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Investigation of Biobutanol Efficiency of *Chlorella* sp. Cultivated in Municipal Wastewater

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Abstract

Many strains of microalgae can grow in wastewaters through their ability to utilize inorganic nitrogen and phosphorus in wastewater. The content of municipal wastewater changes from a location to others. Biofuel production from municipal wastewater has gained huge importance due to progresses in cultivation of microalgae in wastewaters. Biobutanol is produced by the acetone-butanol-ethanol (ABE) fermentation. In this study, we examined the biobutanol production efficiency of *Chlorella* sp. *DEE*006 which is cultivated in the municipal wastewater in flat-photobioreactor. Growth of microalgae was monitored at 680 nm using spectrophotometer and the biomass was also pre-treated with acidic hydrolysis (1 M H_2SO_4). Total carbohydrate and protein contents were measured. Fermented microalgae samples were taken for calculation of biobutanol concentration. We obtained both high biobutanol content (6.23 ± 0.19 g·L^{-1}) and high bioethanol yield 0.16 ± 0.005 g (g sugar)$^{-1}$. 50% wastewater had the highest biomass concentration (1930 ± 11 mg/L) among the wastewaters with five various concentrations. It had the highest biomass productivity with 0.28 ± 0.001 g L^{-1}d^{-1}. Also, it obtained the highest carbohydrate and protein concentration with 0.80 ± 0.02 gL^{-1} and 0.95 ± 0.01 gL^{-1}, respectively. According to our results, *Chlorella* sp. *DEE*006 can be used for large scale biobutanol production in the future.

Keywords

Microalgae, *Chlorella*, Biobutanol, Biofuel, Municipal Wastewater

1. Introduction

Biofuel is a renewable energy resource and it has gained great importance in recent years because of depletion of fossil fuel. Biobutanol is a kind of biofuel and it (C_4H_9OH) has four carbons including colourless alcohol. It is used as solvent, organic synthesis and extractant [1]. Biobutanol has several advantages compare to other biofuels. Biobutanol is more advantageous than bioethanol and biomethanol due to its energy density and similarity to gasoline. It can be used in fuel engines as a blend with diesel without any modification. Biobutanol has half the heat of vaporization of that of ethanol. This property is important for engine initiation at sub-zero temperatures. In addition, biobutanol has a lower vapour pressure and lower volatility. This maintains more effective storage and transport [2] [3]. Microalgae are third generation biofuels and they have superior properties compare to first and second generation biofuel. Microalgae have no competition with agricultural crops for land needed. They can grow fast and generate huge amount of biomass. Also, conversion technology of microalgae to biomass has simple structural properties compared to second generation biofuel [4] [5]. Biobutanol is produced by the acetone-butanol-ethanol (ABE) fermentation. In this process, microalgal biomass is used as a substrate. Generally, *C. acetobutylicum* is selected for anaerobic digestion [3]. It produces butyric and acetic acids through a process called acidogenesis, followed by solventogenesis, where butanol, acetone and ethanol are synthesized. Biomass has to have rich carbohydrate content for effective biobutanol production. Starch which is storage component of carbohydrate is located in plastids in microalgae. Moreover, cellulose, the cell wall component of microalgae, contributes carbohydrate content in microalgae [2] [6]. Production of acetate and butyrate reduce the culture pH and it causes to solvent production. This reaction results in an acetone, butanol and ethanol ratio of 3:6:1 [7]. Microalgae are basically useful to decrease the concentration of inorganic nitrogen and phosphorus in the wastewaters. Many strains of microalgae can grow in the wastewaters through their ability to utilize inorganic nitrogen and phosphorus in the wastewater. Thus, cultures of microalgae are useful for wastewater treatment as a tertiary process [8]. Developing of urbanization and increasing of urban population led to huge amount of municipal wastewater. The content of municipal wastewater changes from a location to others. It includes organic wastes, human wastes, nutrients and household chemicals. Compared with industrial and agricultural wastewater, there is less nitrogen and phosphorus in municipal wastewater [9]. Biofuel production from municipal wastewater has gained huge importance due to progress in cultivation of microalgae in wastewaters. Chlorophytes is one of the largest phyla of microalgae. These microalgae have been displayed to be potential used to a variety of wastewater conditions and very efficiency at nutrients removal from wastewater. *Chlorella* species have been carried out for removing of nitrogen and phosphorus from wastewaters in detail [10]. *Chlorella* species maintain a high nutrient pollutions removal and depleted in municipal wastewater [11].

In this study, we examined the biobutanol production efficiency of *Chlorella* sp. *DEE*006 which is cultivated in municipal wastewater in flat-photobioreactor.

2. Materials and Methods

2.1. Microalgae Strain and Cultivation

Microalgae were obtained from YYU-microalgae collection. *Chlorella* sp. *DEE*006 was cultured at 25˚C ± 2˚C and Bold's Basal Medium (BBM) was prepared with some modifications according to Andersen, 2005 [12]. BBM was adjusted to pH 6.8 and checked for control experiments. The municipal wastewater was autoclaved and mixed with Bold's Basal Medium (BBM) before using. The batch cultures of *Chlorella* sp. *DEE*006 were grown at different ratios (v/v) of municipal wastewater (0%, 25%, 50%, 75% and 100% wastewater). Municipal wastewater was obtained from Van, Turkey. The microbial contamination was checked with Thoma cell counting chamber and maintained less than 2% when increasing contamination (up to 2%). More than 2% can lead to bacterial and fungal contamination and this situation can adversely affect growth curves of microalgae.

2.2. Design of Photobioreactor

In this study, batch culture studies were examined in 500 mL of Erlenmeyer flasks consisting of 250 mL of medium. For continuous studies, we designed flat airlift photobioreactor (PBR) (1 L) and air was injected at the bottom for culture mixing with a constant air flow rate of 0.3 $L \cdot min^{-1}$. PBR was illuminated with an external light source at a light intensity of approximately 180 μmol m^{-2} s^{-1}. Before using, PBR was sterilised 30 min with a 5 mM peroxyacetic acid solution.

2.3. Growth Evaluation and Harvesting of Microalgae

Growth of microalgae was monitored at 680 nm using spectrophotometer. Ash free dry weights (AFDW) were obtained for correlation between optical density and dry weight. The algal suspension was grown up to stationary growth phase and harvested by centrifugation at 3000 g for 10 min at 4 C according to Barsanti and Gualtieri [13]. The algal biomass was lyophilized and stored at −20˚C for further analysis. All experiments were performed with three biological replicates.

2.4. Specific Growth Rate Calculation

Specific growth rates of microalgal biomass were calculated by the following equation [14]. Specific growth rate (μ): In $(X_1 - X_2)/(t_2 - t_1)$. Where, X_1: Biomass concentration at the end of the selected time interval in $mg \cdot L^{-1}$; X_2: biomass concentration at the beginning of the selected time interval in $mg \cdot L^{-1}$; $t_2 - t_1$: Time elapsed between the selected time points in days (d). The doubling time was calculated with using Equation below.

$$T_d = 0.693/\mu$$

2.5. Acid Treatment Procedure

Biomass was also treated with acidic hydrolysis (1 M H_2SO_4). Samples were autoclaved at 121˚C for 30 min. After hydrolysis, samples were cooled down to room temperature and suspension was centrifuged at 4000 g for 10 min. The supernatant was taken for sugar content analysis.

2.6. Determination of Total Protein Concentration

Total protein was extracted according to Weis *et al.,* 2002 [15]. Protein concentration of the isolates was calculated according to the Bradford method [16]. Bovine serum albumin (BSA) was used as standard. Absorbance values were measured at 595 nm.

2.7. Determination of Total Carbohydrate Concentration

Total carbohydrate contents of the isolates were determined by the anthrone method with minor modifications [17]. Glucose was used as a standard at various concentrations (10, 40, 60, 100, 150, 200 μg/mL).

2.8. Fermentation of Microalgal Biomass

C. acetobutylicum was selected for biobutanol fermentation. The growth medium of *C. acetobutylicum* included 1 - 40 g of glucose or microalgae biomass, 0.17 g of KH_2PO_4, 0.16 g of Na_2SO_4, 5 g of yeast extract, 1 of tryptone, 0.01 g of p-aminobenzoic acid and 0.01 g of Biotin with pH adjusted to 4.8. The butanol fermentation was carried out at 37˚C.

2.9. Fermentation of Microalgal Biomass

Fermented microalgae samples were taken for calculation of biobutanol concentration. Biobutanol concentrations were measured according to Maiti *et al.*, 2015 [18] spectrophotometrically. All experiments were performed with three biological replicates.

3. Results and Discussion

3.1. General Observations about Municipal Wastewater

In this study, the municipal wastewater was sterilized and the microbial contamination was checked with Thoma cell counting chamber and maintained less than 2%. Throughout our study, municipal wastewater and BBM were mixed proportionally (0%, 25%, 50%, 75% and 100%).

3.2. Evaluation of Growth Curves of Microalgae Grown in Municipal Wastewater

Growth curves of microalgae can change in various mediums and stress conditions. In this study, we plotted growth curves of microalgae grown in different concentrations (0%, 25%, 50%, 75% and 100%) of municipal wastewater. Control group includes components of BBM and shown as 0%. But, 100% wastewater consists of completely municipal wastewater. Growth curves were plotted via absorbance values and biomass concentrations versus time (days). The results were given in **Figure 1**. 50% wastewater displayed the fastest growth (1.49 ± 0.002), reaching stationary phase for growth at nine days. Control, 25%, 75% and 100% of wastewater reached to stationary phase between six and nine days with different absorbance values 1.29 ± 0.001, 1.46 ± 0.001, 1.32 ± 0.001, 0.94 ± 0.001, respectively. The efficient growth of microalgae in wastewater is dependent on many reasons such as the types and the sources of wastewater. Also, the variation is also displayed in the tolerant capacities of various microalga species to specific wastewater conditions [10]. According to literature, our results were similar. Many microalgae strain can be grown in municipal wastewater and they can deplete nitrogen, phosphorus and sulphur until reaching stationary phase. Thus, biomass of microalgae can be applied for wastewater treatment and biofuel production [8] [19].

According to biomass concentrations, microalgae had linearity when cell number and optical density were plotted versus time (days). 50% wastewater had the highest biomass concentration (1930 ± 11 mg/L) among wastewater with five various concentrations. 25% wastewater showed higher biomass concentration (1789 ± 11 mg/L) than those of control (1556 ± 11 mg/L) and 75% wastewater (1567 ± 19 mg/L). Also, 100% wastewater displayed the lowest biomass concentration with (1122 ± 22 mg/L). The results were shown in **Figure 2**. These results are support by Cai *et al.*, 2013 [20]. In this study, they show that growth of microalgae, biomass content and lipid production change significantly in wastewater [20]. Cho *et al.*, 2013 also carried out that *Chlorella* sp. yield the highest biomass production nearly 3.0 $g \cdot L^{-1}$ with 10% anaerobic digestion tanks, and conflux line of the 90% wastewaters combined wastewater as nutrients for microalgae cultivation [21].

Biomass productivity of *Chlorella* sp. *DEE*06 grown in different concentrations of wastewater was calculated according to their maximum biomass concentrations. 50% wastewater had the highest biomass productivity with 0.28 ± 0.001 g $L^{-1}d^{-1}$. 25% wastewater reached to higher stationary phase than control group and 75% wastewater. It showed higher biomass productivity (0.26 ± 0.002 g $L^{-1}d^{-1}$) than those of control (0.22 ± 0.004 g $L^{-1}d^{-1}$) and 75% (0.22 ± 0.002 g $L^{-1}d^{-1}$). 100% wastewater had the lowest biomass productivity (0.19 ± 0.003 g $L^{-1}d^{-1}$). In addition, we calculated specific growth rates of microalgae in different wastewater concentrations.

50% wastewater showed maximum specific growth rate (5.98 ± 0.01 d^{-1}) when compared to control, 25%, 75% and 100%. 100% wastewater had minimum specific growth rate (5.55 ± 0.02 d^{-1}). Also, control, 25%, 50%, 75% and 100% wastewater had 0.120 d, 0.121 d, 0.116 d, 0.119 d and 0.125 d, respectively. The results were given in **Table 1**. The total nitrogen and total phosphorus can fluctuate in medium according to types of wastewater. Some of studies indicated that municipal, agricultural and industrial wastewaters for *Chlorella* species can cause changes of biomass content and productivity [1] [9] [22] [23].

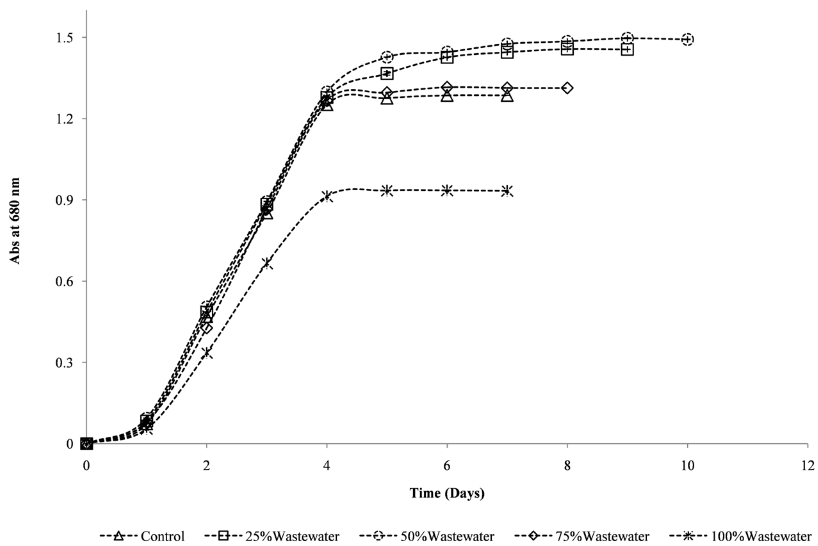

Figure 1. Growth curves of *Chlorella* sp. *DEE*006 cultivated in various concentrations of municipal wastewateras a function of absorbance values at 680 nmvs time (days).

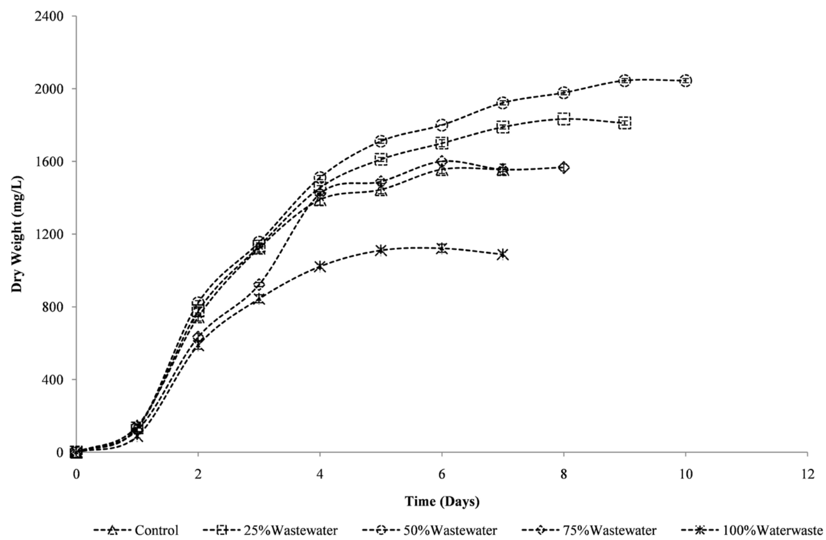

Figure 2. Growth curves of *Chlorella* sp. *DEE*006 cultivated in various concentrations of municipal wastewater as a function of dry weight (mg/L) at 680 nmvs time (days).

Table 1. Biomass concentrations, biomass productivities, specific growth rates and doubling time of *Chlorella* sp. *DEE*006 cultivated in various concentrations of municipal wastewater.

Wastewater concentrations	Biomass concentrations ($g \cdot L^{-1}$)	Biomass productivity ($g\ L^{-1}d^{-1}$)	Specific growth rate (μ) (d^{-1})	Doubling Time (d)
Control	1.56 ± 0.03	0.22 ± 0.004	5.78 ± 0.01	0.120
25% wastewater	1.79 ± 0.01	0.26 ± 0.002	5.75 ± 0.01	0.121
50% wastewater	1.93 ± 0.01	0.28 ± 0.001	5.98 ± 0.01	0.116
75% wastewater	1.56 ± 0.01	0.22 ± 0.002	5.83 ± 0.01	0.119
100% wastewater	1.12 ± 0.02	0.19 ± 0.003	5.55 ± 0.02	0.125

3.3. Carbohydrate and Protein Contents of Microalgae Grown in Municipal Wastewater

We plotted carbohydrate and protein concentration of *Chlorella* sp. *DEE*006 grown in municipal wastewater according to gL^{-1} versus time (day). 50% wastewater had the highest carbohydrate concentration (0.80 ± 0.02 gL^{-1}) on the stationary phase. 25%, 75% and 100% showed lower carbohydrate contents with 0.66 ± 0.01 gL^{-1}, 0.52 ± 0.01 gL^{-1} and 0.37 ± 0.01 gL^{-1}, respectively. In addition, protein contents of *Chlorella* sp. *DEE*006 were plotted and 50% wastewater had the highest protein content (0.95 ± 0.01 gL^{-1}) among other wastewater concentrations. 25% wastewater (0.85 ± 0.01 gL^{-1}) showed the higher protein content than those of 75% (0.71 ± 0.01 gL^{-1}) and 100% (0.50 ± 0.01 gL^{-1}). The results were displayed in **Figure 3**.

These results show parallel to literature. The current studies focused on increasing the biomass and lipid yield of microalgae [24]. Lipid content of microalgae grown in wastewater was not determined in a few studies. Lipid contents of microalgae were lower than 10 mg L^{-1} d^{-1} (10%) [25]. This can cause much more carbohydrate and protein accumulation. Wang *et al.* (2010) carried out *Chlorella* sp. on anaerobically digested dairy manure. The total fatty acid content ranged from 9.0% to 13.7% DW (0.141 - 0.233 $g \cdot L^{-1}$) depending on the wastewater concentration used [1]. We calculated maximum carbohydrate and protein concentrations of microalgae grown in various municipal wastewaters. The highest carbohydrate content was 39.9% ± 1.2% at 50% wastewater. 25% wastewater showed the lower carbohydrate contents (35.8% ± 0.7%). Control, 75% and 100% wastewater had similar carbohydrate contents with 33.8% ± 0.9%, 33.4% ± 0.2% and 33.5% ± 0.5%, respectively. In addition, 25% and 50% wastewater accumulated similar protein contents, 46.2% ± 0.7% and 46.4% ± 0.2% respectively. Control, 75% and 100% had lower protein contents, 45% ± 2.1%, 45.2% ± 0.7% and 44.2% ± 1.2%, respectively. Carbohydrate and protein contents were given in **Table 2**.

3.4. Biobutanol Production

C. acetobutylicum was used for biobutanol fermentation. Initial sugar concentrations (1, 2, 5, 10, 20 and 40 g) were adjusted and glucouse was used for standard. Because *Chlorella sp.DEE*006 grown in % 50 wastewater has the highest biomass productivity and carbohydrate content, it was selected for biobutanol production. Biobutanol content found as 6.23 ± 0.19 $g \cdot L^{-1}$. In addition to this, we carried out its bioethanol yield and it was 0.16 ± 0.005 g (g sugar)$^{-1}$. The results were given in **Table 3**. In the literature, scientific articles related with biobutanol production from microalgae are not enough for evaluation of biofuel on microalgae. Many studies focused on biodiesel production. Yang studied the effects of water usage and life cycle water for biodiesel production and determined nutrients usage of microalgae in this system [26]. Wang examined enhancing bio-butanol production from biomass of *Chlorella vulgaris* JSC-6 with sequential

Table 2. Carbohydrate, protein, lipid and biomass concentrations of *Chlorella* sp. *DEE*006 cultivated in various concentrations of municipal wastewater.

Wastewater concentrations	Carbohydrate (dwt %)	Protein (dwt %)	Lipid (dwt)	Biomass concentrations ($g \cdot L^{-1}$)
Control	33.8 ± 0.9	45 ± 2.1	N.D.	1.56 ± 0.03
25% wastewater	35.8 ± 0.7	46.2 ± 0.7	N.D.	1.79 ± 0.01
50% wastewater	39.9 ± 1.2	46.4 ± 0.2	N.D.	1.93 ± 0.01
75% wastewater	33.4 ± 0.2	45.2 ± 0.7	N.D.	1.56 ± 0.01
100%wastewater	33.5 ± 0.5	44.2 ± 1.2	N.D.	1.12 ± 0.02

Table 3. Biobutanol content (g/L) and Biobutanol yield (g/g sugar) of *Chlorella* sp. *DEE*006 cultivated in 50% concentration of municipal wastewater.

Wastewater concentration	Initial sugar concentration (g/L)	Biobutanol content (g/L)	Biobutanol Yield (g/g sugar)
50% wastewater	40	6.23 ± 0.19	0.16 ± 0.005

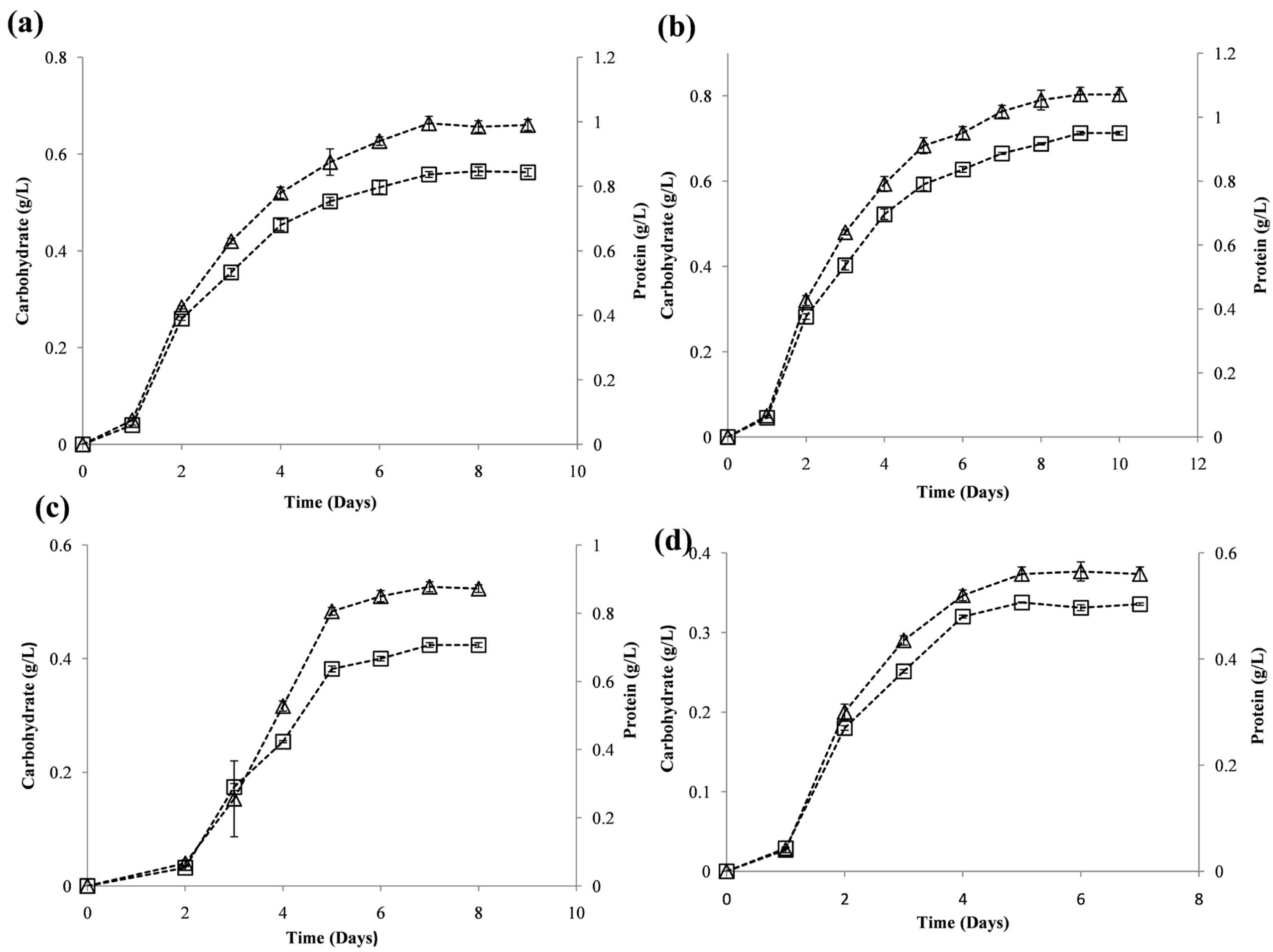

Figure 3. Curves of carbohydrate and protein contents of *Chlorella* sp. *DEE*006 cultivated in various concentrations of municipal wastewater as a factor of dry weight (gL^{-1}) versus time (days). (a) 25%, (b) 50%, (c) 75% (d) 100% of wastewater concentrations. Carbohydrate and protein content were shown as triangular and square in the figures, respectively.

alkali pretreatment and acid hydrolysis and found that the optimal NaOH and H_2SO_4 concentrations used for the pretreatment were 1% and 3% (w/v), respectively for high bio-butanol production [27].

4. Conclusion

The biobutanol production efficiency of *Chlorella* sp. *DEE*006 which is cultivated in municipal wastewater in flat-photobioreactor was carried out in the present study. They gave the various days because microalgae reached to stationary phase in the different days. Various concentrations of wastewater affect stationary phases and give different biomass concentration. We obtained both high biobutanol content (6.23 ± 0.19 g·L^{-1}) and high bioethanol yield 0.16 ± 0.005 g (g sugar)$^{-1}$. 50% wastewater had the highest biomass concentration (1930 ± 11 mg/L) among wastewater with five various concentrations. It had the highest biomass productivity with 0.28 ± 0.001 g L^{-1}d^{-1}. Also, it obtained the highest carbohydrate and protein concentration with 0.80 ± 0.02 gL^{-1} and 0.95 ± 0.01 gL^{-1}, respectively. According to our results, *Chlorella* sp. *DEE*006 can be used for large scale biobutanol production in the future.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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