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Kinetics and modeling of microalga *Tetraselmis* sp. FTC 209 growth with respect to its adaptation toward different trophic conditions

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ABSTRACT

Biomass and lipid production of microalga, *Tetraselmis* sp. FTC 209, isolated from local coastal waters was evaluated for photoautotrophic, mixotrophic and heterotrophic metabolism. Four non-linear growth models (logistic, logistic with lag, modified Gompertz, Baranyi–Roberts) were assessed for prediction of culture performance. Using statistical criteria, the Baranyi–Roberts model was chosen to estimate the growth kinetics values. Walne's mariculture medium was redesigned based on the concept of elemental balance to support higher density photoautotrophic growth. Biomass capacity of at least 100% was fixed for all micronutrient components while a sequential increase of 10% was imposed on deficient macronutrients. Results indicate that the new medium (W-30) managed to achieve a maximum cell concentration (X_{max}) of 1.505 g/L and maximum lipid content (P_{max}) of 376 mg/L (~25% of dry cell weight), representing 1.8 and 3.7-fold improvements, respectively, relative to Walne's basal. Light and dark cultivations with glucose supplementation revealed that *Tetraselmis* sp. FTC 209 favors mixotrophic over heterotrophic mode in terms of cell growth and lipid production. Compared with the baseline photoautotrophic W-30 medium, cultivation with addition of 30 g/L glucose produced the highest X_{max} at 8.08 g dry cell weight/L. This effectively provides significant algal biomass and lipid productivity of 404 mg dry cell weight/L per day and 90.9 mg lipid/L per day, respectively.

1. Introduction

Microalgae are ubiquitous aquatic dwellers of enormous scale. The most recent survey, tallying 72,500 species from the global literatures, with a dedicated on-line algal database, is now available (http://www.algaebase.org) [1]. The marine ecosystem displays phenomenal assemblages of species that proliferate abundantly in the littoral zones of the world, in contrast with their freshwater relatives [2]. Yet, this natural resource is relatively underutilized, despite existing exploitation as food, fodder and chemical derivatives. Currently, the potential for algal biofuels to assure energy security is being actively scrutinized by scientific and industrial communities alike. Manipulation of marine species to produce a renewable triacylglyceride (TAG) feedstock illustrates commercial sense, given that it avoids direct competition between resources needed for potable water and irrigation of land-based crops.

Oceanic water exists naturally as complex aqueous suspension of at least 50 elements, with sodium and chloride ions as dominant components. Even so, adaptation as a growth medium requires additional enrichment, usually in the form of inorganic salts in favor of higher biomass levels. The exact nutrient requirements for innumerable species have yet to be fully identified. Media are either developed based on the chemistry of water from natural habitats or the stoichiometric composition of algal biomass grown under regular physiological conditions [3]. Preferably, a medium should be tailored to match the stoichiometric make-up of individual species to improve growth and generation of the desired product. This is best approached by elemental balance analyses [4]. The requisite bioelements for the growth of a photosynthetic microalga has been identified as comprising N, P, K, Mg, Ca, S, Fe, Cu, Mn, and Zn [5].

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Photoautotrophy requires solar and adenosine triphosphate (ATP) to convert CO_2 into organic molecules. However, biomass is significantly improved in species that assimilate carbon (organotrophy). In the absence of light, some are even capable of rendering themselves into facultative heterotrophs. Nonetheless, the number of strains able to utilize organic substrates is limited. *Chlorella* for instance, is thoroughly established and has been the most commonly cited genus over the past three decades [6]. Current focus should include other genera with notable metabolic shift ability. Microalgae exhibiting plurality in metabolisms have been garnering widespread interest recently. Adapting native strains provides an avenue for increasing value whilst expanding the pool of existing species capable of alternative trophism [7].

The marine *Tetraselmis* is a unicellular flagellate with a lipid embodiment of 15–23% when cultured in photoautotrophic system [8]. At present, cultivation methods used in hatcheries for *Tetraselmis* spp. mainly depend on polybags or transparent glass-fiber columns (up to 500 L) that are kept indoors with artificial lighting [9]. Some species of *Tetraselmis* such as *T. chui*, *T. tetrathele*, and *T. subcordiformis* have the ability to assimilate glucose but none was found viable on acetate [10]. Only the commercially important *T. suecica* was prominently featured in ensuing studies on trophic modification [11,12]. However, the microalga stock culture must first be free of any foreign microbial contaminants, especially of fungal or bacterial origin in order to successfully execute the trophic switching experiments. The removal of such tenacious microbial epibionts from medium or algal cell bodies amongst others will greatly help the recovery of distinctively pure natural bioactive compounds from microalgae, or further maximizing the efficiency of photobioreactor culture to act as consumers of anthropogenically released CO_2 in atmospheric air. Axenicity is often difficult to achieve in large scale. It is far easier to render the algal cultures free from bacterial or mold infestation on smaller forms [13]. In the past, a combination of penicillin, streptomycin and nystatin had been demonstrated on the commercially important *Chlorella vulgaris* and *Chlorella pyrenoidosa* strains [14] while a one-shot treatment on agar plate that mixed together ampicillin, cefotaxime, and carbendazim was proven successful to clean *Chlamydomonas reinhardtii* [15].

This study investigated the potential of a local isolate, *Tetraselmis* sp. FTC 209 as lipid-producer. The approach covered axenic isolation, strain identification, and analysis of kinetic models that accurately describe the *Tetraselmis* growth. Although the kinetics aspect has been the subject of much bacterial researches, definitive modeling of microalgae growth is rarely tackled. Photoautotrophic performance was assessed for a set of media redefined using the elemental balance technique, and subsequently compared with the possibility of mixotrophic (photoheterotrophic) and heterotrophic modes.

2. Materials and methods

2.1. Microalga strain

The indigenous phytoplankton classified as *Tetraselmis* sp. FTC209 was previously isolated from coastal waters in close proximity to the Centre of Oceanograph and Marine Aquaculture Studies, Institute of Bioscience, Universiti Putra Malaysia. The marine research station is located near the shore of Teluk Kemang Beach, Port Dickson, Negeri Sembilan, Malaysia (Latitude: 2° 28′ 0.0” N; Longitude: 101° 50′ 59.99” E).

2.2. Axenic isolation and inoculum preparation

Broad-spectrum antibiotics were chosen for isolation and maintenance of pure *Tetraselmis* cells. An antibiotics cocktail consisting of 100/25 μg/mL ampicillin/streptomycin dosage is reasonably well-tolerated by chlorophyte microalgae [16]. Fungal contamination proved to be severe in the early subculturing of cells. This problem was solved by adding generic fungicide to the solid medium; Carben-50® (Dynamics Specialty Product, Malaysia) and Benocide® (Hextar Chemicals, Malaysia). Each contains 50% (w/w) carbendazim or benomyl. The effectiveness of the two fungicides was evaluated from 10 to 50 μg/mL in solid agar. The antibiotic and antifungal mixture was introduced to solidifying Walne's agar medium. Agar plates streaked with cells were incubated under 12:12 h, light (L): dark (D). Axenic status was confirmed by cultivating into 5 g/L glucose, 1 g/L yeast extract medium, or plating 100 μL of culture onto nutrient agar, incubated at 25 °C for 48–72 h. Purified cells were grown in 250 mL flasks containing 100 mL Walne's medium, incubated in illuminated orbital shaker (IS-971R, Lab Companion) at 130 rpm and 27 °C. Liquid cultures were later maintained by aseptic transfer of 10% (v/v) inoculum into fresh, sterile medium at 2-week intervals. A variation of Walne's medium was fortified with 5 g/L glucose to promote cell adaptation to the heterotrophic metabolism.

2.3. DNA extraction

Nucleic acid extraction was performed according to a procedure described for plant system [17]. A freshly prepared and pre-warmed extraction buffer (600 μL) composed of 1.4 M NaCl, 100 mM Tris–HCl (pH 8), 20 mM EDTA (pH 8), 2% sodium dodecyl sulphate (SDS), 2% activated charcoal, and 2% β-mercapthoethanol was added to 100 mg of wet cells from a week-old culture. The cells–buffer mixture was homogenized and left to react at 60 °C for 30 min aided by gentle mixing at 10 min intervals. It was cooled to room temperature and 600 μL chloroform:isoamyl alcohol (Chl:IAA at 24:1 v/v solution) was added, mixed and centrifuged at 18,320 rcf for 6 min. The translucent supernatant containing crude DNA was separated and washed with Chl:IAA to produce clear solution. After a further centrifugation step (18,320 rcf for 6 min), the supernatant (350 μL of the upper phase) was transferred to a new microtube and gently mixed with 150 μL of 5 M NaCl. Iso-propanol was added at about 2/3 the volume of solution and slowly mixed by gently inverting the tube several times until DNA filaments became visible. This step may be facilitated by cooling on ice for 20 min. The mixture was then centrifuged at 21,250 rcf at 4 °C for 20 min. The resultant supernatant was discarded and the pellet was washed with 80% ethanol and was re-centrifuged at 21,250 rcf for 5 min. The DNA pellet was left to dry for not more than 5 min and resuspended in 100 μL sterile double distilled water. The DNA solution was then mixed with 3.5 μL RNAse, and incubated for 30 min at 37 °C. The quantity and quality of the purified genomic DNA was determined by electrophoresis using 0.8% agarose gel.

2.4. Sequencing and phylogenetic analysis

Inference of *Tetraselmis* sp. taxon up to the species level was attempted by 16S analysis. The primer pair was reportedly constructed through aligning the nucleotide sequences of major species in commercial aquaculture, resulting in the design of a degenerate primer set (forward: 5′-CGA AAG CCT GAC **R**GA GCA ATA-3′, reverse: 5′-TAA GGG GCA TG**M** TGA CTT GAC GT -3′) permitting amplification independently of their evolutionary distance [18]. PCR was performed in a total volume of 20 μL containing 50 ng of DNA template, 2 μL of 10× buffer, 0.4 μL of 10 mM dNTP, 0.8 μL of each primer (10 μM), and 1 unit of Taq-polymerase (NEB). The amplification program comprised initial denaturation at 94 °C for 5 min, 30 cycles of denaturation (95 °C for 30 s), primer annealing (T_a of 58 °C for 30 s) and extension (72 °C for 30 s), followed by a final extension step at 72 °C for 10 min prior to a 4 °C hold.

Table 1
Cellular quota of macro and micronutrients of *Tetraselmis* on a percent dry weight basis [3] and calculated biomass capacities of individual bioelements contained in 1 L Walne's medium.

Macronutrient	Average weight fraction, w_i (%)	Biomass capacity,ϕ (%)
Carbon	81.626	ND
Nitrogen	12.442	0.525
Phosphorous	1.059	1.931
Sulfur	1.455	0.070
Potassium	1.959	–
Magnesium	0.188	–
Calcium	1.176	–
Micronutrient	**Average weight fraction, w_i (%)**	**Biomass capacity,ϕ (%)**
Iron	7.451×10^{-3}	14.276
Manganese	7.520×10^{-3}	4.386
Boron	1.200×10^{-4}	1938.756
Zinc	9.844×10^{-4}	405.447
Copper	1.107×10^{-3}	182.107
Cobalt	2.256×10^{-4}	869.594
Molybdenum	9.229×10^{-5}	850.801

ND: not determined.

The sequencing steps of template DNA was carried out by 1st Base Laboratories (Malaysia). The forward and reverse sequences were confirmed through the CAP contig assembly program in BioEdit Sequence Alignment Editor (Version 7.1.9). The sequence was then compared with NCBI depositories through a BLAST search. The top 15 rDNA sequences were retrieved, aligned, and assigned a phylogenetic relationship to *Tetraselmis* sp. FTC209. Sequence alignments and phylogenetic analysis were performed based on the UPGMA (unweighted pair group method with arithmetic mean) clustering procedure with 1000 bootstrap using CLC Main Workbench (version 6.8.1, CLC Bio A/S, Denmark).

2.5. Medium formulation

2.5.1. Autotrophic medium modification

Seawater at salinity 3.2% and pH 8.2 was "aged" in the dark at 4 °C for at least a week after on-site collection. It was then subjected to double filtration, first using a Whatman No. 4 filter to remove coarse sediments and then by vacuum filtration (0.45 μm cellulose nitrate) to entrap planktonic materials. Filtered seawater was then autoclaved at 121 °C, 15 psi for 20 min prior to cultivation. For the selection of basal medium, *Tetraselmis* with species such as *T. suecica* favored Walne's over other media, i.e., ES and F/2 Guillard [19]. Further evaluation of the medium to promote a higher cell density was carried out through elemental balancing. This technique determines the maximum biomass capacities of each bioelement by considering its amount (g) in 1 L growth medium and the weight fraction of the respective bioelement in the *Tetraselmis* cell [20]. Biomass capacity, ϕ (%) of bioelement *i*, is calculated according to Eq. (1):

$$\phi_i(\%) = \frac{m_i}{w_i} \times \frac{1}{W_{\text{dry mass}}} \times \frac{1}{\rho_{\text{cell,fresh}}} \quad (1)$$

where m_i is the weight of bioelement *i* in a liter of Walne's medium, w_i is the average weight fraction of bioelement *i* in dried biomass (Table 1), $W_{\text{dry mass}}$ is the mass portion of algal cells and $\rho_{\text{cell,fresh}}$ is the density of fresh cells. $W_{\text{dry mass}}$ was taken as 0.25 and $\rho_{\text{cell fresh}}$ was found to be 1.01 g/mL [20].

The computed biomass capacity for each bioelement is also shown in Table 1. Results indicate the absence of K, Mg, Ca and an apparent deficit of all macronutrients. Similar to most standard media, Walne's formulation has inadequate Fe and Mn [4]. Other micronutrients were supplemented in excess. Medium was modified as follows; the biomass capacity of trace metals considered to be insufficient, i.e., ϕ_{Fe} and ϕ_{Mn} were increased to 100% to avoid becoming the limiting nutrients. The feasibility of different autotrophic media was investigated on the basis of sequential increases of 10% in biomass capacities (ϕ) of all macronutrients from 10% to 50%. First, K and P were simultaneously enriched by substituting NaH_2PO_4 with mono and dibasic potassium phosphates. Ca and Mg were added through $CaCl_2{\cdot}2H_2O$ and $MgSO_4{\cdot}7H_2O$, respectively. S was initially available in minute quantities in $CuSO_4{\cdot}5H_2O$. At this point it was also complemented by $MgSO_4{\cdot}7H_2O$. However, this still translates to a ϕ_S of 17.1%, even if the medium has ϕ_{Cu} and ϕ_{Mg} at 100%. Satisfying the S requirement without an unnecessary surplus in Cu or Mg was achieved by introducing $(NH_4)_2SO_4$. Hence, balancing the N quota took account of the small quantities of ammonium ions present, in addition to nitrate. Increases in Na_2-EDTA were to proportionally match the extra Fe in the medium. Citric acid was introduced as a solubilizer for the Ca salt. Media redesigned in this manner were denoted W-10 to W-50 (Table 2).

2.5.2. Mixotrophic and heterotrophic media

Mixotrophic or heterotrophic cultivation differs from autotrophic in which the medium was enhanced by organic carbon. In this section, the modified W-30 medium was used as a basal medium supplemented with various initial concentrations of glucose. The autotrophic and mixotrophic runs were conducted in a diurnal cycle of 12 h (L): 12 h (D). Heterotrophic cultivations proceeded in a 24 h dark environment.

2.6. Stirred tank photobioreactor

All experiments were conducted in a 2.0 L glass stirred tank bioreactor (Biostat B, B. Braun Biotech, Germany). The bioreactor was fitted with three 14 W cool daylight T5 lamps (OSRAM) encircling the unit at 60° separations, providing an even illumination of 2280 ± 250 lux (Easy View EA30, Extech Instruments). The fluorescent irradiance was equivalent to a photosynthetic photon flux of 30 ± 3.3 μmol/m^2/s. Photobioreactor was inoculated with 10% (*v*/*v*) of two-week-old inoculum to achieve a working volume of 1.5 L. The cell number was about 32×10^6 cells/mL prior to inoculation. The temperature of the culture was maintained at 28 ± 1 °C while air flow rate was fixed at 1.5 L/min (1.0 vvm). A single Rushton turbine (diameter 0.052 m) was used for agitation. The "just suspended" speed, N_{js}, which was the minimum agitation to maintain off-bottom cells suspension, was adopted in all experimental runs. Through visual observation, a tip speed of 0.409 m/s (150 rpm) was determined to be a suitable N_{js} to avoid settling of circulating algal cells. The initial pH was adjusted to 6.5–7.0. DOT and pH were uncontrolled but recorded continuously throughout the run.

2.7. Analytical methods

During the cultivation, 20 mL samples were withdrawn at 48 h intervals. Aliquots were centrifuged at 980 rcf for 5 min. Supernatant was decanted and cells were resuspended, recentrifuged and washed with 0.5 M ammonium formate to remove excess seawater salts, followed by distilled water. Dry cell weight per volume was determined gravimetrically after cells were lyophilized overnight in a pre-weighed sample vials. The lyophilization procedure consists of pre-freezing (−30 °C) at 1 bar for 4 h, sample preparation for 15 min at 1 bar, main drying run (30 °C) for 20 h at 0.001 mbar and concluded with final drying at 0.0001 mbar for 15 min (Epsilon 1-8D, Martin Christ, Germany).

Lipid was extracted from 300 to 400 mg of lyophilized cells according to a method modified from Folch et al. [21]. Cells were first pulverized and weighed, followed by adding 4 mL methanol containing 500 ppm butylated hydroxytoluene (BHT), 2 mL

Table 2
Composition of Walne's basal and medium formulated using an elemental balance (mg/L).

Component	Walne's	W-10	W-20	W-30	W-40	W-50
$NaNO_3$	100	1746	3490	5237	6982	8727
$(NH_4)_2SO_4$	–	125	251	377	503	629
NaH_2PO_4	20	–	–	–	–	–
KH_2PO_4	–	120.5	241.0	361.6	482.1	602.6
K_2HPO_4	–	66.1	132.2	198.3	264.4	330.5
$MgSO_4{\cdot}7H_2O$	–	48	96	144	193	241
$CaCl_2{\cdot}2H_2O$	–	110	218.3	327.4	436.5	545.6
$FeCl_3{\cdot}6H_2O$	1.3	9.1	9.1	9.1	9.1	9.1
$MnCl_2{\cdot}4H_20$	0.36	4.4	4.4	4.4	4.4	4.4
$Na_2EDTA{\cdot}2H_2O$	45	200	200	200	200	200
H_3BO_3	33.6	33.6	33.6	33.6	33.6	33.6
$C_6H_8O_7$	–	20	20	20	20	20
$ZnCl_2$	2.1	2.1	2.1	2.1	2.1	2.1
$CuSO_4{\cdot}5H_2O$	2	2	2	2	2	2
$CoCl_2{\cdot}6H_2O$	2	2	2	2	2	2
$Na_2MoO_4{\cdot}2H_2O$	0.5	0.5	0.5	0.5	0.5	0.5
Thiamine HCl	0.02	0.02	0.02	0.02	0.02	0.02
Cyanocobalamine	0.01	0.01	0.01	0.01	0.01	0.01
Biotin	2×10^{-4}	2×10^{-4}	2×10^{-4}	2×10^{-4}	2×10^{-4}	2×10^{-4}

chloroform and 0.4 mL water. The mixture was homogenized and disrupted in an ultrasonic bath (Thermo-10D, Thermoline) for 15 min. Chloroform (2 mL) and water (2 mL) were added and the mixture was vortexed for 60 s. After centrifugation and siphoning off the upper phase, the lower chloroform phase containing lipid was collected in a pre-weighed vial. Organic solvent was heated to 62 °C and purged with a nitrogen stream. Total lipid was also determined gravimetrically.

Residual glucose in the supernatant was analyzed using a biochemistry analyzer (YSI 2700 Select Biochemistry Analyzer, YSI, Ohio). Intracellular chlorophyll *a* was determined via a trichromatic method [22]. The cell pellet (from centrifugation of 3–5 mL culture) was first treated with 0.2 mL dimethylsulfoxide, coupled with sonication for 5 min, followed by mixing with 2.8 mL of 90% acetone. Cell disruption was resumed for another 5 min before the mixture was kept overnight in the dark for pigment extraction to take effect. The extracts were then recentrifuged for 5 min and chlorophyll *a* in the supernatant (mg/L) was determined spectrophotometrically and calculated using Eq. (2):

$$\text{Chl}\,a = 11.85\,x(\text{OD}_{664} - \text{OD}_{730}) - 1.54\,x(\text{OD}_{647} - \text{OD}_{730}) - 0.08\,x(\text{OD}_{630} - \text{OD}_{730}) \quad (2)$$

Lipid content was also profiled by transesterifying it into fatty acid methyl ester (FAME) according to the technique described by O'Fallon et al. [23]. Oil sample (≈30 μL) was placed in a 15 mL centrifuge tube together with 0.7 mL of 10 N KOH (dissolved in water) and 5.3 mL methanol. The mixture was then incubated in a 55 °C water bath for 1.5 h, accompanied by vigorous shaking at every 20 min interval for reactants to properly permeate, dissolve, and hydrolyze the bio-oil. Subsequently, the tube was cooled to room temperature under running tap water and added with 0.58 mL 24 N H_2SO_4. The tube was again mixed by inversion, and with the presence of precipitated potassium sulfate, was incubated again at 55 °C in water bath for another 1.5 h with hand shaking at every 20 min. After another round of cooling under tap water, 3 mL of hexane was added and tube was vortexed for 5 min. The hexane layer containing FAME was placed in chromatography vial to be analyzed by gas chromatography or stored under −20 °C.

FAME sample was analyzed with Agilent 7890A (California, USA) gas chromatography equipped with flame ionization detector (FID). Separation is achieved using capillary column BPX70 70% cynopropyl polysilphenylene-siloxane (SGE Analytical, Ridgewood, Victoria, Australia), 30 m in length, an ID of 0.32 μm, and using N_2 as carrier gas at a flow rate of 5.7 mL/min. The temperatures of injector and detector were set at 250 and 280 °C, respectively, and the split ratio of 15:1 was used. Oven temperature was programmed as follows: holding at 1 °C for 4.6 min, 100 to 170 °C at a rate of 10 °C/min, 170 to 230 °C at a rate of 1.5 °C/min and hold at 230 °C for 7 min, 230 to 250 °C at a rate of 30 °C/min and then hold at 250 °C for 1 min. Fatty acid components were identified by comparing their retention times with the standards (37 Components Mix, Supelco Analytical, Pennsylvania, USA).

Using the same chromatography results, fundamental indicators related to the quality of biodiesel, i.e., cetane number (CN), saponification value (SV) and iodine value (IV) were possible to predict through empirical models [24] as shown by Eqs. (3)–(5):

$$\text{CN} = 46.3 + \frac{5458}{\text{SV}} - (0.225 \times \text{IV}) \quad (3)$$

$$\text{SV} = \frac{\sum(560 \times N)}{M} \quad (4)$$

$$\text{IV} = \frac{\sum(254 \times D \times N)}{M} \quad (5)$$

where D is the number of double bonds, M is the molecular mass and N is the percentage of each fatty acid constituent.

2.8. Kinetic models assessment and statistical analyses

Four models; logistic, logistic with lag, modified Gompertz, and Baranyi–Roberts were used to evaluate the growth kinetics of *Tetraselmis* sp. FTC 209. The time-dependent increase in microalgae population following the logistic function is expressed by Eq. (6):

$$X(t) = \frac{X_0 e^{(\mu_{\max}\cdot t)}}{\left[1 - \left(\frac{X_0}{X_{\max}}\right)\left(1 - e^{(\mu_{\max}\cdot t)}\right)\right]} \quad (6)$$

where X_0 and $X_{\max}$ are the initial and maximum cell concentrations (g/L) and $\mu_{\max}$ refers to the maximum growth rate (h^{-1}). A newer version of the logistic includes an additional term related to the lag phase, λ(h) as in Eq. (7):

$$X(t) = X_0 + \frac{(X_{\max} - X_0)}{\left[1 + \exp\left\{\left(\frac{4\mu_{\max}}{(X_{\max} - X_0)}\right)(\lambda - t) + 2\right\}\right]} \quad (7)$$

Eq. (7) was the result of 'reparameterization' of empirical models by Zwietering et al. [25] which incorporate microbiologically

relevant meanings to their mathematical parameters. The derivation was also applied to the classical Gompertz model (Eq. (8)):

$$y = A \cdot \exp\left\{-\exp\left[\frac{\mu_{\max} exp(1)}{A}(\lambda - t) + 1\right]\right\} \quad (8)$$

Eq. (8) is designated the "Modified Gompertz", and A refers to the asymptote of maximal value at the y-axis. Eq. (8) can be treated as a new model because the current form is more applicable to biological systems. In this work, Eq. (8) is rewritten to Eq. (9) to include the relevant biomass concentration terms:

$$X(t) = X_0 + (X_{\max} - X_0)\, exp\left\{-\exp\left[\frac{\mu_{\max} exp(1)}{(X_{\max} - X_0)}(\lambda - t) + 1\right]\right\} \quad (9)$$

A different family of mechanistic growth models developed by Baranyi [26] has a geometrically different curve profile depicting a quasi-linear segment for the exponential phase. The specific growth rate takes the form of a first-order differential equation:

$$\mu(t) = \frac{1}{x}\frac{dx}{dt} = \mu_{\max}\alpha(t) f(t) \quad (10)$$

where α (t) is an 'adjustment function' describing the inoculum adaptation inside bioreactor, α(t) is monotonically increasing; $0 \le \alpha \le 1$ and $\lim_{t\to\infty} \alpha(t) = 1$ by following:

$$\alpha(t) = \frac{P(t)}{P(t) + K_p} = \frac{q(t)}{1 + q(t)} = \frac{q_0}{q_0 + e^{-\mu_{\max} \cdot t}} \quad (11)$$

The quotient q_0 represents the physiological state of the inoculum. It may be proportional to the concentration of a critical substance, P(t) that causes a bottleneck in the process of cells adjusting to their new environment, and $\mu_{\max}$ is influenced by P(t) according to Michaelis–Menten kinetics. The f(t) function in Eq. (10) refers to the end-of-growth inhibition. It is monotonically decreases with $f(0) = 1$ and $\lim_{t\to\infty} f(t) = 0$. Most dynamics models describe f(t) by a logistic inhibition function as shown in Eq. (12):

$$f(t) = 1 - \left(\frac{x}{x_{\max}}\right) \quad (12)$$

Explicit solution to the differential Eq. (10) transposed to a linear expression:

$$X(t) = X_0 + \mu_{\max} B(t) - \ln\left(1 + \frac{\exp^{\mu_{\max} \cdot B(t)} - 1}{\exp^{(X_{\max} - X_0)}}\right) \quad (13)$$

where

$$B(t) = t + \frac{1}{\mu_{\max}} \ln\left(\exp^{(-\mu_{\max} \cdot t)} + \exp^{(-\mu_{\max} \cdot \lambda)} - \exp^{(-\mu_{\max}(t+\lambda))}\right) \quad (14)$$

Levenberg–Marquadt algorithm was used to solve Eqs. (6), (7), (9) and (13), fitted to the growth data of *Tetraselmis* sp. FTC 209. The fitting procedure was performed on Sigmaplot version 11.0 (Systat Soft Inc., CA, USA). In addition to the standard correlation coefficient (R^2), additional model validation may include statistical criteria as a function of predicted values (pred), experimentally observed values (obs), the mean of observed values (mean obs) and the number of samples [27]:

Root mean square error (RMSE):

$$\mathrm{RMSE} = \left[\frac{\sum(\mathrm{obs} - \mathrm{pred})^2}{n}\right]^{\frac{1}{2}} \quad (15)$$

Standard error of prediction (% SEP):

$$\%\mathrm{SEP} = \frac{100}{\mathrm{mean\_obs}} \times \left[\frac{\sum(\mathrm{obs} - \mathrm{pred})^2}{n}\right]^{\frac{1}{2}} \quad (16)$$

Bias factor (B_f):

$$B_f = 10^{\frac{\sum \log(\mathrm{pred}/\mathrm{obs})}{n}} \quad (17)$$

Accuracy factor (A_f):

$$A_f = 10^{\frac{\sum |\log(\mathrm{pred}/\mathrm{obs})|}{n}} \quad (18)$$

Calculated kinetic parameters of different cultivation media were analyzed using a one-way ANOVA. The Tukey's HSD method was chosen for post hoc tests for each dataset (SAS ver. 9.1, SAS Institute, USA). All statistics were based on a confidence level of 95%, and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Decontamination of Tetraselmis strain

Initially, the starting culture was heavily contaminated with airborne bacteria and molds. These contaminants did not affect the physiological function of *Tetraselmis* as a photosynthetic gas exchanger. However, a pure culture is needed for growth kinetic studies, maintenance of stock cultures, and metabolic experiments. Separation of *Tetraselmis* cells from contaminants, especially fungi, is a particularly difficult task. Microscope inspection identified the fungi as belonging to the *Aspergillus* group. Vigorous growth of fungi could easily ruin a culture in a couple of days. A one-shot solution was tested for the *Tetraselmis* sp. FTC 209. It was found that microalga showed a considerably slow growth but nevertheless subsisted on plates supplemented with the ampicillin/streptomycin cocktail, and Carben-50® or Benocide® to a certain concentration. Serial sub-culturing was tedious, given that distinctive colonies suitable for transference were only discernable at 3–4 weeks incubation. *Tetraselmis* tolerance and the contaminant susceptibility toward treatment were visually scored against separate isolates plated onto control agar (Table 3). Emphasis was on the suitable fungicidal range, because it was a major concern, especially in our laboratory, which was constantly exposed to airborne spores. This is quite serious for microbiology facilities located in humid areas or older buildings [28].

Treatment using Carben-50® and Benocide® effectively eradicated the threat at 30 μg/mL but at the expense of noticeable inhibition to the microalga, where growth was restricted to one or two peripheries of the streaked plate. In terms of appearance, microalga colonies retained their green pigments even at reduced growth rate. *Tetraselmis* may initially thrive on plates with higher concentrations of fungicide. In spite of this, bleaching occurred during the third week of incubation. Carben-50® proved to be potent for inhibiting fungal growth at lower concentration (10 μg/mL). Cleaned *Tetraselmis* stock was obtained following serial re-streaking of a single colony onto 20 μg/mL Carben-50® treated plates at least two times in succession before being re-incubated onto normal Walne's agar.

Benomyl and carbendazim are both systemic fungicides of benzimidazole carbamate group. Their mode of action requires metabolite binding to microtubuli and causing mitotic arrest of fungal cell division, followed by inhibition of cytokinesis. Fungicide toxicity has a selective heightened effect on fungal microtubules compared with mammalian cells [29]. Hyphae elongation is visibly suppressed. It would appear as a single speck on spots where spores had germinated on the agar plate. The application of

Table 3
Observation of *Tetraselmis* and fungal contaminant growth on plates with different concentrations of fungicides.

Microorganism	Fungicide	Dosage (μg/mL)					
		1	10	20	30	40	50
Tetraselmis sp. FTC 209	Carben-50	+++	+++	+++	++	–	–
	Benocide	+++	+++	+++	++	+	–
Fungal contaminant	Carben-50	+++[a]	+[b]	–	–	–	–
	Benocide	+++[a]	++[a]	++[b]	–	–	–

+++ Unimpeded growth (against control), ++ growth with observable inhibition, + growth with substantial inhibition, – no growth.
[a] Morphology in the form of branching mycelium.
[b] Morphology akin to stunted hyphae.

analytical-grade carbendazim at very low concentration (1 μg/mL) is sufficient to control fungal contamination without observable effects on *Chlamydomonas reinhardtii* [28]. Carben-50®, Benocide®, or any related trade synonyms that are more common for the treatments of post-harvest diseases on fresh fruits and vegetables (e.g., anthracnose, stem-end rot and blue mold) provided equally satisfactory results at slightly higher doses. Most are marketed as a 50% wettable powder, and pose to be much cheaper and easily available alternatives. To date, their application in algal aquaculture remains scant.

3.2. Identification of Tetraselmis strain

Under light microscopy, *Tetraselmis* appears as a bright green, solitary and ellipsoidal thecate cell. The genus is most distinguishable by the four flagella protruding from an apical depression of the cell. Photomicrograph measurement on 100 individual cells led to an algal mean length of 13.10 ± 1.3 μm (data not shown). To identify the strain and its taxonomic relationship with the existing sequences in NCBI database, a partial 16S-like region of rDNA genes (860 bp) was amplified by PCR and sequenced. 16S rRNA genes are applied by the majority of researchers to deduce the relationships among higher taxa of organisms, from families upwards. Its homologue 18S rRNA may serve the same purpose and is nuclear-encoded [18]. Notwithstanding that nuclear genes have a higher degree of conservation with respect to organelle genes, the primer selected was considered adequate to amplify the genomic portion of the SSU ribosomal gene in the plastid region. Fig. 1 shows a UPGMA tree considering among-site rate variation based on 16S rRNA sequences of different eukaryotes belonging to the so-called crown taxa. Homology searches showed that the studied microalga was clustered in the same group as *Tetraselmis* species, having similar phenotypes, which defines the same species for the sample at both morphological and molecular levels. Furthermore, similarity of the sample is shown to be much closer to strain *Tetraselmis striata* SAG 41.85 than the rest of the *Tetraselmis* species clustered in the same group, with the highest bootstrap score (confidence level), confirming phylogenetic reliability of the clustering procedure.

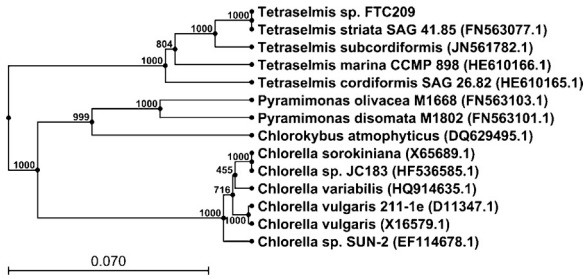

Fig. 1. Phylogenetic relationship of isolate *Tetraselmis* sp. FTC 209. Numbers above each node denote confidence levels generated from 1000 bootstrap.

3.3. Growth model assessment

The four models' fits with data from a run using basal Walne's are shown in Fig. 2. The logistic model appears to skip the lag phase. Additionally, its simulation line represents the averaged values of observed data. The logistic with lag term, modified Gompertz and Baranyi–Roberts models produced closer agreement with the actual data. The goodness of fit or accuracy indices of the four predictive models are presented in Table 4. First, the correlation coefficient, R^2 of all models are respectably above 95%. Auxiliary statistical validation is therefore required. RMSE is a good measure of accuracy in which a low value indicates the best model agreement. Logistic with lag term and Baranyi–Roberts models possess very low RMSE (0.0317 and 0.0216, respectively). Bias factor, B_f, is another way to quantify the relative departure between model and observed values. A perfect agreement will result in unity, while a B_f that is higher or lower than 1.0 indicates overprediction or underprediction of observed data. B_f for all models was good, considering it fell within 0.9–1.05. The model is still acceptable for B_f ranges from 0.7–0.9 or 1.06–1.15 but consequently rejected if <0.7 or >1.15 [27]. The accuracy factor, A_f, represents the difference between the means of observed and simulated data. Again, a low value is preferred, because this gives a higher capacity of correctness. The Baranyi–Roberts model provides the lowest A_f (1.0351) and the lowest %SEP that directly correlates to the residual. Based on the statistical evaluation, it is suggested that the Baranyi–Roberts model matches closest to the data describing microalga growth and its accuracy is followed closely by the logistic with lag term.

Baranyi–Roberts model [26] was first derived as a dynamic predictor of food pathogen growth but it has increasingly found its predictive capacity applied over a much wider scope. It was only recently that phycological studies made use of the model for algal growth estimation; examples include a study on *Aphanothece microscopica* growth on wastewater [27] and screening of microalgae strains with potential for biodiesel [30]. The three sigmoidal-type models tested are quite robust for describing the growth kinetics of a wide range of microorganisms. Nevertheless, the logistic function is more suited for simulating growth increments that are several-fold in magnitude, evident from its original development for predicting human population dynamics [31]. The function is less likely to converge toward the end-value of biomass (X_{max}) gained at such a small degree (<1 g/L). The other possible drawback may be that the sigmoid curve has an inflection point at the mid-exponential section. This is inappropriate for describing the exponential growth phase, given that the relationship between the logarithms of biomass concentration with time is supposedly linear by definition. A possible limitation arises from the use of sigmoidal-type models might result in overestimation of μ_{max} and λ [32].

3.4. Effect of modified medium on photoautotrophic culture

The growth profiles of *Tetraselmis* sp. FTC 209 grown in six variations of photoautotrophic media are shown in Fig. 3. The mesh

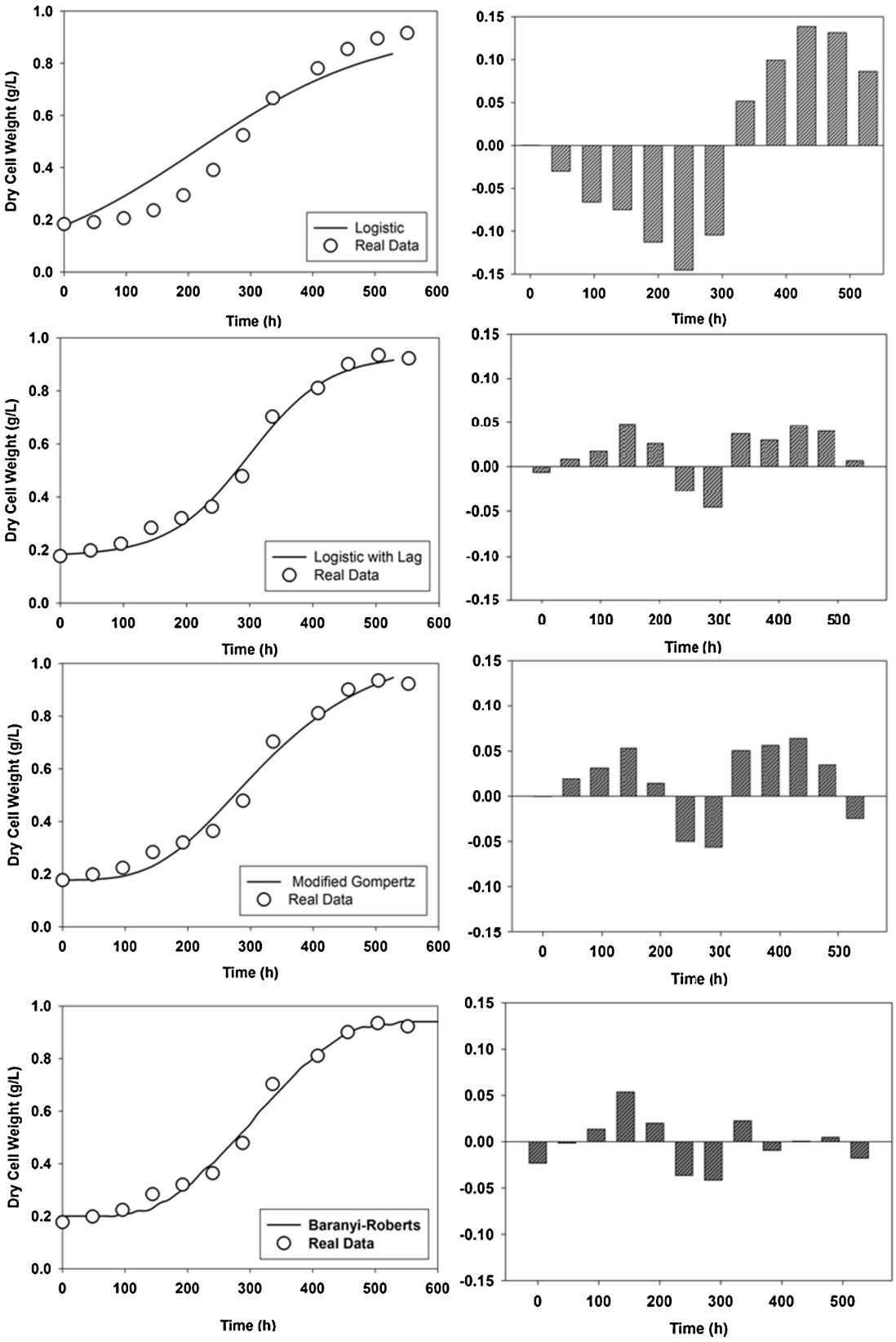

Fig. 2. Growth of *Tetraselmis* sp. FTC209 in Walne's basal. (Solid line) simulated growth from kinetic models, (bar graph) residual error between observed and predicted data.

surface plot portrays the conformity of closest matched model values with observed data. The related kinetic data is also compiled in Table 5. Generally, nutrient enrichment led to significant changes in biomass concentration ($p < 0.0001$). *Tetraselmis* biomass could be enhanced to as high as 1.8 times for culture grown in W-30 medium compared with that in Walne's medium (X_{max} of 0.827 g/L). The newly inoculated cells underwent a lag phase of 4–6 days, depending on the types of media used. The lag phase is

Table 4
Performance indices of the four growth models for *Tetraselmis* sp. FTC 209.

Error model	Logistic	Logistic with lag	Modified Gompertz	Baranyi–Roberts
R^2	0.957	0.991	0.981	0.988
RMSE	0.0529	0.0317	0.0494	0.0216
B_f	1.0442	0.9999	0.9288	0.9988
A_f	1.1163	1.0667	1.1167	1.0351
% SEP	12.0067	6.0267	9.3872	4.9119

Table 5
Kinetic data of *Tetraselmis* sp. FTC 209 in photoautotrophic mode.

Kinetic parameters	Autotrophic media					
	Walne	W-10	W-20	W-30	W-40	W-50
X_o (g dcw/L)	0.218 ± 0.076^{a}	0.200 ± 0.065^{a}	0.257 ± 0.081^{a}	0.283 ± 0.028^{a}	0.315 ± 0.085^{a}	0.346 ± 0.018^{a}
X_{max} (g dcw/L)	0.827 ± 0.127^{c}	$1.042 \pm 0.124^{b,c}$	1.481 ± 0.043^{a}	1.505 ± 0.165^{a}	1.360 ± 0.069^{a}	$1.257 \pm 0.024^{a,b}$
λ (h)	$127.47 \pm 27.50^{a,b}$	$113.08 \pm 26.94^{a,b}$	$111.73 \pm 15.70^{a,b}$	94.41 ± 9.00^{b}	98.74 ± 13.65^{b}	168.66 ± 9.35^{a}
μ (h^{-1})	0.0023 ± 0.00023^{c}	$0.0031 \pm 0.00086^{a,b,c}$	0.0045 ± 0.00095^{a}	$0.0042 \pm 0.00021^{a,b}$	$0.0027 \pm 0.00021^{b,c}$	$0.0030 \pm 0.00028^{b,c}$
Pr (mg dcw/L/day)	39.39 ± 6.02^{e}	57.87 ± 7.06^{d}	$82.26 \pm 2.414^{a,b}$	94.08 ± 10.30^{a}	$75.54 \pm 3.857^{b,c}$	$62.87 \pm 1.210^{c,d}$
P_{max} (mg lipid/L)	101.15 ± 18.25^{c}	153.96 ± 18.78^{c}	281.32 ± 8.26^{b}	376.02 ± 14.16^{a}	259.10 ± 13.23^{b}	226.78 ± 4.36^{b}
Pr (mg lipid/L/day)	4.82 ± 0.73^{e}	8.55 ± 1.04^{d}	15.63 ± 0.45^{b}	20.89 ± 2.28^{a}	$14.40 \pm 0.74^{b,c}$	$11.34 \pm 0.21^{c,d}$
P_{max}/X_{max} (mg/g)	122.29 ± 26.67^{b}	147.80 ± 71.49^{a}	189.98 ± 13.55^{a}	249.79 ± 43.18^{a}	190.56 ± 27.63^{a}	180.36 ± 76.75^{a}

Mean values in a row followed by the same superscript letter were not significantly different ($P < 0.05$).

a consequence of intracellular phenomenon. It can be described as a two-compartment growth model in which cell only starts to divide following i) the evolution of all chromosomal materials, and ii) synthesis of critical enzymes or metabolites needed for cell division through absorption of nutrients from the surrounding medium [33]. Cultivation with Walne's basal produced a lag of 5.3 days (127.5 h) and the lowest μ_{max} (0.0023 h^{-1}). At $\phi = 10\%$ for all macronutrients (W-10), the lag phase was shortened by 14 h.

Cultivation was further improved by incrementally enriching the macronutrients. The shortest lag phase was observed in W-30 culture (94.4 h). The low cell growth rate was therefore, partly due to a lack of nutritive resources. For instance, the bioelements absent in Walne's medium, e.g. Mg is an absolute requirement because it is a constituent of chlorophyll and an essential cofactor/activator of major reaction, i.e., nitrate reduction, CO_2 fixation and nucleic acid synthesis; Ca influences the signaling of flagellar apparatus; and K is needed for osmotic regulation and is a cofactor of numerous enzymes [2].

Differences in specific growth rate, biomass concentration and productivity in the photoautotrophic cultivations were negligible for W-20 and W-30 runs. Both recorded a 95.6% improvement in growth rate ($\mu_{max} \approx 0.004\ h^{-1}$) in relation to the basal Walne's, where the X_{max} achieved was 1.48 to 1.50 g dcw/L, with productivities of 82.26 and 94.08 mg dcw/(L/day), respectively. It was noted that cultures exhibited a regressive growth trend in W-40 and W-50 medium. This may be attributed to microalga having a negative response to heightened nitrate concentration. Growth of *Tetraselmis* sp. FTC209 was inhibited when the nitrate concentration exceed 60 mM, though stoichiometric calculation dictated that the medium could fulfill more than 30% of the cellular N requirement. *T. suecica* showed a lower tolerance at 25 mM of nitrate [34]. Generally, excess nitrate can be stored in cells without detrimental effect [35], but its reduced form nitrite can act as protonophore that may inhibit ATP synthesis and stimulating ATP hydrolysis, resulting in low cell growth [36]. It took 9–12 days for *Tetraselmis* cultured with the modified media to reach the stationary phase and 20 days with Walne's basal. A similarly lengthy growth profile was also observed during the screening of wild-type *Tetraselmis* spp. cultured with standard mariculture medium [37]. Thus, it is quite common for algae farming to operate a 30-day cycle for the highest possible harvest. It shows here that redesigned medium provides a higher yield and possibly halves the growth duration, which could potentially increase productivity per operational year.

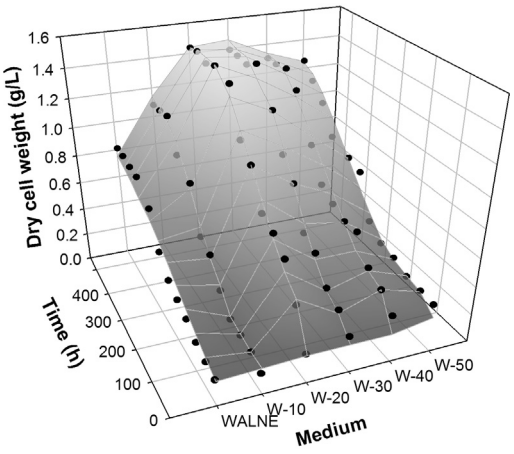

Fig. 3. Biomass concentration of *Tetraselmis* sp. FTC 209 as a function of cultivation time for different autotrophic media. Mesh plot represents model values as predicted by Eq. (10).

Low cell titers in each sample contributed to experimental limitation, hindering detail time course profiling of lipid content and hence, the kinetic calculation of product formation. Nevertheless, lipid production was previously established as a non-growth associated process, whereby accumulation began at the onset or late-end of the stationary phase, depending on the strains [5]. Lipid yield at the end of the experiment (500 h) was highest in W-30, with P_{max} of 376.02 mg/L, accounting for ~25% of the cellular content and a productivity of 20.89 mg lipid/L day. These represent 3.7 and 4.3 times the improvement respectively, over Walne's medium. For additional comparison, this study can be viewed as further expanding the compositional range of earlier work by Danquah *et al.* [3], which attempted a similar design principle to culture *T. suecica* in polybags. Understandably, medium is the only controllable variable in the polybag system, and the culture environment is not as tightly regulated as it is in a stirred tank photobioreactor. Their algal medium was the result of a single level design restricted to three types of bioelement (ϕ_N, $\phi_S = 5\%$ and $\phi_P = 10\%$). X_{max} and P_{max} in this case were 1.29 g/L and 108.7 mg/L for the particular *Tetraselmis* species. Biomass obtained from this study was slightly higher than the authors' data but a three-fold increase in lipid yield was achieved.

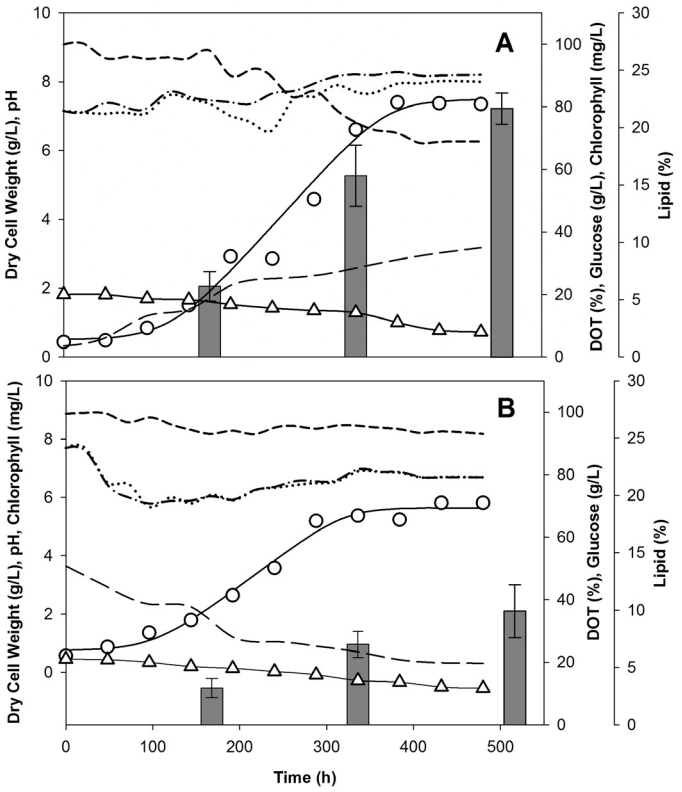

Fig. 4. Time course data for (A) mixotrophic and (B) heterotrophic culture using W-30 medium supplemented with 20 g/L glucose. (Empty circle) biomass, (solid line) simulated data, (empty triangle) glucose, (short dash line) DOT, (long dash line) chlorophyll content, (dotted line) pH in early morning, (dash-dot line) pH in late evening and (bar graph) percentage of cellular lipid.

3.5. Effect of different trophic conditions

Mixotrophic and heterotrophic cultivations were tested as means to circumvent the low biomass productivity of the photoautotrophic mode. Typical time courses of cultivation using W-30 medium with 20 g/L glucose for both conditions are shown in Fig. 4. *Tetraselmis* sp. FTC209 exhibited a certain capacity for adaptation to new medium conditions and lighting regimes. In both cases, inoculation was followed by an acclimatization of 3 to 4 days prior to cells entering the exponential phase. Results show a general increase in X_{max} to 7.151 and 5.646 g dcw/L, as well as μ_{max} of 0.0258 and 0.0178 h^{-1} for mixotrophic and heterotrophic cultures, respectively. The trend of a higher differential increased in biomass per unit time, was similarly observed in all light-assisted cultures against dark cultivation while utilizing the same medium. Nonetheless, both metabolisms generally supported greater growth than the non-glucose-supplemented culture, evidently from substantial improvements in μ_{max} and X_{max} by 473% and 382%, respectively, under mixotrophic, and by 295% and 282%, respectively, with heterotrophic cultures relative to the W-30 autotrophic run. The μ_{max} of *Tetraselmis* can be high as 0.013 h^{-1} when strictly cultured photosynthetically in an optimized medium [3].

There has been only one study that directly compared *Tetraselmis* performance under different trophic conditions. Day and Tsavalos [12] reported that growth of *T. suecica* was higher under mixotrophic mode, with a μ_{max} of 0.031 h^{-1} as opposed to 0.028 h^{-1} in dark cultivation and a final cell yield of 16.4 g/L. The findings corroborated with results of the present study, pointing to higher values of kinetic parameters for the mixotrophic mode. A study by Azma et al. [10] on the heterotrophy of *T. suecica*, however, did not furnish specific details on the growth kinetics, but only reported a very high final X_{max} of 28.8 g/L. A compilation of kinetic data on commercially important strains by Lee [38] implied that, while heterotrophic metabolism is either inferior or comparable to photosynthetic cultures, mixotrophic metabolism has an advantage, whereby its μ_{max} is consequently the sum total of the specific growths of photoautotrophic and heterotrophic metabolisms. An energetic study on the fractional contribution of light and glucose on *Chlorella pyrenoidosa* growth revealed that 63% of ATP was produced from photosynthesis activity [39]. Thus, illumination is the major source of ATP generation needed for synthesis of new cells in the early stage of mixotrophy before mutual shading occurs as a result of increased biomass density.

The culture pH dropped in the early hours and gradually increased over time. The initial reduction was attributed to the consumption of $(NH_4)_2SO_4$, because uptake of ammonium requires less energy than that of nitrate [40] but adopting ammonium as a sole N source may cause a sharp reduction in the medium pH, and severely reduced growth and biomass yield. Nitrate consumption, on the contrary, increases the culture pH [6]. This took place when $(NH_4)_2SO_4$ had been used up. Ammonium being the end product of nitrate reduction, causes preliminary feedback inhibition and repression of nitrate uptake [5]. The differences in daily pH for mixotrophic culture relate to cell respiration of the previous dark cycle, whereas fixation of CO_2 throughout the day contributes to hydroxide ion build-up in the medium [2]. Insignificant differences in the pH profiles were observed in morning and evening sampling for dark cultivation. The DOT levels for both trophisms were above 70% saturation throughout the cultivation. This shows that oxidative and photo-respiration of cells could decrease the dissolved oxygen tension in the culture, though not as drastically as those of fungal or bacterial fermentations.

Mixotrophic culture broth gradually turned dark green, whereas heterotrophic was mostly dull yellow at the termination of cultivation. The difference was correlated to a gross shift in cellular pigment content. Chlorophyll *a* in heterotrophic culture was greatly reduced to a final value of 0.31 mg/L, about 0.9% of the total chlorophyll *a* observed during mixotrophic cultivation (35 mg/L). Chlorophyll *a* was produced at a linear rate when light was available. The end of experiments showed that mixotrophic *Tetraselmis* cells retained similar lipid percentage but the light-starved culture saw a two-fold decrease when compared to the photoautotrophic run. Lipid content of above 20% w/w from the mixotrophic run is considered feasible for biofuel production [8]. The dearth of chlorophyll complex in light-limited cultures was implicated in the low levels of fatty acids, the building blocks for membrane lipids and storage TAG [12]. Deprivation of light prevents the energized state of photosynthetic electron transport, a driver for ATP, NADPH formation, and potentially reduced the pyruvate pool, a major photosynthate that is converted to acetyl CoA. The latter component is a precursor for de novo fatty acid synthesis within chloroplasts before eventual export to the cytosol and subsequent conversion to TAG in the endoplasmic reticulum [41].

Different initial glucose concentrations also affect microalgae growth (Table 6). There exists a trend of increasing μ_{max}, X_{max} and P_{max} for concentrations from 5 to 30 g/L ($p < 0.001$). A possible substrate inhibition was encountered with a 40 g/L glucose medium. Mixotrophic culture apparently outperformed the heterotrophic counterparts, whereby X_{max} of 8.08 g dcw/L and P_{max} of 1817.92 mg lipid/L were observed in the run using 30 g/L glucose. This corresponds to 37.7% more biomass and a 5-fold increased in lipid over that of the heterotrophic culture. Biomass productivity was encouraging at 404 mg dcw/L day. Nevertheless, it is the overall lipid productivity that determines the costs of a cultivation process. With comparable oil content, the optimal mixotrophic culture results in a Pr_{lipid} of 90.9 mg lipid/L/day, which is approximately 4 times the productivity obtained from the photoautotrophic W-30

Table 6
Kinetics of *Tetraselmis* sp. in mixotrophic and heterotrophic growth cultivated with different initial glucose concentration.

Kinetic parameters	Mixotrophic					Heterotrophic				
	5	10	20	30	40	5	10	20	30	40
X_o (g dcw/L)	0.308 ± 0.036^{b}	0.349 ± 0.026^{a,b}	0.414 ± 0.057^{a,b}	0.498 ± 0.042^{a,b}	0.440 ± 0.126^{a,b}	0.611 ± 0.030^{a,b}	0.650 ± 0.009^{b}	0.480 ± 0.093^{a,b}	0.384 ± 0.101^{a,b}	0.479 ± 0.040^{a,b}
X_{max} (g dcw/L)	1.750 ± 0.048^{f}	2.500 ± 0.171^{f}	7.151 ± 0.263^{b}	8.080 ± 0.090^{a}	6.652 ± 0.340^{b,c}	2.055 ± 0.119^{f}	4.694 ± 0.672^{e}	5.645 ± 0.128^{d}	5.868 ± 0.256^{c,d}	2.319 ± 0.091^{f}
λ (h)	90.22 ± 12.37^{b,c,d}	108.53 ± 8.20^{a,b,c}	99.85 ± 10.09^{b,c,d}	99.78 ± 13.42^{b,c,d}	129.97 ± 6.992^{a}	78.41 ± 6.17^{c,d}	85.89 ± 12.44^{b,c,d}	69.43 ± 7.27^{d}	95.47 ± 8.12^{b,c,d}	115.40 ± 3.33^{a,b}
μ (h^{-1})	0.0049 ± 0.00027^{d}	0.0083 ± 0.00086^{c,d}	0.0258 ± 0.00196^{a,b}	0.0289 ± 0.00369^{a}	0.0226 ± 0.00126^{a,b,c}	0.0057 ± 0.00134^{d}	0.0112 ± 0.00082^{c}	0.0178 ± 0.00177^{c}	0.0204 ± 0.00102^{b,c}	0.0082 ± 0.00140^{d}
Pr_{cell} (mg dcw/L/day)	97.21 ± 2.72^{f}	156.21 ± 10.66^{e}	397.29 ± 14.47^{a}	404.00 ± 4.50^{a}	332.55 ± 17.02^{b}	102.78 ± 5.75^{f}	234.72 ± 33.62^{d}	282.25 ± 6.41^{c}	293.40 ± 12.78^{b,c}	115.96 ± 4.55^{e,f}
P_{max} (mg lipid/L)	388.22 ± 10.85^{e,f}	512.09 ± 34.94^{e}	1548.20 ± 54.75^{b}	1817.92 ± 20.25^{a}	908.86 ± 46.51^{c}	341.70 ± 19.10^{f}	722.38 ± 13.47^{d}	559.25 ± 16.455^{e}	346.681 ± 15.10^{f}	162.07 ± 6.3^{g}
Pr_{lipid} (mg lipid/L/day)	21.57 ± 0.60^{e}	28.45 ± 1.94^{d}	86.01 ± 3.10^{a}	90.90 ± 1.24^{a}	50.49 ± 2.58^{b}	18.98 ± 1.06^{e}	36.12 ± 5.17^{c}	26.63 ± 0.45^{d,e}	19.29 ± 5.16^{e}	8.10 ± 0.32^{f}
P_{max}/X_{max} (mg/g)	221.88 ± 21.87^{a}	204.84 ± 13.35^{a,b}	216.55 ± 13.66^{a,b}	224.99 ± 10.21^{a}	138.54 ± 13.98^{c,d}	166.23 ± 52.98^{a,b,c}	153.88 ± 29.16^{b,c,d}	99.07 ± 23.07^{d,e}	62.41 ± 9.28^{e}	69.88 ± 12.88^{e}

Mean values in a row followed by the same superscript letter were not significantly different ($P < 0.05$).

run. Because lipid productivity depends on both intracellular oil and biomass accumulation, this result is acceptable in the case of *Tetraselmis* sp. FTC 209.

Conventional wisdom has been to starve the microalga of N source to tip the balance in favor of lipid production [4]. The consequence would be higher lipid output but at the expense of growth. This would somewhat diminish the economic feasibility of algal oil, because a high lipid content is normally associated with low biomass productivity. As such, taking the route of increasing biomass also contributes to a satisfactory result. Adopting glucose-supplemented media has a sound basis and is quite practical for *Tetraselmis* since the current large scale cultivations are mostly conducted indoor [9]. This will inevitably rule out interference from uncontrolled environmental factors, only that the existing closed column bioreactors will require some minor auxiliary modification through incorporation of in situ sterilization system to permit a bacterial-free operation throughout its run.

Glucose is seen as a safer choice of C source when compared to acetate or ethanol, mainly due to their inherent corrosiveness or high flammability in production line, and because glucose is readily accepted as substrate for producing metabolites of higher-value, in cases where the strain in question is meant to produce bioproducts requiring regulatory approval [42]. Past studies had demonstrated that mixotrophy significantly improves the growth of *Chlorella* sp. (both marine and freshwater species), *Nannochloropsis* sp. and *Chaetoceros* sp., giving rise to a supposition that as photosynthesis and oxidative glucose metabolism occurs concurrently in cells grown under mixotrophic mode, it is quite possible for photosynthetic activity to be further stimulated by the presence of glucose, most probably owing to the local increased of CO_2 released from glycolysis activity [43]. Glucose can be considered as a final product of photosynthesis, and this also allows for an assumption that any photosynthetic microorganism must be able to incorporate it into its cellular metabolism. It is reasonable to expect that the incorporation is rather straightforward [44]. Moreover, Chojnacka and Noworyta [45] did not observe any photoinhibition effect on *Spirulina* sp. growth when exposed to a high light intensity (in excess of 10,000 lux) under mixotrophic as opposed to photoautotrophic condition. They had implicated this as either due to the protective influence of glucose on algal cells or an apparent shift had occurred in the photoinhibitory light intensity.

3.6. Fatty acid profile

Comparison on fatty acid methyl ester (FAME) profile for *Tetraselmis* cells grown under mixotrophic and heterotrophic cultivation is shown in Fig. 5. A total of 26 fatty acids from both set of oil samples were successfully identified through GC analysis. The dominant fatty acids extracted from mixotrophic cells were palmitic acid (C16:0) (18.5%), followed by oleic acid, (C18:1n9c) (15.7%), stearic acid (C18:0) (9.3%), and γ-linolenic (C18:3n6) (6.3%). The strain was found to produce an evenly proportion of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), each accounting to 39.4% and 39.2% of the total FAME, respectively, with polyunsaturated fatty acids (PUFA) making up the remaining fraction. A similar fatty acid proportion could be deduced from the heterotrophic bio-oil sample, whereby the dominant constituents were palmitic acid (C16:0) (24.4%), followed by oleic acid, (C18:1n9c) (16.5%), but exhibiting higher content of γ-linolenic (C18:3n6) (10.7%), and cis-10-heptadecenoic acid (C17:1) (6.0%). Its SFA percentage of 39.7% was comparable to the mixotrophic sample. Nonetheless, a slight increase of unsaturation and longer fatty acid chain length was detected in the heterotrophic algal oil with PUFA of 28% was registered from the chromatogram. By extending the range of FAME identification outside the typical chain lengths of C16 to C22, it was discovered that uniquely for mixotrophic culture,

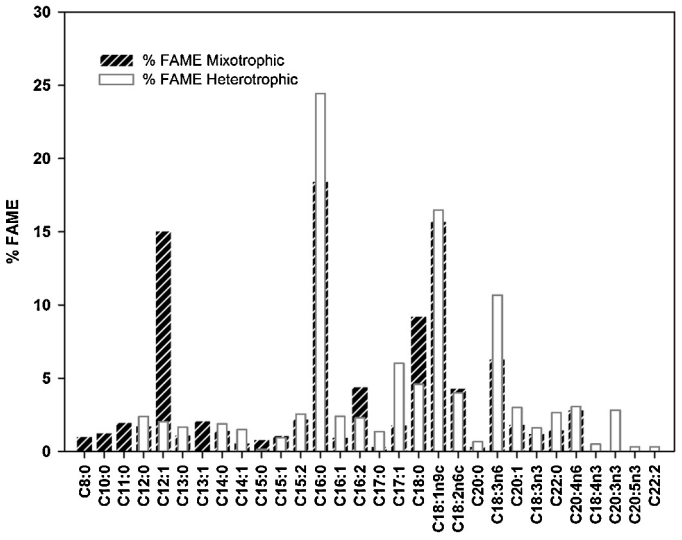

Fig. 5. Fatty acids profile of *Tetraselmis* sp. FTC 209 expressed in percent of total FAME.

this strain could also substantially produce lauroleic acid (C12:1) (15.1%). An unusual isoform, this uncommon MUFA is a natural metabolite of lauric acid commonly used for formulation of pharmaceutical preparations, cosmetics or personal care products. The occurrence of this unique short chain fatty acid in other *Tetraselmis* species has not been reported in literatures elsewhere.

In term of biofuel quality, the bio-oil from mixotrophic mode was nonetheless more suitable since the percentages of SFA and MUFA were much closer to 80% as opposed to heterotrophic at 71%. Higher saturation in carbon atoms is preferred for oxidative stability of biodiesel during storage since PUFA chains, i.e., esters of linoleic and linolenic acids are very much susceptible to auto-oxidation reaction with O_2 in ambient air. The composition of both γ-linolenic and linolenic acid for mixotrophic bio-oil were estimated at 7.5%, whereas it was 12.3% for the heterotrophic oil. As such, the heterotrophic bio-oil barely passed the 12% acceptance limit specified by the European Standard EN14214 (2004) for automotive fuels [46].

At this juncture, by considering the algal oil from mixotrophic culture as better candidate for possible biofuel application, additional empirical calculation of oil quality yielded a saponification value (SV) of 148.48. The high SV indicates that the oils in question are mostly normal triglycerides. On the other hand, iodine value (IV) is the crude measurement of the total unsaturation and all the existed double bonds whether in MUFA and PUFA chains. The theoretical IV was 104.38, which was well below the EN 14214 maximum limit of 120 g I_2/100 g oil. This safety ceiling is necessary by reason of heating oil with higher IV would initiate polymerization of glycerides, thus leading to formation of undesirable deposits, or deterioration in the case of lubricating oil [47]. Cetane number (CN) is a dimensionless descriptor of the ignition quality of diesel fuel, which is analogous to octane number for gasoline. It is widely considered as a prime indicator of biodiesel quality. The index relates to time delay in the auto-ignition of fuel following its introduction into combustion chamber. The shorter the time taken for ignition, the higher the CN would be. Most of biodiesels have CN values within high 40 s to lower 60 s. CN of this *Tetraselmis* oil was approximately 59.57. This index was reckoned as satisfactory since it has theoretically surpassed the widely accepted standards; ASTM D975 dictates that petroleum diesel should meet a minimum of 40, while ASTM D6751 with specific to biodiesel prescribes a higher acceptance at 47. Both EN 14214 and the Australian Standard 2000 are viewed as slightly stringent by imposing the minimum CN value at 51 [46].

4. Conclusions

The application of antibiotics and agriculture-grade fungicides is an inexpensive way to simultaneously eliminate bacterial and fungal contamination in *Tetraselmis* sp. FTC 209. Subsequently, phylogenetic analysis had identified the strain as highly similar to *T. striata*, while Baranyi–Roberts model was found to be sufficient for describing the microalga growth through statistical validation. This study highlighted the significance of cellular composition in developing a medium capable of supporting higher cell density via elemental balance technique. The strain affinity toward organic substrate in addition to light also helps to increase the small number of *Tetraselmis* species capable of metabolic switching, with good potential as lipid producers that may subsequently be used in biodiesel production.

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