# The Use of a Consortium of *Chlorella vulgaris* and *Pseudomonas putida* for Bioremediation of Artificial Sugar Wastewater


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**Abstract.** Sugar wastewater can be a serious problem for environment because of high organic content namely nitrogen concentration. Other problems caused by the waste are unpleasant colour and an inferable smell. The appropriate treatment is needed for solving the problems. The growth of algae in the pool of waste was suggested as the propertreatment for sugar wastewater. The aim of this research was to study the use of *Chlorella vulgaris* for the treatment of artificial waste water. *Chlorella vulgaris* was cultured in artificial wastewater having low pH.*Pseudomonas putida* was used to supply $CO_2$ for photosynthesis process in a lagoon tank and it was added at the second day. Polyacrylate polyalcohol was also introduced to provide buoyancy effect of the medium All of the experiments were conducted in a rectangular tank using the artificial illumination and it was also equipped by air pump to supply oxygen and circulate the waste in the tank. pH, Dissolved Oxygen (DO), Optical Density (OD), Chemical Oxygen Demand (COD) and Total Nitrogen (TN) were measured daily during the experiments. COD was successfully reduced up to 6900 mg/l and 29% of COD removal at the fourth day. The low pH experiments demonstrated the effectiveness of a consortium of algae and bacteria at TN removal from wastewater by reducing the TN from 70 mg/l to below 35 mg/l. According to the above results, it can be concluded that the consortium of *Pseudomonas putida* and *Chlorella vulgaris* was applicable as one of alternatives for sugar wastewater treatment.

**Keywords:** *Chlorella vulgaris*, *Pseudomonas putida*, bioremediation, wastewater

## Introduction

Microalgae as a green source havetaken much interest from researchers regarding its ability as the solver for environmental problems. The ability of capturing $CO_2$ and converting it to renewable energy of algae has taken researchers' attention. Microalgae as the base of the food chain can also be the solution for wastewater treatment. Algae also feature widely tolerant to the mineral composition of the environment, so it will contribute well to the waste treatment especially for sugar wastewater.

Photosynthesis takes importance place for growing algae. It needs light from solar radiation or other sources, $CO_2$, water and inorganic salts. Temperature for the best result of algae growth also should maintain to 20-30$^0$C. The $CO_2$ concentration becomes the factor which limits biomass yields. The $CO_2$ demand is stoichiometrically about 1.7 kg CO2/kg dry biomass (Pousted and Shcoub, 2009). Microalgae uses photosynthesis to convert solar energy into chemical energy and it is stored in the form of lipids, carbohydrates, protein, etc. The energy is then converted to biofuels which means biofuels are primarily a form of solar energy.

Mixed systems of microalgae and bacteria are the best solution to industrial wastewater treatment. Oxidative bacteria will utilize oxygen ($O_2$) produced from photosynthesis to their respiratory process and produce $CO_2$that symbiotically will be used in photosynthesis process. Besides, algae also can remove the inorganic salts in the waste and it will reduce chemical oxygen demand (COD) of the waste.

The Kyoto Protocol is one of the most influential agreements in moving to use biomass as one of the alternatives substituting fossil fuels. In May 2008, 182 parties renewed this protocol, including the EU as a party in its own right and have made a commitment to reduce greenhouse gas emissions. Microalgae can be seen as $CO_2$ decrease for greenhouse gas emissions control. Department of Food and Rural Affairs (DEFRA) published a UK biomass strategy in 2007, which focused on potential applications of biomass for energy and transport. The report mentioned about central role of biomass in complying with the EU target of 20% renewable energy by 2020. The UK also proposes for at least 60% cuts in $CO_2$ emission by 2020 (Charlton *et al.*, 2006).

Algae are the bottom of the food chain and also they are the principal producers of oxygen on Earth. Algae are known to have several advantages that make them potentially greener fuel feedstock than farmed crops such as, algae are considered to be a very efficient biological system for harvesting solar energy for the production of organic compounds, cultivation of microalgae avoids the increase of farm land required for oleaginous plant, algae can be grown on arable land that cannot be used by other crops. Microalgae also can use sea or brackish water as a media to live. Algae can also convert carbon dioxide (waste) into a high density liquid form of energy (natural oil) (Amin, 2009; Gross, 2008;Campbell, 2008).

Algae based wastewater treatment processes could potentially offer many advantages that is the ability of the algae cell to assimilate the organic carbon (heterotrophic growth) as well as inorganic nutrients such as nitrogen and phosphorous for their growth (Wang et. al, 2005). It was efficiently removed nitrogen and phosphorus shown in Mallick (2008), as well as in metal ion depletion. Microalgae as the alternative for wastewater treatment is also will result in savings of the precious freshwater resources. Removing nitrogen and carbon from wastewater, microalgae can help reduce the eutrophication in the aquatic environment (Balat and Balat, 2010). In the treatment, algae will use abundant dissolved $CO_2$ in the photosynthesis process and also nutrient in the wastewater as the media to grow and produce oxygen that later it will be used by bacteria in the symbiosis.

The previous study showed that *Chlorella vulgaris* can reduce COD and TN from the high pH of artificial sugar wastewater (Zuhra, 2011). This experiment tried to combine the using of the algae and bacteria called *Pseudomonas putida.*

The aim of this research is to investigate the use of a consortium of *Chlorella vulgaris*and *Pseudomonas putida*for bioremediation of sugar wastewater, especially in removing contaminant from artificial sugar wastewater. The objectives of the research are to conduct the treatment of artificial sugar waste water in lagoon tank using *Chlorella vulgarisand* to characterize the physicochemical of waste water including pH, DO, COD, Total Nitrogen and optical density.

## Materials and Methods

*Chlorella vulgaris,* provided from Culture Collection of Algae and Protozoa SAMS Research Services Ltd, Scottish Marine Institute (Argyll, Scotland, Catalogue number CCAP 211/79), previously carried out in Blue Green 11 (BG 11) medium until the required amount for carrying out the lagoon tank experiments was obtained. Trace elements consisting of metals (Boron, Manganic, Zinc, Copper, Molybdenum and Cobalt) were also added to the BG-11 solution in order to support photosynthetic electron transport as mention by Raven etal. (1999) in Wang (2008).

The experiment for culturing *Chlorella vulgaris* was run out in three Erlenmeyer flasks 250 ml. Two flasks were added by 5 ml each of *Chlorella vulgaris* and another was added by 10 ml of medium and it was diluted in 100 ml of distilled water. The BG -11 medium was added two millilitres of each millilitre of algae culture every day. A ridge air tube was put in the culture medium. Two pieces of 30 W white standard fluorescent lamps were arranged vertically, at 20 cm distance from the surface of the flasks which continuously provide light. Those lamps assured an even and continuous illumination in range light intensity between 2000-3000 lux on the surface of three culturing flasks. These lamps used provided light spectrum that was similar to that of natural light from sun. pH, Dissolved Oxygen (DO), temperature, light intensity and optical density (OD) were checked daily and BG-11 medium was also added every morning. The system need to be run out for 7-10 days until the colour was changed to darker green showing the growth of the initial culture of algae. The light intensity was measured using a light intensity digital lux meter DT-1309 Jenway. pH was measured by 370 Jenway. DO concentration was measured using a portable AP74 Dissolved Oxygen Acumet Fischer Scientific. The OD of the algae at 658 nm was measured as the cell density indicator using a spectrophotometer (UV mini 1240 UV/VIS Spectrophotometer).

*Pseudomonas putida* was purchased from American Type Culture Collection, Manassas, USA with ATTC number 47054. The medium was prepared by adding 20 g of LubriaBertani (LB) (Fluka) to 1 litre of distilled and deionised water. The prepared medium was then sterilized for 2 hours at 160$^0$C in the oven. The medium was needed to cool down for 50$^0$C in a water bath. The 5-6 ml of sterilised LB medium was poured into petri dish plates and let the plates cool down and completely solidified. The plates then should put in the lab bench drawer to sterilise them from any forms of contamination. The bacterial vial was opened and withdrawn 1 ml of the cell line with 1 ml pipette. The cell line was transferred aseptically via sterile loop into the broth tubes. All tubes were incubated at 37$^0$C using hot plates for a week. The control of temperature and the measurement of DO and pH were done daily for four days.

The laboratory scale lagoon tank was made from polyvinyl chloride glass. A lighting system comprised of two unit of lamps Marine White 40W, fluorescent lamps was mounted about 200 mm above the reactor to give a light intensity of 2000-3000 lux. Temperature was held at 20±4$^0$C. The variation in temperature came from the lights warming the tank. The wastewater was allowed continuously exposure of light. Gentle mixing of the wastewaters was provided by air pump a rate 110 rpm and it also circulate the wastes.

The composition of the chemicals of artificial sugar wastewater (SWW) were preparing based on the ratio of C : N : P. Glucose was weight as much as 6 g/l, ammonium carbonate was 0.31 g/l and potassium dihydrogen phosphate was 0.031 g/l and the waste also prepared as much as 13 l. 0.9 g of polyacrylate-polyalcohol (Fluka) was fed to the four ports in the lagoon tank. Forty ml of algae from the final step were taken from the flask and added then by 10 ml of BG-11 and 10 ml of SWW. All of the solutions were mixed well in four Petri dishes and then poured the contents at four points in the lagoon tank. *Pseudomonas putida* were added at the second day. Samples from the reactor were drawn daily and characterized. The observation was taken for taking the reading of DO, LI, temperature, and pH for the next four days. Wastewater samples which were collected from the tank were analysed for COD, and TN was measured using Hach Large LT200 based on standard methods

## Results and Discussion

The initial characteristics of wastewater are reported in Table 1. The waste contain of the ratio of C : N : P used was 100 : 50 : 0.5. The initial COD of the wastewater was quite high and the initial pH also contributes a good condition for *Chlorella vulgaris* to grow. At the third day*, Pseudomonas putida* was added to the tank to supply $CO_2$ from its respiration process. The wastewater was evaporated and the height of the liquid in the tank was reduced around one cm daily and it will be corrected the value of COD and TN from the measurement. This phenomenon was caused by continually heating of light.

Table 1 Initial characteristics of artificial sugar wastewater.

| Wastewater Characteristics | Value |
|---|---|
| COD (mg/l) | 8150 |
| Total Nitrogen (mg/l) | 72.8 |
| OD (Abs) | 0.11 |
| DO (mg/l) | 8.0 |
| pH | 7.4 |
| Temperature ($^0$C) | 20 |
| LI (lux) | 1400 |
| Viscosity (cp) | 1.0 |
| Height of liquid (cm) | 14.7 |

### *Chemical Oxygen Demand (COD) of Artificial Sugar Wastewater*

Chemical Oxygen Demand (COD) measures the oxygen equivalent of the organic matter content of a sample that is can oxidize by a strong chemical oxidant. COD of the wastewater in this experiment was measured using closed reflux method and the values were then determined by colorimetric method. At the experiment, the decrease of COD was clearly seen in the Fig 1. COD was 8150 mg/l when the experiment was started and then decrease gradually until reaching about 6400 mg/l at the third day. Furthermore, *Pseudomonas putida*was introduced after taking the measurement of the third day. The COD slightly increases at the fourth day because bacteria were still in adaptation phase that it may not produce $CO_2$ optimally. It was also the contribution of carbon from the medium of bacteria. When the experiment was stopped at the fifth day, COD value of wastewater was about 4900 mg/l and the removal efficiency was about 38%. This result suggests that *Chlorella vulgaris* and *Pseudomonasputida* interact with each other in removing organic nutrients from the wastewater in this lagoon treatment system. Actually, the process should continue until the system reaches the steady state condition where the consortia of algae and bacteria cannot degrade the contaminant in the wastewater. It will be shown by the same result for COD measurement for three days.

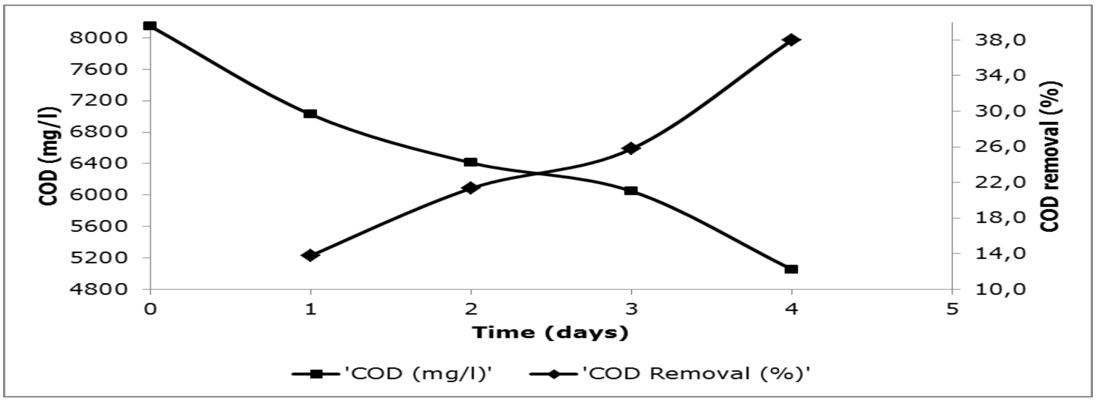


Figure 1. Removal efficiency and mean concentration of COD in lagoon tank

***Dissolved Oxygen (DO) Concentration and Optical Density (OD) of Artificial Sugar Wastewater***

At the experiments, the value of DO gradually decrease from about 8 mg/l to 4 mg/l. It reached the lowest value at the third day. *Pseudomonassp* was introduced at that day when the bacteria also consume $O_2$. DO decrease steadily because of the presence of *Pseudomonas sp*. The bacteria effectively consumed oxygen produced from the photosynthesis process. Optical density (OD) of the culture shows there was the growth of culture daily. Optical Density (OD) of the *Chlorella vulgaris* at 658 nm was measured daily using a spectrophotometer (UV mini 1240 UV-Vis Spectrophotometer Shimadzu) as the absorbance. This method was a very quick and easy test that clearly illustrated the growth of algae throughout the experiment.

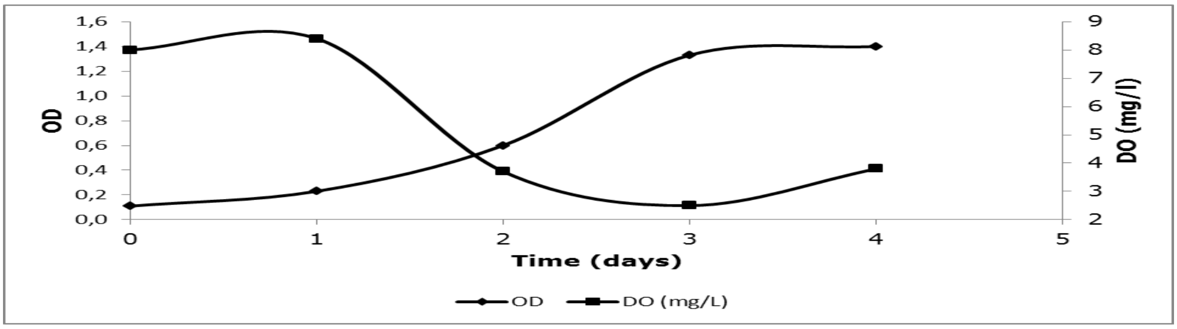


Figure 2. Optical Density (OD) and Dissolved Oxygen (DO) in lagoon tank

***Total Nitrogen (TN) of Artificial Sugar Wastewater***

Nitrogen is one of the main nutrients for algal growth. Wastewater can supply the need of nitrogen for algal growth as well as it will reduce total nitrogen concentration as contaminant in the waste.

Figure 3 describes total nitrogen reduce significantly from about 75 to about 32 at the fourth day. It decreases sharply at the second day and then it reduces slightly until the end of this experiment. These results show that even the greenery of Chlorella cannot be seen but it was a small growth of algae that can reduce the total nitrogen concentration up to 26% of removal.

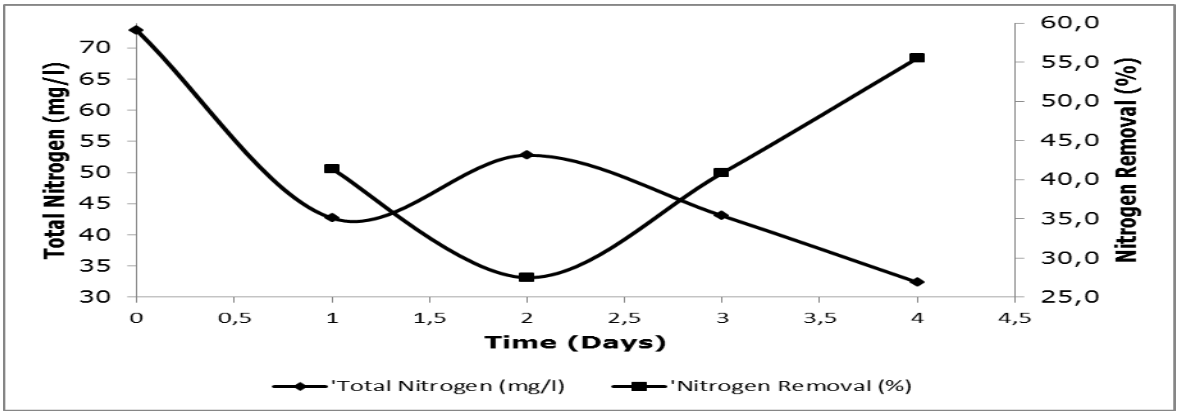


Figure 3. Total nitrogen and nitrogen removal in lagoon tank.

***Light Intensity (LI) and pH***

Exposure of wastewater to the light has a major effect on the temperature and rate of photosynthesis by algae and this process consequently affects the DO and pH of the culture medium. Light was provided continuously through the experiment and the measurement was taken from several points in the tank. The light in the surface of liquid is higher that if the measurement was taken at the bottom of the surface. Fig 4 showed the light intensity measurement at the first day was about 1400 lux of light and it changed in the range 1400-1500 lux. The light intensity has provided enough energy to the medium, so photosynthesis process was occurred in the tank. It was shown by the growth of algae that it can be seen clearly algae grown at the bottom of the reactor. The temperature for two experiments was in a range of $20^0$C to $25^0$C. The initial wastewater has the lowest temperature and it increases to $25^0$C. So, the effect of light intensity for increasing the temperature of the wastewater cannot observe clearly in these experiments.

The pH of the lagoon tank affects many of the biochemical processes associated with algal growth and metabolism. The pH of the first experiment was recorded as shown in Figure 4. In this experiment, pH of initial wastewater is about 7.5. It decreased gradually from 8 to 6 at the fifth day. The $CO_2$ concentration resulted from the respiration of the bacteria affect the decrease of the pH.

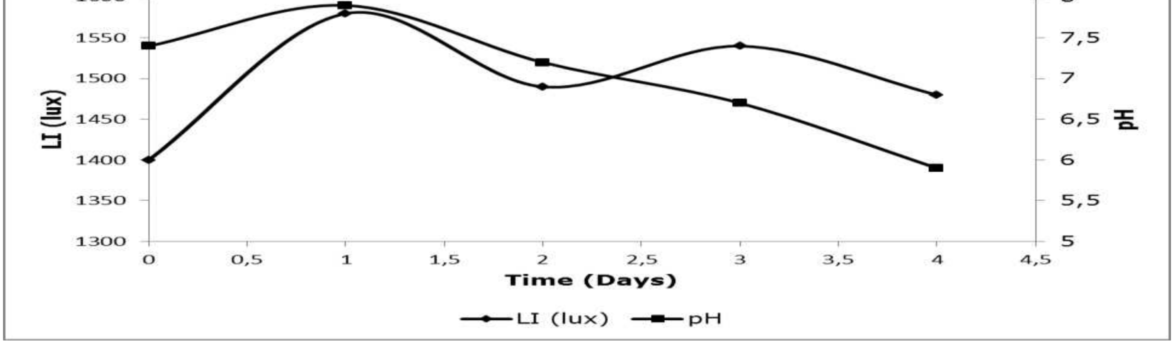


Figure 4 pH and Light Intensity in lagoon tank

## Conclusions

A consortium of *Chlorella vulgaris* and *Pseudomonas putida* was studied in this work. *Chlorella vulgaris* was grown in artificial wastewater having high dissolved nitrogen and high organic carbon. In this study, the consortiumin artificial sugar waste water successfully reduced COD where COD decrease up to 4900 mg/l and 38% of COD removal. The physiochemical properties recorded in this study as follow: the pH values varied from 7.5 to 6. The DO values ranged from 4 to 8, and the highest light intensity was 1400 lux. Based on optical density data, this research showed a slight increase of OD from 0.11 to 0.20 of absorbance at the fourth day. OD has increased from 0.11 to 1.6 of absorbance when the lagoon tank was stopped at the fifth day. The experiment demonstrated the effectiveness of a consortium of algae and bacteria at TN removal from wastewater by reducing the TN from 70 mg/l to below 35 mg/l. The removal of TN reached more than 55% when the bacteria were introduced in the tank. This results showed the potential cost savings of using wastewater when compared to the added of fertilizers in raceway ponds.

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