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Generation of Biodiesel and Carotenoids from Rhodotorula Glutinis using Sweet Sorghum Juice

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Generation of biodiesel and carotenoids from *Rhodotorula glutinis* using sweet sorghum juice

By

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The growth of *Rhodotorula glutinis* in sweet sorghum juice in three levels of three factors of temperature, carbon to nitrogen ratio and pH was evaluated. Accompanying of this growth was the generation of lipids converted to fatty acid methyl ester (FAMEs) and carotenoids. The optimized condition for maximum biomass and carotenoid accumulation was determined to be at 25C, pH of 5.5 and carbon to nitrogen ratio of 10. This condition yielded 22.7 g/L biomass with specific growth rate of 0.213 hr^{-1}. At this condition the carotenoids generation was also maximum with 2.6 mg/gram biomass, comprising of torularhodin, beta-carotene and torulene. The accumulation of lipids following generation of biodiesel was highest at same temperature and pH but carbon to nitrogen ratio of 70, generating 96.3 mg of FAMEs/gram of biomass containing methyl ester of oleic acid, linoleic acid, palmitic acid, stearic acid and linolenic acid.

DEDICATION

This work is dedicated to my beloved husband-Emmanuel and children-Erastus and Elianna

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I am sincerely thankful to my major professor, Dr. Darrell Sparks, who gave me a chance to make myself worthwhile for the last two years. For his support, for his time and sticking with me until the end, I am in deep gratitude. To Dr. Ashli Brown-Johnson, for the advice and continuous belief in me. For showing me, how to reach my goal without too much drama, keeping my cool- thank you! To Dr. Hernandez, for giving me an opportunity to start all this continuing study, for keeping the doors opens not just for me but for the entire family.

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Lastly, to Almighty God-for always finding a way for me.

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CHAPTER I

INTRODUCTION

Biodiesel

Triacyclglycerols (TAG) are esters of fatty acids bound to glycerol which comprises microbial oil, vegetable oil and animal fats. TAG is transesterified by reacting with alcohol, usually methanol in the presence of a catalyst, a strong base or an acid to produce a glycerol and three molecules of alkyl esters. When this mixture of fatty acid methyl esters meets the strict specifications of ASTM D6751 it will be known as biodiesel. Biodiesel varies in fatty acids composition depending on the feedstock used, which can affect its characteristic and performance. The properties affected by fatty acids composition include cetane number, cold-flow, viscosity and oxidative stability [1]. These properties are dictated by the chain length and degree of unsaturation of the fatty acids composition. Biodiesel has properties very close to petroleum diesel, which makes it suitable to be used as a substitute. Its miscibility with petroleum diesel allows it to be mixed in any ratio. It is known as “clean diesel” because it is safe and biodegradable, and since it is partially combusted, it produces less air pollutants than petroleum diesel. Additionally, it offers several advantages compensating its higher price than petroleum diesel, it has higher flash point, which is advantageous for safe handling and storage and has excellent lubricity.

The enormous demand of fuel source has leads to great interest for alternative sources for fuel production from lignocellulosic biomass (grasses and trees) and municipal waste. Additional sources are derived from animal and plant oils or the microbial oil produced by algae, certain bacteria and yeast. These alternative sources provide a significant increase in energy and economic security as well as positive environmental impacts. As shown in the projection in 2011 Annual Energy Outlook (Figure 1), the dependence of the United States on foreign liquid fuel significantly decreases because of the increase in domestic production of liquid fuels including biofuels. By 2035, it is predicted to further decrease because of wide interest to alternative sources. Figure 2 presents the growth of the biofuels production from 2001 to 2011 proving the improvement over the last ten years.

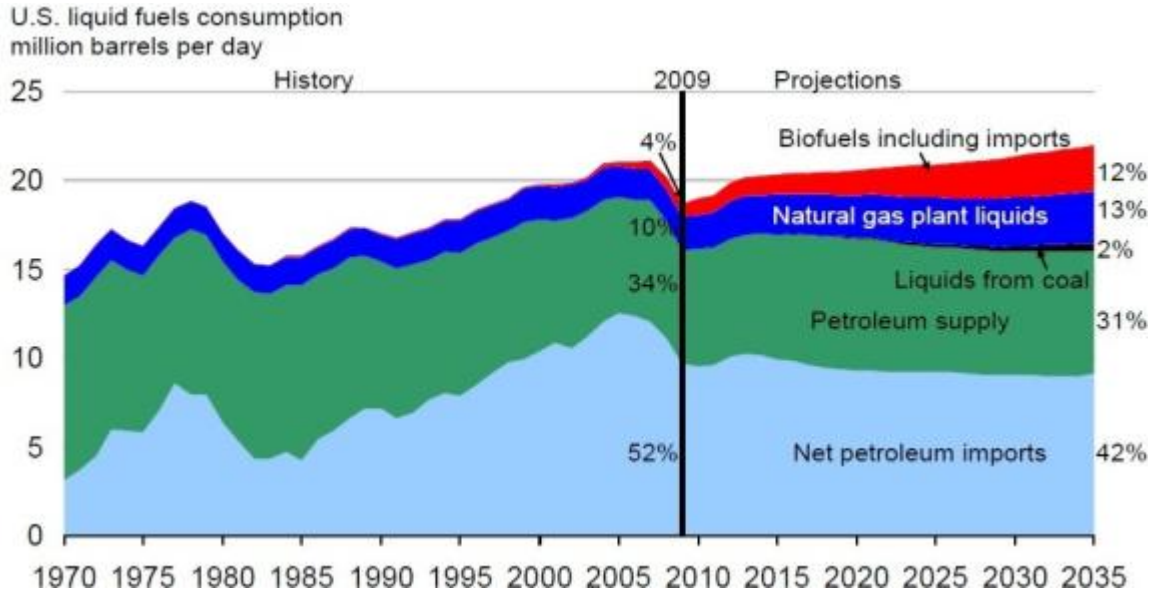


Figure 1 US fuel consumption sources from 1970 to 2009 including the prediction until 2035.

(http://www.eia.gov/neic/speeches/newell_12162010.pdf)

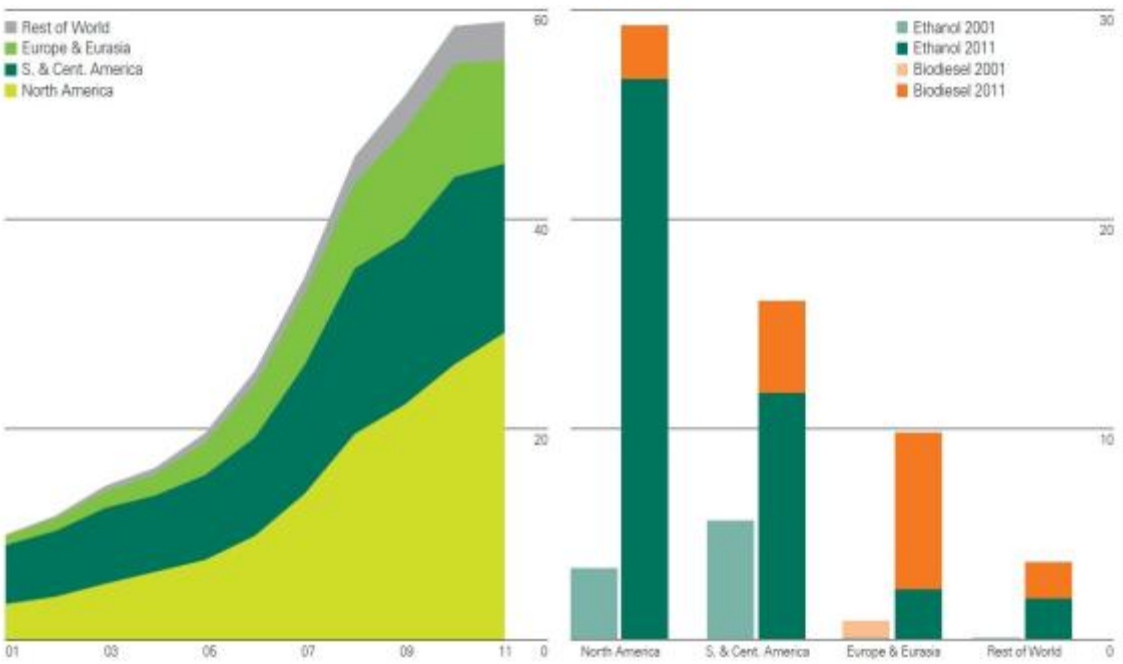


Figure 2 World biofuel production from 2001 to 2011

(BP Statistical Review of World Energy, June 2012)

Carotenoids

Carotenoids are yellow to red pigments of aliphatic or alicyclic structure, composed of isoprenoids joined in such a manner that the isoprenoid units are reversed at the center of the molecule so that the two methyl groups are in a 1,5 positional relationship and the remaining nonterminal methyl groups are in a 1,6 positional relationship [2, 3] (See Figure 3). There are over 600 carotenoids fully characterized which function mainly as natural antioxidants but are widely used in industrial applications as pigments and nutritional supplements. For this reason, there is wide interest for carotenoid production for commercial use. Carotenoids are widely distributed in nature, mostly plants that exhibit coloration from yellow to red. But these sources

offers disadvantages due to seasonal and geographic variability and one of the alternative sources is microorganisms like bacteria, fungi, yeast and algae.

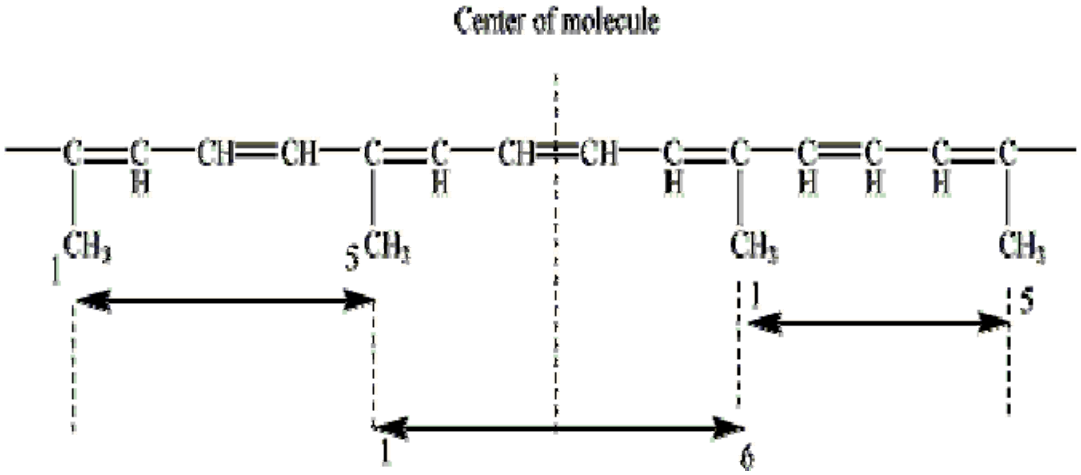


Figure 3 Arrangement of methyl groups in isoprenoid units

(Goodwin, 1954)

Rhodotorula glutinis

Rhodotorula glutinis is capable of producing lipid fractions of triglycerides and carotenoids. This organism stores excess carbon as triglycerides and produce carotenoids to protect them from light and oxygen. At different stages of growth and cultural conditions, the lipid amount varies [4] and carotenoids are produced, usually β-carotene, γ-carotene, torulene and torularhodin. It has capability to adapt in the environment's changes and grow at wide range of pH but has maximum growth at pH 5.5. Presented in Figure 4 is the electron micrograph of a resting cell showing the lipids in the cytoplasm and the *Rhodotorula glutinis* grown in sweet sorghum juice.

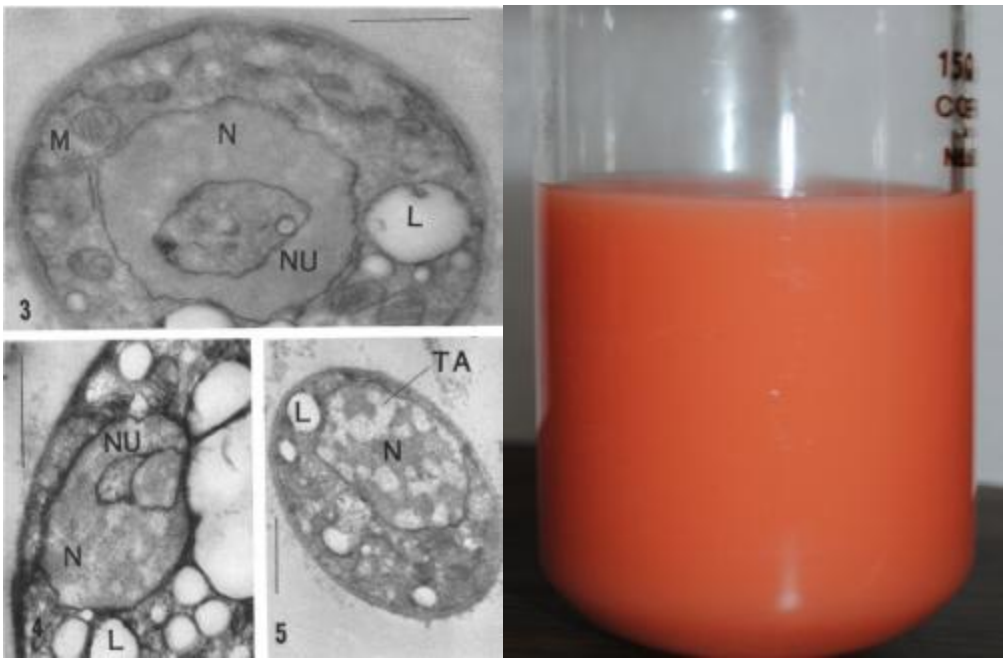


Figure 4 (Left) Electron micrograph of resting cell of *R. glutinis* (Thyagarajan, 1962), (Right) *R. glutinis* in culture medium containing sweet sorghum juice

Sweet Sorghum

Sweet sorghum [*Sorghum bicolor* (L) Moench] is a graminaceous crop providing both stalk and grains for food. The high sugar content of its stalk makes it comparable to sugarcane; hence, it is a promising alternative for bioethanol production. It can also be used as fodder, and in India- as bedding, roofing, fencing and paper. The major advantage of this crop over energy crops widely grown, like sugarcane and corn, is in how it cultivated. It is drought tolerant and requires a minimum amount of nitrogen during cultivation. Additionally, it was reported that the level of nitrogen for growth isn't significant on the sugar content of the plant [5], and excessive amount of nitrogen can reduce the quality of the juice [6]. The time to achieve full crop growth is also shorter,

allowing the use of land more efficiently. The crop duration for sweet sorghum is 4 months versus the time needed to cultivate sugarcane which is as long as 12 months [7]. Furthermore, sweet sorghum is more resilient compared to sugarcane with respect to abiotic stress [8].

The sugar composition of sweet sorghum depends primarily on variety and location but all contain three sugars: fructose, glucose and sucrose. Sucrose being the major sugar with varying content from 46 %- 91 % [9].

The United States is the largest producer of sweet sorghum juice. Several studies have been done to develop varieties of sweet sorghum to improve production and resistance to abiotic stress. In the southern part of the country, where the weather is relatively warm, which is favorable for the cultivation of sweet sorghum, different varieties were developed as early as the 1960's; the recommended varieties are Dale, M81E, Brandes and Theis. The M81E variety (Figure 5) was released from the U. S. Sugar Crops Field Station in Meridian, Mississippi in 1981. It is the best variety with respect to the gross yield of stalks, sugar and fermentable sugar per acre. Furthermore, it was developed to be resistant to anthracose and red stalk rot, which are common diseases of sweet sorghum [10].

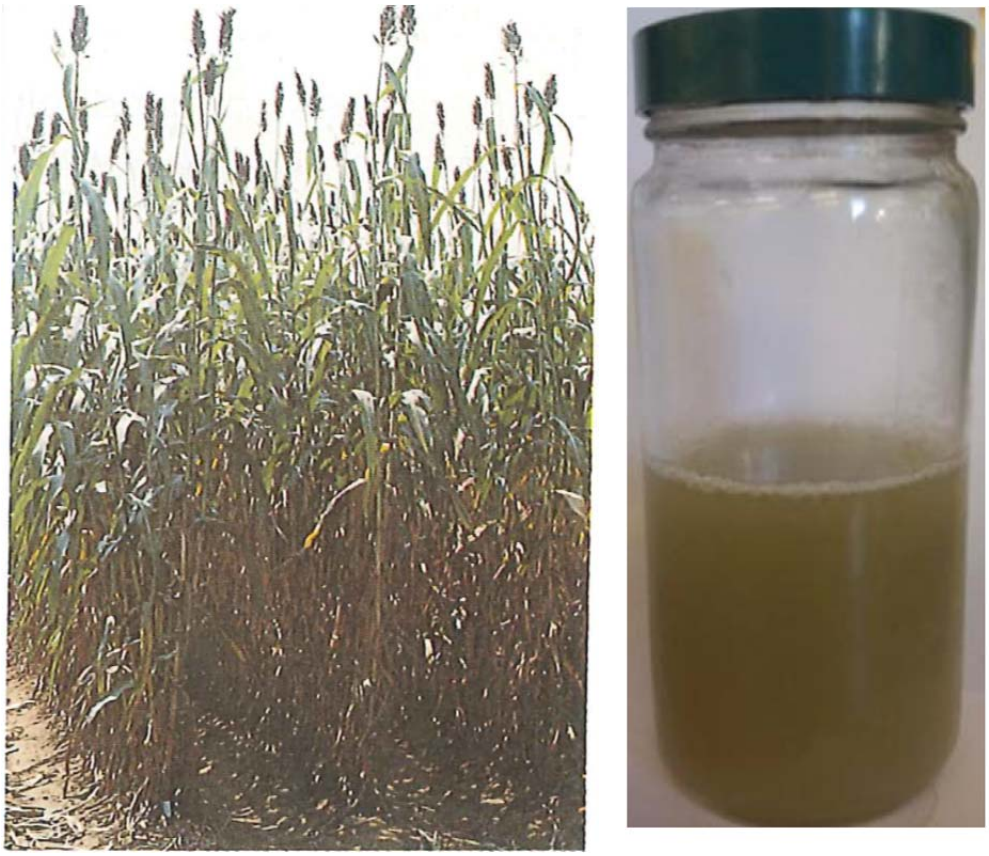


Figure 5 Typical M81E sweet sorghum plant (left) and filtered juice (right) (http://msucares.com/crops/sorghum/m81e_description.pdf)

CHAPTER II

REVIEW OF LITERATURE

Rhodotorula glutinis Cultivation

Rhodotorula glutinis is one of the yeast commonly used for carotenoids production because of its unicellular nature and high growth rate yield using a wide range of carbon and nitrogen sources for its cultivation making it possible for convenient large-scale fermentation. This microorganism can assimilate various simple sugars, (pentoses and hexoses) and even some disaccharides. Among the simple sugars, galactose, although seldomly used, gave the highest cell growth yield compare with glucose and fructose [11]. Furthermore, some pentoses can also be used as a substrate; however, the yield is significantly lower than the hexoses. Sucrose and maltose can also be employed as a carbon source but maltose resulted to half as much as sucrose cell growth yield but lactose has unable to support the growth of this microorganism [11]. This capability permitted several researchers to use cheaper carbon sources from different agro-industrial origin such as sugarcane juice, whey ultrafiltrate, grape must, sugarcane molasses, sweet potato extract, fermented radish brine, enriched rice bran by solid state fermentation [12-20] and activated sludge. Several nitrogen sources can also be employed for the cultivation of *R. glutinis*, from inorganic to organic nitrogen and hydrolyzed mung bean waste flour, the organic nitrogen sources such as yeast appears to be favorable for high biomass yield [17, 21]. The favorable growth conditions for several factors has been

reported by several researches: the low carbon to nitrogen ratio favors the growth of *R. glutinis*[21], for the optimum temperature, it ranges from 25-30°C, which gave the highest maximum specific growth rate and sugar utilization rate [11, 17, 22] and the pH is optimum at 5.5-6 [11, 17]. Furthermore, the growth of the *Rhodotorula glutinis* was observed to grow faster with irradiation of LED lamps [23] which probably due to faster transport of nutrients.

Lipid Accumulation

Some microorganism can produce lipids as much as 70% of its cell biomass and these microorganisms are called oleaginous yeasts [24]. Microbial lipid accumulation has been investigated for over a century as a source of oils and fats. Although the oil accumulated from yeast and fungi are the same as plant fats and oils, it has advantages over this source; these oils are not use for human consumption, which is the primary concern with plant oils. Additionally, microorganisms are not affected by seasonal changes [25], and can assimilate several sources of carbon making culturing more economically feasible [26]. Figure 6 describes the typical profile of the lipid accumulation, where the lipid accumulation starts when the all the nitrogen has been utilized and there is still carbon available for the microbial consumption. But often times the supplication of nitrogen is necessary for initial growth of the microorganism which is typically with carbon to nitrogen ratio of 40 to 50:1 [26]. The exhaustion of the nitrogen supply while there is still carbon available is the determining step of lipid synthesis accumulating triacyclglycerols as oil droplets. Ratledge hypothesized that the limitation of nitrogen favors increase the AMP deaminase resulting to a depletion of cellular AMP concentration. The decrease of AMP cellular concentration leads to the decrease in

activity of isocitrate dehrydrogenase involve in citric acid cycle in the mitochondrion. Thus, the backward reaction of isocitrate to citrate is highly favorable. The high concentration of citrate concentration in mitochondrion allows the transfer of citrate to cytosol where ATP:citrate lyase converts the citrate to acetyl CoA. Continuous supply of acetyl-CoA is used for fatty acid synthesis [24].

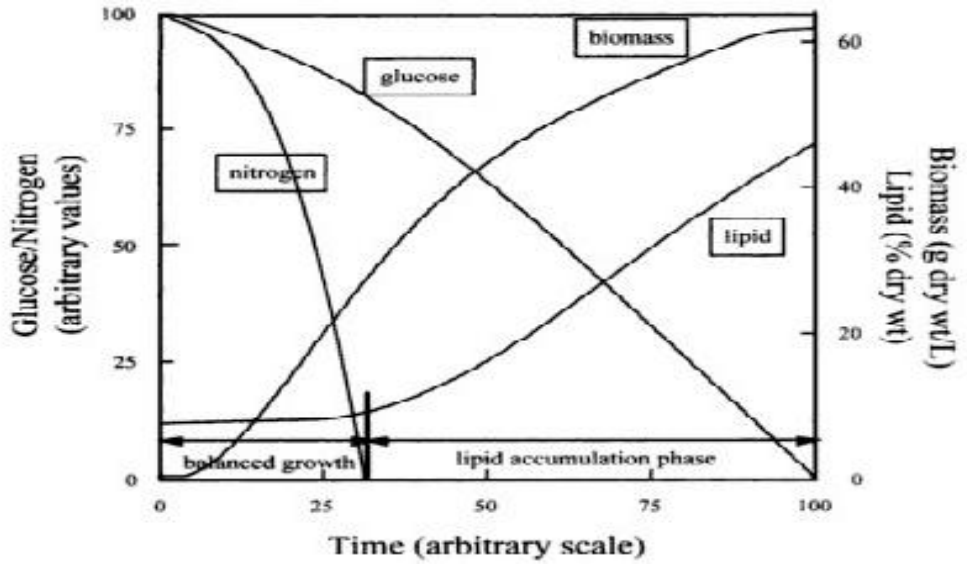


Figure 6 Ideal relationship of lipid accumulation in an oleaginous microorganism (Cohen and Ratledge, 2005)

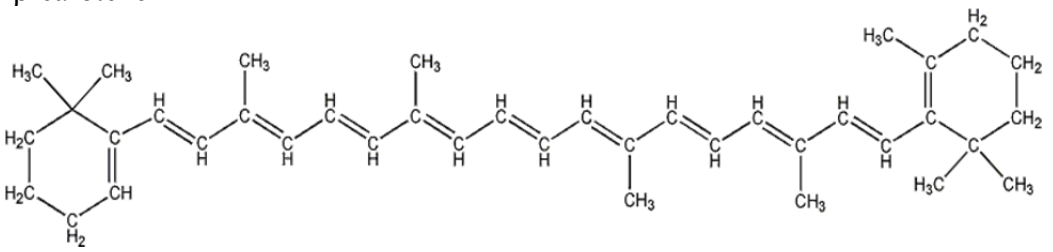
Rhodotorula glutinis is one of the known oleaginous yeast cultivated to produce lipids. Several studies have been done reporting the accumulation of lipids in different strain of *Rhodotorula* using pure glucose [27, 28], cheap feedstock like sugar cane molasses [29], lignocellulosic material hydrolysate such as wheat straw [30] and even wastewater [31]. The accumulated lipids range from 15-40% of the dried biomass depending on the dilution rate in continuous culture. These lipids upon transesterification produce fatty acid methyl esters (FAMEs) which are mostly palmitic acid methyl ester

(C16:0), stearic acid methyl ester (C18:0), oleic acid methyl ester (C18:1) and linoleic acid methyl ester (C18:2) [27, 28]. Several factors dictate the accumulation and composition of lipids from *R. glutinis,* such as, the effect of irradiation of LED lamp in cultivation resulted in faster growth but the lipid accumulation was lower than without the presence of the light [23]. The unsaturation of the lipids is different at lower temperature because the lipid unsaturation is affected by the response of the microorganism to the decrease functionality of the biological membranes[32].

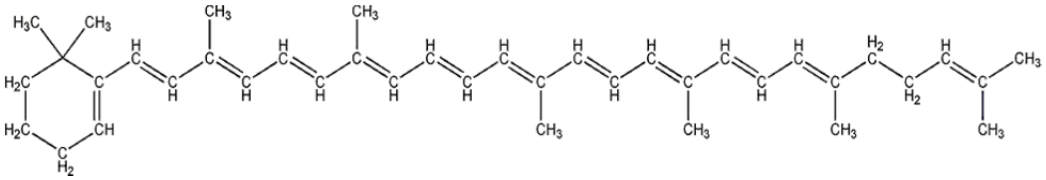
Carotenogenesis

Carotenoid production from microorganism has been an interest for more than 50 years. Several microorganisms, particularly some classes of fungi have been reported for the presence of significant amount of carotenoids. A few red yeast species of *Rhodotorula, Rhodosporidium, Sporidiobolus, Sporobolomyces, Cystofilobasidium, Kockovaella* and *Phaffia* were found to contain carotenoids [33]. The mechanisms of carotenogenesis of these microorganism are known to depend on the biochemical makeup of the microorganism's environment conditions and cultural stress applied during cultivation [34], therefore the carotenoids composition can be possibly manipulated. One of the most studied species is *Rhodotorula glutinis*; it is reported to contain four major carotenes such as β-carotene, γ- carotene, torulene and torularhodin [35]. The structures of these are shown in Figure 7.

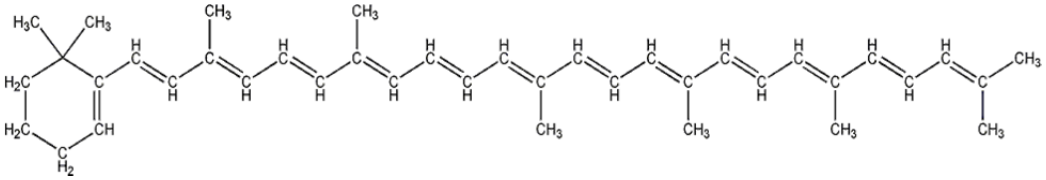
a. β-carotene



b. γ-carotene



c. torulene



d. torularhodin

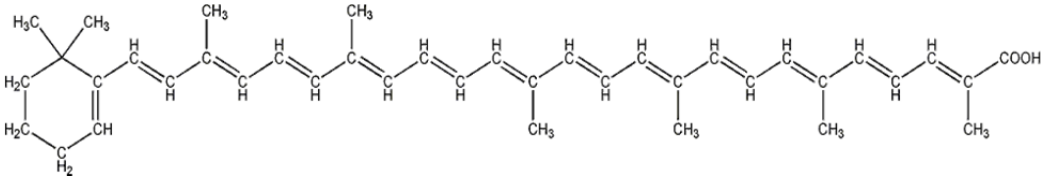


Figure 7 Structures of four major carotenoids extracted from *Rhodotorula glutinis*

Of all these carotenoids, β-carotene is the most important carotenoid economically. It is known to have many uses including as an antioxidant and a vitamin A precursor. It was speculated that due to its antioxidant activity, it can reduce the risk of diseases especially of certain cancers but the studies have been inconsistent. Several studies have shown β-carotene as a chemoprotective agent in fruits in vegetables. Out of

15 epidemiologic studies conducted, 11 showed a significant effect of β-carotene intake in lowering the risk of lung cancer but based on two interventional trials done by taking high doses of β-carotene as dietary supplement, there were no effect observed on the nonsmokers and may in fact cause a harm to the smokers [36] This was hypothesized to be caused by the asymmetric cleavage of β-carotene which lessen the level of retinoic acid leading to a higher level of cell proliferation in smokers. Aside from its value to human health, it is also widely used in industrial applications in food, beverages, chemical, pharmaceutical, agriculture and environment not only because of its antioxidant activity but as food coloring agent. It comprises more than 20% of the total carotenoids available in the market and in 2018 has a predicted market value of $334 million which was recorded to have market value of 261 million in 2010 [37]. About 90% of of β-carotene available in the market is produced through chemical synthesis by β-ionone, but the chemical synthesis is being regulated because of the possibility of causing harmful effects to human body.

Another major product is torularhodin. A group of researchers had conducted a study about its scavenging activity towards peroxyl radicals, which cause the lipid oxidation and eventually leads to the damage of cell membrane. They reported that torularhodin is consumed first before β-carotene when peroxyl-producing radicals agent was added [38].

Since animals cannot synthesize these carotenoids and humans acquire them through diet, the production is really of great interest to researchers particularly through utilizing of microbial sources which offer great advantage and one of widely-studied microorganism is *Rhodotorula glutinis.*

As mentioned, the composition of the carotenoids produced in the biosynthetic pathway depends entirely on the microorganism species, growth medium composition and cultivation conditions. Several species of *Rhodotorula* accumulate different carotenoid composition as when grown in same cultivation medium [39]. Generally, the growth conditions and medium composition were varied upon screening of best microorganism.

The production of carotenoids using *Rhodotorula glutinis* employing different cultivation conditions have been exploited by several researchers for more than a decade. One of the factors investigated was the cultivation temperature; based on the reports, at lower temperature, 5°C, the composition of the carotenoids obtained was predominantly β-carotene [15, 22, 35, 40, 41]. The significant increase in β- carotene ratio leading to decrease in torulene and torularhodin was due to the temperature dependence of the enzymes responsible for the synthesis of these carotenoids. The β-carotene synthetase has higher concentration at lower temperature as opposed to the torulene synthetase which is active at higher temperature, leading to the blocking of torulene and torularhodin production in carotenoids biosynthesis pathway by favoring the dehydration of γ-carotene to β-carotene as shown in Figure 8, which is the determining step for the production of either β-carotene or torulene and eventually torularhodin [40, 42, 43]. One of the reports also noted that although the maximum biomass yield was obtained at 30°C, the carotenoid production per gram of dried sample was higher at 25°C [41], as opposed to the report of Simpson, et al. [40] that the carotenoid accumulation per gram of cell dried mass was the same at two different level of temperature. Another factor is the pH of the cultivation medium, the optimum pH for the growth of *R. glutinis* ranges from 5.5-6.5,

carotenoid production is highest at around this pH range, when the pH is lower and higher than this range, it was observed that there was a decrease in total carotenoid production [41]. The carbon to nitrogen ratio was found to have no effect on cellular pigmentation but affected the biomass production of the *R. glutinis* which leads to more carotenoids synthesis. A carbon to nitrogen ration of 70 has lower volumetric production of carotenoids compared with the carbon to nitrogen of 10 since the latter produces for biomass [21]. The aeration rate of the cultivation also affects the composition of the carotenoids. It was reported that the application of high aeration rate lead to the highest total carotenoid production [18, 28, 41].

The medium composition is also a great factor in carotenogenesis. One of these factors is the carbon source. The consumption of glucose leads to production of more β-carotene while, fructose and sucrose produces more torulene [21]. Several studies have been reported using agro- industrial carbon sources to grow *Rhodotorula glutinis*, sugar cane juice, whey ultrafiltrate, grape must, sugarcane molasses, sweet potato extract, fermented radish brine even using and enriched rice bran by solid state fermentation [12-20]. Squina, et al [13] used sugarcane as a carbon source and produced more torulene than β-carotene when the sugar concentration was lower and used peptone and yeast extract as the nitrogen source, but torularhodin and β-carotene were not significantly affected by growth medium composition. Whey ultrafiltrate was co-cultivated with lactic acid bacteria such L*actobacillus helveticus* which resulted in parallel growth of the carotenoids and cell mass, and the torularhodin was the highest carotenoid obtained and total carotenoids was maximum at the early stage of stationary phase [14]. This co-cultivation was necessary since *R. glutinis* cannot assimilate lactose which is the main

sugars found in whey ultrafiltrate. Several raw materials such as grape must, glucose syrup, beet molasses soy bean flour extract and maize flour extract were used as the carbohydrate source for one specific strain of *R. glutinis*. The use of different carbohydrate sources for cultivation significantly impacted the extracted amount of total carotenoids and composition of individual carotenoids, which was predominantly torularhodin but the carbohydrates were analyzed by total carbon and not individual sugars contained in the raw materials [15].

In terms of the effect of the nitrogen source, hydrolysed nitrogen sources produced more β-carotene than inorganic sources, while ammonium nitrate resulted in more carotenoids than ammonium sulfate and ammonium chloride [21]. Additionally, the use of hydrolyzed mung bean waste flour was reported to be a good source of nitrogen in combination with sweet potato extract [17].

Aside from the nitrogen sources, the addition of an inhibitor, such as light exposure and solvents like alcohol were also reported to affect the total carotenoids accumulation and the individual carotenoids composition suggesting that the cultivation medium was truly complex and not fully established on what are really the favorable growth nutrients needed to fully optimize carotenoid accumulation. Matelli also reported that re-suspension of the biomass in distilled water for 24 hours yielded a higher accumulation of β-carotene of as much as 2.5 mg/g biomass, which was 150% more compared with those kept in the cultivation medium for the same amount of time [12].

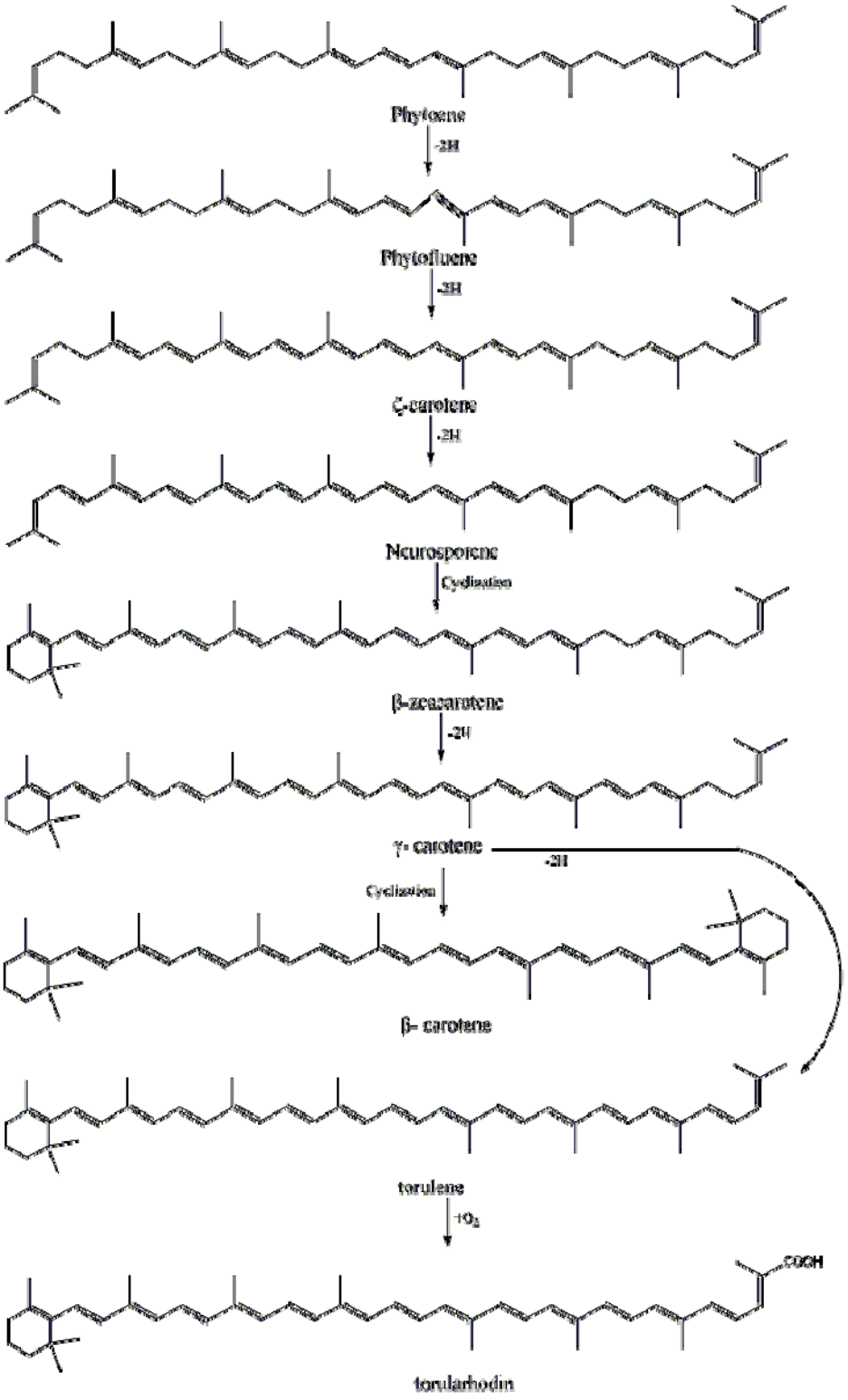


Figure 8 Biosynthetic pathway of carotenogenesis

(Simpson, 1954)

Aside from employing different growth media and modifying cultural conditions, mutagenesis can also alter the behavior of microorganism. Treatment of *R. glutinis* with high hydrostatic pressure doubled the β-carotene content to more than twice as much as the regular strain [44]. Stress factors can be applied to microorganism to produce certain carotenoids. The wide research on the proteomics involved in the biosynthetic pathway of carotenogenesis makes it possible to manipulate the desired carotenes accumulated. Several studies have been conducted to characterize the genes and proteins involved in the biosynthesis pathway of formation of important carotenoids in conjunction with genetic and metabolic engineering to improve the productivity and even the selectivity of the pathway to produce the desired carotenoids formed [34]. These manipulations are possible since all carotenoids are synthesized from formation of isopentenyl pyrophosphate to the formation of the first colorless carotenoids, phytoene. There are several biosynthetic pathways proposed leading to the formation of different acyclic and cyclic carotenoids, the biosynthetic pathway illustrated in Figure 8 was the biosynthetic pathway proposed by Simpson et al in 1968, leading to formation of β-carotene, α-carotene, torulene and eventually torularhodin, which are major carotenoids accumulated in cultivation of *R. glutinis.*

CHAPTER III

RESEARCH OBJECTIVES

The primary objective of this study was to evaluate the growth of *Rhodotorula glutinis* supplying sweet sorghum juice as primary carbon source which contains three sugars: fructose, glucose and sucrose. This objective was met by varying three level-three factors of temperature, pH and carbon to nitrogen ratio. The evaluation includes:

(1) determination of the cultural conditions favorable for growth of *Rhodotorula glutinis* yielding the maximum carotenoid and biodiesel with respect to the three factors of temperature, pH and carbon to nitrogen ratio.

(2) estimation of the optimization parameters of the three factors using general factorial design by ADX interface of SAS® software.

(3) evaluation of the effects of each factor as well as factor interactions towards growth and products accumulation.

CHAPTER IV

MATERIALS AND METHODS

Reagents and Chemicals

All reagents and standards were purchased from Sigma-Aldrich (Sigma–Aldrich, St. Louis, MO) and all the organic solvents were purchased from Fisher Scientific (Fisher, Scientific, Pittsburg, PA). All chemicals were used as received without further purification.

Sweet Sorghum Juice

The sweet sorghum juice was obtained from Alternative Crops Research in Mississippi State University (Mississippi State, MS). The raw extracted juice was filtered filter paper Whatman No. 4 to eliminate the coarse particles.

Rhodotorula glutinis

Rhodotorula glutinis was from American Type Culture Collection (Manassas, VA) and kept in glycerol at -80°C.

Rhodotorula glutinis Cultivation

Optimization of Three-Level Factors

A 1mL of *Rhodotorula glutinis* in glycerol was precultured in 50 mL medium prepared by mixing 10.0 g/L of glucose, 5.0 g/L of peptone and 3.0 g/L of yeast extract with pH adjusted to 5.5 for 24 hours. An aliquot of 2 % of second preculture inoculum

was used for growing of microorganism containing 50.0g/L of total sugars from sweet sorghum juice, previously analyzed using liquid chromatography (method discussed below) containing 17 % total sugars (13 % fructose, 22 % glucose and 65 sucrose) with ammonium sulfate (depending on Carbon to Nitrogen ratio of 10:1, 40:1 or 70:1 by mass), 0.5 g/L of magnesium sulfate, 5.5 g/L of dibasic potassium phosphate, 3.7 g/L of monosodium phosphate and 1.0 g/L yeast extract.

The yeast was cultivated in Sartorius Stedim BioStat® Bplus bioreactors (Sartorius, Bohemia, New York) each being equipped with a 2.0 L jacketed uni-vessel. The pH was adjusted to 2.5, 5.5 and 8.5 initially and was monitored but not controlled during experimentation. Additionally, pO_2 was maintained at 60 %. The microorganism was grown and maintained at different temperature: 20°C, 25° and 35°C. The foaming was prevented by addition of nonoil, polypropylene-based Antifoam 204 concentrate (Sigma–Aldrich, St. Louis, MO). The cultivation was done in seven days.

Effect of Partial Pressure of Oxygen

The optimized condition was applied to the same cultivation conditions and medium mentioned above with pO_2 setting of maintained at 40 %, 80 % and 100 %.

Pretreatment of Cultivation Medium

The 2 x 10 mL collected media were centrifuged using Sorvall RC-5B at 5000 rpm for 20 minutes, the supernatant was analyzed for sugar content and the biomass was washed with deionized water, centrifuged again and freeze-dried using a Labconco Freeze Drier (Labconco, Kansas City, MO). Afterwards, the samples were weighed to

determine cell mass present. Separate samples of the cell cultures were collected for carotenoid and fatty acids extractions.

Sugar Analysis

The supernatant was filtered using a 0.45µm Nylon syringe filter and analyzed using an Agilent 1100 liquid chromatography (Agilent Technologies, Santa Clara, CA) equipped with a Varian (Agilent) 385-LC evaporative light scattering detector (Agilent Technologies, Santa Clara, CA). The 2 µL of sugar samples were eluted on Phenomenex Luna® 250 x 4.6 mm 100 Å column (Phenomenex, Torrance, CA) using acetonitrile: water (6:4) with flowrate of 0.7 milliliters per minute. The concentrations of individual sugars were calculated based on the standard curve obtained from different concentrations ranging from 2.5 – 25.0 mg/L of the three sugars standards (glucose, fructose and sucrose)

Nitrogen (as Ammonium) Analysis

The supernatant was passed through a 1 mL-C18 Sep Pak® cartridge filter followed by a 0.45 µm nylon filter and analyzed using a Dionex ICS-3000 ion chromatograph (Dionex, Sunnyvale, CA) equipped with 5×250 mm IonPacCS16 column with a 5×50 mm IonPacCG16 guard column. The sample injection volume was 25 µL was eluted with 36 mmol/L of KOH and operated at a flow rate of 1 mL/min at 30 °C. The concentration of nitrogen was calculated based on the standard curve obtained from different concentrations ranging from 0.08- 50 mg/L of nitrogen (as ammonium) standard.

Carotenoid Extraction and Determination

A quantity of 100 mg of dried biomass was extracted using the solvent used by Park, et al [45], 4.0 mL dimethyl sulfoxide, 4.0 mL acetone, 2.0 mL petroleum ether and 2.0 mL saturated sodium chloride. The ether layer was separated and allowed to evaporate using a turbovap LV with nitrogen supply. The extracted carotenoids were dissolved in mobile phase consisting of ethyl acetate:2-propanol:acetonitrile (20:40:40) and quantified using Agilent 1100 liquid chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector using 450 nm wavelength UV light. The carotenoids were separated using a Restek® Ultra C18 250 x 4.6 mm 100 Å column (Restek, Bellefonte, PA). The concentration of carotenoids were calculated using the calibration curve obtained from different concentration ranging from 0.001 to 0.01 mg/ml of the β-carotene standard (Sigma–Aldrich, St. Louis, MO) and the remaining two carotenoids concentration were calculated with the reference to the response factor obtained from β-carotene.

Fatty Acid Methyl Esters Derivation and Analysis

A separate portion of 100 mg dried biomass was extracted using modified Bligh Dyer [46] extraction method. The chloroform layer was allowed to evaporate at 50°C using Turbovap LV with nitrogen supply. The Bligh Dyer extract was reacted with 2% H_2SO_4 in methanol for two hours at 60°C. The solution was cooled and neutralized with a solution containing 3 % (w/v) $NaHCO_3$ and 5 % (w/v) NaCl. The fatty acid methyl esters (FAMEs) generated were extracted with hexane and then the hexane was evaporated again using Turbovap LV, the FAMEs extracted were re-dissolved in toluene containing 200 ppm of 1,3 dichlorobenzene as internal standard and 100 ppm of

butylhydroxytoluene as an antioxidant. Two microliters of rediluted FAMEs in toluene were injected at 260°C in splitless mode into an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a 30 m x 0.25 mm Restek Stabilwax® DA capillary column (Restek, Bellefonte, PA) and detected using flame ionization detector. The concentration of the FAMEs was calculated using the calibration curve obtained from several concentration of FAMEs mixture containing C8:0-C24:0 methyl esters (Sigma–Aldrich, St. Louis, MO).

Statistical Analysis

For all the statistical analysis the software used was SAS®. The general factorial design was employed using the ADX interface for the optimization analysis and General Linear Model for comparison of data.

CHAPTER V

RESULTS AND DISCUSSION

Rhodotorula glutinis Cultivation

The cultivation of *Rhodotorula glutinis* using sweet sorghum juice as a carbon source was investigated with respect to different cultivation temperature, initial pH and carbon to nitrogen ration by mass with supply of 60% aeration. Using the logistic model proposed by Weiss and Ollis [47, 48]

$$\frac{dX}{dt} = \mu_{\max} X(t)\left(1 - \frac{X(t)}{X_{max}}\right) \tag{1}$$

$$X(t) = \frac{X_o e^{\mu_{max} t}}{\left[1 - \left(\frac{X_o}{X_{max}}\right)\left(1 - e^{\mu_{max} t}\right)\right]} \tag{2}$$

where *dX/dt* is the rate of biomass growth, μ_{max} is the maximum specific growth rate, *X(t)* is the concentrations of non-lipid biomass at any time t, X_{max} corresponds to maximum biomass reached in a run and X_0 is the initial non lipid biomass. The logistic model was found suitable for the kinetic growth of biomass with R^2 of more than 0.9.

The specific growth rate (μ_{max}) of and maximum biomass (X_{max}) of *R. glutinis* was calculated at all combinations of the three factors of temperature, initial pH and carbon to nitrogen ratio (Table 4, Appendix A).

Using the ADX interface of SAS® software, the quadratic response plot of the maximum biomass using the main and interactive effect of the three factors, temperature, pH and carbon to nitrogen ratio was determined using the model given by:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j} \sum \beta_{ij} X_i X_j + \varepsilon \quad (3)$$

where k is the number of factors, β_0 is the constant term, β_1 is the linear coefficient of factor *i* and β_{ii} is the quadratic coefficient of the factor *i* and β_{ij} is the interactive effect coefficient for factor *i* and factor *j*.

Using the model in Equation (3), the coded model for the maximum biomass using with k = 3, which were the temperature (*t*), pH (*h*) and carbon to nitrogen ratio (*c*) was given by

$$\textbf{Maximum Biomass} = 20.53391 - (2.794395t + 0.586484c + 0.900798h) + 2.534185ch - (3.668079t^2 + 4.902211h^2) \quad (4)$$

which was plotted in Figure 9. This means that the maximum biomass has a constant term of 20.335 g/L of non-lipid biomass and all three factors have negative linear influence to the non-lipid biomass. Furthermore, the quadratic interactions of pH and temperature have negative effect to the response but the interaction between carbon to nitrogen ratio and pH positively affect the non-lipid biomass. And the statistical analysis also showed that the interaction between these three factors does not significantly affect the biomass accumulation.

The optimized condition obtained of these three factors are: temperature at 25°C, pH of 5.5 and C:N ratio of 10. Using the least square means analysis with respect to the maximum biomass and specific growth rate (μ_{max}) as shown in Table 1, the optimized

condition was compared to other four three-factor combinations yielding high biomass. Although the maximum non-lipid biomass obtained from the optimized condition is not significantly different with other three-factor combinations, the specific growth rate (μ_{max}) was significantly different with the rest of the three factor combinations. This concludes that this condition is the most favorable for *R. glutinis.*

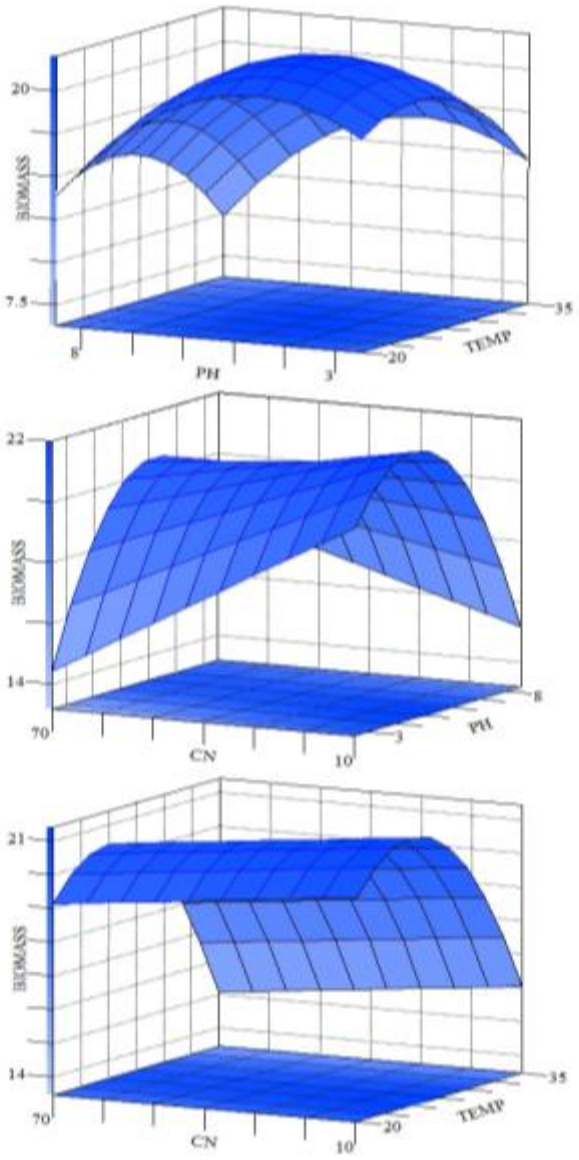


Figure 9 Surface response plot of non-lipid biomass at optimized condition of three factors

Table 1 Specific growth rate and maximum biomass comparison of the high yielding non-lipid biomass using logistic model

Temperature °C	C:N	pH	Maximum Biomass (g/L)	Specific growth rate ($hour^{-1}$)
25	10	5.5	22.6913[a]	0.2131[a]
20	10	5.5	22.2855[a]	0.0880[b]
20	40	8.5	20.1013[b]	0.1332[c]
20	40	5.5	19.5040[b]	0.1330[c]
20	10	2.5	19.2759[b]	0.0490[d]

Note: Same letter means not significantly different

Although the factor interaction effect is observed in biomass accumulation, the general main effects of the factors was observed clearly with respect to maximum biomass obtained and specific growth rate, which is presented in Figure 10. The main factors were evaluated using the most favorable condition from least mean square analysis obtained from Table 5 of Appendix A. The effect of the carbon nitrogen ratio on non-lipid biomass at three levels was not significantly different although the biomass obtained decreases as the nitrogen amount decreases. Both pH and temperature greatly affect the non-lipid biomass, although the highest level at 35°C showed the lowest growth of microorganism, this temperature level achieved the fastest stationary phase at its most favorable pH and C:N ratio condition, the difference of the calculated maximum biomass at 20°C and 25°C was negligible but the stationary phase was achieved at faster rate with 25°C than the lower temperature. For the three pH level, generally at pH 5.5 the growth is favorable for the microorganism.

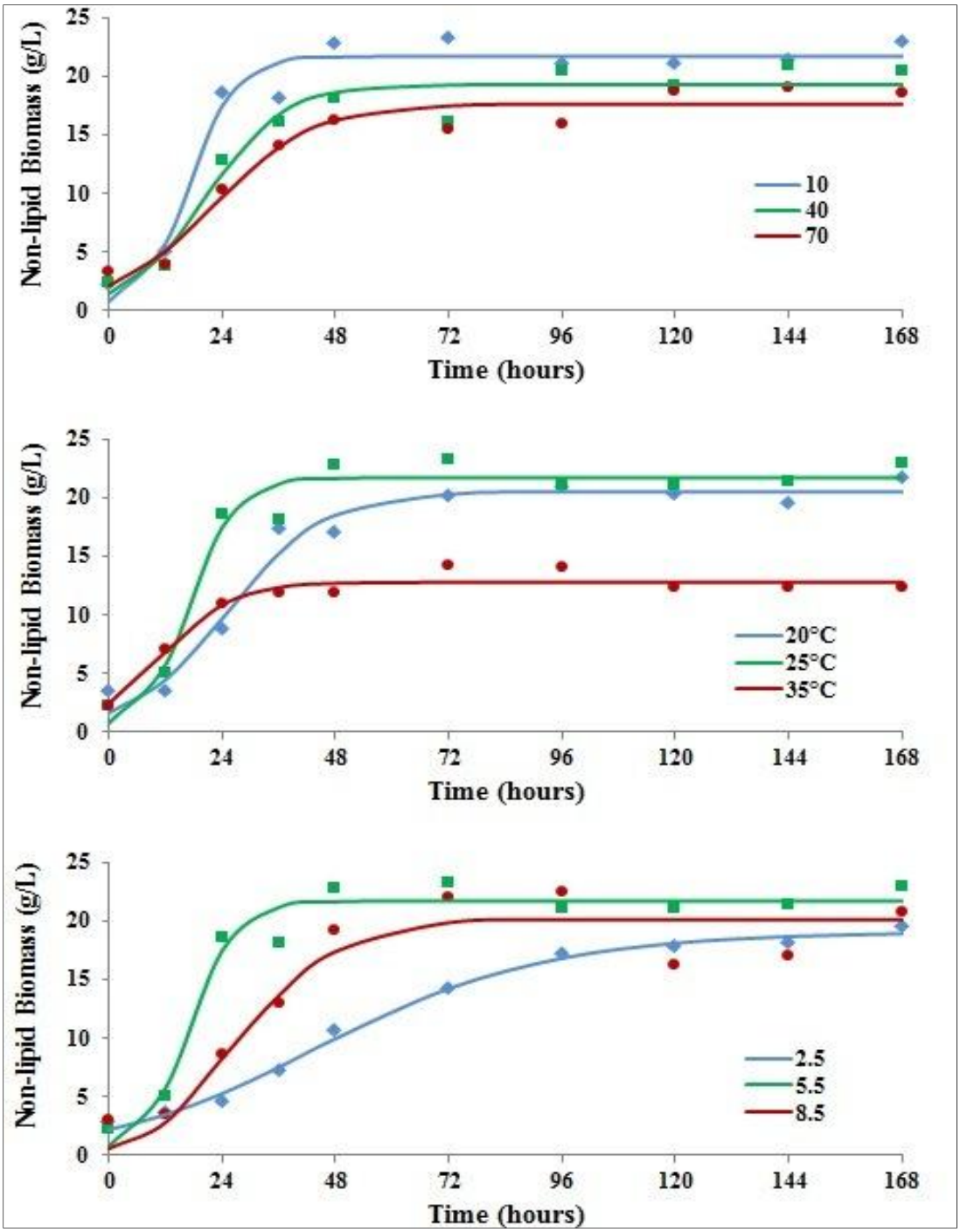


Figure 10 Typical effect of carbon to nitrogen ratio (top) temperature (middle) and pH (bottom) on non-lipid biomass

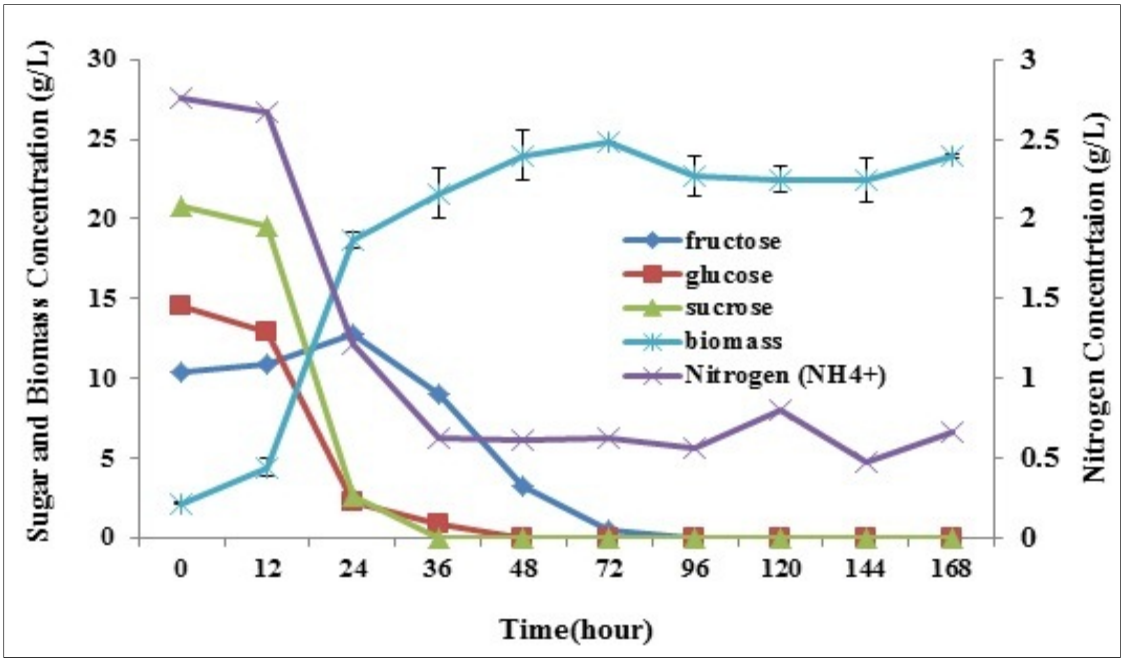


Figure 11 Individual sugar, nitrogen utilization and biomass accumulation of *R. glutinis* grown in sweet sorghum juice at optimized condition

The utilization of sugars and nitrogen and the accumulation of biomass at optimized condition were graphed in Figure 11. The breakdown of glucose and sucrose were observed as fast as 24 hours of cultivation, and complete utilization of both sugars was observed by 36 hours, whereas fructose was assimilated longer than the other two sugars. The utilization of all three sugars appeared to be simultaneous and the increase of both glucose and fructose concentration during the cultivation of *R. glutinis* were observed as sucrose concentration decreases. The sugar utilization was clearly observed at lower temperature where the growth of the microorganism is slowest as presented by Figure 12. The continuous decrease of sucrose means breaking down of this disaccharide into simple sugars of fructose and glucose, resulting in an increase or at least a stabilization of both sugar concentrations. Furthermore, the slow assimilation of sugars

indicates that the activity of the enzymes degrading both sucrose and glucose are greater compared with the fructose since the fructose degradation was barely detected. It is also important to note that stationary phase is achieved when the carbon source has been exhausted and the nitrogen concentration started to stabilize but 100% utilization of nitrogen was not achieved.

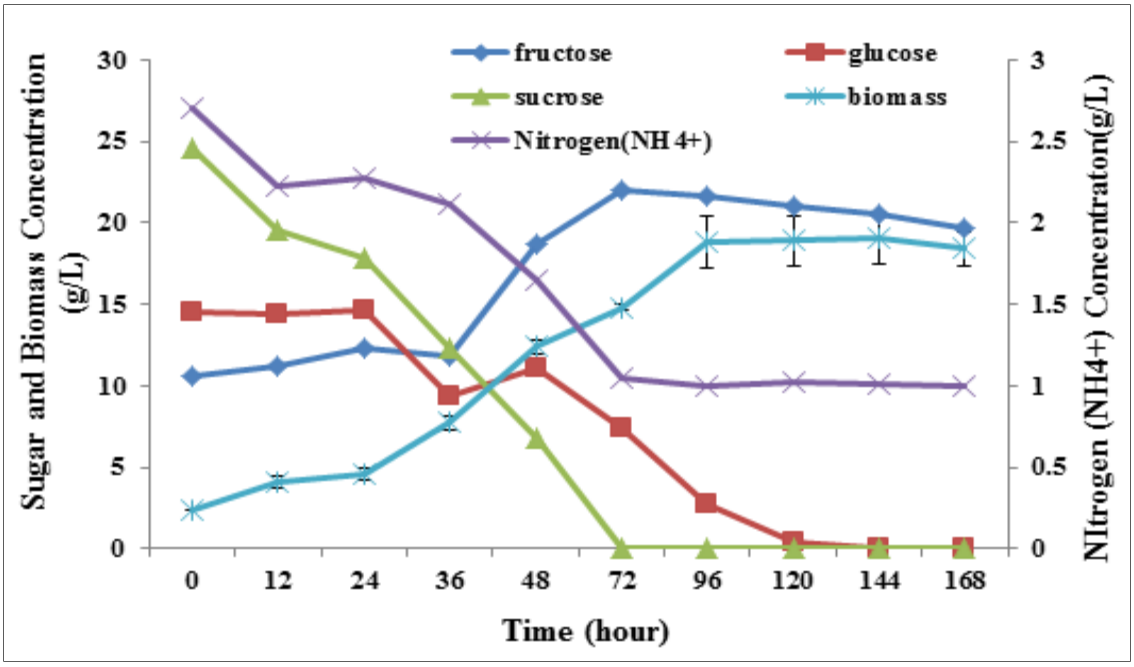


Figure 12 Individual sugar, nitrogen utilization and biomass accumulation of *R. glutinis* grown in sweet sorghum juice at 20°C; C:N-10 and pH 2.5

The optimized condition was used with several pO_2 levels: 40%, 80% and 100%. The biomass obtained from these cultivation conditions is shown in Figure 13. The effect of amount of oxygen starting from 60% to 100% with the accumulated biomass is not significantly different but the 40% pO_2 was not adequate to achieve the maximum biomass. Although the maximum biomass attained by the three levels of pO_2 from 60-

100% was comparable, the specific growth rate of the three levels showed a slight increase as the level of pO_2 increases (See Table 2). This was in conformation with the observation reported by Aksu, that the cell dry mass increases as the aeration increases which possibly enhances the mass transfer of substrate, products and oxygen [41].

Table 2 Maximum biomass and specific growth rate at different pO_2 levels

pO_2 level (%)	Maximum Biomass (g/L)	Specific growth rate ($hour^{-1}$)
40	12.3050	0.0370
60	22.6914	0.2131
80	20.1254	0.2321
100	20.4257	0.2783

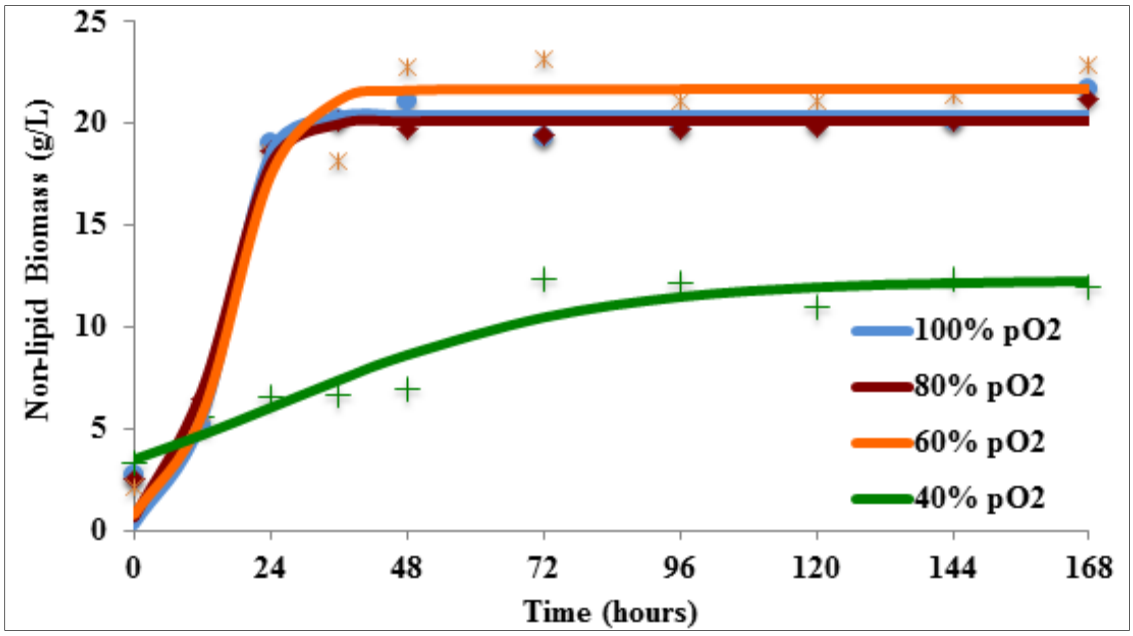


Figure 13 Non-lipid biomass of optimized conditions in different levels of pO_2

Products Identification

Identification of Individual Fatty Acid Methyl Esters

The individual extracted fatty acid methyl esters are identified using the standard of FAMEs mixture containing C8:0-C24:0 methyl esters from Sigma–Aldrich (St. Louis, MO). A sample chromatogram is presented below (Figure 14).

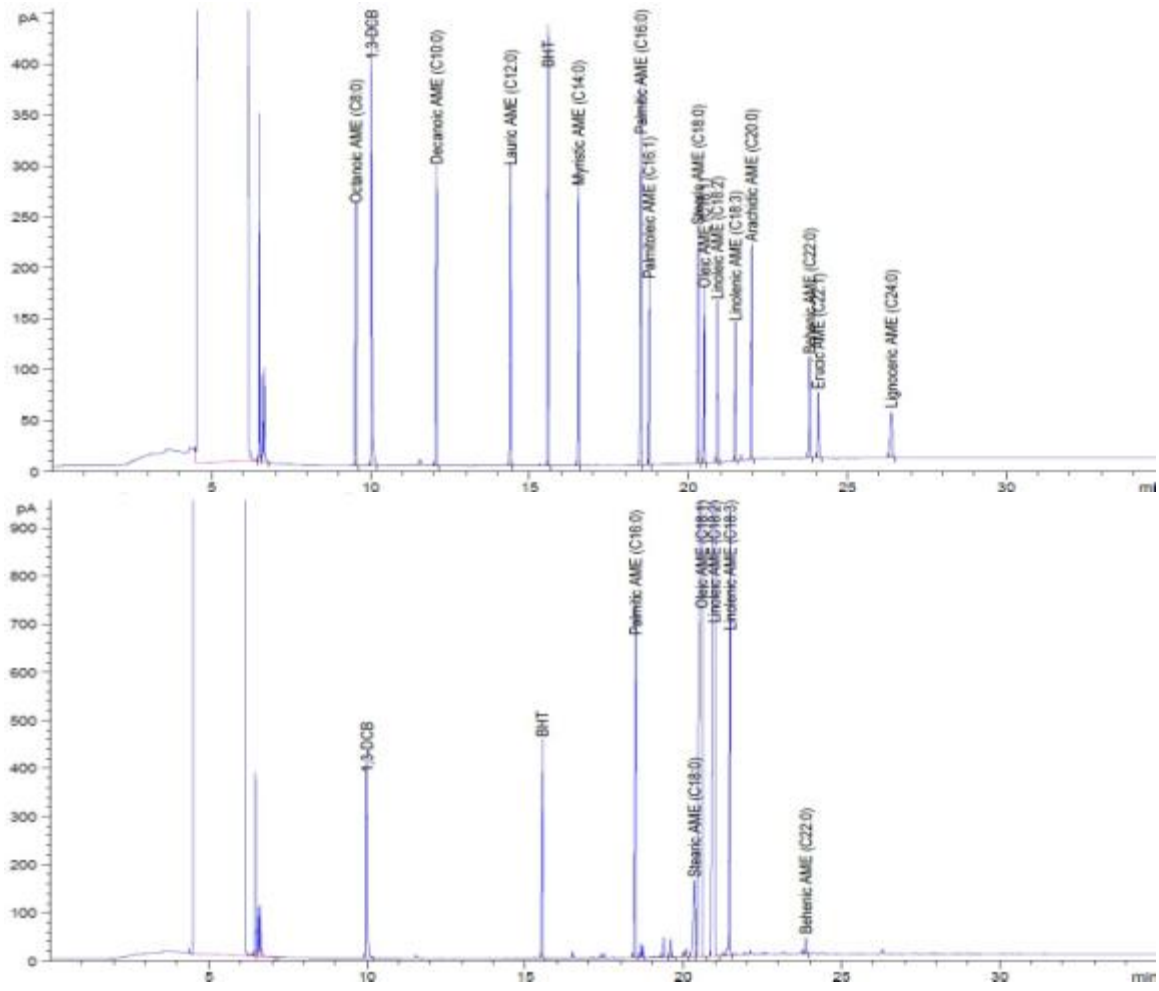


Figure 14 Sample chromatogram of standard FAMEs (above) and extracted FAMES from *Rhodotorula glutinis*

Identification of Individual Carotenoids

The use of dimethyl sulfoxide as a preliminary solvent softened the dried biomass by disrupting the yeast cells, making the extraction with organic solvent easier supporting the optimization done by Park, et al, who claimed that this solvent had the greatest extraction efficiency on carotenoids extraction among all other solvents used [45]. The extracted carotenoids were identified through liquid chromatographic analysis, presented in Figure 15a-b, these three peaks were identified with reference to the β-carotene standard from Sigma Aldrich®. To confirm the identity of the remaining two peaks, the extracted carotenoids were subjected to gel chromatography followed by ultraviolet-visible spectrophotometric analysis of the eluents which is presented in Figure 15c. The recovered carotenoids upon dissolution with petroleum ether showed absorption maxima at 467 nm, 501 nm and 537n m. On the other hand, the spectrophotometric analysis of both torulene and β-carotene in petroleum ether which had maxima at 454 nm, 480 nm and 514 nm appeared for the former and at 425 nm, 449 nm and 476 nm for the latter [49]. This identification is confirmatory to majority of the research done with cultivation of *Rhodotorula glutinis* which also reported three carotenoids (β-carotene, torulene and torularhodin) with varied composition depending on the strain of the microorganism and the cultivation conditions.

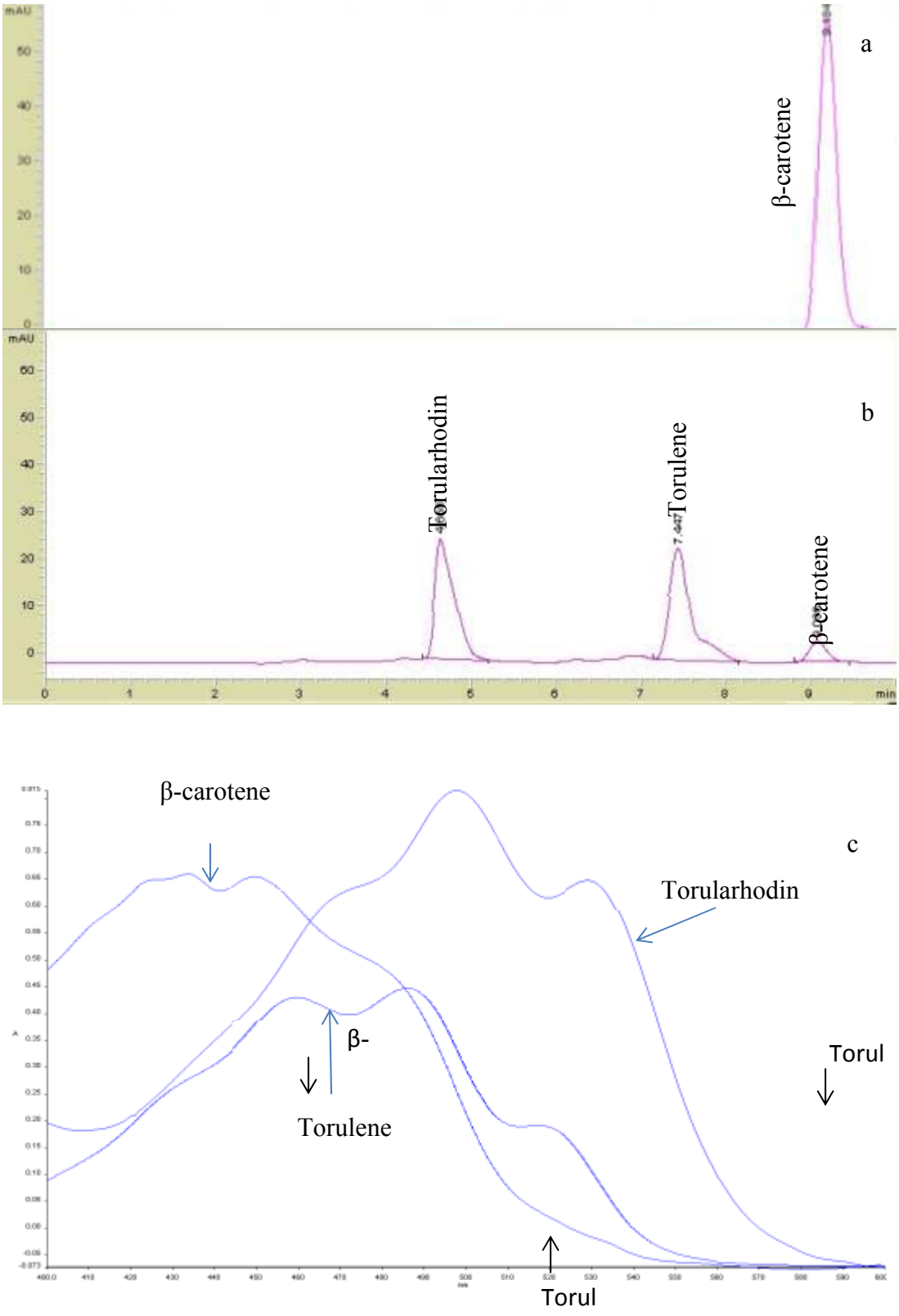


Figure 15 Chromatogram of β-carotene (a) extracted carotenoids (b) and UV-Vis wavelength scan of eluted carotenoids

Product Accumulation

The carotenoids and fatty methyl esters were modeled using the mixed growth associated product formation describe by Luedeking-Piret equation:

$$\frac{dP}{dt} = \beta X + \alpha \frac{dX}{dt} \tag{5}$$

which relates the product formation as proportional to the growth and non- growth (stationary phase) cell formation, where P is the product concentration and X is the cell growth concentration, t denotes the time and α and β are growth and non-growth coefficients, respectively which are dependent on cultivation conditions. Evaluating the β at stationary phase gives a *dX/dt=0*.

$$\beta = \frac{dP/dt \quad (stationary \quad phase)}{X_{\max}} \tag{6}$$

$$P(t) = P_o + \alpha X_o \left\{ \frac{e^{\mu_m t}}{\left[1 - \left(X_o / X_m\right)\left(1 - e^{\mu_m t}\right)\right]} - 1 \right\} + \beta \frac{X_m}{\mu_m} \ln\left[1 - \frac{X_o}{X_m}\left(1 - e^{\mu_m t}\right)\right] \tag{7}$$

Biodiesel Accumulation

Equation (7) was used for the modeling of the total fatty acid methyl esters (FAMEs) conversion of accumulated lipids by *Rhodotorula glutinis* at various temperature, pH and carbon to nitrogen ratio. Since *R. glutinis* accumulates lipids in form of triacylglycerol [50], the lipids accumulated can be assumed as the same weight with the FAMEs generated. The result of the seventh day total FAMEs at each three-factor combinations was used to optimize the cultivation conditions with respect total FAMES.

Employing the general factorial design, the same equation for biomass optimization as presented in Equation (3) was also used for the modeling and predicting the fit for the analysis. The response plot for the three factor interactions with total FAMES as the response is shown in Figure 16. The three factor analysis was found to have insignificant interaction to affect the biodiesel accumulation but the interaction of the carbon to nitrogen ratio and pH manifest an effect to the biodiesel synthesis. Moreover, both quadratic effect of temperature and pH showed a significant effect towards the biodiesel synthesis. The equation describing the response plot optimization is given by:

$$\textbf{Total FAMEs} = 0.881848 - (0.06322t + 0.\ 0607011c + 0.08494h) + 0.155548ch - (0.530941t^2 + 0.237874h^2) \quad (8)$$

The optimized condition for the biodiesel production is at pH 5.5, 25°C and carbon to nitrogen ratio of 70. It has been reported that lipids start to accumulate when the nitrogen content starts to diminish and when the carbon source is still available [24]. This limitation of nitrogen is crucial since it directs the flow of reaction to the lipid synthesis by formation of acetyl-CoA [24].

The optimized condition lipid and biomass accumulation condition is shown in Figure 17. At this condition, the nitrogen supply is already exhausted after 24 hours, where there is still carbon supply available when the lipid started to accumulate but the biomass has not yet reached the maximum biomass. The lipid content obtained after at seventh day was just 14.3%. This is relatively low compared to other reports, but this could be due to fast exhaustion of carbon. Also the maintained oxygen imparts the low lipid accumulation levels, which was observed by Yen, et al. The high dissolved oxygen content has lower lipid accumulation compared with low dissolved oxygen, which can be

explained by the retardation of cell growth at lower dissolve oxygen resulting to the enhancement of lipid accumulation [51].

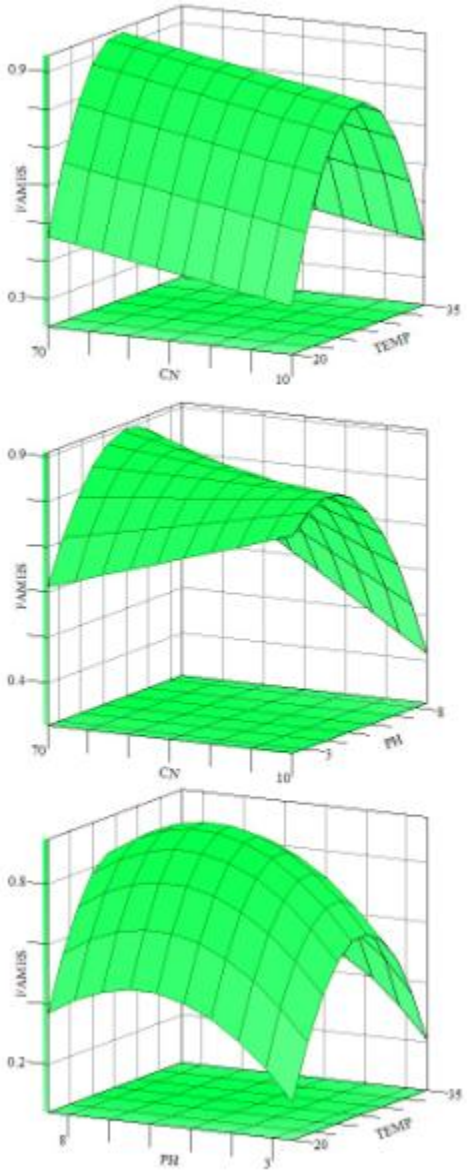


Figure 16 Surface response plot of total fatty acids methyl ester at optimized condition

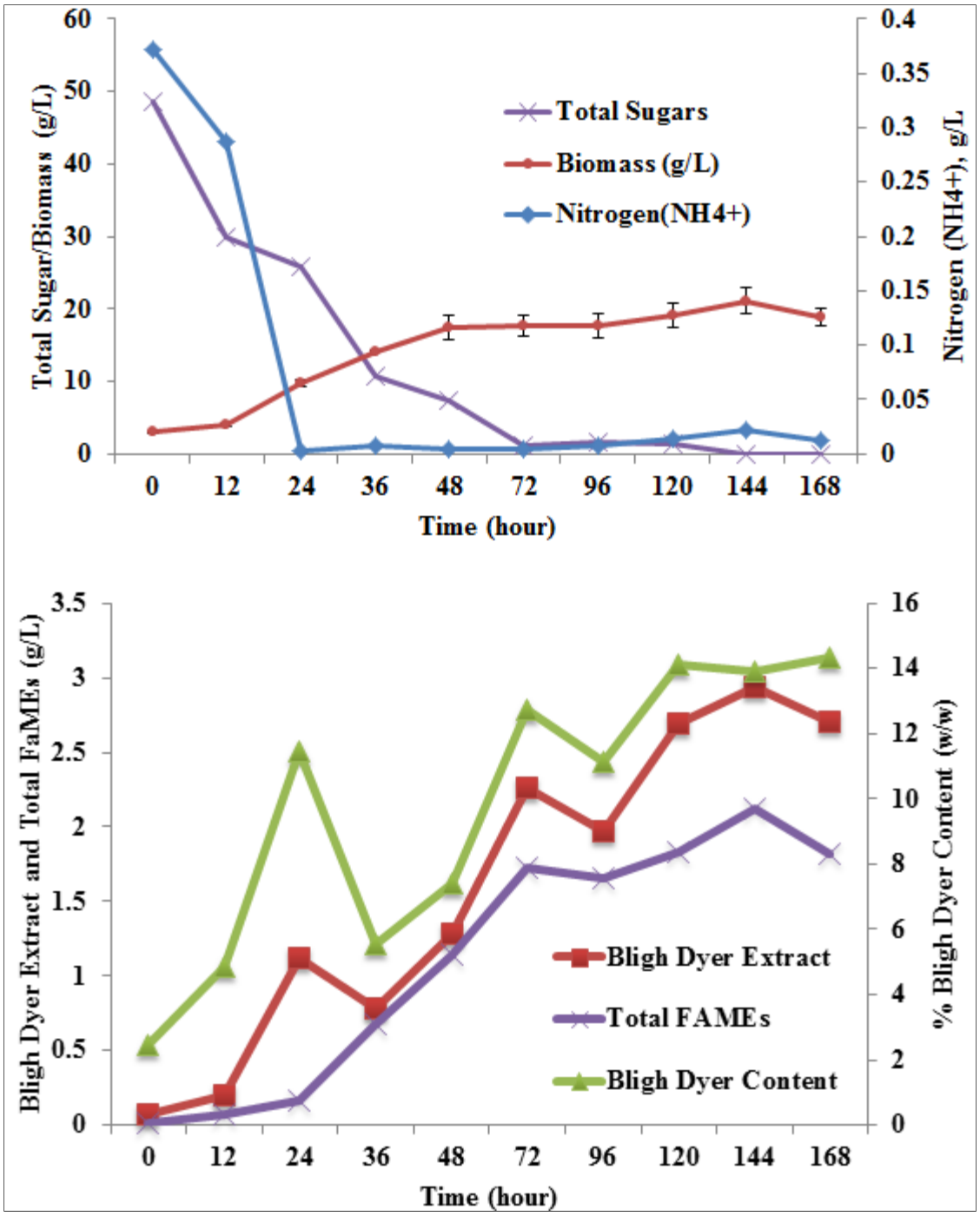


Figure 17 Bligh Dyer extract and FAMEs synthesis at optimized condition

The conversion of the extracted lipids to fatty acid methyl esters from the start of the cultivation to the seventh day is shown in Figure 18. At the end of the cultivation

period, the cellular concentration of the total FAMEs was 96.3 mg/gram biomass comprised of 20.3 % palmitic acid methyl ester (C16:0), 7.6 % stearic acid methyl ester (C18:0), 39.6 % oleic acid methyl ester (C18:1), 27.9 % linoleic acid methyl ester (C18:2) and 2.9 % linolenic acid methyl ester (C18:3). The fatty acid methyl esters components change as time progressed. The graph shows that the oleic acid was relatively a very small component at the start of the cultivation and eventually became the major component of the FAMEs towards the end of the cultivation. Stearic acid methyl ester also gave a similar pattern although the increase was relatively small. The increase in lower unsaturated FAMEs resulted in lowering of palmitic acid and the highly unsaturated linolenic acid. This is comparable to the FAMEs profile reported using *R. glutinis* [30, 52]

The high ratio of oleic acid methyl ester in the total FAMEs converted would impart a good characteristic to the biodiesel generated since this particular fatty acids balance the cetane number and cold flow properties. Also, the mixture of both saturated and unsaturated methyl esters in the FAMEs would give a good balance towards the oxidative stability and cold flow property.

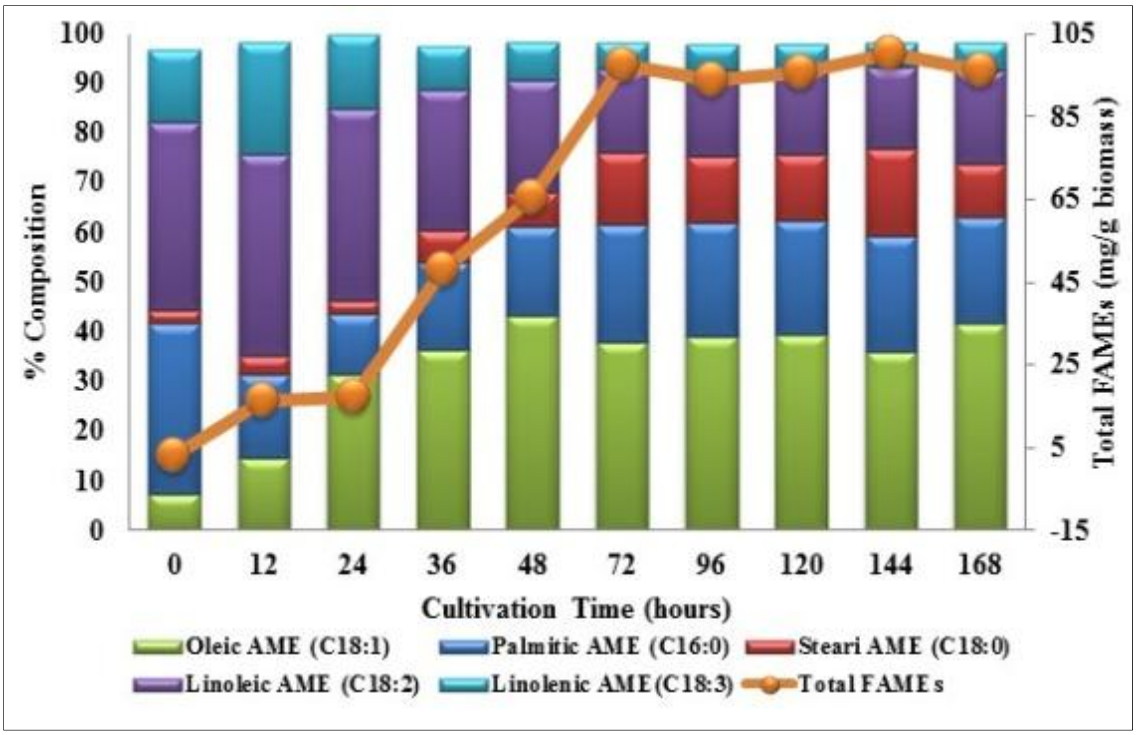


Figure 18 Fatty acid methyl ester components at different cultivation time

The fatty acid methyl ester profile at cultivation condition of pH of 5.5, temperature of 25°C and carbon to nitrogen ratio of 10 at partial pressure of 60% is shown in Figure 19, the total FAMEs reached as much as 33.2 mg/gram of biomass after 24 hours of cultivation but there was no increase observed until the seventh day of the cultivation. This can be probably because the carbon of the system was already exhausted after 24 hours and there was still traceable amount of nitrogen in the medium. The fatty acid methyl ester profile was also consistent from the start of cultivation until the seventh day, totaling 90% of combined linoleic AME and oleic AME.

At different level of partial pressure of oxygen, no effect was observed in the composition and cellular amount of total FAMEs, except at pO_2 level of 40 %, which generated 11.4 mg FAMEs/gram biomass.

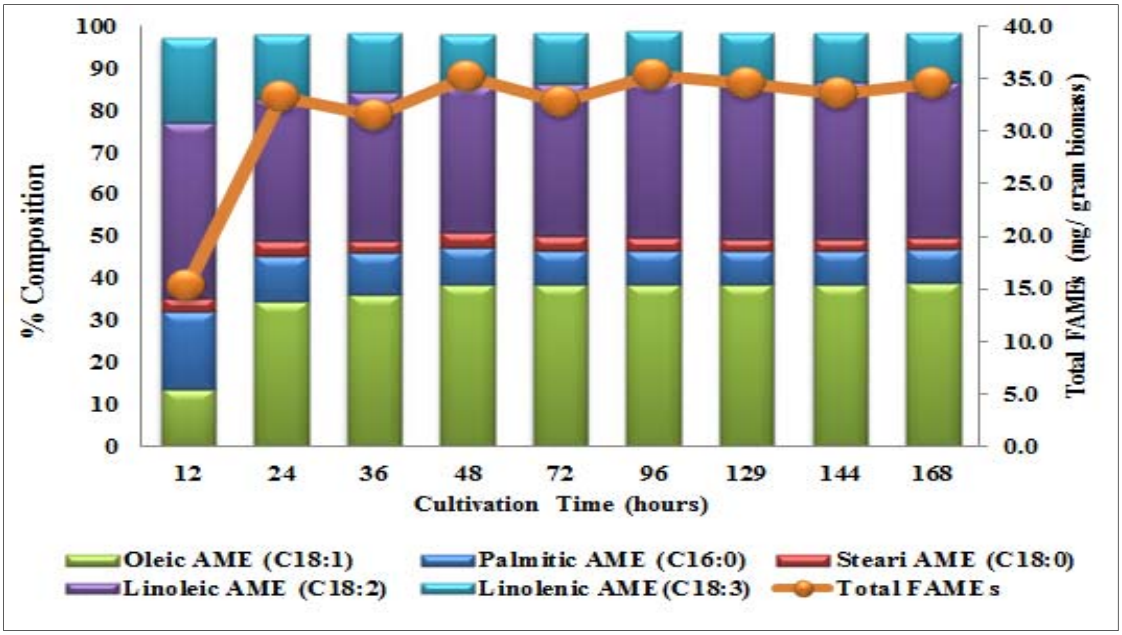


Figure 19 Fatty acid methyl ester components at different cultivation time (pH-5.5, temperature 25 °C, C: N ratio-10 and pO_2 = 60%)

Carotenoid Accumulation

Using Luedeking-Piret equation given in Equation (7), the total accumulated carotenoids at each three factors combination of three levels of carbon to nitrogen ratio, temperature and pH, totaling 27 combinations were modeled and the carotenoids obtained at seventh day of cultivation at each combination were optimized using the general factorial design. The same model used at biomass optimization (Equation 3) was used to predict the response plot of the accumulated carotenoid plot presented in Figure 20. The predicted model fit of the three factors carbon to nitrogen ratio (c), pH (h) and temperature (t) with respect to the volumetric accumulated carotenoid is given below:

$$\textbf{Total Carotenoids} = 0.042573 - (0.005412t + 0.005252c + 0.008588h) + 0.003944ht - (0.02267t^2 + 0.015679h^2) \quad (9)$$

There was no significant interaction with the three factors but the surface response plot showed the two-factor interaction of pH and temperature with respect to the carotenoid accumulation. The analysis also showed the same negative linear effect of the three factors to the accumulated total carotenoids. Additionally, the quadratic effect of both temperature and pH show a decrease in the total carotenoids. It can be observed that as the temperature approached both 20°C and 35°C, the carotenoids decreased. The same effect was observed with pH 2.5 and 8.5. Interestingly, the carbon nitrogen ratio was found not to be a significant factor with respect to the accumulated total carotenoid. The accumulation of carotenoids was found to be directly proportional to the cell dried weight per liter of the cultivation medium. The optimized condition was the same obtained from cell dried weight optimization, at pH 5.5, carbon to nitrogen ratio of 10 and temperature of 5.5. This observation was in agreement with other studies done by other researchers [21, 41]. The volumetric and cellular accumulations were both optimum at this condition.

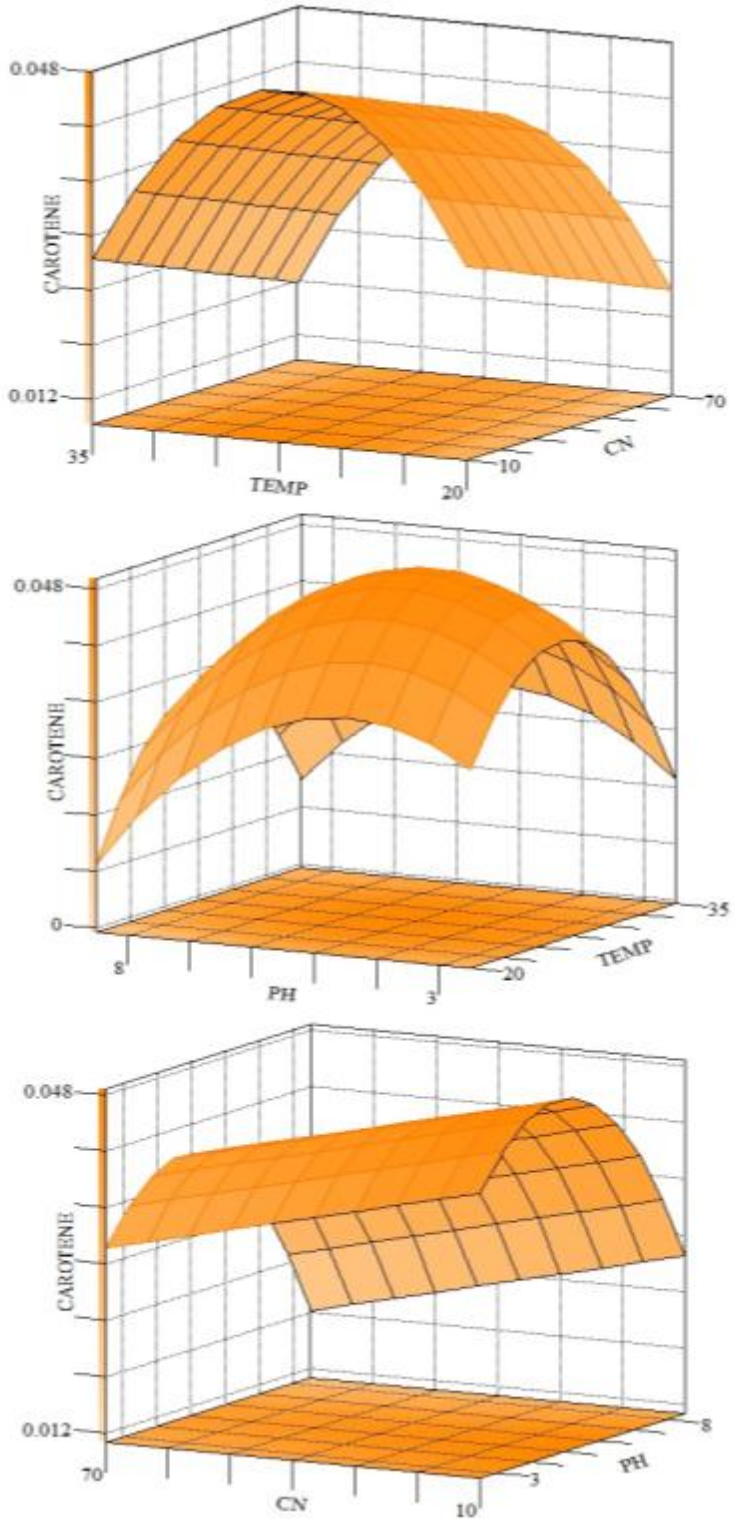


Figure 20 Surface response plot of carotenoids accumulation at optimized condition

As identified the major carotenoids obtained are torularhodin, torulene and β-carotene. Torularhodin constitutes as much 96% of the total carotenoids; the remaining 4% is shared by both torulene and β-carotene. Various reports have been made acclaiming torularhodin as the dominant carotenoids; groups of Frengova and Peterson reported to have as much as 67 % of torularhodin [14, 39] while the group of Buzzini was much as 79 % of torularhodin [15]. The present study is the highest torularhodin percentage so far. This could be dependent on the strain of the *R. glutinis* employed and possibly due to the maintained dissolved oxygen of the system throughout the cultivation period which is first explained by Sakaki, who claimed that the high production of torularhodin is due to the high aeration of the culture medium. The biosynthesis of high amount of torularhodin compare with the β-carotene is to protect the membrane from oxygen stress [38, 53]. Also, the torulene concentration was almost constant for the entire cultivation period, indicating the continuous conversion of torulene to torularhodin, which proved that the synthesis of torularhodin was highly favored at this condition. The absence γ-carotene can be explained by relatively high amount of torularhodin. Simpson et al, reported that the amount of increase in torularhodin was as much as the decrease of γ-carotene concentration [40].

The utilization of both sugar and nitrogen was examined with the accumulation of individual carotenoids at the optimized condition. The total consumption of sugars was observed after 72 hours and when the accumulation of both torularhodin and β-carotene started to stabilize as shown in Figure 21. This is logical since there is no source of carbon available for further synthesis of carotenoids. The nitrogen on the other hand, was not 100% utilized which the consumption stopped when the microorganism reached the

stationary phase. The total cellular carotenoid extracted was 2.6 mg/g biomass containing as much as 96.1 % torularhodin, 3.4 % β-carotene and 0.5 % torulene.

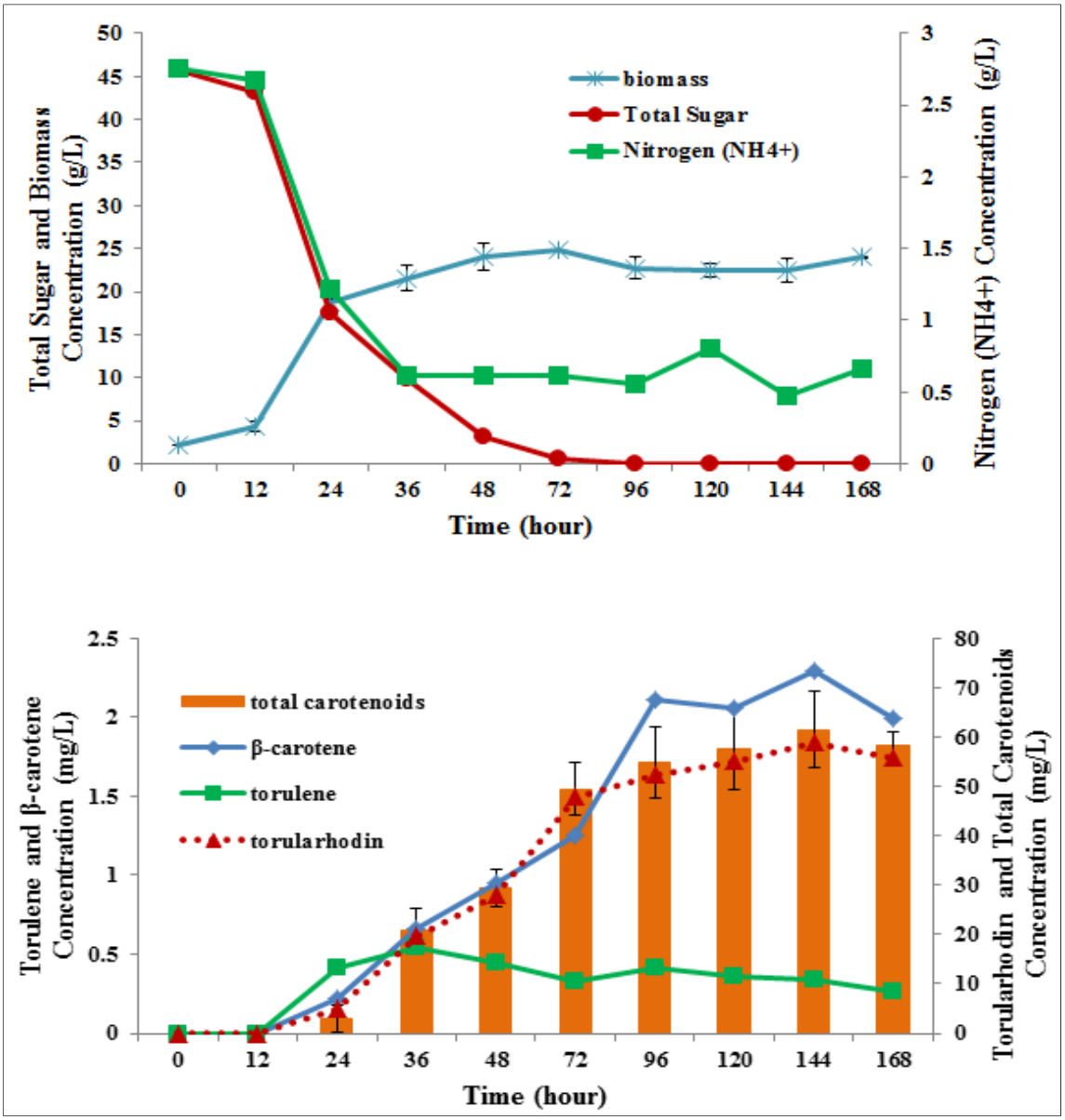


Figure 21 Utilization of total sugar and nitrogen and accumulation of carotenoids at optimized condition

The production of β-carotene was found to be comparable with the studies reported using glucose as substrate and untreated strain of *R.* glutinis as shown in Table 3 except with the carbon source from sucrose, which was relatively high which could be affected by the strain of *R. glutinis* used and also the total carotenoids was not reported. Additionally, it had been reported that at low temperature conditions are favorable for β-carotene production, but from the results that were obtained from 20°C, the behavior wasn't exhibited. This might due to the fact that torularhodin was highly favorable carotenoid.

Table 3 Comparison of cellular concentration of β-carotene

β-carotene (μg/g dried biomass)	Carbon source	Reference
74	glucose	[28]
22.9	glucose	[35]
64	glucose	[40]
130	sucrose	[54]
2500	sugarcane	[12]
55	glucose	[27]
88	This study	

The effect of the aeration with the optimized condition with respect to volumetric carotenoids obtained was investigated by supplying 40%, 80% and 100% pO_2. Figure 22 displays the effect of the increase in partial pressure of oxygen in the cultivation medium. The 100% pO_2 showed a fast accumulation of torularhodin, which further support that oxygen is the main factor that dictates the favorable synthesis of torularhodin while the pO_2 level decreased, the torularhodin synthesis also decreased. The proportion of β-carotene increased with the decrease of pO_2 level. Although the proportion of β-carotene

obtained at 40% was higher, there was a significant decrease of total carotenoid synthesized.

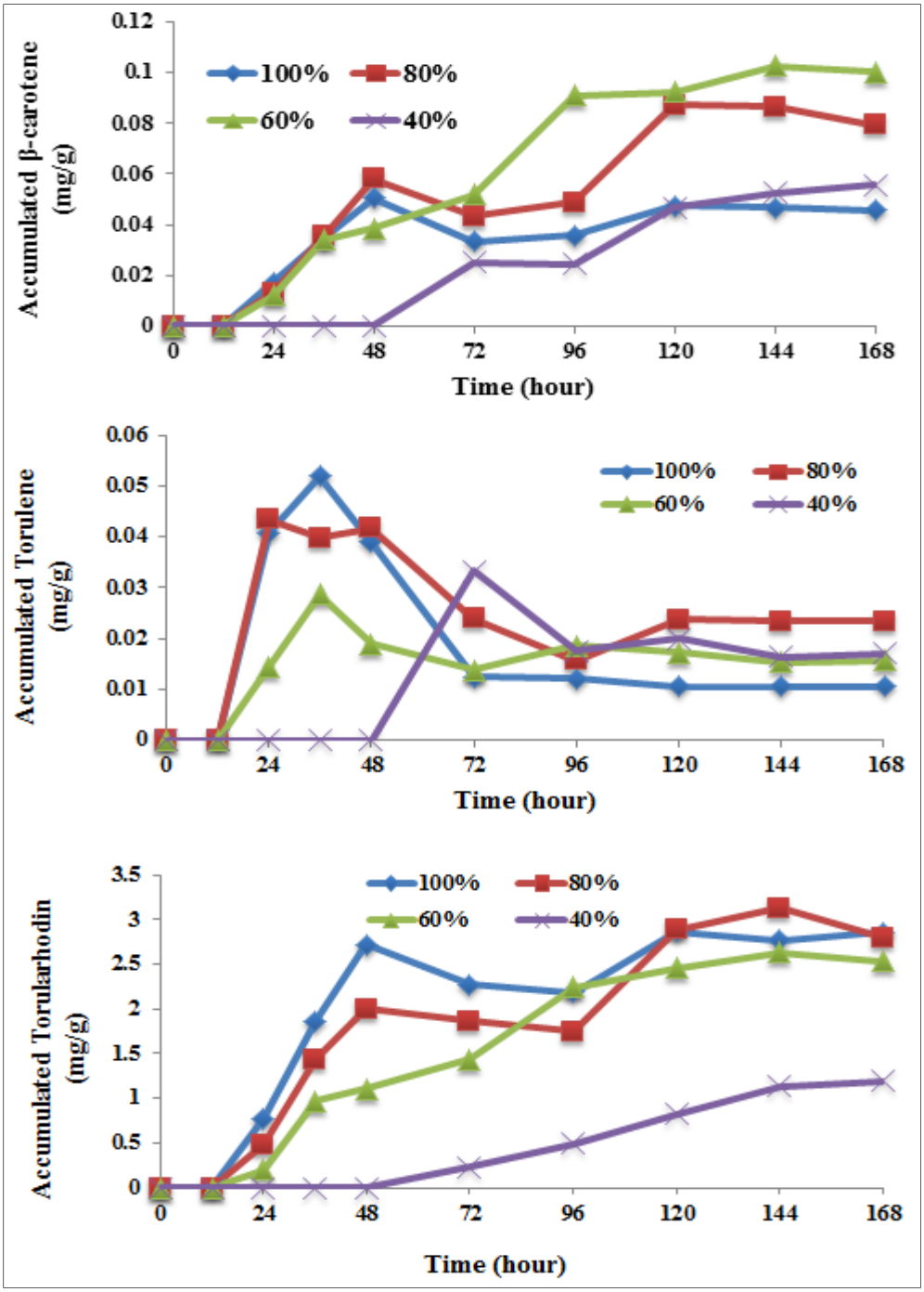


Figure 22 Effect of different levels of pO_2 on individual carotenoids accumulated from *Rhodotorula glutinis*

CHAPTER VI

CONCLUSION

The cultivation of *Rhodotorula glutinis* using sweet sorghum juice as the sole carbon source produced a high yield of biomass, which yielded both lipids and carotenoids. The optimization of the cultivation conditions with respect to carotenoids and total fatty acid methyl ester yield were investigated using three levels of three variables of pH, temperature and carbon to nitrogen ratio. For both products the pH favorable condition was 5.5 and temperature of 25°C, which are the optimum pH and temperature for the growth of *R.glutinis,* the carbon to nitrogen ratio obtained that was favorable for volumetric total carotenoids was 10 while for the total FAMEs was 70. This was expected since carotenoids are known to have directly proportional relationship with the growth of *R. glutinis,* on the other hand the result for total FAMES was also anticipated since it was well explained that the mechanism of lipids synthesis is initiated with nitrogen limitation. At the end of seventh day the total volumetric carotenoid extracted was as much as 58.1247 mg/L comprises 96.1% torularhodin, 3.4% β-carotene and 0.5% of torulene. Moreover, the lipids obtained at this time were only 10%, which, were converted to total FAMEs. Both optimizations showed that the interaction of these three factors does not affect the amount of both carotenoids and FAMEs yield.

The proportion of torularhodin in the total carotenoids extracted was as much as 96.1 %, which was explained by Sakaki that the high oxygen content in the cultivation

medium favored the torularhodin biosynthesis. Additionally, maintaining 100% pO2 setting in the cultivation condition showed that there was a fast synthesis of torularhodin when the level of oxygen was increasing further supported this. However at high a carbon to nitrogen ratio of 10, the increase of dissolved oxygen content in the cultivation medium does not affect the lipid synthesis.

Sweet sorghum as a feedstock for microbial oil could be promising if the cultivation condition of *R. glutinis* is controlled especially with respect to the carbon to nitrogen ratio. The growth rate is relatively fast, which is proportional to the biomass yield, by feeding additional sweet sorghum juice as soon as the growth is achieved would possibly lead to generation of higher amount of lipid.

The cultivation of carotenoids using sweet sorghum generated a relatively high amount of carotenoids but the ratio of β-carotene was relatively low, channeling the pathway towards the production of β-carotene could be encouraging because of the high biomass yield which could be done by not purging air into the system. Instead the used of the impeller would be sufficient to the growth of the microorganism. Also, mutagenesis or genetic modification could increase the β-carotene accumulation of this microorganism given that the source is readily available and cheap.

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APPENDIX A

BIOMASS KINETICS DATA AND COMPARISON

Table 4 Kinetics parameters for biomass modeling

Temperature (°C)	Carbon to Nitrogen Ratio	pH	Initial Biomass(X_0)	Maximum Biomass(Xm)	Maximum specific growth rate(μ_{max})	R^2
20	10	2.5	2.317	19.276	0.049	0.988
20	10	5.5	1.856	22.286	0.088	0.973
20	10	8.5	2.135	7.086	0.083	0.892
20	40	2.5	2.542	17.486	0.034	0.936
20	40	5.5	3.034	19.504	0.133	0.970
20	40	8.5	0.399	20.101	0.133	0.956
20	70	2.5	1.172	11.178	0.076	0.953
20	70	5.5	1.107	17.235	0.099	0.988
20	70	8.5	0.458	13.238	0.129	0.870
25	10	2.5	2.250	19.067	0.044	0.997
25	10	5.5	0.687	22.691	0.213	0.982
25	10	8.5	3.982	14.666	0.015	0.865
25	40	2.5	0.220	16.378	0.175	0.990
25	40	5.5	1.249	19.276	0.129	0.979
25	40	8.5	0.934	16.700	0.112	0.966
25	70	2.5	1.570	17.033	0.078	0.967
25	70	5.5	2.252	17.622	0.089	0.977
25	70	8.5	0.130	16.675	0.224	0.956
35	10	2.5	2.350	10.961	0.008	0.780
35	10	5.5	1.813	13.845	0.076	0.973
35	10	8.5	1.449	5.798	0.073	0.822
35	40	2.5	2.291	13.617	0.039	0.914
35	40	5.5	2.451	12.89	0.131	0.978
35	40	8.5	2.267	7.842	1.934	0.646
35	70	2.5	2.152	3.220	1.934	0.505
35	70	5.5	4.202	19.020	0.087	0.914
35	70	8.5	2.567	10.036	0.212	0.753

Table 5 Least square means of biomass

				Max LSMean	Temperature (°C)	Carbon to Nitrogen Ratio	pH
A				22.691	25	10	5.5
A				22.286	20	10	5.5
	B			20.101	20	40	8.5
	B	C		19.504	20	40	5.5
	B	C	D	19.276	20	10	2.5
	B	C	D	19.276	25	40	5.5
E	B	C	D	19.067	25	10	2.5
E	B	C	D	19.020	35	70	5.5
E	F	C	D	17.622	25	70	5.5
E	F	C	D	17.486	20	40	2.5
E	F		D	17.235	20	70	5.5
E	F			17.033	25	70	2.5
	F	G		16.700	25	40	8.5
	F	G		16.675	25	70	8.5
	F	G		16.378	25	40	2.5
		G	H	14.666	25	10	8.5
			H	13.845	35	10	5.5
			H	13.617	35	40	2.5
I			H	13.238	20	70	8.5
I	J		H	12.890	35	40	5.5
I	J	K		11.178	20	70	2.5
	J	K		10.961	35	10	2.5
		K		10.036	35	70	8.5
			L	7.842	35	40	8.5
			L	7.086	20	10	8.5
			L	5.798	35	10	8.5
M				3.220	35	70	2.5

Note: Same letters means are not significantly different

APPENDIX B

SAS OUTPUT FOR OPTIMIZATION

SAS® Output for Biomass Optimization

Fit Statistics for BIOMASS

	Master Model	Predictive Model
Mean	14.99501	14.99501
R-square	75.08%	72.00%
Adj. R-square	69.28%	68.42%
RMSE	2.80329	2.8421
CV	18.69481	18.95364

Predictive Model for BIOMASS

Coded Levels(-1,1):

```
BIOMASS = 20.53391 - 2.794395*TEMP - 0.586484*CN - 0.900798*PH
          - 3.668079*TEMP*TEMP + 2.534185*CN*PH - 4.902211*PH*PH
```

Uncoded Levels:

```
BIOMASS = -26.384 + 3.21398*TEMP - 0.174416*CN + 4.565021*PH - 0.06521*TEMP*TEMP
          + 0.028158*CN*PH - 0.54469*PH*PH
```

Effect Estimates for BIOMASS

	Master Model			
Term	Estimate	Std Err	t	Pr > |t|
TEMP	-2.794395	0.467215	-5.98096	<.0001
CN	-0.503146	0.471368	-1.06742	0.2917
PH	-0.87217	0.471368	-1.8503	0.0711
TEMP*TEMP	-3.668079	0.927101	-3.9565	0.0003
TEMP*CN	0.750038	0.561908	1.334806	0.1890
TEMP*PH	0.2576494	0.561908	0.458526	0.6489
CN*CN	-1.473185	0.80924	-1.82046	0.0757
CN*PH	2.5291151	0.577306	4.380895	<.0001
PH*PH	-4.902211	0.80924	-6.0578	<.0001
TEMP*CN*PH	-0.045634	0.688194	-0.06631	0.9474

Effect Estimates for BIOMASS

	Predictive Model			
Term	Estimate	Std Err	t	Pr > |t|
TEMP	-2.794395	0.473683	-5.89929	<.0001
CN	-0.586484	0.473683	-1.23813	0.2218
PH	-0.900798	0.473683	-1.90169	0.0634
TEMP*TEMP	-3.668079	0.939936	-3.90248	0.0003
TEMP*CN				
TEMP*PH				
CN*CN				
CN*PH	2.5341855	0.580141	4.368221	<.0001
PH*PH	-4.902211	0.820444	-5.97507	<.0001
TEMP*CN*PH				

Figure 23 ADX fit statistics for maximum biomass

SAS® Output for FAMEs Optimization

ADX Report for FAMEs

Fit Statistics for FAMES

	Master Model	Predictive Model
Mean	0.356667	0.356667
R-square	54.59%	52.32%
Adj. R-square	44.03%	46.23%
RMSE	0.308422	0.302288
CV	86.47333	84.75371

Predictive Model for FAMES

Coded Levels(-1,1):

```
FAMES = 0.881848 - 0.06322*TEMP + 0.060701*CN - 0.08494*PH - 0.530941*TEMP*TEMP
        + 0.155548*CN*PH - 0.237871*PH*PH
```

Uncoded Levels:

```
FAMES = -6.36905 + 0.510713*TEMP - 0.007482*CN + 0.193285*PH - 0.009439*TEMP*TEMP
        + 0.001728*CN*PH - 0.02643*PH*PH
```

Effect Estimates for FAMES

	Master Model			
Term	Estimate	Std Err	t	Pr > |t|
TEMP	-0.06322	0.051404	-1.22987	0.2254
CN	0.0550585	0.051861	1.061664	0.2943
PH	-0.080711	0.051861	-1.5563	0.1270
TEMP*TEMP	-0.530941	0.102001	-5.20526	<.0001
TEMP*CN	-0.050782	0.061822	-0.82142	0.4159
TEMP*PH	0.0380599	0.061822	0.615638	0.5414
CN*CN	0.0567031	0.089034	0.636873	0.5276
CN*PH	0.1485599	0.063516	2.338938	0.0241
PH*PH	-0.237871	0.089034	-2.67169	0.0106
TEMP*CN*PH	-0.062891	0.075716	-0.83062	0.4108

	Predictive Model			
Term	Estimate	Std Err	t	Pr > |t|
TEMP	-0.06322	0.050381	-1.25482	0.2157
CN	0.0607009	0.050381	1.204828	0.2343
PH	-0.08494	0.050381	-1.68593	0.0984
TEMP*TEMP	-0.530941	0.099972	-5.31087	<.0001
TEMP*CN				
TEMP*PH				
CN*CN				
CN*PH	0.1555478	0.061704	2.520856	0.0152
PH*PH	-0.237871	0.087263	-2.7259	0.0090
TEMP*CN*PH				

Figure 24 ADX fit statistics for maximum FAMEs

SAS® Output for Carotenoids Optimization

ADX Report for CAROTENE

Fit Statistics for CAROTENE

	Master Model	Predictive Model
Mean	0.016769	0.016769
R-square	75.98%	72.49%
Adj. R-square	70.39%	68.98%
RMSE	0.010425	0.010671
CV	62.16731	63.63554

Predictive Model for CAROTENE

Coded Levels(-1,1):

```
CAROTENE = 0.042573 - 0.005412*TEMP - 0.005252*CN - 0.008588*PH
           - 0.02267*TEMP*TEMP + 0.003944*TEMP*PH - 0.015679*PH*PH
```

Uncoded Levels:

```
CAROTENE = -0.2458 + 0.02048*TEMP - 0.000175*CN + 0.01148*PH - 0.000403*TEMP*TEMP
           + 0.000175*TEMP*PH - 0.001742*PH*PH
```

Effect Estimates for CAROTENE

	Master Model			
Term	Estimate	Std Err	t	Pr > |t|
TEMP	-0.005412	0.001737	-3.11482	0.0033
CN	-0.004999	0.001753	-2.85163	0.0067
PH	-0.008588	0.001753	-4.89916	<.0001
TEMP*TEMP	-0.02267	0.003448	-6.57527	<.0001
TEMP*CN	0.0022793	0.00209	1.090767	0.2814
TEMP*PH	0.0039443	0.00209	1.887562	0.0658
CN*CN	-0.002346	0.003009	-0.77969	0.4398
CN*PH	0.0034486	0.002147	1.606309	0.1155
PH*PH	-0.015679	0.003009	-5.20995	<.0001
TEMP*CN*PH	-0.002923	0.002559	-1.14207	0.2597

Effect Estimates for CAROTENE

	Predictive Model			
Term	Estimate	Std Err	t	Pr > |t|
TEMP	-0.005412	0.001779	-3.04295	0.0038
CN	-0.005252	0.001779	-2.953	0.0049
PH	-0.008588	0.001794	-4.78613	<.0001
TEMP*TEMP	-0.02267	0.003529	-6.42356	<.0001
TEMP*CN				
TEMP*PH	0.0039443	0.002139	1.844011	0.0715
CN*CN				
CN*PH				
PH*PH	-0.015679	0.00308	-5.08975	<.0001
TEMP*CN*PH				

Figure 25 ADX fit statistics for maximum carotenoids

APPENDIX C

NUTRIENTS UTILIZATION AND BIOMASS ACCUMULATION PROFILE AT DIFFERENT FACTORS COMBINATIONS

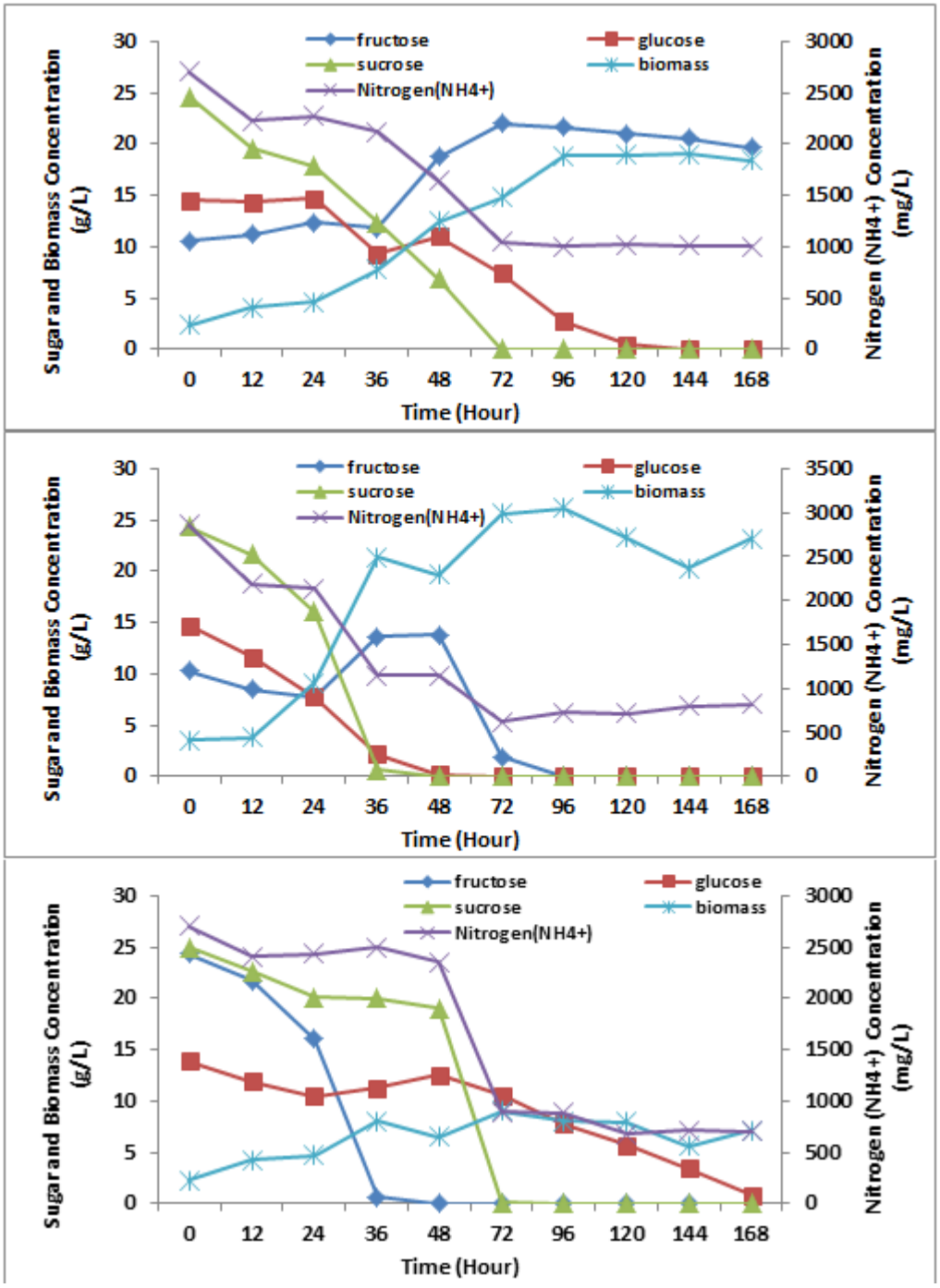

Figure 26 Nutrients utilization and biomass accumulation at C:N of 10 and temperature of 20°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

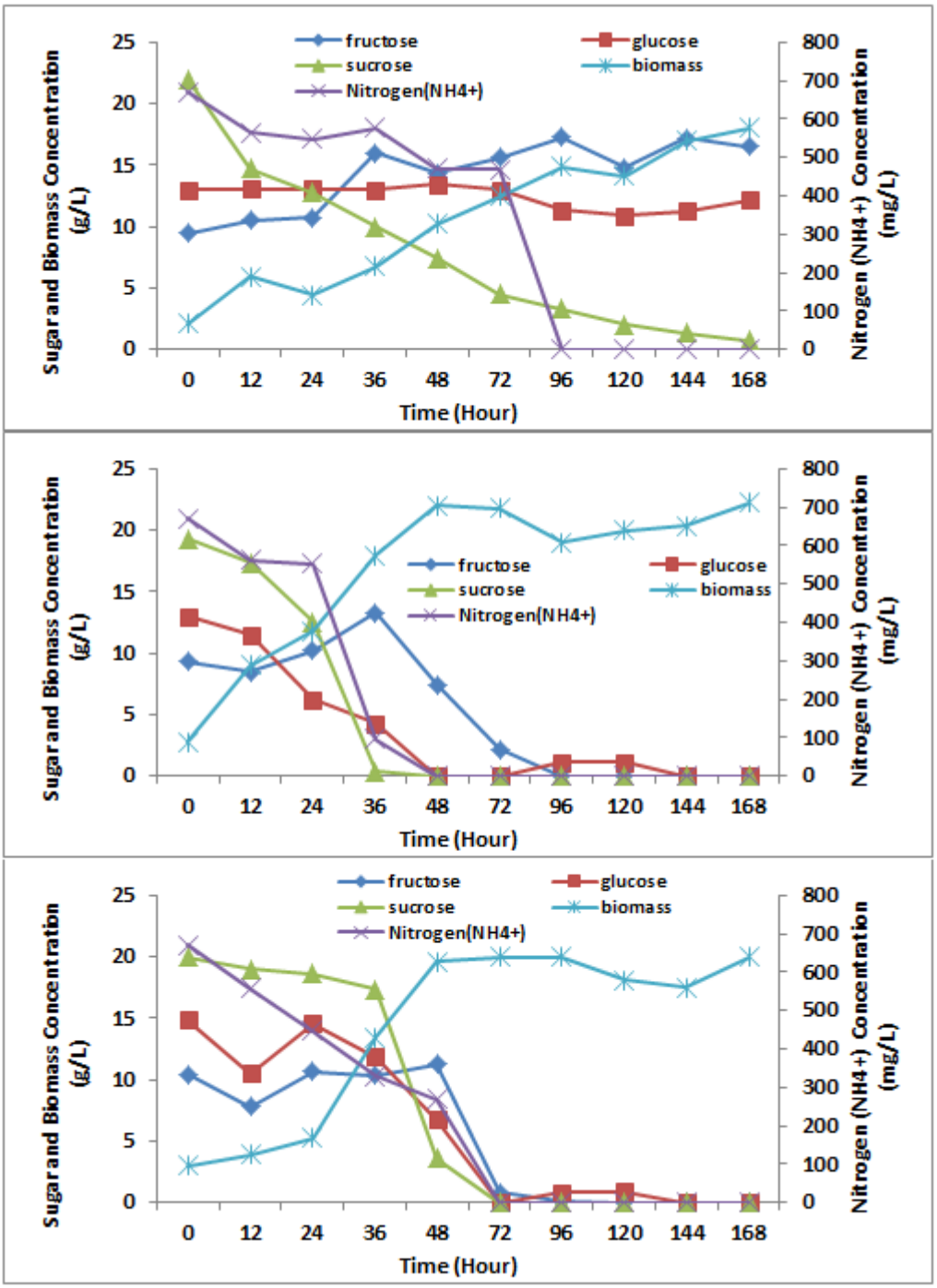

Figure 27 Nutrients utilization and biomass accumulation at C:N of 40 and temperature of 20°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

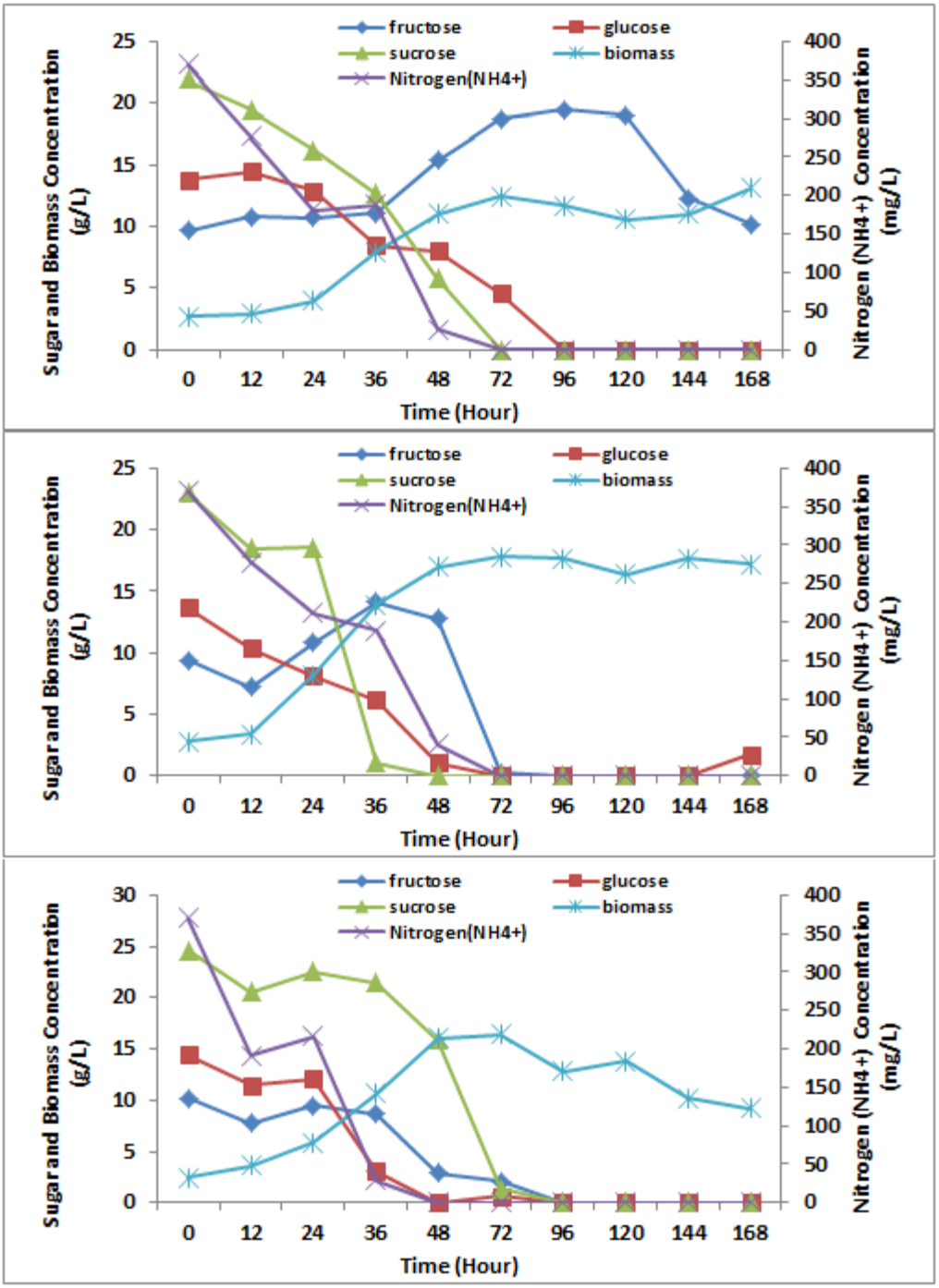

Figure 28 Nutrients utilization and biomass accumulation at C:N of 70 and temperature of 20°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

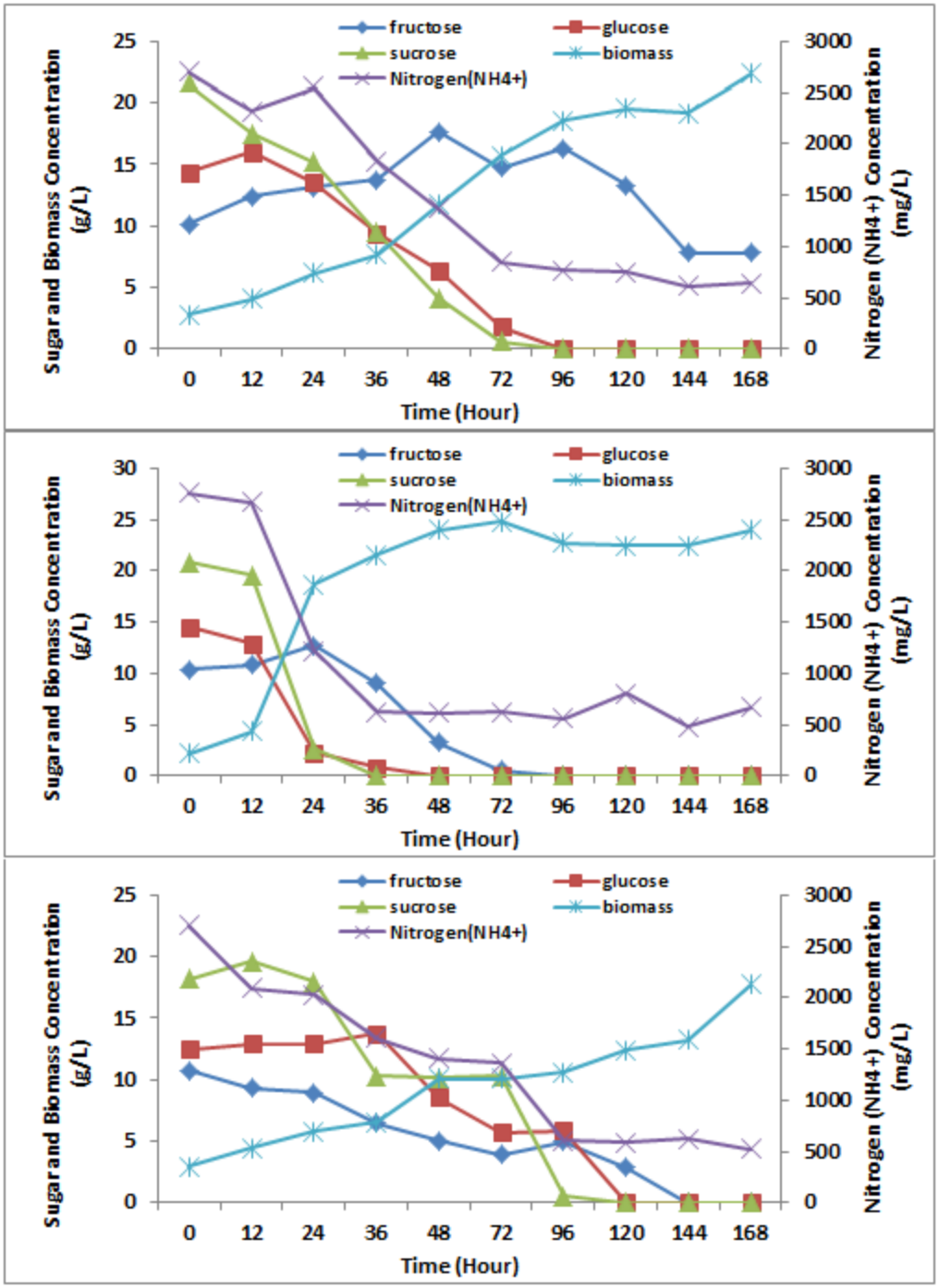

Figure 29 Nutrients utilization and biomass accumulation at C:N of 10 and temperature of 25°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

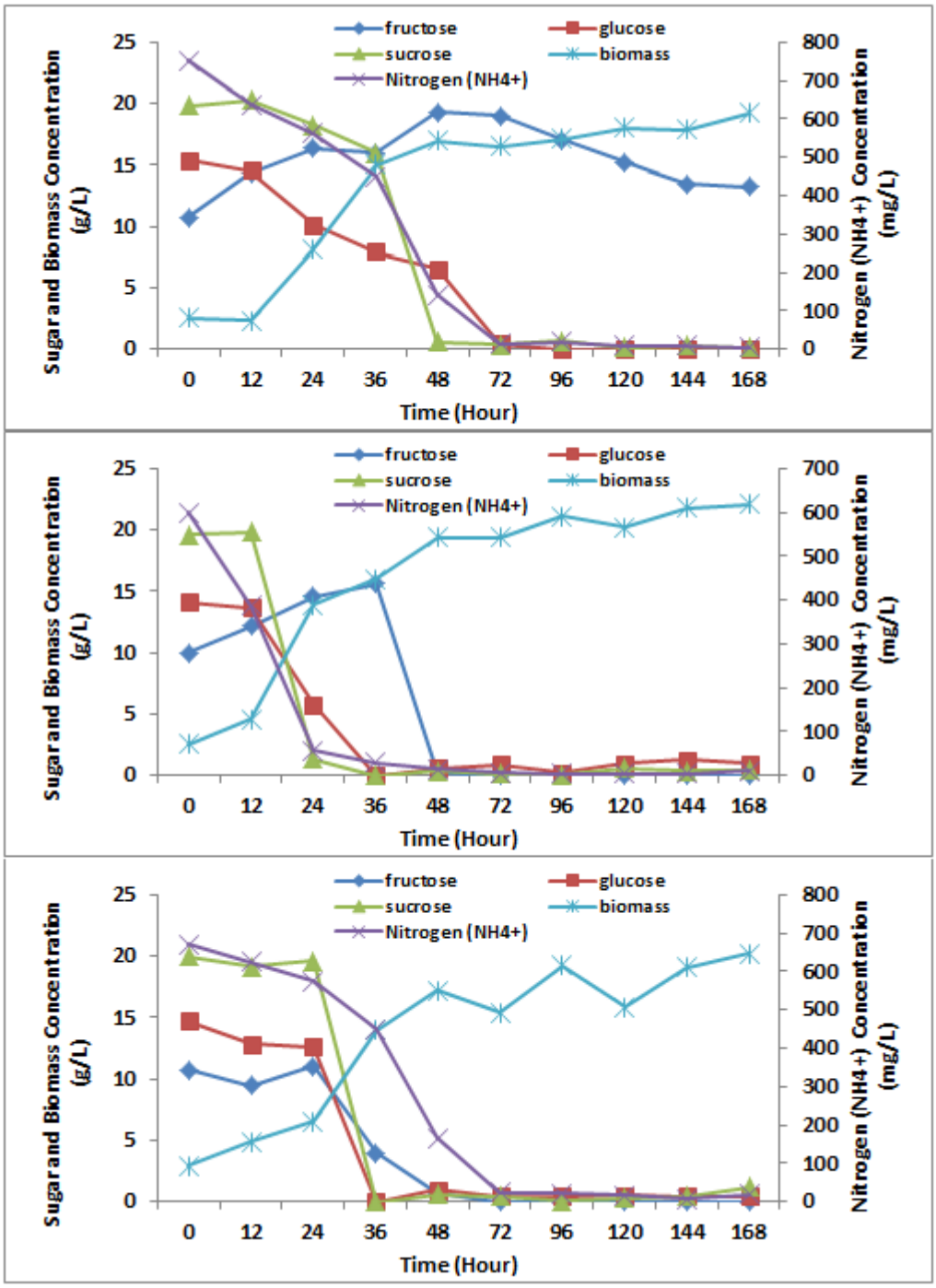

Figure 30 Nutrients utilization and biomass accumulation at C:N of 40 and temperature of 25°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

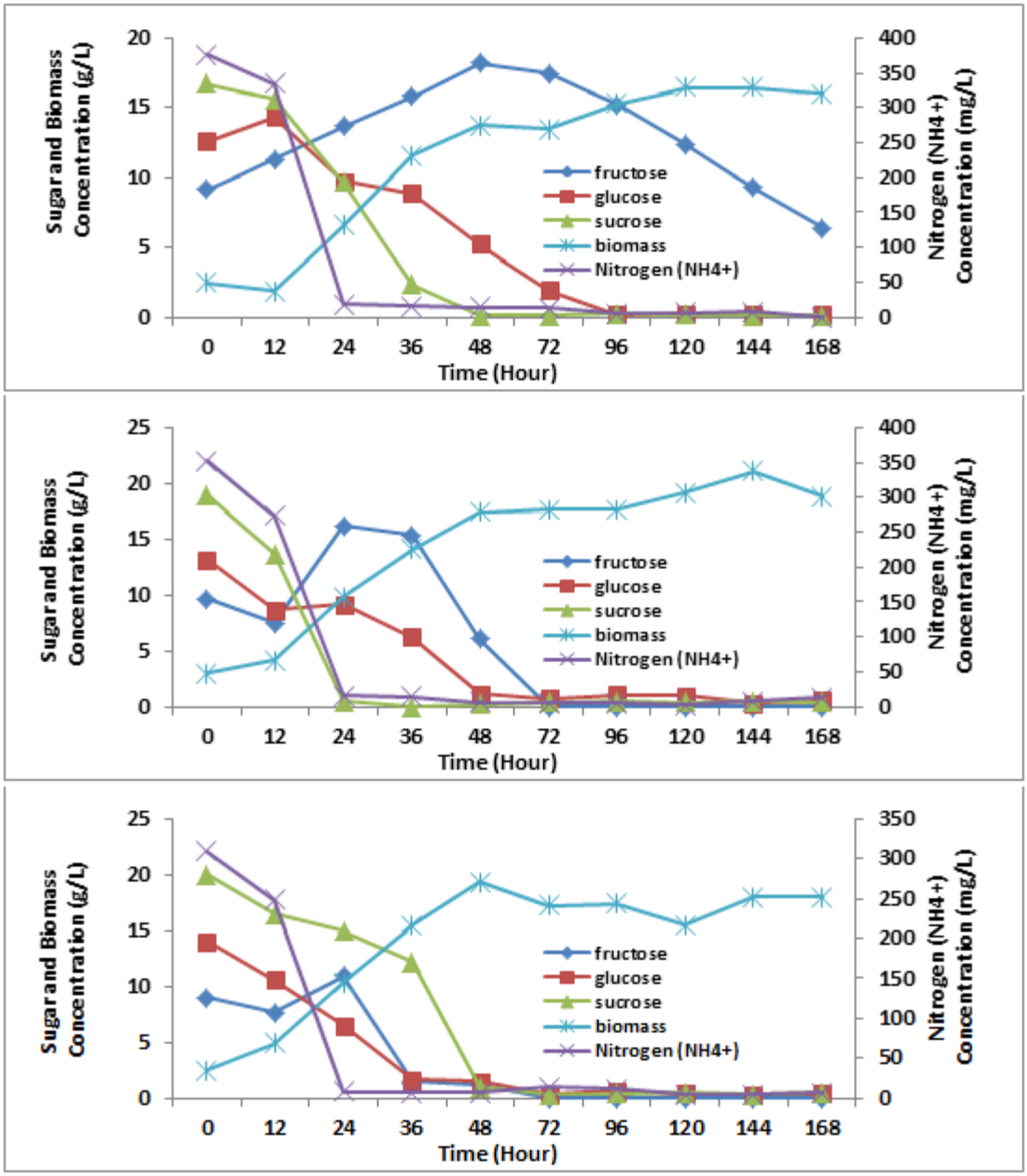

Figure 31 Nutrients utilization and biomass accumulation at C:N of 70 and temperature of 25°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

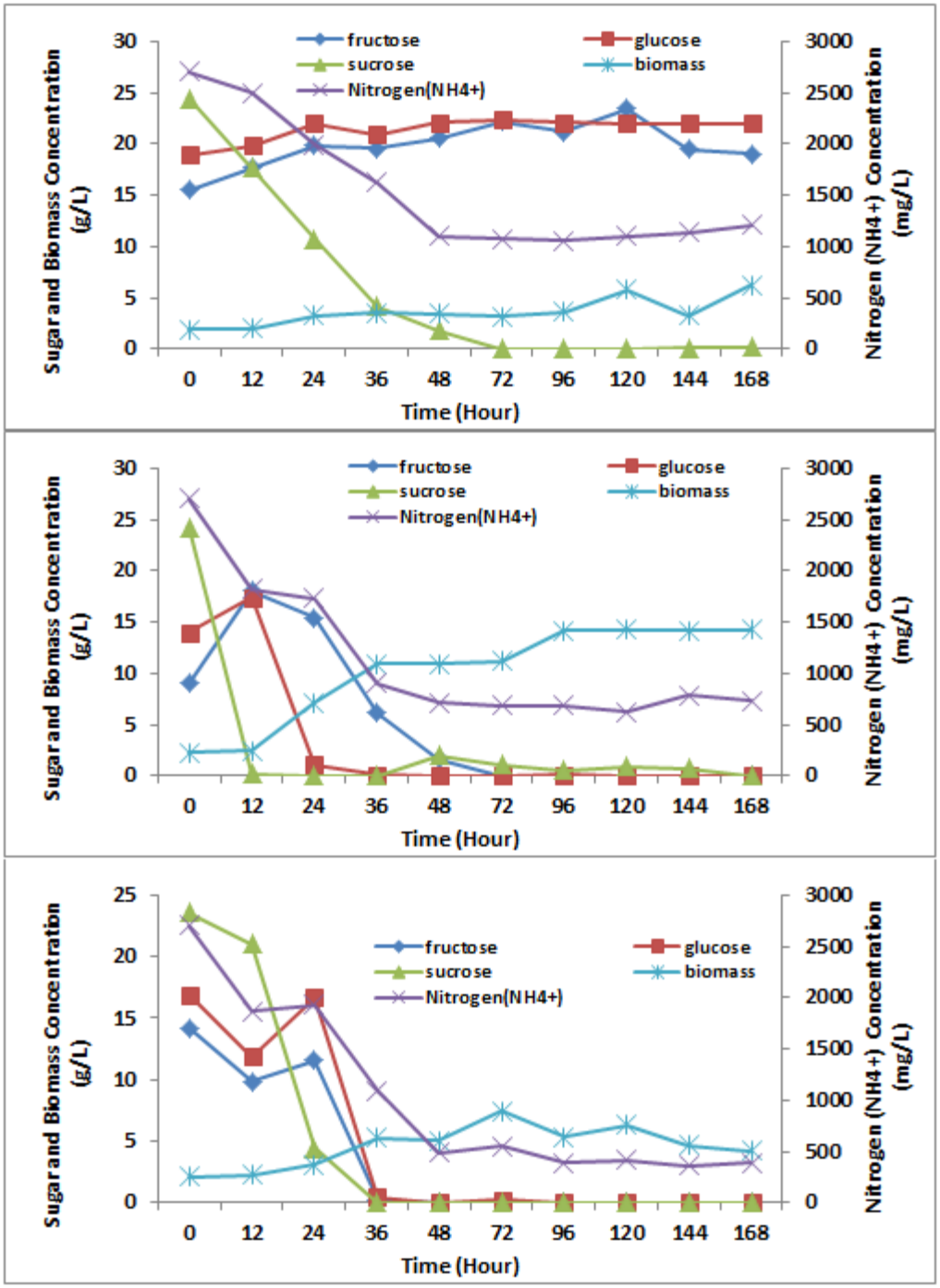

Figure 32 Nutrients utilization and biomass accumulation at C:N of 10 and temperature of 35°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

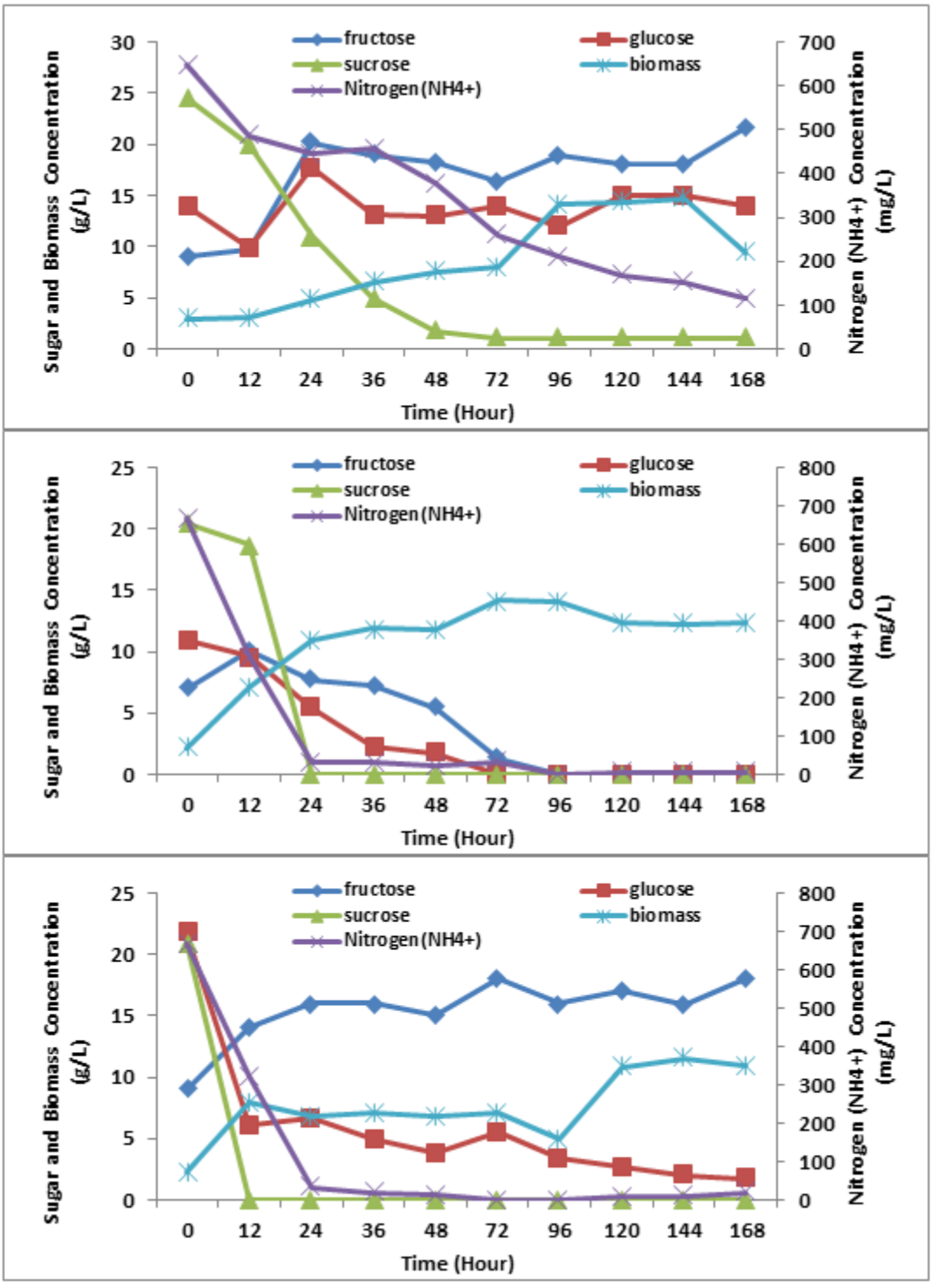

Figure 33 Nutrients utilization and biomass accumulation at C:N of 40 and temperature of 35°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

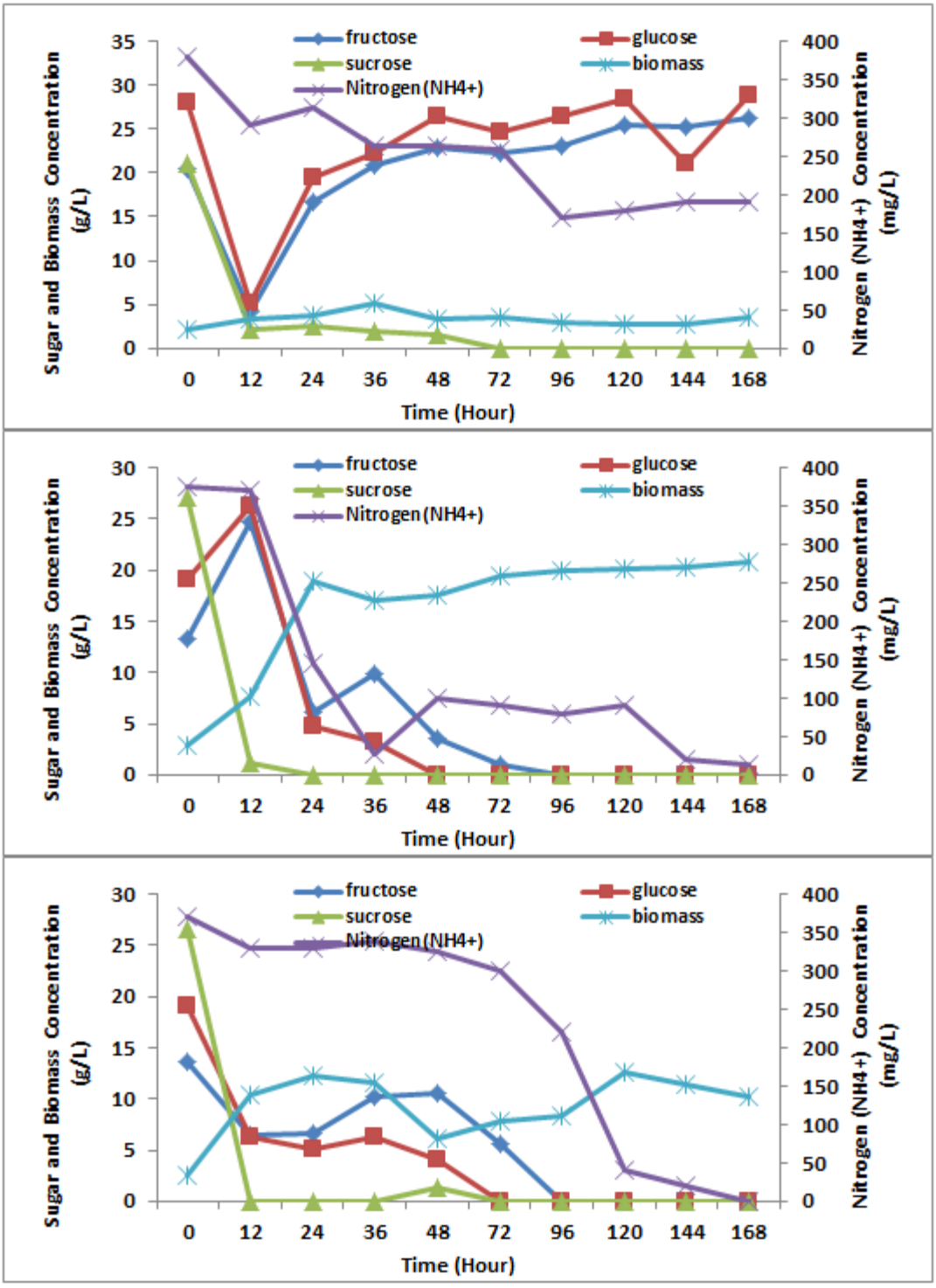

Figure 34 Nutrients utilization and biomass accumulation at C:N of 70 and temperature of 35°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

APPENDIX D

CAROTENOIDS ACCUMULATION PROFILE AT DIFFERENT FACTORS COMBINATIONS

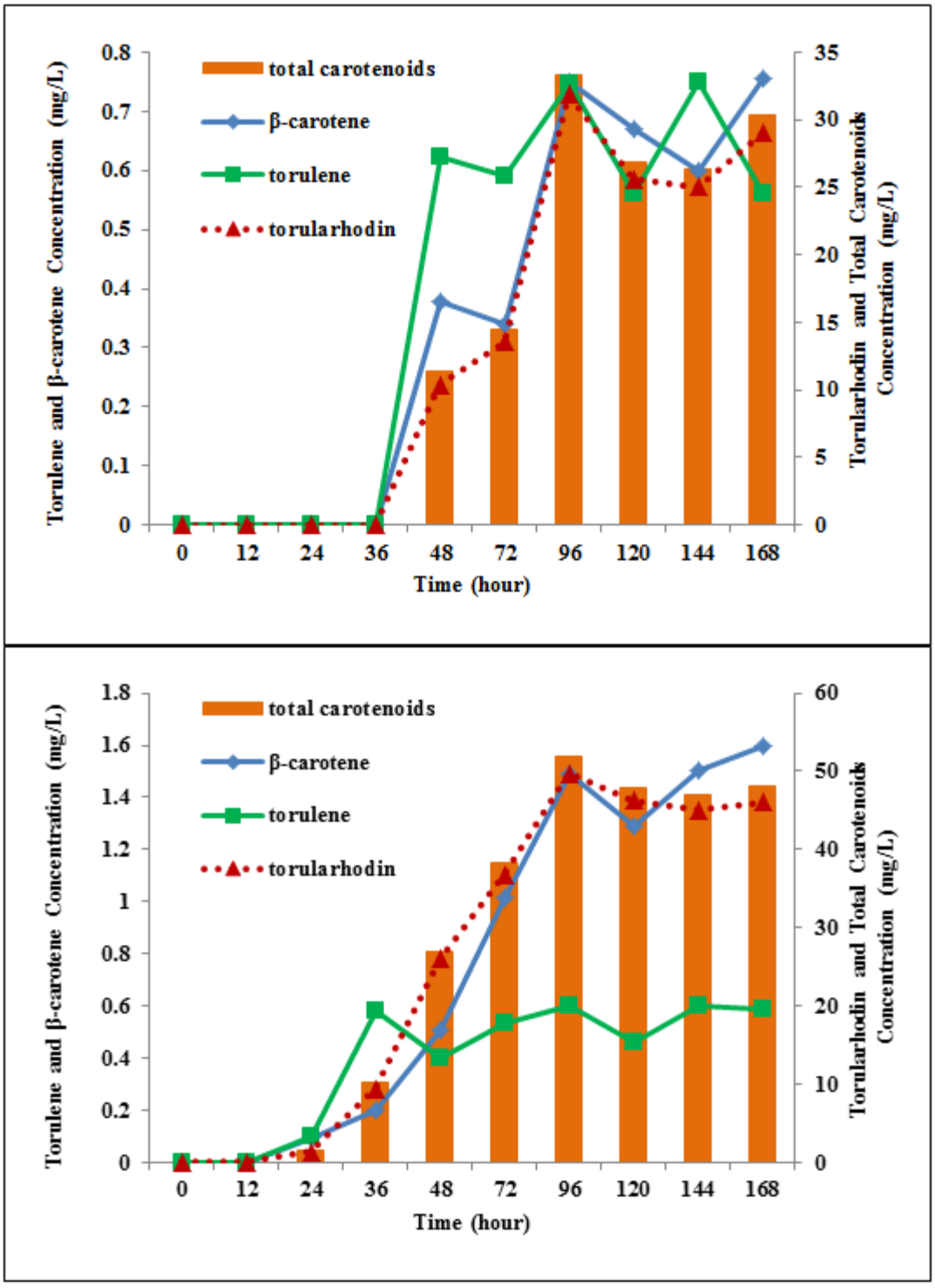

Figure 35 Carotenoid accumulation at C:N of 10 and temperature of 20°C pH 2.5 (top) 5.5 (bottom) and 8.5 (no carotenoid accumulation)

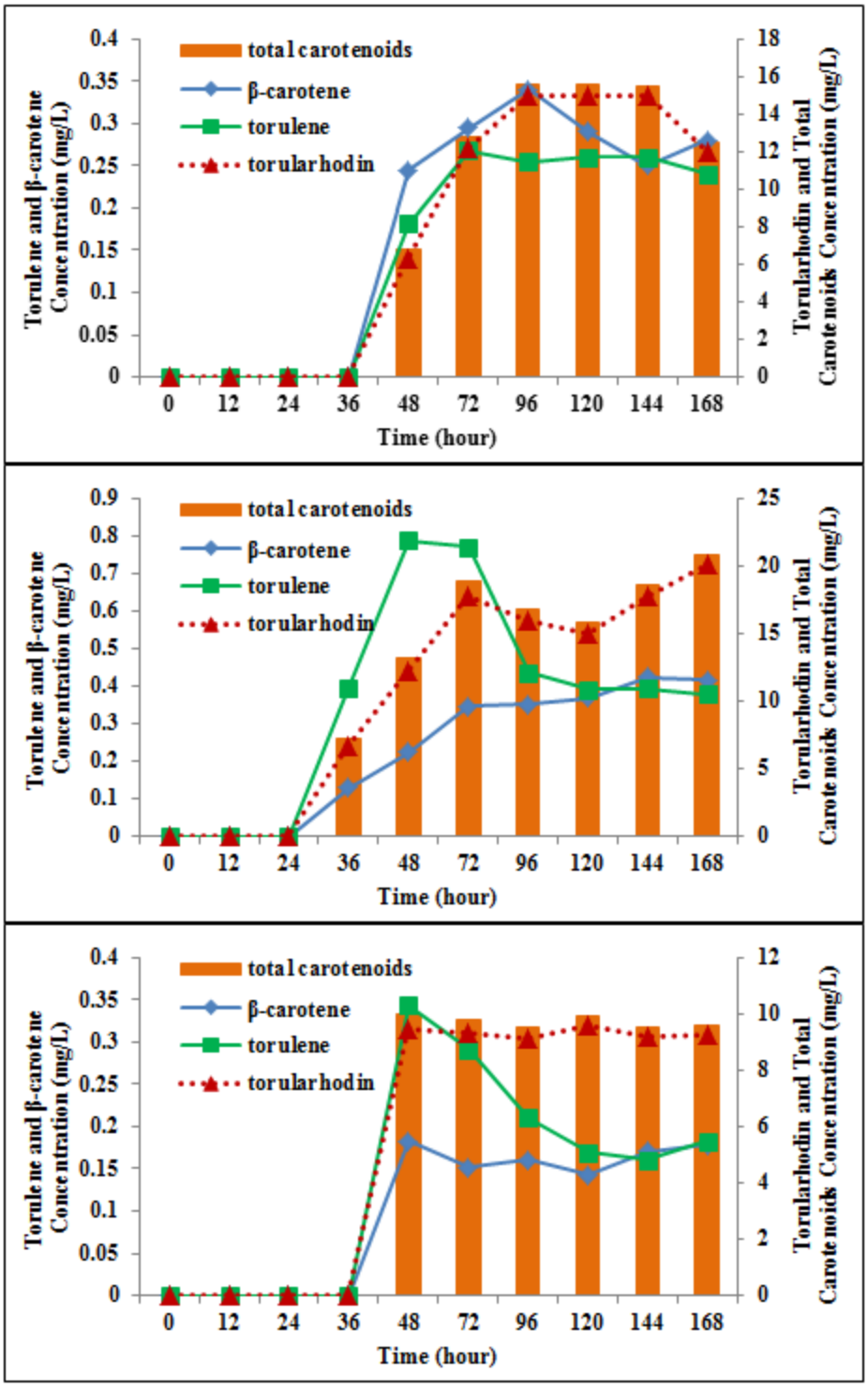

Figure 36 Carotenoid accumulation at C:N of 40 and temperature of 20°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

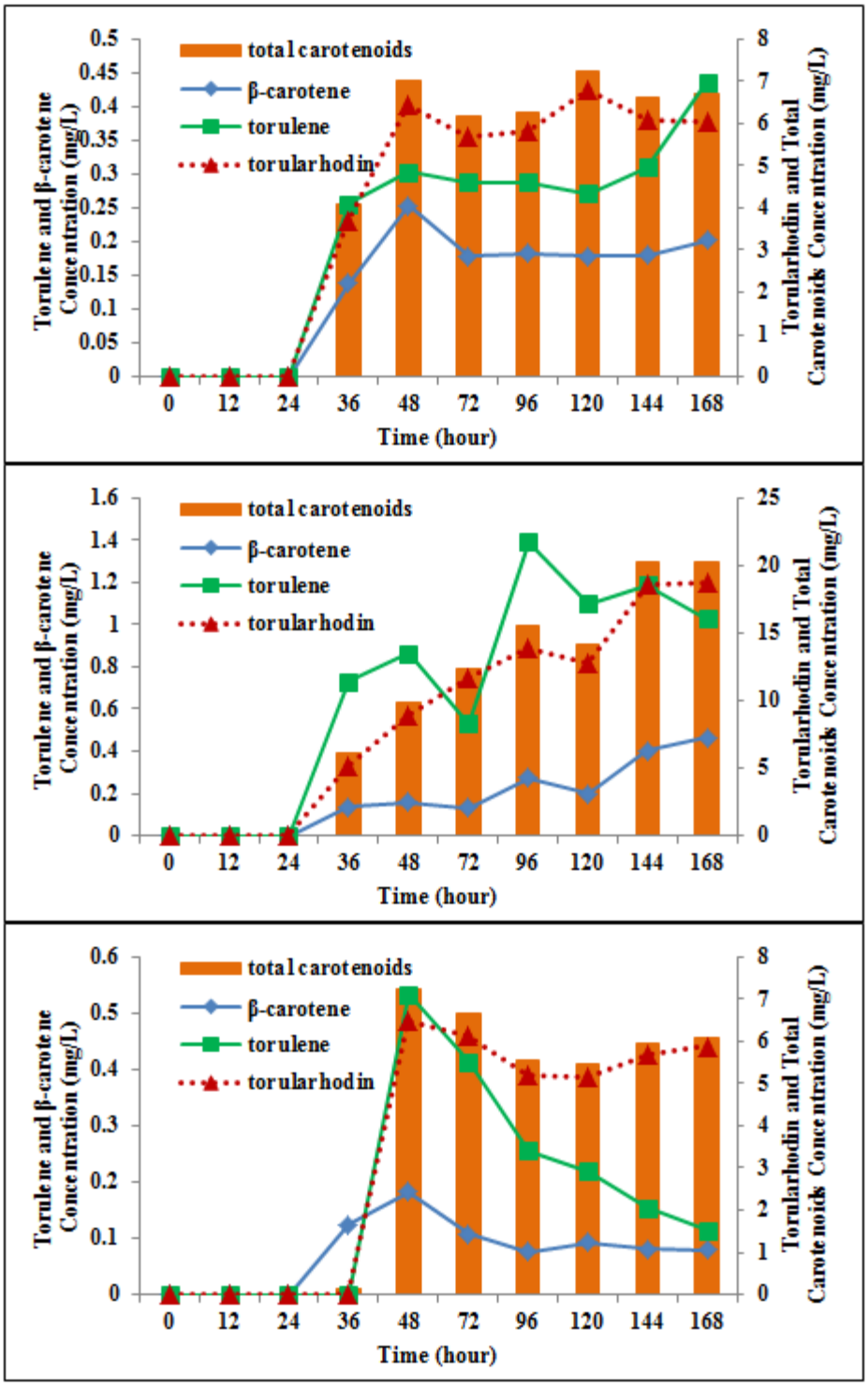

Figure 37 Carotenoid accumulation at C:N of 70 and temperature of 20°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

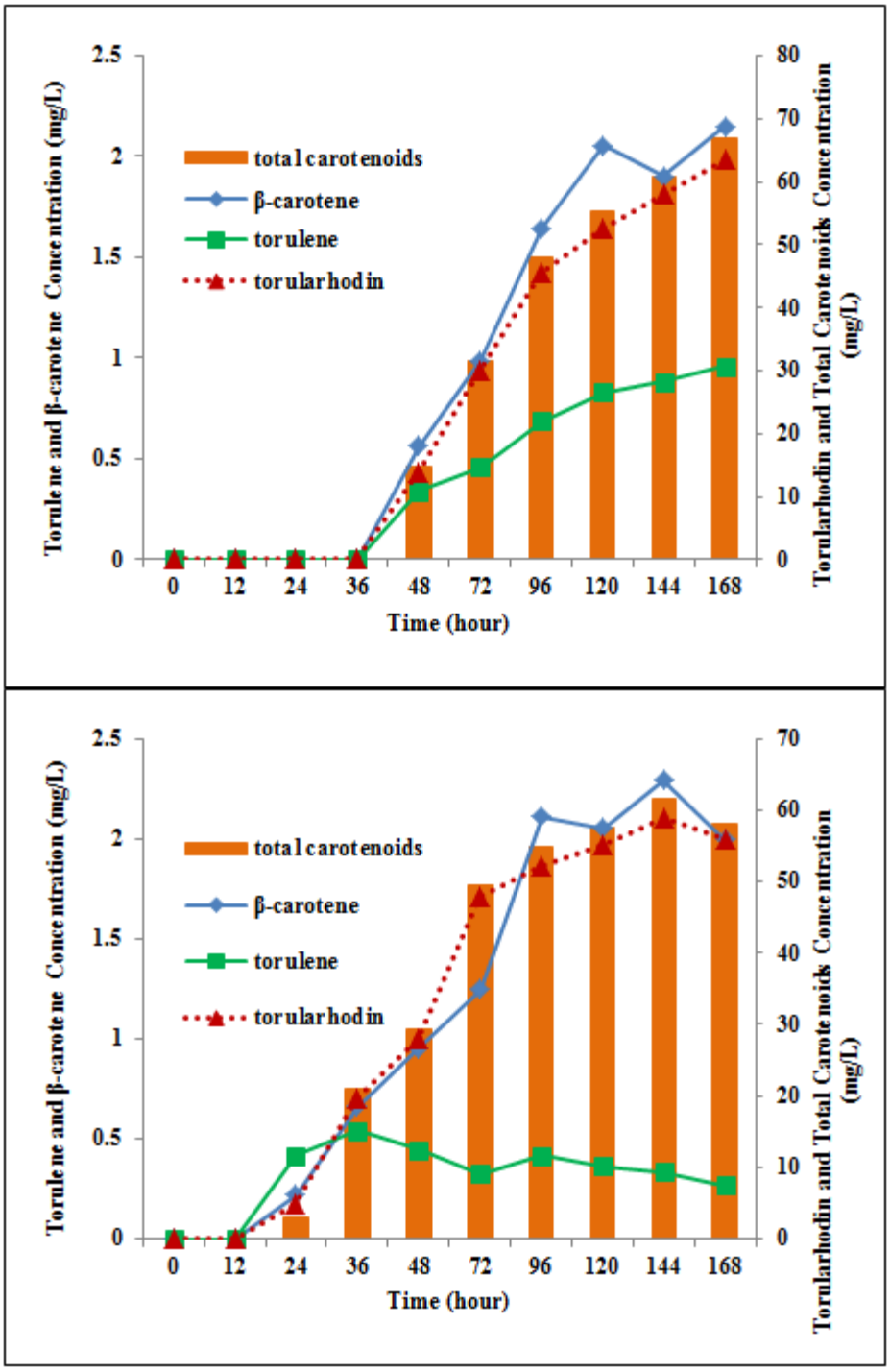

Figure 38 Carotenoid accumulation at C:N of 10 and temperature of 25°C pH 2.5 (top) 5.5 (bottom) and 8.5 (no accumulation)

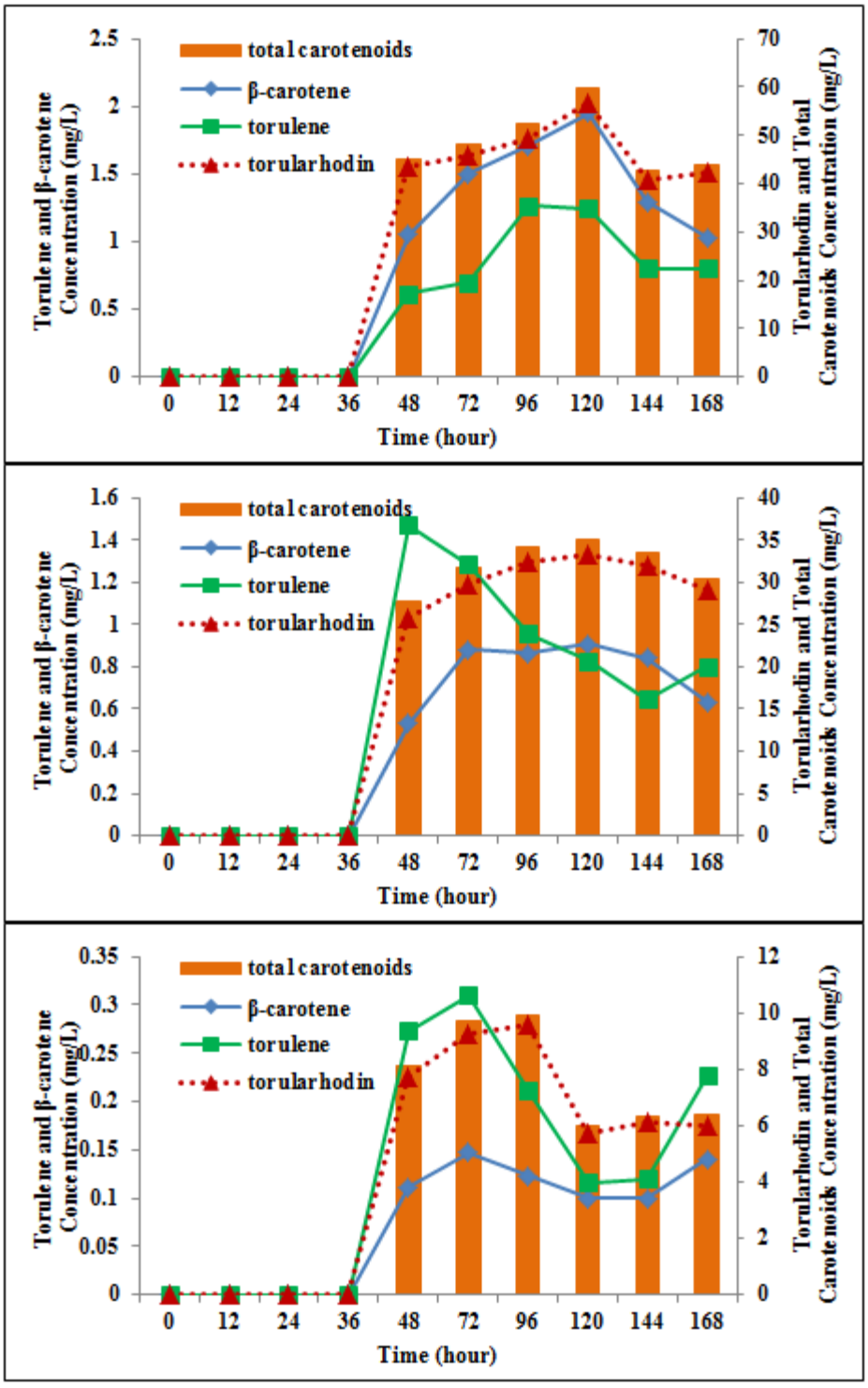

Figure 39 Carotenoid accumulation at C:N of 40 and temperature of 25°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

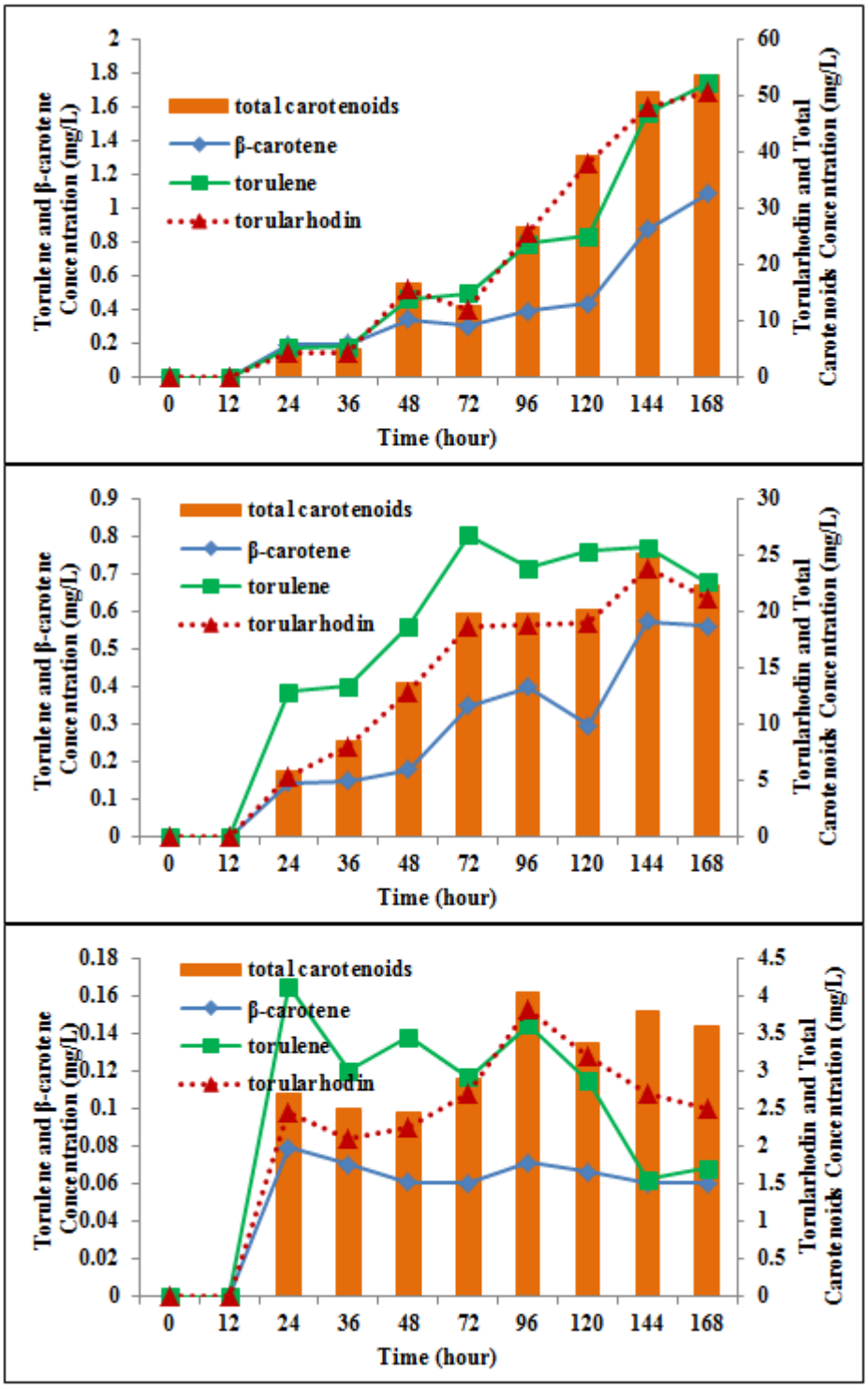

Figure 40 Carotenoid accumulation at C:N of 70 and temperature of 25°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

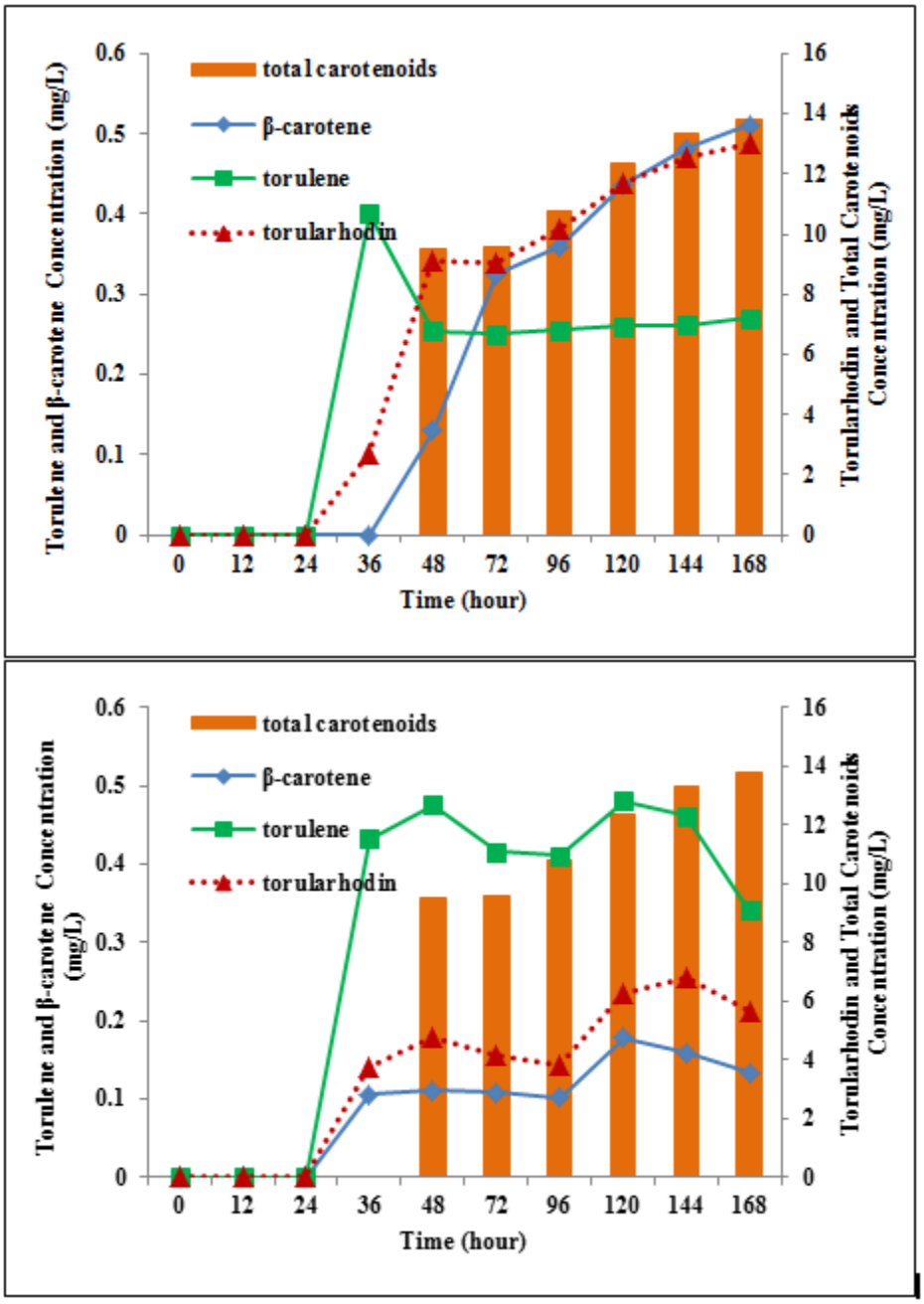

Figure 41 Carotenoid accumulation at temperature of 35°C pH 5.5 with C:N of 10 (top) and 70 (bottom)

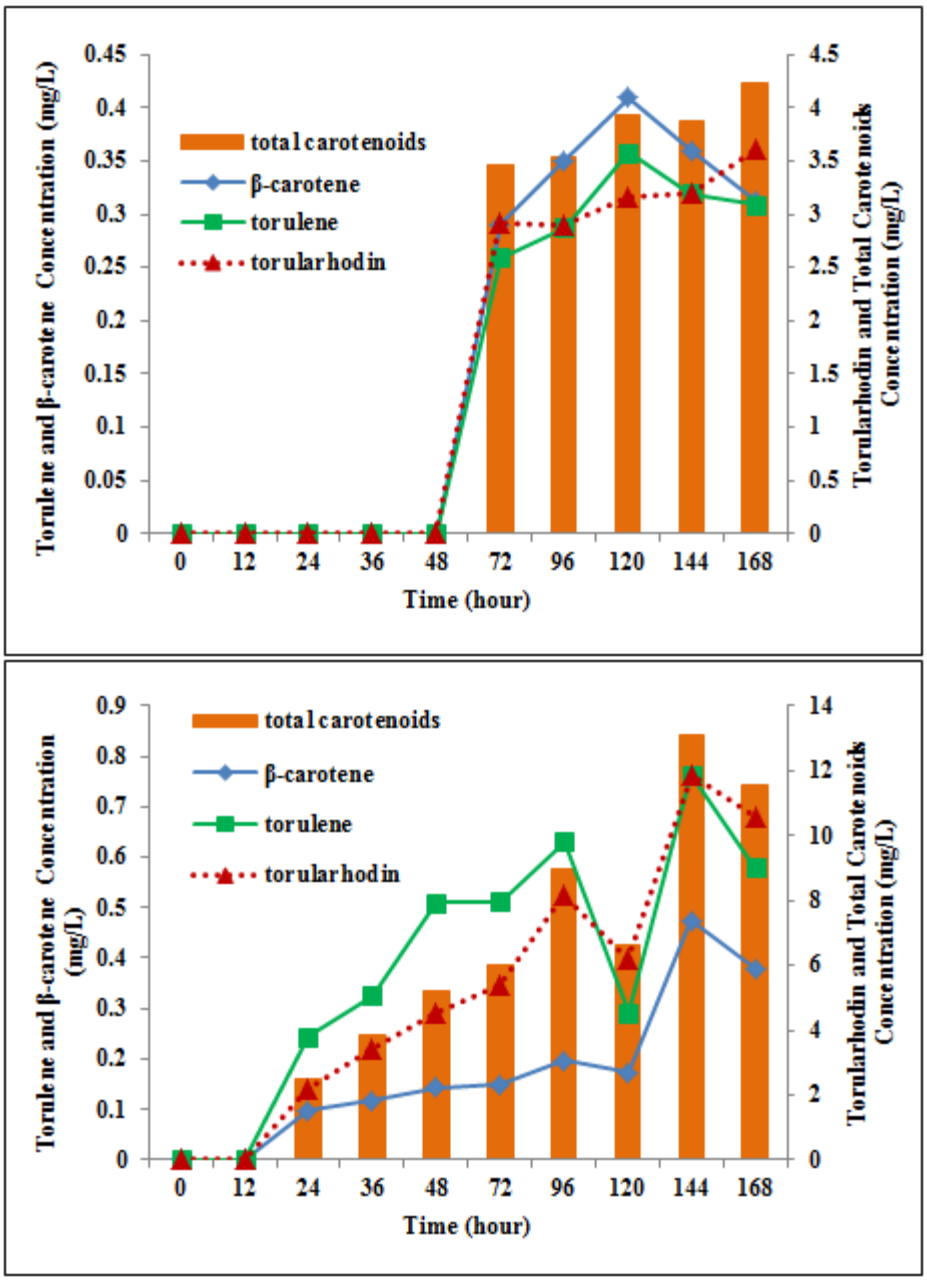

Figure 42 Carotenoid accumulation at C:N of 40 and temperature of 35°C pH 2.5 (top) 5.5 (bottom)

APPENDIX E

FAMEs ACCUMULATION PROFILE AT DIFFERENT FACTORS COMBINATIONS

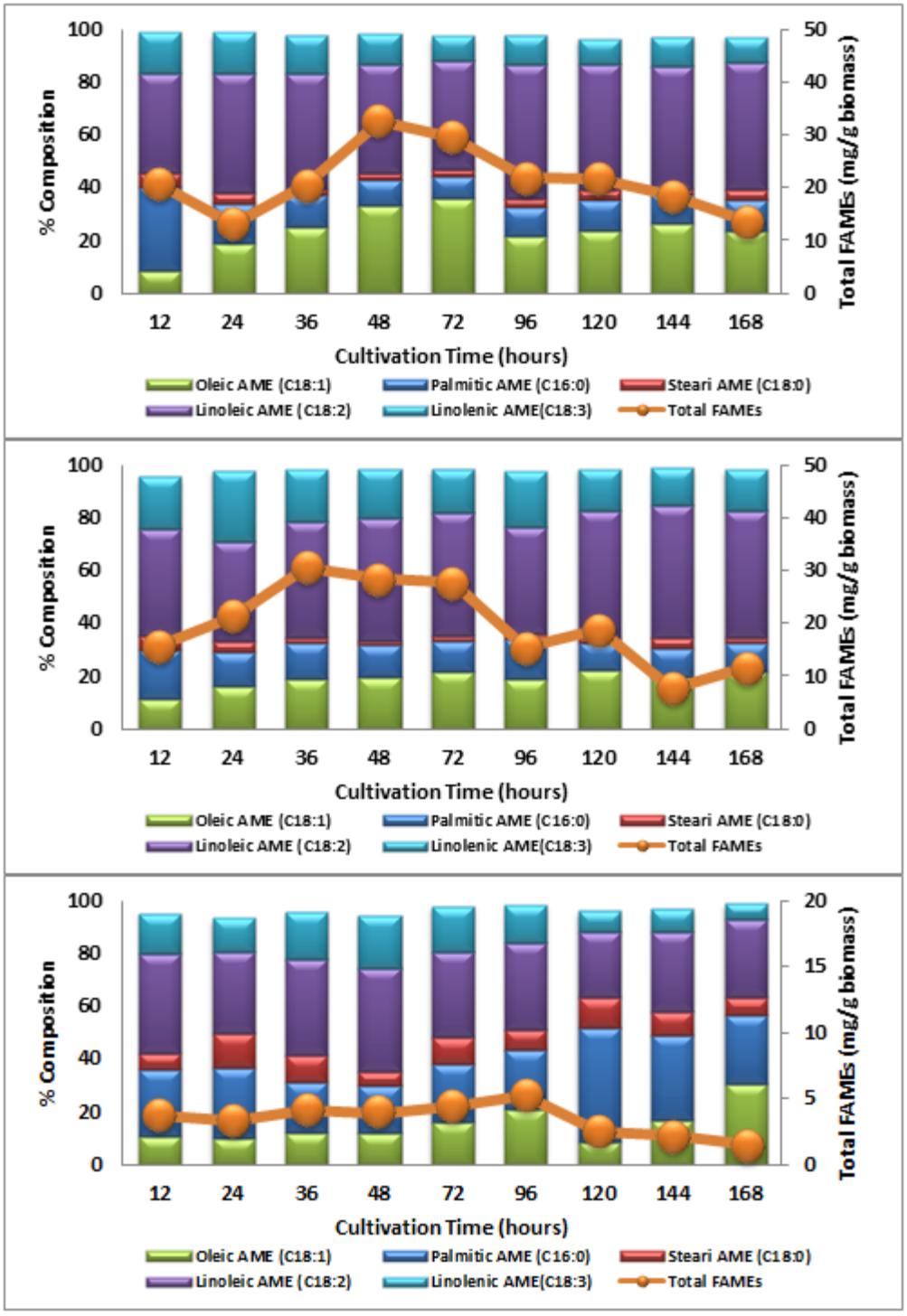

Figure 43 Fatty acid methyl ester accumulation at C:N of 10 and temperature of 20°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

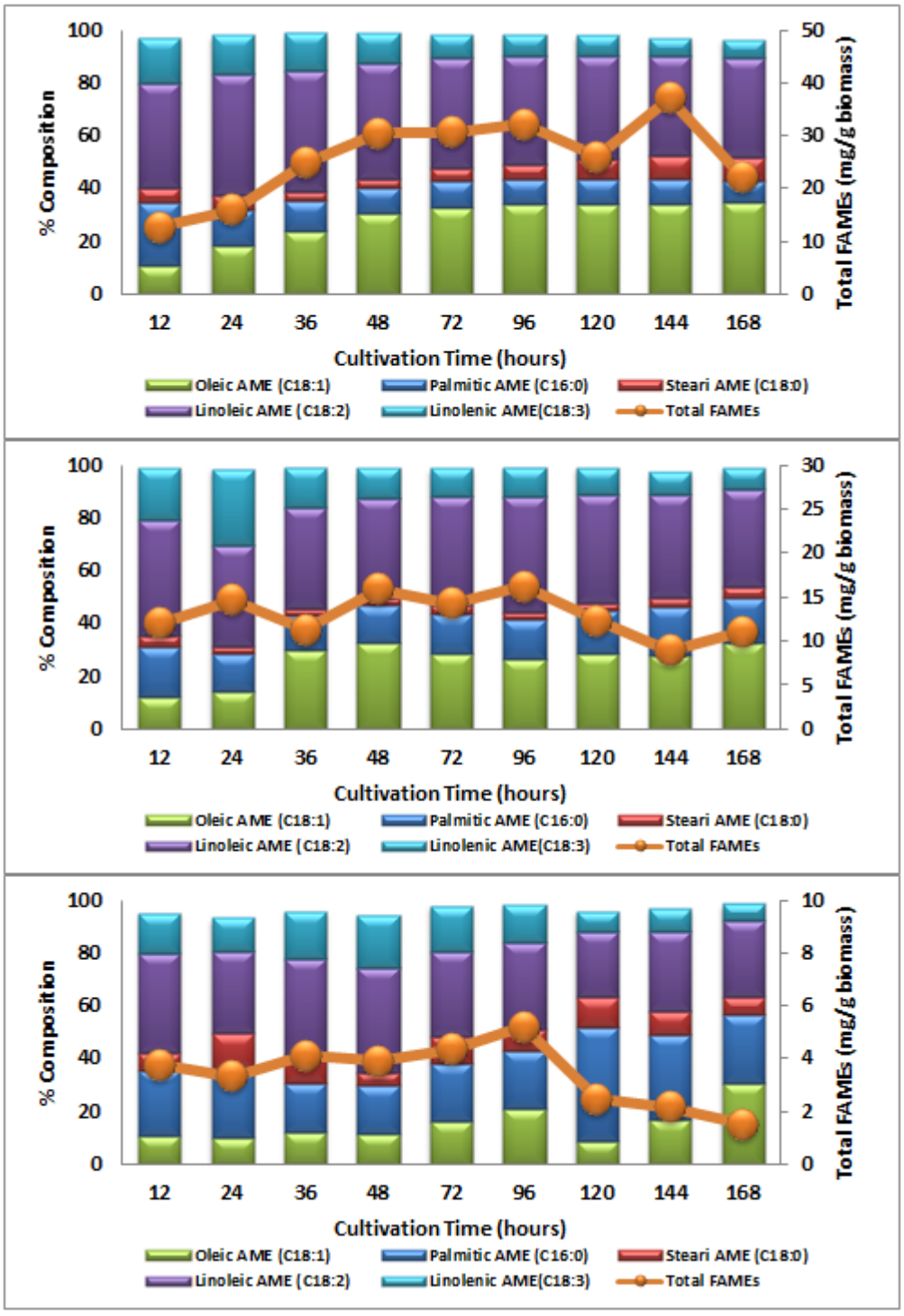

Figure 44 Fatty acid methyl ester accumulation at C:N of 40 and temperature of 20°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

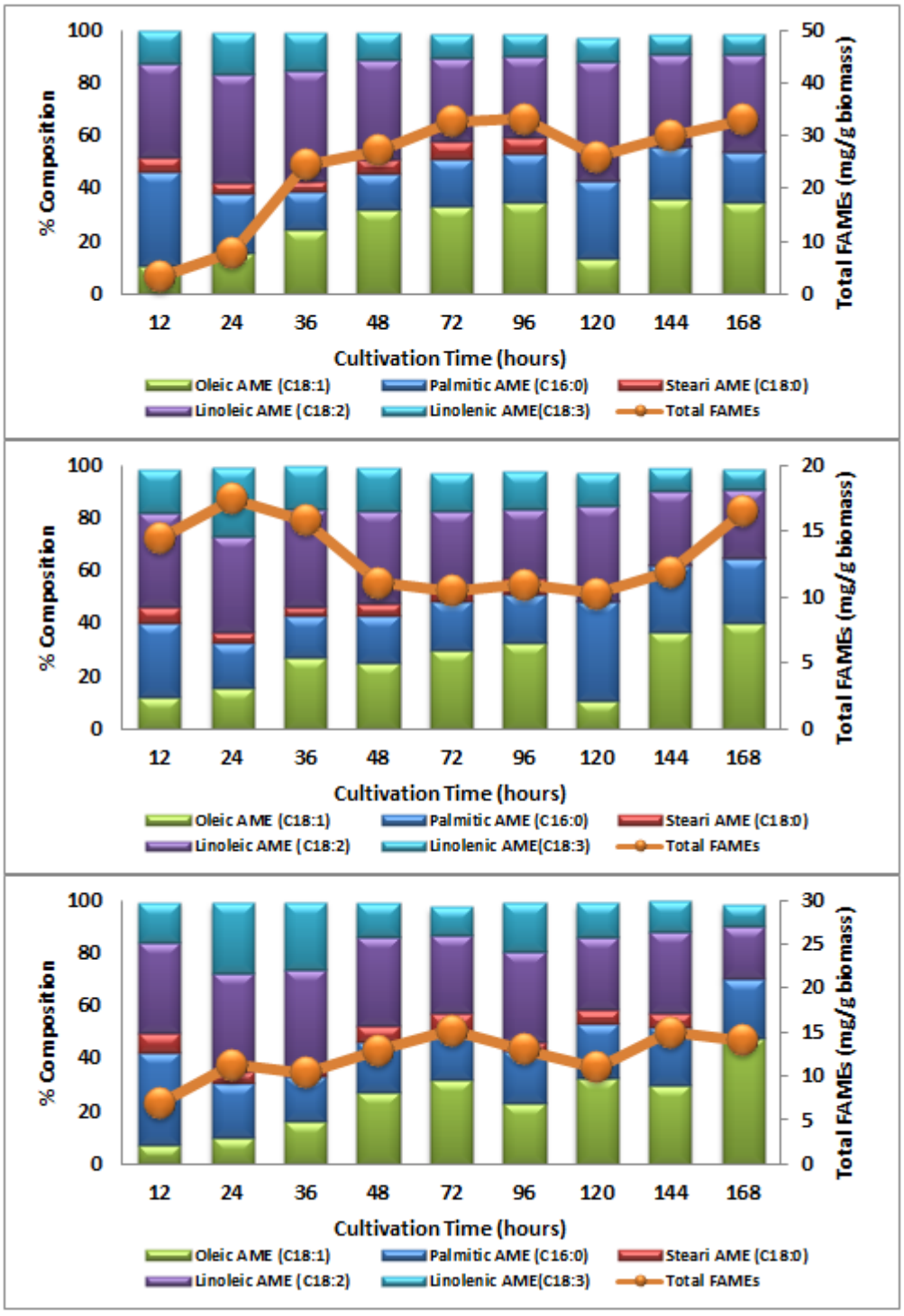

Figure 45 Fatty acid methyl ester accumulation at C:N of 70 and temperature of 20°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

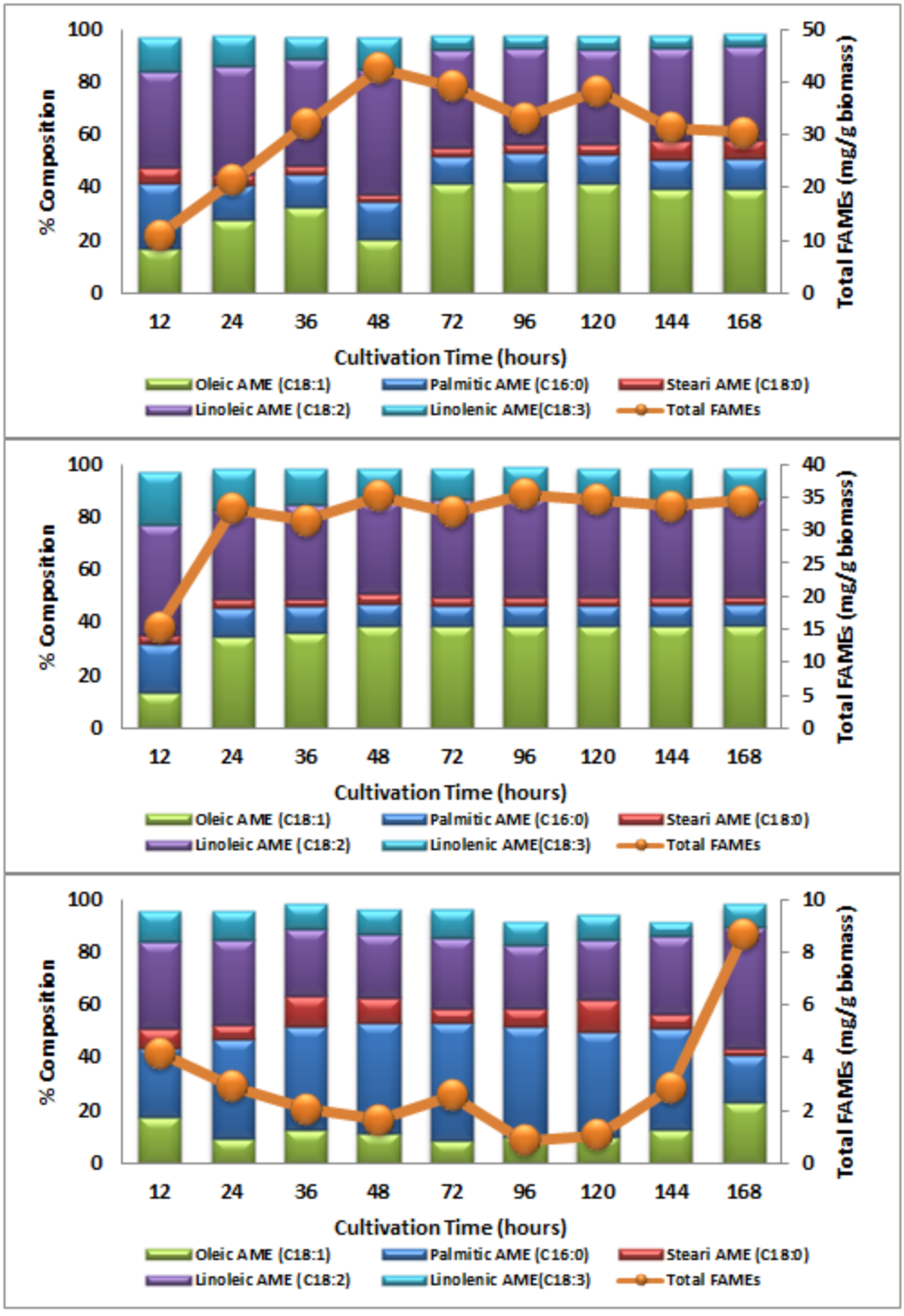

Figure 46 Fatty acid methyl ester accumulation at C:N of 10 and temperature of 25°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

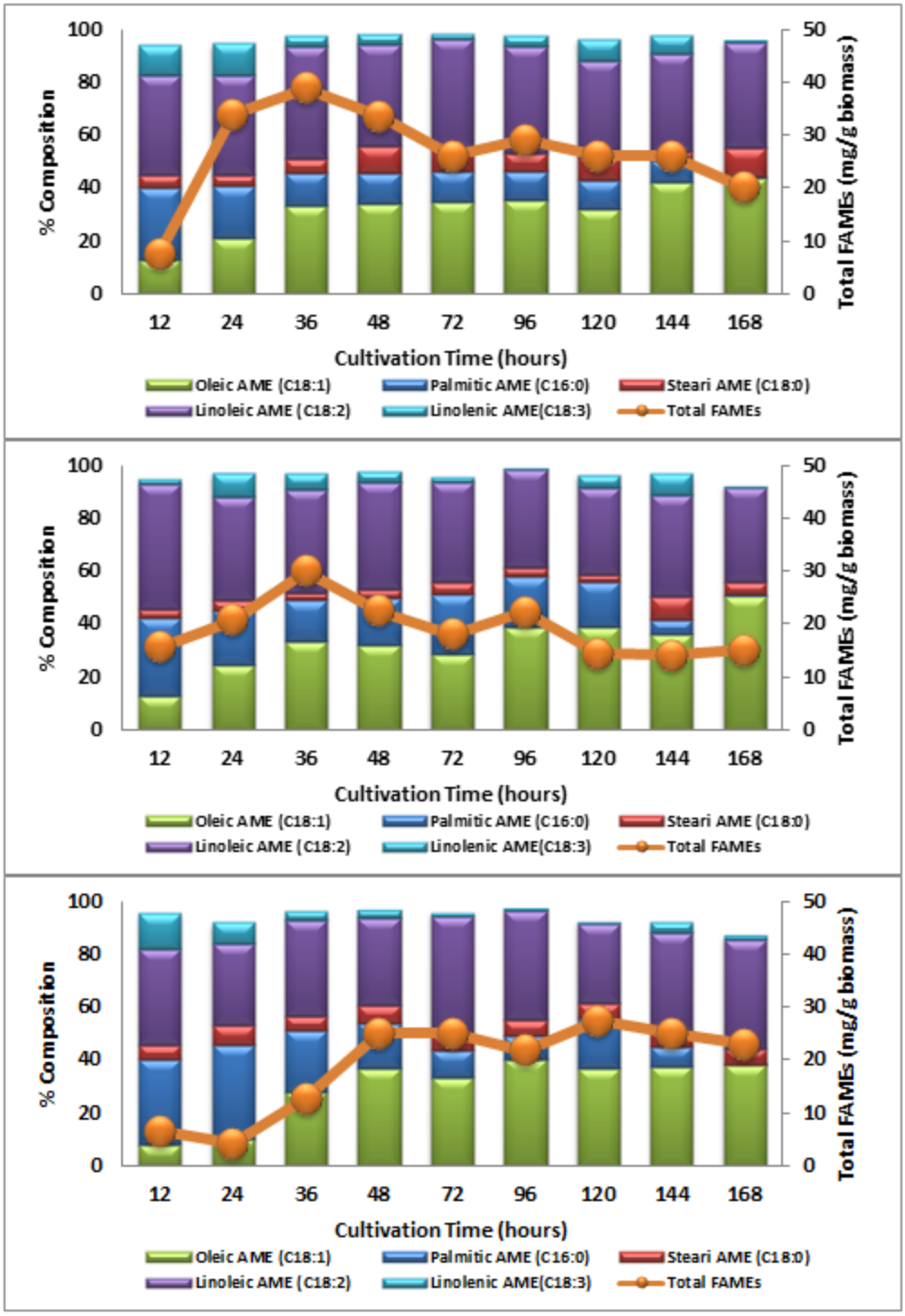

Figure 47 Fatty acid methyl ester accumulation at C:N of 40 and temperature of 25°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

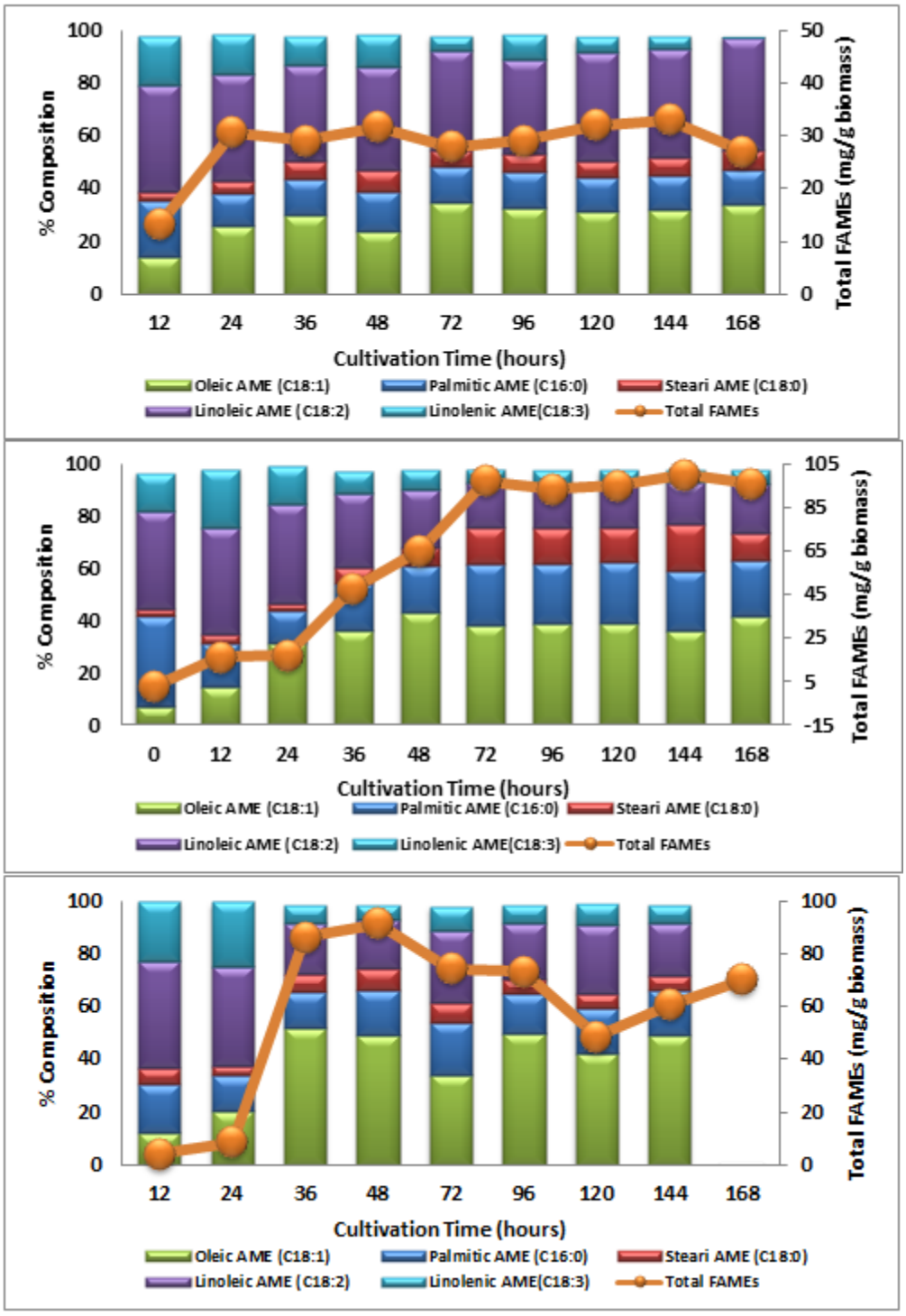

Figure 48 Fatty acid methyl ester accumulation at C:N of 70 and temperature of 25°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

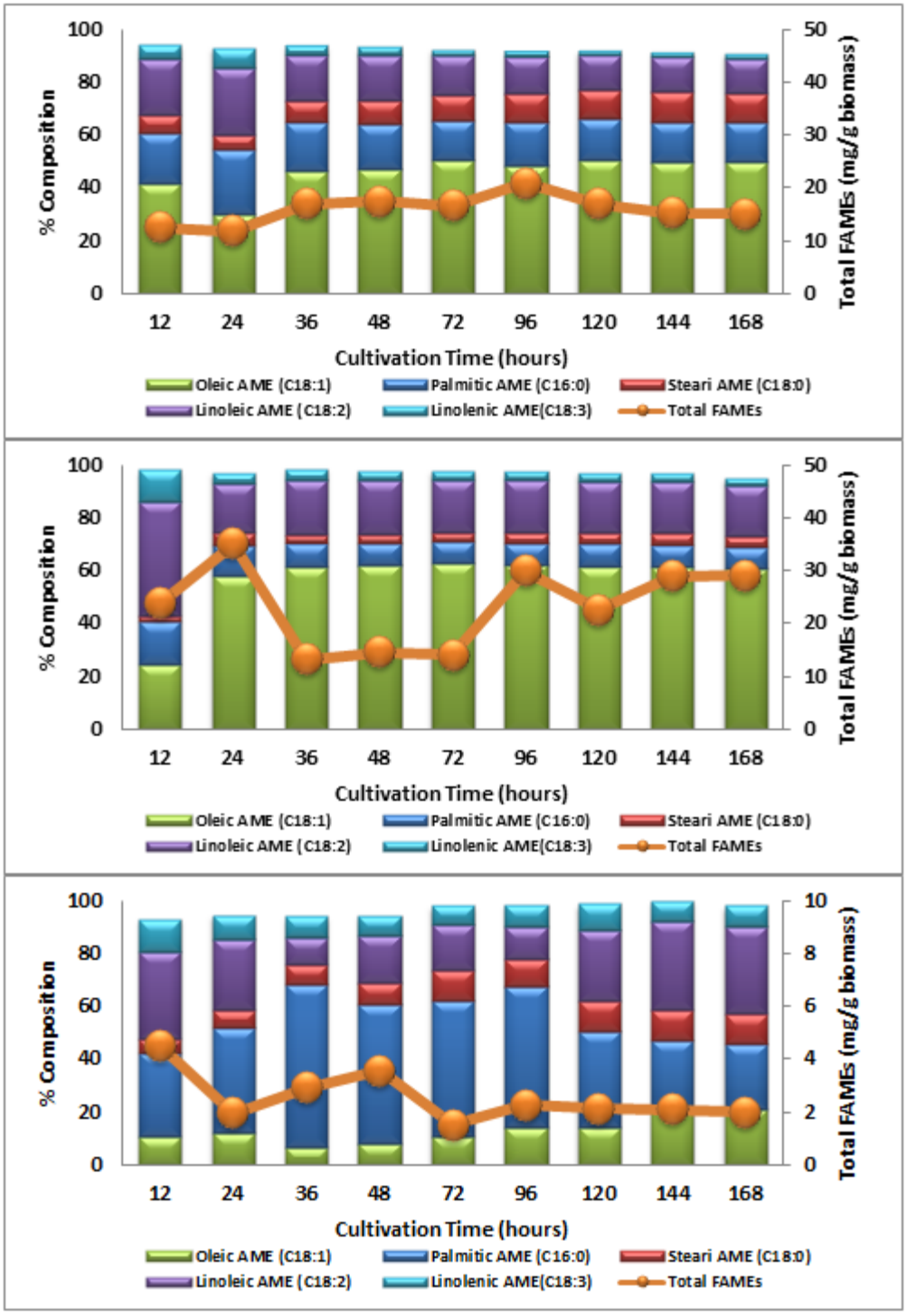

Figure 49 Fatty acid methyl ester accumulation at C:N of 10 and temperature of 35°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

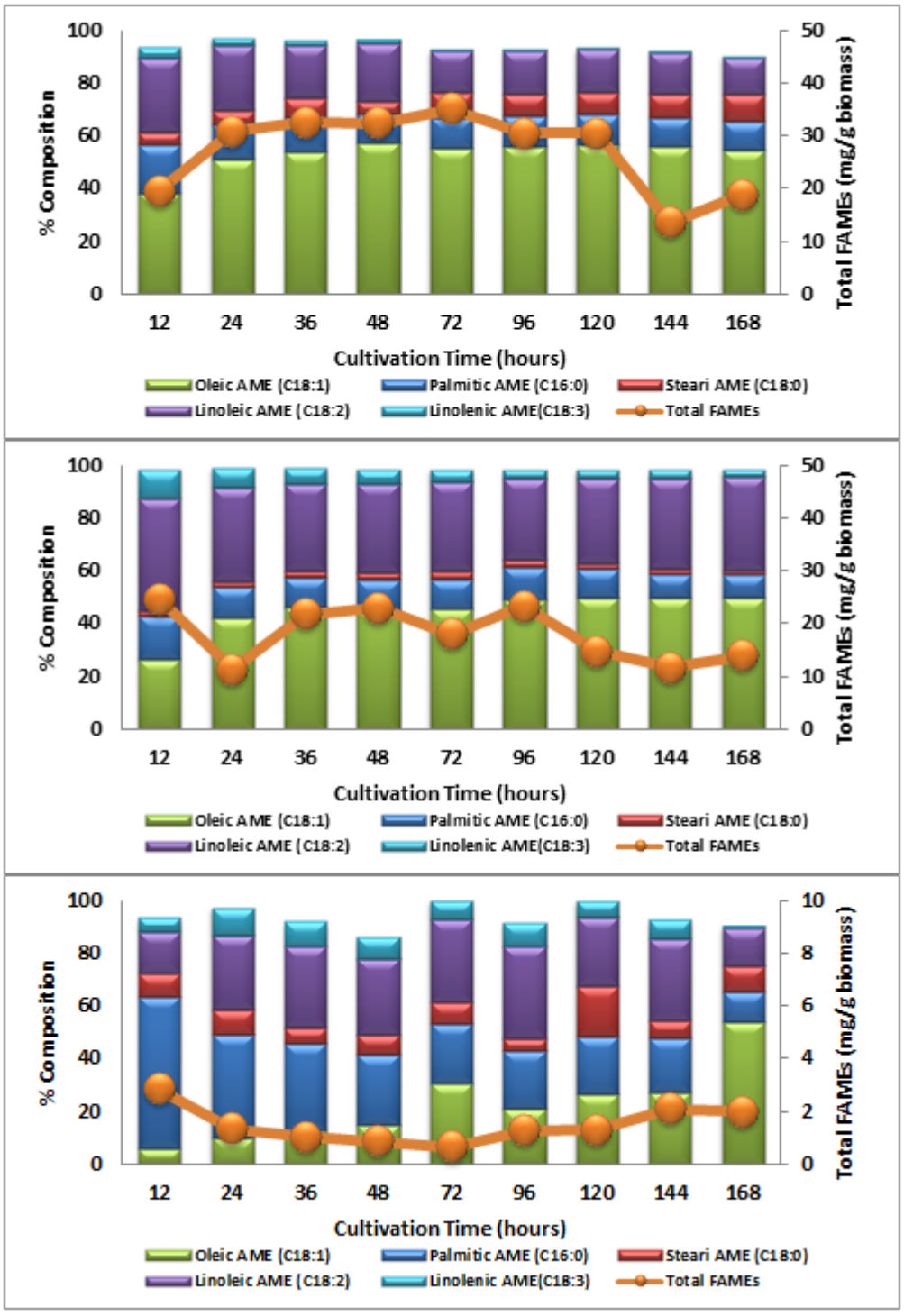

Figure 50 Fatty acid methyl ester accumulation at C:N of 40 and temperature of 35°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)

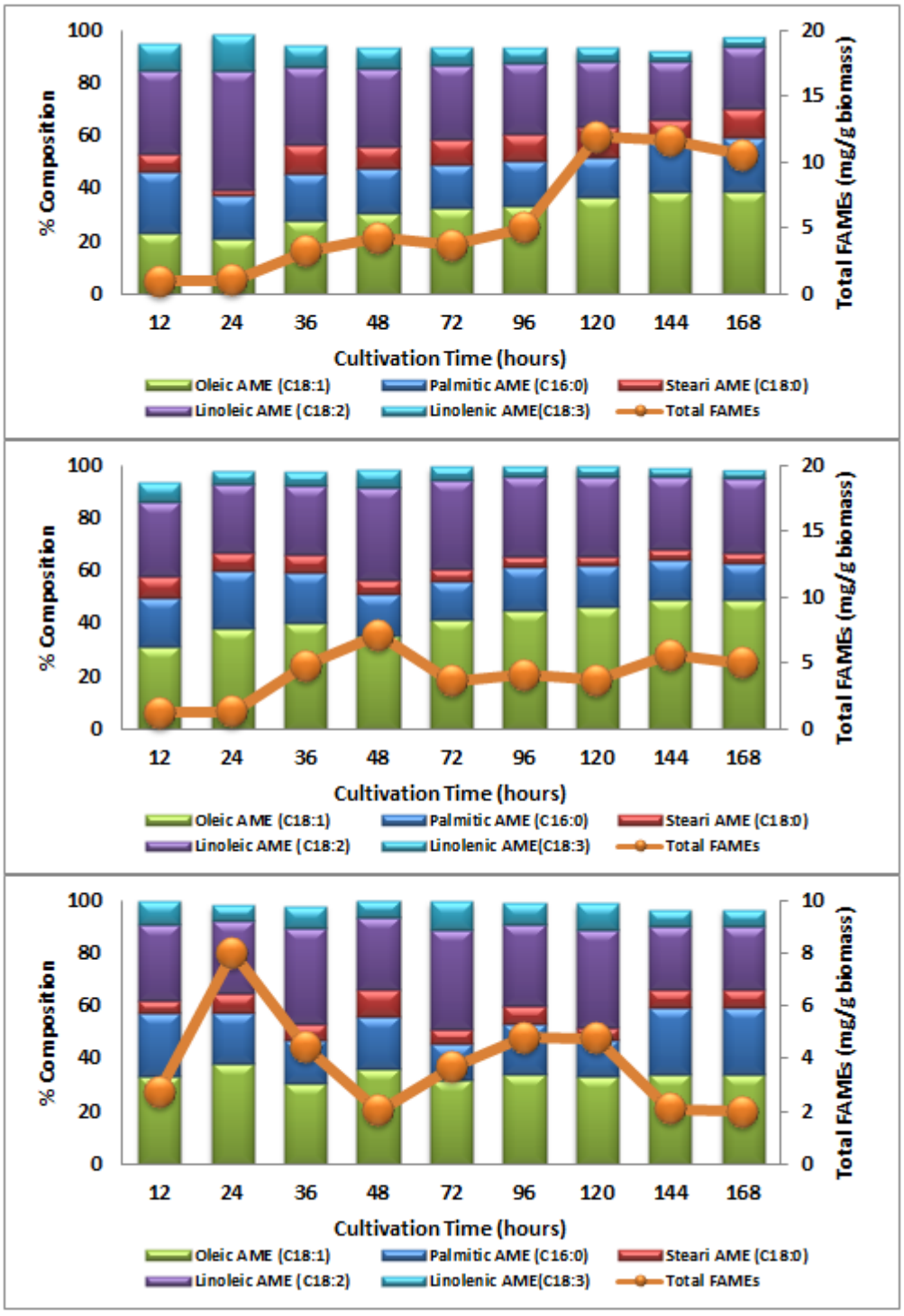

Figure 51 Fatty acid methyl ester accumulation at C:N of 70 and temperature of 35°C pH 2.5 (top) 5.5 (middle) and 8.5 (bottom)