shown in Figure 2-3 below measures the productivity of a protein production process on a per area basis.

Figure 2-3 Protein yield per hectare for whole algae protein and conventional sources of proteins. Taken from [6].

Due to its high growth rates and high protein contents, microalgae have one of the highest protein yields per unit area of land.

2.2 Microalgae

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that are able to live in a wide range of environmental conditions, both aquatic and terrestrial [7].

Microalgae have immense potential for the production of biofuels and bioproducts. They have high growth rates (doubling times as low as 7-8 hr [8]) and high concentrations of valuable compounds therefore, they have high productivities. Microalgae could generate between 58700 and 136 900 L/ha/yr of biodiesel (30 or 70% lipid content) compared to 5366 L/ha/yr for the best terrestrial biodiesel crop, palm oil [7]. Other valuable compounds are pigments, vitamins, and antioxidants to generate nutraceuticals, pharmaceuticals, functional foods and food additives, farm and aquaculture feed, biofuel, cosmetics and bioplastics [7,9,10].

The price volatility of crude oil and the expected future price increase, combined with the urge to reduce greenhouse emissions, has renewed interest in the production of biodiesel from microalgae recently [11]. After the World War II, concerns regarding rapid population growth and food shortages sparked interest in microalgae and early works in the 1950s showed good potential. However, advances in traditional agriculture and significant scale-up challenges made the microalgal technology less attractive [6]. In addition, its utilization as a source of protein in mainstream foods was greatly hampered by its colour, flavour, and lack of consistency.

2.3 Microalgae applications

2.3.1 Food supply and health foods

By mid-21st century, the human population is expected to reach around 10 billion people and food production must increase by 50% by then [12]. Currently, about one billion people already have insufficient protein intake [13]. And it is predicted that conventional sources of protein would be insufficient and alternative sources of protein have to be found [13]. Plant proteins are currently the main source of protein

intake worldwide for food and feed [14]. Health concerns could give a boost to plant-based sources of protein [14]. Switching from animal protein to plant protein is more sustainable [15,16]. In Germany for example, switching from an omnivore diet to a vegan one can reduce food-based greenhouse gas emissions by 50% [14]. Further exploiting the crop cultivation process, which is already highly optimized, could aggravate environmental problems such as deforestation, loss of biodiversity, and land degradation. Food production accounts for 20-30% of total environmental impact and 30% of global greenhouse gas emissions [14]. Current plant protein and animal protein production systems are not sustainable hence, in the long term, a novel source of protein is needed [15,16].

Microalgae can present as a more sustainable alternative source of protein. Based on WHO/FAO/UNU recommendations on the essential amino acids (EAAs) requirements, microalgae have favourable amino acid profiles [5]. They are also a good source of essential minerals, vitamins, and antioxidants.

2.3.2 Aquaculture feed

Aquaculture, the farming of aquatic organisms, is one of the fastest growing food-producing sectors [17]. The production of fish meal and fish oil as feeds for aquaculture is predicted to reach its maximum sustainable yield within the next decade, driving up their prices drastically [18,19]. Hence, an alternative aquafeed is needed to meet the high demands from aquaculture [20,21].

Microalgae can reduce or even replace conventional aquafeeds. Microalgae have good nutritional qualities, are able to grow in a wide range of habitats, and can improve the growth and resistance to diseases of organisms farmed in aquaculture [5,19,22,23].

2.3.3 Biofuels

Biofuels that come from food crops have disadvantages such as low land use efficiency, low lipid content, high water requirements, and food-fuel competition [24]. The use of microalgae yields oil at a fraction of the land required for conventional crop-based biodiesel sources [24]. In addition, microalgae avoids the food versus fuel debate, or using valuable cropland for cultivation [24]. Biofuel production by microalgae can reduce the net emissions of carbon, be easily implemented, and act as buffer for oil prices [25].

2.4 Microalgae cultivation and production

2.4.1 Cultivation modes and configurations

Phototrophic cultivation

Phototrophic mode of cultivation is the growth mode most commonly employed where the microalgae are grown in transparent photobioreactors or in open ponds [26–28]. Artificial lighting or natural sunlight is absorbed by the microalgae and is converted through photosynthesis to chemical energy. Light is the source of energy while inorganic carbon is the carbon source. Inorganic carbon in the form of carbon dioxide is used as the source of carbon and can be converted with the help of the chemical energy to other useful molecules for cell growth and maintenance. As the concentration of cells increases however, the light penetration into the culture decreases exponentially. Hence, this mode of cultivation suffers from low biomass concentration and productivity [29–31]. Many research are focused on the design of

the photobioreactors, either to better utilize the natural sunlight or to employ artificial lighting more efficiently.

Organic carbon source is not required, CO₂ can be sequestered, and this mode is less expensive than heterotrophic and mixotrophic cultivation [32]. Disadvantages include low cell concentration, light source requirement, and may be photo-inhibited at high light intensities [25,33].

Heterotrophic cultivation

In heterotrophic cultivation, the organic compounds added act both as the carbon source and the source of energy. And since in this cultivation, light is not required, it does not suffer from light attenuation issues and thus, higher biomass productivities and concentrations can be achieved. In addition, lipid content under heterotrophic cultivation is generally at par or higher than that under phototrophic mode, leading to even greater lipid productivity and lower cost of downstream processing. Microalgae can be cultivated in well-established fermenters used to grow other microbials such as yeast and bacteria [34]. Organic compounds that could be utilized include sugar, organic acids, and even cheaper sources such as corn powder hydrolysate and glycerol. However, the cost of heterotrophic cultivation is a lot higher than phototrophic cultivation because of the addition of organic substrates and the expensive bioreactors [35,36]. It has been said that this mode of cultivation is not feasible (at least for biodiesel production), unless the residues can be sold at a high price and intracellular oil can be completely extracted. [36]. Another drawback of this mode of cultivation is that it suffers from contamination issue especially from bacteria, which may affect the

quantity and quality of the desired products [33]. Achieving sterility is therefore crucial for this mode of cultivation, which further adds to the cost.

Mixotrophic cultivation

In mixotrophic cultivation, both inorganic and organic carbon are utilized. Organic carbon is used as the source of energy and carbon while inorganic carbon is used along with light as the source of energy [37,38]. This is not to be confused with photoheterotrophy, which requires light for the assimilation of organic carbon. The specific growth rate of microalgae under mixotrophic cultivation is roughly the sum of that under phototrophic and heterotrophic cultivation [39]. The two metabolic processes (photosynthesis for phototrophy and respiration for heterotrophy) may have synergistic effects and enhance productivity [40–43]. Since organic compounds can be used, the growth of microalgae does not strictly depend on light availability. Light availability instead complements the organic substrates, leading to improved growth rates, faster growth cycle, reduced biomass loss due to pure respiration during dark period of phototrophy and hence, increased biomass productivity [44,45]. Lipid content may also be increased, leading to an even higher lipid productivity [46]. Moreover, the CO₂ released during respiration can be reused for photosynthesis unlike in heterotrophy mode, which means lower CO₂ emission [11,32]. Mixotrophy yields higher productivities compared to heterotrophy given identical organic carbon supply [41,43]. Mixotrophic cultures are also less sensitive to lighting conditions, which will be advantageous for high density cultivation or when utilizing wastewater which is usually more opaque, where usually light penetration becomes a limiting factor [47]. In mixotrophic cultivation, the chlorophyll content is lesser than that under phototrophic

mode since chlorophyll is not needed when energy can be obtained from the organic compounds through respiration [48–50]. This reduction in chlorophyll content is especially useful in biodiesel production since chlorophyll is known to interfere with the transesterification reaction [32]. Mixotrophic/heterotrophic cells also have higher oleic acid content, known to improve biodiesel quality [51].

2.5 Current status

2.5.1 Economic and technological bottlenecks

High production costs of microalgae and contamination issues continue to hamper the use of microalgae as feed for aquaculture [5,52,53]. Further cost reductions are needed for products aimed at the commodities market [52]. Crop loss, a commonly overlooked factor, increase the economic risk and could threaten the industry of microalgal cultivation [52,54].

Properties such as high protein contents, favourable amino acid profile, presence of omega-3 fatty acids, vitamins, minerals, and antioxidants make microalgae a valuable food and feed resource [55]. Low productivity and high operating costs however, meant that the economics of the process is not yet viable or economical [56]. At the scales achieved, biomass productivity is still insufficient and downstream processing costs are still too high.

2.5.2 Addressing bottlenecks

One way to lower the production costs of microalgae is to improve their growth rate or productivity. Different strategies have been explored for increasing the productivity of

microalgae such as nutrient limitation, genetic engineering, and heterotrophic/mixotrophic mode of cultivation. Nutrient limitation for example, can increase the accumulation of lipids in cells. However, the growth of the cells is usually affected and may result in a net decrease in productivity instead [57]. In the case of genetic engineering, only a few positive results have been reported so far [58,59], and little is known about the risks and effects of genetically modified (GM) microalgae on human health and the environment [60]. The stability of genetically modified microalgae is also questionable. Moreover, the use of GM microalgae faces many strict legislations and is generally viewed with scepticism by the public [61].

Heterotrophic/mixotrophic mode of cultivation on the other hand, offers the possibility of significantly increasing the productivity of microalgae to drive down the costs of production [62–65]. However, this strategy incurs additional costs and suffers from contamination issues since it involves the supplementation of organic substrates such as glucose, acetate, glycerol, *etc.* [60,62,66–68].

Contamination greatly impedes the commercialization of microalgae biomass production as it is often responsible for culture crashes [52,69,70]. These contaminants include bacteria, fungi, viruses, and grazers such as protozoa, rotifers, and copepods. They can compete with microalgae for nutrients, produce toxic compounds that kill the microalgae, or consume the microalgae, thereby causing a loss of productivity [71].

Various methods to control contamination have been tested. Direct engineering solutions such as filtration and sonication have been shown to be effective but can be expensive and time consuming to apply in large scale [52]. Chemical control by adding antibiotics, probiotics, or disinfectants can affect the growth of microalgae [72], and they are also expensive and not environmentally friendly [52]. The use of biological control such as predators can be problematic too as these predators consume

both the grazers and microalgae [52]. Manipulating the growth environment such as salinity or pH of the media requires comprehensive understanding of the adaptive range of microalgae and contaminants [52]. Mixed-species cultivation may be vital for culture stability and can help improve variability in biomass production [54]. However, the search for suitable species is not straightforward and more ecological work needs to be done towards understanding this strategy.

2.5.3 Use of plant products

Recently, plant extracts have been gaining increasing interest in aquaculture due to their bioactive molecules with growth-promoting, immunostimulatory, antibacterial, antipathogenic, and antiparasitic effects [73,74]. For example, Shalaby et al. showed that when 3% garlic powder was incorporated into the diet of Nile tilapia, the average weight gained per fish was 35.5% higher than that of control [75]. In addition, Lee et al. found aqueous extracts of garlic (62.5 – 500mg/mL) to be effective against the Gram positive and Gram negative bacteria tested, which included *Staphylococcus aureus* and *Vibrio vulnificus* [76].

In agriculture, plant extracts have also attracted increasing attention due to their growth-promoting, immune stimulating, and antimicrobial properties, potentially replacing the antibiotics commonly used [77]. An *in vivo* antimicrobial efficacy study by Citarasu showed 300 ppm plant extracts to have similar effect as antibiotic on the gut microorganisms of chicken, significantly lowering the number of the pathogenic bacteria *E. coli* and *Clostridium perfringens* compared to control [78]. In the same study, when antibiotic or 300 ppm plant extracts were administered, the body weight

of the chickens increased by 4.7% and 8.1% respectively, compared to control.

Another study by Shalaby et al. showed an increase in weight gain of broiler chicks by about 13% when 200 ppm of thyme extract was incorporated into its diet [79].

3 Use of herbs/spices to improve the growth rate of

Chlorella sp.

3.1 Introduction

Microalgae have diverse potential applications such as biofuels, aquaculture feed, wastewater treatment, and production of nutraceuticals and pharmaceuticals [23,80]. However, the relatively high production costs and the vulnerability to bacterial contamination limit the applications only to high value products such as aquaculture feeds, pharmaceuticals and nutraceuticals [5,65,81].

The growth-promoting, immunostimulatory, and antimicrobial properties of phytonutrients can be incorporated into microalgae cultivation and potentially reduce both the production costs and bacterial contamination. A thorough literature investigation however, yielded no work on the use of phytonutrients or herbs and spices in microalgal cultivation. Hence, this study aims to investigate the feasibility of using herbs and spices to improve the productivity of microalgae. Screening of several herbs and spices in the form of aqueous extract and dried powder was first performed. The growth characteristics as well as biochemical composition, such as fatty acid, protein, and carbohydrate contents of microalgae were also investigated.

3.2 Materials and methods

3.2.1 Herbs and spices

All herbs and spices were purchased from local supermarket chain NTUC Fairprice, Singapore. The list of herbs and spices tested were: onion powder (SingLong, Malaysia), basil leaves (MasterFoods, Australia), garlic powder (MasterFoods,

Australia), rosemary leaves (SingLong, Malaysia), and turmeric powder (McCormick, Australia).

Aqueous extract preparation – The aqueous extracts of various herbs and spices were prepared by adding 1 g of the as purchased herb or spice into 20 mL of deionized water (DI) before mixing for 1 hr using a tube rotator (Stuart SB3). The resulting solutions were then mixed with DI water based on the ratio shown in Table 3-1 to obtain aqueous extracts of various concentrations. **Dried powder preparation** – Onion, garlic, rosemary, and turmeric powder purchased were used as purchased. Basil leaves were grounded in a mortar using a pestle before use.

Table 3-1 Different concentrations of herbs and spices added in aqueous extract form.

Herbs and spices	Volume of extract (mL)	Volume of DI water (mL)	Total volume (mL)	Concentration , %w/v
Rosemary, basil, garlic, turmeric, onion	1.25	3.75	5	1.25
Rosemary, basil, garlic, turmeric, onion	2.5	2.5	5	2.5
Garlic, turmeric, onion	3.75	1.25	5	3.75

3.2.2 Microalgae and culture conditions

Chlorella sp. (ATCC 14854) was grown in modified R-medium as shown in Table 3-2 below [82]. In the case of aqueous extract, 5 mL of aqueous extract of various

concentrations were added to centrifuge tubes together with *Chlorella sp.* seed culture to make up a total working volume of 35 mL. The starting cell concentration was standardized at 2 mil cells/mL. Tubes were rotated using the tube rotator at 15 rpm and kept at 28 °C with continuous light illumination of 55 $\mu\text{mol}/\text{m}^2\text{s}$. In the case of dried powder, 0.01 g of the dried powder was added to 100 mL working volume of seed culture with starting cell concentration of 2 mil cells/mL in 250 mL Erlenmeyer flasks. Flasks were kept in a shaking incubator (Labwit ZWYR-240) at 28 °C and 180 rpm with continuous light illumination of 75 $\mu\text{mol}/\text{m}^2\text{s}$.

Table 3-2 Composition of R-medium used to grow *Chlorella sp.*

Composition	Concentration (mg/L)
sodium citrate dihydrate	500
ferric chloride hexahydrate	10
calcium chloride dihydrate	53
magnesium sulfate heptahydrate	900
ammonium nitrate	600
potassium dihydrogen phosphate	200
potassium phosphate trihydrate	393
sodium acetate	1804.7
boric acid	1
zinc sulfate heptahydrate	1
manganese sulfate monohydrate	0.303
cobalt chloride hexahydrate	0.2
sodium molybdate dihydrate	0.2
copper sulfate pentahydrate	0.0625

3.2.3 Analytical methods

Microalgae biomass was harvested by centrifugation (LaboGene ScanSpeed 1580R) at 1500 rpm for 3 min, washed and oven-dried at 50 °C.

3.2.4 Measurement of microalgae cell concentration

Cell count was performed daily using a haemocytometer and an optical microscope (Optika). Measurements were done at least in duplicate.

3.2.5 Protein content

Protein content was determined using Lowry's method, with some minor modifications [83]. Briefly, 0.2 mL of 24% trichloroacetic acid was added to 5mg ($\pm 5\%$) of dried biomass and heated at 95 °C for 1.5 hr. 0.6 mL of DI water was then added and the mixture was centrifuged at 8100 rpm for 20 min. The supernatant was discarded and the pellet was re-suspended in 0.5 mL of Lowry D reagent at 55 °C for 2 hr. Lowry D is made up of Lowry A, B, and C in the proportions 48:1:1. Lowry A is 2% w/v sodium carbonate in 0.1N sodium hydroxide. Lowry B is 1% w/v sodium potassium tartrate tetrahydrate and Lowry C is 0.5% w/v copper sulphate pentahydrate. After re-centrifuging at 8100 rpm for 20 min, 0.05 mL of the supernatant was mixed with 0.95 mL of Lowry D. After 10 min, 0.1 mL of 1N Folin-Ciocalteu reagent was added. After 30 min, the absorbance was read at 750 nm. Bovine serum albumin (BSA) was used as the standard.

3.2.6 Fatty acid content

Fatty acid content was determined following the *in situ* direct transesterification method [84]. Briefly, 0.3 mL of 5% HCl in methanol was added to 8mg ($\pm 5\%$) of dried biomass and heated for 1 hr at 85 °C. After the reaction, 1 mL of hexane was added and the mixture was left to stand for at least 1 hr at room temperature. 250 μ g of glyceryl triheptadecanoate (C17-TAG) was used as the internal standard. The fatty acid methyl ester (FAME) in the hexane layer was then quantified by GC-MS (Agilent 5973N, HP 5-MS column, 30 m $\times$ 0.25 mm $\times$ 0.25 mm) at a rate of 20 °C min⁻¹ from 70-300 °C and hold for 1 min at 230 °C using He as the carrier gas at a constant flow rate of 1.5 mL min⁻¹.

3.2.7 Carbohydrate content

Carbohydrate content was determined, with some minor modifications, according to [83]. Briefly, 2 mL of 1M H₂SO₄ was added to 5mg ($\pm 5\%$) of dried biomass and heated at 90 °C for 2 hr. After centrifuging, 0.1 mL of the supernatant was then mixed with 2.5 mL concentrated sulfuric acid and 0.5 mL phenol. After 20 min, the absorbance was read at 485 nm. Glucose was used as the standard.

3.3 Results and discussion

3.3.1 Screening of herbs and spices

Aqueous extracts of herbs and spices were introduced into cell cultures of *Chlorella* sp. and the cell concentration was monitored for 9 days. Figure 3-1 (a) shows the growth curves of *Chlorella* sp. with different concentrations of onion extract added

(1.25, 2.5, and 3.75% w/v). It is found that the addition of aqueous onion extract, regardless of its concentration, increased the growth rate and

(a)

(b)

Figure 3-1 Growth curves of *Chlorella sp.* with (a) different concentrations of onion extract added and with (b) different herbs and spices added in aqueous extract form

final cell concentration of *Chlorella sp.* significantly. The final cell concentrations with the addition of aqueous onion extract were 4-6 times higher than that of control. It is also observed that 3.75%w/v onion extract concentration has a lower growth rate than both 1.25%w/v and 2.5%w/v onion extract concentrations in the initial days. However, the growth rate for 1.25%w/v and 2.5%w/v onion extract concentrations reduces around day 3 while for 3.75%w/v onion extract concentration, the growth continued at about the same rate. This leads to a higher final cell concentration in

3.75% w/v onion extract concentration than that of 1.25% w/v and 2.5% w/v onion extract concentrations. The final cell concentrations for control, 1.25% w/v, 2.5% w/v, and 3.75% w/v onion extract concentration were 44, 169, 191, and 242 mil cells/mL, respectively.

Similar observations were also found for other herbs and spices and the extract concentrations that provided the highest growth for *Chlorella sp.* were identified.

Figure 3-1 (b) shows a comparison of the growth performance of *Chlorella sp.*

supplemented with the optimal extract concentration of various herbs and spices tested in this study. It can be seen from Figure 3-1(b) that the choice of herbs and spices has significant influence on the growth performance of *Chlorella sp.* 1.25% w/v extract concentration was found to be the most suitable for turmeric, basil, and rosemary while 3.75% w/v extract concentration was the most suitable for onion and garlic. The use of rosemary extract inhibited the growth of *Chlorella sp.* while the use of onion extract increased the cell concentration at the end of the growth phase to about six times that of control. The use of extracts of herbs and spices also had an effect on the specific growth rate and the duration of the growth phase to different extents. A summary of the most suitable extract concentration, specific growth rate, day on which the exponential phase ends, and cell concentration at end of exponential phase is shown in Table 3-3. In brief, onion extract provided the highest growth rate and highest cell concentration at the end of the exponential phase, followed by garlic, turmeric, basil, control, and rosemary.

Table 3-3 Specific growth rate, day of end of exponential phase, and cell concentration at end of exponential phase for different extracts of herbs and spices. For each herb and spice, the extract concentration shown gives the maximum cell concentration at end of exponential phase.

Herbs and spices	Extract concentration (% w/v)	Specific growth rate (d ⁻¹)	Day exponential phase ends	Cell concentration at end of exponential phase (mill cells/mL)
onion	3.75	0.79	7	233.8
garlic	3.75	0.65	8	171.3
turmeric	1.25	0.67	6	108.8
basil	1.25	0.63	6	88.1
control	NA	0.40	8	47.4
rosemary	1.25	0.33	9	40.3

Supplementation of herbs and spices in the form of dried powder was also conducted and the cultures of *Chlorella sp.* were observed for 5 days. The growth performance shown in Figure 3-2 indicates that the effect of most herbs and spices on microalgae was inhibitory, opposite to the growth promotion observed when extracts of herbs and spices were used. The control sample had the highest growth rate in the initial 3 days. Beyond day 3, the onion powder-supplemented sample had the highest growth rate, providing a marginally higher cell concentration than that of control. The samples supplemented with the other herbs and spices had growth that were suppressed. In fact, the relative performances of the herbs and spices in both the aqueous form and in the dried powder form are reasonably similar, except in the case of turmeric and basil.

Figure 3-2. Growth curves of *Chlorella sp.* with different herbs and spices added in dried powder form (0.01 g).

The contrasting difference in growth performance of *Chlorella sp.* with the introduction of herbs and spices in different forms is believed to be due to the higher concentration of growth-inhibiting compounds derived from the herbs and spices. During photosynthesis, reactive oxygen species (ROS) is constantly produced. These ROS can cause cell damage through oxidation of lipids, amino acids and deactivation of specific enzymes [85]. Antioxidants in the form of enzymes and molecules were found to improve the growth of microalgae by promoting the recovery of cells that have been damaged by ROS or to enhance the elimination of ROS from cells [86].

However, ROS is also needed for vital cellular functions such as cell signalling, gene expression, and even in enzyme chemistry [87–90]. A high concentration of antioxidants hence, can disrupt the normally functioning cell regulatory networks due to the imbalance in ROS concentration, thereby inhibiting the growth of microalgae. Herbs and spices contain phenolic compounds such as phenolic acids, flavonoids, and tannins that contribute to their antioxidant capacity [85]. Table 3-4 shows that herbs and spices that were more inhibitory to the microalgae such as basil and rosemary contain higher amounts of phenolics. In addition to causing an imbalance in ROS concentration, phenolic compounds can chelate with metal ions present in the medium and decrease the bioavailability of essential nutrients such as calcium, iron, magnesium [91].

Table 3-4 Reported phenolic content of the herbs and spices used [92]. Moisture contents were measured to convert the basis from fresh weight basis to dry weight.

Herbs and spices	Phenolic content	
	mg/100 g fresh weight	mg/100 g dry weight
Onion	102.83	-
Garlic	87.04	-
Turmeric	-	2117
Basil	231.8	4317.89
Rosemary	1082.43	2518.67

The difference in concentration of phenolic compounds between the aqueous extract and dried powder forms of herbs and spices used could be attributed to a few possible reasons. It is anticipated that a majority of the phenolic compounds remain undissolved or remain bound to the powder used [93,94]. These phenolic compounds may take hours or even days to dissolve completely [95]. The preparation of aqueous extracts of herbs and spices was carried out for 1 hr whereas in the case of dried powder, the powder was present in the growth medium for the entire experimental duration. The growth medium of microalgae also contains metal ions that can coordinate with the phenolic compounds, thereby increasing their solubility [96]. The microalgae can also produce extracellular enzymes to free the phenolic compounds that are bound to the plant components [97]. All these reasons lead to the belief that the aqueous extracts had a lower concentration of growth-inhibiting phenolic compounds. Since most aqueous extracts of herbs and spices promote the growth of microalgae, the results are used for further analysis.

3.3.2 Underlying effect of aqueous extracts of herbs and spices

Glucose has been demonstrated to be able to serve as a carbon source for microalgae to achieve high cell concentrations [98]. With an attempt to determine the underlying causes of the increase in microalgae growth with the introduction of extracts of herbs and spices, the nutrient composition of the different herbs and spices used were obtained from the database of United States Department of Agriculture (USDA) and tabulated in Table 3-5. It is noted that the sugar contents are in agreement with the growth performance shown in Figure 3-1 (b). It is also to note that rosemary is the only herb (or spice) tested that does not contain sugar. Figure 3-3 shows a plot of the

cell concentrations reached at end of exponential phase against the sugar contents in the herbs and spices used.

Figure 3-3 Comparison of cell concentration achieved at end of exponential phase with the amount of sugar presents in different extract concentration of herbs and spices added.

Table 3-5 Nutrient composition of the different herbs and spices used in powder form [99].

Nutrient (g per 100 g dry weight)	Onion	Turmeric	Rosemary	Garlic	Basil

Water	5.39	12.85	9.31	6.45	10.35
Protein	10.41	9.68	4.88	16.55	22.98
Total lipid	1.04	3.25	15.22	0.73	4.07
Carbohydrate	79.12	67.14	64.06	72.73	47.75
Fiber	15.2	22.7	42.6	9	37.7
Sugars	6.63	3.21	0*	2.43	1.71

*Not provided and safe to assume as 0.

A growth comparison between 3.75% w/v onion extract concentration and an equivalent amount of glucose was also performed. It can be seen in Figure 3-4 that the growth of *Chlorella sp.* supplemented with 3.75% w/v onion extract concentration is able to sustain the exponential growth for 7 days while that supplemented with glucose only supported an exponential growth period of 2 days. Though the specific growth rate of the glucose supplemented culture is higher than that of 3.75% w/v onion extract concentration, the cell concentration on the 7th day in onion extract culture is more than double that of the glucose-added culture.

Figure 3-4 Comparison of growth curves of *Chlorella sp.* supplemented with 3.75% w/v onion extract concentration (0.0124g), glucose (0.0175g), and control.

It is believed that the superior growth performance of *Chlorella sp.* using onion extract compared to glucose is contributed by the antibacterial, antipathogenic, and antiparasitic effects of phytonics present in herbs and spices [73,74,77]. The inhibition of bacteria that are inhibitory to the microalgae, can allow further growth of the microalgae. Hence, the effect of antibacterials in allowing further growth of bacteria was investigated using the antibiotics, ampicillin and cefotaxime. As shown in Figure 3-5, the growth of *Chlorella sp.* was improved with the introduction of antibiotics. The specific growth rate and cell concentration when using antibiotics

were consistently higher than that of control. However, the difference in growth of microalgae between the different antibiotics and its combination was not significant.

Figure 3-5 Comparison of growth curves of *Chlorella sp.* supplemented with antibiotics ampicillin, cefotaxime and combination of ampicillin and cefotaxime.

While the presence of sugar and antibacterials in onion extract are believed to be significant contributors to the superior growth of microalgae, there are also other components in the onion extract that can also promote the growth of microalgae. For example, onion contains organic acids such as malic, citric, and pyruvic acid that can serve as a source of carbon [100–102]. Onion also contains many essential amino

acids such as glutamine, arginine, and lysine that can also be used to support microalgal growth [103].

3.3.3 Effect of aqueous onion extract on the biochemical composition of *Chlorella* *sp.*

3.75% w/v onion extract concentration was found to provide the maximum improvement in microalgae growth. The cells from 3.75% w/v onion extract concentration culture were then harvested and tested for protein, fatty acid, and carbohydrate content. Carbon fixed from photosynthesis is used to synthesise the three major biomolecules proteins, lipids, and carbohydrates. Typically, microalgae contain 30-40% protein, 10-20% lipid, and 5-15% carbohydrate at the end of the exponential phase [104].

Protein content mainly determines the nutritional value of microalgae [19]. As shown in Figure 3-6, the protein contents for control and onion extract 3.75% w/v supplemented culture were found to be 352.3 ± 89.3 mg per g dry weight and 310.7 ± 46.7 mg per g dry weight, respectively. No significant difference was found ($p > 0.05$) although the significant enhancement of microalgae growth in the 3.75% w/v onion extract concentration culture translates to a 450% increase in protein productivity. Protein contents in microalgae is considered to be directly related to the concentration of nitrogen in the culture medium since nitrogen is a key component for protein biosynthesis [105,106]. It indicates that the nitrogen concentration in the culture is sufficient to provide a normal nitrogen condition to sustain the microalgae growth.

Figure 3-6 Protein, fatty acid, and carbohydrate contents for control and onion 3.75% w/v-added culture. Asterisk indicates significant difference compared to control ($p < 0.05$).

Carbohydrates function as storage of energy. These carbohydrates can be used for the biosynthesis of proteins or lipids when required by the cells. The carbohydrate content was determined for control and 3.75% w/v onion extract concentration culture. Figure 3-6 shows a significant increase in the carbohydrate content from 45.9 ± 6.6 mg per g dry weight to 104.0 ± 16.3 mg per g dry weight with the addition of onion extract. While the addition of glucose was reported to increase the carbohydrate content of *Chlorella vulgaris* [107], one of the key conditions is a deficiency of nitrogen in the culture medium. The supply of additional carbon source, in the form of sugar for the case of onion extract, increases the carbon-to-nitrogen ratio. The increase in carbon-to-nitrogen ratio was found to give an effect similar to nitrogen depletion and induces the production of lipids [108]. This induction of lipid production however, is species specific. Certain species accumulate lipids while others accumulate carbohydrates [109]. It is possible that the *Chlorella sp.* used in this work accumulates carbohydrates instead of lipids. The finding is also corroborated by the insignificant difference in

fatty acid contents (see Figure 3-6) when onion extract was added (43.0 ± 6.4 mg per g dry weight compared to 26.5 ± 9.5 mg per g dry weight for control).

3.4 Conclusion

In this study, aqueous extracts of onion, garlic, turmeric, and basil were found to increase microalgal biomass productivity, although to different extents. Notably, onion extract of concentration 3.75% w/v doubled the specific growth rate and increased the cell concentration at end of exponential phase to nearly six times that of control. The carbohydrate content was also found to increase by 127%. Herbs and spices introduced in the form of powder, however, were found to be inhibitory. The difference in effect on growth of microalgae is thought to be due to the difference in concentration of phenolic compounds present in the medium. Herbs/spices show promising potential to increase microalgal productivity.

4 Protein Digestibility Corrected Amino Acid Score

(PDCAAS) determination of *Chlorella vulgaris*

4.1 Introduction

The global population is expected to soar to about 9.7 billion people by 2050 [110].

Current food production rate must increase by 70-100% while protein demand is projected to increase by 80-520% by 2050 [14,111,112]. Currently, plant-based proteins make up the biggest source of proteins (~57%) and considering that their production may have reached its peak, alternative sources of proteins are needed [14,111].

Microalgae (such as *Chlorella vulgaris* and *Spirulina sp.*) have been identified as a potential source of proteins. They have superior yields of proteins and comparable, if not higher, protein contents, compared to conventional protein sources [112].

However, studies regarding characterization of their nutritional properties are limited.

The nutritional quality of a protein is judged based on the efficiency of protein digestion and the presence of essential amino acids. Numerous parameters exist and they are used as criteria by researchers to evaluate protein quality of a microalgae (or any food or feed). Some common parameters are the chemical score (CS), essential amino acid index (EAAI), protein digestibility-corrected amino acid score (PDCAAS), protein efficiency ratio (PER), net protein utilization (NPU), biological value (BV), and many others.

The chemical score (CS) and essential amino acid index (EAAI) scores of a protein are determined based on the EAA concentration profile of a protein relative to egg albumin (considered to have an ideal EAA profile). The difference between the two scores is that EAAI considers the concentration of all EAAs while CS only considers

the most limiting EAA [113]. Protein efficiency ratio (PER), net protein utilization (NPU), and biological value (BV), although a most definitive measure of protein quality, require *in vivo* studies involving feeding trials that are expensive and can raise animal welfare issues. Practical difficulties, coupled with poor method sensitivities of the methods led to the introduction and adoption of the PDCAAS method by FAO/WHO, currently the approved method internationally for assessment of protein quality [114].

Protein digestibility-corrected amino acid score (PDCAAS), while similar to CS and EAAI, is considered an advancement as it takes into account the overall protein digestibility of the protein [113]. Although practical, it has the drawback of assuming the ileal digestibility of protein to be that measured from fecal nitrogen balance, which is incorrect [115]. Other limitations exist and are discussed at the end of this chapter. With regards to these limitations, FAO has proposed a new index to overcome these limitations, called digestible indispensable amino acid score (DIAAS). Although superior to PDCAAS, it is not currently viable for practical use until more human ileal digestibility data on individual amino acids are collected [115].

Therefore, in this chapter, the PDCAAS score is used to determine the protein quality of microalgae (*Chlorella vulgaris*). The PDCAAS score is essentially a product of 1) *in vitro* protein digestibility and 2) limiting amino acid ratio. It can be done *in vitro*, is quick to perform and inexpensive, and has very high correlation to the *in vivo* rat digestion model. In the materials and methods section, the method is first explained. Then, the digestibility value, amino acid ratio, and subsequently the PDCAAS value are determined and compared with that found elsewhere and with other sources of proteins. Lastly, comments and suggestions regarding the daily protein intake

(consuming only microalgae) are also presented before the limitations of the PDCAAS method is provided.

4.2 Materials and methods

4.2.1 Microalgae

Organic *Chlorella vulgaris* powder was purchased from British retailer, Myprotein. The product contains 100% *Chlorella vulgaris* powder. Cells were confirmed to be single cells as viewed under the optical microscope as well as under the scanning electron microscope (SEM). The protein content of the *Chlorella* powder was listed to be 59%.

4.2.2 Reagents

Enzymes (pepsin, trypsin, and chymotrypsin), L-Glycine, casein, and 6 other control powders (A, B, C, D, E, F) were purchased along with the protein digestibility kit from Megazyme, Ireland. Enzymes were prepared fresh and used within 30min of preparation.

All reagents needed for the assay but not provided along with kit were purchased from Sigma-Aldrich and of ACS grade unless stated otherwise. The reagents were 2% ninhydrin, 37% hydrochloric acid, trichloroacetic acid, Tris base, sodium acetate, and ethanol 99% purity.

4.2.3 Dumas method for protein content determination

Protein content was determined by the Dumas method using Vario EL III CHNS Elemental Analyser according to the manufacturer's protocol. The N% was multiplied by a factor of 6.25 to obtain the protein content.

4.2.4 Limiting amino acid ratio

4.2.4.1 LCMS

Amino acid determination commonly involves the acid hydrolysis of samples prior to quantification by LCMS or HPLC. Acidic hydrolysis can be used to quantify leucine, histidine, phenylalanine, valine, isoleucine, lysine, arginine, alanine, proline, glutamic acid, glycine, aspartic acid, and hydroxyproline. Of these 10 amino acids, only the first 6 are essential and phenylalanine is usually quantified together with tyrosine as aromatic amino acids (AAA). Serine and threonine can be quantified using acid hydrolysis as well, although with some losses (5-15%). However, only threonine is essential. Cysteine and methionine are prone to oxidation and are often protected (using performic acid for example) prior to acid hydrolysis. Only methionine is essential, however, and it is usually quantified together with cysteine as sulphur amino acids (SAA). Another essential amino acid is tryptophan, which is destroyed by acid hydrolysis and usually alkaline hydrolysis is done instead [116].

Acid hydrolysis was done according to [117]. Briefly, 100mg of sample was weighed into a 20mL screw-capped test tube with Teflon caps. 7mL of 6M HCl was added to it before the tube was heated in a 95C water bath for 24h. After hydrolysis, HCl was evaporated in an oven dryer at 95C. Once dried, the amino acids were re-dissolved in water. Then, it was filtered through a 0.45um filter and diluted accordingly before the derivatization procedure.

Derivatization and amino acid detection/quantification were done using EZ:faast amino acid analysis (LCMS) kit by Phenomenex. Derivatization and LCMS elution program were done according to manufacturer's instructions.

Briefly for derivatization, sample first underwent a solid phase extraction step to remove interfering substances. Then, sample was derivatized using propyl chloroformate/chloroform with 3-picoline as the catalyst. Derivatized amino acids were then reconstituted in a water-methanol mixture before it is ready for LCMS analysis.

LCMS was done using Agilent 6130 LCMS with electrospray ionisation (ESI), single quadrupole mass analyser, positive ion mode, and 100-650m/s scan range. Column length was 250 x 3.0mm with expected column backpressure of 200-220bar. Injection volume was 1uL with mobile phase flow rate of 0.5mL/min and column temperature of 35C. Two mobile phases were used: A) 10mM ammonium formate in water and B) 10mM ammonium formate in methanol. Gradient elution program was as follows: 68% B to 83% B in 13min followed by 4min of column equilibration at 68% B. Homoarginine, methionine, and homophenylalanine were used as internal standards to account for the various types of amino acids.

4.2.5 *In vitro* digestibility

The calculation of *in vitro* digestibility involves digesting the sample (or 6 controls and casein) using digestive enzymes (pepsin, trypsin, and chymotrypsin) and then quantifying the amount of amino acids released (also known as primary amine concentration), using glycine as the standard. The primary amine concentrations for the 6 controls and casein are then fitted to the corresponding literature values of digestibility using linear regression. The linear regression equation is then used to determine the digestibility values of unknown samples.

4.2.5.1 Standard assay procedure

Sample extraction

The microalgae powder was deemed to be fine enough to be used directly. Hence, no milling or screening is needed for it. 500mg of sample (*Chlorella*, casein, 6 controls, or blank) was weighed and transferred into a 50mL centrifuge tube. The tube was tapped against a surface to ensure that no sample sticks to the side of the wall and thereby not get digested by the enzymes.

To the sample in the tube, 19mL of HCl (0.06N and pH 2) was added and vortexed to mix thoroughly. The sample is then incubated for 30min at 37°C and 300rpm. During this period, pepsin solution (1mg/mL of 0.06N HCl at pH 2) was prepared. After 30mins, 1mL of the pepsin solution is added to the sample and vortexed to mix thoroughly. Sample is then incubated for 1hr at 37°C and 300rpm.

Next, trypsin/chymotrypsin solution (5mg/mL each in 0.001N HCl at pH 3, mixed together) was prepared 30mins before the end of the 1hr incubation. Once the 1hr incubation is over, pH was adjusted to 7.4 by the addition of 1M Tris buffer (pH 7.4). Then, 200uL of the trypsin/chymotrypsin solution was added to the sample and vortexed to mix thoroughly. Sample is then incubated for 4hr at 37°C and 300rpm.

At the end of the 4hr incubation, samples are placed in a boiling water bath for 10min to deactivate the enzymes. After 10mins, samples were allowed to cool to room temperature (within 20mins). 4mL of sample was then transferred to a 15mL centrifuge tube. 1mL of 40% trichloroacetic acid solution was added to it and vortexed to mix thoroughly. Samples were incubated overnight or at least 16 hrs to allow for the proteins to precipitate.

Once complete, 1.75mL of the clear supernatant was transferred to a 2mL centrifuge tube and centrifuged for 10min at 15 000g and ambient temperature. Then, samples are

diluted accordingly (10 to 20-fold dilution) in acetate buffer (50mM at pH 5.5).

Diluted samples are then ready for colorimetric determination.

Preparation of glycine calibration standards

Different concentrations of L-glycine were prepared to be used as calibration standards. 10mM L-glycine was prepared in 50mM sodium acetate buffer adjusted to pH 5.5. The different concentrations of L-glycine standards were diluted accordingly using the acetate buffer. Standards were then treated with the ninhydrin reagent described in the following section before the absorbance is read using a UV/Vis spectrophotometer (Shimadzu UV 2450).

Colorimetric determination of amines

Using a 24-well clear flat-bottomed plastic plate, 0.2mL of sample (or DI water for blank) or standard solution was added together with 0.1mL ninhydrin solution. The lid was placed on the plate and the plate was wrapped with aluminium foil and placed floating on a water bath at 70°C for 35min. After 30min, the plate is left to cool (within 10min). Then, 0.3mL ethanol (50% v/v) was added to the sample and mixed properly using pipette through multiple aspiration. The absorbance is then read at 570nm using sodium acetate buffer as a blank.

4.2.5.2 Glycine standard curve

The absorbance values of L-glycine standards are plotted against the various concentrations. A best fit line is then obtained and used to determine the primary amine concentration of samples.

4.2.5.3 Corrected primary amine concentration

After determining the primary amine concentration based on the L-glycine standards, the value is first corrected for dilution factor (with acetate buffer and with trichloroacetic acid) as shown below, equation 4-1.

$$C2 = C1 * D * 1.25 \quad 4-1$$

Where:

C1 = primary amine concentration (diluted)

D = dilution factor of sample using acetate buffer

1.25 = dilution with trichloroacetic acid prior to overnight incubation

Different amino acids contribute differently to the absorbances measured. For this reason, the primary amine is further adjusted using the equation 4-2 and constants below as provided by the manufacturer, to account for the differences.

$$CN = C2 + \frac{Pro * 2 * 10}{Lys * 0.5 * 10} + (Hist + Arg) * 0.2 * 10 \quad 4-2$$

Where:

C2 = primary amine concentration corrected for dilution by acetate buffer and trichloroacetic acid

Pro, Lys, Hist, Arg = respective amino acid concentration in the original sample

2, 0.5, 0.2 = constants for the various amino acids

4.2.5.4 Data fit to literature values

After obtaining the corrected amino acid concentrations for the control samples (A-F and casein), the values are fitted against the literature values and a linear regression line is obtained. The literature values are supplied in the manufacturer's booklet and are shown below, *Table 4-1*.

Table 4-1 Literature values of *in vivo* rat digestibility.

Matrix	Literature value (mM)
Control A	83
Control B	86
Control C	91
Control D	84
Control E	84
Control F	81
Casein	100

In vitro digestibility

Using the linear regression equation from the previous section 4.2.5.2, the primary amine concentration of an unknown sample can be calculated. The value is then converted to in terms of g, which is the *in vitro* digestibility value, as shown below.

$$In\ vitro\ digestibility = \frac{M * X + B}{100} \quad 4-3$$

Where:

M = slope of linear regression line

X = corrected primary amine concentration, CN

B = y-intercept of linear regression line

100 = conversion from percentage to g

4.2.6 PDCAAS score

The PDCAAS is essentially a score that considers the abundance (or lack) of essential amino acids in a protein source (limiting amino acid ratio) and the availability (or digestibility) of those amino acids to the human body. Hence, the PDCAAS score is a product of both parameters. In addition, each parameter has a maximum (truncated to be exact) value of 1 and so the highest score possible is 1.

4.3 Results and discussion

4.3.1 Dumas method for protein content determination

The results from elemental analysis is as shown in *Table 4-2* below.

Table 4-2 N and protein content of *Chlorella* powder used for *in vitro* digestibility studies.

N% (as determined by analyser)	Protein content% (factor of 6.25)
9.596%	59.975%

A protein content of 59.975% was found for the *Chlorella* powder used in this digestibility study, which is very similar to that listed by the manufacturer on the packaging (59%).

4.3.2 Limiting amino acid ratio

LCMS

The amino acid composition of the proteins found in *Chlorella vulgaris* was analysed qualitatively and quantitatively through the use of LCMS and is shown in Table 4-3 below.

Table 4-3 Amino acid composition of proteins derived from *Chlorella vulgaris* (proteins make up 51.25% of dry biomass)

Amino acid	% total protein
arginine	7.04
serine	3.58
glycine	7.01
threonine	3.89
alanine	13.11
methionine	1.74
proline	6.78
lysine	5.21

aspartic acid	6.68
histidine	2.52
valine	9.89
glutamic acid	6.63
tryptophan	ND
leucine	10.07
phenylalanine	7.27
isoleucine	5.78
cystine	0.20
tyrosine	2.61
total	100

18 amino acids were detected through LCMS except tryptophan which is destroyed during acid hydrolysis. Cystine and methionine on the other hand, are destroyed as well, but to varying degrees depending on the reaction conditions. The protein content found by summing up the individual amino acids was 51.25g/100g dry biomass. This differs from the 60% protein content determined through the Dumas method as the Dumas method also measures nitrogen contributed by non-protein compounds such as inorganic nutrients (nitrates and nitrites) as well as nucleic acids and chlorophyll. Typically, non-protein N contributes to about 15-30% of the total N [118]. Here, non-protein N contributed 14.6% to the total N.

The amino acid composition of *Chlorella vulgaris* determined in this work agrees well with that found elsewhere for *Chlorella vulgaris* and the phylum Chlorophyta [118,119]. Typically, alanine, leucine, valine, aspartic acid, and glutamic acid are the most abundant amino acids accounting for roughly 50% of the total amino acids. In

this work, the five amino acids accounted for 46.4% of the total amino acids.

Elsewhere, the five amino acids accounted for 53.62% and 46.4%, respectively [118,119].

Amino acids typically found in low concentrations are tyrosine, tryptophan, histidine, cystine, and methionine, again in agreement with the LCMS results from this work [118,119]. Of those amino acids, only tryptophan, histidine, and methionine are essential amino acids and hence important in determining the nutritional quality of a protein. According to the WHO/FAO/UNU, a protein is said to be able to meet the daily essential amino acid requirements (of an adult) when 0.66g of the protein consumed per kg of body weight is able to provide sufficient amount of all the essential amino acids [120]. Should any of the essential amino acids lack, more protein ($>0.66\text{g/kg}$ body weight) has to be consumed. For example, if the protein consumed (based on 0.66g/kg body weight) is able to provide only 50% of the most lacking essential amino acid, then the protein intake has to be doubled so as to provide the sufficient amount of the most lacking amino acid.

Different amino acids have different required daily amounts and the ratio between the amino acid amount (in the protein) and the required daily amount is termed the amino acid ratio. The ratio with the lowest value is then termed the limiting amino acid ratio. In the example above, the limiting amino acid ratio would be 0.5. It can be seen then, the importance of this ratio in determining the quality of a given protein. Plant-based proteins typically have insufficient essential amino acids (ratio less than 1), unlike animal-based proteins. Hence, more amounts of plant-based proteins have to be consumed in order to meet the daily requirements. Sometimes, the ratio can go very low such that the amount to be consumed becomes not so practical. That is why it is advised to consume multiple sources of plant-based proteins that complement each

other so that their combination is able to provide sufficient amounts of the entire spectrum of amino acids.

Table 4-4 below shows the amino acid ratios for the different amino acids found in *Chlorella vulgaris*. Note that tryptophan was not measured since it was destroyed by the acidic hydrolysis process. It is instead assumed that tryptophan makes up 2% of the total proteins since the amino acid profile of proteins from microalgae is relatively well preserved across species and conditions of growth [113]. A 2% tryptophan concentration or 20mg/g protein would be more than sufficient to meet the daily requirement of 6mg/g protein.

Methionine, another amino acid commonly found in low concentrations, also makes up about 2% of the total proteins found in microalgae [113]. The methionine concentration found for *Chlorella vulgaris* in this work (19.33mg/g protein) agrees well with the 2% concentration as well, given that cystine's concentration was found to be nearly zero. This considers also the fact that both cystine and methionine were destroyed, at least partially, during the hydrolysis process. This means that the actual concentration of cystine+methionine here could be higher than the 19.33mg/g protein measured. The use of this value leads to methionine+cystine having the lowest or limiting amino acid ratio of 0.88. In fact, plant-proteins generally have insufficient methionine and lysine (not for microalgae) to meet the daily requirement (based on 0.66g protein/kg body weight) [121].

The rest of the essential amino acids were found to be more than sufficient to provide the daily amino acid requirement. Also, assuming the limiting amino acid ratio is indeed 0.88, this means that increasing the daily recommended protein intake by $1/0.88$ or 1.14 times, consuming only microalgae as the source of protein, would be

sufficient to meet the daily amino acid requirement (*i.e.* 0.66×1.14 or 0.75g microalgae protein/kg body weight). Assuming a 60% protein content, for a 65kg adult, this would mean $0.75 \times 65 / 0.6$ or 81.25g of microalgae is needed per day. For comparison sake, with a protein content of 27% and an amino acid ratio of 1, $0.66 \times 65 / 0.27$ or 159g of chicken meat is needed to meet the daily amino acid requirement.

Table 4-4 Amino acid ratios of amino acids found in *Chlorella vulgaris*.

Amino acid	mg/g protein	Adult mg/g protein requirement ¹	Amino acid ratio
threonine	38.92	23	1.69
methionine+cystine	19.33	22	0.88
lysine	52.12	45	1.16
histidine	25.20	15	1.68
valine	98.90	39	2.54
tryptophan ²	20.00	6	3.33
leucine	100.69	59	1.71
phenylalanine+tyrosine	98.82	38	2.60
isoleucine	57.75	30	1.93
Limiting amino acid ratio			0.88

¹ Taken from FAO [120] (amended values from 2007)

² Destroyed during acid hydrolysis, assumed to be 2% of total proteins since amino acid profile of microalgal protein is relatively conserved [113]

4.3.3 *In vitro* digestibility

4.3.3.1 Glycine standard curve

Figure 4-1 Glycine standard curve for amino acid concentration determination

Glycine was used as the standard for quantification of amino acids released during the digestion process. A linear correlation was obtained with R^2 of 0.998.

4.3.3.2 Corrected primary amine concentration

The absorbance value post-digestion of *Chlorella vulgaris* is shown in Table 4-5 below (after 20x dilution). Using the linear regression equation obtained from Figure 4-1, the equivalent glycine concentration was found to be 0.349mM. Equation 4-1 is then used to correct for TCA as well as distilled water dilution prior to measurement by UV/Vis spectrophotometry.

Additionally, the different amino acids present also contribute differently to the absorbance measured and has to be considered. Here, Equation 4-2 is applied using the

different constants for different amino acids. A final corrected amino acid concentration is obtained for microalgae (*i.e.* 23.73mM).

Table 4-5 Absorbance, glycine equivalent concentration, and TCA dilution correction factor of *Chlorella vulgaris* after enzymatic digestion.

Sample	Abs 570nm	glycine equivalent (mM)	Corrected for TCA dilution (mM)	Dilution factor for absorbance measurement	Corrected for dilution factor (mM)	Corrected for different amino acids present (mM)
<i>Chlorella vulgaris</i>	2.013	0.349	0.437	20	8.730	23.730

4.3.3.3 Data fit to literature values

The same steps above are done on casein and 6 other controls as described in Section 4.2.5.4 and their results are tabulated in Table 4-6 below. Casein and the 6 controls are then fitted to literature values of *in vivo* rat digestibility provided by the kit supplier as shown in Figure 4-2 below and the linear regression line is obtained.

Table 4-6 Final corrected amino acid concentration from ninhydrin digestion and literature values of *in vivo* rat digestibility.

Samples	Final corrected amino acid concentration (mM)	Literature values of <i>in vivo</i> rat digestibility, %
---------	---	--

Control A	9.21	83
Control B	10.19	86
Control C	15.55	91
Control D	9.28	84
Control E	8.16	84
Control F	7.49	81
Casein	21.03	100

Figure 4-2 Correlation between *in vitro* digestion measured in this work and literature values of *in vivo* rat digestibility (supplied by manufacturer).

The linear regression line (R^2 of 0.977) is then used to predict the *in vivo* digestibility value of *Chlorella vulgaris* biomass. Using the 23.73mM value found earlier in Table 4-5, this gives *Chlorella vulgaris* a digestibility value of 100% (102.9% actual but truncated to 100% as per PDCAAS convention). This means that most of the proteins of *Chlorella* are readily available and digested by the digestive enzymes to release the amino acids. In comparison to values in literature, the protein digestibility of *Chlorella* varies widely, from 0.27 all the way to 0.97, with values ranging mostly between 0.7 and 0.9 [55]. It is also observed that newer reports on protein digestibility of *Chlorella* are showing higher values, which could mean that the actual digestibility values are indeed on the high side, assuming that the methods of measurement improve and become more accurate over time.

4.3.4 PDCAAS

After obtaining the limiting amino acid ratio from Section 4.3.2 and the digestibility value from Section 4.3.3, the PDCAAS score of the protein (*Chlorella vulgaris*) can finally be determined. The product of digestibility and limiting amino acid ratio gives a PDCAAS value of 0.88 for *Chlorella vulgaris* biomass (see Table 4-7).

Table 4-7 PDCAAS value of *Chlorella vulgaris* biomass used in this work

Digestibility	Limiting amino acid ratio	PDCAAS
1	0.88	0.88

For comparison sake, the PDCAAS value of other sources of protein are shown in Table 4-8 below (along with the results from this work). It can be seen that the

digestibility value of *Chlorella vulgaris* is the highest among the most common sources of proteins. This means that most of the amino acids present in protein are easily digested and made available to the human body for assimilation. The amino acid score, however, is lower than all the other proteins except wheat. This is due to the lack of the essential amino acid methionine. Note that methionine is destroyed during hydrolysis but was not accounted for in this work, although it is said that methionine typically make up about 2% of the total proteins in microalgae, which will still lead to a limiting amino acid ratio of 0.91 [113]. Nevertheless, the PDCAAS score of microalgae is on par with other high-quality protein sources such as beef and soy and thus a valuable source of protein (see Table 4-8).

Table 4-8 PDCAAS value of different sources of protein (data taken from [122]).

Protein	Digestibility	Limiting amino acid score	PDCAAS
Egg	0.98	1.21	1 (truncated from 1.19)
Cow's milk	0.95	1.27	1 (truncated from 1.21)
Beef	0.98	0.94	0.92
Soy	0.95	0.96	0.91
<i>Chlorella vulgaris</i> (this study)	1.00	0.88	0.88
Wheat	0.91	0.47	0.43

Limitations

Firstly, the PDCAAS method of scoring a protein's nutritional value does not give extra credit to proteins with exceptional quality. For example, the PDCAAS value of egg and cow's milk is truncated to 1 (from 1.19 and 1.21, respectively), making it appear to be much less superior than it actually is (*i.e.* less able to make up for the lack of amino acids in other sources of proteins consumed together).

Secondly, the *in vitro* method of quantifying the released amino acids does not consider the antinutritional factors present in foods such as plant fibre or phenolic compounds. This differs from the way the body behaves or assimilates the proteins from a food matrix.

Thirdly, the PDCAAS score overestimates (or does not indicate) the quality of proteins having multiple limiting amino acids (ratio <1). This could lead to the illusion that supplementing the amino acid that is most lacking in a protein source with another protein source (that has sufficient amount of the lacking amino acid) will be sufficient when in fact there are other amino acids that are limiting as well.

4.4 Conclusion

In this chapter, the protein digestibility value and the limiting amino acid score of the microalgae *Chlorella vulgaris* were determined. Protein digestibility was determined by performing *in vitro* enzymatic digestion of the biomass and quantifying the amount of amino acids released. The amount of amino acids released, based on the total amount of amino acids present, is then estimated from a correlation to determine the *in vivo* rat digestibility value. The limiting amino acid score on the other hand, is determined by first acid hydrolysing all the available proteins in the biomass. Then,

the amount of the individual amino acids per weight of the protein is quantified and based on the adult daily requirement of essential amino acids, individual amino acid ratios can be determined. The most lacking amino acid, or the lowest amino acid ratio is identified as the limiting amino acid ratio. This ratio, together with the digestibility of the proteins present, determine the quality (*i.e.* PDCAAS score) of the measured protein. The PDCAAS score of the *Chlorella vulgaris* biomass was found to be 0.88 (digestibility of 1 and limiting amino acid ratio of 0.88). The quality of the protein is therefore considered good and on par with other sources of protein such as beef and soy. Methionine was deemed as the lacking amino acid, which is often the case with plant-based proteins. Nevertheless, only a slightly higher amount (1.14) of protein intake is required (when consuming microalgae as the only source of protein) to meet the daily essential amino acid requirements.

5 Biochemical composition estimation through elemental and stoichiometry analysis

5.1 Introduction

The biorefinery concept to convert microalgae biomass to various value-added products is gaining increasing significance in an effort to improve the economic viability of microalgae-derived commodity products such as biofuels and proteins for food and feed. However, the integration of the various downstream processes is complex and not at a mature stage yet and requires knowledge regarding the biochemical composition and how the various biochemicals partition at each stage or step of the process. This information will also prove invaluable when optimizing the process to achieve various targets (e.g. lowest cost as in industry or highest purity/yield as in research). However, determining the composition of the material/product is a laborious step especially with processes that have multiple steps and streams.

Dried microalgae biomass is typically made up of biochemicals such as proteins, carbohydrates, lipids, nucleic acids, pigments such as chlorophyll and carotenoids, minerals or ashes, and some residual water. Often, the protein content can be estimated most quickly through elemental analysis using the appropriate N-to-protein conversion factor (6.25 is usually used). Then, lipid content is determined through the methods of either Folch or Bligh and Dyer. Carbohydrate content is then finally calculated based on the difference method ($100\% - \text{protein content} - \text{lipid content}$). This is usually done on a moisture-free and ash-free basis.

Sometimes, ash content is ignored which leads to overestimation of the carbohydrate content. A more accurate determination must consider the ash content as it can

constitute a large percentage of the dry biomass (up to 37.4% dry weight) especially when considering marine species or species grown in open conditions [12]. Ashes (or heavy metals) can lead to toxicity if accumulated or consumed in high amounts and thus should not be ignored.

Another biochemical that is often ignored, nucleic acids (mostly RNA) are treated as non-existent even though it can account for a significant proportion of the dry weight. For example, in Chlorophyta and Cyanobacteria, the median RNA content was found to be 5.1 and 8.7, respectively [123]. And even though N is assumed commonly to come entirely from proteins (using the 6.25 conversion factor), non-protein nitrogen (NPN) can account for about 15-30% of the total nitrogen, coming from sources such as chlorophyll, nucleic acids, and amino sugars. The nucleic acid content cannot be ignored if the product is to be used as a food product or ingredient. Microorganisms, whether bacteria, yeast, or microalgae are currently being explored as a potential source of proteins. Nucleic acids consumed in high amounts can lead to health issues such as gout. Therefore, its accurate measurement (or at least an estimation) is of utmost importance so as to design a process to exclude it as much as possible in the final product.

As mentioned earlier in this section, elemental analysis is a rapid (and safe) way of estimating the protein content (using N% value). However, values other than N% are also obtained from the analysis namely C%, H%, and S%. These other values are often discarded or ignored. Ashes content is typically about 5-10% of biomass weight. For more accurate quantification, ashing using combustion chamber or thermogravimetric analysis (TGA) should be done. Through elemental analysis of biochemical constituents and mass balance among the different products/streams, a vast amount of information can be gleaned from this previously untapped data. The last remaining

atom that contributes to the make-up of cells is oxygen, O which can now be obtained by subtracting C, H, N, S, and ashes content from 100% (the remaining atoms, including P, make up less than 1%, and assumed to be negligible).

C is usually the most abundant (~50%) and other elements will be divided by C to obtain ratios H/C, N/C, S/C, and O/C ratios. For every ratio, two approaches are taken, theoretical and experimental to determine the range within which the ratio must and typically exist, respectively. It is noted also that only protein contains S and the N content of nucleic acids is known or fixed (only the N content of proteins has to be estimated).

The objectives of this chapter are to 1) derive an approximate elemental mass composition of the different biochemicals present in microalgae (proteins, carbohydrates, lipids, and nucleic acids), 2) estimate the biochemicals content through least sum of squared errors, and to 3) compare the estimated composition with that measured and calculate the root mean squared error of prediction.

5.2 Method development

5.2.1 Elemental stage

Three main biochemicals make up the bulk of microalgae biomass (proteins, carbohydrates, and lipids). The remaining biomass is usually made up of ash, nucleic acids (RNA mainly), water, pigments (chlorophyll), *etc.* (whose compositions are somewhat known and fixed; ash here is assumed to contain elements other than CHNSOP). In this first stage, the biochemicals are first analysed from a building block standpoint since all three biochemicals are made up of monomer units whose compositions are known. Knowing the building block composition/profile, the

elemental composition of the biochemical can be estimated. Literature values of the biochemical elemental composition were also obtained from other sources of these biochemicals (*i.e.* microalgae/common foods/zooplankton). Using data available from literature on elemental (and biochemical) composition of microalgae biomass grown under a wide range of conditions (hence a wide variation of biochemical compositions), the biochemical composition is predicted using the elemental composition of the biochemicals estimated previously. The predicted composition is then compared with that measured (from literature) and the root mean squared errors are reported to evaluate the prediction model.

5.2.1.1 S/C ratio

Proteins

In most cases, carbon is the main element (typically ~50wt% of biomass). Hence, the ratios (e.g. S/C, H/C, *etc.*) are used for more meaningful comparisons between different biomasses. The S/C ratios of the 18 amino acids are tabulated in Table 5-1 below along with their averages (equi-molar and equi-mass) and that of *Chlorella* species for comparisons sake.

Table 5-1 S/C ratios for various amino acids and that in *Chlorella pyrenoidosa*

amino acid	molecular formula	molecular weight	S/C (mass)	S/C (molar)
alanine	C ₃ H ₇ O ₂ N ₁ S ₀	89	0.0000	0.0000
arginine	C ₆ H ₁₄ O ₂ N ₄ S ₀	174	0.0000	0.0000
aspartic acid	C ₄ H ₇ O ₄ N ₁ S ₀	133	0.0000	0.0000

cysteine	C3H12O2N1S1	126	0.8889	0.3333
glutamic acid	C5H9O4N1S0	147	0.0000	0.0000
glycine	C2H5O2N1S0	75	0.0000	0.0000
histidine	C6H9O2N3S0	155	0.0000	0.0000
isoleucine	C6H13O2N1S0	131	0.0000	0.0000
leucine	C6H13O2N1S0	131	0.0000	0.0000
lysine	C6H14O2N2S0	146	0.0000	0.0000
methionine	C5H11O2N1S1	149	0.5333	0.2000
phenylalanine	C9H11O2N1S0	165	0.0000	0.0000
proline	C5H9O2N1S0	115	0.0000	0.0000
serine	C3H7O3N1S0	105	0.0000	0.0000
threonine	C4H9O3N1S0	119	0.0000	0.0000
tryptophan	C11H12O2N2S0	204	0.0000	0.0000
tyrosine	C9H11O3N1S0	181	0.0000	0.0000
valine	C5H11O2N1S0	117	0.0000	0.0000
Average			0.0790	0.0296
Average (equi-mass)			0.0563	0.0211
<i>Chlorella pyrenoidosa</i> amino acids ¹			0.0304	0.0114

¹ Data taken from [117]

The average S/C ratio for all 18 amino acids is 0.079 (mass) or 0.0296 (molar).

However, every amino acid has a different molecular weight. Assuming equal mass for every amino acid, that is 100/18 or 5.6wt% of total protein, the average S/C comes out to be 0.0563 (mass) or 0.0211 (molar). Also, theoretically, the S/C ratio of amino acids (or proteins) must range between 0 and 0.8889 (mass).

A more accurate ratio can be obtained if the actual amino acid profile is known. Since the amino acid profile of microalgae is said to be relatively preserved, the amino acid profile for *Chlorella pyrenoidosa* is taken as the basis of S/C ratio calculation [113,117]. The S/C ratio was calculated to be 0.0304 (mass) or 0.0114 (molar). Note that if based on the amino acid profile obtained for *Chlorella vulgaris* used in this work (in Chapter 4), the S/C ratio was calculated to be 0.0091 (mass) or 0.0034 (molar). This is lower since the acid hydrolysis step destroys the sulphur amino acids. If the wt% of the amino acids cysteine and methionine is assumed to follow that of the *Chlorella pyrenoidosa*, then the S/C ratio of proteins from *Chlorella vulgaris* is calculated to be 0.0252 (mass) and 0.0094 (molar), which is similar to that of *Chlorella pyrenoidosa*.

An analysis of 22 different common food proteins found an average S/C ratio of 0.0213 (mass) [124]. A total amino acid analysis of 6 species of zooplanktons gave an average S/C ratio of 0.025 (mass) [125].

Overall

A summary of all the S/C (mass only) values is presented in Table 5-2 below.

Table 5-2 S/C (mass) values of proteins and whole food/biomass containing proteins

Basis	S/C (mass)	References
-------	------------	------------

Proteins		
Range (theoretical)	0-0.8889	-
amino acids (average)	0.0790	-
amino acids (equi-mass)	0.0563	-
<i>Chlorella pyrenoidosa</i>	0.0304	[117]
<i>Chlorella vulgaris</i> (in this work)	0.0252	-
22 selected common foods	0.0213	[124]
Amino acids from zooplankton	0.0250	[125]

Since only proteins contain sulphur, the other biochemicals are not considered[125].

5.2.1.2 N/C ratio

Proteins

The N/C ratios of the 18 amino acids are tabulated in Table 5-3 below.

Table 5-3 N/C ratios for various amino acids and that in *Chlorella pyrenoidosa* and *vulgaris*

amino acid	molecular formula	molecular weight	N/C (mass)	N/C (molar)
alanine	C ₃ H ₇ O ₂ N ₁ S ₀	89	0.3889	0.3333
arginine	C ₆ H ₁₄ O ₂ N ₄ S ₀	174	0.7778	0.6667
aspartic acid	C ₄ H ₇ O ₄ N ₁ S ₀	133	0.2917	0.2500
cysteine	C ₃ H ₁₂ O ₂ N ₁ S ₁	126	0.3889	0.3333

glutamic acid	C ₅ H ₉ O ₄ N ₁ S ₀	147	0.2333	0.2000
glycine	C ₂ H ₅ O ₂ N ₁ S ₀	75	0.5833	0.5000
histidine	C ₆ H ₉ O ₂ N ₃ S ₀	155	0.5833	0.5000
isoleucine	C ₆ H ₁₃ O ₂ N ₁ S ₀	131	0.1944	0.1667
leucine	C ₆ H ₁₃ O ₂ N ₁ S ₀	131	0.1944	0.1667
lysine	C ₆ H ₁₄ O ₂ N ₂ S ₀	146	0.3889	0.3333
methionine	C ₅ H ₁₁ O ₂ N ₁ S ₁	149	0.2333	0.2000
phenylalanine	C ₉ H ₁₁ O ₂ N ₁ S ₀	165	0.1296	0.1111
proline	C ₅ H ₉ O ₂ N ₁ S ₀	115	0.2333	0.2000
serine	C ₃ H ₇ O ₃ N ₁ S ₀	105	0.3889	0.3333
threonine	C ₄ H ₉ O ₃ N ₁ S ₀	119	0.2917	0.2500
tryptophan	C ₁₁ H ₁₂ O ₂ N ₂ S ₀	204	0.2121	0.1818
tyrosine	C ₉ H ₁₁ O ₃ N ₁ S ₀	181	0.1296	0.1111
valine	C ₅ H ₁₁ O ₂ N ₁ S ₀	117	0.2333	0.2000
Average			0.3265	0.2799
Average if equi-mass			0.3048	0.2613
<i>Chlorella pyrenoidosa</i> amino acids ¹			0.3162	0.2711

¹ Data taken from [117]

The average N/C ratio for all 18 amino acids is 0.3265 (mass) or 0.2799 (molar).

However, every amino acid has a different molecular weight. Assuming equal mass

for every amino acid, the average N/C comes out to be 0.3048 (mass) or 0.2613 (molar). Also, theoretically, the N/C (mass) ratio of amino acids (or proteins) must range between 0.1296 and 0.7778.

The N/C ratio was calculated to be 0.3162 (mass) or 0.2711 (molar). If the wt% of the amino acids cysteine and methionine is assumed to follow that of the *Chlorella pyrenoidosa*, then the N/C ratio of proteins from *Chlorella vulgaris* used in this work (in Chapter 4) is calculated to be 0.3070 (mass) or 0.2631 (molar), which is similar to that of *Chlorella pyrenoidosa*.

An analysis of 22 different common food proteins found an average N/C ratio of 0.329 (mass) [124]. A total amino acid analysis of 6 species of zooplanktons gave an average N/C ratio of 0.3192 (mass) [125].

Overall

A summary of all the N/C (mass only) values is presented in Table 5-4 below.

Table 5-4 N/C (mass) values of proteins and whole food/biomass containing proteins

Basis	N/C (mass)	References
Proteins		
Range (theoretical)	0.1296-0.7778	-
amino acids (average)	0.3265	-
amino acids (equi-mass)	0.3048	-
<i>Chlorella pyrenoidosa</i>	0.3162	[117]
<i>Chlorella vulgaris</i> (in this work)	0.3070	-

22 selected common foods	0.3290	[124]
amino acids from zooplankton	0.3192	[125]

5.2.1.2 H/C ratio

Carbohydrates

The simplest monosaccharide, glucose, has a molecular formula $C_6H_{12}O_6$. The H/C (molar) is 2. Sucrose, a disaccharide, has the molecular formula $C_{12}H_{22}O_{11}$ with H/C (molar) of 1.8333. It has a lower H/C ratio due to the dehydration reaction that removes a molecule of water when two monosaccharides combine. Accordingly, the H/C ratio declines as the sugar molecules combine to form long chain polysaccharides such as starch and cellulose. Polysaccharides have an H/C (molar) ratio of 1.67 [124]. The H/C (molar) ratio of carbohydrates then must range between 1.67 and 2 or in terms of H/C (mass), between 0.1392 and 0.1667. An analysis of wastewater made up of carbohydrates commonly found in the human diet gave an H/C (mass) ratio of 0.1522 [124]. A carbohydrate (glycogen) analysis of zooplanktons gave an H/C ratio of 0.1396 (mass) [125].

Lipids

Saturated fatty acids have the generic formula $(CH_2)_nO_2$. Palmitic acid (C16:0) for example, has the molecular formula $C_{16}H_{32}O_2$. C16:0 then has an H/C (molar) ratio of 2. When fatty acids combine with glycerol to form triglycerides, each fatty acid loses a molecule of water and the H/C ratio decreases. For example, when 3 molecules of palmitic acid combine with a molecule of glycerol (with loss of 3 molecules of water), the H/C ratio decreases to 1.922. In the case of unsaturated fatty acids, for example palmitoleic acid, the H/C ratio decreases from an initial 1.875 (in free fatty acid form)

to 1.804 (in triglyceride form). The lowest H/C ratio then occurs when polyunsaturated fatty acids combine to form triglycerides (1.42 when DHA or C22:6 forms a triglyceride). The H/C (molar) ratio of lipids then must range between 1.42 and 2 or in terms of mass, between 0.1183 and 0.1667. The average of 18 most common fatty acids gives an H/C ratio of 1.762 (molar) or 0.1468 (mass). The most abundant fatty acids in common foods are usually C16:0 and C18:1, which have H/C (mass) ratios of 0.1602 and 0.1521, respectively (in triglycerides form) [124]. In a separate analysis, the H/C (mass) ratio of wastewater made up of fatty acids from common foods was found to be 0.1532 [124]. A triacylglycerol (triglyceride) analysis of 22 species of zooplanktons gave an average H/C ratio of 0.1488 (mass) [125].

Proteins

The H/C ratios of the 18 amino acids are tabulated in Table 5-5 below.

Table 5-5 H/C ratios for various amino acids and that in *Chlorella pyrenoidosa* and *vulgaris*

amino acid	molecular formula	molecular weight	H/C (mass)	H/C (molar)
alanine	C ₃ H ₇ O ₂ N ₁ S ₀	89	0.1944	2.3333
arginine	C ₆ H ₁₄ O ₂ N ₄ S ₀	174	0.1944	2.3333
aspartic acid	C ₄ H ₇ O ₄ N ₁ S ₀	133	0.1458	1.7500
cysteine	C ₃ H ₁₂ O ₂ N ₁ S ₁	126	0.3333	4.0000
glutamic acid	C ₅ H ₉ O ₄ N ₁ S ₀	147	0.1500	1.8000
glycine	C ₂ H ₅ O ₂ N ₁ S ₀	75	0.2083	2.5000
histidine	C ₆ H ₉ O ₂ N ₃ S ₀	155	0.1250	1.5000

isoleucine	C ₆ H ₁₃ O ₂ N ₁ S ₀	131	0.1806	2.1667
leucine	C ₆ H ₁₃ O ₂ N ₁ S ₀	131	0.1806	2.1667
lysine	C ₆ H ₁₄ O ₂ N ₂ S ₀	146	0.1944	2.3333
methionine	C ₅ H ₁₁ O ₂ N ₁ S ₁	149	0.1833	2.2000
phenylalanine	C ₉ H ₁₁ O ₂ N ₁ S ₀	165	0.1019	1.2222
proline	C ₅ H ₉ O ₂ N ₁ S ₀	115	0.1500	1.8000
serine	C ₃ H ₇ O ₃ N ₁ S ₀	105	0.1944	2.3333
threonine	C ₄ H ₉ O ₃ N ₁ S ₀	119	0.1875	2.2500
tryptophan	C ₁₁ H ₁₂ O ₂ N ₂ S ₀	204	0.0909	1.0909
tyrosine	C ₉ H ₁₁ O ₃ N ₁ S ₀	181	0.1019	1.2222
valine	C ₅ H ₁₁ O ₂ N ₁ S ₀	117	0.1833	2.2000
Average			0.1722	2.0668
Average if equi-mass			0.1635	1.9616
<i>Chlorella pyrenoidosa</i> amino acids ¹			0.1687	2.0243

¹ Data taken from [117]

Tryptophan has the lowest H/C (molar) of 1.0909. When amino acid condenses, a molecule of water is expelled, lowering the H/C ratio. Natural proteins typically have 50-500 amino acid residues [126]. Assuming a protein with 500 residues of tryptophan, this gives a reduced H/C (molar) ratio of 0.9094. On the other hand, cysteine has the highest H/C (molar) ratio of 4. Hence, the H/C (mass) must range between 0.0758 and 0.3333 or H/C (molar) between 0.9094 and 4. The average H/C

ratio for all 18 amino acids is 0.1722 (mass). However, every amino acid has a different molecular weight. Assuming equal mass for every amino acid, the average H/C ratio is calculated to be 0.1635 (mass).

The amino acid profile for *Chlorella pyrenoidosa* is taken as the basis for H/C ratio calculation [113,117]. The H/C ratio was calculated to be 0.1687 (mass) and 2.0243 (molar). If the wt% of the amino acids cysteine and methionine is assumed to follow that of the *Chlorella pyrenoidosa*, then the H/C ratio of proteins from *Chlorella vulgaris* used in this work (in Chapter 5) comes out to be 0.17 (mass).

An analysis of 22 different common food proteins, in the form of amino acids, found an average H/C ratio of 0.1719 (mass) [124]. A total amino acid analysis of 6 species of zooplanktons gave an average H/C ratio of 0.1327 (mass) [125].

Overall

A summary of all the H/C (mass only) values is presented in Table 5-6 below.

Table 5-6 H/C (mass) values of proteins and whole food/biomass containing proteins

Basis	H/C (mass)	References
Proteins		
Range (theoretical)	0.0758-0.3333	-
amino acids (average)	0.1722	-
amino acids (equi-mass)	0.1635	-
<i>Chlorella pyrenoidosa</i>	0.1687	[117]
<i>Chlorella vulgaris</i> (in this work)	0.17	-

22 selected common foods	0.1719	[124]
Amino acids from zooplankton	0.1327	[125]

5.2.1.3 O/C ratio

Carbohydrates

Similar to the H/C ratio, the condensation of monosaccharides to form polysaccharides reduces the O/C ratio due to loss of water molecule. The maximum O/C ratio is then that of monosaccharides such as glucose, which has O/C (mass) ratio of 1.3333. For polysaccharides, O/C (mass) minimum is 1.1111 although the average is 1.1133 [124]. The O/C (mass) ratio then must range between 1.1111 and 1.3333. A study of wastewater made of common carbohydrates found in human diet found an O/C (mass) ratio of 1.2173. A carbohydrate (glycogen) analysis of zooplanktons gave an O/C ratio of 1.1104 (mass) [125].

Lipids

For lipids, the general formula is $(CH_2)_nO_2$. The upper limit for O/C then is when the carbon chain is the shortest (*i.e.* C4:0) and the fatty acid exists as free form. This gives an O/C (molar) ratio of 0.5. On the other extreme, the lower limit of O/C happens when the longest chain fatty acid (*i.e.* C22) exists in triglyceride form, with loss of water. This gives an O/C (molar) ratio of 0.087. The O/C (mass) then must range between 0.116 and 0.6667. The O/C (mass) ratio of wastewater made up of fatty acids from common foods was found to be 0.1573 [124]. Also, the most common fatty acids are usually C16:0 and C18:1, with an O/C (mass) ratio of 0.1573 and 0.14, respectively. A triacylglycerol (triglyceride) analysis of 22 species of zooplanktons gave an average O/C ratio of 0.1449 (mass) [125].

Proteins

The O/C ratios of the 18 amino acids are tabulated in Table 5-7 below.

Table 5-7 O/C ratios for various amino acids and that in *Chlorella pyrenoidosa* and *vulgaris*

amino acid	molecular formula	molecular weight	O/C (mass)	O/C (molar)
alanine	C ₃ H ₇ O ₂ N ₁ S ₀	89	0.8889	0.6667
arginine	C ₆ H ₁₄ O ₂ N ₄ S ₀	174	0.4444	0.3333
aspartic acid	C ₄ H ₇ O ₄ N ₁ S ₀	133	1.3333	1.0000
cysteine	C ₃ H ₁₂ O ₂ N ₁ S ₁	126	0.8889	0.6667
glutamic acid	C ₅ H ₉ O ₄ N ₁ S ₀	147	1.0667	0.8000
glycine	C ₂ H ₅ O ₂ N ₁ S ₀	75	1.3333	1.0000
histidine	C ₆ H ₉ O ₂ N ₃ S ₀	155	0.4444	0.3333
isoleucine	C ₆ H ₁₃ O ₂ N ₁ S ₀	131	0.4444	0.3333
leucine	C ₆ H ₁₃ O ₂ N ₁ S ₀	131	0.4444	0.3333
lysine	C ₆ H ₁₄ O ₂ N ₂ S ₀	146	0.4444	0.3333
methionine	C ₅ H ₁₁ O ₂ N ₁ S ₁	149	0.5333	0.4000
phenylalanine	C ₉ H ₁₁ O ₂ N ₁ S ₀	165	0.2963	0.2222
proline	C ₅ H ₉ O ₂ N ₁ S ₀	115	0.5333	0.4000
serine	C ₃ H ₇ O ₃ N ₁ S ₀	105	1.3333	1.0000
threonine	C ₄ H ₉ O ₃ N ₁ S ₀	119	1.0000	0.7500

tryptophan	C ₁₁ H ₁₂ O ₂ N ₂ S ₀	204	0.2424	0.1818
tyrosine	C ₉ H ₁₁ O ₃ N ₁ S ₀	181	0.4444	0.3333
valine	C ₅ H ₁₁ O ₂ N ₁ S ₀	117	0.5333	0.4000
Average			0.7028	0.5271
Average if equi-mass			0.4769	0.3577
<i>Chlorella pyrenoidosa</i> amino acids ¹			0.5206	0.3905

¹ Data taken from [117]

Tryptophan has the lowest O/C (molar) of 0.1818. When amino acid condenses, a molecule of water is expelled, lowering the O/C ratio. Natural proteins typically have 50-500 amino acid residues [126]. Assuming a protein with 500 residues of tryptophan, this gives an O/C (molar) ratio of 0.0911. On the other hand, aspartic acid or glycine have the highest O/C (molar) ratio of 1. Hence, the O/C (mass) must range between 0.1215 and 1.3333 or O/C (molar) between 0.0911 and 1 (molar). The average O/C ratio for all 18 amino acids is 0.7028 (mass) or 0.5271 (molar). Assuming equal mass for every amino acid, the average O/C comes out to be 0.4769 (mass).

The amino acid profile for *Chlorella pyrenoidosa* is taken as the basis of O/C ratio calculation [113,117]. The O/C ratio was calculated to be 0.5206 (mass). If the wt% of the amino acids cysteine and methionine is assumed to follow that of the *Chlorella pyrenoidosa*, then the O/C ratio of proteins from *Chlorella vulgaris* used in this work (in Chapter 5) comes out to be 0.4993 (mass).

An analysis of 22 different common food proteins found an average O/C ratio of 0.7154 (mass) [124]. A total amino acid analysis of 6 species of zooplanktons gave an average O/C ratio of 0.4442 (mass) [125].

5.2.1.4 Overall

A summary of all the O/C (mass only) values is presented in Table 5-8 below.

Table 5-8 O/C (mass) values of proteins and whole food/biomass containing proteins

Basis	O/C (mass)	References
Proteins		
Range (theoretical)	0.1215-1.3333	-
amino acids (average)	0.7028	-
amino acids (equi-mass)	0.4769	-
<i>Chlorella pyrenoidosa</i>	0.5206	[117]
<i>Chlorella vulgaris</i> (in this work)	0.4993	-
22 selected common foods	0.7154	[124]
amino acids from zooplankton	0.4442	[125]

5.2.2 Biochemical stage

A summary of all the ratios for proteins, carbohydrates, and lipids from Section 5.2.1 is shown in Table 5-9 below, along with the averages. Nucleic acid is included as well and its composition is taken to be that of RNA [125,127].

Table 5-9 Summary of elemental ratios for proteins, carbohydrates, lipids, and nucleic acids

Basis	S/C (mass)	N/C (mass)	H/C (mass)	O/C (mass)	P/C (mass)	Ref.

Proteins						
range (theoretical)	0-0.8889	0.1296- 0.7778	0.0758- 0.3333	0.1215- 1.3333	-	-
amino acids (average)	0.0790	0.3265	0.1722	0.7028	-	-
amino acids (equi-mass)	0.0563	0.3048	0.1635	0.4769	-	-
<i>Chlorella pyrenoidosa</i>	0.0304	0.3162	0.1687	0.6942	-	[117]
<i>Chlorella vulgaris</i> (in this work)	0.0252	0.3070	0.1700	0.6657	-	-
22 selected common foods	0.0213	0.3290	0.1719	0.8347	-	[124]
amino acids from zooplankton	0.0250	0.3192	0.1327	0.4442	-	[125]
Selected for further study	0.0304	0.3162	0.1687	0.6942	-	-
Carbohydrates						
range (theoretical)	-	-	0.1392- 0.1667	1.1111- 1.3333	-	-

10 selected common foods	-	-	0.1522	1.2173	-	[124]
glycogen from zooplankton	-	-	0.1396	1.1104	-	[125]
Average	-	-	0.1459	1.1639	-	-
Lipids						
range (theoretical)	-	-	0.1183- 0.1667	0.116- 0.6667	-	-
17 selected common foods	-	-	0.1532	0.1573	-	[124]
triacylglycerol from zooplankton	-	-	0.1449	0.1488	-	[125]
Average	-	-	0.1491	0.1531	-	-
Nucleic acids (RNA)						
RNA from zooplankton	-	0.4551	0.1154	1.3590	0.2724	[125]
Approx composition assuming 50% G-C base pairs	-	0.4444	0.0833	0.9722	0.2777	[127]
Average	-	0.4498	0.0994	1.1656	0.2751	-

Using the selected/average ratios in Table 5-9, the mass fractions of each element can be determined and is shown in Table 5-10 below.

Table 5-10 Mass fraction of individual element for proteins, carbohydrates, lipids, and nucleic acids (RNA)

biochemical	C	S	N	H	O	P
proteins	0.4526	0.0138	0.1431	0.0764	0.3142	0.0000
carbohydrates	0.4329	0.0000	0.0000	0.0632	0.5039	0.0000
lipids	0.7680	0.0000	0.0000	0.1145	0.1175	0.0000
nucleic acids (RNA)	0.3345	0.0000	0.1504	0.0332	0.3899	0.0920

A complete mass balance of all biochemicals in a biomass is shown in Table 5-11 below. Total proteins, carbohydrates, lipids, RNA, water, and ash are denoted as R, L, B, U, W, and A, respectively. The total C element in the biomass is denoted as C_T , total N element as N_T , and so on.

Table 5-11 Example elemental composition of a biomass with specified biochemical composition

Amount in 100g	biochemical	C	S	N	H	O	P	Others (ash)
R	proteins	0.4526	0.0138	0.1431	0.0764	0.3142	0.0000	-
L	carbohydrates	0.4329	0.0000	0.0000	0.0632	0.5039	0.0000	-

B	lipids	0.7680	0.0000	0.0000	0.1145	0.1175	0.0000	-
U	RNA	0.3345	0.0000	0.1504	0.0332	0.3899	0.0920	-
W	water	-	-	-	0.1111	0.8889	-	-
A	ash	-	-	-	-	-	-	1.000
Overall biomass elemental composition		C _T	S _T	N _T	H _T	O _T	P _T	Q _T

Elemental analyser is commonly used to report N%, hence it is expected that C%, H%, and S% can be obtained easily as well. Ash content, A (or Q_T) can be easily measured and is commonly reported hence it is used in the prediction model. Phosphorus or P_T here, is not as commonly measured and its abundance is about 1% on average. Hence, for simplicity sake, it is assumed to be known (zero) to reduce the number of unknown variables. Oxygen or O_T here can be assumed to be the remaining (*i.e.* 100%-C, H, N, S, and ash). The unknowns left are R, L, B, U, and W. Since the sum (with ash, A) must equal 100%, one balance equation can be obtained, reducing the unknowns to 4. These unknowns are then predicted (through the use of Excel Solver) such that its sum of squared errors are the least. Mathematically, it is expressed as follows:

$$\begin{aligned}
 \text{minimize } f(x) = & (C_{T-\text{predicted}} - C_{T-\text{measured}})^2 + (S_{T-\text{predicted}} - S_{T-\text{measured}})^2 + (N_{T-\text{predicted}} - N_{T-\text{measured}})^2 + (H_{T-\text{predicted}} - H_{T-\text{measured}})^2 + (O_{T-\text{predicted}} - O_{T-\text{measured}})^2
 \end{aligned}
 \tag{5-1}$$

5.3 Method validation

The estimation methods developed in the previous sections are validated in this section using available literature data on biomass composition, both proximate and ultimate analysis. In addition, the analysed biomass (*Nannochloropsis oculata*) was grown under varying conditions (increasing growth period or N-starvation) to induce lipid accumulation and simultaneous protein degradation. The composition ranges sufficiently widely (see

Table 5-12 below) and hence allows for a more compelling validation of the approach.

Table 5-12 Biochemical estimation of *Nannochloropsis oculata* grown under varying conditions to yield a wide range of compositions. The batches were harvested in order of increasing periods of growth (3-14 days) before being freeze-dried for analysis. [128]

Batch	Measured								Predicted			
	C	H	N	O	%Protein	%Lipids	%Carbohydrates	%Ash	%Protein	%Lipids	%Carbohydrates	%RNA
1	46.3	6.4	11.2	30.4	74.7	0.0	19.4	5.9	65.6	11.8	10.8	5.9
2	53.7	7.5	9.6	23.5	58.1	23.0	13.2	5.7	56.2	34.2	0.0	3.9
3	54.6	7.8	8.7	23.5	51.4	30.7	12.3	5.6	51.8	37.0	1.7	3.9
4	59.2	8.8	4.6	24.3	28.0	46.8	22.0	3.2	30.1	49.1	17.6	0.0
5	60.1	8.8	5.4	23.4	32.1	48.8	16.7	2.4	32.6	50.8	12.0	2.2
6	60.3	9.2	4.3	22.8	26.5	51.0	19.0	3.5	28.7	53.1	14.7	0.0
7	62.9	9.3	3.9	21.8	23.4	56.1	18.4	2.1	23.4	59.7	12.9	1.9
8	62.3	9.3	2.8	23.5	17.1	58.7	22.2	2.0	16.8	58.5	21.0	1.7

To evaluate the performance of the estimation (against the measured value), root mean square error was determined as shown in Figure 5-1 below.

(a)

(b)

(c)

Figure 5-1 Scatterplots of measured biochemical content vs. that predicted for a) proteins, b) lipids, and c) carbohydrates. Root mean square error, RMSE and R^2 values are shown as well.

In the case of proteins (Figure 5-1 (a)), the predicted protein content has a RMSE of 3.452. This means, on average, the measured protein content deviates from that predicted by about 3.5% (absolute). This is considered remarkable considering that the within species coefficient of variation, CV% of a similar strain, *Nannochloropsis*, was reported to be 17% or approximately an RMSE of 7.33% (0.17 multiplied by mean of 43.1) here [55], or RMSE of 9.35% here [129].

For lipids, the content measured is lower than that estimated in the early growth phases (when lipid contents are low), then later becomes more similar in the later growth

phases (when lipid contents are high). One reason could be that in the early growth phases, there are more polar lipids (*i.e.* phospholipids) and that the Folch method of lipid extraction (one of the most common lipid extraction methods and that applied in the study where the data is sourced from) is said to not be able to recover polar lipids completely (86%) [130]. When nutrient depletes over time, the polar lipids level drops while non-polar lipids, triacylglycerides (TAGs) increase [131]. The higher recovery rate of non-polar lipids in the later growth phases and the poorer recovery rate of polar lipids in the early growth phases could lead to the observed differences. The RMSE of prediction is calculated to be 6.473%. The within method CV% of the Folch method is 1.24% or RMSE of about 2.3%.

Finally, for carbohydrates quantification, the difference method is most commonly used which can cause large errors since components other than measured proteins, lipids, and ashes are lumped together as carbohydrates. In this work, carbohydrate was also calculated through the difference method, although it considers ash and nucleic acids too. It could be that the lower than expected amount of lipids measured might have led to the higher than expected amount of carbohydrates measured due to the method of accounting used in the study. The RMSE found for carbohydrates in this work (7.518%) is nevertheless reasonable, considering that the within method CV% and RMSE of the Dubois method are 20.6% and 4.42%, respectively.

5.4 Conclusion

In this chapter, the biochemical composition of microalgal biomass was estimated through elemental and stoichiometry analysis. The elemental ratios H/C, N/C, O/C, and S/C were determined for the individual biochemicals proteins, carbohydrates,

lipids, and nucleic acids. The overall elemental composition of the biomass can be measured easily through elemental analyser. These values are then used to estimate the biochemical composition of the biomass. The applicability of the method is then assessed using data available from literature, of biomass grown under varied conditions. It found that the method is able to predict quite accurately the biochemical composition with low values of RMSE or comparable to within methods RMSE. It is nevertheless recommended for the research community to come up with a standard suite of methods, to allow for better comparison and consistency of measurements [132].

6 Conclusion

6.1 Summary of main findings

In the first of this work, the use of herbs and spices were investigated on the growth of microalgae. Different concentrations and different forms of addition were tested. Of the herbs/spices tested, four (aqueous extracts of onion, garlic, turmeric, and basil) were found to have a positive effect on the growth, but to different extends. Most notably, the use of onion extract led to the doubling of the growth rate and increase of the cell density to 6 times that of control. The carbohydrate content was also found to more than double with its addition. Interestingly, the addition of the herbs/spices directly into the culture was found to be inhibitory in general. It is thought to be due to the differential extraction/solubility of phenolic compounds in the two forms of addition (direct powder *vs.* aqueous extract).

In the following work, the protein quality of microalgae was determined through the PDCAAS method. The PDCAAS score consists of two parts: digestibility of the proteins and the limiting amino acid score. For a protein to be considered a good quality protein (*i.e.* high PDCAAS score), the proteins present must not only be easily digestible but also contain sufficient amounts of the essential amino acids needed by the human body. *Chlorella vulgaris* was found to have a digestibility of 1. Methionine was found to be the lacking amino acid, giving a limiting amino acid score of 0.88. The overall PDCAAS score hence is 0.88. This is on par with conventional sources of proteins (beef and soy) and as such, microalgae (or in this case *Chlorella vulgaris* specifically) was found to be a good protein substitute.

Finally, in the last part of this work, a method was developed to estimate the biochemical composition of biomass through elemental analysis of the biomass. An

estimated mass composition of the biochemicals (proteins, lipids, carbohydrates, *etc.*) was first found and this information is then used, along with elemental analysis to predict the biochemical composition of the biomass. The method was done tested using data readily available in literature. The method was able to predict with high accuracies giving root mean squared errors of 3.452%, 6.473%, and 7.518%, respectively for proteins, lipids, and carbohydrates.

6.2 Suggestions for future work

With regards to the work on protein quality, it would be interesting to investigate the quality of the extracted proteins instead (protein concentrate/isolate). The concentration/purification of the proteins should theoretically give a higher limiting amino acid score, meaning a higher PDCAAS score too. However, the process of extraction can play a big role on the quality too. For example, the destruction (or unavailability due to chemical reactions) of amino acids due to harsh conditions (temperature/pressure/chemicals) can reduce the amino acid score instead during the process of protein purification. Mild processing conditions are therefore needed, bearing in mind that it also has to be efficient, cheap, and scalable.

In the same respect, it would be very interesting and highly useful to develop and validate the prediction model not just on the starting biomass but also as the biomass goes through the many steps of a biorefinery process. Biorefinery processes typically steps of cell disruption, extraction, separation/purification, washing, *etc.* The optimization of the process, as a whole, and within individual unit operations, would require a large number of measurements to be done. The use of easy and relatively

quick elemental analyser, coupled with a prediction model would come in very handy and speed up the development process.

7 References

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