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Oil-Biodegradation Potential of Bacterial Communities by using Algae Extract as Bio-Stimulant

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Abstract: ***Bioremediation of hydrocarbon-contaminated soils by bacteria is a frequent process but requires nutrients when the soil is deficient in nitrogen and phosphate. The aim of the present study was to use algae extract as bio-stimulant of hydrocarbon-degrading bacteria. To this end, the algae extract was added to the contaminated soil at three concentrations (pure, 1:10 and 1:100), during the incubation period, at a rate of 15 % of moisture. The controls consisted of soil alone, moist soil, and soil added with Carbon, Nitrogen and Phosphate C:N:P at a proportion of 100:5:0.5. The number of bacteria and the amount of total hydrocarbons in the soil were also studied. The addition of the bio-stimulant to the polluted soil gradually increased the degradation of total petroleum hydrocarbons (TPH) during the experimental period. Thus, at the end of the experiment, the concentration of TPH in the soil had decreased from 2.24 % to 0.37%. Although the degradations obtained in the soil with C, N and P and algae extract at 1:10 and 1:100 were similar, the number of bacteria in the soil with the algae extract was high.***

Keywords: seaweed extracts, hydrocarbons, bio-stimulant

1. Introduction

The demand for petroleum resources continues growing every year, leading to more frequent exploration and transportation of petroleum. In this context, possible oil spills may lead to the accumulation of hydrocarbons in the soils. Oil-contaminated soils can be treated by means of different treatments, including biotreatment, which is achieved by the use of living organisms such as bacteria, fungi, etc. (Acuña and Pucci, 2022). However, for optimal growth, these organisms need nutrients, chemicals, moisture conditions etc. (Cambarieri et al., 2021). Thus, researchers are constantly searching for new products or agents to optimize the process and find the best nutrient and the best moisture conditions for each soil (Acuña and Pucci, 2022). However, when used at high concentrations, most of these agents can have negative effects on bacterial communities. Considering the negative impacts of these products, current regulations are limiting the use of mineral fertilizers and biodegradable chemical products. In the search for more sustainable and environmentally friendly solutions to bioremediation, in the last years, researchers have focused their attention on biologically based products, with algae as a valuable resource for the production and protection of crops due to their bio-stimulating effects on bacterial communities (Gonçalves, 2021).

In Argentine Patagonia, San Jorge Gulf has been impacted by the invasive alga *Undaria pinnatifida* (*Undaria*), a brown seaweed species native to China, Japan, and Korea, and a source of proteins, vitamins, dietary fibers and minerals (Godde et al., 2016). This alga has been used a bio-stimulant in agriculture (El Boukhari et al., 2020) but not for oil biodegradation. Based on the above, the aim of the present study was to investigate the use of algae extract as a soil amendment to be used as a growth stimulator of hydrocarbon-degrading bacteria.

2. Material and Methods

Soil samples

Soil samples were collected in the San Jorge Gulf Basin, from a deposit of soils previously treated by bio-piles. Samples were kept outdoors under extreme climatic conditions (dry arid climate, low rainfall (8-15 mm/month) and a temperature of about (4-8 °C) for a few months. Soil samples were then stored at 8°C in the dark until use.

Analytical methods for soil characterization

The soil samples were characterized according to standard physicochemical parameters and methodologies. Results showed a pH value of 6.68 and electrical conductivity of 3156.27 µS/cm. The physical analysis of the samples showed the following values: a pH of 6.68 and moisture of 5.19%, whereas the chemical analysis showed the following values (ppm): i) cations and anions: chloride 640.8, sulfate 41487.98, carbonate < 1, bicarbonate 144.71, calcium 158.33, magnesium 32.04, nitrite 0.3, nitrate 223.58, ammonium 0.7, and phosphate 2636.82; ii) heavy metals: As 1.80, Ba 1157.6, Cd < 1.0, Zn 38.8, Cu 13.4, Cr < 10.0, Hg <0.2, Ni 6.2, Ag < 2.0, Pb < 10.0, and Se < 0.8, and iii) total petroleum hydrocarbons (TPH) 2.24%.

Algae biomass extract of *Undaria pinnatifida* was obtained from Soriano S.A. (Chubut, Argentina). Its pH values was 5.69, and its chemical analysis showed the following values (ppm): cations and anions: chloride 3972.4 sulfate 674.85, carbonate < 1, bicarbonate 68.6.71, calcium 20, magnesium 600, nitrite 5.17, nitrate 132.88, ammonium 4.26, and phosphate 969.1.

Experimental design and treatments

Treatments consisted of soil with three different concentrations of the algae extract: pure, 1:10 and 1:100, whereas controls consisted of soil alone (SS), soil with 15% w/w moisture (SH), and soil added with C:N:P at a proportion of 100:5:0.5, as controls. All treatments were performed in triplicate with 500 g soil inside a 1-L brown bottle, and incubated for 180 days at room temperature. Samples were taken every month to determine TPH, nutrients and bacterial count.

CO_2-evolution test

The CO_2 produced in the reactors was absorbed in 250-mL gas wash bottles in series, each filled with 2 mL of 3M NaOH. The CO_2- evolution test sampling was performed without changing the absorption bottles. NaOH (4 mL) was removed through the butyl rubber septum by using 5 mL PE syringes to avoid CO_2 influx from the room air to the system. The amount collected for sampling was considered in the calculation of degradation.

Determination of TPH content by Infrared Spectroscopy

The soil TPH concentration was determined by infrared spectroscopy as previously described, by the Environmental Protection Agency method [EPA 418.1]. Essentially, 2 g of each individual sample was dissolved in 10 mL of carbon tetrachloride, phase separated, and percolated through 2 g of silica gel, and the absorbance was then measured at 2930 cm^{-1}.

Enumeration and isolation of aerobic bacteria

Culturable bacteria from each sample were counted using the standard plate dilution method. To this end, 1 g of soil (wet weight) was suspended in 9 mL of sterile physiological water (pH 7.2) and vortexed for 1 min at low speed. Aliquots of 100 µL of undiluted samples, and 10^{-1} to 10^{-6} dilutions were grown on R2A medium (Reasoner and Geldreich, 1985) and hydrocarbons degrading MBM-PGO medium (NaCl 5 g/L, K_2PO_4H 0.5 g/L, $NH_4PO_4H_2$ 0.5 g/L, $(NH_4)_2SO_4$ 1 g/L, Mg SO_4 0.2 g/L, KNO_3 3 g/L, and $FeSO_4$ 0.05 g/L, suspended in distilled water). Next, 30 µL of a 1:1 mixture of petroleum-diesel oil was spread on the surface, and plates incubated at 28^0C for up to 21 days (Pucci and Pucci, 2003).

Statistical Analysis

All data analyses were done using the statistical software Past (Hammer & Harper, 2005) with a maximal Type 1 error rate of 0.05. Kruskal-Wallis non parametric test was used where the assumptions of analysis of variance (ANOVA) were not met.

Statistical analysis

PHC data (TPH and fractions) were transformed to the logarithm base 10 in order to allow the use of parametric statistics. Analysis of Variance (ANOVA) was used. No transformation was suitable for the pH data so a non-parametric statistical analysis (Kruskal–Wallis test) was conducted. Significance was accepted at $\alpha = 0.05$ (95% confidence level) for all statistical analysis.

3. Results

The soil had a neutral pH (6.68), high electrical conductivity (3156 µS cm^{-1}), and low content of heavy metals. Elemental analysis showed that the C:N:P ratio (1150:1:52) was low and that the soil had low moisture. Increases in the N and P content in the microcosms, in relation to the control, were due to the addition of inorganic nutrients and algae extract at different concentrations. The content of aliphatic (12%), aromatic (43%) and polar (45%) hydrocarbon fractions evidenced that readily degradable compounds had already been removed from the soil during the previous biopile treatments in the polluted site. Heterotrophic and hydrocarbon clastic microbial populations quantified by the plate count were relatively high (heterotrophs: 1.6×10^5 CFU g $soil^{-1}$, degraders: 3.5×10^5 CFU g $soil^{-1}$ respectively), indicating that there was an abundant indigenous microbial population with hydrocarbon-degrading capabilities. In the algae microcosm assays, TPH decreased by 80 ± 2.99% after 150 days of incubation. This value was higher than that observed in the soil alone (47.7 ± 1%), and similar to that observed in the soil with C, P and N (83.04± 3.13%). The presence of the algae extract increased the total bacterial heterotrophic population counts in four orders of magnitude, compared to those from the initial soil, up to values of 1.60×10^4 CFU g $soil^{-1}$ and 1.84×10^9 CFUg $soil^{-1}$ after introducing it into the soil.

CO_2 evolution a one-way ANOVA Kruskal Wallis comparing all treatments 1:10 and 1:100 with the control SS, confirmed that the observed differences were statistically significant (P = 6.036 E-15). The differences between the treatment with the algae extract at 1:10 and the control SS demonstrate that the biostimulant may be more effective than the conventional control approach. These improved oil biodegradable results obtained by using algae extract as biostimulant agree with previous similar studies.

Biostimulation of indigenous communities in an oil-polluted soil caused an important increase of the bacterial populations, linked to a significant increase in the biodegradation efficiency of TPH (C_{10}–C_{35}) and polycyclic aromatic hydrocarbons, when compared to a control, but in lower proportion than that observed with chemical products, which produce an increase in the pH.

Of the different algae concentrations used, 1:100 was the optimum one for oil biodegradation by the bacterial consortium studied. Higher algae concentrations led to no significant improvements in bacterial cell growth or in the biodegradation rate. In agreement with that observed in this research, several researchers have reported that the addition of nutrients within a certain limit would not affect cell growth or the process of biodegradation, and that the excess

content of nutrients may even be toxic to cell growth (Acuña et al., 2012, Osinowo et al., 2020; Thavasi et al., 2011). The maximum degradation of oil with algae extract was 83.48% in 150 days, indicating its effectiveness for use in bioremediation applications in soils with nutrient deficiency.

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Table 1: Values of bacterial number, total petroleum hydrocarbons and CO_2 and the beginning and at the end of the experiment

	Dhb CFU/g	R2A CFU/g	TPH (%)	CO_2 mg /kg	% **dgc**
Initial value	$3.50 \times 10^{+05}$	$1.60 \times 10^{+05}$	2,24	-	-
After 150 days					
SS	$1.90 \times 10^{+04}$	$7.00 \times 10^{+04}$	1.17	298.00	47.77
SH	$4.50 \times 10^{+09}$	$6.10 \times 10^{+09}$	0.59	7443.02	73.66
100:5:0.5	$2.20 \times 10^{+04}$	$2.00 \times 10^{+04}$	0.38	21893.46	83.04
EA 1/10	$4.50 \times 10^{+09}$	$2.40 \times 10^{+09}$	0.37	8800.55	83.48
EA 1/100	$7.90 \times 10^{+09}$	$1.21 \times 10^{+09}$	0.44	8980.37	80.36
EP	$7.30 \times 10^{+09}$	$7.10 \times 10^{+09}$	0.68	7699.08	69.64

Soil alone (SS), soil with 15% w/w moisture (SH), and soil added with C:N:P at a proportion of 100:5:0.5, as controls , extract algae (EA), extract algae (100%) degradation porcent (%dgc), hydrocarbons degrading bacteria (Dhb),

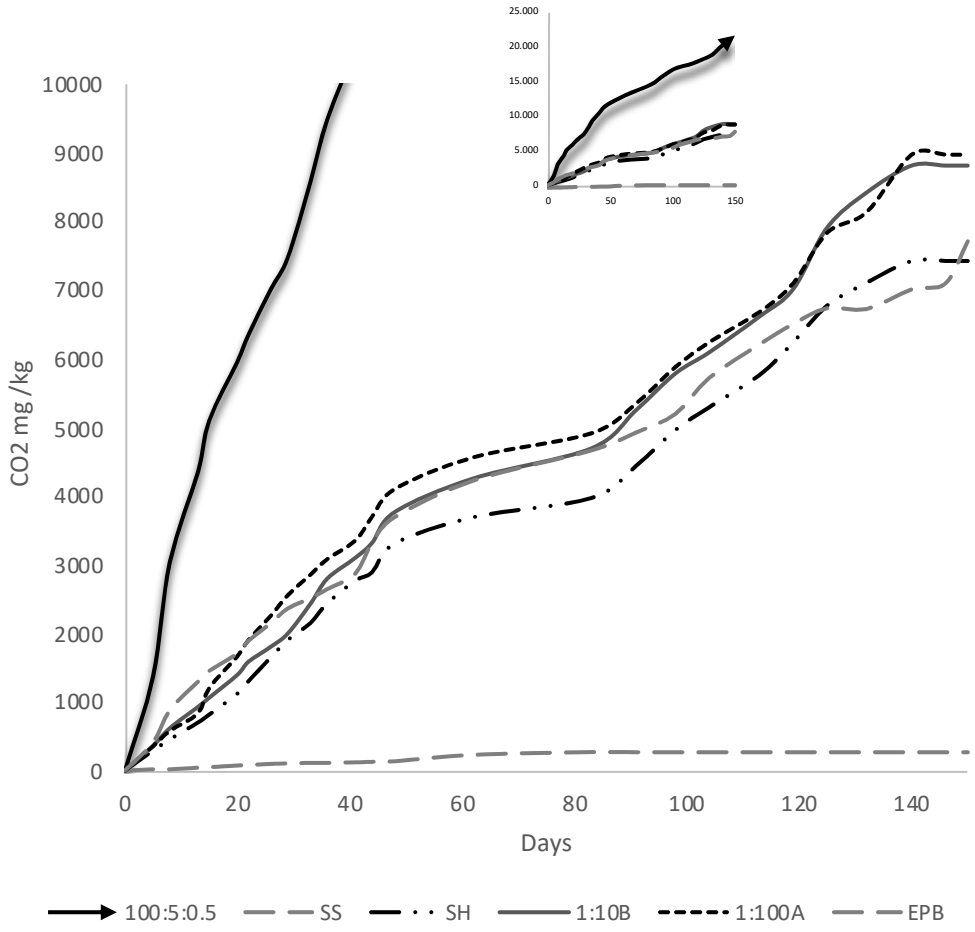

Figure 1: CO_2 evolution

Soil alone (SS), soil with 15% w/w moisture (SH), and soil added with C:N:P at a proportion of 100:5:0.5, EP algae extract 100%, AE algae extract