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Investigation on the feasibility of Chlorella vulgaris cultivation in a mixture of pulp and aquaculture effluents: treatment of wastewater and lipid extraction

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<th>Nomenclature</th>
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<td>DI</td>
<td>Deionized water</td>
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<td>LW</td>
<td>Lake water</td>
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<td>AWW</td>
<td>Aquaculture wastewater</td>
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<td>PWW</td>
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<td>Mixture of pulp wastewater and lake water</td>
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<td>PAWW</td>
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<td>PAWW’BBM</td>
<td>BBM medium was prepared with PAWW instead of deionized water</td>
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<td>PAWW’N+P</td>
<td>Nitrate and phosphate were added to PAWW</td>
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Abstract

In this study, feasibility of *Chlorella vulgaris* cultivation in pulp wastewater (PWW) diluted with lake water (LW) and aquaculture wastewater (AWW) was investigated. The best ratios of PWW and AWW (PAWW) viz., 80% PWW:20% AWW and 60% PWW:40% AWW were selected as microalgal culture medium. Algal growth was investigated with and without addition of macro and micronutrients to the cultivation medium. The highest dry algal weight was observed as 1.31 g/L in 60% PWW:40% AWW without adding micronutrients. Nutrients and organic compounds removal efficiencies by microalga were studied in PAWW. Protein, carbohydrate and lipid percentage of harvested microalga from wastewater and Bold's Basal Medium (BBM) solution were analyzed. Fatty acids analysis revealed that C16 and C18 are the major fatty acids in *C. vulgaris* cultivated in BBM and PAWW. The results of this study revealed that *C. vulgaris* is a potential candidate for PAWW treatment and lipid and carbohydrate accumulation.

**Keywords:** Pulp wastewater (PWW); Aquaculture wastewater (AWW); *Chlorella vulgaris*; Biochemical components; Lipid profile.
1. Introduction

Water pollution, mainly due to anthropogenic activities, has become a serious environmental issue in recent decades. Wastewaters from industrial and agricultural sources contain detrimental contaminants viz., metals, dyes, phenols, detergents, antibiotics, disinfectants, pesticides and nutrients. These contaminants have harmful impact on the health of human beings and ecotoxicological effects on aquatic organisms (Schwarzenbach et al., 2006). Thus, it is necessary to treat wastewater before they enter downstream bodies of water.

Besides water quality issue, energy supply is another major problem that humanity faces in the 21st century. Fossil fuels are considered as the main source of primary energy throughout the world (Likozar et al., 2016). However, due to air pollution and decrease in reserves of fossil oil, replacing an alternative source of cheaper, renewable and environmentally friendly energy is necessary (Šoštarič et al., 2012).

Microalgae can address both these issues, to a significant extent, in an eco-friendly and inexpensive way. Microalgae are found in freshwater, saline water, brackish water and even in wastewater. Most of them grow photoautotrophically in the presence of sun light, CO$_2$ and nutrients (Kuo et al., 2015).

These organisms have their own remarkable advantages, such as fast growth rate, high productivity, non-rerequirement for arable land and accumulation of valuable biomolecules (Maurya et al., 2016).

Nitrogen and phosphorus compounds and the other macro and micronutrients, as the sources of environmental pollution, are considered the main enrichers for cultivation of microalgae (Ruiz et al., 2014). Wastewater treatment using microalgae does not require the use of any harmful and expensive chemicals and the produced microalgal mass can be used further to produce bioenergy.

The produced biodiesel from microalgae is composed by transesterification of microalgal lipid (Likozar and Levec, 2014). Saturated fatty acids such as palmitic (C16:0) and stearic (C18:0) acids of harvested microalgae from wastewater increase cetane number, energy yield and biodiesel quality (Canakci and
Sanli, 2008). The produced biodiesel from algae as clean, renewable and carbon-natural fuel can be considered as an alternative candidate to fossil fuels. Some other useful products like biopolymers, pigments, fertilizers, and biofuel can also be derived from algal biomass, produced in wastewater (Batista et al., 2013).

Attention to selecting the appropriate wastewater(s) and microalga(e) is necessary to conduct a successful wastewater treatment plan using microalgae and consequently producing biofuel from algal biomass (Salama et al., 2017). Different types of wastewaters have different concentrations of nitrogen, phosphorous and carbon components (Liu et al., 2016). Sometimes microalgae cannot grow well in a wastewater due to low or high concentration of some nutrients or toxicity of them. To overcome this problem, manipulation of wastewaters such as diluting or mixing them is needed to accomplish effective cultivation and wastewater treatment.

The wastewater production by pulp industries is the third largest in terms of amount and has harmful effects on the human health and environment (Ashrafi et al., 2015). However, the concentration of carbon is high in pulp wastewater (PWW) but it has low concentration of nitrate and phosphate (Gentili, 2014). In this study, aquaculture wastewater (AWW) was added to PWW and the feasibility of Chlorella vulgaris cultivation in the mixture of PWW and AWW (PAWW) was investigated.

Microalgal growth in PAWW with and without adding different nutrients (individually and together) were studied. The concentrations of nutrients, chemical oxygen demand (COD) and total organic carbon (TOC) in wastewater on the first and final days were measured. Finally, the biochemical compositions and fatty acid methyl esters (FAMEs) quantity of cultivated alga in the mixed wastewater were analyzed. The findings of this study will be helpful for optimization of microalgae cultivation in industrial wastewaters without adding macro and micronutrients to the algal medium.
2. Materials and methods

2.1. Mediums and chemicals

Deionized water (DI), natural lake water (LW), aquaculture wastewater (AWW), pulp wastewater (PWW) mixture of PAW+LW (PLWW) and PWW+AWW (PAWW) were used to prepare media (Table 1). LW and PWW were collected from local lake in Kuopio and paper making company (from recycled fiber plant before wastewater treatment) in Kuopio, Finland, respectively. AWW was prepared from closed recirculating aquaculture system in the Department of Environmental and Biological Sciences, University of Eastern Finland. The following analytical grade chemicals were used to prepare stocks of Bold’s Basal Medium (BBM): NaNO\(_3\) (25 g/L), MgSO\(_4\).7H\(_2\)O (7.5 g/L), NaCl (2.5 g/L), K\(_2\)PO\(_4\) (7.5 g/L), KH\(_2\)PO\(_4\) (17.5 g/L), CaCl\(_2\).2H\(_2\)O (2.5 g/L), ZnSO\(_4\).7H\(_2\)O (8.82 g/L), MnCl\(_2\).4H\(_2\)O (3 g/L), MoO\(_3\) (0.71 g/L), CuSO\(_4\).5H\(_2\)O (1.57 g/L), Co(NO\(_3\))\(_2\).6H\(_2\)O (0.49 g/L), H\(_3\)BO\(_3\) (11.42 g/L), EDTA (50 g/L), KOH (31 g/L), FeSO\(_4\).7H\(_2\)O (4.98 g/L), H\(_2\)SO\(_4\) (Conc.) (1.0 mL).

2.2. Microalgal strain and cultivation conditions

The universal distributed freshwater microalga, C. vulgaris CCAP 211/11B was obtained from the Culture Collection of Algae and Protozoa (CCAP, Scotland, UK). The harvested microalgal pellet after centrifuging at 3000 rpm for 5 min was inoculated in 250 mL bottles including 200 mL medium. Batch cultures were incubated at 25 ± 2 °C under continuous illumination of 85 µmol photon m\(^{-2}\) s\(^{-1}\) provided by white fluorescent lamps for one week. The continuous aeration with 0.04% CO\(_2\) was injected to the medium. Microalga was cultivated in BBM and wastewater media without adjusting initial media pH (7.1 and 5.9-6.3, respectively)

A calibration curve of OD\(_{680}\) vs. dry algal mass (g/L) was established to convert optical density values to dry mass (g/L) as follows (Ji et al., 2016):

\[
\text{Dry algal mass (mg/L): } a \times \text{OD}_{680} + b
\]

where, OD\(_{680}\) is algal density at 680 nm and \(a\) and \(b\) are the constants of the equation.
2.3. Experimental design

2.3.1. Effect of PWW concentration on microalgal growth

In the first step of experiment, LW, AWW, PWW and mixtures of PLWW and PAWW were used instead of deionized water to prepare the algal medium. For this purpose, different dilution percentages of PWW viz., 0, 20, 40, 60, 80 and 100% were prepared by mixing with LW and AWW. The same concentrations of BBM stocks (section 2.1.) were added to PLWW and PAWW as algal medium (Table 1). Dry algal mass was measured to monitor the best medium for *C. vulgaris*.

2.3.2. Cultivation of microalgae with and without adding nutrients

Experimental units with the highest algal growth viz., 60%PWW:40%AWW and 80%PWW:20%AWW were selected to evaluate the feasibility of *C. vulgaris* cultivation in PAWW without adding nutrients. Fifteen experimental units (as shown in Fig. 2 and Table 1) were set up with and without adding macro and micronutrients to the medium. The control culture medium was prepared by adding standard concentrations of BBM stocks to deionized water.

2.4. Wastewater analysis and removal efficiency of pollutants

Wastewater treatment by microalga was conducted in PAWW'BBM, PAWW'N+P and PAWW. The concentrations of nitrate as nitrogen (NO$_3^-$-N), ammoniacal nitrogen (NH$_3$-N), total phosphorus (PO$_4^{3-}$-TNT), chemical oxygen demand (COD), total nitrogen (TN) and total organic carbon (TOC) were analyzed on the first and final day of the experiments. Samples for analysis were taken, centrifuged for 10 min at 7000 rpm and filtered using 0.45 µm membrane filters. Cadmium reduction, Nessler, ascorbic acid and dichromate methods were applied to measure the concentrations of NO$_3^-$-N, NH$_3$-N, PO$_4^{3-}$-TNT and COD, respectively using HACH analysis kits and spectrophotometer (DR 2800 and DR 2010). TOC/TN Analyzer (multi N/C 2100S) were used to analysis the concentrations of TN and TOC.

The removal efficiencies of pollutants were calculated as follow:

$$R\% = \frac{C_i - C_f}{C_i} \times 100$$  \hspace{1cm} (2)
where, \( C_i \) and \( C_F \) are the concentrations of target pollutant on first and final day of experiment, respectively.

2.5. Biochemical components of microalgal biomass

After one-week of cultivation time, algal biomass was collected from three media viz., BBM, PAWW’BBM and PAWW’N+P media and biochemical components (total protein, carbohydrates and lipids) of samples were analyzed. Total protein was extracted and analyzed using a modified method reported by Rausch (Rausch, 1981). Ten mg of dried microalgal powder were re-suspended in 10 mL 0.5 M NaOH. The mixture was vortexed for 1 min and subsequently sonicated for 30 min. Then the solution was incubated in an oven at 100 °C. After 2 h, microalgal solution was centrifuged at 6000 rpm for 5 min. Total protein concentration of supernatant was measured according to Bradford method (Bradford, 1976). Four mL of Bradford reagent was added to 1 mL of supernatant and the absorbance of solution was read at 595 nm after 5 min.

The total carbohydrate concentration was determined by the phenol-sulfuric acid technique as reported by Salama et al. (Salama et al., 2014). Briefly, 10 mg of dried microalgae powder was added to 10 mL deionized water and vortexed for 1 min. The suspension was incubated in a water bath at 90-100 °C for 30 min followed by 30 min sonication. Five mL concentrated sulfuric acid and 1 mL phenol (5%) were added to 1 mL of sample. The mixture was kept in a water bath at 90-100 °C for 5 min. The concentration of carbohydrate in the supernatant was read at 490 nm and compared with glucose standard curve.

To extract algal lipids, 5 mL methanol and 2.5 mL chloroform were added to 100 mg dried microalgal powder. After 1 min vortex, the suspension was sonicated for 30 min. Then microalgal biomass at the bottom of the tube was separated by centrifuging and the supernatant was collected to another tube. The extraction procedure was repeated but with the half amount of solvents. The collected supernatant from the previous step was added to the test tube including microalga. Four mL 1% NaCl and 4 mL
chloroform were added and the test tube was shaken for 5 min at 80 rpm. After centrifuging at 7000 rpm for 5 min, the chloroform phase including lipid (the dark green layer) was carefully collected. Finally, chloroform was evaporated and the weight of lipid was calculated gravimetrically.

2.6. Fatty acid (FA) composition

For determination of lipid content, microalgal biomass was freeze-dried and fatty acid content was expressed at a dry weight (DW) basis. The analysis was based on a modified one-step in-situ transesterification method (IST), according to Levine et al. (Levine et al., 2011). Fatty acid methyl esters (FAMEs) were subsequently analyzed on a gas chromatograph (Agilent Technologies 7890A) equipped with a flame ionization detector (FID), according to the following method: Oven temperature was increased from 40°C (held for 0.5 min) to 195°C at a rate of 25°C/min, from 195°C to 205°C at a rate of 3°C/min and from 205°C to 230°C (held for 1 min) at a rate of 8°C/min. Helium was used as the carrier gas with an average velocity of 30.34 cm/sec. Temperature of injector and detector was set at 250 °C. For the analysis, a capillary column (DB–WAX, 10 m × 0.1 mm × 0.1 μm), as well as a calibration standard (FAMQ-005, AccuStandard) and an internal standard (IS) (C17:0, Sigma) at a final concentration of 50 mg/L, were used. Measurements were carried out in duplicate and mean ±SD values are presented in the results.
3. Results and discussion

3.1. Optimization of microalgal growth in PLWW and PAWW

In this study, wastewater from local pulp and paper making factory was used and investigated for the feasibility of microalgal growth at different concentration of PWW. For this purpose, PWW was diluted with LW and AWW. Fig. 1 shows the dry algal mass (g/L) at different concentrations of PWW (0, 20, 40, 60, 80 and 100%). In case of PLWW, microalgal dry mass increased from 0.51 to 1.21 g/L as PWW concentration increased from 0 to 40%. Then it decreased at higher concentration of PWW (60 to 100%). Productivity of microalgal dry mass at PAWW is illustrated in Fig. 1. As it can be seen from Fig. 1, microalga grew well in different concentrations of PWW. The maximum dry algal mass observed was 1.1 g/L, at 80% PWW concentration. A literature review reveals that based on the characteristics of wastewater, dilution factor can increase or decrease microalgal growth. Sepúlveda et al. (2015) used 0-80% centrate for the production of the marine microalga Nannochloropsis gaditana (Sepúlveda et al., 2015). In their study, the maximum biomass productivity (g/L/day) was observed as 0.4 g/L/day, in the range of 30-50% of centrate. They explained that microalgal growth decreased in centrate concentration lower than 20% and higher than 80%, due to insufficient concentration of nutrients and toxicity of ammonium, respectively. In the current study, microalgal growth in PLWW and PAWW increased as PWW concentration increased. PWW is a complex medium including high contents of BOD, COD, fatty acids, organic and inorganic and nitrogen compounds (Ashrafi et al., 2015). Higher biomass production at higher concentrations of PWW can be related to high concentrations of organic substrate (Hwang et al., 2014). This finding is in agreement with another study in which biomass production was higher at higher concentration of acetate and butyrate-rich wastewater effluent (Hwang et al., 2014).

3.2. The availability of macro and micronutrients
According to the different ratios of PWW:AWW, two ratios with the highest algal dry mass viz. 60%:40% and 80%:20% were selected as microalgal culture medium. To confirm that macro and micronutrients of these mixtures can support algal growth, different experimental units were prepared with and without adding nutrients (Table 1). As derives from Fig. 2, microalgal dry weight, in the experimental units without adding nitrate and phosphate (1, 4, 10 and 13), was significantly lower than the others. Decreasing the concentration of PWW from 80 to 60% and increasing the concentration of AWW from 20 to 40% increased microalgal growth in the experimental units without adding nitrate and phosphate. Although, changing the ratios of wastewaters improved microalgal growth, algal dry weight in the aforementioned experimental units was significantly lower than in case of experimental unit 15 having nitrate and phosphate. It can be concluded that microalgal growth is limited in PAWW due to low concentrations of nitrate and phosphate. Microalgae need phosphorous and nitrogen compounds (mainly nitrate and phosphate) for their growth and intracellular metabolism. Nitrogen compounds are fundamental in the structure of protein and nucleic acids. Phosphorus is also an essential nutrient as it has the key role in formation of adenosine triphosphate (ATP) as energy carrier in algal cells (El-Kassas, 2013). Similar to these results, Gao et al. (2017) reported that nutrients concentration in aquaculture wastewater is insufficient for cultivation of C. vulgaris (Gao et al., 2016). Analysis of PWW (Table 2) revealed that concentrations of nitrate and phosphate are not sufficient for cultivation of microalgae and without adding them, biomass productivity is low. Magnesium (Mg), Sodium (Na) and Calcium (Ca) are the other important macronutrients that microalgae need to grow healthy. The results of C. vulgaris cultivation with and without adding Mg, Na and Ca are presented in Fig. 2. Microalgal dry weight (g/L) in 60% PWW+40% AWW medium without adding Mg, Na and Ca (experimental units 2, 3 and 5) were 0.93, 0.99 and 0.98 g/L, respectively. In the experimental unit 15 including all of BBM stocks, and in experimental unit 11 without adding Mg, Na and Ca, microalgal dry weight was found to be 1.00 and 1.11 g/L, respectively.
Microalga grew well in the medium without adding the above nutrients (Mg, Na and Ca) and even better growth was noticed in the experimental unit having all of BBM stocks. These results clearly show that the concentrations of Mg, Na and Ca in PAWW are enough for the cultivation of *C. vulgaris*. In another study Ji et al. (2015) investigated the growth of *Scenedesmus obliquus* in diluted BBM with food wastewater (FW), sodium chloride and sea water (Ji et al., 2015). They concluded that the reason of the highest dry weight of alga (0.41 g/L) in FW can be related to the presence of Ca, Mg, Mn and Fe in the wastewater.

Microalgal growth and lipid content depends not only on essential macronutrients (nitrate and phosphate) and major ions such as Mg and Ca, but also on micronutrients such as iron, manganese, cobalt, molybdenum, copper and zinc (Sunda et al., 2005). Here, *C. vulgaris* grew in the experimental units without adding trace elements, boric acid, Ethylenediaminetetraacetic acid (EDTA) and Fe (6, 7, 8 and 9, respectively) as well as in the supplemented medium with all of them (experimental unit 15).

The maximum dry biomass of alga was observed as 1.11 g/L (80% PWW+20% AWW) and 1.31 g/L (60% PWW+40% AWW) in the medium without adding micronutrients. This value for the experimental units with the same ratios of PWW:AWW containing all of BBM stocks was 0.93 and 1.00 g/L, respectively. Supplementation of micronutrients, as well as EDTA, are necessary in the algal medium to improve CO₂ fixation, photosynthesis efficiency and biomass production (Singh et al., 2016). Carvalho et al. (2006) showed that biomass concentration of microalgae significantly decreased in the absence of micronutrients viz., Mn, Fe, Co and Zn (Carvalho et al., 2006). Results of this experiment show that the mixture of PWW and AWW used in this study has enough amounts of essential micronutrients to support microalgal cultivation.

### 3.3. PAWW wastewater treatment using *C. vulgaris*

Optimal growth of microalgae relies on the concentration of nitrogen, phosphorus and carbon compounds as essential nutrients (Nayak et al., 2016). The concentration of nitrogen, phosphorus and
carbon compounds in PAWW'BBM (BBM medium prepared with PAWW instead of deionized water), PAWW'N+P (PAWW containing nitrate and phosphate) and PAWW (without adding nutrients) are presented in Table 2. The concentration of COD and TOC in PWW were quite higher than in AWW. However, the concentration of TN and TP in PWW were lower than in AWW. Therefore, their appropriate mixture balanced the concentration of nutrients, increased microalgal growth and enhanced wastewater treatment efficiency. Fig. 3 depicts the removal efficiency of different nutrients and organic compounds in PAWW by *C. vulgaris*. Nutrients removal efficiency in the presence and absence of external sources of nutrients (BBM stocks) was assessed. The highest nitrate removal efficiency was 69.39 ± 6 in the PAWW'BBM medium. Nitrate removal efficiency was not observed in PAWW without adding BBM nutrients and nitrate concentration increased. The reason might be due to nitrification which takes place in the presence of oxygen, reduced nitrogen and nitrifying bacteria (Jiang et al., 2016). The results corroborated with the study of Jiang et al. where nitrate concentration increased in the high-ammonia complex wastewater after five days cultivation of *Monoraphidium* spp. (Jiang et al., 2016). NH$_3$-N removal efficiencies in PAWW'BBM, PAWW'N+P and PAWW were found to be 50.00 ± 2%, 23.44 ± 3% and 15.42 ± 3%, respectively. The equilibrium of NH$_4^+$/NH$_3$ (pKa = 9.25) in aqueous solution is H$_2$O + NH$_3$ ⇌ OH$^-$ + NH$_4^+$ and is governed by pH and temperature (Ji et al., 2014a). NH$_4^+$ and NH$_3$ are the dominant forms of ammonia nitrogen at pH lower than 8.75 and higher than 9.75, respectively (Molins-Legua et al., 2006). As the final pH in this study was 9.43, thus, NH$_3$-N removal could be largely attributed to the presence of *C. vulgaris* in the medium. The overall TN and TP removal efficiencies were ranged from 55.49 to 94.41% in all tested media. Similar removal efficiencies of TN and TP were observed in the PAWW'BBM and PAWW'N+P media, showing that microalga can grow very well in nitrate and phosphate enriched wastewater and uptake nitrogen and phosphorus from PAWW without adding external source of other nutrients. In
general, in different media either BBM or PAWW, removal efficiency of TP was higher than removal efficiency of TN. This observation is in agreement with the results of Ji et al. (Ji et al., 2016). Authors mentioned that high removal efficiency of TP can be related to intracellular uptake of $\text{H}_2\text{PO}_4$ or $\text{HPO}_4^{-}$, precipitation of phosphate as calcium phosphate at basic pH and adsorption to cell surface. As can be seen from Fig. 3, TN removal efficiency was higher than NO$_3$-N removal efficiency that reveals microalga consumes not only inorganic nitrogen but also organic sources of nitrogen. Nutrients removal efficiency in PAWW was significantly lower than in case of PAWW'BBM and PAWW'N+P. This is strongly related with the microalgal growth as it was 0.21, 1.15 and 0.83 g/L, respectively in the mentioned media.

COD indicates the overall concentration of dissolved and suspended organic matter of the wastewater. Higher removal efficiency of COD and lower concentration of organic matter before effluent discharge are important in wastewater treatment plants (Markou, 2015). Removal efficiency of COD and TOC from PAWW by *C. vulgaris* was investigated in this work. COD and TOC removal efficiency by microalga in the three different media tested followed the order PAWW'BBM > PAWW'N+P > PAWW. The highest values of COD and TOC removal efficiencies were observed as 94.41 and 79.38%, respectively in PAWW'BBM. Normally, microalgae through photoautotrophic metabolism utilize light and CO$_2$ to supply their energy and carbon sources. Some microalgal species such as *C. vulgaris* can uptake organic carbon under photoautotrophic cultivation (Mujtaba et al., 2017). Zhu et al. reported that 67.25, 65.81, 76.46, 79.84, 78.18 and 74.29% of COD was removed from the 400, 800, 1300, 1900, 2500 and 3500 mg L$^{-1}$ COD cultures under photoautotrophic condition (Zhu et al., 2013). The findings of the current experiment reveal that *C. vulgaris* is able to use organic carbon and strongly reduces the concentrations of COD and TOC in wastewater.
3.4. Biochemical composition of C. vulgaris in different culture media

Nutrients composition and cultivation conditions can affect both growth and biochemical composition of microalgae (Ji et al., 2014b). The content of proteins, carbohydrate and lipid of microalga cultivated in BBM, PAWW'BBM and PAWW'N+P are depicted in Fig. 4. The percentage of accumulated carbohydrate in BBM and wastewater media were ranged from 43.98 to 49.96%. As compared to BBM medium, the concentration of protein increased in PAWW'BBM and PAWW'N+P media. The highest protein content was observed in microalga cultivated in PAWW'BBM, but it was not significantly higher than in PAWW'N+P. In agreement to this study, the same range of protein concentration of C. vulgaris (from 44 to 46%) was observed for control and experimental groups by other researchers (Marudhupandi et al., 2014). The value of carbohydrate as one of the major component of microalgae is around 12-17% in C. vulgaris under normal conditions (Spolaore et al., 2006). Here, the percentage of carbohydrate varied from 18.32 to 19.09% for different media. In addition, accumulated carbohydrates in microalga cultivated in PAWW can be converted to fermentable sugars and employed as an appropriate bio-resource for production of bioethanol (Pancha et al., 2014). The lipid content of C. vulgaris was varied from 7.95 to 12.10%. The highest lipid percentage was found in BBM medium, 12.10%. It was 1.52 and 1.33-fold higher than in PAWW'BBM and PAWW'N+P. As derives from Fig. 4, lipid concentration was higher in BBM medium while protein concentration was higher in PAWW'BBM and PAWW'N+P media. These findings might be related to higher concentration of TN in PAWW'BBM and PAWW'N+P in comparison with BBM medium. The higher concentration of nitrogen in algae medium will induce the concentration of protein and reduce the concentration of lipids (Martínez et al., 2000; Hsieh and Wu, 2009). The value of biochemical composition of C. vulgaris in PAWW (PAWW'BBM and PAWW'N+P) was more than 75%. Similar results have been reported by other researchers where average concentration of biochemical composition of C. vulgaris, cultivated in diluted monosodium glutamate wastewater, was found to be 77.28% (Ji et al., 2014b).
3.5. Fatty acid profile of *C. vulgaris* in different culture media

Lipids were extracted from harvested algal biomass of BBM, PAWW'BBM and PAWW'N+P media. The major fatty acids of extracted lipid were identified by a GC system. The nine identical FAMEs with different relative content were found in the investigated media (Fig. 5). The chain length of these FAMEs was found to range between C14 and C20 that is considered as the appropriate FAMEs for biodiesel production (Zheng et al., 2012). Similar to these results, Zheng et al. reported FAMEs chain length of *C. vulgaris* in the range of C14 and C20 (Zheng et al., 2012). The total percentage of C16 (hexadecanoic acid) and C18 (octadecanoic acid) FAMEs were found to be 96.3, 96.40 and 95.98% in the algal biomass from the aforementioned media. Mathimani et al. reported that C16 and C18 are the major FAMEs present in *C. vulgaris* (Mathimani and Nair, 2016). The sum of saturated and monounsaturated fatty acids (e.g. 16:0, 16:1 and 18:1) percentage as the preferred fatty acids for producing biodiesel, was higher than 50% in the current study. Saturated fatty acids like C16:0 with high cetane number are more oxidative-stable while monounsaturated fatty acids reduce the freezing point and improve the low temperature properties of biodiesel (Hu et al., 2008). Stressful conditions such as nutrient deprivation, lower growth temperature and higher light intensity can change the fatty acids composition of microalgae (Cho et al., 2016). Insignificant variations in the fatty acid profiles of *C. vulgaris*, both in BBM and PAWW, revealed that PAWW can be used as microalgal medium and preserve diversity of microalgae fatty acids.
4. Conclusions

Algal growth was investigated with and without the addition of macro and micronutrients to PAWW (mixture of PWW and AWW). The highest dry algal weight was observed as 1.31 g/L in 60% PWW:40% AWW ratio without adding micronutrients. Removal efficiency of nutrients and organic compounds was observed in PAWW'N+P as TN (76.56%), TP (92.72%), COD (75.48%) and TOC (70.67%). Proteins, carbohydrates and lipids concentration were high in algal biomass harvested from optimized medium of PAWW'N+P. Microalgal growth was low in PWW due to low concentration of nitrate and phosphate. Mixing PWW with AWW improved algal growth.

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Fig. 1. Biomass productivity of microalga in different concentrations of PWW after seven days of cultivation.
Fig. 2. Biomass productivity of microalga in PAWW with and without adding nutrients after seven days of cultivation.
Fig. 3. Nutrients and organic matters removal efficiencies by cultivation of microalga in PAWW after seven days of cultivation.
**Fig. 4.** Biochemical composition of cultivated microalga in BBM and PAWW after seven days of cultivation.

<table>
<thead>
<tr>
<th>Media</th>
<th>Lipid (%)</th>
<th>Carbohydrate (%)</th>
<th>Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBM</td>
<td>12.10 ± 1</td>
<td>18.32 ± 1</td>
<td>43.98 ± 2</td>
</tr>
<tr>
<td>PAWW'BBM</td>
<td>7.95 ± 1</td>
<td>18.52 ± 2</td>
<td>49.96 ± 3</td>
</tr>
<tr>
<td>PAWW'N+P</td>
<td>9.07 ± 1</td>
<td>19.09 ± 1</td>
<td>47.49 ± 1</td>
</tr>
</tbody>
</table>
Fig. 5. Fatty acid methyl esters (FAMEs) of cultivated microalga in BBM and PAWW after seven days of cultivation.
Table 1. Different experimental units with and without adding nutrients.

<table>
<thead>
<tr>
<th>Media</th>
<th>Experimental units</th>
<th>Percentage of media</th>
<th>BBM stocks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First phase</strong></td>
<td>Distilled water</td>
<td></td>
<td>All BBM stocks</td>
</tr>
<tr>
<td></td>
<td>PLWW: LW:PWW (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0:100</td>
<td>All BBM stocks</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20:80</td>
<td>All BBM stocks</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>40:60</td>
<td>All BBM stocks</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>60:40</td>
<td>All BBM stocks</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>80:20</td>
<td>All BBM stocks</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>100:20</td>
<td>All BBM stocks</td>
</tr>
<tr>
<td></td>
<td>PAWW: AWW:PWW (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0:100</td>
<td>All BBM stocks</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>20:80</td>
<td>All BBM stocks</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>40:60</td>
<td>All BBM stocks</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>60:40</td>
<td>All BBM stocks</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>80:20</td>
<td>All BBM stocks</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>100:20</td>
<td>All BBM stocks</td>
</tr>
<tr>
<td><strong>Second phase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PAWW: AWW:PWW (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>20:80/40:60</td>
<td>Without NaNO₃</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20:80/40:60</td>
<td>Without MgSO₄·7H₂O</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>20:80/40:60</td>
<td>Without NaCl</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>20:80/40:60</td>
<td>Without K₂HPO₄ + KH₂PO₄</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20:80/40:60</td>
<td>Without CaCl₂·2H₂O</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>20:80/40:60</td>
<td>Without Trace elements solution</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>20:80/40:60</td>
<td>Without H₃BO₃</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>20:80/40:60</td>
<td>Without EDTA + KOH</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>20:80/40:60</td>
<td>Without FeSO₄·7H₂O + H₂SO₄ (conc.)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20:80/40:60</td>
<td>Without NaNO₃, K₂HPO₄ + KH₂PO₄</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>20:80/40:60</td>
<td>Without MgSO₄·7H₂O, NaCl, CaCl₂·2H₂O</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>20:80/40:60</td>
<td>Without micronutrients</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>20:80/40:60</td>
<td>Without microelements</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>20:80/40:60</td>
<td>Without BBM</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>20:80/40:60</td>
<td>With BBM</td>
</tr>
<tr>
<td>BBM (control)</td>
<td>-</td>
<td></td>
<td>All BBM stocks</td>
</tr>
</tbody>
</table>
Table 2. Concentrations of nutrients and organic compounds in PWW, AWW and PAWW.

<table>
<thead>
<tr>
<th>Pollutants (mg/L)</th>
<th>PWW</th>
<th>AWW</th>
<th>PAWW'BBM</th>
<th>PAWW'N+P</th>
<th>PAWW</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_3$-N</td>
<td>2.81 ± 0.3</td>
<td>18.83 ± 0.8</td>
<td>29.44 ± 2</td>
<td>25.42 ± 2</td>
<td>5.35 ± 0.4</td>
</tr>
<tr>
<td>NH$_3$-N</td>
<td>3.93 ± 0.3</td>
<td>2.6 ± 0.3</td>
<td>2.9 ± 0.2</td>
<td>3.2 ± 0.2</td>
<td>2.91 ± 0.2</td>
</tr>
<tr>
<td>TP</td>
<td>3.92 ± 0.1</td>
<td>18.25 ± 1</td>
<td>161.14 ± 6</td>
<td>176.01 ± 8</td>
<td>16.75 ± 1</td>
</tr>
<tr>
<td>COD</td>
<td>3530 ± 257</td>
<td>190 ± 13</td>
<td>1420 ± 72</td>
<td>1550 ± 114</td>
<td>1520 ± 63</td>
</tr>
<tr>
<td>TN</td>
<td>11.94 ± 0.9</td>
<td>53.15 ± 4</td>
<td>59.66 ± 4</td>
<td>63.37 ± 7</td>
<td>28.28 ± 2</td>
</tr>
<tr>
<td>TOC</td>
<td>916 ± 45</td>
<td>33 ± 3</td>
<td>521 ± 14</td>
<td>551 ± 44</td>
<td>571 ± 37</td>
</tr>
</tbody>
</table>
Research Highlights

- A mixture of pulp and aquaculture wastewater (PAWW) was used as alga growth medium.
- The maximum algal dry mass was obtained as 1.31 g/L in PAWW.
- High removal efficiency of nutrients was observed by *Chlorella vulgaris*.
- *C. vulgaris* was found to be rich in protein, carbohydrate and lipid.
- Fatty acid analysis revealed that C16 and C18 are the major fatty acids in alga.