Bio-remediation of Most Contaminated Sites by Heavy Metals and Hydrocarbons In Dhiba Port Kingdom of Saudi Arabia Using Chlorella Vulgaris

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Abstract

Dhiba port has a strategic location near the Neom project. Various anthropogenic activities contributed to the discharge of heavy metals and oil spill in the aquatic system and caused environmental pollution. Microalgae are the best microorganisms in aquatic conditions which known to be capable of eliminating contaminants. We investigate the heavy metals and hydrocarbons contaminations exhibited in five sites of port. Our aim is to determine the most contaminated sites then using Immobilized and fresh *Chlorella vulgaris* in bioremediation. The results indicated that Immobilized and fresh *C. vulgaris* have the capabilities of growing in contaminated seawater and were capable of removing heavy metals completely. Immobilized *C. vulgaris* is the most efficient in the degradation of hydrocarbons at site one. Overall, Immobilized *C. vulgaris* is the most effective in removing both heavy metals and hydrocarbon. It is an economic tool due to simplifying harvesting and then retaining for further processing.

Introduction

Since coastal and maritime tourism is a new vital economic activity and pioneer in advancing the economic diversity of the Kingdom of Saudi Arabia, beaches and coastal areas quality are based on the nearby areas' environmental quality, including ports. Ports activities of tourism and transportation, including ship discharges of ballast water, loading and unloading of cargo, and accidental discharge of oil and other chemicals in the sea have various environmental impacts that affect the extent of physiochemical and biological constituents run in the port water (Davis & MacKnight, 1990; Jahan & Strezov, 2017). These impacts are significant, ranging from heavy metal contamination, oil pollution, fecal pollution to the introduction of exotic species through ballast water uptake and discharge (Luna et al., 2019; Niimi, 2004; Sany et al., 2013; Suneel et al., 2019). Due to their high toxicity to the marine environment, several studies have been examining the bioremediation of Polycyclic aromatic hydrocarbons (PAHs) and heavy metals. The term bioremediation refers to the use of biodegradation processes to remove environmental pollutants in a variety of ways (Crawford & Crawford, 1996). The bioremediation of contaminants in the marine environment is carried out mainly by diverse microorganisms. Algae are low-cost sorbents for elimination of oil and can impact the fate and vehicle of spilled oil (Mishra & Mukherji, 2012). Algae have a pivotal role in self-removal of organic pollution in natural water (Şen et al., 1988). Many studies have depicted that algae eliminate nutrients such as nitrogen and phosphorus (Craggs et al., 1996), heavy metals (Tam et al., 2001), toxic hydrocarbon, inorganic toxins, and pesticides (Shashirekha et al., 1997) from enclosing water by adsorption and absorption (Kızılkaya et al., 2012; El-Naggar et al., 2018) of bioaccumulation abilities of the cells (Oswald, 1988). Several species of microalgae have shown an influential role in the remediation of both heavy metals and hydrocarbons. Nweze and Aniebonam (2009) reported the probability of using naturally present algae isolated from a puddle near Nsukka Fire Service Station to remove hydrocarbon from water polluted with petroleum products. Microalga *Chlorella kessleri* could grow at different crude oil concentrations (0.5, 1, and 1.5%), mixotrophically solely and in combination with *Anabaena oryzae* (Hamouda, et al., 2016). Wang et al. (2018) argued that the acclimation process is a potential method of wastewater treatment using *Chlorella vulgaris. C. vulgaris* showed high efficiency of biodegradation under a low concentration of 0.5% and 1% of crude oil. The growth reached a high level even with the 2% of crude oil in an experiment of 15 days (El-sheekh & Hamouda, 2013). *C. vulgaris* can be used for the biodegradation of crude and refined oil in contaminated aquatic environments (Samuel et al., 2020). *Ankistrodesmus braunii* and *Scenedesmus quadricauda* were able to eliminate more than 70% of phenol from olive-oil mill wastewaters within five days (Al-Dahhan et al., 2018). Hamouda et al. (2016) reported that *Scenedesmus obliquus* was able to remove heavy metals Pb, Cd, Cu, and Mn, from wastewater under different conditions. Sharma and Khan (2013) noticed that *Chlorella minutissima* was a better efficient alga in removing heavy metals from polluted habitats than *Scendesmus* spp and *Nostoc muscorum.* Matsunaga et al. (1999) observed that *Chlorella vulgaris* was able to remove 65% cadmium between 7 to 28 days. *Chlorella* sp. removed effectively by 76% to 96% of cadmium and 78% to 94% of nickel under laboratory condition (Rehman & Shakoori, 2004). Other results showed that *C. vulgaris* was able to remove up to 70% and tolerate 200 mg/L of As^{5+} present in the growth medium (Jiang, 2011). (Lee et al., 2020) investigated the optimal alginate bead size for the nutrient removal using *C. vulgaris* and suggested the cell immobilization technology as an efficient technique for the wastewater treatment systems. The main objectives of the current study are to investigate the contamination pattern in Dhiba port marine environment, analyze water in the five sites inside the port related to contaminations of heavy metals and hydrocarbons, and study the potential of fresh alga *C. vulgaris* and immobilized *C. vulgaris* to bioremediate pollutants that exist in the most contaminated two sites in port. It also aims to compare between fresh alga *C. vulgaris* and immobilized alga for efficient remediation of heavy metals and hydrocarbon in the most contaminated two sites and the effects of these pollutants on chlorophyll and carotene content.

Materials And Methods

Sampling location and collection

The study location is Dhiba port (27° 34' N to 34° 33' E), located at the north-western corner of the Kingdom of Saudi Arabia. It is the nearest Saudi port to the Suez Canal and the Mediterranean basin countries ports, including Turkey 593 miles, Greece 491 miles, and 988 miles to the nearest French ports (www.mawani.gov.sa). Thus, it acquires unique importance in its strategic location near the NEOM project, which is the Saudi Crown Prince Mohammed Bin Salman's vision and a centerpiece of Saudi Arabia 2030 Vision (www.neom.com). The registration of vessel arrivals from various ports worldwide showed that a number of 12029 vessels had navigated the port during the period 2005-2019 (Table 1).

Water samples were collected from the water surface on 25th January 2020 from five different Dhiba port locations (Figure 1). For heavy metals and hydrocarbons determination, samples were stored in the dark at a low temperature of 4°C until examination.

Isolation and identification of *Chlorella vulgaris*

The green microalga *Chlorella vulgaris* was isolated from water samples collected from Thuwal beach, Red Sea, Saudi Arabia (22°16'35.0"N 39°05'22.3"E). The isolation was done through a serial dilution technique followed by plating on modified BG-11 medium (Rippka, 1998; Stanier *et al.*, 1971). The alga was based on AlgaeBase (Guiry & Guiry, 2021), and (Bellinger & Sigee, 2010; Stevenson & Lowe, 1986).

Preparation of immobilized microalga in alginate beads

For each flask, 30 ml of algal suspension in its exponential growth phase were harvested by centrifugation at 3000 rpm for 10 min. The supernatant was then decanted, and the volume of sediment was adjusted to 2 ml with sterilized deionized water. After that, the concentrated algal suspension was mixed with 2% (w/v) sodium alginate solution and dropped into a 2% calcium chloride solution using a sterilized burette. Beads were left to harden overnight then rinsed with distilled water.

Estimation of Growth

Optical density

For microalga growth and pigments measurement, alginate beads should be dissolved in 100 ml of 0.1 M sodium citrate solution with pH 5 that was prepared by adding 10 ml of sodium citrate to a specified number of beads at 45 ˚C with stirring, and the beads would dissolve within one hour. Then, the solution was centrifuged at 5000 rpm for 5 min. After that, the supernatant was decanted, and the volume was adjusted to 3 ml with sterilized water. Alga's biomass was determined every three days by measuring the algal suspension's optical density at 600 nm using a SHIMADZU UV-2600 spectrophotometer.

Pigments determination

A known volume of culture was centrifuged at a speed of 3000 rpm for 10 min. After that, the algal pellets were treated with a known volume of methanol, kept in the water bath for 30 min at 55 ˚C, and then centrifuged again. The absorbance of the pooled extracts was registered by SHIMADZU UV-2600 spectrophotometer at 666, 653, and 470 nm. Calculations were made according to the formulae devised by (Costache et al., 2012) for chlorophyll *a*, chlorophyll *b,* and carotenoids.

Experimental Design

Two treatments were conducted triple to study the potential of *C. vulgaris* in the bioremediation of heavy metals and the biodegradation of hydrocarbons. For each treatment, two Erlenmeyer flasks (250 ml) contained 150 ml of sterilized seawater were enriched with nitrogen and phosphate source (0.225 g of $NaNO_3$ and 0.006 g of K_2HPO_4). Under a laminar flow cabinet, three flasks were cultivated with the algal beads, and the other three were cultivated with the residue of 30 ml of centrifuged algal cells of each flask. The cultures were incubated under the conditions of 12:12h light: dark and at 25 ˚C temperature and slight aeration for two weeks (Figure 2).

Chemical parameters analysis

Heavy metals

Laboratory analysis was carried out for heavy metals determination before and after the experiment. The Elements: Aluminum, Antimony, Arsenic, Barium, Beryllium, Cadmium, Chromium, Cobalt, Copper, Iron, Lead, Manganese, Molybdenum, Nickel, Selenium, Silver, Titanium, Vanadium, and Zinc were measured using ICP- OES (Inductively Coupled Plasma-Optical Emission Spectrometer) Agilent Technologies 720 ICP-OES Axial.

Determination of petroleum hydrocarbons

Petroleum hydrocarbons were extracted from 100 ml of seawater of each sample. The pH was adjusted with 1 M HCl to get pH < 3. Organic compounds were extracted via liquid-liquid phase extraction thrice, using 10 ml and 5 ml of dichloromethane (CH_2Cl_2). The organic lower phase was collected, and the moisture was removed by adding about 2g anhydrous sodium sulfate (Na_2SO_4). The clear extract was transferred to a test tube and evaporated with a gentle nitrogen gas stream at room temperature. The sample concentrated to about 10 µL (Suhrhoff & Scholz-Böttcher, 2016). The analysis was performed using a gas chromatograph (GCMS-QP2010 Plus, Shimadzu, Japan) equipped with a mass spectrometer with a fuse-silica capillary column (30 m × 0.25 mm ID × 0.25 µm Rtx®-1, Restek, USA) was used. Helium was used as a carrier gas, and the temperature programming was 60-300 ˚C, 1/5 min. GC-MS internal library search was used to identify the hydrocarbons. The analysis was conducted before and after the experiment.

Statistical Analysis

Experiments were conducted in triplicate and expressed as ± standard error of the mean. The data were compared by analysis of variance one-way and three-way ANOVA. Significance was determined using Duncan's multiple range tests ($p \leq 0.05$). Analysis was carried out using MS Excel (2016) and SPSS (Version 16).

Results And Discussion

Results in Table 1 show the number of vessel arrivals from various ports worldwide that reached Dhiba port. 12029 vessels, through 14 years ago, denoting anthropogenic activities during these years and hence accumulation of waste products. Results in Table 2 show nineteen heavy metals that were investigated in five sites of Dhiba port. The results demonstrated that among nineteen heavy metals were investigated, different concentrations of As, Be, and Se. The Be and Se were found at all five locations. Site no. 1 was contaminated by Sb^{3+} , As^{3+} , Be^{2+} , and Se^{2+} with concentrations 0.03168, 0.04126, 0.08985, and 0.199 ppm respectively where the site no. 3 was contaminated by the previous heavy metals $Sb3^{+}$, As^{3+} , Be^{2+} , Se^{2+} , in addition to Zn^{2+} with concentrations 0.07546, 0.05709, 0.09326, 0.4618, and 0.00979 ppm respectively. The concentrations of metals in surface seawaters varied from one site to another. Zinc metal has been depicted only in the third site, which has a high total concentration of heavy metals (HMs) compared with other sites, so it was chosen for the bioremediation experiment. The hydrocarbons concentrations were estimated before experiment (Table 3). The level of total hydrocarbons ranged from 0.21 ppm to 0.55 ppm. The first site was the most highly polluted with hydrocarbons. It showed particular compounds that were not found in the other sites (1,1,3-Trimethylcyclopentane and Diethyl Phthalate), so it was chosen for the biodegradation experiment.

Estimation of *C. vulgaris* growth

Green microalgae *C. vulgaris* is halotolerant, proliferating, and growing in marine environments and favorably using it for bioremediation and biodegradation experiments. Luangpipat and Chisti (2017) indicated that *C. vulgaris* thrived in a full-strength seawater medium and enhanced lipid productivity by nearly 2-fold compared to freshwater. *Chlorella* sp. is a micro green alga that is usually found in seawater (Maghfiroh et al., 2018). *C. vulgar* was cultivated in a photobioreactor with controlled conditions of Na CL that was extracted from salt from brackish and seawater (Sahle-Demessie et al., 2019). Figs 3a-b shows the suspension *C. vulgaris* and *C. vulgaris* beads growth that measured by optical density at 600 nm. The growth of immobilized cells reached a high level compared to fresh cells. The immobilized cells that were grown in sample one reached the highest growth level in close to eighth days of cultivations, and optical density reached 2.4 nm. In the case of site 3, the maximum growth of alga beads reached at (O.D 1.8 nm) after ten days and at (O.D 0.4 nm) within seven days in case of fresh alga. A plausible explanation for this result is that the third site was mostly contaminated with heavy metals as a result of the negative effect of alga growth, whereas the first site was more contaminated with hydrocarbons. In this case, it is preferred for alga to grow under mixotrophic conditions and use hydrocarbon as the sole carbon source. Melo et al. (2018) proved that when *C. vulgaris* was grown under mixotrophic conditions, the cellular productivity increased, and it becomes more effective to remove agro industrial by-products. The highest growth rate of *C. vulgaris* was obtained when grown under mixotrophic conditions than when grown under photoautotrophic conditions (Abreu et al., 2012). Bansal, (2019) investigated whether the growth of *C. vulgaris* and *C. protothecoides* were promoted under mixotrophic conditions when using glycerol as a carbon source. When *Chlorella* spp. was grown mixotrophically on glucose, it produced superior biomass concentration than heterotrophic and photo-autotrophic conditions (Cheirsilp & Torpee 2012). The results indicated that immobilized cells were higher in growth than suspension cells in both two sites. *C. vulgaris* immobilized by sodium alginate produced higher amount of cells than suspension cells (Abu Sepian et al., 2019; Rushan et al., 2019). Immobilization technique can offer higher micro-algal cell density, which is useful for diminishing lag period (Ide et al., 2016), due to it is less sensitive to stress conditions (Lee et al., 2020). Results in Table 4 showed that the effect of seawater was taken for both sites on chlorophyll-a, content of both immobilized and suspension *C. vulgaris* cells. Chlorophyll-*a* contents are more promotive in suspension *C. vulgaris* that was grown in seawater taken from site 3 within ten days. Contaminations in site 3 were more abundant with heavy metals and less content of hydrocarbons, so the alga was grown under photoautotrophic conditions. Chlorophyll-*a* in autotrophic was promoted by alga growth, which revealed the production of necessary pigments by *C. vulgaris* for photosynthesis, the only pathway for the metabolism of phototrophic microalgae (Mohammad Mirzaie et al., 2016). The same trends were observed for Chlorophyll-*b* contents but within seven days with suspended alga (Table 5). After 10 days of cultivation, Chlorophyll-*a* and *b* were decreased. This may be due to decrease of nutrients content in media such as nitrogen and phosphorus. Chlorophyll contents decrease could be due to decreasing of nitrogen in media (Li et al., 2008). The highest level of carotenoid contents of *C. vulgaris* was 666.14 µg ml^{-1} recorded at day 14 with suspension cells that was grown in seawater sample taken from site one followed by carotenoid contents of *C. vulgaris* that was grown at the same days but in site three. Both sites at the day 14 of growth had the stress conditions such as site three that had most contaminations by metals, site one was mostly contaminated by hydrocarbons, and when incubations period to day 14, the nutrients of media decreased and accumulation of toxic compounds (Table 6). Green alga such as *Chlorella* can be overproducing secondary carotenoids under stress culture conditions like nitrogen limitation, cultivation period and salt stress (Fu et al. 2013; Santhosh et al. 2016). High amount of carotenoids were produced by *S. platensis* after 7 and 11 days of incubation with various concentrations of oil (El-Sheekh & Hamouda, 2013). The three-way ANOVA shown in Table 7 demonstrated the variable among different sites, alga treatments and the incubation periods in relation to Chl *a*, Chl *b* and carotenoid. The results indicated that there were significant interaction among sites, alga treatments (immobilized and suspended), and incubation times in relation to pigments contents in *C . vulgaris*. In site 1 there were significant interactions among the types in treatments (suspension, alga, and immobilized) and incubations periods and also in case of site three (Table 8).

The bioremediation of heavy metals

The results in Table 9 demonstrated that when applied suspension *C. vulgaris* and immobilized *C. vulgaris*, heavy metals were completely disappeared. The removal efficiencies of these metals were affected by their initial concentrations. *C. vulgaris* presented a high efficacy in removing 100% of Sb, As, Se, and Zn. This finding is consistent with the work of (Zou et al., 2020) where their results showed that *C. vulgaris* was highly efficient in removing Se and Cr collectively and separately. The bioremediation process was effective using both suspended and immobilized *C. vulgaris* cells. Thus, our results may also be explained by enhancing growth rate of *C. vulgaris* during exponential phase. This result is in agreement with (Li et al., 2019) who studied the biotreatment of mixed wastewaters with MnO_2 industry by *C. vulgaris*. However, the removal of heavy metals (Cu, Cr, Pb, and Cd) from dyes by *C. vulgaris* was significantly enhanced when endophytic bacterial strain MN17 inoculum was applied (Mubashar et al., 2020). Marine green alga *Chlorella* sp. NKG16014 exhibited the highest elimination of Cd due to cell adsorption and intracellular accumulation (Matsunaga et al., 1999).

Sorption capacities of heavy metals such as Cu, Zn, Cd and Ni by *C. vulgaris* were attained at the lowest biomass concentration (Fraile et al., 2005). The heavy metals in the current study's contamination levels can be correlated to contamination caused by the port activities.

The biodegradation of petroleum hydrocarbons

Results in Table 3 investigated the hydrocarbons that were existent in five sites in Dhiba port. The results demonstrated that the site no .1 was much contaminated by hydrocarbons, so it was shown for applied

C. vulgaris and immobilized *C. vulgaris* for possible bioremediation and cleaning. Results in Figs 4a,b and Table 10 revealed the effect of C*. vulgaris* and immobilized *C. vulgaris* on removing hydrocarbon that exhibited in site one. Both treatments were effective of biodegradation of hydrocarbons but the highest biodegradation rate of hydrocarbons was observed with immobilized *C. vulgaris.* Muñoz et al., (2003) suggested that the microalgae release biosurfactants that could improve phenanthrene degradation. Madadi et al., (2016) recommended using *C. vulgaris* and surfactants to treat wastewaters from petroleum industries. *C. vulgaris* had a high ability in remediation of crude oil hydrocarbons within 14 days (Kalhor et al., 2017). The results showed a complete absence of the previous hydrocarbons and a presence of new compounds. This new compounds may be due to the conversion of hydrocarbons into intermediate compounds (Okoh, 2006) This result is in agreement with El-sheekh and Hamouda, (2013) who proved the ability of the *C. vulgaris* to degrade n-alkane and PAHs. Kalhor et al. (2017) found that *C. vulgaris* owned not only significant resistance against the pollutants but also a high ability in bioremediation of crude oil hydrocarbons. Several studies established the vital role of *Chlorella vulgaris* for the biodegradation of PAHs in ecosystem (Abdel-Shafy & Mansour 2016; Wang & Zhao 2007). Results indicated that immobilized *C. vulgaris* was more efficient to degrade hydrocarbons. This cells immobilization technology would accelerate the nutrient uptake rate of microalgae for improving efficiency of seawater treatment. Immobilized *C. vulgaris* cells under optimal conditions are effectively efficient in eliminating nonylphenol from contaminated water (Gao et al., 2011). Liu et al., (2012) reported that immobilized *Chlorella sorokiniana* GXNN 01 was vital species for use in wastewater treatment. Immobilized *C. vulgaris* was capable of removing NH_4 and N from wastewater (Hameed et al., 2007). Immobilized cells have amplified reaction rates due to superior cell density (Mallick, 2002).

Conclusions

Dhiba's port is a strategic location and one of the most vital ports in Saudi Arabia where humans' activities are expected to be increased when NEOM project will release. There were some contaminations indicated by heavy metals and hydrocarbons appeared in five sites of Dhiba's port. Suspension and immobilized microgreen alga *C. vulgaris* were proved efficiency for bioremediate both heavy metals and hydrocarbons. Immobilized *C. vulgaris* was the most effective in removing heavy metals and also hydrocarbons that existed in two sites than suspension alga, and hence harvesting beads from media are very simple, and could be applied in biofuel production after bioremediation processes.

Declarations

Ethics approval and consent to participate

Not applicable

Consent to Participate

Not applicable

Consent to Publish

Not applicable

Availability of data and materials

The data of table 1 that used to indicate the number of vessels that arrived at Dhiba port from 2005 to 2019, were derived from Saudi Ports Authority (Mawani) website [mawani.gov.sa].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RA designed the experiments. AB collected and analyzed the samples. AB and RA performed the experiments. RA and AM carried out the statistical analysis. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1-10 is only available as a download in the Supplemental Files section.

Figures

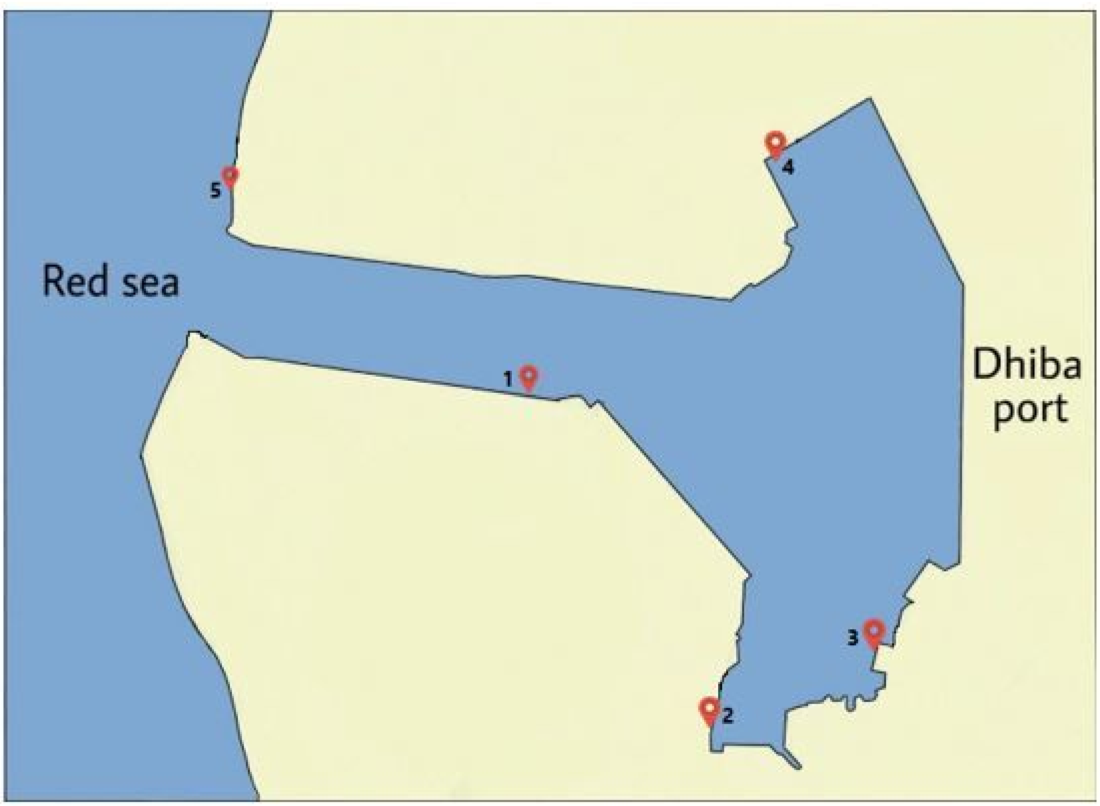

Figure 1

Geographical map focusing on sampling sites in Dhiba port Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

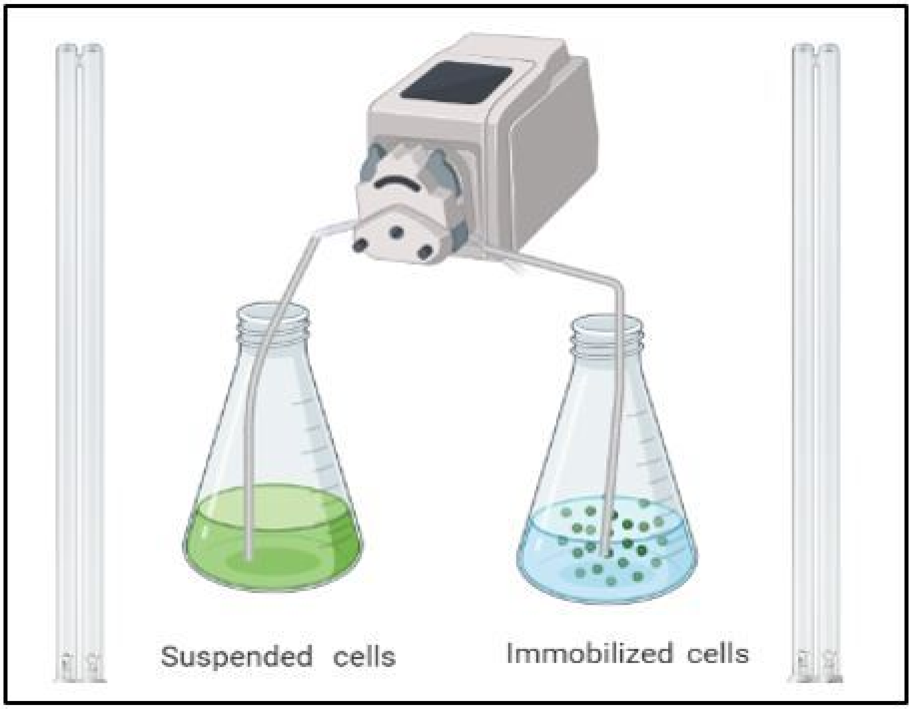

Figure 2

Experiment design

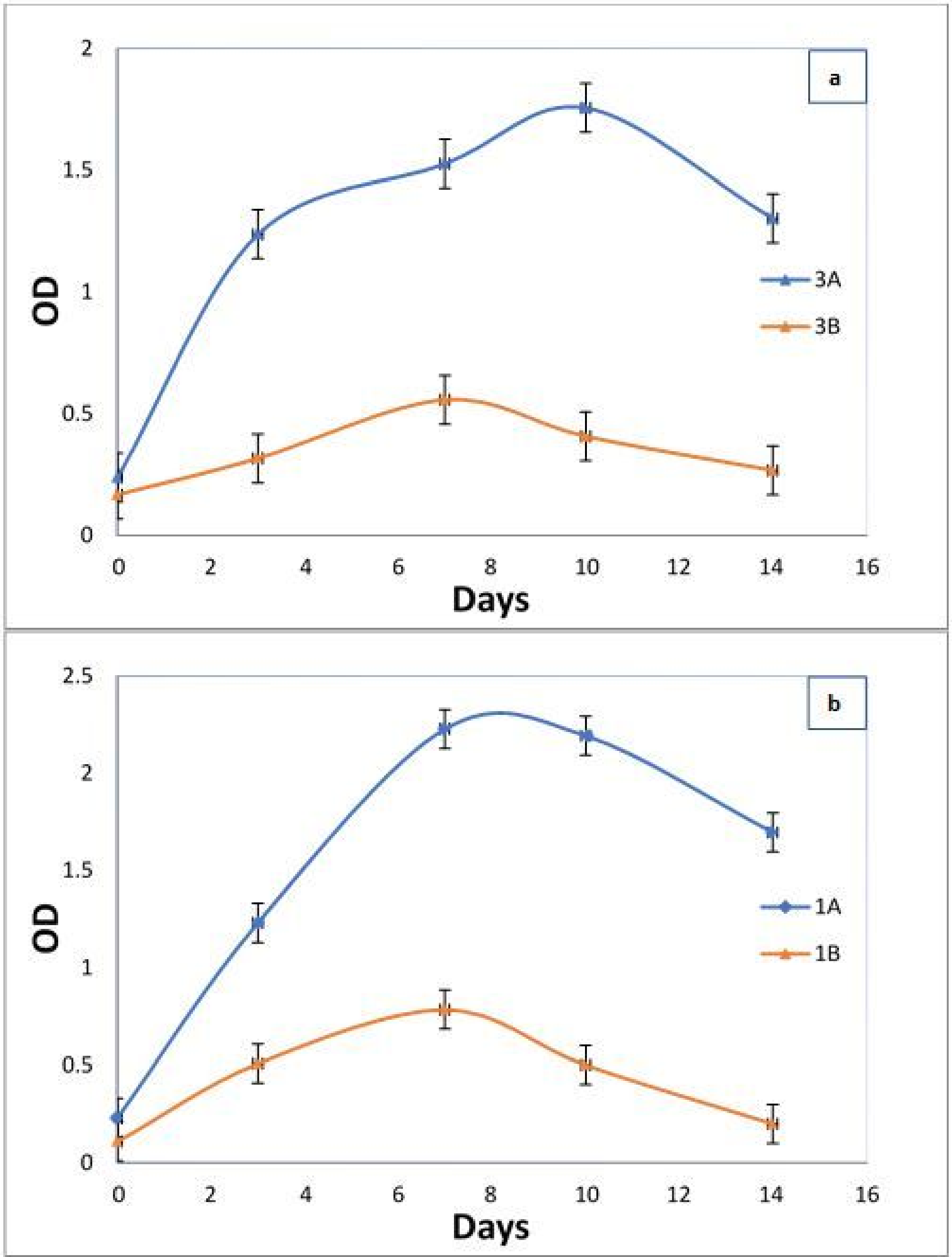

Figure 3

Growth curves of immobilized (A) and suspended (B) Chlorella vulgaris cells measured as optical density 600 nm. (a) Growth on sample one for heavy metals experiment. (b) Growth on sample three for hydrocarbons experiment.

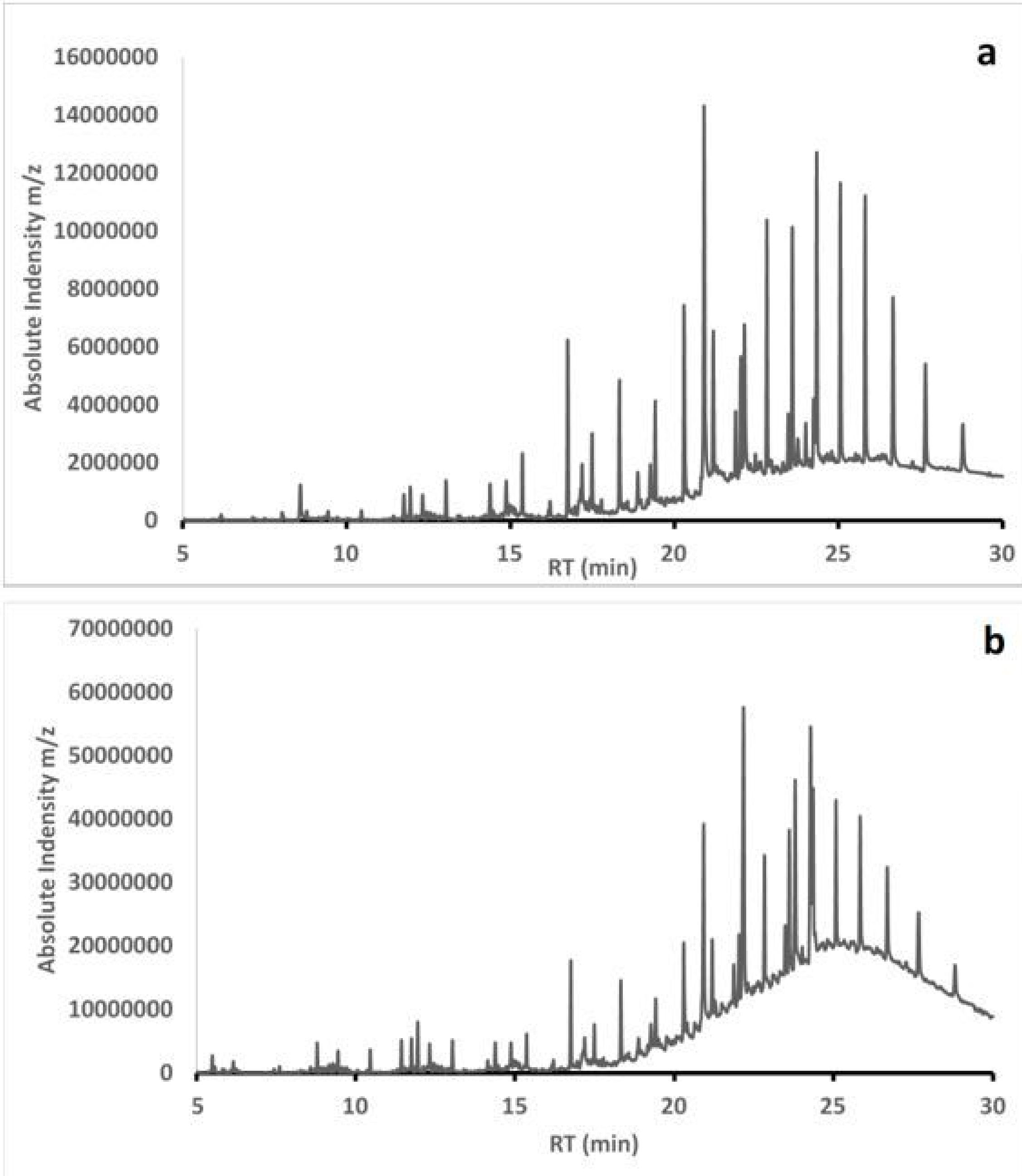

Figure 4

GC/MS chromatogram of residual hydrocarbons after 14 days of incubation. (a) with immobilized C. vulgaris cells. (b) with suspended C. vulgaris cells.

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