Cultivation Strategy for Freshwater Macro- and Micro-Algae as Biomass Stock for Lipid Production

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Abstract. In this research, an algae cultivation strategy was studied. Integrating algae cultivation with wastewater treatment is currently seen as one of the most economical ways of producing algae biomass. A combination of an anaerobic baffled reactor (ABR) and a constructed wetland (CW) was applied for treating domestic wastewater with an additional collection tank for improving effluent quality. The effluent produced from the three stages was used as algae cultivation media and suplemented with 10% bold basal medium (BBM). The results showed both micro- and macro-algae growth and their lipid contents were higher when they were grown in effluent-BBM (9:1 v/v) media. The lipid content of the micro-algae mixed culture was 16.5% while for macro-algae *Oedogonium sp* and *Cladophora sp* it was 6.90% and 6.75% respectively.

Keywords: *algae cultivation; anaerobic baffled reactor; constructed wetlands; domestic wastewater; macro-algae; micro-algae.*

1 Introduction

Algae cultivation for CO_2 mitigation is currently receiving much attention. This is due to its benefits, such as the ability to produce green energy derived from biomass through carbon fixation by photosyntetic organisms [1]. Algae have fast growth capability related to their CO_2 fixation ability: the algae efficiency for athmospheric carbon fixation is 10-50 times higher than that of terrestrial plants. Considering these benefits, potential algae biomass applications for CO_2 reduction, biofuel production, aquaculture feed stock, food supplements, pharmeuchetical and many bioactive compounds are intensively investigated [2].

Existing algae cultivation methods still face problems of having to make the culture process economically viable. One of the problems is the water supply and another problem is that fertilizers for growth nutrition are still expensive. The uneconomical cost is one of the considerations for the feasability of algae

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production at an industrial level [3]. Therefore, an important strategy currently being explored and developed is integrating algae cultivation into wastewater treatment systems. This strategy considerably lowers the cost for solving the problems mentioned [4,5].

The cost of algae biomass cultivation can be reduced by growing the algae in a domestic wastewater treatment system. The biomass produced can be applied for biofuel stock [4,5]. Our previous study [6] demonstrated that the effluent released from the treatment of domestic wastewater undergoes a level of compositional change. This may happen during anaerobic fermentation in the anaerobic baffled reactor (ABR) and further degradation at the constructed wetland (CW) stage. These changes include: the available carbon compounds and nutrients can be used more easily by the algae for growth; the level of suspended solid particles is decreased so optimal penetration of light for algae photosynthesis can be achieved; moreover, the number of predators and pathogenic microorganisms as competitors for the algae will be decreased. All of these conditions are believed to strongly support the growth of the algae.

The use of micro-algae species such *as Scenedesmus sp, Chlorella sp, Scenedesmus sp, Phormidium sp, Botryococcus sp, Chlamydomonas sp* and *Spirulina sp* for domestic wastewater treatment has been widely reported [7]. The ability of *Chlorella vulgaris* in lowering levels of nitrogen in the form of ammonia and/or ammonium ions from wastewater has been reported. Research conducted by Chinnasamy, *et al.* [8] demonstrated the ability of 15 indigenous algae isolates in processing domestic waste with the ability to eliminate persistent organic pollutants (nutrients) by more than 96%. These micro-algae may be suitable for biomass production with domestic wastewater as growth media.

Macro-algae, especially freshwater filamentous algae such as *Oedogonium sp, Cladophora sp, Pytopthora sp, Microspora sp, Micractinium sp*, etc., are interesting candidates. For example *Oedogonium* sp, a filamentous green freshwater macro-algae [9,10] shows rapid growth and high adaptability for growing in a limited nutrient aquatic environment. It has many potential biochemical compositions. It is also known as a mat forming algal species [9,10]; different from micro-algae, it forms bunches of visible floating biomass. These properties are factors that make harvesting the biomass easier, thus there are no additional costs for centrifugation or coagulation during biomass collection, which is one of the largest challenges in algae research and production today.

In this study the potency of domestic wastewater effluent from a combined ABR and CW treatment systems for freshwater mixed culture micro-algae and

filamentous macro-algae cultivation was investigated. The lipid contents of the produced biomass was analysed in view of its potential for biofuel application.

2 Methodology

2.1 Domestic Wastewater Treatment Strategies to Produce Algae Cultivation Media

Domestic wastewater treatment for algae cultivation was conducted through three subsequential stages: (a) primary treatment using a laboratory scale ABR; (b) secondary treatment using a laboratory-scale CW; and (c) using an additional tank for letting the remaining suspended particles in the treated water settle down. The treated water was then collected and used for algae cultivation. An illustration of the schematic processes of the domestic wastewater treatment strategy is presented in Figure 1.

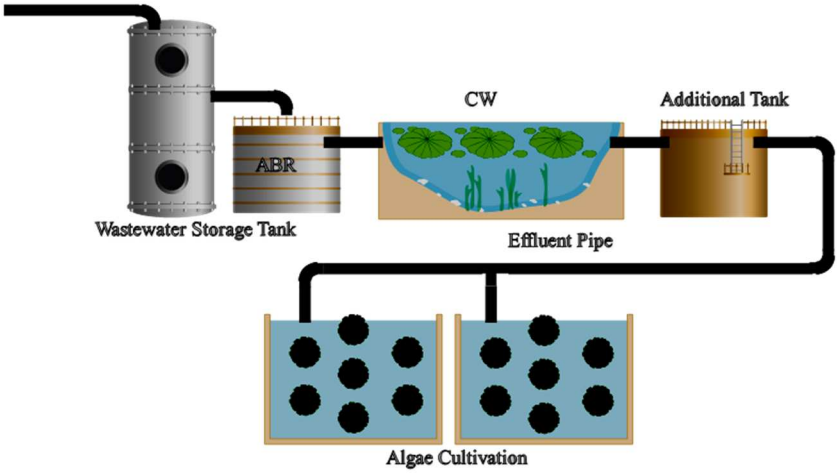

Figure 1 Illustration of domestic wastewater treatment processes for algae cultivation media applied in this study. It consisted of laboratory-scale ABR and CW and an additional tank for domestic wastewater treatment followed by the use of the effluent for algae cultivation media.

The two laboratory-scale bioreactors (ABR and CW) were designed and operated for 60 days. The ABR system was designed with dimensions of 20 x 12 x 100 cm and consisted of 6 compartments. The CW system had dimensions 20 x 12 x 100 cm. It had 4 layers, which consisted of sponge, lime-stone (diameter 3 to 5 cm), and lime-stone (diameter 0.5 to 1 cm). Subsequently sand was moved from the bottom to the top of the bioreactor system. Two types of water plants were used in the system, i.e. water-hyacinth (*Eichornia crassipes*)

and water-lettuce (*Pistia stratiotesL*). The combined bioreactors were operated in a continuous flow system. Domestic wastewater was collected every week and stored in a collection tank and pre-fermented for 24 hours prior to being pumped into the ABR. The biomass of activated sludge used in each of the compartments ranged between 5 to 6 g/L (mixed liquor). The ABR had a 6-L working volume. The wastewater was fed into the ABR at a rate of 0.25 L/hour. No additional chemicals were added during the operational period.

Activated sludge was collected from a swamp in Palembang that has been receiving domestic wastewater for years. The combined ABR and CW system improved ABR performance for removal of organic pollutants in domestic wastewater. ABR effluent was directly introduced into the raceway of the CW system. CW effluent was pumped into the collection tank and directly used for algae cultivation (Figure 1).

Wastewater characteristics such as COD, free ammonia, pH and TSS were measured according to the Indonesian National Standard (SNI) methods for wastewater analyses: SNI6989.2:2009 (COD), SNI 06-6989.3-2004 (TSS), SNI 06-0045-2006 (ammonia). The pH value of the wastewater was measured using a pH-meter (YSI-63).

2.2 Algae Cultivation Methods

Two methods of algae cultivation were applied in this study. The first method was applied for the micro-algae and the second method for the macro-algae.

Micro-algae cultivation steps: a mixed culture of micro-algae was grown by using the collected effluent as growth media. The mixed culture was identified and characterized (see Table 1). Prior to being used, a stock of mixed culture was prepared and subjected to cell density quantification (cells were counted by improved Neubauer haemocytometer counting slide under a light microscope with 400 magnification). For experimental data collection, the algae were grown in a glass bottle with 1 L of one of the following 4 types of culture media: (1) tapwater only medium as control; (2) tap water/bold basal medium (BBM) mix (9:1 v/v); (3) treated water (effluent)/BBM mix (9:1 v/v); and (4) treated water without BBM. The algae were grown outdoors in direct sunlight under natural athmospheric CO_2 (air-pumped) and temperature. The growth conditions such as pH and temperature were measured daily. The algae growth was measured daily by counting cell density.

Macro-algae cultivation steps: the macro-algae used in this study consisted of two species, namely: *Oedogonium sp* and *Cladophora sp* (both belong to the filamentous green macro-algae of the taxonomic group *Chlorophyceace*). The

algae were grown in 5-L containers filled with 1 L of 4 types of culture media as described in above. The filamentous macro-algae stock cultures were filtered with filter paper and weighed (1 g, wet weight) as the seed culture. The algae were grown outdoors as described above. As the control media for the algal growth, a tap water culture medium without BBM was tested and observed.

2.3 Algae Growth Measurement

Algae growth was measured by two different methods: (1) micro-algae cells were counted by improved Neubauer haemocytometer under a light microscope with 400x magnification; (2) macro-algae biomass was measured by weighing the algae biomass filtered by filter paper, the filter paper was dried in an oven at 40 °C and weighed prior to use, one liter of algae biomass was filtered and left air-dried before weighing (for wet weight) and dried in the oven at 40 °C until a stable weight was achieved (for dry weight).

Chlorophylls estimation procedure: 10 mL micro-algae sample was centrifuged at 6000 rpm for 15 minutes. Pellets were extracted by adding 5 ml of 96% ethanol. These were kept in the dark and then centrifuged at 6000 rpm for 15 minutes. The supernatant was measured by optical density. For the macro-algae samples: algae were collected by fishnet and weighed (1 gr). The 1 gr of wet biomass was grounded and mixed with 5 mL of 96% ethanol. The filtrate was collected and mixed with 96% ethanol to make 100 mL of solution. The chlorophyll contents of both micro- and macro-algae were measured at two different wave lengths (i.e. 649 nm and 665 nm). The contents were calculated based on Wintermans and De Mots method in the following Eqs. (1) to (3)[11]:

$$\text{Chlorophyll a} = 13{,}7\ (D\text{-}665) - 5{,}76\ (D\text{-}649)\ \text{ppm} \quad (1)$$

$$\text{Chlorophyll b} = 25{,}8\ (D\text{-}649) - 7{,}60\ (D\text{-}665)\ \text{ppm} \quad (2)$$

$$\text{Chlorophyll total} = 20{,}0\ (D\text{-}649 + 6{,}10\ (D\text{-}665)\ \text{ppm} \quad (3)$$

2.4 Lipid Extraction and Analyses

Micro-algae biomass was harvested after 11 days and centrifuged at 4000 rpm for 5 minutes. Supernatant was discarded, the pellets were collected and dried at 40 °C for 4 hours. The dried pellets were used for lipid extraction. Macro-algae biomass was collected by fishnet, dried and grounded with a coffee grinder. The biomass was used for lipid extraction.

Lipid extraction was conducted as follows: (1) For the macro-algae: 5 g of dried biomass was inserted into a Soxhlet apparatus. The algal biomass was extracted with the Soxhlet apparatus for 8 hours with the addition of 170 mL n-hexane at 90 °C. The extracted lipid was evaporated and then kept at room temperature

for evaporating the remaining solvent. (2) For the micro-algae: 1 gr wet biomass or 0,1 gr dried biomass was suspended in 4 mL methanol and 2 mL chloroform, then microwaved for 1 minute; 2 mL chloroform was added and vortexed for 2 minutes; the mix was centrifuged for 15 minutes at 4500 rpm, supernatant was transferred into a clean erlenmeyer, the pellet was resuspended with (chloroform: methanol – 1:1 v/v) and transferred into a boiling flask and stir-heated at 50-60 °C for 15-20 minutes. It was left at room temperature and centrifuged at 4500 rpm for 10 minutes. Supernatant was collected and vaccuum evaporated. The extract was left in an oven at 60 °C to remove the remaining solvent. The oil produced was then weighed for measuring the total lipid percentage [12]. The extract was analysed for the lipid content using GC-MS. The lipid composition analysis was measured with the GC-MS running condition similar to Sathis and Sims [13].

3 Results and Discussion

3.1 Domestic Wastewater Characteristics and System Performance

The characteristics of the domestic wastewater treated in this study can be summarized as follows: COD (300.5 ± 7.8 mg/L); free ammonia (2.16 ± 0.6 mg/L), pH 7.7 ± 0.3 and TSS (300 ± 10 mg/L). Performance of the combination of ABR and CW over an operational period of 30 days can be explained as follows: average of COD removal (87.9 ± 1.9 %). Other measured parameters from the effluent: TSS (83.5 ± 3.5 mg/L); free ammonia (0.51 ± 0.10 mg/L) and pH (6.9 ± 0.3).

3.2 Algae Cultivation Methods and Growth Profiles

The macro-algae used consisted of two species, namely *Oedogonium sp* and *Cladophora sp*. The mixed culture of micro-algae consisted of 12 species, with population percentages ranging from 0.1% to 42% (Table 1). *Scenedesmus dimorphus* dominated the mixed culture, followed by *Pediastrum sp, Closterium sp, S. Obtusus, S. Quadricauda, S. acuminatus, C.Asteroideu.* The remaining species accounted for less than 1 % of the population in the mixed culture. Typical microalgae compositions were reported in a recent study located in several domestic wastewater treatment plants (WWTP) in Bandung related to this study [14].

The mixed micro-algae culture and both macro-algae species were grown in the four types of culture media (tap water as control, tap water/BBM mix, effluent/BBM mix, and effluent only). These experiments were performed to investigate the effect of culture media on biomass production (cell density for

micro-algae and biomass weight for macro-algae, and also their lipid and chlorophyll contents).

Table 1 Mixed culture of micro-algae and macro-algae culturespecies.

Phylum	Species	Population percentage (%)
	Micro-algae – mixed culture	
Chlorophyta	*Scenedesmus dimorphus*	42
	Pediastrum sp	19,6
	Closterium sp	11,2
	Eudorina sp	8,4
	Scenedesmus quadricauda	3,9
	Scenedesmus obtusus	8,9
	Scenedesmus acuminatus	2,7
	Coelastrum asteroideum	2,3
	Chorella sp	0,8
	Ankistrodesmus sp	0,1
	Selenastrum sp	0,1
	Closterium sp	0,1
	Macro-algae	
Chlorophyta	*Oedogonium sp*	-
	Chladophora sp	-

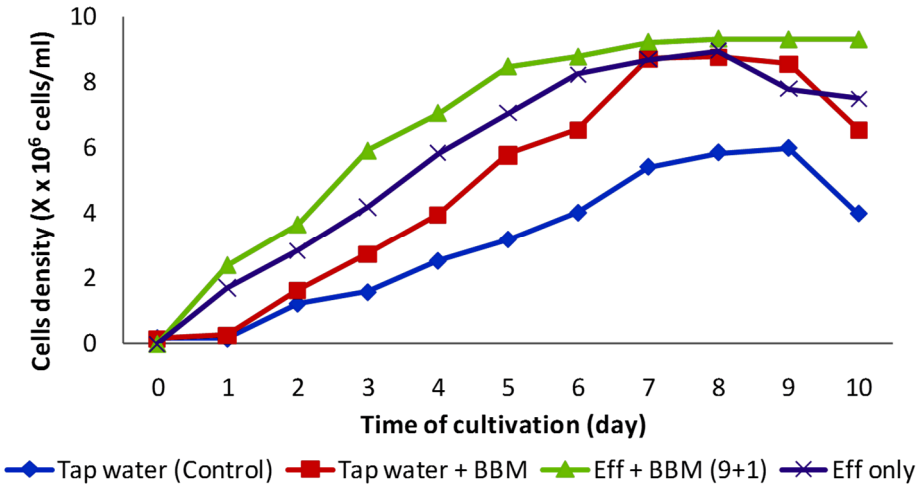

Figure 2 Growth curve of mixed micro-algae culture cultivated in four different culture media: tap water as control, tap water + BBM, effluent produced in this study with the addition of BBM (9:1 v/v), and effluent without BBM.

The growth profile during the ten days period of microalgae cultivation can be seen in Figure 2. There is a significant difference in mixed-culture micro-algae growth in the four types of culture media. The highest cells density was shown by cultivation using the effluent/BBM mix media, followed by the effluent only media and the tap water with addition of BBM media; the effluent only and tap water/BBM media showed a quite similar pattern, especially the cell densities after 7 days of cultivation. The slowest growth happened when they were grown in tap water only media (control).

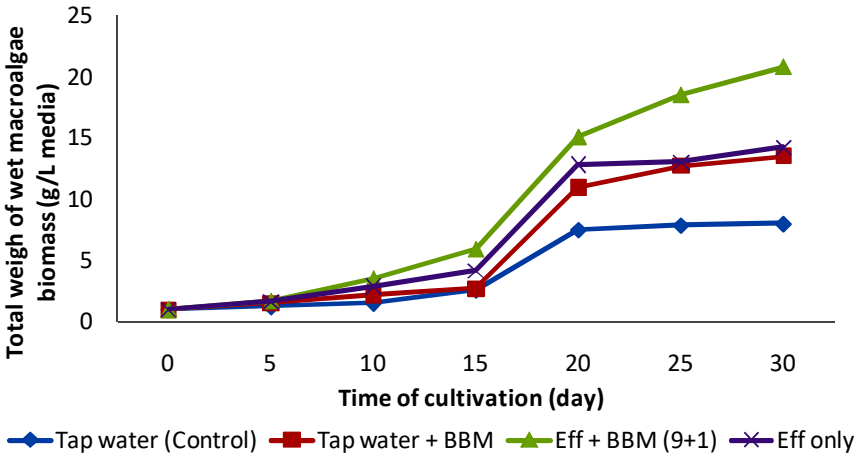

Figure 3 Growth curve of macro-algae *Oedogonium sp* cultivated in four different culture media: tap water as control, tap water + BBM, effluent produced in this study with the addition of BBM (9:1 v/v), and effluent without BBM.

The growth profiles of macro-algae *Oedogonium sp* are presented in Figure 3 and *Cladophora sp* in Figure 4. The growth profile of *Oedogoniumsp* also shows a significant difference in terms of biomass production during cultivation in the four tested media. The highest biomass production was measured for the sample taken from the effluent/BBM mix media, followed by the effluent only media and the tap water/BBM mix media respectively, while the lowest biomass was measured in the sample taken from tap water only media. Similar trends can also be seen in the *Cladophora sp* growth profiles. Only the average biomass weight was slightly lower compared to that of *Oedogonium sp.*

At the final day of the experiment (day 10 for micro-algae and day 30 for macro-algae), the biomasses from all samples were collected and subjected to weights measurement; the data are presented in Table 2. The biomass production showed that the effluent media (both with and without the supplementation of BBM) was suitable for cultivating both macro- and micro-

algae. These results indicate that the effluent media supported the growth of algae biomass; the nutrients availabe are useful for algae growth; the conditions of the effluent after the treatment stages were appropriate to produce algae biomass for further application. In general, quite similar trends were observed in the total weight of the micro- and macro-algae biomasses. The highest biomass weight was measured for the sample taken from the effluent/BBM mix media, followed by the effluent only media and the tap water/BBM mix media, while the lowest weight was measured for the sample taken from the tap water only media.

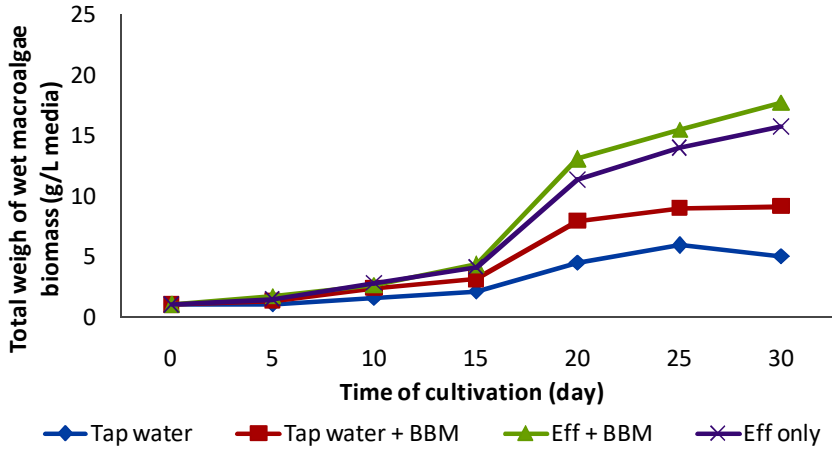

Figure 4 Growth curve of macro-algae *Cladophora sp* cultivated in four different culture media: tap water as control, tap water + BBM, effluent produced in this study with the addition of BBM (9:1 v/v), and effluent without BBM.

Table 2 Wet and dried micro- and macro-algae biomass weight cultivated in four different culture media at the end of the experiment.

Culture media	*Oedogonium sp*		*Cladophora sp*	
	wet weight (g/L)	**dried weight (g/L)**	**wet weight (g/L)**	**dried weight (g/L)**
Tap water (control)	8,01	0,9	5,0	0,6
Tap water + BBM	13,5	1,6	9,1	1,1
Effluent + BBM	20,8	2,4	17,8	2,1
Effluent only	14,2	1,7	15,7	1,9

During growth, the morphological changes and life cycles of the macro-algae were observed. Part of the cycles are presented in Figure 5. The reproduction of filamentous macro-algae generally occurred by vegetative, sexual and asexual

systems; vegetative reproduction occurred when new filament was grown by the development of small pieces of breaking filaments, asexual reproduction occurred by multi flagellate zoospores and by akinetes, while sexual reproduction with female and male sex organs, such as in *Oedogonium sp*, involves the oogonium and antheredium. These observations are useful for further cultivation strategy development (e.g. seed methods for algae cultivation).

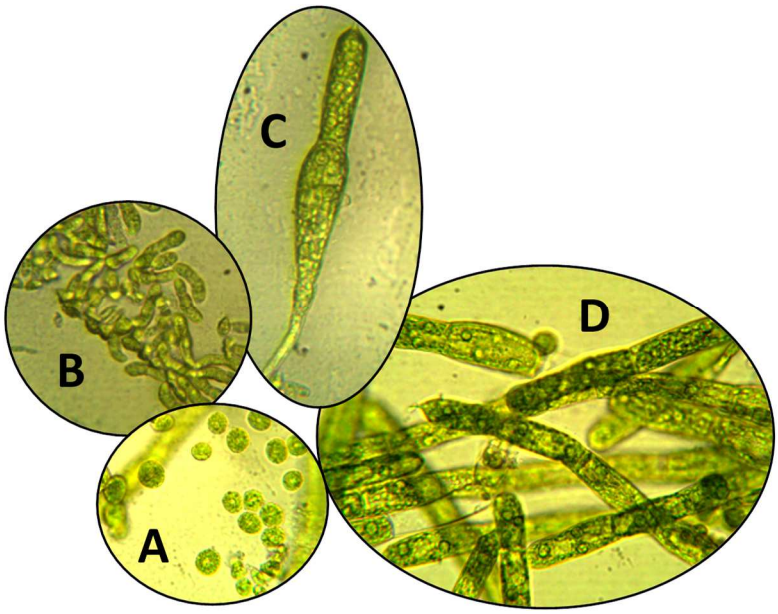

Figure 5 Macroalgae *Oedogonium* sp life cycle: (A) zoospora, (B) young juvenile (C) young juvenile or filament with holdfast, (D) adult filamentous algae.

3.3 Lipid Extraction and Analysis

As has been discused above, microalgae biomass was harvested at day 10 and macroalgae biomass at day 30. The biomasses were harvested when the growth of the biomass had reached a decreasing stage (at the transition stage when the stationary phase of growth had ended and the biomass started to enter the death phase). As reported in many studies, such as Bigogno, *et al.* [15], this is due to the algae biomass accumulating the most lipids in its cells during this stage.

The collected biomass was dried and grounded prior to the extraction process for total lipid measurement. The total lipid and chlorophyll contents of both the micro- and macro-algae studied in this experiment are presented in Table 3. Generally in this study, total lipid (%) of the micro-algae was higher than that of

the macro-algae. The total lipid percentage of mixed culture micro-algae grown in effluent/BBM mix was the highest (16,5%), followed by the samples taken from effluent (15.25%) and tap water/BBM (11.75%) respectively, while the lowest was shown by the sample taken from the tap water only media. Similar patterns were also shown by both macro-algae *Oedogonium sp* and *Cladophora sp*.

Table 3 Growth media, cultivation temperature, total lipid and total chlorophyll content of algal species.

Algae species	Culture media	Temp (°C)	Total lipid (%)	Total chlorophyll (ppm)
Microalgae mixed culture	Tap water (control)	29.4 - 29.6	8.25	33.14
	Tap water + BBM	30.1 - 30.6	11.75	48.28
	Effluent + BBM	30.2 - 30.5	16.50	52.90
	Effluent only	30.0 - 30.5	15.25	50.27
Oedogonium sp	Tap water (control)	29.3 - 29.8	3.75	35.15
	Tap water + BBM	30.2 - 30.4	5.25	50.19
	Effluent + BBM	30.3 - 30.7	6.90	53.23
	Effluent only	30.1 - 30.3	6.25	52.42
Cladophora sp	Tap water (Control)	29.6 - 29.9	3.75	33.31
	Tap water + BBM	30.2 - 30.4	4.25	51.90
	Effluent + BBM	30.1 - 30.3	6.75	53.21
	Effluent only	30.1 - 30.4	6.25	51.43

Table 4 Fatty acids of *Oedogonium sp* analyzed by GC MS.

Retention times (min)	ID/library	Abundance/area (%)
29.67	Eicosapentaenoic acid	7.85
29.75	Hexadecenoic acid	6.05
30.14	Palmitic acid	16.05
30.70	Cetylic acid	3.30
32.88	Octadodecanoic acid	11.26
33.04	Linolenic acid	19.99
33.50	Stearic acid	1.29

Total chlorophyll shows a similar pattern: the highest level was observed in the effluent/BBM mixed media, followed by the effluent only media and the tap water/BBM media, while the lowest is shown by the sample from the tap water only media. The results of this study are in line with several studies reported in the literature: mostly lipid content of micro-algae was higher than that of macro-

algae [16], however the lipid content of some macro-algae species, unfortunately, have yet to be explored. The reason for selecting macro-algae in this study was that the biomass harvesting and post harvesting handling are easier than with micro-algae.

Fatty acid compounds in the *Oedogonium sp* sample analysed by GC MS are presented in Table 4. The highest fatty acid percentage was shown by linolenic acid, followed by palmitic acid and octadodecanoic acid. Other fatty acids, such as hexadecenoic acid, eicosapentaenoic acid, were present at a moderate level, while quite low percentages of cetylic acid and stearic acid were measured.

4 Conclusions

From the experimental data presented in this study it can be concluded that the combination of an ABR and a CW with an additional collection tank were adequate for treating domestic wastewater. The treated domestic wastewater is suitable for algae cultivation media: the addition of 10% of BBM into the effluent increased both macro- and micro-algae growth and their lipid contents. A higher total lipid percentage was shown by mixed-culture micro-algae (16.5%) compared to that of both macro-algae *Oedogonium sp* and *Cladophora sp*. However, the considerably high cost for micro-algae harvesting is still under intensive study, thus further investigation is required to compare the production cost of valuable products from macro- and micro-algae.

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