# ASSESSMENT OF THE FEASIBILITY OF CO-PRODUCTION OF FUCOXANTHIN, OMEGA-3 FATTY ACIDS AND BIOETHANOL FROM MARINE MICROALGAE

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Degree of Master of Science

Department of Chemical and Process Engineering

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Thesis submitted in partial fulfillment of the requirements for the degree Master of Science in Chemical and Process Engineering

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## DECLARATION OF THE CANDIDATE & SUPERVISORS

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## DEDICATION

Dedicated to my parents, family and teachers who have supported me unconditionally.

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## ABSTRACT

The marine microalga *Tisochrysis lutea* is renowned for its ability to synthesize fucoxanthin and docosahexaenoic acid (DHA), which are nutritionally valuable high-value compounds. Although numerous studies in literature have assessed fucoxanthin and DHA production by *T. lutea*, very few have evaluated the feasibility of comprehensively utilizing biomass for co-production of these metabolites within the framework of biorefineries. To this end, the current study focused on the synthesis of fucoxanthin and DHA by cultivation of *T. lutea* under two different initial nitrate concentrations (1x: 882 µM, 3x: 2,646 µM) and three different illuminance levels (LL: 3,750 lux; ML: 7,500 lux; HL: 11,250 lux). The maximum fucoxanthin yield of 8.80 ± 0.30 mg/L (14.43 ± 0.52 mg/g) and DHA yield of 7.08 ± 0.02 mg/L (11.90 ± 0.14 mg/g) were achieved in the 3x HL culture at the end of 16 days of cultivation. Thereafter, a biphasic solvent extraction procedure using ethanol/n-hexane/water (10:9:1 v/v/v) was utilized for co-extraction of 97.96 ± 0.54% fucoxanthin and 74.11 ± 1.49% DHA from 3x HL biomass, and products were separated into two fractions. Fermentation of the residual biomass obtained from co-extraction resulted in a bioethanol yield of 48.49 ± 0.58 mg/g. Thus, results showcase the efficacy of the developed co-extraction procedure and the biorefinery potential of *T. lutea.*

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## LIST OF ABBREVIATIONS

PUFA – Polyunsaturated fatty acid

EPA – Eicosapentaenoic acid

DHA – Docosahexaenoic acid

WHO – World Health Organization

CCAP – The Culture Collection of Algae and Protozoa

PTFE – Polytetrafluoroethylene

LEDs – Light emitting diodes

1x – Standard nitrate concentration of f/2-Si media

3x – Three times the standard nitrate concentration of f/2-Si media

LL – Low light; 3,750 lux

ML – Medium light; 7,500 lux

HL – High light; 11,250 lux

FAMEs – Fatty acid methyl esters

GC-FID – Gas chromatography with flame ionization detection

HPLC – High-performance liquid chromatography

# 1. INTRODUCTION

Microalgae are photosynthetic microorganisms which are capable of synthesizing lucrative high-value compounds such as carotenoids, polyunsaturated fatty acids (PUFA) and polysaccharides, thereby making them a potential feedstock for nutraceutical, pharmaceutical and cosmetics industries [1,2]. Moreover, microalgae possess the capability to accumulate high quantities of lipids and carbohydrates, and are hence viewed as a potential bioresource for biofuel production [3,4]. Nevertheless, as compared to conventional fossil fuels, the production of microalgae-based biofuels entails high production costs, consequently making the process economically infeasible [1,5]. Thus, newfound emphasis has been placed on using microalgae for co-production of high-value metabolites and subsequently producing biofuels from the residual biomass through the biorefinery approach [1,5,6].

In this context, marine microalgae have been identified suitable candidates for co-production of high-value products such as carotenoids and omega-3 PUFA [7]. Particularly, heterokonts and haptophytes such as *Phaeodactylum tricornutum*, *Odontella aurita, Isochrysis* sp. and *Tisochrysis lutea* are known to synthesize the carotenoid fucoxanthin, as well as omega-3 PUFAs such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) [7–10].

Fucoxanthin accounts for approximately 10% of the total carotenoids synthesized in nature [11]. Due to the multitude of beneficial health effects and its market potential, research on the production of fucoxanthin has gained considerable interest [12–14]. Currently, fucoxanthin is produced from brown seaweeds, despite of the low concentration of the carotenoid in dry biomass (< 1 mg/g) [9,15]. Contrastingly, microalgae accumulate fucoxanthin in concentrations which are up to a 100-fold higher than macroalgae [16], reaching up to 59.2 mg/g under controlled culture conditions [9]. Thus, microalgae have been increasingly explored as a promising source for the production of fucoxanthin.

Furthermore, it is noteworthy that the majority of microalgal strains studied for fucoxanthin production are also rich in omega-3 PUFA. For instance, microalgae such as *P. tricornutum* are *O. ariata* produce EPA up to ~35% and ~28% of total fatty acids respectively [15,17], whereas *T. lutea* and *Isochrysis* strains produce DHA up to ~15-17% of total fatty acids [18]. Consumption of omega-3 PUFA such as EPA and DHA impart numerous health benefits to humans, such as the enhancement of cardiovascular health, improved kidney function and growth/development of infants [19]. Although the major commercial source of omega-3 PUFA in the human diet is fish, the development of alternative sources of omega-3 PUFA is essential due to concerns over accumulation of contaminants in fish, decline of wild fish stocks and dietary preferences (i.e. taste, odor, etc.) [20,21]. To this end, being primary producers of EPA and DHA, microalgae have been studied as a viable alternative source of omega-3 PUFA [20]. Thus, considering their biochemical composition, marine microalgal sources can be exploited for co-production of fucoxanthin and omega-3 fatty acids.

### 1.1. Research gap

In the current study, *T. lutea* (previously known as *Isochrysis* aff. *galbana*, T-Iso) was utilized to study the potential of co-producing fucoxanthin and DHA. The aforementioned microalga was selected as the species of interest due to its ability to proliferate under high temperatures and high irradiance prevalent in tropical climates. Since the biochemical composition of microalgae is significantly affected by culture conditions [22], the evaluation of optimum conditions to maximize fucoxanthin and DHA accumulation is important. Previous studies have reported that cultivation of microalgae under low light conditions and nutrient repletion are effective in enhancing the biosynthesis of fucoxanthin and DHA [7,9]. Nonetheless, the response of microalgae to culture conditions is species-specific, and the overall production of target metabolites should be maximized based on a compromise between high biomass production and product accumulation [23].

Whilst numerous studies focusing on simultaneous biosynthesis of fucoxanthin and PUFA have been reported in literature [8,24,25], only a few studies have been performed on co-

extraction of these metabolites from a single biomass feedstock [26,27]. Furthermore, some co-extraction studies focused on obtaining a single crude extract consisting of both PUFA and fucoxanthin [27], whereas others focused on fractionation of the two products within the concept of an integrated biorefinery [26]. Although the simultaneous production and separation of fucoxanthin and EPA from *P. tricornutum* has been reported, a similar study has not been conducted for *T. lutea* [26]. Results of previous studies showcase that the extraction yields of PUFA and fucoxanthin are highly dependent on the microalgal species [27]. Whilst Kim et al. studied the separation of fucoxanthin and lipids from crude fucoxanthin extracts obtained from *Isochrysis* aff. *galbana*, only 75% of the total fucoxanthin was recovered and the DHA yields were not quantified [28]. Therefore, considering this gap in literature, the current study focused on the co-extraction fucoxanthin and DHA from *T. lutea* biomass and separating the two products.

### 1.2. Hypotheses

It was hypothesized that;

- Low light conditions and nutrient repletion would enhance the biosynthesis of fucoxanthin and DHA in *T. lutea*
- A biphasic solvent system comprising of two polar and non-polar phases would enable the efficient co-extraction and separation of fucoxanthin and DHA from *T. lutea*
- Residual biomass could be effectively valorized for production of bioethanol

### 1.3. Objectives

In order to address the research gap, the current study was performed with the following objectives;

- Determination of the optimum growth parameters for improved biosynthesis and productivity of fucoxanthin and omega-3 fatty acids in *T. lutea*
- Evaluation of bioethanol yield from microalgae biomass grown in conditions to maximize productivity of high value compounds

- Development of an extraction process to maximize yields of fucoxanthin, omega-3 fatty acids and bioethanol

Accordingly, in the first phase of the current study, three different illuminance levels and two different initial nitrate concentrations were used to evaluate the effect of culture conditions on production of fucoxanthin and DHA by *T. lutea*. Thereafter, in the second phase of the study, a co-extraction procedure based on a biphasic solvent system was utilized to simultaneously recover fucoxanthin and DHA from microalgal biomass which showcased the highest product yields in the first phase. Finally, alcoholic fermentation was performed to produce bioethanol from residual biomass obtained from the co-extraction process. Hence, the potential for co-production of fucoxanthin, DHA and bioethanol was evaluated within the framework of microalgal biorefineries. No previous study in reported literature has utilized *T. lutea* for the co-production of fucoxanthin, DHA and bioethanol in a biorefinery context.

# 2. LITERATURE REVIEW

## 2.1. Fucoxanthin

Fucoxanthin is one of the most abundant xanthophyll carotenoids found in the marine ecosystem, and are present in brown seaweed and microalgae [29]. Fucoxanthin imparts a distinctive golden-brown color to microalgae due to the formation of light-harvesting complexes in the photosystems [30].

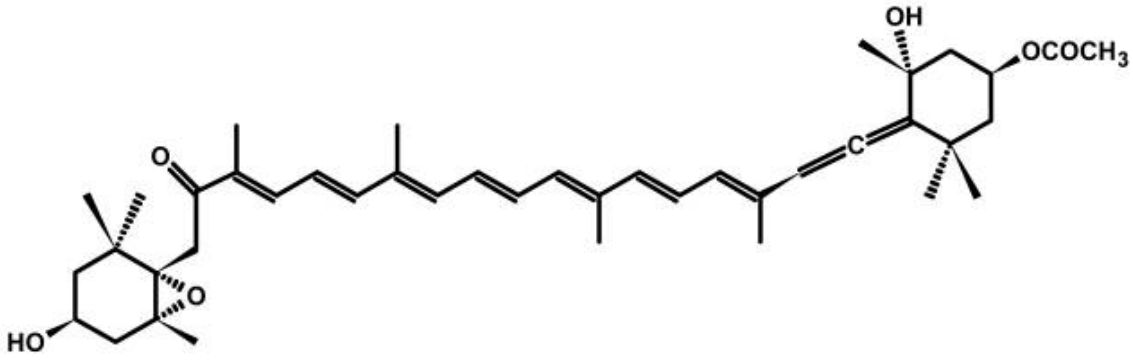


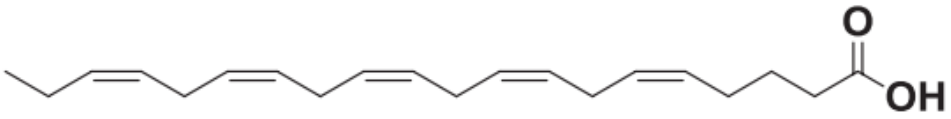

*Figure 2-1: The chemical structure of fucoxanthin* [14]

Fucoxanthin comprises of an unusual allenic bond, as well as epoxy, carbonyl, carboxyl and hydroxyl groups (Fig. 2-1). The structure of fucoxanthin, especially due to the presence of the allenic bond, imparts it with potent antioxidant capacity due to the ability to scavenge singlet molecular oxygen and free radicals [31]. Thus, fucoxanthin possesses numerous bioactive properties, including antioxidant, anti-inflammatory, anti-carcenogenic, anti-obese, antidiabetic, antiangiogenic and antimalarial activities, as reported in literature. Consumption of fucoxanthin is known to have hepatoprotective effects, and improve the health of blood vessels, bones, skin, and eyes [14]. Thus, fucoxanthin has emerged as a compound of commercial interest with therapeutic applications in nutraceutical, pharmaceutical and cosmetics industries [31].

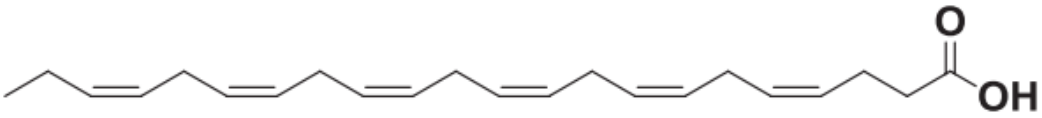
## 2.2. Omega-3 fatty acids

Omega-3 PUFAs, are fatty acids which comprise of multiple double bonds in the carbon chain, the first of which, starting from the methyl end, is between the third and fourth

carbon atom (Fig. 2-2). Long-chain PUFAs, especially EPA and DHA, have been known to impart numerous health benefits, with the improvement of cardiovascular health and reduction of blood cholesterol levels being key among them [19].




Eicosapentaenoic acid (EPA)




Docosahexaenoic acid (DHA)

*Figure 2-2:Structures of omega-3 fatty acids, EPA and DHA* [32]

Nonetheless, the daily intake of omega-3 fatty acids recommended by the World Health Organization (WHO); 250 mg of EPA+DHA, is not satisfied by the majority of the world's population [19]. Primarily, this is because the main source of omega-3 fatty acids in the human diet is fish or fish oil. Due to incessant overfishing, the stock of wild harvest fish is inadequate to satisfy the global requirement of omega-3 fats [19]. Moreover, there is a negative perception regarding the consumption of fish due to dietary preferences and concerns over accumulation of contaminants such as mercury. In contrast, microalgae are a promising source of omega-3 fatty acids which can bypass the negative effects of fish consumption (unsuitability to vegetarians, unsustainability, food safety, etc.), being the primary producers of EPA and DHA in the marine ecosystem [20].

### 2.3. Microalgal strains for co-production of omega-3 fatty acids and fucoxanthin

Microalgae are renowned for their ability to synthesize numerous compounds of commercial value, including carotenoids, phycobiliproteins and omega-3 PUFAs [33,34]. Certain microalgal species exhibit the capability to synthesize multiple high-value products, making them a potential feedstock for high-value product based biorefineries.

In this context, marine microalgae capable of synthesizing both fucoxanthin and omega-3 PUFAs have gained the interest of researchers worldwide. Fucoxanthin is a high-value carotenoid which has secured a market value of $ 40,000-80,000/kg, depending on the purity of the final product [35]. Similarly, omega-3 PUFAs, especially EPA and DHA, have a market value of $80-120/kg and have secured applications as high-value nutraceuticals.

*Table 2-1: Microalgal species capable of synthesizing both fucoxanthin and omega-3 fatty acids*

| **Microalgal species** | **Product yields** | **References** |
|---|---|---|
| *Phaeodactylum tricornutum* | Fucoxanthin – 59.2 mg/g<br>EPA – 4.4% (w/w) | [9,36] |
| *Isochrysis galbana* | Fucoxanthin – 6.04 mg/g<br>DHA – 1.7% (w/w) | [10,18] |
| *Odontella aurita* | Fucoxanthin – 18.47 mg/g<br>EPA – 3.16% (w/w) | [15,37] |
| *Chaetoceros gracilis* | Fucoxanthin – 15.4 mg/g<br>EPA – 1.41% (w/w) | [38] |
| *Nitzschia laevis* | Fucoxanthin – 13.6 mg/g<br>EPA – 3.41% (w/w) | [39,40] |
| *Tisochrysis lutea* | Fucoxanthin – 18.23 mg/g<br>DHA – 2.1% (w/w) | [11,41] |

The identification of suitable marine microalgal strains for the production of fucoxanthin and omega-3 PUFAs should be performed with great scrutiny. This is because the overall product yields and cost of production would vary with the microalgal strain, as the optimum culture conditions (temperature, illumination conditions, etc.) and downstream processing requirements are species-specific [7]. Moreover, certain microalgal strains would not be suitable for cultivation in different geographical locations as culture conditions, especially under large-scale outdoor cultivation, varies dramatically from location to location [42]. A few microalgal strains capable of synthesizing both fucoxanthin and DHA is summarized in Table 2-1.

## 2.4. *Tisochrysis lutea* as a producer of DHA and fucoxanthin

The marine haptophyte *T. lutea*, first isolated in Tahiti, French Polynesia, has been identified as an ideal strain for fucoxanthin and DHA production in tropical climates due to its capability of growth under high temperatures (> 30 $^{o}$C) and high irradiance levels [43].

*Table 2-2: Summary of selected studies on the production of DHA and fucoxanthin using T. lutea*

| Study | Target metabolites | Description | Reference |
|---|---|---|---|
| Fucoxanthin and Polyunsaturated Fatty Acids Co-Extraction by a Green Process | Fucoxanthin and DHA | Extraction process for recovery of fucoxanthin and DHA was developed. | [27] |
| Effect of cultivation mode on the production of docosahexaenoic acid by *Tisochrysis lutea* | DHA | Effect of metabolic mode on growth and production of DHA was determined. | [44] |
| Effects of growth phase and nitrogen limitation on biochemical composition of two strains of *Tisochrysis lutea* | DHA | Effect of growth phase (stationary or exponential) and two nitrogen concentrations were determined. | [45] |
| Different DHA or EPA production responses to nutrient stress in the marine microalga *Tisochrysis lutea* and the freshwater microalga *Monodus subterraneus* | DHA | Effect of various nitrogen and phosphorous supply regimes were determined | [46] |
| Process optimization of fucoxanthin production with *Tisochrysis lutea* | Fucoxanthin | Effect of temperature and illuminance level was studied in chemostat controlled cultivation. | [16] |
| Screening of Isochrysis strains for simultaneous production of docosahexaenoic acid and fucoxanthin | Fucoxanthin and DHA | Screening of various strains for co-production of DHA and fucoxanthin. | [8] |

Nonetheless, as observed in Table 2-2, majority of reported literature focuses on the use of *T. lutea* for production of either DHA or fucoxanthin, with very few studies focusing on the production of both compounds in the context of biorefineries. Thus, further studies on the exploitation of *T. lutea* for co-production of both DHA and fucoxanthin is necessary to fully valorize this microalga.

A key advantage in the co-production of these metabolites is that their synthesis in microalgal cells is favored under similar culture conditions. For instance, the synthesis of fucoxanthin and DHA is enhanced under low light conditions. In the case of fucoxanthin, this is because it serves the function of assisting the light-harvesting function of cells, and thus it is overproduced in response to lower light availability as a mechanism to harvest more light for photosynthesis [11]. PUFAs such as DHA are the major fraction of lipids constituent in cellular membranes. Due to the low availability of light, the thylakoid membrane which facilitates the cellular photosystems would expand and thereby increase the cellular PUFA (and consequently, DHA) content [7]. Nonetheless, it is noteworthy that the use of low light conditions may result in lower biomass productivities, and thus reduce the overall productivity of fucoxanthin and/or DHA. Hence, it is important to achieve a compromise between biomass production and product accumulation to maximize the yields of target compounds in microalgae cultures. Moreover, nutrient repletion would enhance the production of both fucoxanthin and DHA, as the functioning of cellular photosynthetic complexes (such as those formed by fucoxanthin and chlorophyll) and the photosystems (in organelles containing membrane lipids) is significantly affected by nutrient stress [8]. Thus, it is evident that similar culture conditions could be exploited to simultaneously enhance the synthesis of both compounds in *T. lutea*.

### 2.5. Production of bioethanol from residual microalgal biomass

Residual microalgal biomass, upon extraction of lipophilic compounds such as lipids and carotenoids, contain high contents of carbohydrates (> 50% w/w). Microalgal starch, glycogen, and cellulose can be easily hydrolyzed and fermented with yeast to yield bioethanol [47]. Although production of bioethanol from microalgae may not be economically feasible, coupling the process with the manufacture of high-value products would enhance the prospects of wholistically utilizing the biomass to produce multiple co-products within the framework of biorefineries. Accordingly, numerous researchers have integrated the production of bioethanol from residual biomass with the manufacture of high-value products [48]. Nevertheless, thorough analysis of techno-economic

considerations is necessary to gauge the feasibility of microalgal bioethanol production as compared to other potential methodologies of biomass valorization.

# 3. MATERIALS AND METHODS

## 3.1. Microalgal species and pre-culture conditions

*T. lutea* (CCAP 927/14) was obtained from The Culture Collection of Algae and Protozoa (CCAP), Scotland, United Kingdom. Seed cultures of *T. lutea* were grown in 1.8 L of modified f/2 media [49], prepared without the addition of $Na_2SiO_3$ (i.e. f/2-Si medium) and with a nitrate concentration threefold that of standard media. The composition of standard f/2-Si medium is given in Table 3-1. The photobioreactors were laboratory glass bottles equipped with GL45 screw caps with 3-port connections (Duran 1129751, Germany) for aeration of cultures and pressure regulation. The ports for aeration and pressure regulation were fitted with 0.22 µm polytetrafluoroethylene (PTFE) membrane filters. The photobioreactors were maintained at room temperature (30 ± 2 °C), and illuminated with cool white light emitting diodes (LEDs) at 7,500 lux under a light/dark cycle of 16:8 hrs.

*Table 3-1: Composition of f/2-Si medium* [50]

| **Component** | **Final concentration (µM)** |
|---|---|
| Seawater (30-35 ppt salinity) | - |
| $NaNO_3$ | 882 |
| $NaH_2PO_4 \cdot H_2O$ | 36 |
| $FeCl_3 \cdot 6H_2O$ | 11.7 |
| $Na_2EDTA \cdot 2H_2O$ | 11.7 |
| $MnCl_2 \cdot 4H_2O$ | $9.10 \times 10^{-1}$ |
| $ZnSO_4 \cdot 7H_2O$ | $7.65 \times 10^{-2}$ |
| $CoCl_2 \cdot 6H_2O$ | $4.20 \times 10^{-2}$ |
| $CuSO_4 \cdot 5H_2O$ | $3.93 \times 10^{-2}$ |
| $Na_2MoO_4 \cdot 2H_2O$ | $2.60 \times 10^{-2}$ |
| Thiamine·HCl | $2.96 \times 10^{-1}$ |
| Cyanocobalamin | $2.05 \times 10^{-3}$ |
| Biotin | $3.69 \times 10^{-4}$ |

### 3.2. Cultivation of *T. lutea* under different initial nitrate concentrations and illumination conditions

*Table 3-2: Culture conditions utilized for cultivation of T. lutea*

| Photobioreactor | Nitrate concentration (µM) | Illuminance level (lux) |
|---|---|---|
| 1x LL | 882 | 3,750 |
| 3x LL | 2,646 | 3,750 |
| 1x ML | 882 | 7,500 |
| 3x ML | 2,646 | 7,500 |
| 1x HL | 882 | 11,250 |
| 3x HL | 2,646 | 11,250 |

The effect of initial nitrate concentration and illuminance level on biosynthesis of fucoxanthin and DHA was studied in 1 L laboratory glass bottle photobioreactors with GL45 3-port screw caps and 800 mL working volume. Seed cultures of *T. lutea* in the exponential growth phase were centrifuged for 15 min at 1,500 × g (Eppendorf 5804 R, Germany). Autoclaved sweater was used to wash the harvested biomass prior to inoculation in photobioreactors at a concentration of approximately 0.1 g/L.

The effect of two nitrate concentrations was studied by varying the amount of nitrate stock solution added to f/2-Si medium. Accordingly, the standard nitrate concentration (1x) and three times the standard nitrate concentration (3x) were used. Three levels of illumination corresponding low light (LL); 3,750 lux, medium light (ML); 7,500 lux and high light (HL); 11,250 lux were utilized for growth experiments. Table 3-2 shows the summary of the six different culture conditions used in the current study. Aeration, pressure compensation, temperature and photoperiod were unchanged from the conditions mentioned in section 3.1. The increase of biomass density with culture time was observed visually, as per the images in Fig. 3-1.

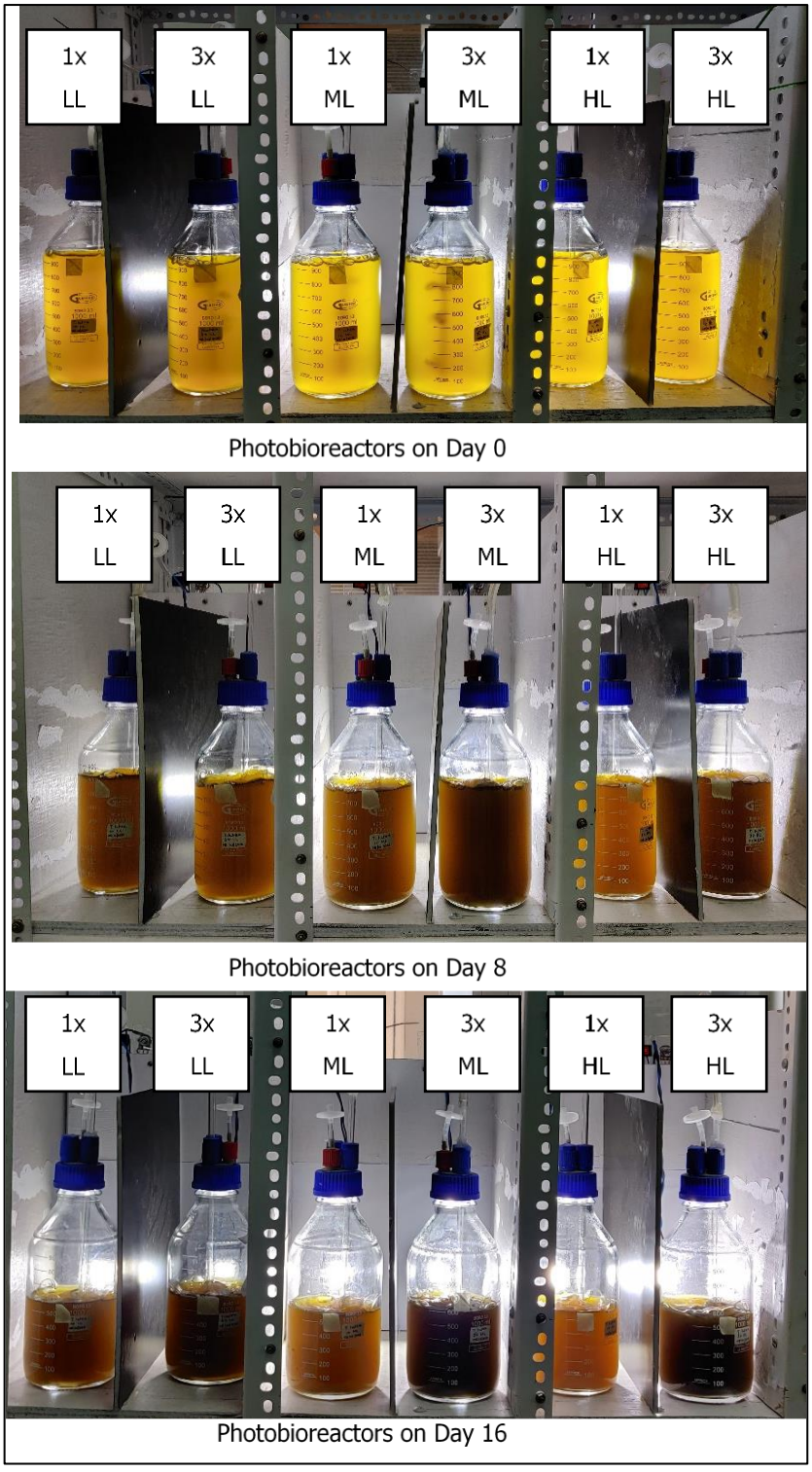



*Figure 3-1:Biomass production in T. lutea cultures cultivated under various initial nitrate concentrations and illuminance levels*

### 3.3. Measurement of biomass concentration

The biomass concentration of microalgae cultures was evaluated every 2 days by filtration of 10 mL culture aliquots through dried and pre-weighed glass microfiber filter papers (Whatman GF/C, 1.2 µm pore size, ⌀47 mm). Thereafter, the filters with biomass were dried at 60 ˚C in a laboratory oven (Memmert UN110, Germany) for 24 hrs. The culture concentration of biomass was estimated by measurement of the net increase in the weight of filter papers.

### 3.4. Estimation of nitrate concentration in culture media

The nitrate concentration of cultures was assessed every 2 days. Culture aliquots of 5 mL were centrifuged for 5 min at 10,000 × g to remove biomass. 0.22 µm nylon syringe filters were used to filter the supernatant, which were subsequently subjected to dilution by 10-20 times using autoclaved seawater. Thereafter, the nitrate concentration was estimated by measurement of absorbance at 220 nm [51]. Fig 3-2 illustrates the calibration curve prepared using $NaNO_3$ for analysis of nitrate content in the media.

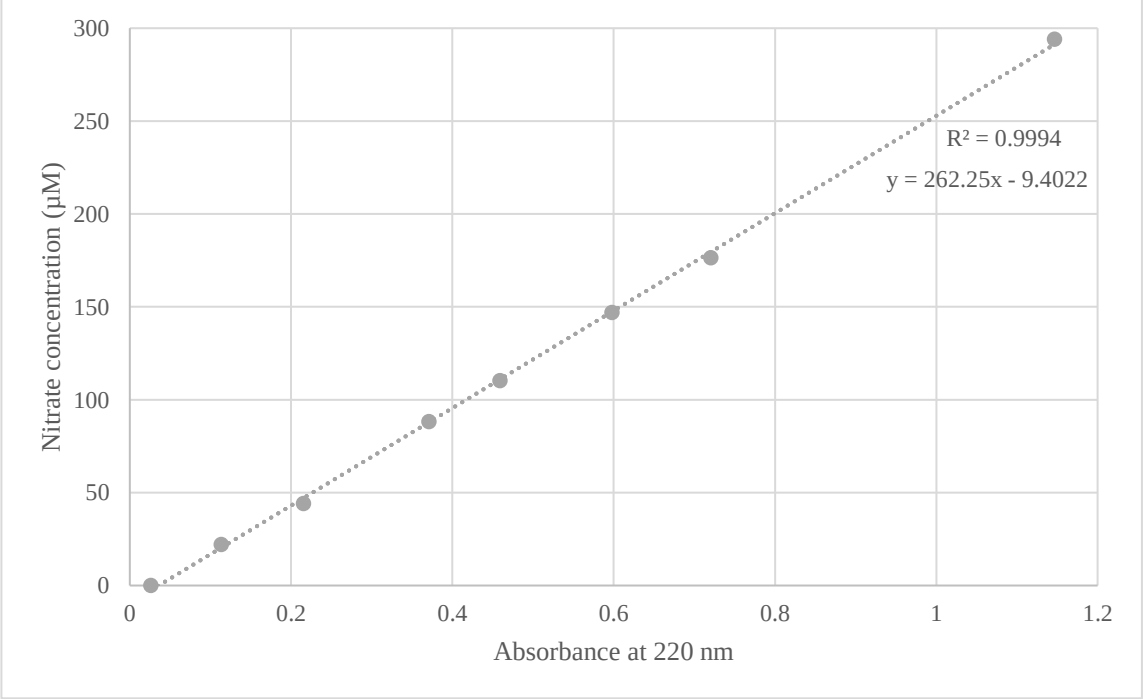



*Figure 3-2: Calibration curve used for spectrophotometric determination of nitrate concentration*

### 3.5. Extraction of lipids and fucoxanthin

For lipid and fucoxanthin analysis, biomass in 10 mL of cultures were collected on days 4, 8, 12 and 16 on GF/C glass microfiber filters which had been pre-combusted at 450 ˚C for 2 hrs.

The Bligh and Dyer method [52], with slight modifications, was used for the extraction of lipids. A solvent mixture of 1:1 (v/v) chloroform/methanol was added to the harvested microalgae. Following a total extraction time of 24 hrs, 0.8% NaCl was added to form a final solvent ratio of 10:10:9 (v/v/v) chloroform/methanol/0.8% NaCl, and the samples were vortexed for 30 secs. Thereafter, centrifugation was performed for 5 min at 1,200 × g to induce phase separation. The nonpolar layer comprising of extracted lipids was added to a new centrifuge tube.

Fucoxanthin was extracted for 1 hr using 4 mL of ethanol in a water bath (Memmert WNB7, Germany) set at 30 ˚C [15,28]. Thereafter, the samples were centrifuged at 1,200 × g for 5 min, and the extracts were transferred to a new centrifuge tube.

All extractions of lipids and fucoxanthin were repeated twice and the solvents were pooled together to ensure the complete recovery of desired compounds.

Crude extracts of microalgal lipids and fucoxanthin are shown in Fig. 3-3.

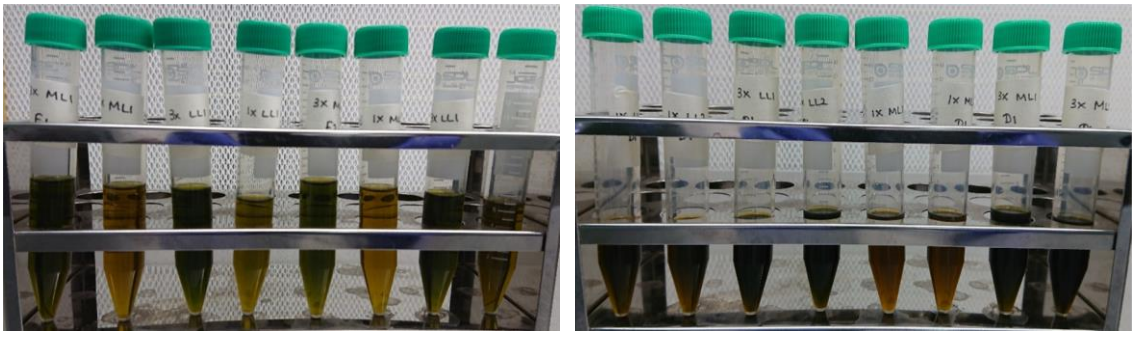


*Figure 3-3:Crude extracts of fucoxanthin (left) and microalgal lipids (right)*

### 3.6. Quantification of DHA content in microalgal cultures

An internal standard (heptadecanoic acid; C17:0; Sigma Aldrich H3500) was added to each sample obtained as per the lipid extraction procedure described in section 3.5.

Thereafter, the lipid extracts were evaporated under a gentle stream of nitrogen. Fatty acid methyl esters (FAMEs) were synthesized by transesterification of lipids. Transesterification was performed via heating of lipids and 5 mL of methanol containing 1% (v/v) sulfuric acid overnight at 50 °C in a water bath [53]. n-hexane was used to extract FAMEs from crude esterified samples according to the procedure described by Breuer et al [54]. The samples were filtered through 0.22 µm PTFE syringe filters and analyzed in a gas chromatograph (Agilent 7980A, USA) with a flame ionization detector (GC-FID). A DB-23 column (60 m × ⌀0.25 mm × 0.15 µm) was used for the analysis. Samples were injected to the column using an injection volume and inlet temperature of 1 µL and 250 °C respectively. The pressure of the carrier gas, Helium (He), was maintained at 230 kPa with a 1:50 split ratio. The oven temperature program of 50 °C for 1 min, 25 °C/min up to 175 °C, 4 °C/min up to 230 °C, 230 °C for 5 min was used. The FID detector temperature was 280 °C. FID was operated with air, hydrogen ($H_2$) and He flow rates of 450 mL/min, 40 mL/min and 30 mL/min respectively. The column was calibrated with the Supelco 37 Component FAME Mix (CRM47885) prior to analysis of samples.

### 3.7. Quantification of fucoxanthin content in microalgal cultures

*Table 3-3: Mobile phase flow in HPLC analysis of fucoxanthin*

| Elution time | Methanol | Water |
|---|---|---|
| 0-1 min | 80% | 20% |
| 1-8 min (gradient) | 100% | 0% |
| 8-20 min | 100% | 0% |
| 20-22 min (gradient) | 80% | 20% |
| 22-30 min | 80% | 20% |
| Post run - 1 min | 80% | 20% |

The crude fucoxanthin extracts obtained as per section 3.5. were filtered through nylon syringe filters (0.22 µm). The fucoxanthin content in the extracts were determined at 448 nm in a Zorbax Eclipse Plus C18 column (150 mm × ⌀3 mm × 3.5 µm) using an Agilent

Infinity II 1260 high-performance liquid chromatography (HPLC) system. A sample injection volume of 5 µL and solvent flow rate of 0.3 mL/min was employed.

Methanol (A) and water (B) were used as the mobile phase, and the elution flow was controlled as in Table 3-3. Initially, the solvent flow of 80% A and 20% B was maintained for 1 min, followed by the increment of A up to 100% at 8 min via gradient flow. The flow maintained at 100% A and 0% B from 8-20 min, after which it was reverted to 80% A and 20% B by 22 min using gradient flow. Thereafter, the elution was maintained at 80% A and 20% B up to 30 min, followed by a post-run hold at the same solvent ratio for 1 min.

All-trans fucoxanthin standard (Sigma-Aldrich 16337) was used to calibrate the column at fucoxanthin concentrations of 10-100 µg/mL.

### 3.8. Determination of carbohydrate content in biomass

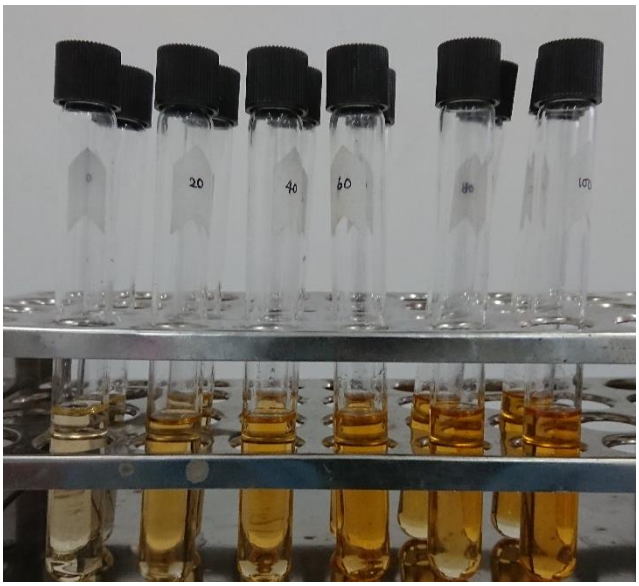



*Figure 3-4:Color development in sugar hydrolysates upon the addition of phenol-sulfuric reagent for determination of carbohydrate concentration*

The carbohydrate concentration in biomass was evaluated on day 16 of cultivation. 10 mL culture samples were centrifuged for 10 min at 4,500 × g to harvest biomass, and the supernatant was removed. Acetic acid (0.5 mL) was added to harvested biomass, and heated for 20 min at 80 °C in a water bath. Thereafter, pigments were extracted using

acetone (10 mL). Acetone-treated samples were subjected to centrifugation for 10 min at 3,500 × g, and the supernatant was removed. The resulting biomass was treated 4 M trifluoroacetic acid (2.5 mL) and incubated for 4 hrs at 95 °C to obtain a microalgal sugar hydrolysate. The hydrolysate was centrifuged for 5 min at 10,000 × g to separate and remove the residual biomass [55]. The solution was cooled, and the carbohydrate content was assessed as per the phenol-sulfuric method [56]. The red-orange color developed by sugar hydrolysates upon addition of the phenol-sulfuric reagent is depicted in Fig. 3-4.

The calibration curve prepared using glucose for analysis of carbohydrate concentration is given in Fig 3-5.

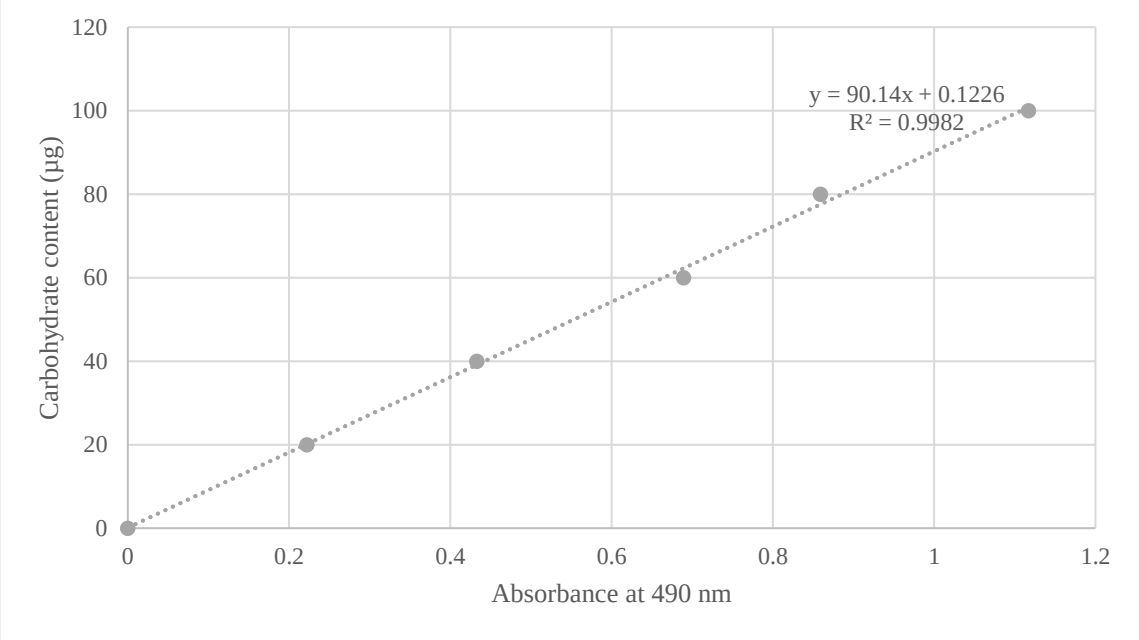



*Figure 3-5: Calibration curve used for spectrophotometric determination of carbohydrate concentration*

### 3.9. Co-extraction of DHA and fucoxanthin

*T. lutea* biomass synthesized under the nitrate concentration and illuminance level which demonstrated the highest fucoxanthin and DHA yields was utilized for the co-extraction experiment of fucoxanthin and DHA. A biphasic solvent system of ethanol/n-hexane/water (10:9:1 v/v/v) was used for the extraction procedure [28]. Briefly, 35 mL of culture was collected on pre-combusted glass microfiber filters (as described in section

3.5.). Thereafter, 2 mL of ethanol and 1.8 mL of n-hexane was added to biomass and extraction was performed for 2, 4, 8 and 16 hrs. Distilled water was added to form a final ratio of 10:9:1 v/v/v of ethanol/n-hexane/water and induce phase separation. The samples were vortexed for 30 secs and centrifuged at 1,200 × g for 5 min. The nonpolar phase was analyzed for DHA content whilst the hydroalcoholic phase was analyzed for fucoxanthin content as described in section 3.6 and section 3.7 respectively.

### 3.10. Fermentation of residual biomass

The residual biomass of the samples which exhibited the best co-extraction performance of fucoxanthin and DHA, was hydrolyzed as per the procedure mentioned in section 3.8. The hydrolysate was enriched with 10 g/L peptone and 5 g/L yeast extract. The pH of the hydrolysate was adjusted to 5.5 and transferred to a 100 mL glass bottle equipped with a 3-port GL45 screw cap, with one port for aeration and two ports for pressure regulation. All openings were fitted with PTFE membrane filters (0.22 µm). The hydrolysate and glass bottle, inclusive of all ports and connections were sterilized by autoclaving. Thereafter, the hydrolysate was purged with nitrogen through the aeration port and inoculated with 5% (v/v) *Saccharomyces cerevisiae* ($OD_{600}$ ~3).


*Figure 3-6:Seed cultures of S. cerevisiae prepared for fermentation of residual T. lutea biomass (left) and fermentation apparatus (right)*

Fermentation was performed by at 30 ˚C in a shaking incubator operated at 180 rpm for 72 hrs [57]. The seed cultures of *S. cerevisiae* prepared for the fermentation experiments and the fermentation apparatus is shown in Fig. 3-6.

Ethanol in the fermentation broth was extracted using 3 mL of ethyl acetate, which was added to 2 mL of fermentation broth. The samples were vortexed for 30 sec, followed by centrifugation at 10,000 × g for 5 min for phase separation. The ethanol in the ethyl acetate extracts were analyzed by GC-FID in a DB-624 column (30 m × ⌀0.32 mm × 1.8 µm). The sample injection volume was 0.3 µL. He was used as the carrier gas, while $H_2$ was used as the fuel and nitrogen ($N_2$) was used as the makeup gas for FID.

### 3.11. Statistical analysis

All experimental analyses were duplicated. Analysis of variance (ANOVA) was used to determine the statistical significance of input variables was determined on a 95% confidence interval ($p<0.05$). All ANOVA analyses were performed in Microsoft Excel 2016, using the DataAnalysis toolpack.

# 4. RESULTS AND DISCUSSION

## 4.1. Biomass production of *T. lutea* under different initial nitrate concentrations and illuminance levels

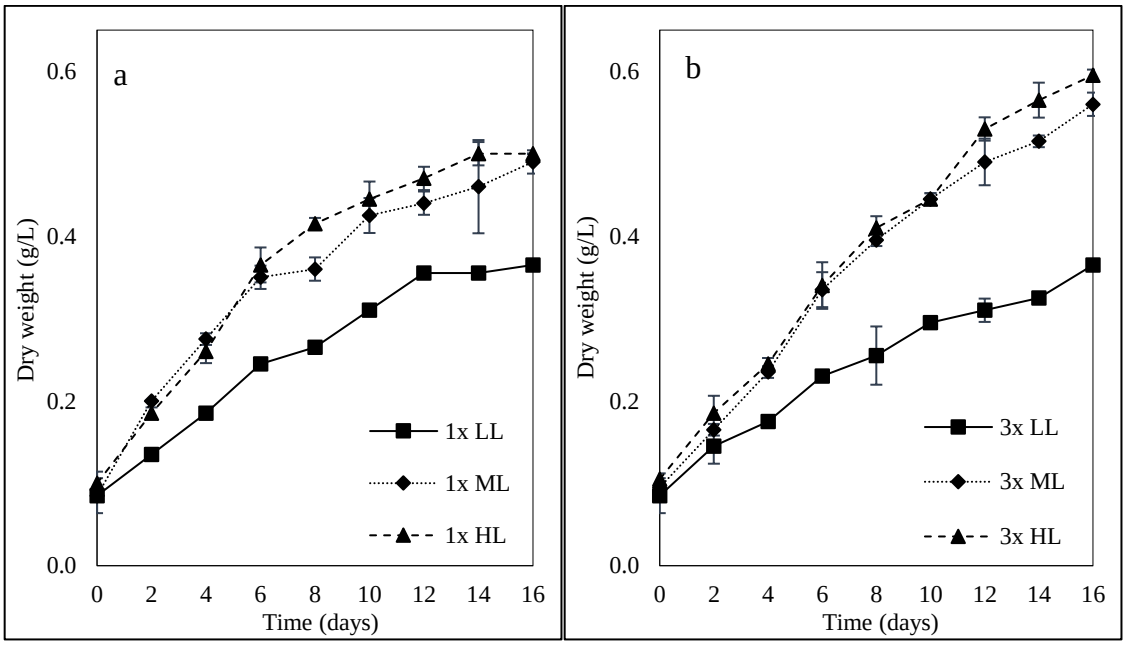



*Figure 4-1: Biomass concentrations of T. lutea grown under different illuminance levels (low light LL: 3,750 lux; medium light, ML: 7,500 lux; and high light, HL: 11,250 lux) and initial nitrate concentrations (1x: 882 µM; 3x: 2,646 µM) over the 16-day cultivation period (a) 1x cultures (b) 3x cultures*

The biomass concentration in the 1x cultures of *T. lutea* increased from the day 0 to 6, and the growth rates were reduced thereafter (Fig. 4-1(a)). It was evident that 1x cultures had reached the stationary phase by the end of cultivation (day 16). In contrast, continued biomass growth was observed in 3x cultures even during day 16 of cultivation (Fig. 4-1(b)). The difference in growth of 1x and 3x cultures could be attributed to nitrate availability, as demonstrated by the nitrate consumption curves (Fig. 4-2). As observed in Fig. 4-2(a) the nitrate concentration in all 1x cultures had reached minimal concentrations by day 8, whereas 3x cultures showed the presence of significant concentrations of nitrate available for the consumption by microalgae throughout the period of cultivation. Almutairi [58] reported that the growth rate *T. lutea* in nitrogen-free free culture was

68.4% lower than the control. Similar results were observed in the current study upon nitrogen depletion, indicating that the nitrate availability in the cultures had a significant inhibitory effect on biomass production during prolonged cultivation of *T. lutea* ($p<0.05$). However, it is noteworthy that some studies have reported higher biomass growth at nitrogen starvation conditions for wild type of *T. lutea* [59,60].

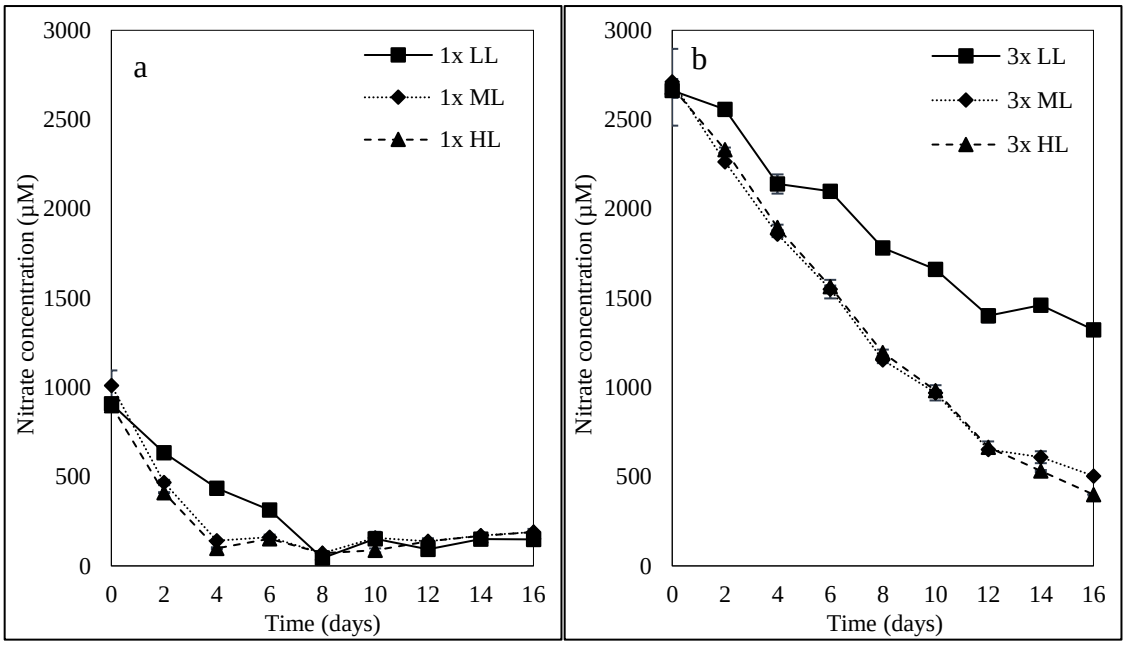



*Figure 4-2: Variation of nitrate concentration in the culture media under different illuminance levels (low light LL: 3,750 lux; medium light, ML: 7,500 lux; and high light, HL: 11,250 lux) and initial nitrate concentrations (1x: 882 µM; 3x: 2,646 µM) over the 16-day cultivation period (a) 1x cultures (b) 3x cultures*

Moreover, it was the observed that the biomass growth in cultures illuminated with ML and HL were notably higher than LL throughout the period of cultivation ($p<0.05$), in both 1x and 3x nitrate supplemented cultures (Fig. 4-1). Final biomass concentration of both 1x LL and 3x LL cultures were similar (0.37±0.01 g/L and 0.36±0.01 g/L respectively), demonstrating that they were light limited. Interestingly, microalgae in 1x LL culture consumed only 759.21 µM of nitrate while microalgae in 3x LL consumed 2208.53 µM of nitrate to produce, approximately, the same amount of biomass. A possible reason for this may be the cultivation of seed cultures in 3x medium, thereby resulting in a high initial

nitrogen quota in inoculated biomass in both 1x LL and 3x LL cultures. Charrier et al., 2015, [61] has identified four genes encoding putative high-affinity nitrate/nitrite transporters (TlNrt2), using in silico analysis from transcriptomic data concerning *T. lutea.* They have established that the expression of TlNrt2.4 gene is related to internal nitrogen quota level than external nitrogen concentration. Accordingly, given the high initial nitrogen quotas, the growth mechanism of 1x LL cultures may have shifted to consume intracellular nitrogen whereas the nitrogen metabolism of 3x LL culture may have been concerned with the extracellular nitrogen concentration.

The biomass growth curves of 1x ML and 1x HL cultures show similar patterns, and the final biomass concentration is approximately equal in both cultures. Similarly, temporal biomass growth in 3x ML and 3x HL overlaps each other up to day 10 of cultivation. Biomass production in the 3x HL culture showed a comparatively higher growth afterwards. These results indicate that the increment in illuminance level from ML to HL resulted in diminishing returns, possibly due to light saturation [62]. Thus, it is possible that ML would be cost effective due to the low energy consumption while providing similar biomass yields as HL cultures. Nonetheless, the specific electrical energy consumption for lighting should be assessed to confirm the most cost effective illumination conditions [63].

Ultimately, the maximum final biomass concentration of 0.60±0.01 g/L was achieved under 3x HL, which incorporated the highest illuminance level and highest initial nitrate concentration employed in the current study.

### 4.2. Accumulation of DHA under different initial nitrate concentrations and illuminance levels

Fig. 4-3 shows the variation of DHA concentration of cultures with time. Interestingly, 1x cultures had higher DHA concentrations as compared to the 3x cultures of the corresponding illuminance level, throughout the period of cultivation. The only exceptions to this observation were seen in HL cultures on day 4 and day 16, and in LL cultures on day 8. Although the accumulation of DHA in the genus *Isochrysis* is generally

favored under nutrient repletion, the converse phenomenon has also been observed in some strains. Sun et al. [64] observed that two *Isochrysis* strains showed opposing behavior during nutrient limitation, with the DHA accumulation being enhanced in *Isochrysis* CCMP462. The increment of DHA under nutrient limitation could be attributed to re-partitioning of DHA from membrane lipids to neutral lipids/triacylglycerols [64,65]. Whilst membrane lipids exist in complex structures in microalgal cells, triacylglycerols are commonly deposited in plastidial or cytosolic lipid droplets [7]. Consequently, the higher extractability of DHA in the form of neutral lipids as compared to complex membrane lipids would have resulted in increased DHA concentrations observed in 1x cultures [66]. Nevertheless, despite of the higher DHA concentrations in 1x cultures, the effect of initial nitrate concentration on DHA concentration was not statistically significant ($p>0.05$), except on day 12 of cultivation.

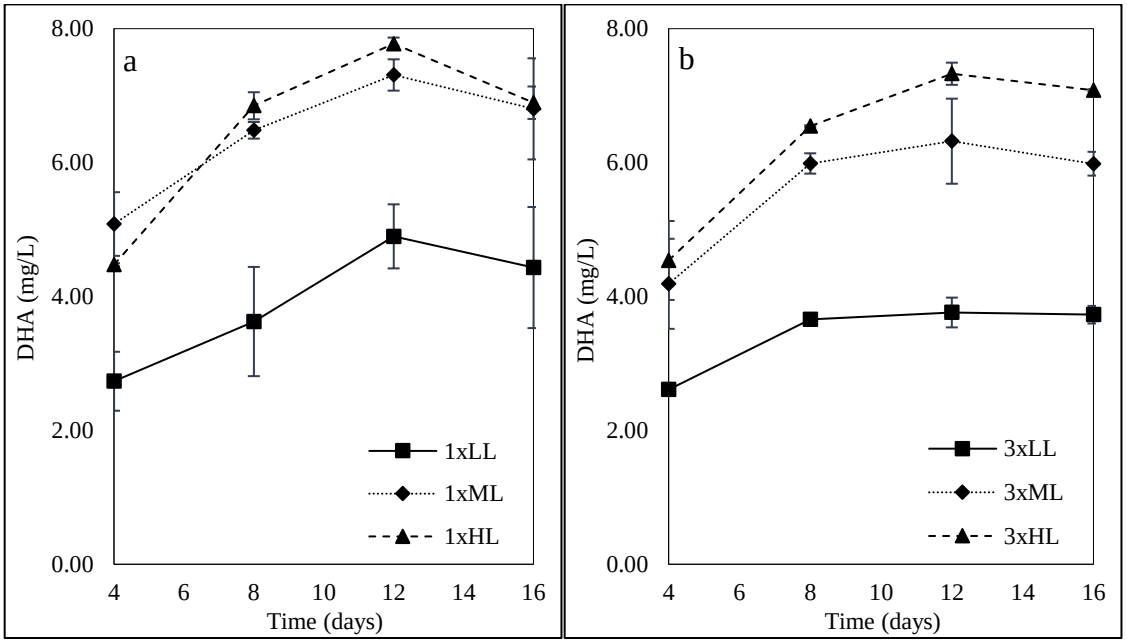



*Figure 4-3: Docosahexaenoic acid (DHA) concentrations of T. lutea grown under different illuminance levels (low light LL: 3,750 lux; medium light, ML: 7,500 lux; and high light, HL: 11,250 lux) and initial nitrate concentrations (1x: 882 µM; 3x: 2,646 µM) over the 16-day cultivation period (a) 1x cultures (b) 3x cultures*

Moreover, it was evident that the DHA concentrations of cultures were strongly correlated with the biomass concentrations, as cultivation under higher illuminance levels resulted in significantly higher DHA yields ($p<0.05$). Additionally, the weight fractions of DHA in microalgal biomass was higher under high illuminance levels, as shown in Fig. 4-4. Similar results were reported by Tzovenis et al. [41], who observed an increment in DHA content by weight at higher photon flux densities. Since high illuminance levels generally promote the accumulation of triacylglycerols in microalgae [67], it is possible the higher DHA contents reported under high illuminance levels in the current study could also be explained by the restructuring of membrane lipids as neutral lipids and the ensuing of increment of extractability.

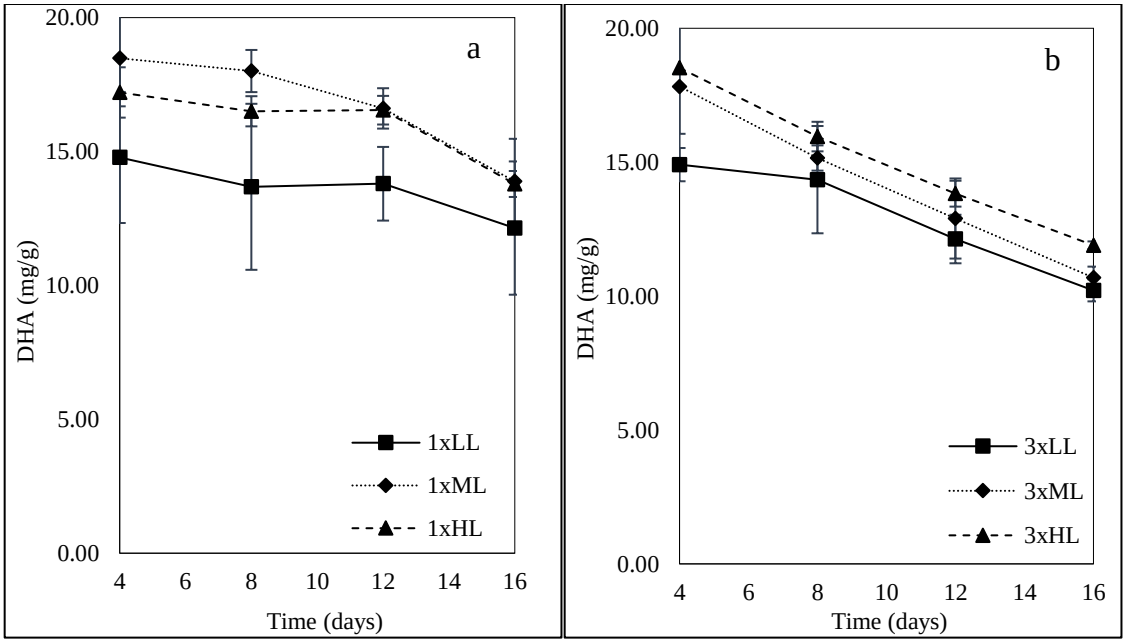



*Figure 4-4: Variation of cellular docosahexaenoic acid (DHA) content of T. lutea grown under different illuminance levels (low light LL: 3,750 lux; medium light, ML: 7,500 lux; and high light, HL: 11,250 lux) and initial nitrate concentrations (1x: 882 µM; 3x: 2,646 µM) over the 16-day cultivation period (a) 1x cultures (b) 3x cultures*

However, it is noteworthy that the weight fraction of DHA in microalgal biomass (Fig. 4-4), showed a continuous reduction from the onset of cultivation. Thus, it is evident that DHA accumulation in biomass was reduced as nutrients were consumed from the growth

media during batch cultivation. Previous studies have shown that *T. lutea* favors the synthesis of saturated fatty acids, as opposed to PUFAs, under nutrient limited conditions [45]. Thus, the results of the current study showed that maintenance of nutrient replete conditions by continuous/intermittent nutrient supplementation may be necessary to obtain *T. lutea* biomass with high cellular contents of DHA.

Ultimately, the maximum DHA concentration on day 16, 7.08 ± 0.02 mg/L, was achieved in the 3x HL culture. Nevertheless, the DHA content in biomass produced under the 3x HL condition was only 11.90 ± 0.14 mg/g, which was lower than the weight fraction of DHA in all 1x cultures. The observed result further stressed the importance of achieving high biomass productivities to maximize the yield of target compounds, since 3x HL cultures also exhibited the highest biomass concentration on day 16.

### 4.3. Accumulation of fucoxanthin under different initial nitrate concentrations and illuminance levels

The variation of fucoxanthin concentration in cultures with time is shown in Fig. 4-5. The effect of nitrate concentration in the media on fucoxanthin concentration of cultures was not statistically significant ($p>0.05$) during day 4 and day 8. In contrast, illuminance level had a significant impact on the concentration of fucoxanthin on day 4 and day 8. This could be attributed to the differences in biomass concentration of cultures, or differences in weight fraction of fucoxanthin in biomass (as the overall concentration of fucoxanthin is the product of biomass concentration and weight fraction of fucoxanthin in biomass). The weight fraction of fucoxanthin was similar in all cultures during day 4 (Fig. 4-6), thereby suggesting that biomass concentration had the more prominent effect on fucoxanthin concentration of cultures. Similarly, the fucoxanthin content in 3x cultures (7.49-8.10 mg/g) did not differ significantly on day 8. In contrast, the fucoxanthin content by weight in 1x cultures decreased from 10.74 ± 0.39 mg/g to 6.39 ± 0.21 mg/g (Fig. 4-6(b)) with the increase in illuminance level from LL to HL. Similar observations of low-light cultivation enhancing fucoxanthin accumulation in biomass has been reported in

literature [16]. Fucoxanthin, being a light-harvesting carotenoid, is accumulated in response to the lower availability of light [9].

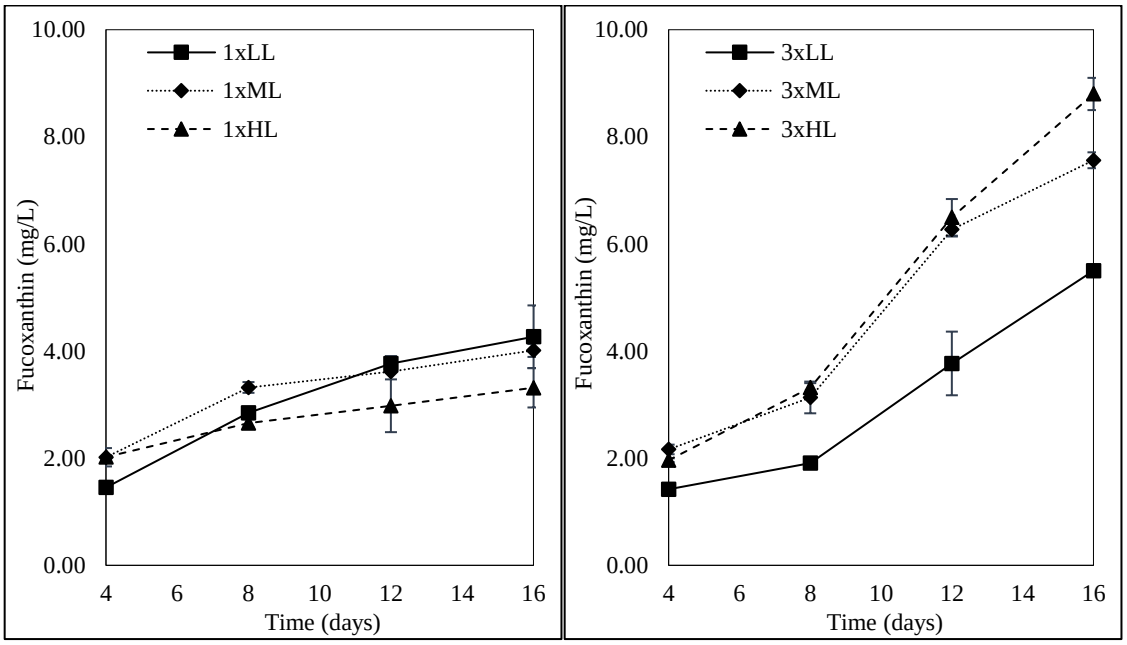



*Figure 4-5: Fucoxanthin concentrations of T. lutea grown under (low light LL: 3,750 lux; medium light, ML: 7,500 lux; and high light, HL: 11,250 lux) and initial nitrate concentrations (1x: 882 µM; 3x: 2,646 µM) over the 16-day cultivation period (a) 1x cultures (b) 3x cultures*

The effect of nitrate concentration in the media on fucoxanthin yield proved to be statistically significant during prolonged cultivation, as per the results reported for day 12 and day 16. Following the depletion of nitrate in 1x cultures during day 8, a notable increase in fucoxanthin concentration of cultures were not observed (Fig. 4-5(a)). In contrast, the fucoxanthin concentration of 3x cultures increased markedly with prolonged cultivation, as observed in Fig. 4-5(b). Fucoxanthin is a carotenoid which serves the function of a light-harvesting carotenoid by formation of complexes with chlorophyll a/c and apoproteins, thereby aiding in the photosynthetic mechanism of microalgae [10,26]. It has been reported that photosynthetic complexes, such as those formed by fucoxanthin, are sensitive to nutrient stress [8]. Thus, the lower fucoxanthin concentrations in 1x cultures during prolonged cultivation is most likely due to the stress conditions caused by nutrient depletion.

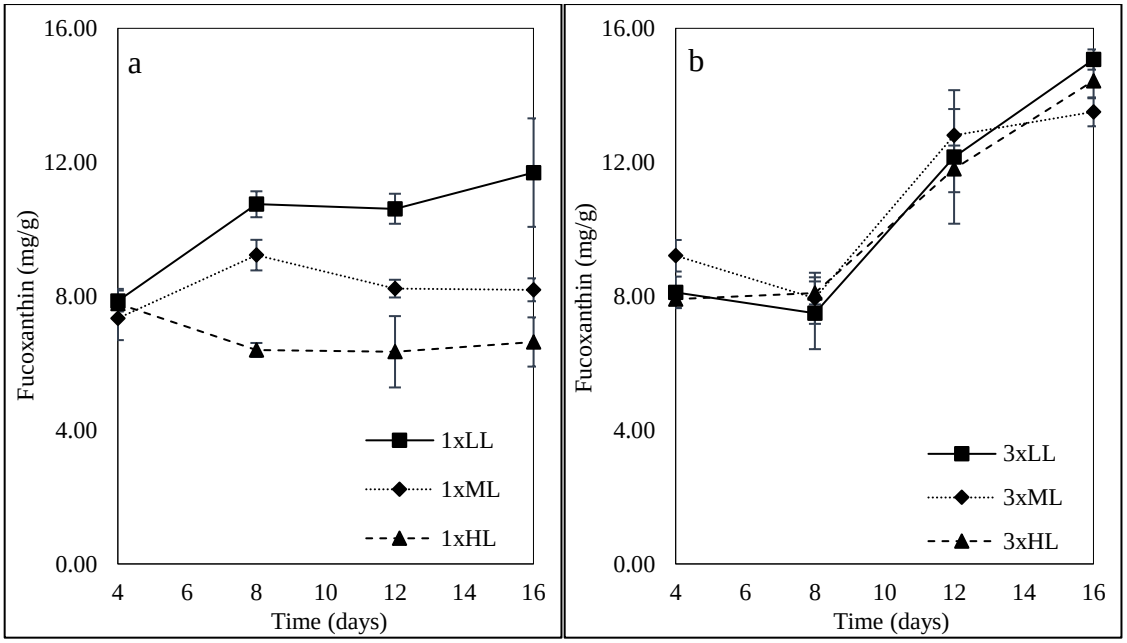



*Figure 4-6: Variation of cellular fucoxanthin content of T. lutea grown under different illuminance levels (low light LL: 3,750 lux; medium light, ML: 7,500 lux; and high light, HL: 11,250 lux) and initial nitrate concentrations (1x: 882 µM; 3x: 2,646 µM) over the 16-day cultivation period (a) 1x cultures (b) 3x cultures*

Hence, the use of nutrient repletion and low illuminance levels were confirmed as effective strategies to enhance fucoxanthin accumulation in *T. lutea*. Nonetheless, the use of lower illuminance levels for fucoxanthin accumulation would in turn reduce the biomass production in microalgal cultures, and consequently lower the overall fucoxanthin concentration. Therefore, achieving a compromise between biomass production and fucoxanthin accumulation is necessary to maximize product yields. This was evident by the results of the current study, wherein the fucoxanthin concentration of 3x LL was only 5.50 ± 0.03 mg/L, despite achieving the maximum cellular accumulation of 15.07 ± 0.30 mg/g. Accordingly, the maximum fucoxanthin concentration of 8.80 ± 0.30 mg/L was achieved in the 3x HL culture, with a cellular accumulation of 14.43 ± 0.52 mg/g.

Nonetheless, it is noteworthy that the production of both fucoxanthin and DHA in the current study could be enhanced significantly [8,16]. The limitation in the reported

biomass production is likely the reason for the lower product yields thus obtained in the current study. Hence, it would be of paramount importance to develop strategies to improve biomass production, such as the efficient design and operation of photobioreactors, and optimization of cultivation conditions.

### 4.4. Co-extraction of fucoxanthin and DHA from *T. lutea*

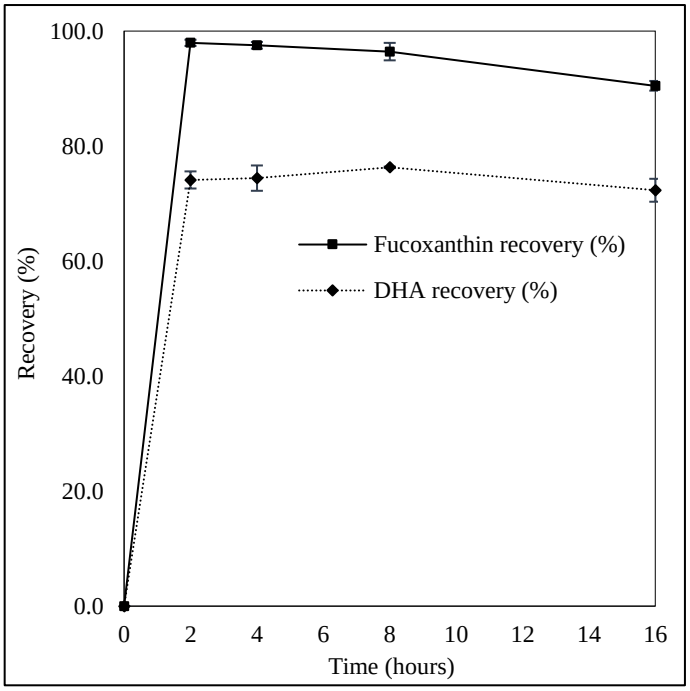



*Figure 4-7: Time-variation of fucoxanthin and docosahexaenoic acid (DHA) recovery during co-extraction with 10:9:1 (v/v/v) ethanol/n-hexane/water*

Fig. 4-7, shows yields of fucoxanthin and DHA during the co-extraction procedure. Most of the fucoxanthin (97.96 ± 0.54%) was recovered within 2 hrs of extraction. Previous studies also suggest that majority of fucoxanthin is recovered within a relatively short time period. Kim et al [28], achieved the maximal fucoxanthin recovery within 5 min extraction for *T. lutea*. In addition, Gallego et al [68], recovered 132.81 mg carotenoids per g of extract from *T. lutea* using pressurized ethyl acetate at 40 °C using one static extraction cycle (20 min). In a study by Gonçalves de Oliveira-Júnior et al [69], fucoxanthin recovery of 1186.7 mg/100 g of extract with >99% purity has been achieved using microwave-assisted extraction and solvent (ethanol or acetone) extraction. The comparatively low

extraction times were exhibited during the extraction of fucoxanthin from other microalgal species as well, with a yield of 0.69 mg/g achieved after 60 min of supercritical fluid extraction from *P. tricornutum* [70]. As shown in Fig. 4-7, prolonged extraction after 2 hrs significantly reduced fucoxanthin recovery ($p<0.05$), possibly because fucoxanthin is a thermolabile and photosensitive compound [71]. Similarly, 74.11 ± 1.49% of DHA was recovered within 2 hrs, and only marginal increments were observed after 2 hrs of extraction ($p>0.05$). Hence, 2 hrs was chosen as the most suitable co-extraction time for fucoxanthin and DHA from *T. lutea*.

Studies reported in literature have often separately quantified fucoxanthin and DHA content in *T. lutea* biomass. In comparison, only a few studies have evaluated co-extraction of the compounds. For example, the study by Delbrut et al. [27], evaluated co-extraction of fucoxanthin with PUFAs in *T. lutea* and *P. tricornutum*. 100% fucoxanthin and DHA recovery was achieved in *T. lutea* within 1 hr of extraction, whereas *P. tricornutum* required 8 hrs to recover 95% fucoxanthin and 89% EPA. This suggests that co-extraction processes should be optimized for each microalgal species. The shorter extraction times in *T. lutea* could be attributed to the absence of a cell wall. Hence, it does not require extensive cell wall disruption prior to extraction of target metabolites, and conventional solvents would be sufficient to conduct the extraction [28].

It is noteworthy that the study by Delbrut et al. [27] did not focus on the separation of PUFAs and fucoxanthin and recovered all compounds of interest into a single crude extract. Contrastingly, few studies on separation of PUFA and fucoxanthin have been reported in literature. For instance, a microwave assisted extraction process was utilized to extract fucoxanthin and EPA from *P. tricornutum*. However, product yields of the extraction process were lower as compared to the reported content in biomass [26]. In contrast, *T. lutea* may be a better candidate as feedstock for production of fucoxanthin and PUFA due to easier extractability [27]. Kim et al. [28], studied fractionation of fucoxanthin and lipids to a hydroalcoholic phase and nonpolar n-hexane phase respectively. However, fucoxanthin recovery was only 75%, whilst DHA recovery was not reported. In contrast, current study which utilized a modified methodology adopted

from Kim et al., showcased 97.96 ± 0.54% recovery of fucoxanthin and 74.11 ± 1.49% recovery of DHA within 2 hrs. Kim et al. [28], obtained a crude ethanol extract which was subsequently evaporated and resuspended in 90% ethanol, which was followed by addition of n-hexane to form a ratio of 10:9:1 (v/v/v) ethanol/n-hexane/water. In contrast, ethanol and n-hexane were added to biomass in the ratio of 10:9 from the onset of extraction in the current study. The non-requirement of an intermediate solvent removal step in the methodology developed in the current study would be advantageous in terms of process economics. Moreover, the use of a polar/nonpolar solvent system would have aided in the recovery of both polar and nonpolar compounds [72]. In the current study, phase separation was induced by the addition of water to form the ratio of 10:9:1 (v/v/v) ethanol/n-hexane/water. Moreover, in contrast to Kim et al. [28], who used multiple extraction steps, only a single extraction step was used in the current study for product recovery. Thus, it can be concluded that the biphasic solvent extraction procedure used in the current study would be an efficient way to extract and separate fucoxanthin and DHA from *T. lutea* feedstock.

### 4.5. Bioethanol production from residual biomass

The possibility of further valorizing the residual biomass from co-extraction of fucoxanthin and DHA was evaluated via alcoholic fermentation to produce bioethanol.

Table 4-1 shows the carbohydrate concentrations obtained from final biomass of *T. lutea* cultures. The higest carbohydrate concentration (104.7±0.6 mg/L, 209.4±1.3 mg/g) was observed in 1x HL biomass, while 3x LL biomass contains lowest value (32.8±1.0 mg/L, 90.0±3.1 mg/g). Carbohydrate concentations are significantly higer in 1x cultures than 3x cultures ($p<0.05$). Similarly, da Costa et al [45], has observed higher cabohydrate content in wild type of *T. lutea* under nitrogen reduced conditions. In addition, Almutairi [58], remarked that the nitrogen depletion will increase carbohydrate content due to the inhibited synthesis of nitrogen containing molecules such as proteins, DNA, and chlorophyll [73]. The influence of high illuminance level on the carbohydrate production also statistically significant ($p<0.05$), although the effect was not as prominent as

compared to the effect of nitogen depletion. Accordingly, a slight increase in carbohydrate concentration under high illuminance levels was observed in both 1x and 3x cultures. Although the accumulation of carbohydrate under high illuminance levels have been reported in literature [74], it is noteworthy that the cellular response to illumination conditions would be species-specific.

*Table 4-1: Final carbohydrate concentrations and of cellular carbohydrate content of T. lutea grown under different illuminance levels (low light LL: 3,750 lux; medium light, ML: 7,500 lux; and high light, HL: 11,250 lux) and initial nitrate concentrations (1x: 882 µM; 3x: 2,646 µM)*

| PBR | Carbohydrates (mg/L) | Carbohydrates (mg/g) |
|---|---|---|
| 1xLL | 72.7±3.2 | 199.2±9.5 |
| 1xML | 102.9±3.2 | 210.0±8.9 |
| 1xHL | 104.7±0.6 | 209.4±1.3 |
| 3xLL | 32.8±1.0 | 90.0±3.1 |
| 3xML | 52.0±1.3 | 92.8±3.3 |
| 3xHL | 62.6±0.3 | 105.2±1.4 |

Although microalgae biomass is a promising feedstock for the third generation bioethanol production [3,75], few studies have been conducted using species of the genus *Isochrysis*. Despite the highest cabohydrate conentration in the current study being observed in the 1x HL culture, biomass obtained from 3x HL was considered for bioethanol production as it yielded the maximal concentrations of the desired high-value products (i.e. fucoxanthin and DHA). The selection was based on the biorefiney concept, wherein multiple co-products are manufactured from a single feedstock. Following the co-extraction of fucoxanthin and DHA from 3x HL biomass, residual biomass was fermented to obtain a bioethanol yield of 48.49 ± 0.58 mg/g. The product yield suggests that alternative biochemical and thermochemical process routes for biofuel production such as hydrothermal liquefaction, pyrolysis and anaerobic digestion could be explored, and most suitable conversion methods should be selected on the basis of energy efficiency and economic considerations [76].

## 5. CONCLUSION

Initial nitrate concentration and illumination level were identified as parameters that could have a significant effect on the biosynthesis of fucoxanthin and DHA in *T. lutea.* Hence, the effect of these parameters on the simultaneous production of fucoxanthin and DHA in *T. lutea* was investigated in the present study. On day 16 of cultivation, the maximum DHA and fucoxanthin concentrations of 7.08 ± 0.02 mg/L and 8.80 ± 0.30 mg/L, were achieved in the 3x HL culture. Moreover, the highest biomass concentration of 0.60±0.01 g/L was also achieved under 3x HL, showcasing the importance of enhancing biomass production to maximize target metabolite yields. Furthermore, the procedure developed in the current study for co-extraction of fucoxanthin and DHA using a biphasic solvent system of ethanol/n-hexane/water (10:9:1 v/v/v) was successful in the recovery of 97.96 ± 0.54% of fucoxanthin and 74.11 ± 1.49% of DHA within 2 hrs of extraction. Thus, as per the hypothesis, it can be concluded that the use of biphasic solvent systems comprising of two distinct polar and nonpolar phases (as employed in the process developed in the current study) would be an efficient way to extract and separate fucoxanthin and DHA from *T. lutea*. Nonetheless, further studies are required to assess the economic feasibility and scalability of the process thus developed. Moreover, the possibility of further valorizing the residual biomass from co-extraction of fucoxanthin and DHA was evaluated via alcoholic fermentation to produce bioethanol, and bioethanol yield of 48.49 ± 0.58 mg/g was obtained. However, comprehensive assessment of the techno-economic feasibility and life cycle analysis is required prior to the selection of most favorable biorefinery routes for valorizing *T. lutea* biomass.

## 6. PUBLICATIONS

M. Premaratne, V.C. Liyanaarachchi, P.H.V. Nimarshana, T.U. Ariyadasa, R.A. Attalage, Co-production of fucoxanthin, docosahexaenoic acid (DHA) and bioethanol from the marine microalga *Tisochrysis lutea*, Biochemical Engineering Journal, manuscript no: BEJ-D-21-00326R1, revised manuscript submitted and under review.

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