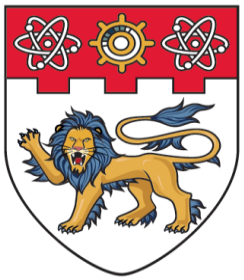

NANYANG TECHNOLOGICAL UNIVERSITY
SINGAPORE

TRANSFORMING OKARA INTO A MICROALGAE CULTURE MEDIUM

KIM JAEJUNG

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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TRANSFORMING OKARA INTO A MICROALGAE CULTURE MEDIUM

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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

Master of Engineering

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Date Professor William Chen

Authorship Attribution Statement

This thesis contains material from 2 papers published in the following peer-reviewed journals in which I am listed as a co-author.

Part of the introduction is published as:

1. Tan YX, Mok WK, Lee J, **Kim J,** Chen WN. Solid state fermentation of brewers' spent grains for improved nutritional profile using *Bacillus subtilis* WX-17. ***Fermentation***. 5, 52. *2019*
2. Mok WK, Tan YX, Lee J, **Kim J**, Chen WN. A metabolomic approach to understand the solid-state fermentation of okara using *Bacillus subtilis* WX-17 for enhanced nutritional profile. ***AMB Express***, 9, 60, 2019.

The contributions of the co-authors for the paper ". Solid state fermentation of brewers' spent grains for improved nutritional profile using *Bacillus subtilis WX-17*" are as follows:

Professor Chen WN conceived the motivation behind the project. Mok WK and Tan YX designed and performed the experiments as well as analysed the data. Kim J provided technical advice on experimental set-up and data analysis. Mok WK and Tan YX wrote the manuscript. Dr Lee J, Kim J and Professor Chen WN revised the manuscript. All authors read and approved the final manuscript.

The contributions of the co-authors for the paper "A metabolomic approach to understand the solid-state fermentation of okara using *Bacillus subtilis* WX-17 for enhanced nutritional profile" are as follows:

Tan YX and Mok WK performed the experiments and wrote the manuscript. Lee J commented on the experiment and manuscript. Kim J provided technical assistance. Professor Chen WN reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Date KIM JAEJUNG

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Abstract

Okara is a soybean residue generated in the soymilk and tofu production. Despite the rich nutritional content of okara, majority of it is underutilized and discarded due to its insolubility. In this study, solid-state fermentation with food-grade fungi was utilized to solubilize the nutrients in okara. The fermented okara was then used as a nutrient-rich culture medium for the microalgae *Phaeodactylum tricornutum* (*P. tricornutum*). The results showed significantly higher biomass production in the fermented okara medium (0.52 g L^{-1}) as compared to the conventional medium F/2 (0.25 g L^{-1}), leading to a 2-fold increment. Moreover, fucoxanthin productivity remarkably increased by 4.8-fold from 0.24 mg $L^{-1}d^{-1}$ to 1.17 mg $L^{-1}d^{-1}$. The fatty acid composition of the cells showed that saturated fatty acids and monounsaturated fatty acids comprised greater proportion of the cells grown in F/2 medium whereas polyunsaturated fatty acids comprised greater proportion of the cells grown in fermented okara medium. This study demonstrates an innovative and low-cost strategy of using fermented okara as a nutrient-rich substrate for achieving high-density algae cultivation and for increasing the production of high-value compounds in a sustainable way.

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1. Introduction

1.1 Okara production and nutrition composition

Okara, a soybean residue, is a by-product generated from the soymilk and tofu production (Fig. 1). High demand for soybean-derived products particularly in the Asian countries lead to huge quantities of okara production annually across the world. For example, the amount of okara generated in China, Japan and Korea is about 2,800,000 tons, 800,000 tons, and 310,000, respectively, every year (B. Li, Qiao, & Lu, 2012).

Figure 1. Schematic diagram depicting the production of okara in the soy and tofu manufacturing process.

Majority of the okara generated every year is discarded and only a minor portion of okara is used as an animal feed, leading to economic loss (B. Li, Qiao, & Lu, 2012). Yet, okara contains high nutritional value as shown in Table 1. Generally, it consists of 55% fiber, 26% protein, 10% lipid, 4% carbohydrate and abundant minerals and vitamins (Van der Riet, Wight, Cilliers, & Datel, 1989). Much research has been conducted to utilize the rich nutrient content in okara (B. Li, Qiao, & Lu, 2012; O'Toole, 1999; Van der Riet, Wight,

Cilliers, & Datel, 1989). However, applications of okara is limited because of its high insolubility attributed to fiber content. Okara is a lignocellulosic biomass covered by rigid cell walls consisting of cellulose, hemicellulose, lignin and pectin, which hinders accessibility to its useful nutrients, particularly proteins. To fully utilize the valuable nutrients entrapped in okara, a method to hydrolyze the okara is needed.

Table 1: Percentage fiber, protein, carbohydrate reportedly found in okara (g/100g dry weight) (Van der Riet, Wight, Cilliers, & Datel, 1989)

Fiber	Protein	Lipid	Carbohydrates
52.8-58.1	25.4-28.4	9.3-10.9	3.8-5.3

1.2 Valorization of okara

In order to recover the useful nutrients in okara, various treatment methodologies such as enzymatic treatment and high-pressure treatment have been attempted (Kasai, Murata, Inui, Sakamoto, & Kahn, 2004). For example, a study hydrolyzed okara with various enzymes including pectinase, cellulase, and lacchase resulting in 72.3% yield. Another study combined autoclaving with enzymatic treatments such as cellulases and two pectinases to effectively solubilize the okara, which led to 83-85% yield (Kasai, Murata, Inui, Sakamoto, & Kahn, 2004). However, the use of commercial enzymes is rather costly. Alternatively, "Biological pre-treatment would be a more environmentally friendly option as it does not require chemicals or solvents, and has the added advantage of not generating toxic compounds (Sindhu, Binod, & Pandey, 2016). For agricultural by-products, solid state

fermentation commonly serves as a biological pre-treatment method, which is also convenient and economical. This is because SSF requires less energy, produces less wastewater, and hence is overall more environmentally friendly (Pandey, 2003). For instance, solid-state fermentation has been applied to brewers' spent grains (BSG), which are underutilized food waste materials produced in large quantities from the brewing industry. The components of BSG consist of the barley malt grain husks, pericarp, and seed coat layers of the grains (Mussatto, Dragone, & Roberto, 2006). BSG are lignocellulosic materials that contain cellulose, non-cellulosic polysaccharides, and lignin (Mussatto, Dragone, & Roberto, 2006). Also, BSG were found to be abundant in proteins, essential amino acids, fiber, and phenolic compounds (Mussatto, 2014). Nutrients such as lipids, fatty acids, and polyphenols were also found to be present in BSG (Fărcaş, Socaci, Dulf, Tofană, Mudura, & Diaconeasa, 2015). The predominant lipids identified were triglyceride and the fatty acids were linoleic, palmitic, oleic, α-linoleic, and stearic acids. Other fatty acids, including myristic and vaccenic acids, were present in lower amounts (Fărcaş, Socaci, Dulf, Tofană, Mudura, & Diaconeasa, 2015). One of the main challenges in the utilization and extraction of useful components in BSG is that the proteins and nutrients are bound to the cellulose and hemicellulose. As such, one study utilized solid state fermentation of BSG using *Bacillus subtilis* WX-17, a strain isolated from commercial natto (Tan, Mok, Lee, Kim, & Chen, 2019). This showed significantly enhanced nutritional content in BSG as compared to unfermented BSG. In total, 35 metabolites showed significant difference, which could be categorized into amino acids, fatty acids, carbohydrates and tricarboxylic acid cycle intermediates. Pathway analysis revealed that glycolysis was upregulated, as indicated by the drop in the level of carbohydrate compounds. This shifted the metabolic

flux particularly towards the amino acid pathway, leading to a 2-fold increase in the total amount of amino acid from 0.859 ± 0.05 to 1.894 ± 0.1 mg per g of BSG after fermentation. Also, the total amount of unsaturated fatty acid increased by 1.7 times and the total antioxidant quantity remarkably increased by 5.8 times after fermentation." Solid-state fermentation can also be applied to okara given its abundant nutrient. For instance, a recent study showed that "fermentation of okara with *B. subtilis* WX-17 improved its overall nutritional content (Mok, Tan, Lee, Kim, & Chen, 2019). The total amino acids content increased from 3.04 ± 0.14 mg/g in unfermented okara to 5.41 ± 1.21 mg/g in okara fermented with *B. subtilis* WX-17. Total fatty acids content increased from 153.04 ± 5.10 to 166.78 ± 2.41 mg/g okara, after fermentation. Antioxidant content (DPPH) also increased by 6.4 times after fermentation."

While these studies have employed bacteria for fermentation, fungi are also an attractive microorganism that secretes abundant enzymes including proteases, lipases and cellulolytic enzymes that can break down complex biomasses such as okara. In particular, filamentous fungi secrete polysaccharidases such as exoglucanses, endoglucanses and β-glucosidases which can catabolize fiber and release fermentable sugars including glucose and galactose. At present, various research has been conducted to ferment okara to improve nutritional value and hence increase the potential of okara as human or animal feed. For instance, okara is fermented with the fungus *Rhizopus oligosporus* to produce the traditional Indonesian food called tempeh (Yogo, Ohashi Kunihiko Terakado, Harada, Nezu, Hara, Tagawa, et al., 2011). 15g of dried Tempeh was then used as animal feed for 2 weeks. Throughout the intake period, the animal feces contained significantly higher amount of short-chain fatty acids as well as microbiota such as *Bacillus* and *Bifidobacterium* were significantly

increased, demonstrating the nutritional advantage of fermented okara (Yogo, et al., 2011). These studies have demonstrated that microbial fermentation was effective in recovering useful nutrients from the food by-product.

2.3 Microalgae as a source of high-value compounds

Microalgae have attracted significant attention as a green and renewable source of high-value nutrients such as polyunsaturated fatty acids (PUFAs) and carotenoids. However, the high production cost has limited the commercial potential of microalgae. To promote the competitiveness of microalgae, extensive studies have searched for methods to reduce the production cost while increasing the yield of bioactive metabolites. Numerous studies have reported increased biomass and lipid yield for some microalgae species when the culture medium was supplemented with organic substrates such as glucose and glycerol. This mode of cultivation, mixotrophic cultivation, is based on the synergistic effect of light and organic substrate (Bhatnagar, Chinnasamy, Singh, & Das, 2011; Garcí, Sevilla, Fernández, Grima, & Camacho, 2000; Garcıa, Mirón, Sevilla, Grima, & Camacho, 2005; Y. Li, Zhou, Hu, Min, Chen, & Ruan, 2012; Liang, Sarkany, & Cui, 2009). Another approach that has been widely studied is to use wastewater as a medium to culture microalgae (Ellis, Hengge, Sims, & Miller, 2012; Y. Li, Zhou, Hu, Min, Chen, & Ruan, 2011; Woertz, Feffer, Lundquist, & Nelson, 2009) which may significantly reduce the overall production cost.

Given the high protein content of okara of 15.2-33.4% (Singh, Meena, Kumar, Dubey, & Hassan, 2015), we hypothesized that the rich protein content in okara could serve as a nitrogen source for cultivation of microalgae. Microbial biotransformation of the protein

into low molecular weight fractions of peptides and amino acids may serve as a free amino nitrogen source to microalgae. For instance, a study hydrolyzed the food waste using fungi to recover glucose, free amino nitrogen and phosphate (Pleissner, Lam, Sun, & Lin, 2013). Subsequently, they utilized the obtained hydrolysate to cultivate microalgae. Microalgae was able to grow in the food waste hydrolysate and the cells contained significantly more proteins and lipids relative to that in the conventional medium. Such result indicates that the food waste hydrolysate was able to serve as a nutrient-rich culture medium.

Among the numerous microalgae species, the marine microalgae *Phaeodactylum tricornutum* (*P. tricornutum*) particularly have garnered much interest as a sustainable source of high-value nutrients. It is rich in omega-3 fatty acids, particularly EPA which provides important health benefits such as lowering blood cholesterol, inflammation, preventing cardiovascular and coronary heart diseases (Yongmanitchai & Ward, 1991a). Currently, marine fish is the major source of EPA and the increasing consumption of EPA as a dietary supplement have been imposing burden on the available live fish stock. As such, *P. tricornutum* is a promising alternative as a sustainable source of EPA. In addition, *P. tricornutum* has high content of fucoxanthin, a major carotenoid in diatoms (Kim, Jung, Kwon, Cha, Um, Chung, et al., 2012). Fucoxanthin plays a role in transferring light energy into the chlorophyll-a for photosynthesis. It displays extensive health benefits such as anti-obesity, anti-cancer, antidiabetic.

The objective of the study was to recover the nutrition enclosed in the okara and utilize it as a culture medium for growing microalgae. Considering okara's high nitrogen content, we hypothesized solid-state fermented okara media is an excellent low-cost and nutrient-

rich substrate to culture microalgae. We selected *Phaeodactylum tricornutum* (*P. tricornutum*) as the algae of interest to extract high-value nutrients such as fucoxanthin and eicosapaentanoic acid (EPA). Overall, the study presents a sustainable production of high-value nutrients from microalgae using okara as a substrate.

2. Materials and Methods

2.1 Algal culture

Phaeodactylum tricornutum (*P. tricornutum*) UTEX646 was obtained from the Culture Collection of Algae at The University of Texas at Austin (UTEX), USA. *P. tricornutum* was cultured under continuous white fluorescence light at an irradiance of 50 μmol photons $m^{-2}s^{-1}$ at 20 ± 1℃ with shaking at 140 rpm and sub-cultured every 5 days.

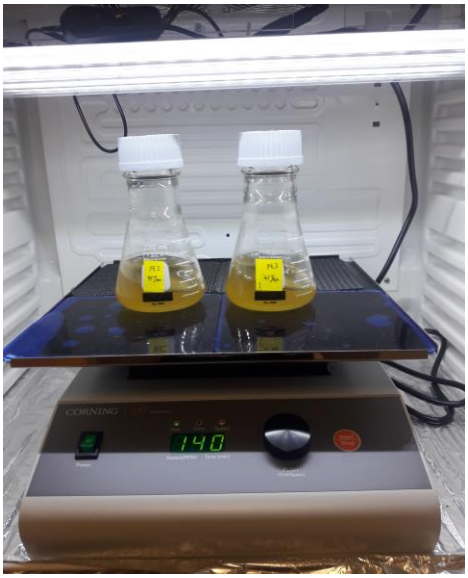

Figure 2. Microalgae culture setup in a chilling incubator with white fluorescence light and an orbital shaker.

P. tricornutum was cultured in conventional medium (F/2) and fermented okara medium where exponentially growing cells were used for inoculation to make experimental cultures with initial OD_{540} of 0.1.

F/2 was prepared using artificial seawater (ASW) by adding seasalt (Sigma, USA) into de-ionized water and stirring for 2 days until the seasalt was fully dissolved and reach the salinity of 30 ppt. ASW was then filter-sterilized through 0.22 μm membranes and enriched with concentrated F/2 without silicon (Guillard, 1975). For fermented okara medium, *P. tricornutum* was cultivated in 20% v/v fermented okara medium prepared with ASW and allowed to acclimate for 3 months before conducting any subsequent analysis.

2.2 Determination of algal growth

P. tricornutum biomass concentration was estimated by converting the absorbance of the culture at 540 nm to dry-weight using an acquired equation:

$$DCW\ (mg/L) = 0.1794\text{x}$$

Where x is the optical density measured at 540 nm. The equation was constructed by determining the dry-weight by centrifuging certain volume of culture broth at 10,000×g and washing twice with water followed by lyophilization for 48 hours.

2.3 Fermented okara media preparation

Fresh okara were provided by Vitasoy International Singapore Pte Ltd (Singapore) and stored at -20 ℃ until usage. Solid-state fermentation of okara and extraction of the nutrients to form fermented okara medium were performed (Kiran, Salakkam, Trzcinski, Bakir, & Webb, 2012). The prepared fermented okara medium was filter-sterilized and stored at -20 ℃ until further usage.

2.4 Nutritional analysis of fermented okara media

Nutritional analysis was carried out by an accredited analytical laboratory of ALS Technichem Pte Ltd (Singapore). Nitrogen content was analyzed by the Kjeldahl method (P. L. Kirk, 1950) and the carbohydrate content and phosphorus content according to the Kirk and Sawyer (S. Kirk & Sawyer, 1991) methods.

2.5 Quantification of fucoxanthin

Quantification of fucoxanthin was carried out with using Agilent 1100 high performance liquid chromatography equipped with a photodiode array detector and C_6 reverse phase column. Methanol and Methanol/1M Ammonium acetate (70:30) were used as the mobile phases with a flow rate of 1 mL/min using the gradient as follow:

Table 2: HPLC-DAD solvent gradient set-up for fucoxanthin quantification

Time (min)	%A (Methanol/1M Ammonium acetate)	%B (Methanol)
0	95	5
80	5	95
81	5	95
82	95	5
100	95	5

Detection was recorded at 445 nm. Fucoxanthin standard (Sigma, USA) was used to produce the standard curve.

2.6 Staining of lipid content in algal cells

Algal culture was washed with PBS mixed with 6 ul Nile red solution and incubated for 5min in the dark. Cells were observed using a fluorescence microscope (Nikon, Japan) with a 450-490 nm excitation.

2.7 Lipid production measurement

3mL of chloroform:methanol 2:1 (v/v) with 10 μL of tripentadecanoin (20mg/mL), 10 μL of heptadeacnoin (10mg/mL) as an internal standard were added to the lyophilized algae biomass. The sample was then homogenized using a bead grinder (Fast Prep, MP Biomedicals, USA) at 6.5 ms^{-1} for 40 seconds for 8 cycles, with 60 seconds of cooling in between each cycle to minimize oxidation. Subsequently, 1mL of 0.9 % NaCl was added and centrifuged at 10,000×g for 10 mins. The bottom phase was then extracted and evaporated with N_2 gas and to complete dryness under vacuum using rotary evaporator at 30 ℃. The dried mass was measured and the lipid content of *P. tricornutum* was calculated.

2.8 Fatty acid measurement

To measure the individual fatty acid composition, fatty acids were transesterified into fatty acid methyl ester (FAME) by adding 500 μL of boron-trifluoride and incubating at 95 ℃ for 20 mins. After cooling the samples to room temperature, 300 μL of hexane and 300 μL of saturated NaCl was added and centrifuged at 10,000×g for 10 mins. The upper phase was then transferred into a glass vial for analysis using gas chromatography-mass spectrophotometry 7890A-5975C (Agilent Technologies, USA) equipped with HP-5MS UI column (30 m x 250 μm x 0.25 μm). The ion source temperature and injector temperature were set at 230 °C and 250 °C, respectively. The oven temperature was kept at 80 °C for 1 min and then raised to 250 °C at the rate of 7 °C/min and held for 8 mins. Injection volume of 1 μL was used. Data were scanned from 35 – 600 m/z. NIST08 mass spectral library was

used to identify the fatty acids and only those matching with 80% similarity index and above were selected. Fatty acids were normalized with respect to the internal standards.

2.9 Statistical analysis

All the biochemical analysis of the microalgae cells was performed in triplicates and the values were reported as means ± standard deviation. Statistical analysis was performed using student's t-test, and means were compared at a significance level of $p < 0.05$

3. Results and Discussion

3.1 Okara fermentation and media preparation

When okara was incubated with spores of the fungi, white filaments formed, and the color turned grey with incubation time (Fig. 3), indicating successful adherence of the fungi onto the surface of okara and growth via fermentation of okara. The fermented okara was then immersed in ASW to extract the nutrients into the medium. Finally, the media was filter-sterilized instead of autoclaving, to avoid exertion of high heat and pressure that may denature any valuable nutrients.

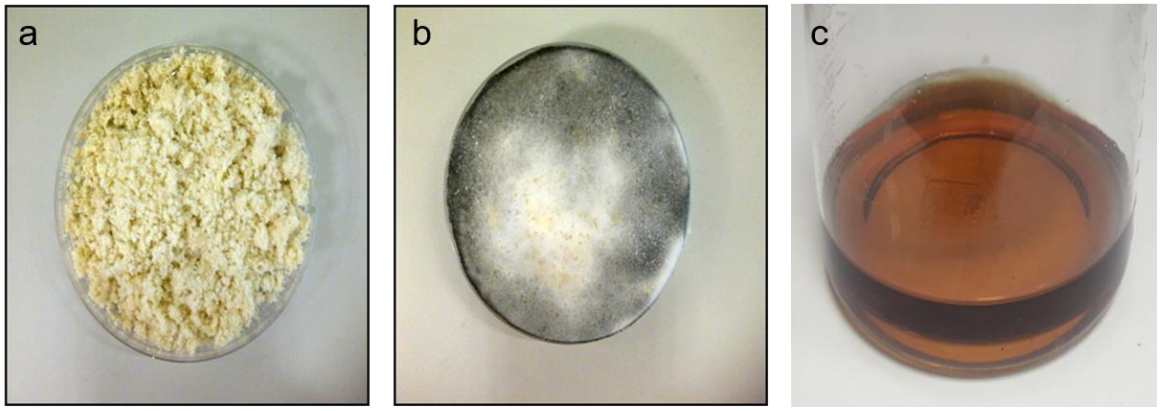

Figure 3: Preparation of fermented okara media: (a) Unfermented and (b) fermented okara on petri plate and prepared (c) fermented okara media.

3.2 Growth of P. tricornutum in fermented okara media and comparison to conventional media

The functionality of the fermented okara media in growing microalgae was demonstrated by measuring the optical density of the culture with incubation time (Fig. 4).

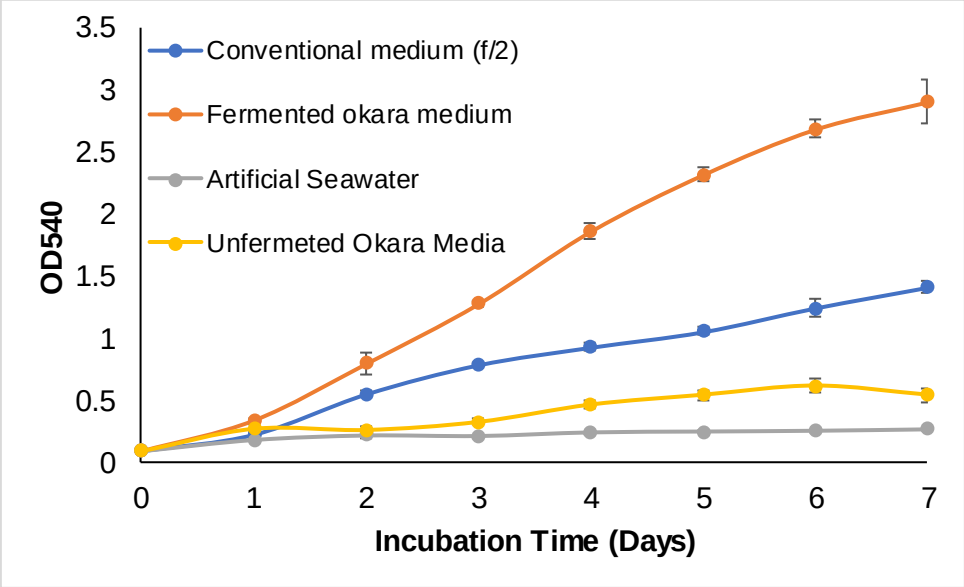

Figure 4: Growth curve of *P. tricornutum* in conventional media (f/2), fermented okara media, artificial seawater and unfermented okara in terms of optical density.

As optical density depends on the turbidity of the culture, we converted the parameter to biomass concentration to accurately quantify the microalgae growth. This was done by first creating a standard curve between optical density and its respective biomass concentration as shown in Figure 5.

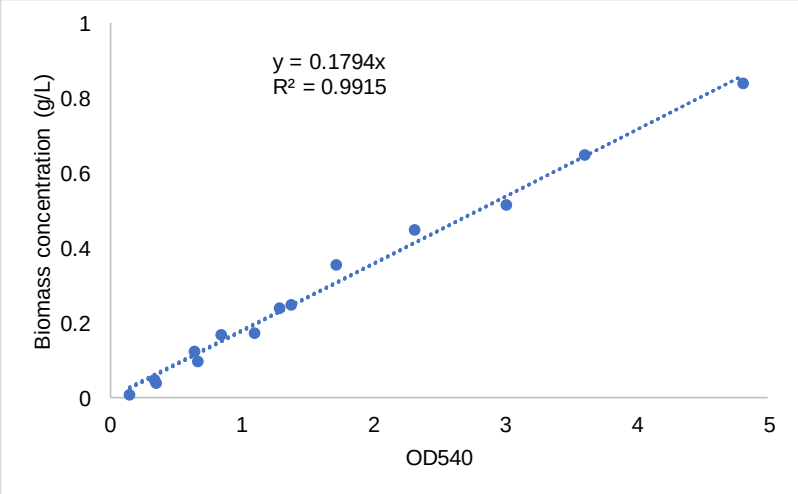

Figure 5. Standard curve between optical density of the culture measured at 540 nm and its respective biomass concentration.

As shown in Figure 6, the growth profile of *P. tricornutum* in fermented okara media and F/2 medium was clearly different, in which the cells grew significantly better in the fermented okara media compared to F/2 medium throughout the incubation period ($p < 0.05$).

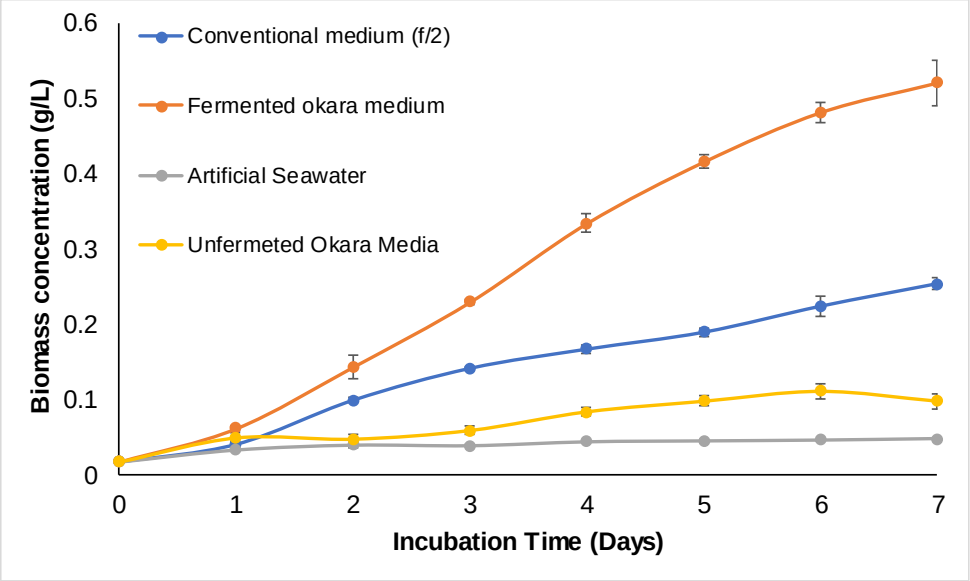

Figure 6. Growth curve of *P. tricornutum* in conventional media (f/2), fermented okara media, artificial seawater and unfermented okara in terms of biomass concentration.

In the fermented okara medium, maximum growth rate of 0.086 $gL^{-1}d^{-1}$ while 0.058$gL^{-1}d^{-1}$ was found for F/2 medium. The maximum biomass produced from the fermented okara medium (0.52gL^{-1}) was approximately 2-fold more as compared to the f/2 medium (0.25gL^{-1}) at the end of the fermentation. Our result clearly demonstrates the superior functionality of fermented okara medium in cultivating microalgae as compared to the conventional medium. In addition, our preliminary studies have shown that the *P. tricornutum* cells could not grow under undiluted fermented okara medium, given the extraordinary amount of nitrogen and phosphorus. The medium was hence prepared with a dilution factor of 5 in ASW for growing *P. tricornutum*. This also adds value in terms of economic perspective, where little amount of okara (10 g, wet mass) can produce approximately 200 mL of

fermented okara medium. Another noteworthy point is the difference in the unfermented and fermented okara media in culturing microalgae. The *P. tricornutum* biomass obtained in unfermented okara medium was substantially lower as compared to fermented okara medium. This highlights the crucial role of fermentation in effectively utilizing soybean residue, possibly by hydrolyzing macromolecules into small molecules that enables microalgae to readily utilize the simplified form of nutrients. For instance, we reported in our previous study that fermentation of okara using *Bacillus subtilis* WX-17 led to approximately 2-fold increase in amino acid content from 3.04 mg/g to 5.41 mg/g of okara compared to that without fermentation. As such, fermentation may enhance the amount of small free amino nitrogen molecules and serve as an effective nitrogen source for cultivating *P. tricornutum* cells. A similar trend was found in a study (Cooray, Lee, & Chen, 2017) which fermented another type of food by-product, brewers' spent grain (BSG), a protein and fiber rich by-product produced from the beer manufacturing industry. The authors reported increased amino acid content in fermented BSG media compared to unfermented BSG media. It was then showed that the fermented BSG media could be used to support the growth of the nitrogen-consuming yeast *Rhodosporidium toruloides,* whereas no obvious growth was seen in the unfermented BSG media. This demonstrates that fermentation is an effective methodology for improving the nutritional content of food by-products including okara. In order to investigate the nutrients behind the excellent growth of the cells in fermented okara media, nutritional composition of the media was analyzed.

3.3 Fermented okara media nutrient composition

The nutrient composition of the obtained fermented okara media was characterized and compared to F/2 media, as shown in Table 3. The fermented okara media contained considerable amount of carbohydrate (3g/L), while no carbohydrate is present in F/2 media. Remarkable amount of nitrogen of 1500mg/L and phosphorus of 140 mg/L were found in the fermented okara medium, which is 122-fold and 144-fold more as compared to the f/2 media, respectively. Such high concentration of the key nutrients for microalgae contained in the fermented okara media may be the reason behind the excellent growth of *P. tricornutum* cells.

Table 3: Nutrient composition in fermented okara media and F/2 media

Parameter	Fermented Okara media	F/2[a]	Fold Change
Carbohydrates (mg L^{-1})	300	0	N.A.
Nitrogen (mg L^{-1})	150	12.3	X12
Phosphorus (mg L^{-1})	14	0.1389	X12

[a] F/2 nutritional composition was obtained from the supplier

For instance, carbohydrate present in the fermented okara media can serve as an organic carbon source. This allows the microalgae to utilize respiration as an additional route for carbon fixation besides photosynthesis, and synergistically carry out the two different metabolic processes to promote growth. Such mixotrophic condition have been reported to result in higher microalgae growth rate and biomass production than under

photoautotrophic condition, consistent with our result (Cerón Garcí, Fernández Sevilla, Acien Fernandez, Molina Grima, & García Camacho, 2000). Also, various nutrients have been known to affect the microalgae growth in different ways. Nitrogen is well known as a crucial nutrient by affecting nucleic acids, amino acids, proteins, pigments synthesis and even cellular carbon accumulation (Yodsuwan, Sawayama, & Sirisansaneeyakul, 2017) while phosphorus is known to influence cellular division (Chauton, Olsen, & Vadstein, 2013). Thus, the substantially higher amount of nitrogen and phosphorus in the fermented okara media as compared to f/2 media may have contributed to the increased biomass production. One study (McClure, Luiz, Gerber, Barton, & Kavanagh, 2018) varied the culture condition and reported that 10 x f/2 medium enhanced the *P. tricornutum* biomass yield to the largest extent followed by f/2 medium with 10 x nitrate addition and lastly f/2 media without any nutrient supplementation. This suggest that various nutrients including nitrogen supplementation to f/2 medium can promote microalgae growth. To better understand the physiological response of the *P. tricornutum* cells associated to photosynthesis, we analyzed the pigment contents such as chlorophyll and fucoxanthin production.

3.3 Fucoxanthin Production

Chlorophyll and fucoxanthin production of *P. tricornutum* in fermented okara media and F/2 medium was determined (Table 4). When *P. tricornutum* cells were cultured in the fermented okara media, remarkably high chlorophyll content of 76.5 mg g^{-1} was found as compared to the 15.5 mg g^{-1} found in the f/2 media. Similarly, higher fucoxanthin content

of 15.3 mg g^{-1} was found in the fermented okara media as compared to 4.9 mg g^{-1} found in the f/2 media. Overall, the fucoxanthin productivity increased by 5-fold from 0.24 mg $g^{-1}d^{-1}$ to 1.17 mg g^{-1} d^{-1}.

Table 4: Quantification of fucoxanthin

Parameters	Fermented Okara media	F/2	Fold Change
Chlorophyll content (mg g^{-1})	76.5 ± 5.60	15.5 ± 4.1	X5
Fucoxanthin content (mg g^{-1})	15.3 ± 0.6	4.9 ± 1.2	X3
Fucoxanthin productivity (mg $L^{-1}D^{-1}$)	1.17 ± 0.04	0.24 ± 0.04	X5

The notably high fucoxanthin production in the fermented okara media may be attributed to its rich nutrient content, especially nitrogen. A recent study (McClure, Luiz, Gerber, Barton, & Kavanagh, 2018) observed significant increase in fucoxanthin concentration, from 23.2 mg g^{-1} to 59.2 mg g^{-1} in *P. tricornutum* after enriching the culture medium with additional nitrate. Another study (Xia, Wang, Wan, Li, Hu, & Zhang, 2013) reported a three-times in fucoxanthin concentration from 6.71 to 18.14 mg g^{-1} in *O. aurita* after nitrate supplementation. A separate study (Guo, Liu, Yang, Sun, Lu, Liu, et al., 2016) also reported two-fold increase in fucoxanthin concentration from 6 to 12mg g^{-1} in C. cryptica. An interesting study (Gómez-Loredo, Benavides, & Rito-Palomares, 2016) reported that fucoxanthin concentration in *P. tricornutum* and *I. galbana* was greater in the cells cultured using Conway medium compared to f/2 medium. All the reported studies suggest the close

relationship between the nutrient concentration of the culture medium and fucoxanthin production.

The high biomass concentration of *P. tricornutum* in the fermented okara media may also have promoted the production of fucoxanthin in the cells. Increasing cell density causes the culture broth opaquer, which then reduces the level of transmitted light reaching the cells. As light progressively becomes the limiting factor, cells may stimulate fucoxanthin production in response given the fucoxanthin's primary role as a 'light-harvesting' pigment. The large impact of light on fucoxanthin production of *P. tricornutum* have also been reported by others. One study reported 4-fold increase in fucoxanthin concentration (42.8 mg g^{-1}) of *P. tricornutum* at low light intensity (100 μmol photons m^{-2} s^{-1}) compared to that at high light intensity (210 μmol photons m^{-2} s^{-1}, 9.9 mg g^{-1}) (McClure, Luiz, Gerber, Barton, & Kavanagh, 2018). This suggest that higher production of fucoxanthin in microalgae cells as a compensation to reduced light intensity, consistent with our result.

Fucoxanthin is an accessory pigment that forms a complex with chlorophyll in the *P. tricornutum*, giving algae culture a brown colour. In relation, the substantially higher fucoxanthin content of *P. tricornutum* combined with the greater cell density in the fermented okara media led to a darker brown colour culture than that in the F/2 medium as shown in Fig. 7.

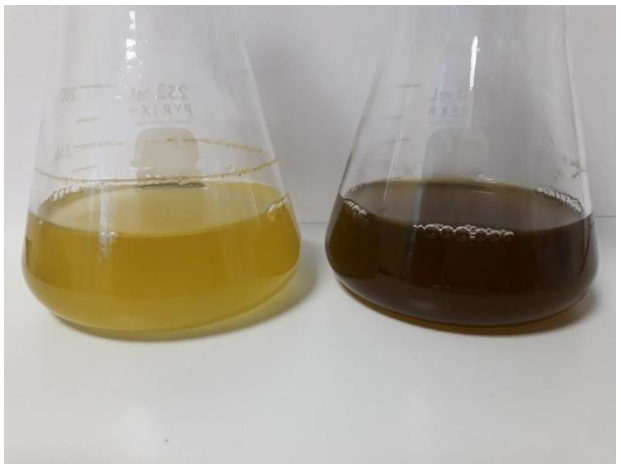

Figure 7: Culture solution of *P. tricornutum* in F/2 (Hodgson, Henderson, Sargent, & Leftley) and fermented okara media (right).

3.4 Fatty Acid Production

The lipid in the algae cells were stained to compare the influence of the respective culture mediums on the lipid production of the cells (Fig. 8). It appears that more lipid is contained in the algae cells harvested from the fermented okara media compared to that of the F/2 medium.

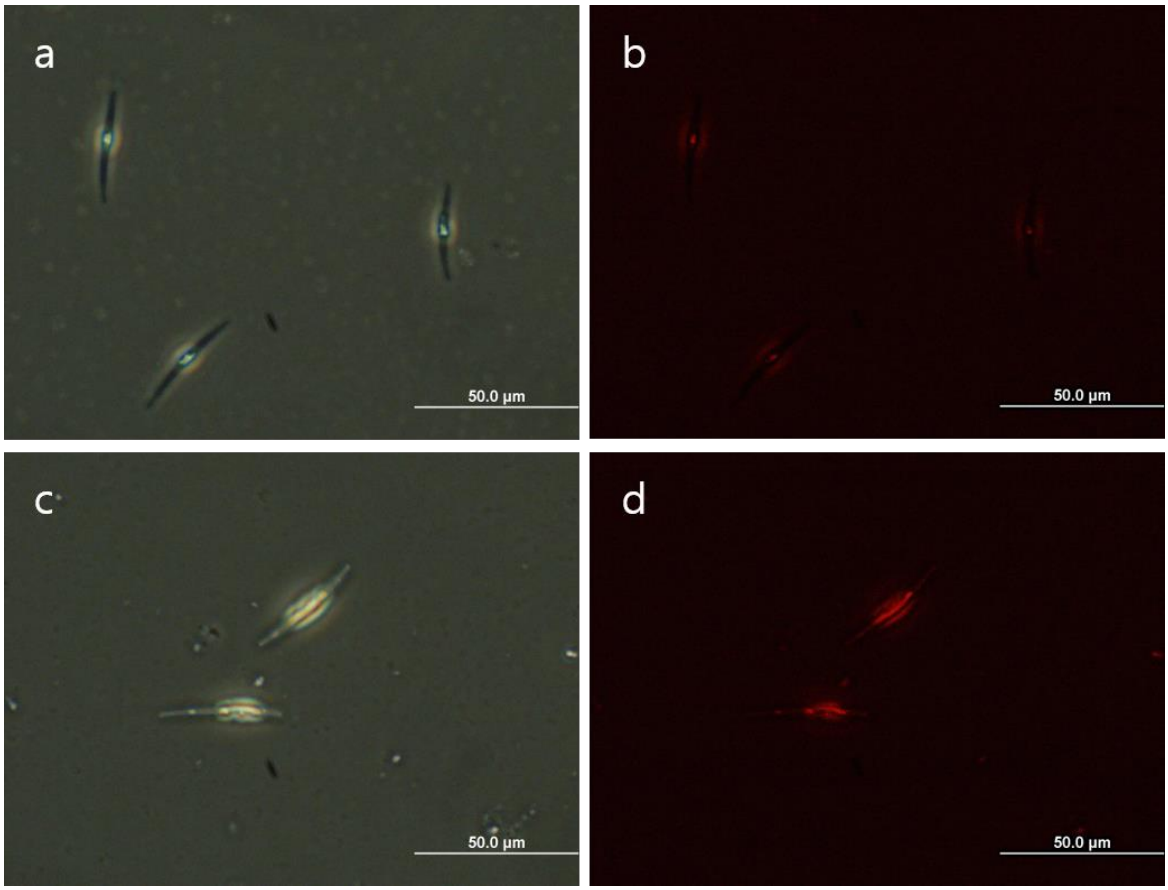

Fig. 8: Algae harvested from F/2 medium under a: bright field; b: fluorescence (lipid stained in red). Algae harvested from fermented okara media under c: bright field; d: fluorescence (lipid stained in red)

Fatty acid composition of *P. tricornutum* cells were investigated (Fig. 9). The profile of the major fatty acids in the *P.tricornutum* cells were similar under both culture conditions, namely C16:0, C16:1, C16:3, C18:0, and C20:5. However, the relative fatty acid abundance differed. Saturated fatty acids (SFAs) C14:0, C16:0, and mono-unsaturated fatty acid (MUFA) C16:1 comprised greater proportion of the total fatty acid than in the

photoautotrophic cell whereas polyunsaturated acids (PUFAs) C16:3 and C20:5 (EPA) was vice versa.

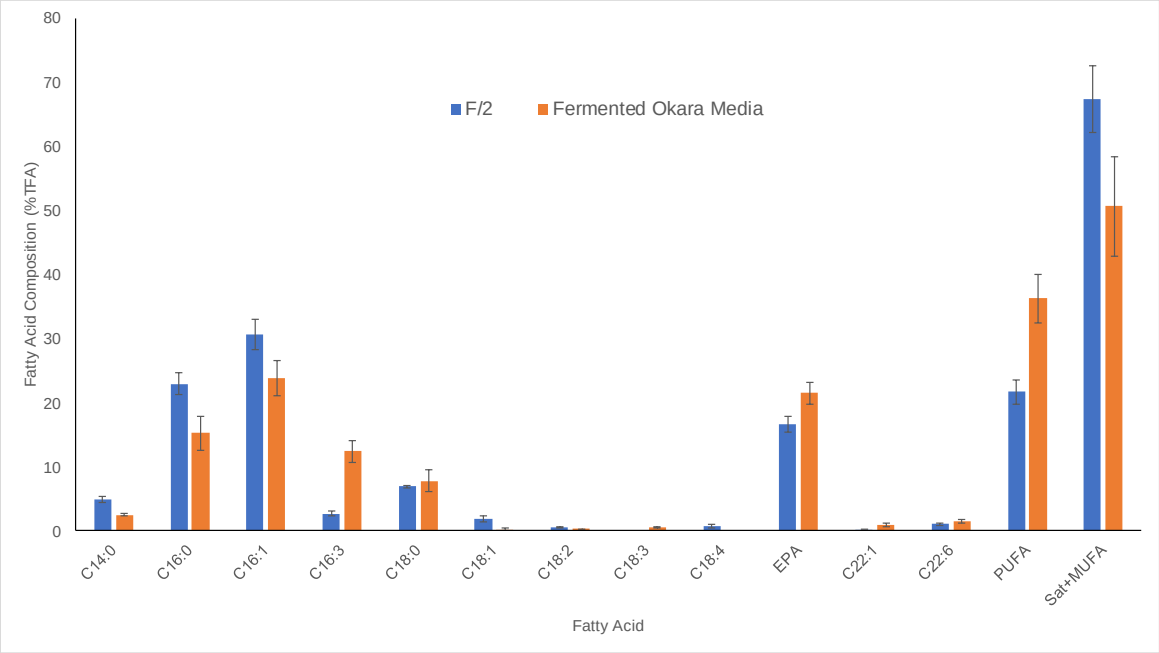

Figure 9. Fatty acid composition of *P. tricornutum* grown in F/2 and fermented okara media.

SFAs and MUFAs are found mainly in the neutral lipids in the form of triacylglycerides that are responsible for energy storage in the microalgae (Hodgson, Henderson, Sargent, & Leftley, 1991). As such, the higher amount of SFAs and MUFAs in the cells under the f/2 media suggest the physiological response towards energy storage. On the other hand, PUFAs are one of the principal components of polar lipids that forms the structural component of the cell, including the photosynthetic membrane of the chloroplast (Grima, Pérez, Camacho, Sevilla, & Fernandez, 1994). In relation, the particularly high levels of unsaturation in the cells under mixotrophic condition in our study may be to support the

high chlorophyll content, favored by the high nitrogen concentration in the fermented okara media. This is in line with other studies which reported increased proportion of PUFAs in the algae in culture media containing high nitrogen levels (Yongmanitchai & Ward, 1991b).

While it is widely known that nitrogen stress leads to higher lipid content, it has been similar for our case. This may be due to the presence of carbohydrates, leading to greater availability of carbon in the fermented okara media which may support the cells to produce more lipids (Roessler, 1990).

4. Conclusion and Future Perspective

4.1 Conclusion

In this study, soybean residue (okara) produced in large quantities was explored as a potential substrate for microalgae cultivation. Solid-state fermentation with food-grade fungi was employed to release the nutrients entrapped in the okara and catabolize the macromolecules into its simpler forms. Subsequently, the nutrients were extracted into artificial seawater, creating fermented okara media. The microalgae *P. tricornutum* showed excellent growth in the fermented okara media, yielding biomass concentration of 0.52 gL^{-1} that was found to be 2-fold more as compared to that in the conventional media F/2 (0.25 gL^{-1}). Analysis of the nutritional content of the fermented okara media showed remarkably higher nitrogen, phosphorus and carbohydrate concentration in comparison to F/2 media, which may explain the superior functionality in supporting microalgae growth. The fermented okara media also promoted the fucoxanthin production, leading to 5-fold higher

productivity as compared to F/2 media. Higher proportion of saturated fatty acids and monounsaturated fatty acids were found in cells grown in F/2 medium while higher proportion of polyunsaturated fatty acids were found in cells cultured in fermented okara medium. Our results successfully demonstrate that low cost okara can serve as a nutrient-rich microalgae culture medium, capable of enhancing the production of high-value nutrients.

4.2 Future Perspective

To further increase the commercial potential of this technology, supercritical fluid extraction could be explored for the downstream process (Fig. 10). Supercritical fluid extraction is a green processing technology that involves minimal organic solvent usage. Moreover, it is highly selective and efficient in extracting lipids and carotenoids and also allows complete separation between the solvent and the compound of interest.

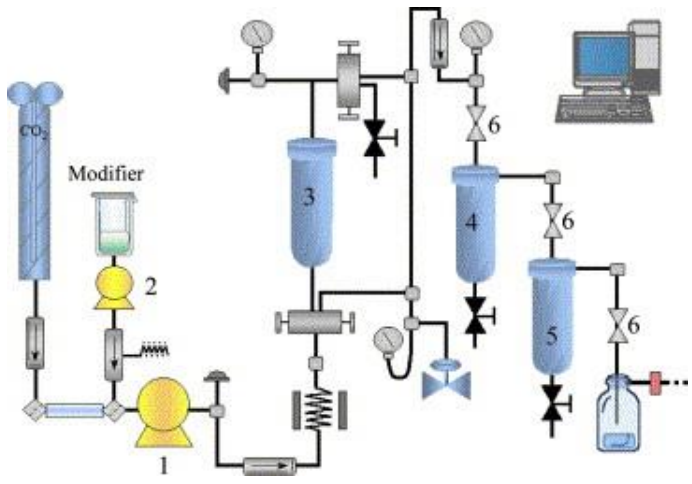

Figure 10: Schematic diagram of supercritical fluid extraction machine (Herrero, Cifuentes, & Ibañez, 2006)

5. References

Bhatnagar, A., Chinnasamy, S., Singh, M., & Das, K. (2011). Renewable biomass production by mixotrophic algae in the presence of various carbon sources and wastewaters. *Applied Energy, 88*(10), 3425-3431.

Cerón Garcí, M., Fernández Sevilla, J., Acien Fernandez, F., Molina Grima, E., & García Camacho, F. (2000). Mixotrophic growth of Phaeodactylum tricornutum on glycerol: growth rate and fatty acid profile. *Journal of Applied Phycology, 12*(3), 239-248.

Chauton, M. S., Olsen, Y., & Vadstein, O. (2013). Biomass production from the microalga Phaeodactylum tricornutum: nutrient stress and chemical composition in exponential fed-batch cultures. *Biomass and bioenergy, 58*, 87-94.

Cooray, S. T., Lee, J. J., & Chen, W. N. (2017). Evaluation of brewers' spent grain as a novel media for yeast growth. *AMB Express, 7*(1), 117.

Ellis, J. T., Hengge, N. N., Sims, R. C., & Miller, C. D. (2012). Acetone, butanol, and ethanol production from wastewater algae. *Bioresource technology, 111*, 491-495.

Fărcaş, A. C., Socaci, S. A., Dulf, F. V., Tofană, M., Mudura, E., & Diaconeasa, Z. (2015). Volatile profile, fatty acids composition and total phenolics content of brewers' spent grain by-product with potential use in the development of new functional foods. *Journal of cereal science, 64*, 34-42.

Garcí, M. C., Sevilla, J. F., Fernández, F. A., Grima, E. M., & Camacho, F. G. (2000). Mixotrophic growth of Phaeodactylum tricornutum on glycerol: growth rate and fatty acid profile. *Journal of Applied Phycology, 12*(3-5), 239-248.

Garcıa, M. C., Mirón, A. S., Sevilla, J. F., Grima, E. M., & Camacho, F. G. (2005). Mixotrophic growth of the microalga Phaeodactylum tricornutum: influence of different nitrogen and organic carbon sources on productivity and biomass composition. *Process Biochemistry, 40*(1), 297-305.

Gómez-Loredo, A., Benavides, J., & Rito-Palomares, M. (2016). Growth kinetics and fucoxanthin production of Phaeodactylum tricornutum and Isochrysis galbana cultures at different light and agitation conditions. *Journal of Applied Phycology, 28*(2), 849-860.

Grima, E. M., Pérez, J. S., Camacho, F. G., Sevilla, J. F., & Fernandez, F. A. (1994). Effect of growth rate on the eicosapentaenoic acid and docosahexaenoic acid content

of Isochrysis galbana in chemostat culture. *Applied Microbiology and Biotechnology, 41*(1), 23-27.

Guillard, R. R. (1975). Culture of phytoplankton for feeding marine invertebrates. In *Culture of marine invertebrate animals*, (pp. 29-60): Springer.

Guo, B., Liu, B., Yang, B., Sun, P., Lu, X., Liu, J., & Chen, F. (2016). Screening of diatom strains and characterization of Cyclotella cryptica as a potential fucoxanthin producer. *Marine drugs, 14*(7), 125.

Herrero, M., Cifuentes, A., & Ibañez, E. (2006). Sub-and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review. *Food Chemistry, 98*(1), 136-148.

Hodgson, P. A., Henderson, R. J., Sargent, J. R., & Leftley, J. W. (1991). Patterns of variation in the lipid class and fatty acid composition of Nannochloropsis oculata (Eustigmatophyceae) during batch culture. *Journal of Applied Phycology, 3*(2), 169-181.

Kasai, N., Murata, A., Inui, H., Sakamoto, T., & Kahn, R. I. (2004). Enzymatic high digestion of soybean milk residue (okara). *Journal of agricultural and food chemistry, 52*(18), 5709-5716.

Kim, S. M., Jung, Y.-J., Kwon, O.-N., Cha, K. H., Um, B.-H., Chung, D., & Pan, C.-H. (2012). A potential commercial source of fucoxanthin extracted from the microalga Phaeodactylum tricornutum. *Applied biochemistry and biotechnology, 166*(7), 1843-1855.

Kiran, E. U., Salakkam, A., Trzcinski, A. P., Bakir, U., & Webb, C. (2012). Enhancing the value of nitrogen from rapeseed meal for microbial oil production. *Enzyme and microbial technology, 50*(6-7), 337-342.

Kirk, P. L. (1950). Kjeldahl method for total nitrogen. *Analytical Chemistry, 22*(2), 354-358.

Kirk, S., & Sawyer, R. (1991). *Pearson's composition and analysis of foods*: Longman Group Ltd.

Li, B., Qiao, M., & Lu, F. (2012). Composition, nutrition, and utilization of okara (soybean residue). *Food Reviews International, 28*(3), 231-252.

Li, Y., Zhou, W., Hu, B., Min, M., Chen, P., & Ruan, R. R. (2011). Integration of algae cultivation as biodiesel production feedstock with municipal wastewater

treatment: strains screening and significance evaluation of environmental factors. *Bioresource technology, 102*(23), 10861-10867.

Li, Y., Zhou, W., Hu, B., Min, M., Chen, P., & Ruan, R. R. (2012). Effect of light intensity on algal biomass accumulation and biodiesel production for mixotrophic strains Chlorella kessleri and Chlorella protothecoide cultivated in highly concentrated municipal wastewater. *Biotechnology and bioengineering, 109*(9), 2222-2229.

Liang, Y., Sarkany, N., & Cui, Y. (2009). Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnology letters, 31*(7), 1043-1049.

McClure, D. D., Luiz, A., Gerber, B., Barton, G. W., & Kavanagh, J. M. (2018). An investigation into the effect of culture conditions on fucoxanthin production using the marine microalgae Phaeodactylum tricornutum. *Algal Research, 29*, 41-48.

Mok, W. K., Tan, Y. X., Lee, J., Kim, J., & Chen, W. N. (2019). A metabolomic approach to understand the solid-state fermentation of okara using Bacillus subtilis WX-17 for enhanced nutritional profile. *AMB Express, 9*(1), 60.

Mussatto, S. I. (2014). Brewer's spent grain: a valuable feedstock for industrial applications. *Journal of the Science of Food and Agriculture, 94*(7), 1264-1275.

Mussatto, S. I., Dragone, G., & Roberto, I. C. (2006). Brewers' spent grain: generation, characteristics and potential applications. *Journal of cereal science, 43*(1), 1-14.

O'Toole, D. K. (1999). Characteristics and use of okara, the soybean residue from soy milk production a review. *Journal of agricultural and food chemistry, 47*(2), 363-371.

Pandey, A. (2003). Solid-state fermentation. *Biochemical Engineering Journal, 13*(2-3), 81-84.

Pleissner, D., Lam, W. C., Sun, Z., & Lin, C. S. K. (2013). Food waste as nutrient source in heterotrophic microalgae cultivation. *Bioresource technology, 137*, 139-146.

Roessler, P. G. (1990). Environmental control of glycerolipid metabolism in microalgae: commercial implications and future research directions. *Journal of Phycology, 26*(3), 393-399.

Sindhu, R., Binod, P., & Pandey, A. (2016). Biological pretreatment of lignocellulosic biomass–An overview. *Bioresource technology, 199*, 76-82.

Singh, A., Meena, M., Kumar, D., Dubey, A. K., & Hassan, M. I. (2015). Structural and functional analysis of various globulin proteins from soy seed. *Critical reviews in food science and nutrition, 55*(11), 1491-1502.

Tan, Y. X., Mok, W. K., Lee, J., Kim, J., & Chen, W. N. (2019). Solid State Fermentation of Brewers' Spent Grains for Improved Nutritional Profile Using Bacillus subtilis WX-17. *Fermentation, 5*(3), 52.

Van der Riet, W., Wight, A., Cilliers, J., & Datel, J. (1989). Food chemical investigation of tofu and its byproduct okara. *Food Chemistry, 34*(3), 193-202.

Woertz, I., Feffer, A., Lundquist, T., & Nelson, Y. (2009). Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for biofuel feedstock. *Journal of Environmental Engineering, 135*(11), 1115-1122.

Xia, S., Wang, K., Wan, L., Li, A., Hu, Q., & Zhang, C. (2013). Production, characterization, and antioxidant activity of fucoxanthin from the marine diatom Odontella aurita. *Marine drugs, 11*(7), 2667-2681.

Yodsuwan, N., Sawayama, S., & Sirisansaneeyakul, S. (2017). Effect of nitrogen concentration on growth, lipid production and fatty acid profiles of the marine diatom Phaeodactylum tricornutum. *Agriculture and Natural Resources, 51*(3), 190-197.

Yogo, T., Ohashi Kunihiko Terakado, Y., Harada, Y., Nezu, Y., Hara, Y., Tagawa, M., Kageyama, H., & Fujisawa, T. (2011). Influence of dried okara-tempeh on the composition and metabolites of fecal microbiota in dogs. *International Journal of Applied Research inVeterinary Medicine, 9*(2), 176.

Yongmanitchai, W., & Ward, O. P. (1991a). Growth of and omega-3 fatty acid production by Phaeodactylum tricornutum under different culture conditions. *Applied and environmental microbiology, 57*(2), 419-425.

Yongmanitchai, W., & Ward, O. P. (1991b). Growth of and omega-3 fatty acid production by Phaeodactylum tricornutum under different culture conditions. *Appl. Environ. Microbiol., 57*(2), 419-425.