*https://doi.org/10.33472/AFJBS.3.1.2021.58-66* ISSN: 2663-2187

**African Journal of Biological Sciences**

Journal homepage: http://www.afjbs.com

**Research Paper** **Open Access**

# Removal of nitrogen and phosphorus from agro-industrial wastewater by using microalgae collected from coastal region of peninsular Malaysia

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**Article Info**

Volume 3, Issue 1, January 2021
Received : 03 April 2020
Accepted : 08 October 2020
Published : 01 January 2021
*doi: 10.33472/AFJBS.3.1.2021.58-66*

## Abstract

The potential of microalgae as a source of renewable energy based on wastewater has received increasing interest worldwide in recent decades. A freshwater microalga *Chlorella vulgaris* was investigated for its ability to remove both nitrogen and phosphorus from three industrial wastewaters which were diluted in microalgae in two different proportions (namely, 50% and 75%). *C. vulgaris* grew fastest under 75% palm oil mill, and showed an maximum cell density (0.408 ± 0.012 g/L) for Palm Oil Mill Effluent (POME) wastewater, followed by riboflavin manufacturing wastewater (0.402 ± 0.083 g/L), and fertilizer industrial wastewater (0.320 ± 0.074 g/L), indicating the levels of nitrogen and phosphorus greatly influenced algal growth. Low removal efficiency for total nitrogen (TN) (11.35 ± 0.07% – 51.31 ± 0.03%) and total phosphorus (TP) (31.25 ± 0.24% – 93.62 ± 0.16%) was observed. *C. vulgaris* grew well when TP concentration was very low, indicating that this might be not the limiting factor to algal growth. The results suggest the potential of removing nutrient from wastewater by microalgae cultivation as production feedstock.

***Keywords:*** *Chlorella vulgaris, Microalgae, Nutrients removal, N removal, P removal, Wastewater treatment*



## 1. Introduction

Over recent decades, high wastewater amounts have been produced, mostly caused by activities of anthropogenic, like industrialization, global urbanization, and agricultural practices ((Bhuyar *et al*., 2019a). The repeated wastewater disposal in the absence of enough and appropriate treatment may lead to some severe pollution problems. Eutrophication phenomenon is a part of the serious problems related to the effluents

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discharge continuously into the water bodies. The peripheral effluents from wastewater contain nutrients, commonly phosphorus and nitrogen. This situation is accountable for the depletion of oxygen can produce pollutants such as ammonia, and key species lost, contributing in the total deterioration of freshwater ecosystems (Renuka *et al*., 2013). Thus, effective wastewater treatment methods are needed, have potentials for the nitrogen and phosphorus reduction in wastewater before dispensing into public usage.

Industrial system is one of the sources where the wastewater comes from. Despite of how industrial wastewater is treated, the 'end product' is called an effluent. To obey with the environmental protection laws, some specific things must be removed from the wastewater. This includes organic matter, pathogens, inorganics (such potassium, sodium, calcium, nickel, lead and zinc), and nutrients (most notably nitrogen and phosphorus) in tertiary wastewater treatment. The treated industrial wastewater can then be discharged in safe into water bodies, applied to soil or land, or even can be reused in some plant operations.

Industrial wastewater has multiplex components and high degree of toxic compounds. In general, wastewater from industries are remediated by using a sort of hazardous chemicals for the pH correction, removal of sludge, as well as the removal of color and odor (Oilgae, 2010). Referred to published reports, industrial wastewater is not appropriate for the growth of microalgae. Anyhow, several studies have exemplified the microalgae-formed remediation feasibility of various specific industrial wastewater like chemical fertilizer industry, animal manure from livestock industry, and palm oil industry (Chinnasamy *et al*., 2010; and Bhuyar *et al*., 2019c).

Phosphorus and nitrogen are nutrients that are aquatic ecosystems natural parts. Besides that, nitrogen is also the most plentiful component in the air we breathe. Both nutrients encourage the algae growth and other aquatic plants, which supply food and domain for shellfish, fish, and compact organisms that live in water. Too much phosphorus and nitrogen contents in water cause pollution of nutrient in groundwater (millions of people in Malaysia use it as drinking water source) (Ramli *et al*., 2020a). This can be harmful, even at the low-level contents. Babies are unsafe to a nitrogen-formed compound (nitrates) in drinking water. The excess of nitrogen in the atmosphere can create pollutants like ozone and ammonia that can harm our capability to breathe, cause visibility limitation and affect the growth of plant. When excessive nitrogen from the atmosphere returns to earth, it can cause harmful to the health of soils, jungles, and waterways.

The main approaches of this study are about bioactivity of microalgae. The isolated microalgae were studied about their mechanisms in removing phosphorus and nitrogen from industrial wastewater. The optimization of these two nutrients in industrial wastewater seems to be great significance to raise the compounds removal efficiency. The demand of wastewater treatment is high, especially in various industrial wastewaters. Therefore, the aims of this study were to isolate and identify the potential microalgae species for the polishing of residual nutrients of nitrogen and phosphorus from industrial wastewater and study the effectiveness of microalgae in reducing the amounts of nitrogen and phosphorus from industrial wastewater samples by using Ultraviolet-visible Spectrophotometer.

## 2. Materials and methods

### *2.1. Preparation of algae culture medium*

Recent studies from Bhuyar *et al*. (2020c) indicate that BG-11 culture medium is a widely used medium for freshwater algae that can be used as a biomarker for the ecological screening of Cd-contaminated waters including *Chlorella vulgaris*. The modified BG-11 medium was prepared based on methods used by Stanier *et al.* (1971). After prepared, the BG-11 medium was autoclaved and adjusted to pH 10.1 before using.

### *2.2. Microalgae samples collection*

In this study, the microalgae samples were collected from Berkelah Waterfall, East Coast region of Peninsular Malaysia uses 5 $\mu$m plankton net microalgae at the subsurface level of the water.

### *2.3. Isolation of microalgae*

Water samples were suspended in a 500 mL of distilled water. The supernatant was transferred to BG-11 solid culture medium (Zamani *et al*., 2011), and the petri dishes were stored in a culture room under constant illumination (4150 lx) with white fluorescent lamps at 25 ± 2 °C. After colonization, the isolation and purification were performed using the plate agar method to obtain unialgal cultures. The microalgal cells were grown at room temperature in liquid BG-11 medium with air bubble pump. For spread-plate technique, each sterilized

plastic petri plate (100 × 15 mm) contains approximately 25 mL of agarized medium in semi-solid condition. 1 mL of diluted sample was transferred to agar plate by using pipette technique and spread evenly on the surface of media with applied aseptic technique (sterile condition). For streak-plate technique, grown microalgae colonies were streaked on new agar plates under sterile conditions for further isolation. The streaking method was repeatedly done until single algal species was obtained.

### *2.4. Identification and screening microalgae*

Algae strains were isolated and differentiated based on the morphological examinations of colonies like color, shape and size of colonies once it grew well on the agar plate. The morphological structures were observed through Olympus B × 53 Fluorescence Research Microscope in Central Laboratory of Universiti Malaysia Pahang. Cells and sub microscopic cellular components were identified with high degree specificity. Each isolate in the colonies was labeled and picture was snapped at magnification of 100X. The two different microalgae were morphologically identified based on the manual 'Microalgae Identification for Aquaculture' (Bhuyar *et al*., 2020a; and Saengsawang *et al*., 2020). The identification was based on their morphology, color, shape and physical characteristic of microalgae.

### *2.5. Pre-cultivation of microalgae*

The microalgae were pre-cultured in a 500 mL Erlenmeyer flask of BG-11 culture medium. pH was adjusted at 10.1 as it was the optimum growth of the chosen strain of microalgae, *C. vulgaris* (Gong *et al*., 2014; and Bhuyar *et al*., 2020d). The culture was cultivated under light condition with a regimen of 24 h at 25 + 2 °C of temperatures with air (sole source of inorganic carbon) was supplied to culture by Atman AT-702 air pump. The culture was shaken by hand twice a day. The culture was then transferred into a new 2 L Erlenmeyer flask and BG-11 medium was added until the total culture volume reached 1 L. The culture was examined daily for growth approximately 2-3 weeks.

### *2.6. Industrial wastewater samples collection*

Wastewater samples were collected from three different locations in Melaka and Pahang. The wastewater samples were collected from Palm Oil Mill Effluent (POME), riboflavin manufacturing wastewater, and fertilizer industrial wastewater. The effluent wastewater samples were filtered through a 0.22-$\mu$m pore size membrane. The industrial effluent wastewater samples were kept in white-colored plastic bottles to prevent light penetration which may help in the growth of algae (Beavington, 1977; and Bhuyar *et al*., 2020b). The wastewaters were then autoclaved at 121 °C for 15 min. The wastewater samples were labeled with AA, BB, and CC.

### *2.7. Wastewater and algae mixture preparations*

The filtrated wastewater samples and cultivated microalgae were mixed in certain amounts for each kind of industrial wastewater. The industrial wastewater samples were diluted with microalgae into two different levels labeled as 50% (200 mL of microalgae and 200 mL of industrial wastewater), and also 75% (100 mL of microalgae and 300 mL of industrial wastewater). The proportions of wastewater were 50% and 75% for industrial effluent wastewaters, respectively mixed with microalgae. The total mixture of microalgae and industrial wastewater were 400 mL and measured by using measuring cylinder.

### *2.8. Microalgae cultivation in wastewater*

The cultures were shaken by hand twice a day. The results were observed and examined every five days for 30 days. All treatments were in a biological incubator and the cultural conditions were the same as the pre-culture conditions.

### *2.9. Determination of total nitrogen concentration*

Sa'id and Mahmud (2013) mentioned that the determination of total nitrogen (TN) concentration was done for each sample by following the spectrophotometric technique. In the preparation of reagents, chemicals of analytical grade purity and distilled-deionized water were used.

All glass wares were cleaned with detergent and rinsed with water and acetone before drying in an oven at 105 °C. 10 mL of the sample was pipetted into a 50 mL volumetric flask. 10 mL of 13 N sulphuric acid was added and mixed with swirling, the flask was allowed to come to a thermal equilibrium in cold water bath (0-10) °C. 0.5 mL of brocine-sulfanilic acid was added and diluted to the mark with deionized water, the solution was then placed on the 100 °C hot water bath for about 25 min for maximum color development, the

flask was then cooled to room temperature. The absorbance was read at 410 nm by using Thermo Scientific GENESYS 10S UV-Vis Spectrophotometer including the blank. This procedure was repeated on the other samples including the standard solutions for making standard calibrations.

### *2.10. Determination of total phosphorus concentration*

The determination of total phosphorus (TP) concentration was done for each sample by following the spectrophotometric technique. In the preparation of reagents, chemicals of analytical grade purity and distilled-deionized water were used. All glass wares were cleaned with detergent and rinsed with water and acetone before drying in an oven at 105 °C. 50 mL of sample was pipetted into a 500 mL volumetric flask, 5 mL of Ammonium molybdate solution and 3.0 mL of ascorbic acid were added with swirling, the mixture was diluted to the mark with deionized water and was allowed to stand for 30 min for maximum color development, the absorbance was then read at 660 nm by using Thermo Scientific GENESYS 10S UV-Vis Spectrophotometer including the blank. This procedure was applied for the remaining samples and the standard solutions.

## 3. Results and discussion

### *3.1 Removal of TN by various proportions of C. vulgaris*

Based on Figure 3.1, Samples Mixture AA (75%) was the highest removal ratio of TN with 51.31 ± 0.03% of TN removal, while Samples Mixture CC (50%) was the lowest removal ratio of TN with 11.35 ± 0.07%. In this study, *C. vulgaris* was insufficiently removing nitrogen content of the Sample Mixture AA, BB, and CC with different proportions (50%) and (75%). The *Chlorella sp.* showed lower removal ratios of TN in effluent wastewater.

Many studies demonstrated in absorbing 45-97% for nitrogen, 28-96% for phosphorus and in reducing the chemical oxygen demand (COD) by 61-86% from different type of wastewater such as textile, sewage, municipal, agricultural and recalcitrant (Bhuyar *et al*., 2019b; Feng *et al*., 2011; Lau *et al*., 1996; Lim *et al*., 2010; Silva-Benavides Torzillo, 2012; Valderrama *et al*., 2002; and Yun *et al*., 1997).

Interestingly, the higher concentration of wastewater led to the higher removal ratio of TN. Under (75%) proportion wastewater conditions, removal ratios of TN from the effluent wastewaters were higher than (50%) of proportion as shown in Figure 3.1. The result in this study may result in the insufficient of using *C. vulgaris* to remove nitrogen from industrial wastewater. The majority of dissolved inorganic nitrogen was in the form of $NH_4$-N in influent and $NO_x$-N in effluent wastewaters, respectively. Most $NH_4$-N and $NO_x$-N were removed by *Chlorella sp*., but for undissolved nitrogen was ineffective. In industrial effluent wastewater, the removal effect of $NH_4$-N was not as good as in influent wastewater, but similar results were reported by González *et al*. (1997).

In general, algae use nitrogen to build nucleic acids and proteins. Nitrogen is essential for building up the algal cells' components, such as genetic material, enzymes, proteins, hormones, vitamins, alkaloids, amides, and energy transfer molecules. Thus, nitrogen is the second most abundant element making up 6-10% of dry weight of green algae *Chlorella*. In recent studies, Grobbelaar (2013) proved that nitrogen content ranges from 1-10% of the cell dry weight. Andersen (2013) proved that carbon is the predominant element of *Chlorella* and accounts for approximate 50% of cell dry weight. Most species of microalgae can utilize both organic and

**Table 3.1: Total nitrogen removal ratio in samples mixture of microalgae and industrial wastewater AA, BB, and CC with different proportions (50%, and 75%) removed by *C. vulgaris***

| Sample | Initial value ($mg.L^{-1}$) | Final value ($mg.L^{-1}$) | Removal ratio (%) |
|---|---|---|---|
| AA (50%) | 18.26 ± 0.14 | 14.83 ± 0.09 | 18.78 ± 0.05 |
| AA (75%) | 26.74 ± 0.02 | 13.02 ± 0.07 | 51.31 ± 0.03 |
| BB (50%) | 14.36 ± 0.08 | 12.12 ± 0.07 | 15.60 ± 0.02 |
| BB (75%) | 22.19 ± 0.04 | 12.84 ± 0.04 | 42.14 ± 0.03 |
| CC (50%) | 11.45 ± 0.08 | 10.15 ± 0.04 | 11.35 ± 0.07 |
| CC (75%) | 12.79 ± 0.07 | 8.81 ± 0.08 | 31.12 ± 0.03 |

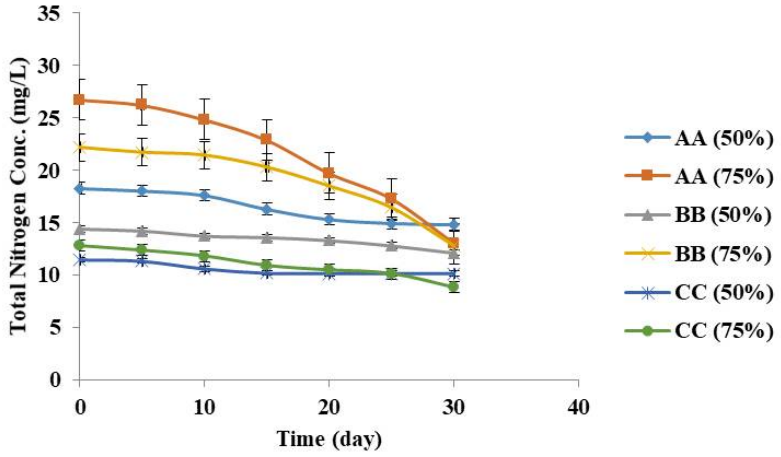


**Figure 3.1: Graph of TN concentration in industrial wastewater and microalgae samples mixture AA, BB and CC at different proportions (50%, and 75%) for 30 days**

inorganic nitrogen. For inorganic nitrogen, eukaryotic microalgae can only assimilate nitrite, nitrate, and ammonium / ammonia (Jia and Yuan, 2016). Hongyang *et al*. (2011) reported the removal of nitrogen (77.8%) from soybean processing wastewater by using *Chlorella pyrenoidosa*. Ruiz-Marin *et al*. (2010) reported $NH^{+}_{4}$-N removal (100%) for carrageenan-immobilized *S. obliquus* and also reported nitrogen removal of 82% for alginate-immobilized *C. vulgaris* cultivated in urban wastewater.

### *3.2 Removal of TP by various proportions of C. vulgaris*

Based on Table 3.2, samples mixture AA (75%) was the highest removal ratio of TP with 93.62 ± 0.16% of TP removal, while Samples Mixture BB (50%) was the lowest removal ratio of TP with 31.25 ± 0.24%. In this study, *C. vulgaris* was sufficiently removing phosphorus content of the Sample Mixture AA, BB, and CC with proportions (75%). Higher TP removal was observed for the samples mixture at proportion (75%) than sample mixture at proportion (50%) as they the samples contained more amount of industrial wastewater (proportion) that contained much amount of phosphorus. Phosphorus is an important nutrient for algae production and plays several roles in microalgae as a constituent of phospholipids (for cell membranes) and adenosine triphosphate (to carry energy for cell functions), among other functions (Cunningham *et al*., 2010). Boyd and Musig (1981) have shown that planktonic communities adsorbed 5-100% (average 41%) of 0.30 mg/L additions of orthophosphate within 24 h. In other studies, Cunningham *et al.* (2010) have also reported that the maximum removal of phosphorus from wastewater was about 51%. It was observed that 98.4% of total phosphorous was removed which is higher than removal rate reported in previous studies (Bhuyar *et al*., 2020d).

Hongyang *et al*. (2011) reported the removal of phosphorus (88.8%) from soybean processing wastewater by using *Chlorella pyrenoidosa*. Many studies demonstrated the remarkable potential of *C. vulgaris* in fixating up to 74% carbon dioxide when grown in a photobioreactor (Keffer and Kleinheinz, 2002), and in absorbing 45-97% nitrogen, 28-96% phosphorus and in reducing the COD by 61-86% from different type of wastewater such as textile, sewage, municipal, agricultural and recalcitrant (Aslan and Kapdan, 2006). Microalgae provide a pathway for the removal of vital nutrients (nitrogen and phosphorus), carbon dioxide, heavy metals and pathogens present in wastewaters and necessary for their growth. Thus, a faster growth rate accompanied by an elimination of water-contamination level is a promising and advantageous process. Hence, *C. vulgaris* is considered as one of the best microalgae for bioremediation of wastewater with an impressive potential to completely remove ammonium and sometimes modest potential to eliminate phosphorus present in the medium (González *et al*., 1997).

Industrial wastewater effluent consists of large materials amount like soluble organic, inorganic, insoluble inorganic materials, macro solids, microbes and toxin. If this wastewater is used as a culture medium, microbes present in it will compete for microalgae growth and nutrition. Hence, ultraviolet light source, autoclaving and centrifugation are the different types of pre-treatment methods used to remove suspended solids and algae feeding organism such as protozoa and bacteria. Wastewater filtration and sterilization has also been carried out in many previous studies, although it has been shown that these treatments can sometime change the content of nutrients (Sawayama *et al*., 1992; and Ramli *et al*., 2020b).

Essentially, the primary step to eliminate the unwanted materials from wastewater is processing. By processing, it is ensured that only the soluble fraction of the waste like carbon, nitrogen and phosphate are used for the microalgae cultivation. This kind of processing depends on the wastewater characteristics. For removal of nutrients from wastewater, single strain or compound of microalgae along with bacteria growth-promoting microalgae are used (Sriram and Seenivasan, 2012). Wastewater samples were collected from secondary effluent of the wastewater treatment, before chlorination; so autoclaving was necessary to eliminate bacteria and pathogens (Ramli *et al*., 2020b). The *Chlorella sp*. cultivated in the autoclaved centrate showed higher growth rate than the raw centrate (Li *et al*., 2011). After sterilizing the wastewater, phosphorus level in it gets reduced and it might have effect on the algal growth. In order to eliminate the influence of microorganisms, filtered and autoclaved wastewater samples were used in this study.

**Table 3.2: Total phosphorus removal ratio in samples mixture of microalgae and industrial wastewater AA, BB, and CC with different proportions (50%, and 75%) removed by *Chlorella vulgaris***

| Sample | Initial value (mg.L$^{-1}$) | Final value (mg.L$^{-1}$) | Removal ratio (%) |
|---|---|---|---|
| AA (50%) | 0.26 ± 0.02 | 0.05 ± 0.02 | 80.77 ± 0.08 |
| AA (75%) | 0.47 ± 0.05 | 0.03 ± 0.01 | 93.62 ± 0.16 |
| BB (50%) | 0.08 ± 0.02 | 0.06 ± 0.01 | 25.00 ± 0.76 |
| BB (75%) | 0.13 ± 0.00 | 0.04 ± 0.02 | 69.23 ± 0.04 |
| CC (50%) | 0.16 ± 0.03 | 0.11 ± 0.02 | 31.25 ± 0.24 |
| CC (75%) | 0.30 ± 0.04 | 0.05 ± 0.02 | 83.33 ± 0.09 |

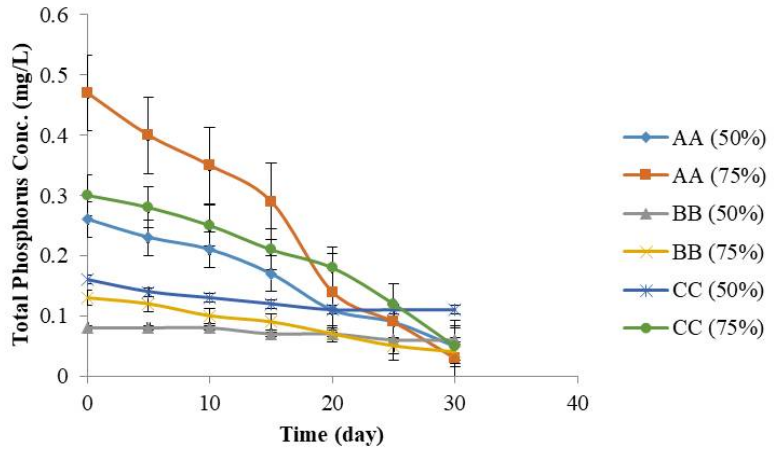


**Figure 3.2: Graph of TP concentration in industrial wastewater and microalgae samples mixture AA, BB and CC at different proportions (50%, and 75%) for 30 days**

## 4. Conclusion

In the present study, we have investigated the effect of optimal concentration of *C. vulgaris* for the removal of nutrients such as nitrogen and phosphorus in industrial wastewater obtained from Berkelah Waterfall, Gambang. The obtained results showed that 11.35 ± 0.07%-51.31 ± 0.03% of TN, and 31.25 ± 0.24%-93.62 ± 0.16% of TP were removed from three different sources of industrial wastewater. TP were removed more effectively than TN. The optimal ratio of *C. vulgaris* density concentration caused an enhancement of its removal efficiency. The Samples Mixture AA was determined to have more nutrients removal, compared to the other two Samples Mixture BB and CC. Therefore, industrial wastewater from POME had better concentration than the other two wastewaters for effective nutrients removal by *C. vulgaris* in industrial wastewater. In addition, there were more nutrients removed after 30 days by the proportion of (75%) which contained 300 mL of industrial wastewater and 100 mL of microalgae than the proportion of (50%) which contained 200 mL of

industrial wastewater and 200 mL of microalgae; hence, the process in this study seems to be slow for nutrients removal, especially for the nitrogen removal from industrial wastewater. The preferred ratio for the nutrient's removal is 75% of industrial wastewater mixed with microalgae.

## Acknowledgment

The authors gratefully acknowledged Universiti Malaysia Pahang for the financial assistance through the Internal Research Grant No. RDU190337 and Flagship Grant No. RDU182205. Author [Prakash Bhuyar] is thankful to UMP for providing Doctoral Research Scholarship DRS as a financial support.

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**Cite this article as:** Prakash Bhuyar, Fakhrul Farez, Mohd Hasbi Ab. Rahim, Gaanty Pragas Maniam and Natanamurugaraj Govindan (2021). Removal of nitrogen and phosphorus from agro-industrial wastewater by using microalgae collected from coastal region of peninsular Malaysia. *African Journal of Biological Sciences.* 3(1), 58-66. doi: 10.33472 / AFJBS.3.1.2021.58-66.