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Removal of Hydrocarbons from Wastewater by *Chlorella vulgaris* and Observation of the Biochemical Effects on the Algae

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ABSTRACT

The current study aimed to use the biological treatment (Phycoremediation) for sewage water, where the green alga *Chlorella vulgaris* was used in the treatment process and to improve water quality by removing some of the pollutants contained in the water, the most important of which are hydrocarbon pollutants, and to note the changes in the biochemical properties of the algae, such as the SOD enzyme and CAT enzyme and total chlorophyll before and after the treatment process. The treatment process took place between wastewater and green algae when the latter reached a stable stage to ensure its high treatment capacity and the best possible life span. Where the measurements were made for the characteristics of the treated water in addition to the biochemical measurements of the algae on the 4th and 8th day of the biological treatment. A study of the growth curve of C. vulgaris showed that the growth phase started on day 6, reached stability on day 12, then reached the death phase on day 19, and continued to decline until the death of the alga. The results of the study showed the value of what was contained in the wastewater before the treatment process, as it was as follows: total hydrocarbons (5.38 mg/l), as for the biochemical properties of algae before exposure to wastewater, they were as follows: superoxide dismutase enzyme (1.2197 U/mg), catalase enzyme (0.6023 U/mg), total chlorophyll (6.1503 mg/g). After the wastewater treatment process was completed, the high ability of green algae to remove hydrocarbon pollutants from the water was shown, at a rate of 72.3–64.5%, respectively. The results of the study showed the effect of sewage water on some physiological characteristics of algae, as it showed an increase in the activity of SOD after 4 days of treatment to reach 1.33 U/mg, while the activity decreased on the 8th day to reach 1.289 U/mg, as for the CAT enzyme It appeared in the same way as the previous enzyme, as its effectiveness increased after 4 days of treatment to reach 0.6916 U/mg, and decreased on the last day to reach 0.5476 U/mg, while with regard to the value of chlorophyll, the value of chlorophyll a decreased to reach 1.9473 mg/g in the last day, while on the contrary, the value of chlorophyll b increased to reach 4.5369 mg/g in the last day, while for total chlorophyll its value increased to reach 6.4842 mg/g in the last day.

Keywords: *Chlorella vulgaris*, algae, hydrocarbons, CAT, SOD.

INTRODUCTION

One of the main problems of surface and groundwater pollution alike is domestic and industrial wastewater that is thrown directly into rivers because it is often untreated or partially treated as a result of the lack of treatment plants or their inefficiency. This problem exists in many countries, especially Iraq (United Nations Educational, 2015). Liquid wastes that are thrown directly or indirectly into water bodies often cause serious problems for living organisms because they are not treated or treated insufficiently (Mohamad et al., 2017). There are many pollutants in wastewater, most of which are minerals, nutrients, hydrocarbons, heavy elements, pesticides, and others (Emparan et al., 2019). Hydrocarbons are among the most important sources of energy in the current era and because of Increasing industrial activities and the world's need for energy, the great need for the use of crude oil emerged, and thus the problems of global pollution with hydrocarbons increased, bearing in mind that crude oil is a complex compound that often consists of hydrocarbons, which constitute 95% of its components, in addition to other elements (Catania et al., 2020). Thus, with the increase in human activities, the complexity of liquid waste increased, so the need to find ways to treat wastewater increased, which must be environmentally friendly, applicable, easy, inexpensive, and effective at all times. Recently, the technologies used in wastewater treatment have increased, some of which depend on physical processes, some of which depend on chemical processes, or that use both methods with us (Bes-Piá et al., 2002). Others depend on biological methods. One of the best technologies used to treat wastewater at the present time is the methods that depend on biological treatment, as it is characterized by being hygienically safe and available in nature, in addition to being easy to apply and low cost, as it depends in its work on microorganisms such as bacteria and fungi and algae (Wang et al., 2016). Biological processes that rely on algae in bioremediation have advantages that exceed their peers from other biological processes that depend on different microorganisms, because they have a high potential to remove and treat water polluted with nitrogen, carbon and phosphorus, in addition to the fact that they do not need chemicals and do not need energy. (Emparan et al., 2019). The best types of algae that have a high capacity for processing are the green algae that belong to Chlorophyta, being cheap, renewable and available in all parts of the world. These advantages made them superior to many other types of algae in bioremediation processes (Gharbani, 2018).

MATERIALS AND METHOD

Algae isolates used in the study

The green alga Chlorella vulgaris used in the treatment was obtained from the College of Science / University of Babylon in the form of pure algae isolates and classified by using microscopy. Which were then incubated under optimal conditions of light intensity of 50 µE/cm^2/sec, light/dark time 16:8, and temperature 2 ± 25 °C using an incubator dedicated to the growth of algae inside the laboratory (Chia et al., 2013).

Preparation and Sterilization of C. vulgaris Growth Media

For the purpose of developing and propagating green algae farms, the culture medium known as chu-10 was prepared, which was modified by Kassim et al. (1999) Table 1. The medium was prepared in the form of stock solutions and kept in the refrigerator at a temperature of 4 °C upon completion of its preparation. Without sterilization until use. 2.5 ml of all stock solutions are taken and collected in a 1 L beaker, after which the volume is completed using distilled water, after which the sterilization process is carried out using the autoclave. except for the stock solution (k_2HPO_4) that was added alone then. By adding a few drops of either 0.01 standard hydrochloric acid (HCl) or 0.01 standard sodium hydroxide solution, the pH was changed from 6.8 to 7, using a pH meter after calibrating it with standard solutions.

Biological wastewater treatment

The wastewater treatment process was started by combining polluted water with algae grown in specific proportions after the green algae reached the stationary phase, by filling glass beakers with a capacity of 1 liter and placing in it 850 ml of waste water and adding 150 ml of waste water to it. This process was done by three replications in addition to the control coefficient that contains waste water only without

Table 1. Chemicals used in the preparation of media chu-10 modified by (Kassim et al., 1999)

Item	Chemical	g/l
1	$MgSO_4.7H_2O$	10
2	NaCl	30
3	$CoCl_2.6H_2O$	0.004
4	Na_2CO_3	8
5	Na_2-EDTA	4
6	$MnCl_2.4H_2O$	0.02
7	$(NH4)6MO_7O_{24}.4H_2O$	0.028
8	$NaNO_3$	8
9	$ZNSO_4.\ 7H_2O$	0.224
10	$CaCl_2$	16
11	$CUSO_4.5H_2O$	0.08
12	H_3BO_3	0.288
13	$FeCl_3$	0.32
14	K_2Hpo_4	4
15	Demineralized water	1L

the addition of algae. After that, these treatments were preserved in the container designated for cultivation. Samples were taken from the farms during specified periods after 4 and 8 days to conduct physical, chemical and biological tests and measure concentrations of heavy metals and total hydrocarbons (Al-Rubaie, 2003).

Estimation of total hydrocarbons

In order to extract the total hydrocarbons from the water, the fluorescence method was used by the spectrofluorometer (UNEP, 1989). Where 1 liter of waste water was taken and 10 ml of carbon tetrachloride was added to it, then an electric mixer was used until the sample was well mixed and blended for 30 minutes, after which the organic layer was separated by a separation funnel, then the sample was passed on a column containing wool to remove the remainder then the concentration of hydrocarbons was measured by a spectrofluorometer and the concentration was determined according to the following equations:

$$C = \frac{X \times D \times 1000}{Q \times L} \qquad X = \frac{Y - 1.37838}{28.14496} \tag{1}$$

where: C – concentration of total hydrocarbons; Y – sample reading – solvent reading; D – dilution factor; Q – the amount of sample injected into the device

Estimation of physiological and biochemical indicators

Total Chlorophyll estimation

The method described by (Parry et al., 2014) was used by taking 1 g of the algae extract and mixing it with 10 ml of acetone 80% in a glass test tube and keeping it for twenty hours at a temperature of 4 degrees Celsius in the dark, then it was shaken again and left as well. For a period ranging between 1–2 hours under the same conditions, then it is centrifuged at a speed of 3000 revolutions per minute for ten minutes, then the filter is taken and 1–2 drops of hydrochloric acid (0.1N) are added to it, and the filter is taken to measure the absorbance by a spectrophotometer at a wavelength of 663, 645 nm, and the chlorophyll concentration was calculated through the following equations:

$$\text{Chlorophyll a (mg/gm)} = \frac{12.7\,A633 - 2.69\,A645}{a \cdot 1000 \cdot w} \times V \tag{2}$$

$$\text{Chlorophyll b (mg/gm)} = \frac{22.9\,A645 - 4.68\,A663}{a \cdot 1000 \cdot w} \times V \tag{3}$$

$$\text{Chlorophyll (mg/gm)} = \frac{20.2\,A645 - 8.02\,A633}{a \cdot 1000 \cdot w} \times V \text{ Total} \tag{4}$$

where: A645 – absorbance at wavelength 645; A663 – absorbance at wavelength 663; V – final volume of extract in milliliters; W – weight of plant tissue in grams; a – the length of the light path in the cell (1cm).

Determination of enzyme activity Superoxide dismutase (SOD)

Based on the method adopted by Marklund & Marklund (1974), 50 µl of algae extract was taken, added to it 1000 µl of Tris-buffer solution and 1000 µl of parallel solution, and compared with the change in the absorbance of the control solution containing distilled water instead of the extract using a spectrophotometer on 420 nm wavelength, filtered with distilled water only representing blank, and the efficiency is calculated according to:

$$\text{SOD activity (units)} = \frac{\%inhibition/50\% \times reaction volume}{total test\ period} \tag{5}$$

where: Inhibition % – the percentage inhibition of pyrogallol reductase which is calculated from the Equation 6 below:

$$\%\,\mathrm{I} = \frac{sample\ (A-B) \times 100}{control\ (A-B)} \tag{6}$$

A and B represent the difference between the first and second absorbances after 2 minutes

Determination of activity of the enzyme catalase

The method described by Hadwan & Kadhum Ali (2018) was used to estimate the activity of the CAT enzyme, where 100 µl of algae extract was mixed with 900 µl of phosphate buffer and 2000 µl of hydrogen peroxide, the sample was mixed well and then left in a water bath at a temperature of 37 °C. After that, 2000 µl of ammonium vanadate reagent was added, The spectrophotometer was used to calculate the activity of the catalase enzyme at a wavelength of 420 nm through:

$$\text{Catalase Activity of test kU} = \frac{2.303}{t} * log\frac{S^o}{S} \tag{7}$$

where: S^o is the absorbance of the standard tube- and S is the absorbance of the model test tube; t represents time.

RESULTS AND DISCUSSION

Stages of cultivation of *Chlorella vulgaris*

The growth ability of *C. vulgaris* was studied throughout the current experiment. The moss was grown in a laboratory under optimal conditions of temperature, light intensity, and ideal ventilation, and the moss was grown using the Chu-10 culture medium modified by (Kassim et al., 1999), whose components are shown in Table 1. The results showed that the growth period of the alga continued for 19 days, which was divided into several phases, starting with the introductory phase, which lasted from day 1 to day 5, then the exponential increase phase, which began on day 6 and ended on day 11, then followed by the stabilization phase, which began on day 12 to day 18, after which the algae reached the stage of death, which started from day 19. Based on a spectrophotometer's measurement of the density of algal cells at a wavelength of 540 nm, several phases were identified. The growth curve of algae during the course of the investigation is shown in Figure 1.

Total hydrocarbons

C. vulgaris recorded high removal rates for total hydrocarbons from wastewater (Fig. 2). The moss had a clearance rate of 64.5% on day 4 of the biological treatment, with a value of 1.908 mg/l, and this percentage increased to 72.3% on day 8 of the biological therapy, with a value of 1.493 mg/l. No elimination percentage was noted for the duration of the biological treatment period for the control treatment. Numerous earlier research that examined the efficiency of microalgae in the degradation of total hydrocarbons shown their efficacy in the remediation of hydrocarbon contaminants (Ghosal et al., 2016). In contrast, a separate study found that *Chlorella vulgaris* has a lot of promise for purifying water contaminated with crude oil. The elimination rate was between 88% and 94% effective (Xaaldi Kalhor et al, 2017). Despite the fact that it has been shown that on day 14 of therapy, *Chlorella vulgaris* is capable of degrading 98% of petroleum hydrocarbon contaminants from the water (Das & Deka, 2019).

Effects of wastewater on the physiological parameters of *C. vulgaris*

Total chlorophyll

Chlorophyll is the green molecule that is the main component of the photosynthesis process carried out by green algae, as it absorbs light in addition to using carbon dioxide and water to form energy for photosynthetic organisms (Khaleghi et al., 2012). There are two types of chlorophyll, chlorophyll a and chlorophyll b, and both act as photoreceptors in photosynthesis (Falk et al., 1996). The case study showed a slight increase in total chlorophyll content of *C. vulgaris* after treating the wastewater on the 8th day of treatment, which is an expected result due to the availability of the necessary elements for the formation of chlorophyll. According to research by Kazem (2017), treating two different kinds of household and industrial wastewater increased the amount of total chlorophyll in *Oscillatoria* sp. Dash et al. (1999) indicated that the chlorophyll content was increased when Westiellopsis prolifica was exposed to a mixture of domestic and industrial wastewater with a clear increase in

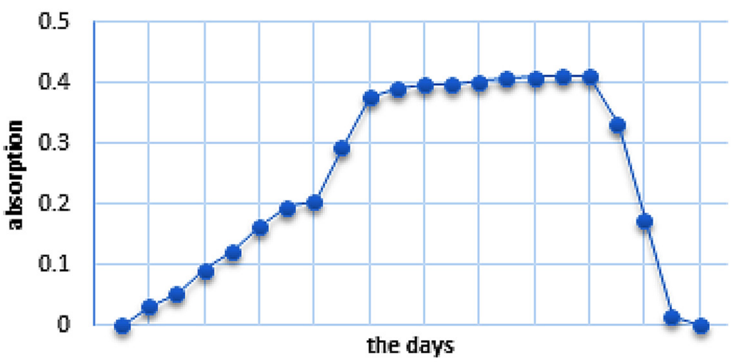

Fig. 1. Optical density of algae *Chlorella vulgaris* during the days of growth

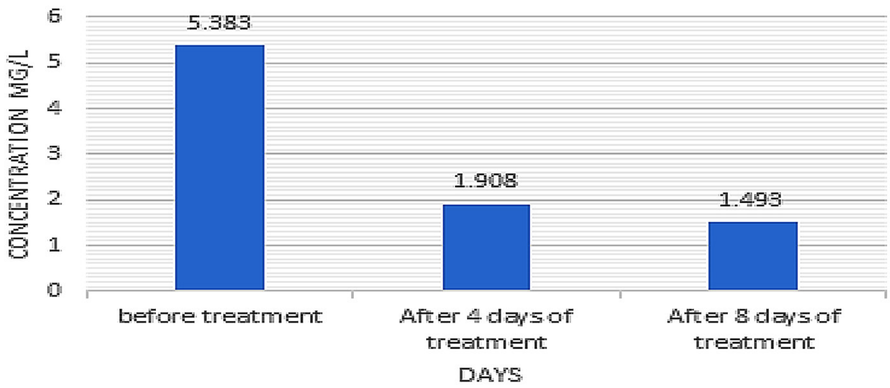

Fig. 2. Total hydrocarbons before and after treatment

the growth rate. However, a decrease in the total chlorophyll values of algae was observed in the first days of treatment, on day 4 specifically. The high concentrations of the pollutants may have inhibited the production of chlorophyll in the first days of the treatment, but after its decline, the pigment returned to rise, as we noticed on the 8th day of the biological treatment. Saikia et al. (2011) indicated that high concentrations of pollutants inhibit the production of pigments in Oscillatoria chlorina algae, where it was observed that the chlorophyll content decreased in the alga when exposed to high concentrations of paper mill waste. Brahmbhatt et al. (2013) confirmed that *Oscillatoria* sp. showed a high efficiency in removing heavy metals, but this was offset by a low total chlorophyll content.

Catalase (CAT) enzyme

CAT is present in all living organisms, as it has been found in many cells of animals, plants, fungi, bacteria, and algae (Costa, 2020). This enzyme provides protection for living organisms because it is responsible for breaking down hydrogen peroxide and changing it into two harmless elements, water and oxygen. as it is known that peroxide is toxic to cells due to its ability to oxidize membrane proteins and lipids (Goyal & Basak, 2010; Nicholls et al., 2000). Specific plant locations, including the cytoplasm, mitochondria, plastids, and peroxisomes, contain the CAT enzyme. (Willekens et al., 1995). The results of the case study showed a change in the activity of the enzyme CAT during the treatment period of wastewater by *C. vulgaris* algae, where the alga recorded a significant increase in the activity of the enzyme on the 4th day of treatment, which is the highest value of the activity of the enzyme obtained throughout the treatment period. Previous studies showed this increase, as the increased activity of the CAT enzyme in algae is evidence of its high efficiency in removing H_2O_2 and its high tolerance to various pollutants (Chakraborty et al., 2010). El-Naggar et al. (2014) showed that algae exposed to heavy metals such as copper and cadmium lead to an increase in the activity of CAT enzyme. Another

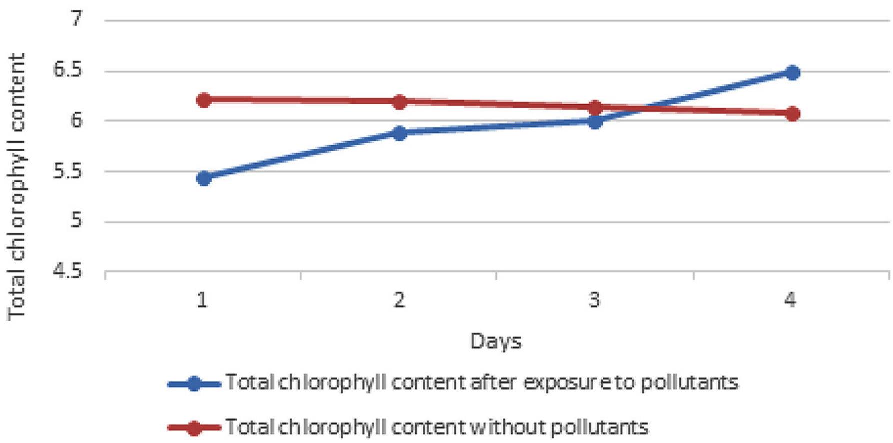

Fig. 3. Chlorophyll content after exposure to pollutants

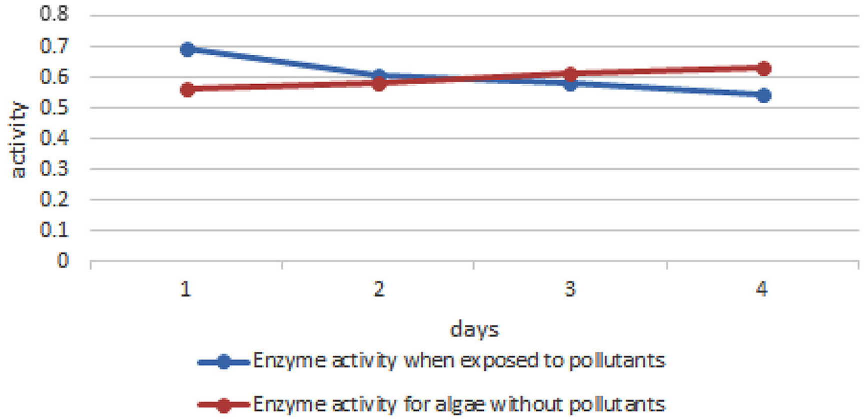

Fig. 4. Enzyme catalase activity after exposure to pollutants

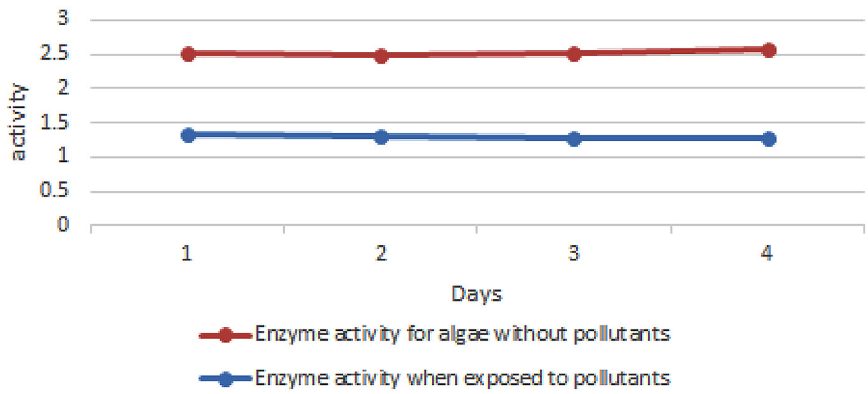

Fig. 5. Enzyme superoxide dismutase activity after exposure to pollutants

study showed an increase in the activity of enzymatic antioxidants, including CAT, when *Chlorella vulgaris* was treated with heavy metals such as copper (Kebeish et al., 2014). While a decrease in activity was observed on the 8th day of treatment, where Qureshi (2007) demonstrated that a reduction in the activity of the CAT enzyme occurs when oxidative damage is increased and exposure duration is prolonged. Another study found that heavy metal treatment boosted the CAT enzyme's activity in Anabaena sp, but that this activity of the enzyme reduced as the quantity of heavy metals rose (Sultan et al., 2007).

Superoxide dismutase (SOD)

SOD is one of the most important antioxidant enzymes found in all different living organisms. It serves as ROS's primary line of defense (Janknegt et al., 2007). This enzyme provides protection against reactive oxygen species or reduces them by converting them into O_2 and H_2O_2, and due to the toxic effect of hydrogen peroxide on the cell, here comes the role of the catalase enzyme, which removes toxicity by converting it into oxygen and water (Kachout et al., 2009). According to the study's findings, *C. vulgaris'* SOD enzyme activity has somewhat increased, where the maximum activity was observed on the 4th day of treatment. Where Blokhina et al. (2003) showed that the stronger the oxidative damage, the greater the activity of the SOD enzyme, which is between the high activity in the first days of treatment evidence of the presence of large quantities of pollutants, as Kazem (2017) confirmed that the values of the SOD enzyme for algae used in the treatment. Domestic and industrial wastewater did not decrease during the treatment period. Chen et al. (2019) also noted that *Chlorella vulgaris* was superior to all species in SOD enzyme activity throughout the wastewater treatment period.

CONCLUSIONS

The current study demonstrated the efficiency of *C. vulgaris* to grow excellently in wastewater and not to have any undesirable effects despite the presence of different levels of pollutants in the wastewater. The study also showed the efficiency of biological treatment using *C. vulgaris* in improving the physical and chemical properties of wastewater within a short period of time. The study also confirmed the efficiency of *C. vulgaris* in removing total hydrocarbon pollutants from wastewater. In addition, the case study showed the role of biochemical characteristics of algae, such as SOD, CAT, and chlorophyll, in helping algae to resist and remove various pollutants.

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