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CO_2 Sequestration Using Sodium Hydroxide and Its Utilization for *Chlorella sorokiniana* Biomass Production

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Abstract. Chlorella is widely used for its fast growth rate and easy cultivation with 14–30% lipid content and 36–59% dry weight. Thus, sodium hydroxide is used to increase carbon consumption, biomass, and metabolites productions in microalgae. This study was conducted to observe the effect of sodium hydroxide addition on biomass and metabolites production in photoautotrophic cultivated *Chlorella sorokiniana*. Microalgae *C.sorokiniana* (LIPI12-Al016) was obtained from the culture collection of Microalgae and Bioprocess Engineering Research Group laboratory, National Research and Innovation Agency. Then, the microalgae were cultivated in media with various concentrations of sodium hydroxide. Biomass production was measured by gravimetry, and carbon consumption was measured by acid-alkalimetry. Sodium hydroxide 60 mM gave the best growth, maximizing average carbon consumption to 691.8 $mg.L^{-1}$ and biomass production to 598.3 $mg.L^{-1}$. The utilization of NaOH in the medium did not increase the metabolites content, except for protein. Carbohydrate was the dominant metabolite among the others. Fatty acids profile mainly composed of C16 and C18 fatty acids, which are favorable for biodiesel production. These results gave an overview of the potency of microalgae *C. sorokiniana* as a CO_2 mitigation agent and alternative sources of energy and nutrition.

Key words: biomass, CO_2 consumption, *C. sorokiniana*, metabolites, sodium hydroxide

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INTRODUCTION

Excessive carbon dioxide (CO2) gas emission in the atmosphere due to the rapid industrial revolution has become a crucial issue of environmental concern. The CO2 emission from human activities contributes 68% in greenhouse gas emission, and around 40% of it was generated by fossil fuel power plants in 2015 (Cheng et al., 2019; Kwak et al., 2016). The average CO2 concentration was also 40% higher than in the 1800s and will be about twice as high in the 2050s (Song et al., 2019; Tu et al., 2019). The global average temperature increase can also reach 2°C in 2050 if CO2 gas is not reduced by more than 50% nowadays (Kwak et al., 2016). One of the methods to reduce CO2 gas emission in the atmosphere is through conversion into organic material with biological mitigation process (Kassim & Meng, 2017; Molazadeh et al., 2018). Microalgae have been widely studied as a CO2 gas mitigation agent because it has various potential capabilities.

Microalgae have a faster growth rate and can absorb 10-50 times more CO2 gas than terrestrial plants with 36–59% dry cell weight (Lakshmikandan et al., 2019; Tu et al., 2019).It generally can live as colonies or solitary in open waters with sunlight such as lakes, rivers, or the sea (Cheng et al., 2019; Li-beisson et al., 2019). Most microalgae species are autotrophic and photosynthetic, producing complex carbon compounds from CO2 and light energy (Cheng et al., 2019). However, it is still the main concern because 80-90% of dissolved CO2 in the autotrophic system is possible to get back to the atmosphere (Nayak et al., 2013). The use of sodium hydroxide (NaOH) solution in the cultivation medium can be a method to strengthen CO2 retention (Nayak et al., 2013; Song et al., 2019). The improvement in autotrophic cultivation also promotes biomass production and makes more possibilities for the use of microalgae.

Different species of microalgae can produce various types of useful metabolic products. Some pigments are used as food coloring because it is safe and healthy for the human body (Neto et al., 2018). Important polysaccharides have also shown health-promoting effects for medical uses, such as reducing blood lipids (Ru et al., 2020). The alternative food, feed, or even fuels become more promising by utilizing microalgae that contain large amounts of lipid (Praharyawan et al., 2016; Zhu et al., 2016). Some microalgae species such as Botryococcus braunii can accumulate lipid > 75% of dry cell weight (Yeesang & Cheirsilp, 2014). Under certain environmental conditions, microalgae can change the biosynthetic pathway from synthesizing membrane compounds to the accumulation of storage lipid (Ajjawi et al., 2017). Many studies have been carried out to increase the amount of lipid content in microalgae cells.

Chlorella is better known in many studies as microalgae for protein and lipid content production (Ma et al., 2017). *Chlorella* is easy to cultivate with a fast growth rate and 14–30% total lipid content under optimal nutrition (Kong et al., 2020). Moreover, the total protein content in *Chlorella* can be up to 43% dry weight according to the growth conditions, and 12-55% for total carbohydrate content (Ru et al., 2020). The involvement of NaOH solution also facilitates cell growth by absorbing CO_2 into bicarbonate ions (HCO_3-) (Yu et al., 2021). However, HCO_3– as weak acid can lower the intracellular pH of the cytoplasm at a high concentration of dissolved

Table 1. The chemical composition of four different cultivation media from the NIES collection

Concentration ($mg.L^{-1}$)	TAP	F2	AF6	BG11
Tris (hydroxymethyl) aminomethane	2420	-	-	-
Acetic acid	1049.1	-	-	-
Na_2CO_3	-	-	-	20
Citric acid	-	-	2	6
Fe-citrate	-	-	2	6
NH_4Cl	400	-	-	-
NH_4NO_3	-	-	22	-
$NaNO_3$	-	75	140	1500
$NaH_2PO_4 \cdot 2H_2O$	-	6	-	-
K_2HPO_4	119	-	5	40
KH_2PO_4	60.3	-	10	-
$MgSO_4 \cdot 7H_2O$	100	-	30	75
$CaCl_2 \cdot 2H_2O$	40	-	10	36
$Na_2SiO_3 \cdot 9H_2O$	-	10	-	-
$Na_2EDTA \cdot 2H_2O$	5	0.44	0.5	1
$ZnSO_4 \cdot 7H_2O$	2.2	0.0021	0.011	0.0222
H_3BO_3	1.14	-	-	0.286
$MnCl_2 \cdot 4H_2O$	0.506	0.018	0.018	0.181
$FeCl_3 \cdot 6H_2O$	-	0.316	0.098	-
$FeSO_4 \cdot 7H_2O$	0.499	-	-	-
$CoSO_4 \cdot 7H_2O$	-	0.0012	-	-
$CoCl_2 \cdot 6H_2O$	0.161	-	0.002	-
$Co(NO_3)_2 \cdot 6H_2O$	-	-	-	0.0049
$CuSO_4 \cdot 5H_2O$	0.157	0.0007	-	0.0079
$Na_2MoO_4 \cdot 2H_2O$	-	0.0007	0.00125	0.039
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.11	-	-	-
Thiamine HCl	-	0.1	0.01	-
Biotin	-	0.0005	0.002	-
Vitamin B_{12}	-	0.0005	0.001	-
Vitamin B_6	-	-	0.001	-

CO2 (Ma et al., 2017). Meanwhile, the insufficient CO2 concentration tends to cause an inhibition in some important photosynthetic enzymes (Tu et al., 2019). The NaOH solution requires more research for the optimal amount of concentration.

This study was conducted to observe the effect of NaOH addition on biomass and metabolites production in photoautotrophic cultivated Chlorella sorokiniana. The growth of microalgae and the consumption of dissolved CO2 concentration were calculated to determine the optimal amount of NaOH in the cultivation medium that provided the highest carbon absorption and biomass production with autotrophic cultivation method. Protein, carbohydrate, lipid, fatty acid, and chlorophyll content in cultivated biomass was also measured to observe microalgae metabolism under the treatment. The results of this study are expected to be useful for the developing potential microalgae as a CO2 mitigation agent and alternative sources of energy and nutrition.

METHODS

Preparation of Cultivation Medium

Sorokiniana (LIPI12-Al016) was obtained from the laboratory culture collection of Microalgae and Bioprocess Engineering Research Group, National Research and Innovation Agency (BRIN), Bogor, Indonesia. The microalgae were cultivated in 24 well microplate containing TAP, BG11, AF6, and F2 to determine the best daily cultivation medium. The chemical composition of each medium is shown in Table 1. Optical density was measured at 750 nm (OD750) for 7 days using SpektraMax® Paradigm® Multi-Mode Microplate Detection Platform. As the stock culture, microalgae were cultivated in optimal medium with an initial OD750 of 0.1. Microalgae were precultured from stock culture for working culture every week. After a month, working culture from the third week became a new stock culture replacing the old one. Microalgae were cultivated in 24 well microplate with pH media between 2-12 to investigate the pH optimum for cultivation media. Room temperature was between 30-33 °C and light intensity around 2000 lux continuously. Cultivation media was prepared by flowing CO2 gas into NaOH solution at flow rate of 1 L/min until pH became 6-7. The optimal medium without carbon source composition was then added to the solution. Cultivation medium with NaOH 20 mM was referred as NaOH20 medium, NaOH30 for NaOH 30 mM, etc.

Effect of NaOH Concentration

The microalgae were cultivated in 1000 mL media with different NaOH concentrations, i.e 30 mM, 60 mM, and 120 mM. These concentrations are based on previous screening results with NaOH concentrations ranging from 20-60 mM (unpublished data). In addition, NaOH120 medium was then included in this treatment to describe the excess concentration. NaOH 120 mM was also the approximately equivalent value of carbon concentration in the TAP medium. For daily sampling, 20 mL cultures were centrifuged at 6500 ×g for 5 minutes. The pellets were dried in the oven for gravimetric measurement of dry weight and supernatant was titrated with PP indicator for acid-alkalimetric measurement of CO_2 concentration.

Protein Content

5 mg biomass powder was extracted with 0.5 mL phosphate buffer 1 M and 0.5 mL glass beads 0.2 mm (Montone et al., 2018). The extract mixture was then stirred with a bead beater at 900 ×g for 2×300 seconds and centrifuged at 3500 ×g for 2 minutes. The protein extract in phosphate buffer was used to measure the protein content. Total protein content in microalgae cells was measured using a method referred to the Bradford assay method (Merck). About 0.02 mL protein extract was added with 1 mL Bradford protein kit and vortex for 10 seconds. After standing in room temperature for 5 minutes, the absorbance was measured at 595 nm. BSA was used as the protein standard.

Carbohydrate Content

Total carbohydrate content in microalgae cells was measured using a method referred to the DuBois phenol-sulfate method (Kim et al., 2020). First, 0.1 mg biomass powder was added with 1 mL water, 1 mL phenol 5%, and 5 mL sulfuric acid, then vortex for 10 seconds. After standing in room temperature for 30 minutes, the absorbance was measured at 490 nm. Glucose was used as the carbohydrate standard.

Lipid Content

Total lipid content in microalgae cells was extracted using a method referred to the Bligh-Dyer method (Sierra et al., 2017). The mixture of the chloroform and methanol (1:1, v/v) was used as the extraction solvent. First, 100 mg biomass

powder was added with 3 mL solvent and then sonicated at 40 kHz for 2 hours. Next, the crude extract was separated, and 3 mL solvent was added again. After being sonicated for 2 hours, the extraction process was continued by maceration for 24 hours. Water was then added to the crude extract (1:1, v/v). The mixture was vortex for 30 seconds and centrifuged at 1500 ×g for 5 minutes. Finally, the chloroform layer at the bottom was taken and evaporated at room temperature. The weight was measured by gravimetry.

Chlorophyll Content

The total chlorophyll content was measured by the spectrophotometric method (Amin et al., 2018). Dry extract in a 1.5 mL microtube resulting from lipid extraction was added with 1 mL of methanol and stirred using a vortex for 60 seconds. The content of chlorophyll-α and chlorophyll-β was calculated as the difference between absorbance at wavelengths of 665.2 nm and 652 nm (Amin et al., 2018).

$$C(a) = 16.29 \times A_{665.2} - 8.54 \times A_{652}$$

$$C(b) = 30.68 \times A_{652} - 13.58 \times A_{665.2}$$

C (a) is the chlorophyll-α content, and C (b) is the chlorophyll-β content. A665.2 is the absorbance at a wavelength of 665.2 nm, and A652 is the absorbance at a wavelength of 652 nm.

Fatty acids analysis

The fatty acids in the biomass powder were transesterified directly into fatty acid methyl esters (FAME) according to the Lewis method (Quilodran et al., 2020). A mixture of chloroform, hydrochloric acid, and methanol (10:1:1, v/v) was used as a transesterification reagent. 20 mg of biomass powder was added with 3 mL reagent in a screw tube. The mixture was stirred with a vortex for 10 seconds and heated at 90°C for 120 minutes. After that, it was cooled at room temperature, and then 1 mL of water was added and stirred with a vortex for 10 seconds. FAME was extracted by adding 3 x 2 mL of hexane and separated after stirring with a vortex for 10 seconds. FAME was identified using GC-MS by submitting the samples to Integrated Laboratory of Bioproduct, BRIN through E-Layanan Sains.

RESULTS AND DISCUSSION

Media Optimization

The highest OD750 on C. sorokiniana growth was seen in the TAP medium, followed by BG11, F2, and AF6 (Figure 1). Moreover, the highest specific growth rate (μ) was also seen in TAP medium with μ equal to 0.696 day-1, followed by 0.538 day-1 in BG11, then 0.491 day-1 in AF6, and 0.408 day-1 in F2 (Table 2). A high specific growth rate (μ) indicates the doubling time of the exponential phase. If this phase can be reached fast, the environment is suitable for microalgae biomass growth. The fastest exponential phase of C. sorokiniana growth was seen in TAP medium on the second day of growth, followed by BG11, AF6, and F2 on the third day, respectively. Meanwhile, the carbon source in the chemical composition of the TAP medium does not bind to other essential nutrients and will not affect by the modification in this study (Table 1). Therefore, the TAP medium was chosen as a basal medium for stock, preculture, and treatment since it also

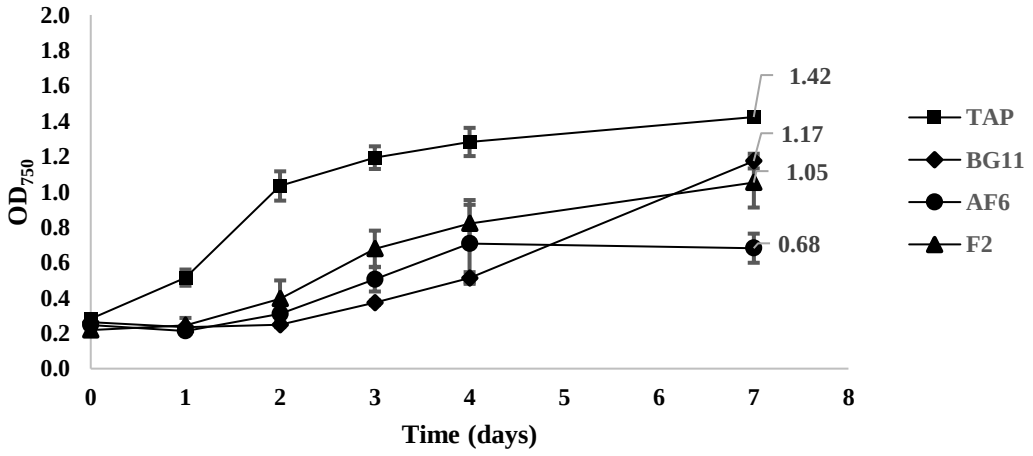

Figure 1. The effect of four different cultivation media on *C. sorokiniana* growth

Table 2. The specific growth rate (day^{-1}) of *C. sorokiniana* on four different cultivation media.

Time (Days)	TAP	F2	AF6	BG11
0	0.000	0.000	0.000	0.000
1	0.606	-0.117	-0.148	0.117
2	**0.696**	0.058	0.372	0.480
3	0.144	**0.408**	**0.491**	**0.538**
4	0.072	0.317	0.336	0.190
7	0.035	0.277	-0.012	0.083

demonstrates the highest and fastest growth for *C. sorokiniana*.

Effect of pH on *C.* sorokiniana growth

pH is critical in microalgal cultivation because it significantly affects microalgal metabolism. It correlates with the availability and solubility of CO_2 and nutrients. High pH limits CO_2 availability and inhibits cell growth (Ma et al., 2017; Wang & Lan, 2018). A similar effect of low pH, where this condition will interfere with the microalgae growth (Ma et al., 2017). However, both conditions, whether high or low pH, are ideal for suppressing undesired contaminants (Ma et al., 2017; Qiu et al., 2017)

sorokiniana can grow at pH 6-10 with the highest OD_{750} at pH 8 (Figure 2). Ma et al.(2017) mentioned that pH 6.5 in *C. sorokiniana* cultivation medium with CO_2 addition provided a low possibility of contamination by protozoa. Thus, the pH of cultivation medium was adjusted between 6-7 to prevent contamination at the beginning of growth and minimize the increase of pH medium due to nutrient consumption during microalgae growth. Another reason was that by adjusting pH to 6-7, more CO_2 gas was added to the culture, providing more carbon for microalgae growth.

Effect of NaOH Addition

The highest dry weight biomass production on the seventh day of *C. sorokiniana* growth was 598.3 mg.L^{-1} as seen in NaOH60 medium, followed by NaOH30 and NaOH120. Furthermore, the highest carbon consumption was 691.8 mg.L^{-1} as also seen in NaOH60 medium, followed by NaOH120, and NaOH30 (Figure 3). Carbon consumption was determined by acidic-alkalimetric titration method, based on the absorption reaction equation between CO_2 gas and NaOH, as in equations (1) and (2) (Molazadeh et al., 2018; Yu et al., 2021).

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+ \quad (1)$$

$$CO_2 + NaOH \leftrightarrow HCO_3^- + Na^+ \leftrightarrow CO_3^{2-} + 2Na^+ \quad (2)$$

The addition of NaOH in culture medium can strengthen CO_2 retention (Nayak et al., 2013). The NaOH60 medium provides a more stable

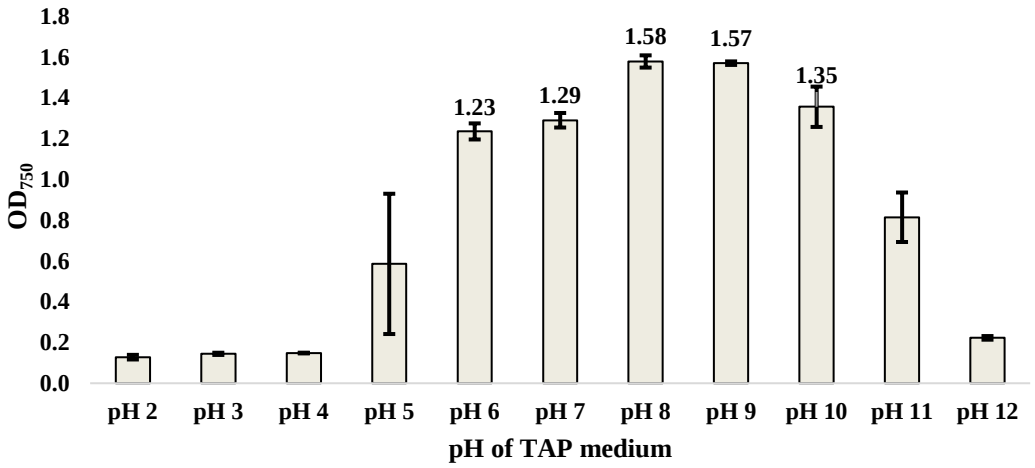

Figure 2. The effect of different pH media on *C. sorokiniana* growth

Table 3. The specific growth rate (day^{-1}) of *C. sorokiniana* on three different NaOH concentration

Time (Days)	NaOH30	NaOH60	NaOH120
0	0.000	0.000	0.000
1	**0.853**	**1.214**	0.413
2	0.349	0.578	**0.472**
3	0.309	0.332	0.402
4	0.192	0.182	0.443
7	0.063	0.059	0.075

carbon source of HCO3− from CO2 absorption to transport into cells. Therefore, NaOH 60 mM was used for further analysis because it offered the highest biomass production and carbon consumption. Meanwhile, the exponential phase of NaOH60 medium was seen on the second day of growth with μ (day-1) equal to 1.214 (Table 3).

Metabolites content

The result showed that carbohydrates were the highest metabolite produced by *C. sorokiniana* in both media. TAP and NaOH60 generated 565.3 mg.g^{-1} and 422.7 mg.g^{-1}, respectively. This was followed by the production of lipid and fatty acid. Meanwhile, the content of protein and chlorophyll was quite low in all conditions (Table 4).

The utilization of NaOH in the medium did not increase the metabolites content, except for protein. This result indicated that NaOH is more appropriate for biomass production. On the other hand, the control medium without NaOH addition likely focus on metabolites rather than biomass production. Adding NaOH increases the dissolved inorganic carbon in the culture, thereby providing better growth conditions. It is a common phenomenon that biomass production could not be in line with the production of metabolites.

Fatty acids profile

The fatty acid profile is considered to determine the biodiesel quality, especially when the microalgae strain generates unsaturated fatty acids (Qiu et al., 2017). As shown in the result (Table 5), C. sorokiniana accumulated 16 and 18 carbon fatty acids, which are suitable for biodiesel production (Nayak et al., 2013). C. sorokiniana cultivated in the TAP medium primarily produced C16:2n6, C18:2n6 (linoleic acid), and C18:3n6 (α-linolenic acid).

In NaOH60 medium, C. sorokiniana accumulated monounsaturated fatty acids (MUFAS) such as C18:1n9 (oleic acid); C18:1n7 and polyunsaturated fatty acids (PUFAS) such as C16:2n6 ; C18:2n6 (linoleic acid); and C20:3n6 (homolinolenic acid). The presence of oleic acid could be an indicator of biodiesel quality (Nayak

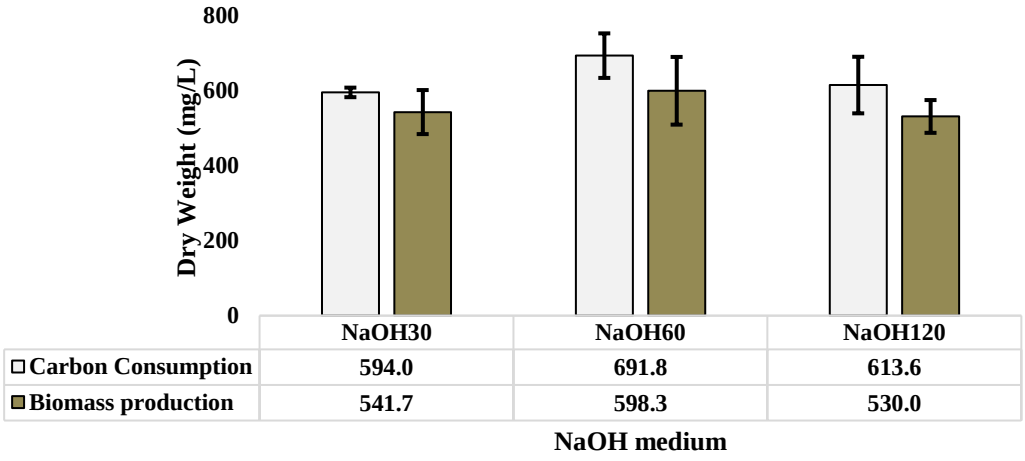

Figure 3. The effect of NaOH concentration on biomass production and carbon consumption

Table 4. Metabolites content of *C. sorokiniana* in TAP and NaOH60 media

	Metabolites Content ($mg.g^{-1}$)				
	protein	carbohydrate	lipid	chlorophyll	fatty acid
TAP	17.7	565.3	246	21.2	210
NaOH60	24.5	422.7	210	8.6	150

et al., 2013). C. sorokiniana in both media also accumulated palmitic acid (C16:0), known as saturated fatty acids (SFAs). Overall, the percentage of unsaturated fatty acids is much higher than saturated fatty acids (Figure 4). Based on the result, NaOH60 possesses more unsaturated fatty acids, especially PUFAs. These kinds of fatty acids not only benefit the energy sector but also in health applications such as diet or functional food (Katiyar & Arora, 2020).

The fatty acid profiles correlate with culture pH. According to Qiu et al. (2017), an increase in pH levels from 6.5 to 8.5 decreased SFAs and MUFAs. On the other hand, the PUFAs fraction increases. The characteristics of unsaturated fatty acids (MUFAs and PUFAs) are lower melting points than SFAs. MUFAs such as 18:1 also much stable against oxidation, thereby increasing the flow properties of biodiesel and lowering the compaction temperature (Nzayisenga et al., 2020). Moreover, MUFAs was reported to be the most favorable for biodiesel production, and this fraction was heavily accumulated by Chlorella at pH 6.5. In addition, biodiesel generated from Chlorella that is cultivated at lower pH is usually appropriate with diesel standard.

The use of alkaline solutions to increase CO2 retention in microalgae cultivation has been carried out using different microalgae or with different concentrations and types of alkaline solutions (Nayak et al., 2013; Song et al., 2019; Yu et al., 2021), but optimization of NaOH concentration to bind CO2 in photoautotrophic cultivated C. sorokiniana has never been done before. The results of this study enrich knowledge about the potential of microalgae C. sorokiniana as a CO2 mitigation agent and an alternative source of energy and nutrition.

CONCLUSION

The utilization of NaOH 60 mM concentration in autotrophic cultivation medium enhanced *C. sorokinian*a biomass production to 598.3 $mg.L^{-1}$. The amount of dissolved carbon consumption was also enhanced to 691.8 $mg.L^{-1}$ due to increased CO_2 retention. NaOH addition in the medium did not increase the metabolites' content, except for protein. The fatty acids profile of *C. sorokiniana* in NaOH60 medium primarily consists of MUFAs and PUFAs. It can be concluded that NaOH60 gave a better result than the TAP medium because it accumulated more various unsaturated fatty acids that are potentially used as energy sources and functional food. Further research needs to be conducted to optimize metabolites production in *C. sorokiniana*.

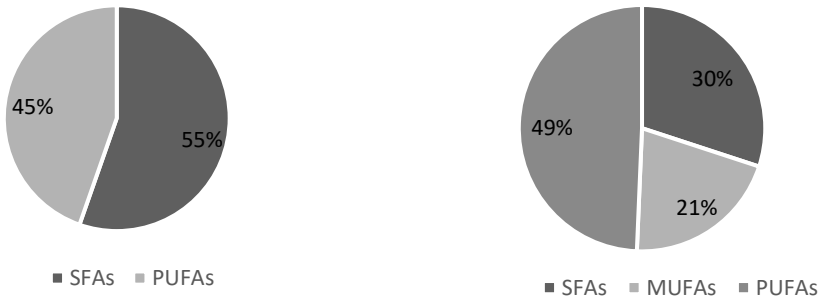

Figure 4. Fatty acids profile of *C. sorokiniana* in TAP (left) and NaOH60 medium (right)

Table 5. Fatty acids profile of *C. sorokiniana* in TAP and NaOH60 media

Fatty Acids	TAP	NaOH60
C16:0	34.83	26.79
C16:2n6	7.58	12.45
C18:1n9		12.23
C18:1n7		6.16
C18:2n6	14.34	19.03
C18:3n6	6.16	
C20:3n6		12.52

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