Recovery of proteins from piggery wastewater using *Scenedesmus* spp. photobioreactors

Mako Tagawa

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University of Valladolid

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Abstract

Algal-bacteria process is a low-cost and environmentally friendly alternative process for wastewater treatment. Currently, Algae biomass as biofuels and high value-added compounds have attention to be produced with wastewater treatments. The macromolecular composition of this biomass grown in wastewater treatment photobioreactors is a key information in order to select alternatives of valorization. The presence of bacteria and the wastewater components such as high concentration of carbon, nitrogen and heavy metals or the environmental conditions influence remarkably the algal biomass compositions. In this study, we set up a photobioreactors system and studied the evolution of algae-biomass, and the efficiency of wastewater treatment. We aimed to clarify the fluctuations of biomass based on green algae *Scenedesmus* spp. grown in non-sterilized piggery wastewater (PWW) and in synthetic water. Results from small 3L laboratory scale indoor photobioreactors were compared with 360L outdoor pilot plant thin layer (TL) photobioreactors and closed tubular (TB) reactor located in Almeria using also *Scenedesmus* species.

Four laboratory experiments, 3L small open photobioreactors (15.8 cm depth, 15.5 cm internal diameter) were designed and performed with high repeatability. They were placed inside a water bath under artificial lights, 1053 ± 32 μmol m^{-2} sec^{-1}, 12h light: 12h dark. Scenedesmus sp. was used as inoculum and two reactors were fed with Modified Bristol medium (MBM) and other two with PWW. Feeding rate was set 500 mL d^{-1} (Hydraulic retention time; HRT 6 day), and pH 8 was maintained in the photobioreactors by supplying CO_2. The removal rate of COD and TN were 83.6 % and 74.5%, respectively, in reactors treating PWW. The dissolved oxygen (DO) concentration in PWW resulted 0 mg L^{-1} while DO 14.8-17.1 mg L^{-1} were determined in MBM condition. This process did not require oxygen supply to remove pollutants by bacteria. It is thought that low-cost operation was possible due to the presence of bacteria for algae based wastewater treatment.

The results showed high protein productivity on algae-bacteria biomass grown in PWW, related with high growth rate of microalgae. TSS during steady state achieved values of around 0.5 L^{-1} with synthetic medium and 1.8 g L^{-1} with PWW, respectively.

Biomass compositions analysis showed similar values between MBM and PWW in laboratory reactors. In PWW feed photobioreactors, biomass with 54.6 % of proteins and 14.7 % of lipids was obtained. In MBM feed photobioreactors, biomass with 51.6 % of proteins and 14.2 % of lipids was obtained. Combining biomass growth and composition, protein productivities of 0.97 g L^{-1} were obtained from non-sterilized PWW and 0.27 g L^{-1} from MBM. However, biomass from TL and BL photobioreactor achieved

high carbohydrate content 43.5%, 43.0% of proteins contents, 9.6% of lipids contents, probably because the different light precedence and intensity. The pigments contents in PWW were lower than medium substrate (MBM, BM) in both of reactors. Chl-*a,* Chl-*b*, Carotenoids were 9.4%, 3.2%, 2.6% in MBM and 1.5%, 0.8%, 0.3% in PWW in laboratory reactors. The presence of heavy metals in PWW probably worked as the oxidative stress. Oxidative stress can degrade protein and lipids such as pigments. But these results were required further research.

The extracted protein contents by enzymatic hydrolysis were maximum 12% with Protamax, 90 min of retention time in PWW biomass from TL photobioreactor. Lipids contents showed similar values before the extraction. Therefore, the all of extraction methods were not enough for the algae samples in PWW.

Introduction

I. Importance of Wastewater treatment

Recent years, the world's water shortage is growing by increasing in population. While 1.4 billion km^3 of water exist on the earth, only 0.76% (1,064,000 km^3) is available to humans, which is 70% used for agriculture (Sato et al., 2013). On the other hand, about 2 million tons (about 0.002 km^3) of water are used daily by humans and discharged as wastewater in municipal, domestic, agricultural and industrial activities. These effluents are drained into the river after wastewater treatment. Developed countries treat about 70% of wastewater, developing countries treat 28 to 38% and undeveloped countries about 8% (Sato et al., 2013). Therefore, strict environmental regulations are forcing the transition to advanced wastewater treatment. Current wastewater treatment uses aerobic biological and physical techniques for most wastewater treatment. However, alternative technologies that have lower costs and lower environmental impact are required (Li et al., 2015).

II. Piggery wastewater treatment with algae-bacteria processes

An algal-bacteria process with high organic matter removal rate can be mentioned as an alternative technology for wastewater treatment with low cost and low environmental impact (Cuellar-Bermudez et al., 2017). In this process, the algae use contaminants such as N, P, and carbon to grow and produce oxygen (Muñoz and Guieysse, 2006; Dalrymple et al., 2013). Organic matter is removed by bacteria to consume oxygen which is produced by algae. Also, carbon dioxide produced by bacteria in the waste solution is consumed by algae. Algae also can remove heavy metals. This has been reported to improve removal efficiency compared to treatment with bacteria alone.

In general, agricultural wastewater is characterized by high N, P content compared to other drainages (Mobin and Alam, 2014). In Europe, many swine wastewater containing high concentrations of organic matter, nutrients, solids, and heavy metals are released. The annual production of pork in Europe is 23.5 million tons, and it is indicated that it is the world's second-largest pig producer in 2015. Therefore, the amount of swine drainage is 214.34 million to 342 million cubic meters (4-8 L^{-1} d^{-1} pig) annually. Recently, piggery wastewater (PWW) treatment by algae-bacteria process have a potential to remove harmful substances environmentally and economically (García et al., 2017; 2018).

III. Cultivation conditions and species selection

Efficient and cost-effective large-scale cultivation of algae is essential for the success of

utilization of algae biomass (Mobin and Alam, 2014). It is depends on the reactor design and algae species used (Chen et al., 2015). Open raceway ponds use sunlight as energy source and the depth is usually no more than 30 cm deep to allow for efficient penetration of light (Mobin and Alam, 2014). Closed photobioreactor have three advantages of reducing the water loss due to evaporation and culture dilution due to precipitation or rain and improvement of carbon dioxide gas contact efficiency (Kumar et al., 2015). Open photobioreactor is more suitable for operation and scale-up and lower cost than closed photobioreactor (Muñoz and Guieysse, 2006). Long term operation with PWW under optimum environmental conditions were conducted in high rate algal ponds (Godos et al., 2009).

Mixed native algae species were recommended to use in large-scale production in wastewater treatment because of their robust and high productivity (Chen et al., 2015). It is usually difficult to prioritize algae with slow growth rates due to the size of the algae and bacteria. Therefore, the selection of algal species has been considered to maintain high algal productivity. *Scenedesmus* species can predominant due to their rapid growth rate in algae-bacteria process (Abeliovich and Weisman, 1978; Park et al., 2012)

IV. The utilization of biocomponents from microalgae biomass with wastewater treatments

In addition to removal of pollutants by algae-bacteria process, cost reduction of the treatment process by useful utilization of algal biomass is attentional (Mobin and Alam, 2014). Algae biomass have been investigated to improve the economics of biorefinery (Chew et al., 2017). Algal biomass is known for its higher production per unit area than land plants. Now that alternative fuels for petroleum are being sought, the use of algae can be considered as one of the important solutions that contribute to greenhouse emissions. From these facts, it has been studied to use algal biomass of algal-bacteria coexistence process for bioethanol and biodiesel (Sydney et al., 2011).

Microalgae have attention from two aspects of the resource for producing bioethanol by bacteria and the direct utilization of biocomponents such as the food, chemicals, medical products, cosmetics, nutritional supplements (Chew et al., 2017). Microalgae can convert CO_2 to carbohydrate, lipids, pigments, high-values materials with the light energy. However, the waste-treated microalgae biomass are difficult to use for food because it is impossible to prevent the contamination of pathogens (Muñoz and Guieysse, 2006). Also, the lipid content of algae in wastewater is reported as low as 0.9-31% in several species compared to pure-cultures (Table 1).

From these reason, it is expected the couple utilization of biofuels and protein utilization. Protein are structured peptides which is binned amino-acids. Recently, soy bean is of interest as a source of bioactive peptides (Minh, 2015; Thi et al., 2017). Also, hydrolyzed protein may be used as an ingredient in fire extinguishers. There are possibility to use protein source from algae biomass for them.

V.   Presence of bacteria and nutrient elements in wastewater

The use of wastewater in algal-bacterial processes are significantly different in elemental composition, light environment, and dissolved oxygen concentration in each wastewater.

While bacteria exist in the wastewater, they consume dissolved oxygen (DO) with pollutants. Since the medium has used as a substrate, DO have been accumulated or maintained at 100% air saturation in the photobioreactors (Peng et al., 2013). High concentrations of DO in the culture medium cause algae growth stress (Marquez et al., 1995; Raso et al., 2012). The effect of low DO is not clear, but this situation can occur in wastewater where bacteria are present. Changes in DO concentration may also differ depending on the reactor design (Peng et al., 2013).

PWW contains high concentrations of nutrients and numerous metals such as Zn, Cu, Cr, Hg, Pb, and metalloids As. PWW inhibit or promote the growth of microalgae are used as diluted to alleviate the limitation of the light availability (Chen et al., 2015). Metals are typically used as growth promoters and (De la Torre et al., 2000; Abe et al., 2008) bio-absorption by algal cells can occur (Kaplan et al., 1987). Non-essential heavy metal such as Hg, As, Pb, Cr cause negative effects in microalgae cells. The metals effect on microalgae growth and biomass were reviewed in Miazek et al., 2015 Also, the protein extraction should be considered to use the hydrolyzed protein for an ingredient. The extraction method are different with the sample components. These samples contain several types of bacteria and algae and also metals.

Objective

Currently, coupled process of wastewater treatment using photobioreactors and valorization of the algal biomass grown in these systems are a remarkable research focus.   The investigation for algal biomass production under the combined conditions of the high pollutants and the bacteria exist in PWW is required. . We aimed to evaluate the algae biomass based on green algae*Scenedesmus* spp.

grown in non-sterilized piggery wastewater and in synthetic water.

In this study, we set up the laboratory scale of *Scenedesmus* spp. photobioreactor to feed both of PWW and synthetic water. We evaluate the efficiency of our system for wastewater treatment with algae biomass production. We analyze the effect of elements compositions (pollutants) on algae growth and biomass. We compare two types of enzyme, Protamax and Alcarase with optimum retention time for proteins fraction valorization.

Materials and Methods

I. Experimental set-up and operation (Experimental system)

Continuous cultures were designed in this experiment. Forth 3L small open photobioreactors (15.8 cm depth, 15.5 cm internal diameter) were settled inside a water bath under artificial lights (Fig. 1). The light intensity 913 ± 23 μmol m^{-2} sec^{-1} and 1053 ± 32 μmol m^{-2} sec^{-1} were measured by A LI-250A light meter (LI-COR Biosciences, Germany) in pre-culture and the experiments. The water inside the water bath was circulated by Compatible Control CC1 (Huher, Germany) to maintain temperature 25°C to prevent becoming high temperature by light. 12h light: 12h dark condition was chosen for light condition. Feeding rate was set 500 mL d^{-1} (Hydraulic retention time; HRT 6 day) by using pomp 2058 (WATSON-MARLOW, United Kingdom). The water immersion pumps are used for mixing inside reactors. The cultures of pH were automatically maintained at pH 8 by a Crison multimeter M44 control unit (Crison Instruments, Spain) which supplying CO_2 during all of the experiments. Optimal pH 8 for nutrient removal and algal growth were chosen from reference (Muñoz and Guieysse, 2006).

For the design of supply nutrients in continuous culture with *Scenedesmus* spp., pre-culture were conducted in all of reactors from initial day to 44th day (Day44 in Fig. 4). Bristol medium which was added trace metal of F/2 medium was used as substrate until Day 16. In pre-culture, TSS/VSS showed a similar value between R1, R2, R3, R4. TSS in all of the reactors increased from 0.07 ± 0.01 to 0.46 ± 0.09 g L^{-1} until the Day 8. However, the growth limitation of *Scenedesmus* sp. were conformed from Day 15 to Day 19 while TN concentration in effluent decreased (Appendix). Therefore, the new medium was used from Day 17, named modified Bristol medium (MBM; Table 2). TSS/VSS increased again after Day 22 because MBM which is two times higher $NANO_3$ medium were used. TSS/VSS showed the steady state after 22nd day (Day 22 in Fig. 4). VSS during steady state in pre-culture were

averaged 0.56±0.24, 0.74±0.15, 0.56±0.09, 0.35±0.18 g L-1 in R1, R2, R3, R4, respectively. In the experiment, MBM (Table 2) and 10% PWW were started to feed as substrate, when algae were steady state in pre-culture (the initial day; Day 44 in Fig.4).

The samples of thin layer (TL) open photobioreactor (5 cm depth) with 10%PWW, and closed tubular (TB) photobioreactor with Bristol medium were used for comparing biomass compositions in different photobioreactor. These reactor were settled for continuous culture outside. More details for TL photobioreactor, TSS were 1.3-1.4 g L^{-1} with TOC/ IC/ TN 45.0, 18.6, 441 mg L^{-1}, Cu /Zn /As /Pb /Cr /Hg 1.7, 13.9, 0.1, 0.03, 0.4, 0.0008 mg L^{-1} . Dilution rate of 0.3 d-1 (HRT 3.3 day) was considered (Morales-Amaral et al., 2015).
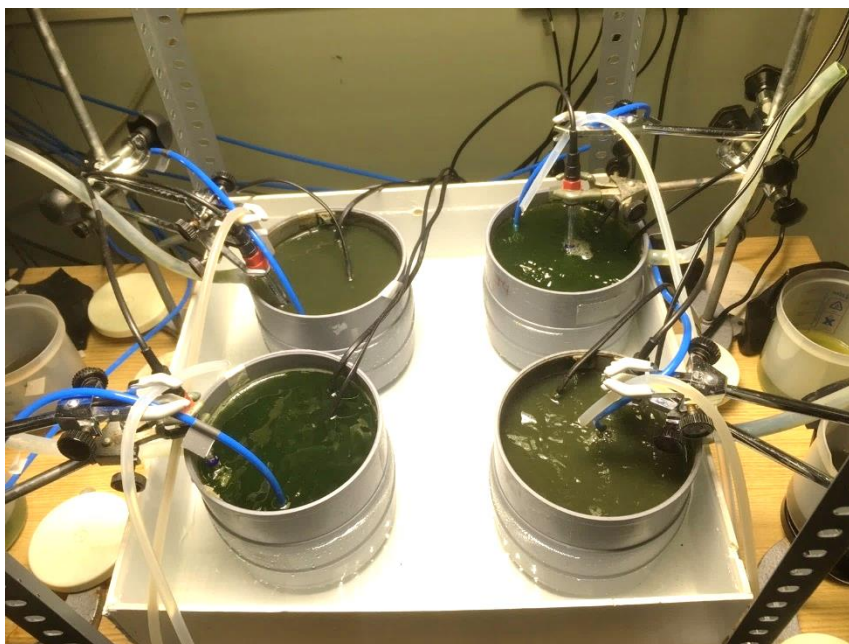

Fig.1 Photograph of forth open photobioreactors and experimental set-up. After 44 day of preculture, the experiment was conducted for 20 day. In the experiment, PWW or MBM were fed as substrate. Left side upper reactor is R2, right side below is R3 in PWW conditions. Left side below is R1 and right side upper one is R4 in MBM conditions.

II. Algal-bacterial inoculum, medium and piggery wastewater

Scenedesmus spp. were inoculated at initial day in pre-culture (Day 0 in Fig.4). They were pre-cultured until Day 44, the steady state is obtained in TSS. All of the cultures were mixed at an initial day of the experiment (Day 44 in Fig. 4) and gained 1.03±0.21 g L^{-1} of TSS.

MBM containing (mM) $NaNO_3$ 5.88; $CaCl_2 \cdot 2H_2O$ 0.17; $MgSO_4 \cdot 7H_2O$ 0.3; K_2HPO_4 0.43; KH_2PO_4 1.29; NaCl 0.43; $FeCl_3 \cdot 6H_2O$ $1.17\times10\text{-}2$; Na_2EDTA $2H_2O$ $1.17\times10\text{-}2$; $CuSO4$ $5H_2O$ $3.93\times10\text{-}5$; $Na_2 MoO_4$ $2H_2O$ $2.60\times10\text{-}5$; $ZnSO4$ $7H_2O$ $7.65\times10\text{-}5$; $CoCl_2$ $6H2O$ $4.20\times10\text{-}5$; $MnCl_2$ $4H_2O$ $9.10\times10\text{-}4$ were fed to R1, R4, respectively. The TOC/IC/TN concentration in MBM averaged 4.03 ± 0.60, 0.70 ± 0.20, 73.07 ± 11.45 mg L^{-1} during experiments (Table 2).

10% PWW were fed to R2, R3, respectively. Dilution factor of 10% PWW was selected from previous researches which were succeed to keep predominant of algae (García et al., 2018). 10%PWW were prepared by centrifuge the PWW 10000 rpm, 10 min and mixed with water. The DOC/IC/TN concentration in 10%PWW averaged 1371.20 ± 126.31, 240.53 ± 86.09, 437.31 ± 114.87 mg L^{-1} during experiments (Table 2). Even if PWW were centrifuged, small amounts of solids (TSS 0.65 ± 0.17g L^{-1}; VSS $98.45\pm1.75\%$ in 10% PWW) were contained. 10% PWW absorbed 2.33 ± 0.06 m^{-1} of light at 680nm while MBM didn't absorb any wavelength of light. absorbance of 10% diluted PWW was shown in Fig. 1.

III. Sampling procedure

Environmental conditions except for light were checked every day in the morning and afternoon (9:30 and 18:30). DO were measured by OXI 330i oximeter and pH were measured by a Crison M44 multimeter and a Crison pH 28 meter. Influents and effluents flowrate were measured every time when effluent samples were recovery one or two times per week. Samples were saved for analysis of TSS/VSS and optical density and liquid phase analysis. Then, residual samples were centrifuged 10000 rpm, 5 min by Legend RT+ (Thermo Scientific, United States). The pellet was saved in a deep freezer at - 80°C until the time when samples were lyophilized for cellular compositions analysis.

IV. Analytical procedures

Total suspended solids (TSS) /Volatile suspended solids (VSS) concentrations were determined by the gravimetric method according to internal analytical standards from the Instrumental Techniques Laboratory (LT1 - UVa). Light absorbance from 350nm to 800nm were measured by UV-VIS spectrophotometer UV-2550 (Shimadzu, Japan). Samples were filtered by 47 mm Glass fiber filter (Fisher Scientific, EU) and TOC, IC, TN concentrations in liquid phase were determined by a TOC-V CSH analyzer equipped with a TNM-1 module (Shimadzu, Japan). Heavy metals in PWW were analyzed, Cu/Zn contents were analyzed with Multielemental Quantitative analysis by Optical

emission spectroscopy (ICP-OES), As/Cr/Pb with Multielemental Quantitative Analysis by Mass spectrometry (ICP-MS) and Hg with Direct analysis by Atomic absorption spectroscopy (DMA-80). Biomass analysis were conducted during the steady state because they were needed to get enough biomass amount for analysis. Microscopic analysis was conducted with samples before biomass analysis.

Total lipids were extracted from dry biomass. 200 mg of lyophilized dry samples were measured and mixed with Al_2O_3 in a ratio 1:1 and grind manually for 15 minutes each sample. Weigh 200mg (100mg biomass) in falcon in duplicate and extracted lipid with 3mL of chloroform-methanol 2:1. Mix and centrifuge 5 min at 3600 rpm. The supernatants were corrected as many times as necessary, until the color is clear, and the precipitate turns white-ash which means pigments have been totally removed. Add 3mL of 0.1M HCl and 0.3 mL of $MgCl_2$ 0.5% to separate proteins in collected supernatants. Mix and centrifuge 5 min at 3600 rpm and recovery the black phase of supernatants which is lipid extractants. Pasteur glass pipettes were used for transfer extractants to the pre-weighted glass tubes. Organic chemicals except for lipids in glass tubes were evaporated in a fume hood and total lipids were weighted after 24 hours. Total lipids contents were determined by equation (1).

$$\text{Lipid content } (\%)(^W/_W) = \frac{\text{Weight}_{\text{lipids+tube}} \text{ (g)} - \text{Weight}_{\text{empty tube}} \text{ (g)}}{\text{Weight}_{\text{dry samples}} \text{ (g)}} \cdots (1)$$

Pigments were analyzed with the dry biomass which is already grinded and mixed 1:1 (w/w) of biomass and alumina in lipids analysis. Weight 4 mg of sample in Eppendorf which is covered with tin foil in triplicate. After 1 mL of methanol were added to each, vortex were used each samples for 1 min. The samples were heated at 60°C for 30 min in the thermo block and let them reach room temperature. The samples were stored in the fridge (4°C) for 24 hours. Next day, centrifuge samples at 10000 rpm for 3 min and recover the supernatant. These supernatant were measured the absorbance in scan mode from 200 to 700 nm with spectrometer. Chlorophyll a (Chl-a), Chlorophyll b (Chl-b), Carotenoids content in methanol were calculated the following equation, and pigments concentration were calculated with the equation (2).

$$\text{Chl a} = 16.72A_{665.2} - 9.16A_{652.4}$$
$$\text{Chl b} = 34.09A_{652.4} - 15.28A_{665.2}$$
$$\text{Carotenoids} = (1000A_{470} - 1.63C_a - 104.96C_b)/221$$

$$\text{Pigments content (\%)} \left(\sideset{^W}{_W}{}\right) = \frac{\text{Concentration of pigment (mg L)} \times \text{Volume}_{MeOH} \text{ (mL)}}{\text{Weight}_{\text{dry samples}} \text{ (g)}} \cdots (2)$$

Organic nitrogen assay were conducted by the Total Kjeldahl nitrogen method and converted to protein content by nitrogen-protein conversion factor 5.95 (López et al., 2010). 100 mg of dry sample and 25 mL of distilled water were mixed in each digestion tube with one dielectric piece. Add 3mL of H_2SO_4 98% two times (to avoid heat up) and one Kjeldahl catalyst (Cu) tablet which is 0.3% in $CuSO_4 \cdot 5H_2O$. Digestion tubes in the digestor connect the form collector and proceed program 20 min up to 150℃. After the samples being checked no form foams, program 370℃ for 60 min were proceed. If the digestion of samples were not completed, the program at 370℃ repeated two or three times with adding water and H_2SO_4 3mL.

Digested samples were distillated by Kjel Flex K-360 (Fisher Scientific, United States). 0.05M or 0.1M H_2SO_4 were added until the color change from green to purple. Added volume were used for gaining nitrogen content in equation (3).

$$\text{Nitrogen content (\%)} = \frac{(NH_4^+ (mol\ L^{-1} \times Added\ volume\ (L) \times 14.007\ (g\ mol^{-1}\ N)}{Weight_{dry\ samples}\ (kg)} \cdots (3)$$

Carbohydrate were analyzed from the dry biomass. Weight 300 mg of samples in a 250 mL Erlenmeyer flask. Add H2SO4 72% (w/w) 3 mL and cover the flask with tin foil. Put the flasks in a thermostatic bath at 30℃ for 1 hour. Take out the flasks from the bath and add 84 mL of distilled water to each flask in order to dilute sulfuric acid from 72% to 4% (w/w), and mixed gently. Covered again with more tin foil and put the flasks in the autoclave, Liquids 121℃ for 59 min. Take the flasks out from the autoclave and let them reach room temperature. Filtrate sample through 0.22 μm filters into HPLC vials. Freeze until analysis.

V. Enzymatic hydrolysis for protein extractions

The enzymatic hydrolysis were conducted with two different types of protease, Protamax and Alcalase (both kindly donned by Novozymes) and three different retention time which are 90, 120, 240 min. Dry biomass 12.5 g were mixed with mili-Q water 250 mL (1:21 w/w ratio) in each bottles. pH was adjusted to 6.5 for Protamax, and 8.0 for Alcalase using 1M NaOH or HCl as required. The

bottles were placed on the rotary shaker until the temperature reached 50℃ and 1:100 ratio of enzyme: substrate (125 mg) of Protamex or 5:100 of enzyme: substrate (0.625 mL) of Alcalase were added. Bottles were stirred at 200 rpm and 50℃, for the required reaction time (90 or 120 or 240 min). pH was adjusted again after hydrolysis to the original optimum pH of each enzyme. Samples were centrifuged at 18500×g, 4 ℃ for 15 min. Save both of the supernatant (the sample contains extracted protein) and the pellets in deep freezer -85℃. Lyophilize of pellets were conducted to get the dry samples.

To get the dry samples of supernatants, the rotavapor were used over 2 hours at 50℃ to remove water from the samples. The samples were saved in deep freezer – 85℃ again. Lyophilize of sample were conducted to get the dry samples for the supernatants

VI. Calculation

TN removal rate (%) in both conditions of MBM and PWW and TOC/IC removal rate (%) in PWW were calculated according to equation (3) in order to know the efficiency for wastewater treatment.

$$\text{RE (\%)} = \frac{\left(C_{feed} \times Q_{feed}\right) - \left(C_{effluent} \times Q_{effluent}\right)}{\left(C_{feed} \times Q_{feed}\right)} \times 100 \cdots (3)$$

The optical density was subjected to regression analysis with the explanatory variable as optical density (680 nm) and the target variable as TSS. At this time, the intercept was set to 0. This calculation was performed in order to know if optical density is an appropriate alternative to TSS analysis.

The estimated extracted protein contents in supernatants were calculated from the protein contents in peletts by using the result of Kjeldahl methods before extraction process, 50.9% of protein contents:

$$\text{Estimated extracted protein contents in supernatants (\%)}$$
$$= 50.9\ (\%) - protein\ contens\ in\ peletts\ (\%)$$

VII. Statistic analysis

Wherever possible, results were expressed in the form of mean ± standard deviation. The Tukey-Kramer test was used to test for differences between multiple groups (1% level of significance) by software, Statcel 3.

Results

TSS/VSS, Optical density

In the experiments, TSS in R2 and R3 increased after starting to feed PWW while TSS in R1 and R4 maintained value from pre-culture. TSS/VSS showed a steady state after the 11th day (Day 55 in Fig. 4) and continuous culture were maintained in all of reactors. TSS during steady state was averaged from 14, 17, 20th day (Day 58, 61, 64 in Fig. 4) and valued 0.55±0.13, 1.83±0.09, 1.71±0.65, 0.50±0.17 g L^{-1} in R1, R2, R3, R4, respectively. VSS during steady state were averaged from 11, 14, 20th day (Day 55, 58, 64 in Fig. 4) and valued 0.62±0.09, 1.67±0.09, 1.83±0.43, 0.61±0.11 g L-1 in R1, R2, R3, R4, respectively. The data of optical density (680nm) were compared to TSS (Fig. 5). Correlation coefficients between optical density and TSS in MBM and PWW were 0.67 and 0.78, respectively. Regression analysis was performed on each data to show a regression equation of y = 0.3023x, y = 0.3346x.

pH, Temperature, Evaporation rate, DO concentration

pH ranged from 7.08 to 8.0 in all reactors and showed a similar value between morning and afternoon. The temperature of all reactors during the experiment were higher values than during preculture and ranged from 20.1℃ to 31.2℃ in all days (Fig. 2). During the experiment, the temperature of all reactors was also different between morning and afternoon (Table 3). Evaporation rate were averaged 22.9%, 25.3%, 23.3%, 25.0% in R1, R2, R3, R4 respectively.

The operational conditions during steady state were showed in Table 3. DO concentration showed different values between morning and afternoon (Fig. 3). In the pre-culture, averages of DO concentration were 16.0, 15.6, 15.6, 16.5 mg L^{-1} in the morning and 18.9, 16.7, 18.8, 19.5 mg L^{-1} in the afternoon in R1, R2, R3, R4 (Table 3). However, these values decreased until >7.5 mg L^{-1} before starting experiments. In the experiments, average of R1, R2, R3, R4 were 11.6, 0.50, 0.18, 10.3 mg L^{-1} in the morning and 14.1, 0.30, 0.0, 12.3 mg L^{-1} (except for Day 0) in the afternoon respectively.

TOC/IC/TN

TOC, IC and TN concentration of R2 and R3 in the influent were higher than R1 and R4. (Fig. 6-a/b). TOC and IC concentration of R1 and R4 in the effluents increased with TSS while the decrease in R2 and R3 occur in PWW conditions (Fig. 6-a/b). TC concentration in effluents showed 48.1, 48.0 mg L^{-1} in R1, R4 and 446.3, 405.6 mg L^{-1} in R2, R3 while the influents concentrations were 4.99, 5.20,

1350, 1420 mg L^{-1} in R1, R4, R2, R3, respectively. The removal rate of TOC/TN in PWW condition resulted in 91.4% and 74.5%, respectively (Fig. 6-c).

TN concentration in effluents was lower than influents in all of the reactor (Fig. 6). TN concentration in effluents showed 31.9, 28.29 mg L^{-1} in R1, R4 and 134.1, 164.9 mg L^{-1} in R2, R3 while the influent concentrations were 61.2, 58.8, 341.2, 320.2 mg L-1 in R1, R4, R2, R3, respectively. Removal rate (RE) of TN between all of the reactors showed similar values during pre-culture and the experiment (Appendix Fig. 12)

Microscopy analysis

Periodical microscopy analysis of biomasses revealed that *Scenedesmus acuminatus* was the predominant microalgae specie in the inoculum samples (Fig.7-e). The sample at 17th and 20th (Day 61 and 64 in Fig.4) during steady state were revealed that *Scenedesmus* spp. were predominant microalgae species in all of reactors during steady states. *Scenedesmus dimorphus* were mainly observed in the R2 and R3 (Fig. 7-b,c). *Scenedesmus obliquus* were mainly observed in the R1 and R4 (Fig. 7-a,d).

Biomass compositions in our photobioreactors and TL photobioreactor

Biomass compositions during steady state were showed similar values between MBM and PWW in our small reactors (Fig.8). Only the Lipid contents were ranged from 11.9% to 16.4%, protein contents were ranged from 51.1% to 55.0%, ash contents were ranged from 11.5 % to 13.6% in MBM and PWW conditions. Carbohydrate in pre-culture was averaged 20.9 ± 5.03 % (Fig. 8). The carbohydrates of samples in the both of MBM and PWW conditions were analyzed but the data were not stable. Therefore, the carbohydrate content was estimated to be the total biomass amount minus the lipid and protein, ash content. The protocol should be checked for the carbohydrate analysis.

In TL reactor, the carbohydrate contents in PWW showed 43.5 % while 32.2 ± 0.14 % were observed in Bristol medium(BM) in TB reactor(Fig. 8). The protein content of PWW conditions in TL reactor was predominantly low compared to BM in TB reactor, MBM and PWW in our reactors (Tukey-Kramer,0.01<P). There was no significant relationship between the lipid content of all of samples in all of reactor. Chl-*a*, Chl-*b*, Carotenoids contents in PWW conditions were lower than medium conditions in both of our reactor and TL reactors (Fig. 9).

The extracted protein contents of *Scenedesmus* spp. in PWW by enzymatic hydrolysis

The extracted protein contents showed almost lower than 10% in both of Alcarase and Protamax (Fig.11). The extracted lipids contents were also low in all of samples (Fig. 11). We got several problems in the results. First problem, the samples were difficult to be homogenized after lyophilized and before analysis (Fig. 13). Secounds, the values had big differences between samples in duplicate. Therefore, we couldn't get the average of each samples. However, the samples by Alcarase extraction seems higher than Protamax extracted samples for both of protein and lipids.

The protein contents in the pellets after enzymatic extraction and estimated protein contents in the supernatants

Almost protein contents were remained in the pellets and not extracted (Fig. 14-a). As for lipids contents, the lipid contents were 9.4 % before extraction process so all of lipids contents were not extracted. The reason that lipid contents in all of samples were higher than 9.4 % (Fig. 14-b) is due to the technique mistake. The recovery of lipids which contain small particles were difficult in the lipids extraction process. Probably, the data of lipids contents showing the weight of small particles with lipids.

The extracted protein contents were estimated 12% with Protamex, 90 min conditions as a maximum (Fig 14-c). The amount of the remain of protein content were showed difference with enzyme and retention time. In the extraction by Protamax hydrolysis, longer retention time showed the reduction of protein extraction. In the extraction by Alcalase hydrolysis, longer retention time showed more amounts of protein extraction.

### Productivity of biochemical components in our reactors

Lipid and protein productivity in PWW conditions were higher than MBM conditions (Fig. 10). MBM conditions showed similar values with pre-culture. Lipid and protein productivities were 0.26 and 0.97g L^{-1} in PWW conditions, 0.08 and 0.27 g L^{-1} in MBM conditions, 0.08 and 0.34 g L^{-1} in pre-culture. The productivity of pigments in PWW were lower than MBM conditions even if the growth rate in PWW was higher than MBM (Fig. 9). The productivity of Chl-*a*, Chl-*b*, carotenoids in PWW conditions were 49.3, 17.0, 13.4 mg L^{-1} while 26.9, 13.9, 5.6 mg L^{-1} in MBM conditions.

Discussion

I. Operational conditions of our small photobioreactors

TSS during steady state was averaged from 14, 17, 20th day (Day 58, 61, 64 in Fig. 4) and valued 0.55±0.13, 1.83±0.09, 1.71±0.65, 0.50±0.17 g L^{-1} in R1, R2, R3, R4, respectively. R1 and R4 have similar results and R2 and R3 other, there is a high repeatability in this system, which proves the good performance of our experimental set-up. RE in R2 and R3 were calculated for determining the water quality of this photobioreactor (Fig. 6-c). COD and TN RE of >83.6 % and> 74.5% were obtained with PWW treatments in this study. In this experiment, the RE of COD were higher than in TN compared to the previous study. Average COD and TN RE of 76 ± 11% and 88 ± 6% were reported in a previous study (Godos et al., 2009).

Operational conditions during steady state of experiments were described in Table 3. The DO concentration in PWW were maintained 0 mg L^{-1} while DO 14.8-17.1 mg L^{-1} were produced in MBM conditions (Table 3, Fig. 3). In both conditions, photosynthesis occurred by algae in the reactors (equation 4; Peng et al., 2013). 8~32 mg L^{-1} (100~400% air saturation) of DO accumulation is commonly observed in microalgal cultures (Peng et al., 2013). DO are usually accumulated in photobioreactor while the consuming rate might be different between reactor design which has different light availability.

$$nCO_2 + nH_2O + light \rightarrow (CH_2O)_n + nO_2 \cdots (4)$$
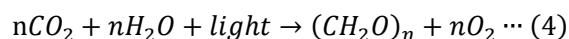
However, low DO concentration in PWW conditions suggest that oxygen reacted to biodegrade organic pollutant by aerobic bacteria. Bacteria remove DO by their respiration (Muñoz and Guieysse, 2006; Dalrymple et al., 2013). Since wastewater contains bacteria, the algae-bacteria process is showing the reduction of oxygen in a photobioreactor, high-rate oxidation ponds (Abeliovich and Weisman, 1978). Therefore, this reduction in DO concentration suggest that bacteria consumed oxygen and proved that algae could provide the oxygen for the reduction of COD in the reactors.
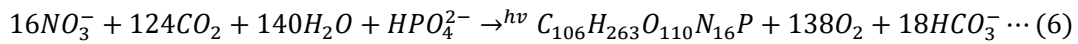
In this experiment, DO supply by algae was effective. DO concentrations in MBM conditions suggest that algae in PWW could supply the oxygen approximately <17.1 mg L^{-1} in R2 and R3. This process did not require the oxygen supply to remove organic matter in the reactor. It is thought that low-cost operation for wastewater treatment was possible due to the presence of algae.

II. The growth of microalgae in algae-bacteria process

In the experiment, TSS/VSS in PWW conditions were higher than MBM conditions during steady

state (Fig. 4). Even if the substrate of 10% PWW contained 0.59 ± 0.27g L^{-1} of TSS affect to the data of TSS in PWW conditions, the result of TSS in PWW show higher values than MBM conditions. From this reason, it was considered that the growth rate of algae in PWW was higher than MBM. Microalgae biomass increase by consuming elements such as nitrogen, phosphate, carbon source with light energy (equation 5,6;Dalrymple et al., 2013). Generally, the growth of microalgae in NH_4^+ is faster than NO_3^- (Harrison et al., 1990). It is possible to consider that higher concentration of NH_4^+ promote the growth of algae in PWW conditions because MBM doesn't have NH_4^+.

The differential of TN concentration between influent and effluent were 29.3, 30.5 mg L^{-1} in R1, R4 and 207.2, 155.3 mg L^{-1} in R2, R3, respectively (Fig. 6-b). These differential values prove that microalgae and bacteria in PWW conditions consumed nutrients more than MBM conditions.

$$16NH_4^+ + 92CO_2 + 92H_2O + 14HCO_3^- + HPO_4^{2-} \rightarrow^{hv} C_{106}H_{263}O_{110}N_{16}P + 106O_2 \cdots (5)$$
$$16NO_3^- + 124CO_2 + 140H_2O + HPO_4^{2-} \rightarrow^{hv} C_{106}H_{263}O_{110}N_{16}P + 138O_2 + 18HCO_3^- \cdots (6)$$

In MBM conditions, accumulated DO might limit the growth of algae in the reactors. Bacteria in PWW conditions might produce higher CO_2 concentration with higher DO concentration. High CO_2/O_2 ratio affected to fix carbon source (Kliphuis et al., 2011). Bacteria help algae growth by CO_2 production in the symbiotic consortium of bacteria-microalgae (Abeliovich and Weisman, 1978), and not only because of higher elements concentration in the substrate. The DO removal in PWW may promote photosynthesis of algae and enhance consuming nutrients by algae from substrate.

Also, the consumption of organic matter may have also resulted in increased growth rates. The growth of *Scenedesmus* varies by species. Some species can consume the specific organic matter in the wastewater while others cannot.

III. The relationship between Optical density and TSS

The maximum optical density in MBM is 3.09, whereas, in the PWW, the maximum optical density is 4.53 (Fig. 5). These data proved that the presence of microalgae in PWW even if the substrate of 10% PWW absorbed 2.33 ± 0.06 m^{-1} of light at 680nm. This result followed the TSS data which showed the over 0.59 ± 0.27g L^{-1}, the concentration of 10%PWW substrates.

The results of a single regression analysis of the TSS with optical density showed a difference between MBM and PWW, the objective variables showed 0.3023 and 0.3346 in MBM and PWW (Fig. 5). The optical density at 680nm was considered to be an appropriate alternative to TSS analysis.

Generally, Chl-*a* pigment which absorb light at 680 nm, the maximum peak is an indicator of algal concentration. However, it has become clear that it is necessary to use different regression equations when determining TSS using optical density.

The optical density in PWW showed higher values than values in MBM. It was considered that the optical density at 680 nm in MBM is only showing the growth of microalgae while the growth of microalgae and the increase of DOC from 10% PWW are showing in PWW conditions. 10% PWW absorbed 2.33 ± 0.06 m^{-1} of light at 680nm while Medium didn't absorb any wavelength of light. Therefore, it is considered that the difference in regressions was depended on the substrate.

Also, it might be required that the over 2.33 ± 0.06 m^{-1} of optical density in PWW is used another single regression analysis because they will show only the growth of microalgae like MBM conditions.

IV. Biomass composition of *Scenedesmus* spp. in PWW treatment

The biomass compositions were compared between PWW and MBM from our small reactors and TL reactor in Almeria university. Generally, the protein contents increased with high concentration of nitrogen source and NH_4^+ are more efficiency compared to NO_3^- (Piorreck et al., 1984). It seems lower lipid content than protein content when use wastewater as a substrate (Table 1). However, the protein contents of *Scenedesmus* spp. in PWW and MBM were showed similar compositions during steady state in our reactor (Fig. 8). The presence of ammonia oxidizing bacteria (AOB) and nitrate bacteria (NOB) may convert from NH_4^+ to NO_3^- in our reactors. The RE of TN showed similar values between MBM and PWW in our reactors. Also, Lipid contents showed <16.4% in both of PWW and MBM. For biofuel production, a lipid content exceeding 20% are considered to be necessary while some researcher suggest 40% (Dalrymple et al., 2013). Therefore, the protein contents in algae biomass will be required to use with biofuels. It is suggested that the algae biomass have same compositions under same conditions such as pH, temperature, light intensity even if DO and feed compositions were different.

The biomass samples of same microalgae in TL and BL photobioreactor were analyzed to compare the algae biomass compositions in the different photobioreactor design and environmental conditions (Fig. 8). Probably, because the high light intensity in TL photobioreactor, the protein contents in PWW was lower than other samples. Also, the carbohydrate contents in PWW condition of TL reactor was higher than other biomass compositions (Fig. 8). It is considered that the carbohydrate was increased due to the increase of light. TL reactor was outside and under higher light

intensity. Generally, sun light can reach 2000 μ mol m^{-2} sec^{-1} outside and height on this reactor is very low.

There are several possibilities as the reason that the carbohydrate increase. The first possibility is that a N deficiency has occurred (Zhu et al., 2014). The carbohydrate content increased in *Chlorella vulgaris* with 10% PWW under heterotrophic and mixotrophic conditions due to N depletion in batch-culture (Wang et al., 2015). It also can be considered the supply of light enhance the photosynthesis which absorb N source. Generally, the growth limitation by nutrient deficiency also occur under the light limitation (Harrison et al., 1990). However, it is unlikely that our result occurred in a nutrient rich substrate, MBM and PWW.

It may depend on the supply of CO_2 by bacteria because the carbohydrate in MBM didn't increase under the high light due to TL photobioreactor (Fig. 8). The presence of bacteria in PWW is thought to promote algal photosynthesis by release from high DO concentrations. From this reason, the result suggested that the combined effect of high light and elements compositions in PWW. This situation can change the ratio of C/N assimilation rate.

The second possibility is the presence of heavy metals in PWW. PWW contains zinc (Zn), copper (Cu), arsenic (As) and the removal of Zn by algae was confirmed in previous study (García et al., 2018). Algae can alleviate the toxicity of heavy metal by carbohydrate. When the light is increased, the high absorption of heavy metals were observed in previous studies. The algae may accumulate the carbohydrate in order to alleviate the toxic of heavy metals under the high light. Therefore, it is considered that the high absorption of heavy metal occurred in PWW of TL photobioreactor due to high light, and increased carbohydrate content to alleviate the toxic of heavy metal.

Also, the results of pigments might be affected by the metals. The pigments contents in PWW conditions were decreased in both of our reactors and TL reactor (Fig. 9). Our PWW contains some metals Cu, An also heavy metal such as As, Cr, Pb, Hg (Table 2). Absorption of metals inside cells via metal transport systems occurred in microalgae cells (Monteiro et al., 2012). Metal in microalgae cells can cause formation of reactive oxygen species (ROS) such as hydroxyl ($\cdot$ OH), superoxide anion ($O_2 \cdot ^-$), singlet oxygen (O_2*) and hydrogen peroxide (H_2O_2) (Pinto et al., 2003; Miazek et al., 2015). ROS can oxidize protein, lipids such as Chl-a, Chl-b, carotenoids. The capacity of initial metals concentration depends on species. The decrease in pigments content were observed with Cu, Zn, Hg in the previous study (Küpper et al., 1998; Zhou et al., 2012). About Zn^{2+}, it might not be a stress because *Scenedesmus obliquus* could remove 836.5 mg g^{-1} of Zn^{2+} with the initial concentration 10-

75 mg L^{-1}. Oxidative stress can be overcoming by antioxidants enzymes in the low concentration of metal (Tripathi et al., 2006).

V.   The extraction of PWW treated microalgae biomass for protein products

The results of extracted protein and lipids contents were considered to have error because the data were difference between duplicate samples (Fig 11). The both of protein and lipids contents were very low in the results even if they have error in the duplicate. There are two possibilities from these results. First idea, it is considered that the supernatant samples had big amounts of carbohydrate which can make low contents of lipids and proteins. Seconds, the evaporation of NH_4^+ occurred during the process of evaporator (Fig 12). NH_4^+ can dissolved in water and evaporate when the water contents maximum NH_3. Third, the samples were not homogenized before analysis (Fig. 13). We need to know what the problem and the real values of the enzymatic extraction was. Therefore, the protein and lipids contents in the pellets samples were analyzed (Fig. 14).

The maximum 12% of extracted protein contents was estimated to calculate the values of Protamex, 90 min. This value was different result from the extracted protein and lipid contents (Fig. 11) but the common results were lower values than the results of Kieldahl. The suit extraction methods were required for algal biomass because the protein contents by Kjeldahl methods showed 50.9% of protein contents (Fig. 11). The idea for the improvements of this extraction are cell broke. To get the higher extraction, we can break the cell due to ultrasonicate or grind or using chemicals because microalgae have strong cell wall. The one of the problem was temperature because the enzyme were putted in the bottle at 40°C. The maximum temperature of rotary shaker was 60 degree and took time to be the samples 40°C for over 1 hour. The experiments were conducted lower temperature due to the technic problem. It might affect the data which were lower extraction. We need to try the different ratio of enzyme and samples, temperature for the enzyme reactions to know the suit operation (Silva et al., 2014; Thi et al., 2017). Also, the relationship between heavy metals and enzymatic hydrolysis were required to know (Li et al., 2016).

VI. Productivity of chemical components in *Scenedesmus* spp.

Lipid and protein productivity in PWW were high compared to MBM conditions because VSS in PWW conditions were three times higher than MBM conditions (Fig. 10). Unfortunately, the pigments productivity in PWW were lower than MBM conditions. Biomass composition varies with algal species. For example, in the culture using urban wastewater in India, the lipid content when culturing

Euglena sp. is reported to be 24.6% (w / w) higher than *Spirogyra* sp. 18.4% and *Phormidium* sp. (Ramachandra et al., 2013).

Higher protein content (averaged 52.8±1.8 %) than lipid showed that the potential of utilization of protein with carbohydrate in this species. The protein in algae biomass have an attention because they have Emulsifying action　(Ba et al., 2016) and antioxidant action (Samarakoon and Jeon, 2012). In addition to their use as biofuels, the use of proteins as functional antioxidants and the usefulness of high value-added peptides have been reported (Samarakoon and Jeon, 2012; Medina et al., 2015; Ejike et al., 2017). Biomass productivity are explained by the growth rate of algae and the contents of biochemical components in each wastewater and species (Pittman et al., 2011). Our results showed that the biomass productivity in algae-bacteria process of PWW treatment was higher than MBM due to higher growth rate of algae. The maximum lipid productivity of 110.56 mg^{-1} L^{-1} d^{-1} in *Chlorella zofingiensis* showed by Zhu et al., 2013 when initial COD of PWW was diluted to 1900mg/L. The protein productivity 0.97 g L^{-1} in PWW were higher than lipids and carbohydrate in this experiment.

Conclusions

In this study, the cultivation of *Scenedesmus* spp. with non-sterilized PWW were evaluated in small scale photobioreactors. The results showed the combined effect of the presence of bacteria and the elements compositions such as high concentration of nutrients and heavy metals on algal biomass. The algae-bacteria process by using PWW enhanced the growth rate of algae and obtained higher protein productivity 0.97 g L^{-1} in PWW than MBM conditions. In the algae-bacteria process, bacteria showed the DO removal in the reactors and proved the higher growth of microalgae with low cost for wastewater treatment. As for algal biomass compositions, decrease of pigments may be occurred due to the oxidative stress of heavy metal. The highest protein content compared to lipids was considered that have a potential of utilization of protein contents with carbohydrates in this species. The results of enzymatic hydrolysis were estimated the maximum 12% protein contents with Protamax, 90 min. Future research were required about the optimization of cultivation conditions with heavy metals, the protein extraction method for biochemical products.

Acknowledgments

Table

Table 1 Comparison of lipid content of microalgae in medium and various wastewater

Species	Cultivation condition	Substrate	Lipid content (% of DCW)	Reference
Chlorella protothecoides	Heterotrophic		50.3-57.8	Xiong et al. (2008)
Dunaliella tertiolecta ATCC 30929	Phototrophic		60.6-67.8	Takagi et al. (2006)
Nannochloropsis sp.	Phototrophic		35.7	Rodolfi et al. (2009)
Scenedesmus obliquus	Phototrophic		17.7	Gouveia and Oliveira (2009)
Scenedesmus quadricauda	Phototrophic		18.4	Rodolfi et al. (2009)
Scenedesmus sp. DM	Phototrophic		21.1	Rodolfi et al. (2009)
Scenedesmus obliquus		Municipal (Secondary treated)	31.4	Martinez et al. (2000)
Scenedesmus sp.		Agricultural (fermented swine urine)	0.9	Kim et al., (2007)
Scenedesmus sp.		Artificial wastewater	12.8	Voltolina et al. (2010)
Chlorella sp.		Agricultural (dairy manure with polystyrene foam support)	9	Johnson and Wen (2010)
Chlorella sp.		Agricultural (digested dairy manure, 20 × dilution)	13.6	Wang et al. (2010, inpress)
Dunaliella tertiolecta		Industrial (carpet mill, untreated)	15.2	Chinnasamy et al. (2010)

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Table 2. The compositions of substrate Modified Bristol medium (MBM) and 10% PWW

Elements compositions	MBM (in R1,R4)	10% PWW (in R2, R3)
TOC (mg L^{-1})	4.03 ± 0.60	1371.20 ± 126.31
IC (mg L^{-1})	0.70 ± 0.20	240.53 ± 86.09
TN (mg L^{-1})	73.07 ± 11.45	437.31 ± 114.87
N-NH$_4^+$ (mg L^{-1})	-	216*
As (mg L^{-1})	-	0.005*
Cr (mg L^{-1})	-	0.02*
Cu (mg L^{-1})	-	1.5*
Pb (mg L^{-1})	-	0.002*
Zn (mg L^{-1})	-	3.1*
Hg (mg L^{-1})	-	0.0002*

Table 3 Operational conditions of steady state in the experiments (Day 16-22)

Parameters		MBM		PWW	
		R1	R4	R2	R3
Light intensity (μmol m^2 s^2)		1028.0	1029.5	1097.5	1058.4
pH		7.83	7.82	7.92	7.92
Temprature (°C)					
	Morning	25.8	25.8	25.6	25.5
	Afternoon	30.6	30.5	30.8	30.9
DO (mg/L)					
	Morning	16.4	14.8	0.03	0.02
	Afternoon	17.1	16.8	0.06	0.03
Evaporation rate (%)		22.9	25.0	25.3	23.3
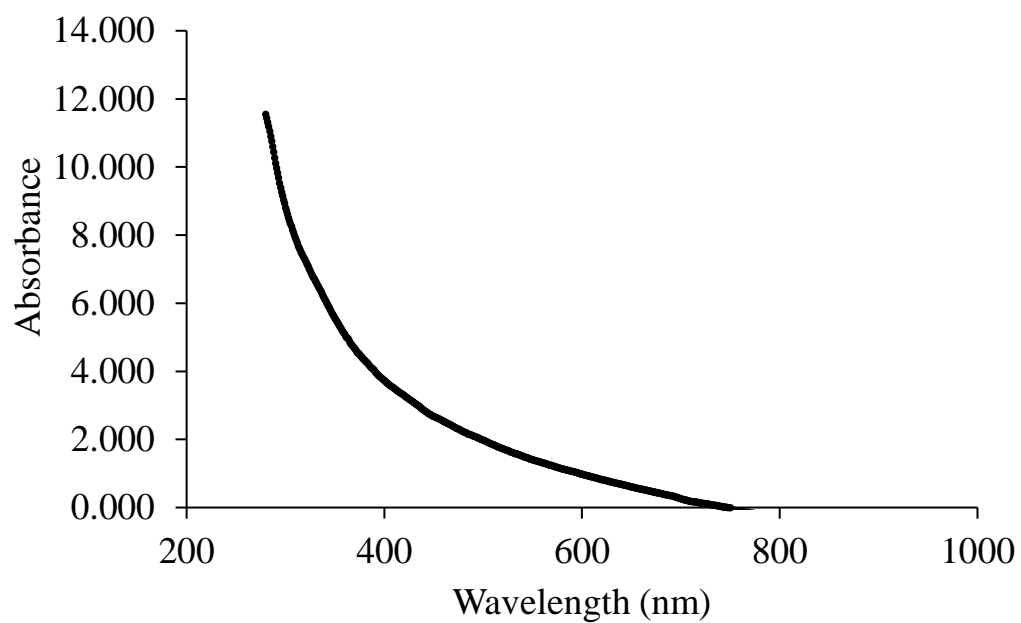
Figure

Figure 1. Light absorption spectrum of 10% diluted PWW. Wavelength 750 nm (No absorption by Chl. a) was used as zero for normalization.
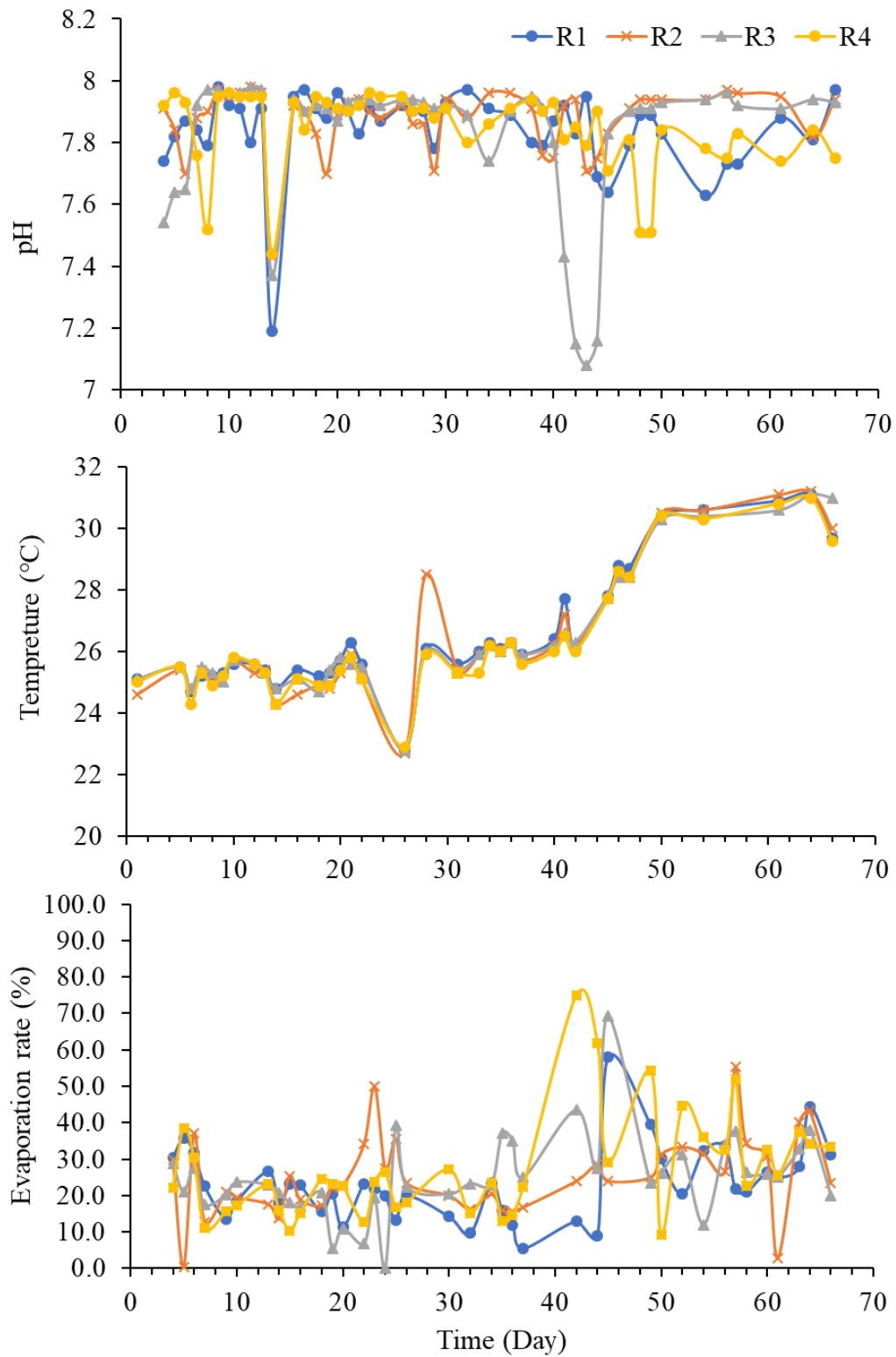
Figure 2. Time course of operational conditions in pre-culture and the experiment. The experiments were conducted from Day44 to Day 64.
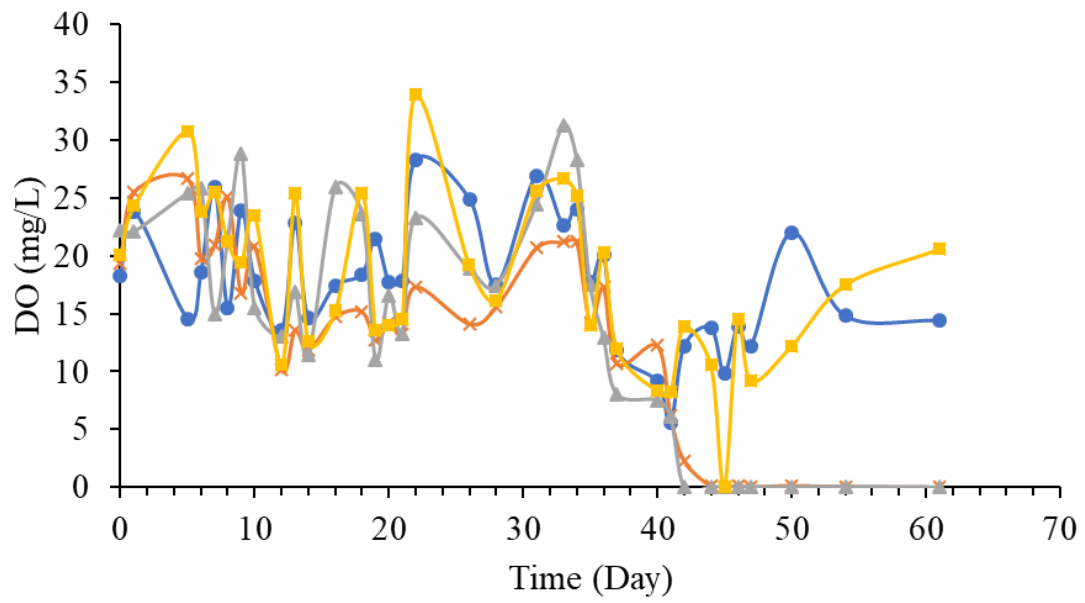
Figure 3. Time course of DO concentrations in pre-culture and the experiment. The experiments were conducted from Day44 to Day 64. R1 and R4 were cultured with MBM, R2 and R3 were cultured with 10% PWW.
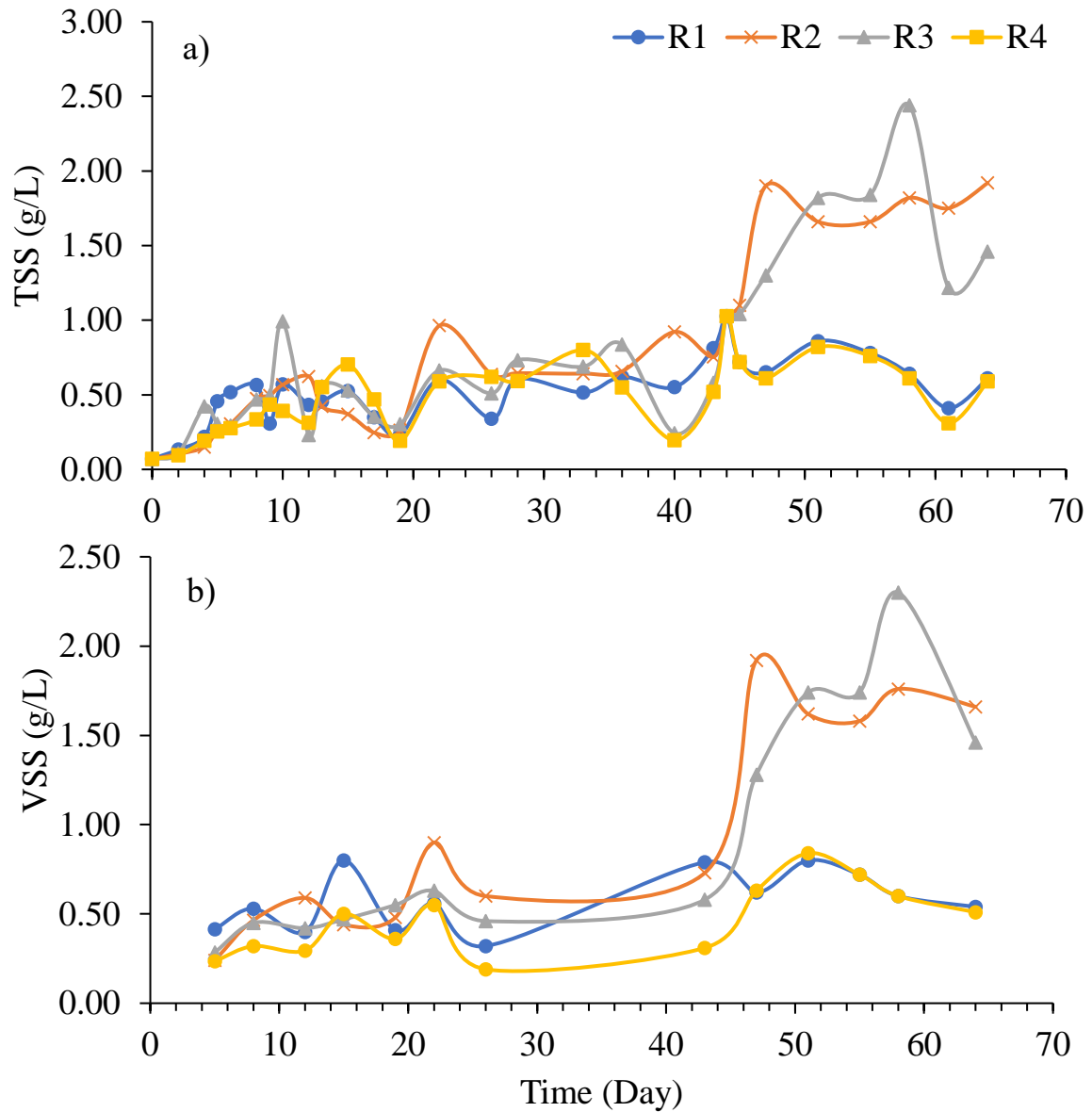
Figure 4. Time course of (a) Total suspended solids (TSS) and (b) Volatile suspended solids (VSS). The experiments were conducted from Day44 to Day 64.
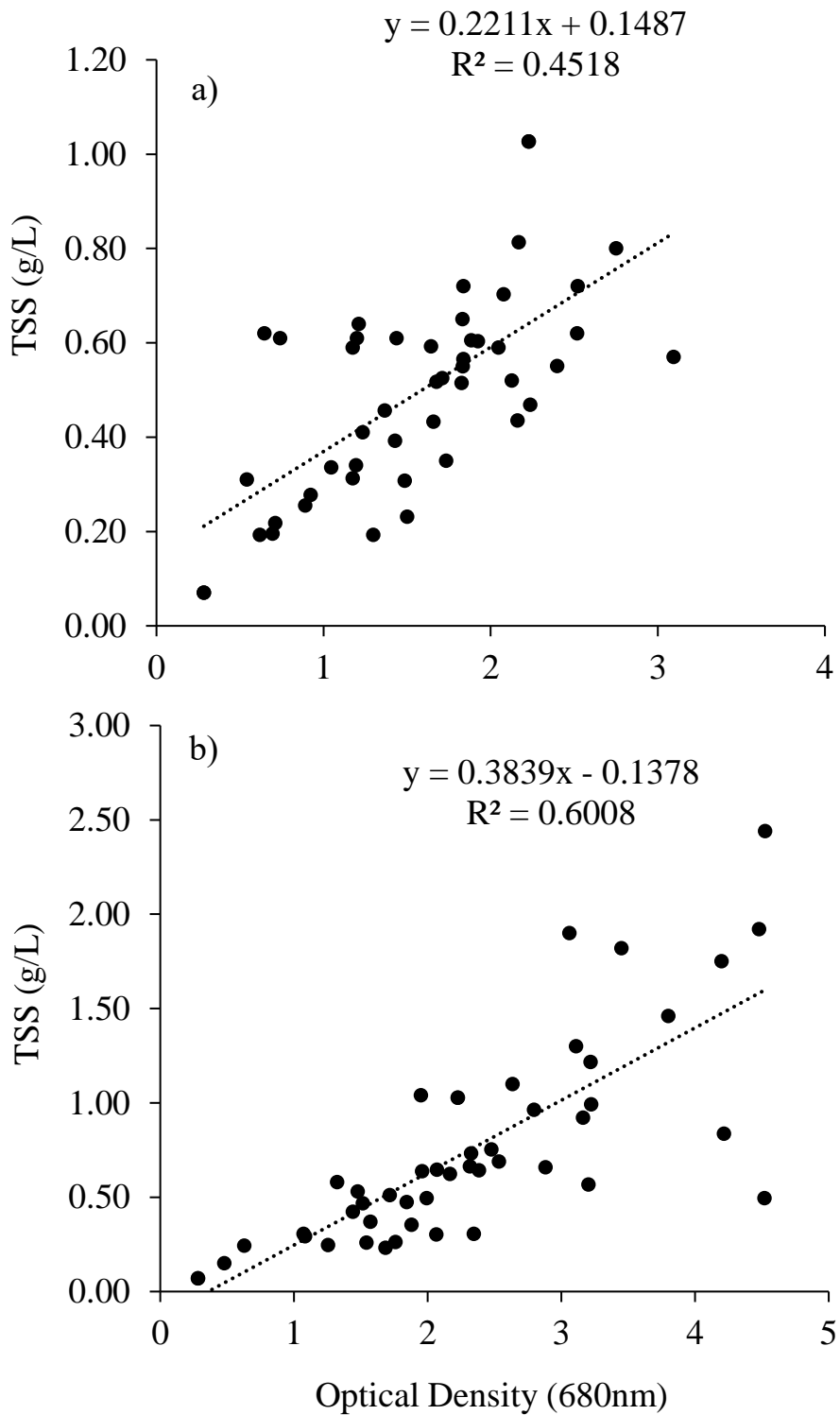
Figure 5. Relationship between TSS and optical density by single regression analysis. a) MBM conditions b) PWW conditions
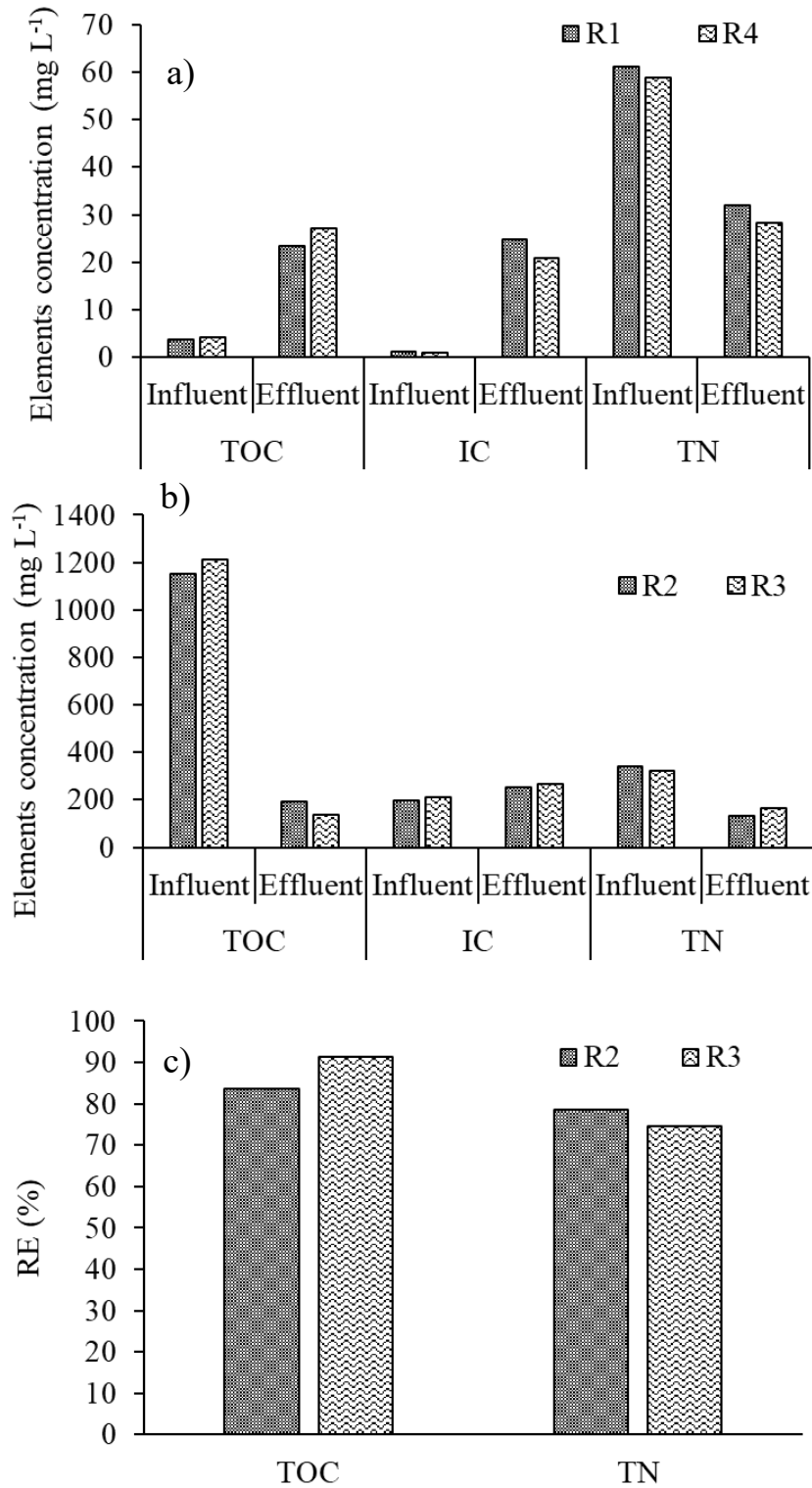
Figure 6. a) The concentration of TOC/IC/TN in the influent and effluents at Day 61 (17th day in the experiment).b) The removal rate of TOC/TN at Day 61 (17th day in the experiment) in PWW conditions.
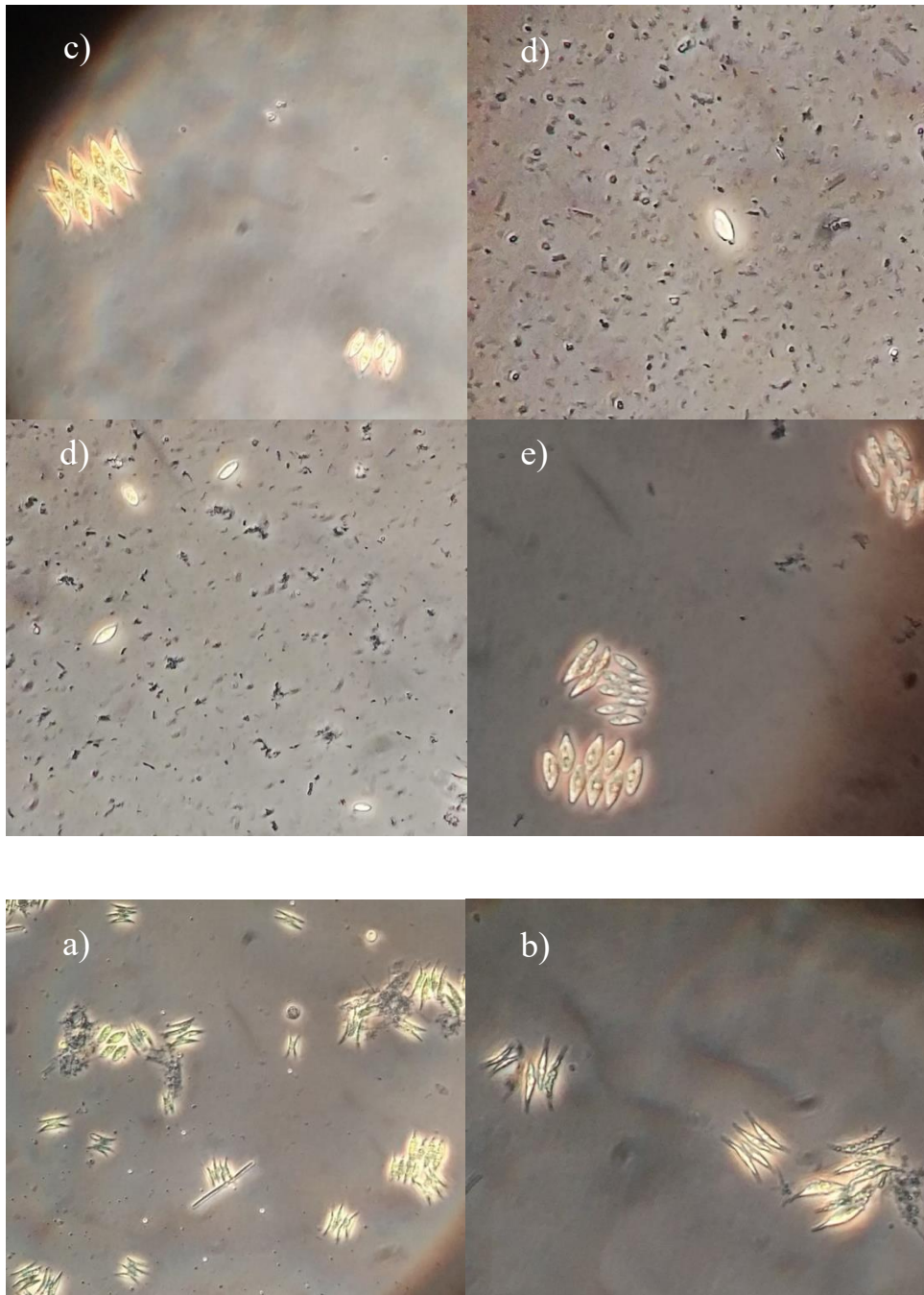
Figure 7. Microscopy analysis were conducted at 17th and 20th (Day 61 and 64 in Fig.4) during steady state. a) R1 b) R2 c) R3 d) R4, e) inoculums samples d) The mixed sample of all of the reactors at the initial day (Day44 in fig. 4).
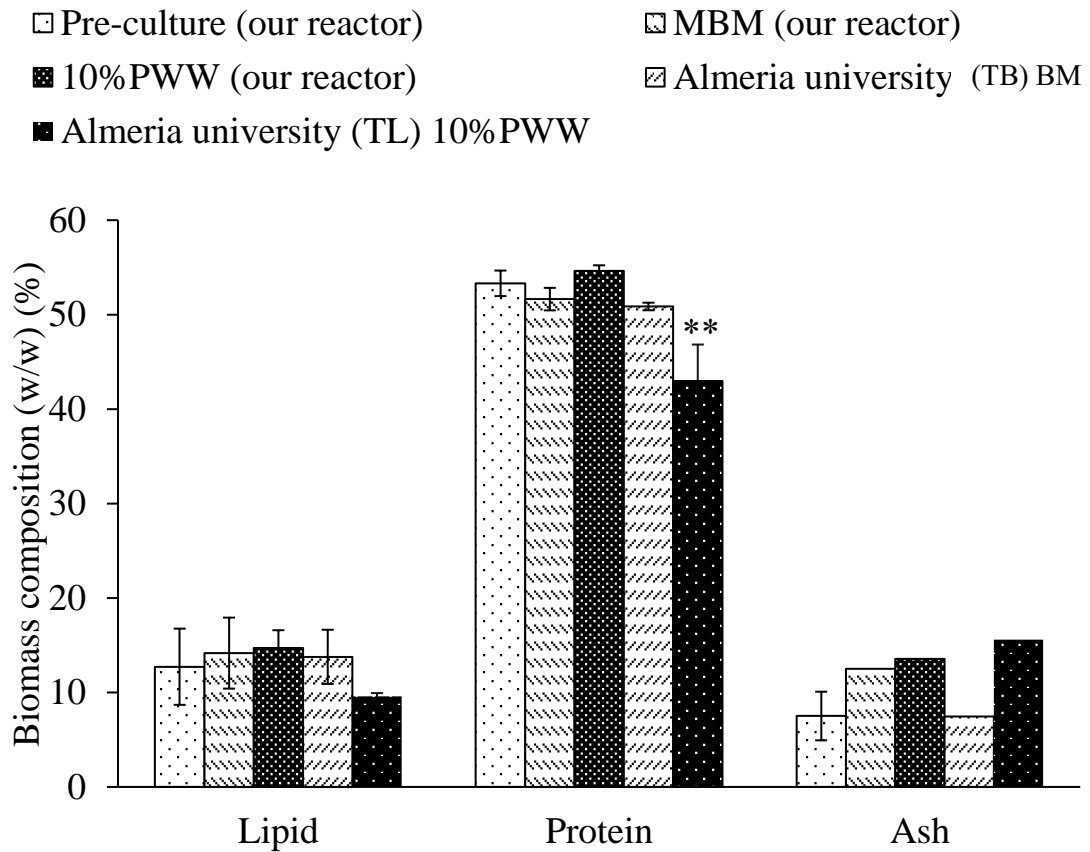
Figure 8. The biomass compositions of our reactor and Thin-layer (TL), Tubular reactor (TB) photobioreactor in Almeria university. Lipid, protein and Ash contents were analyzed. The mark ** showing the significant difference (Turkey Kramer, $p < 0.01$)
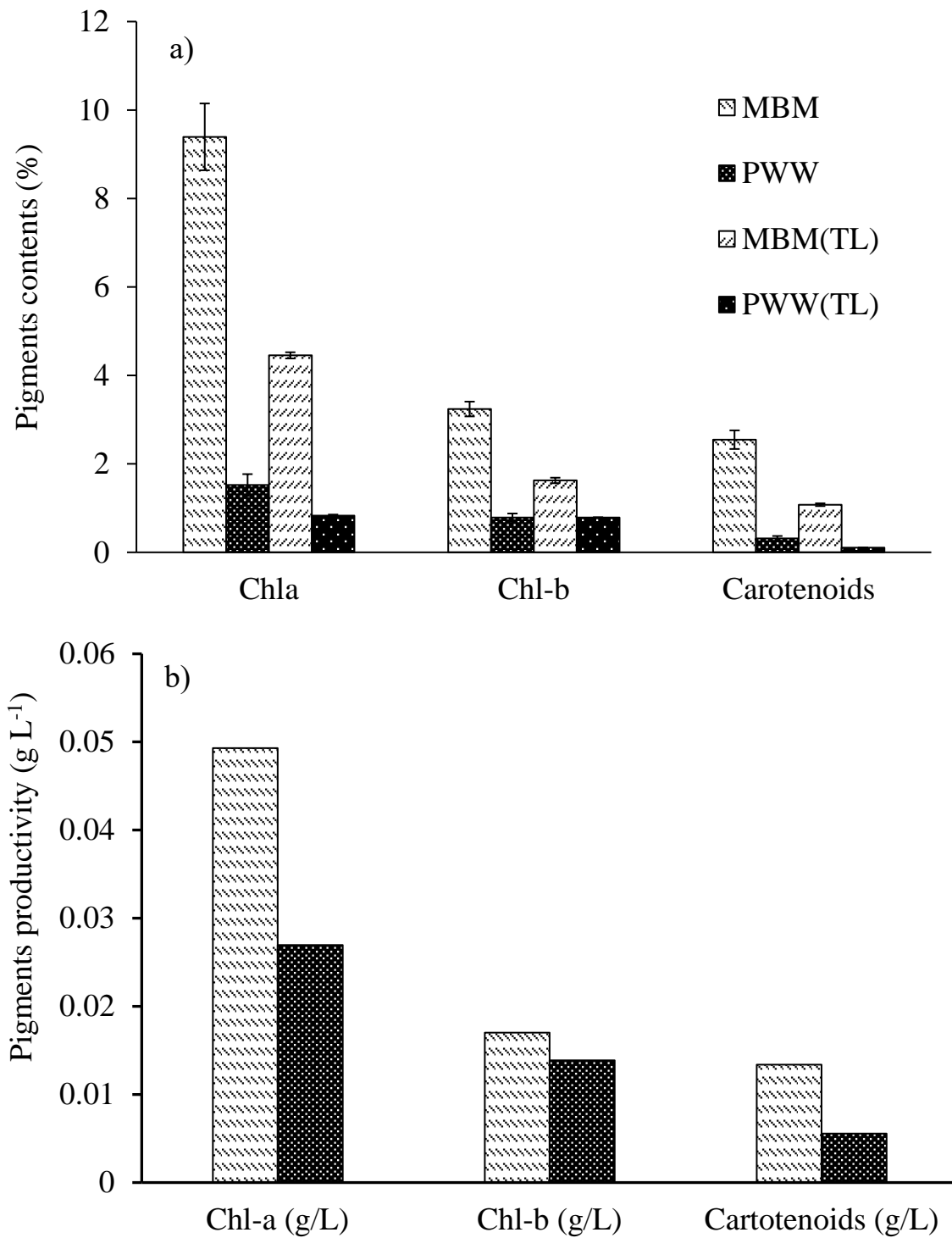
Figure 9. a) The pigments contents of our reactor and TL photobioreactor in Almeria university. b) The pigments productivity in our reactors.
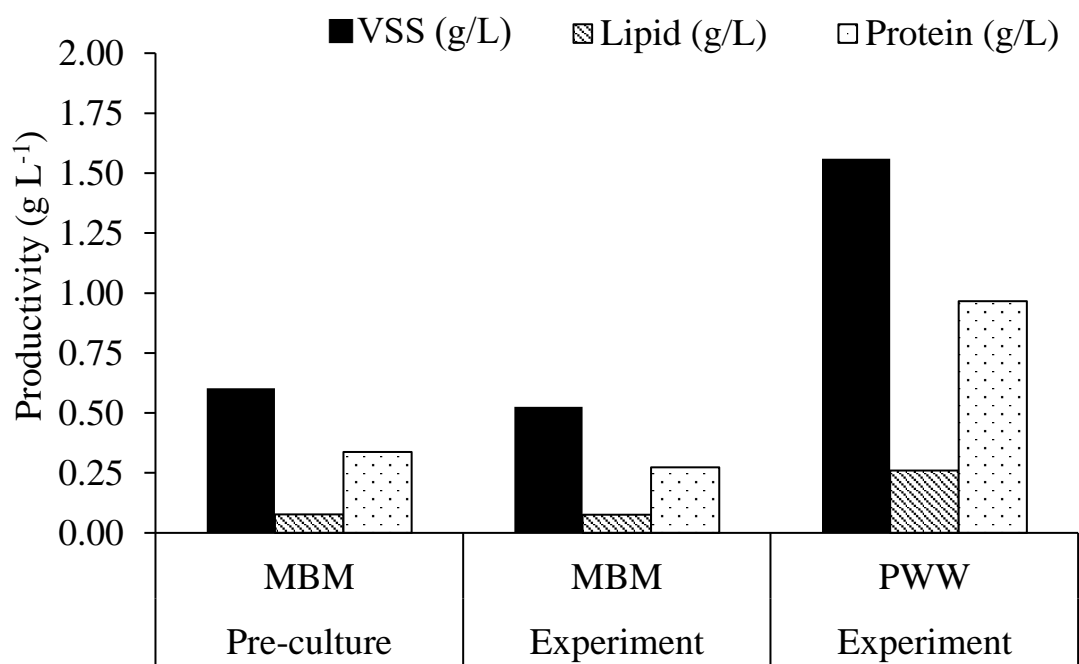
Figure 10. The biomass productivity (VSS) and the productivity of biochemical compounds, lipids and protein in our reactors.
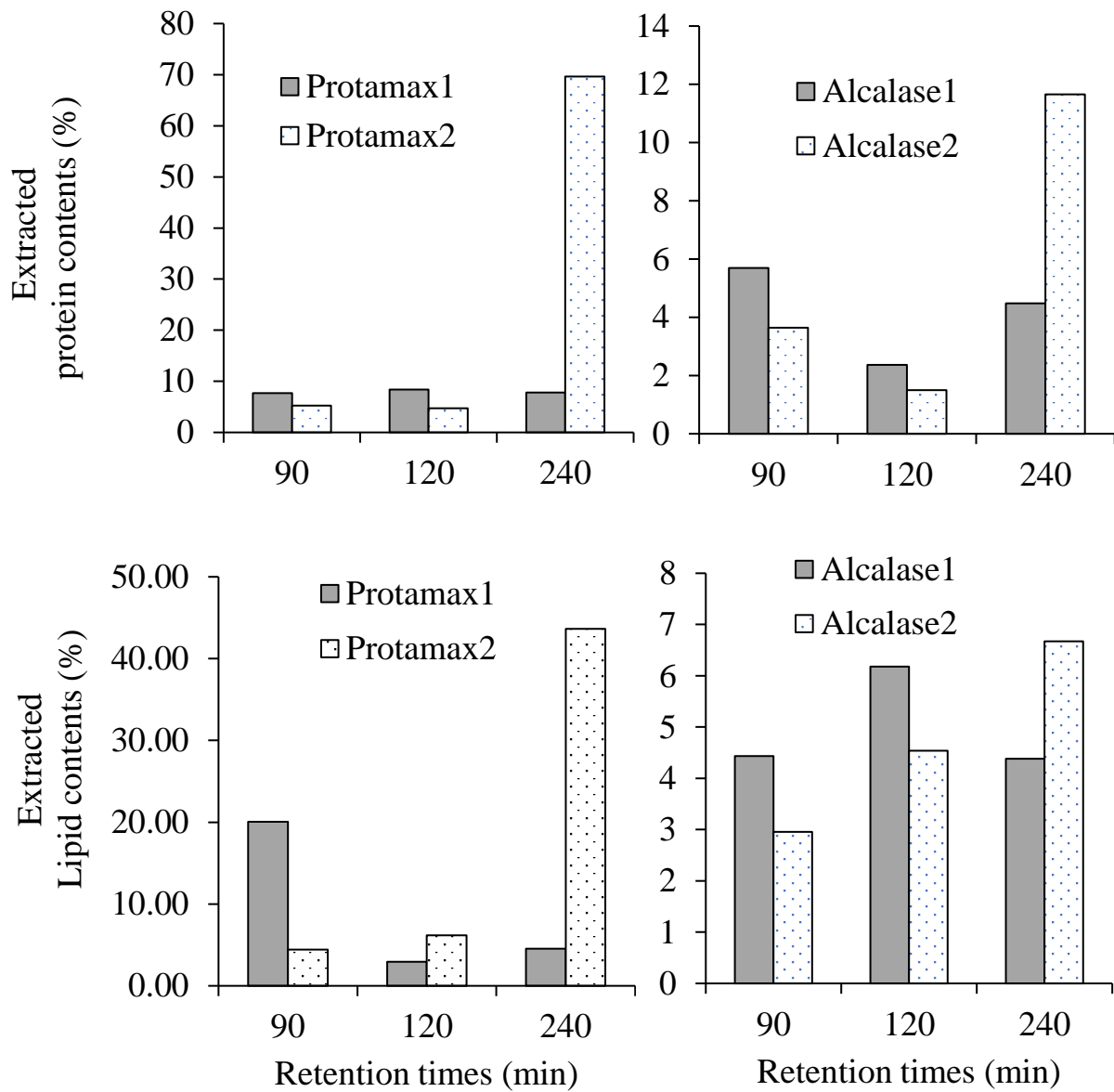
Figure 11. The protein and lipids contents in the supernatants after Alcalase or Protamax enzymatic extraction of PWW biomass in TL photobioreactor.
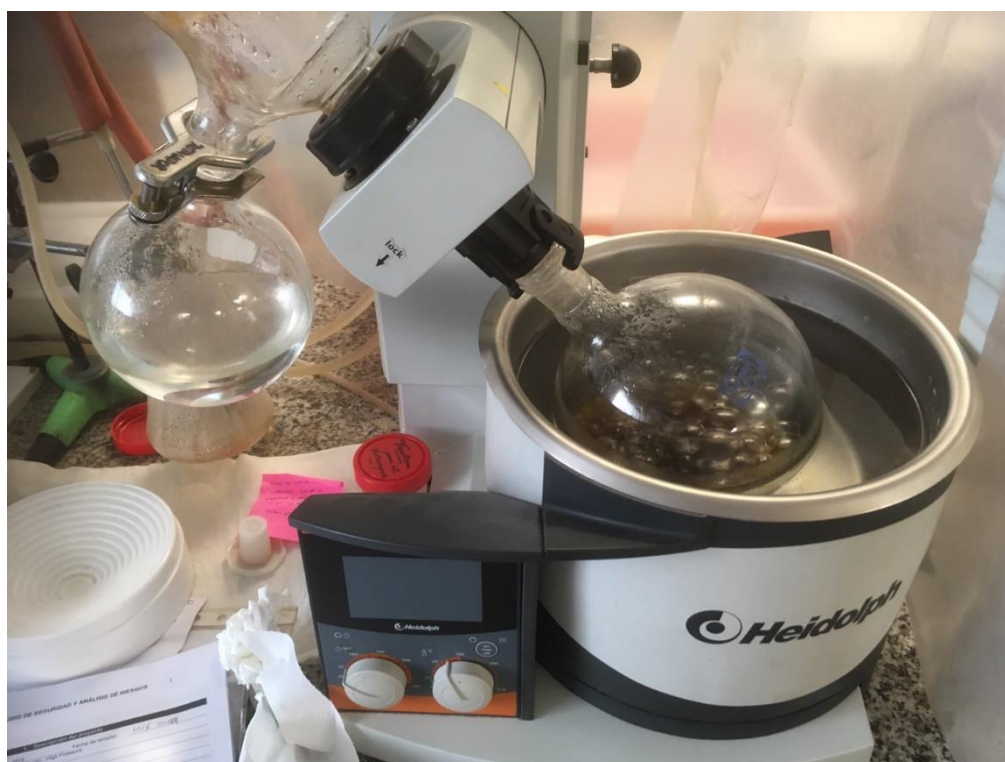
Figure 12. The water evaporation process of the supernatants samples by the rotavapor. The evaporation were conducted with the temperature 50℃, over two hours.

Figure 13. The supernatants dry samples of PWW biomass in BL and TL photobioreactor after lyophilize and Alcalase or Protamax enzymatic extraction. These samples were analyzed for protein and lipids contents.
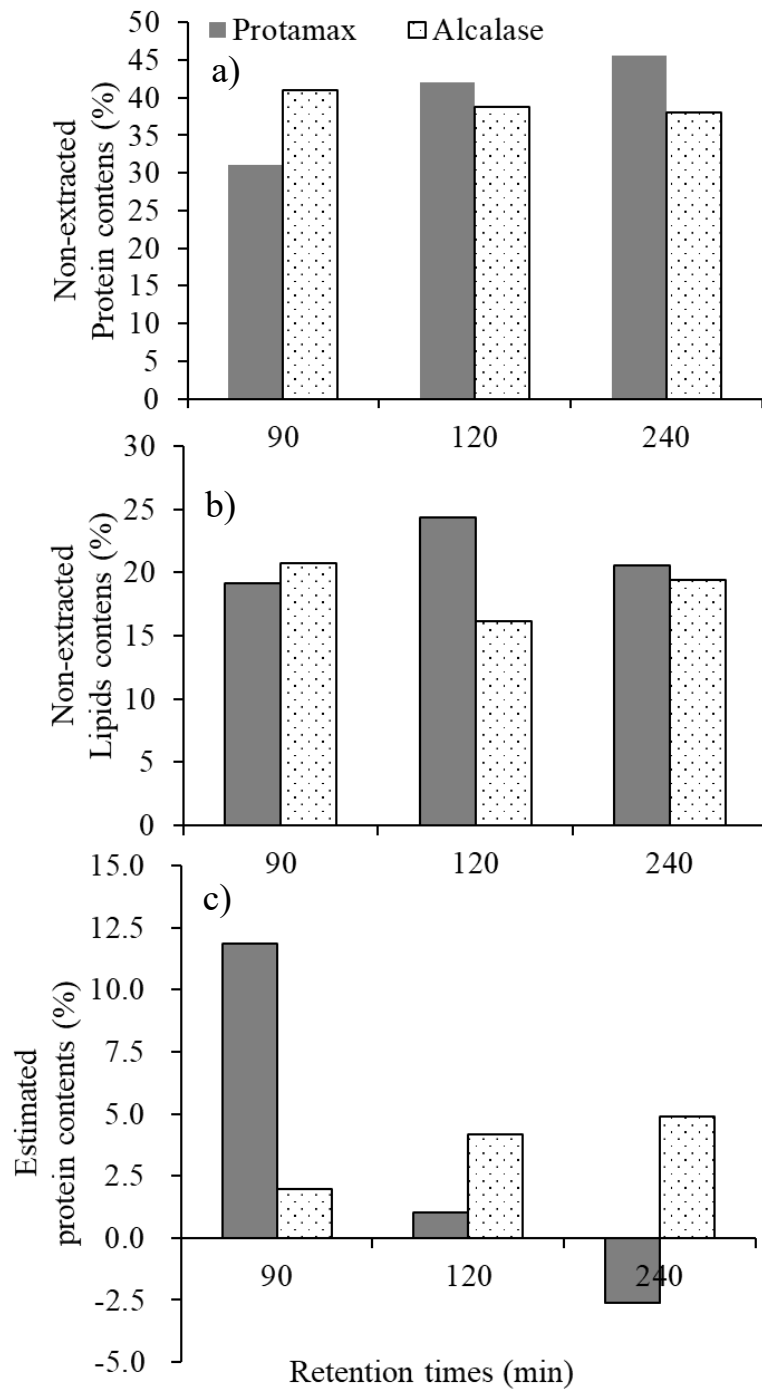
Figure 14. The result of enzymatic extraction of PWW biomass in TL photobioreactor. a) The protein contents in pellets after the extraction.b) The lipids cotents in peletts after the extractions.c) The estimated protein contents in the supernatants after the extractions.

Appendix

Table 4. Light intensities in pre-culture and the experiment.

Light intensity	Pre-culture	Experiments
R1	886.2	1028
R2	936.8	1097.5
R3	901.5	1058.4
R4	927.9	1029.5
Ave.	**913.1**	**1053.35**
S.D	**23.37**	**32.59**

Table 5. The elements compositions of Modified Bristol medium (MBM)

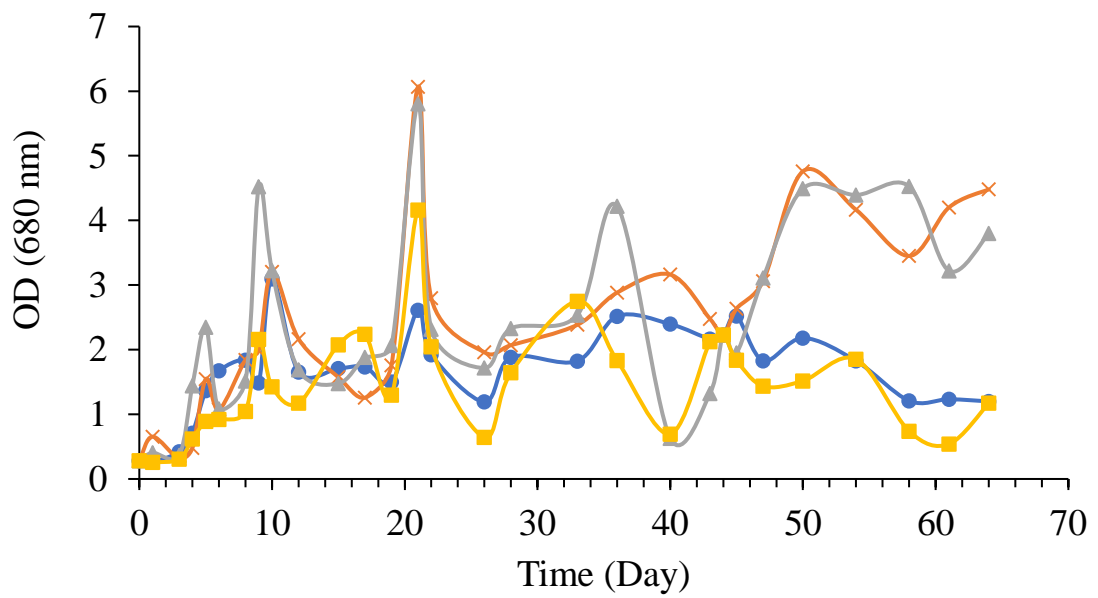| Elements compositions	mM
$NaNO_3$	5.88
$CaCl_2 \cdot 2H_2O$	0.17
$MgSO_4 \cdot 7H_2O$	0.3
K_2HPO_4	0.43
KH_2PO_4	1.29
NaCl	0.43
FeCl3 6H2O	1.17×10^{-2}
Na2EDTA 2H2O	1.17×10^{-2}
CuSO4 5H2O	3.93×10^{-5}
Na2 MoO4 2H2O	2.60×10^{-5}
ZnSO4 7H2O	7.65×10^{-5}
CoCl2 6H2O	4.20×10^{-5}
MnCl2 4H2O	9.10×10^{-4}

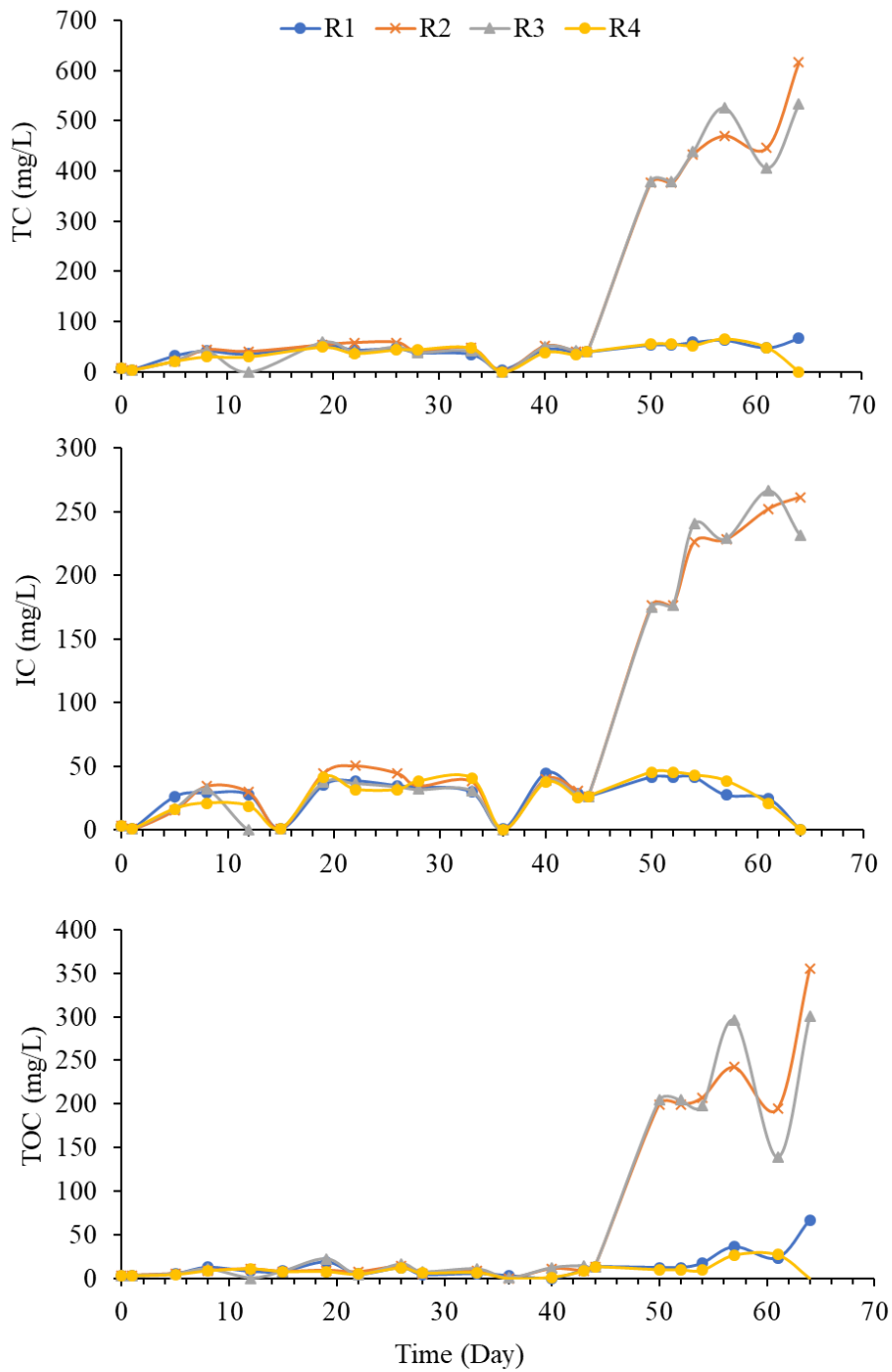Figure 12. The time course of optical density at 680 nm in the all of experiment.

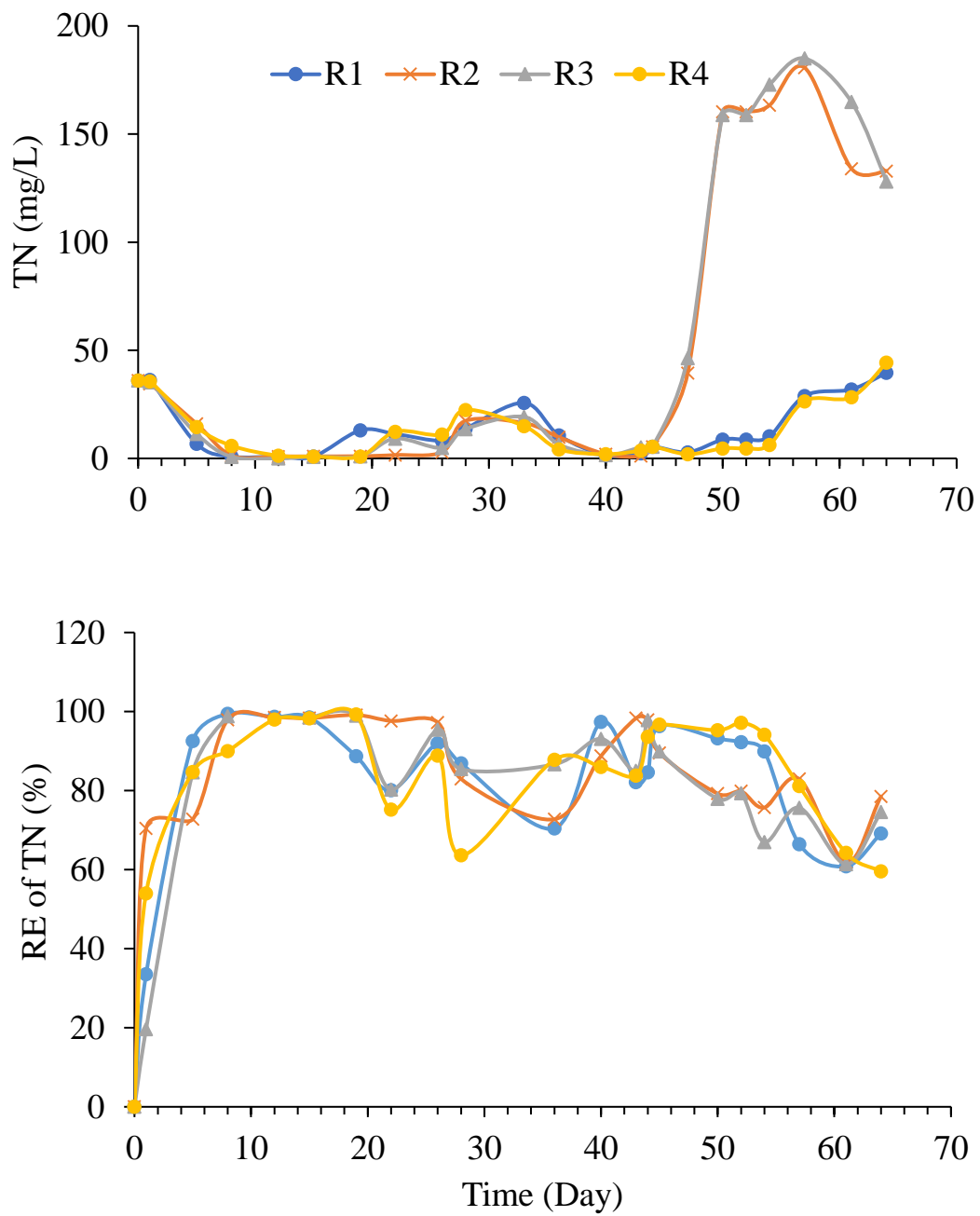Figure 13. The concentration of TC/IC/TOC in the effluents in R1, R2, R3, R4, respectively.

Figure 14. The concentration and removal rate of TN in the all of experiment.

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