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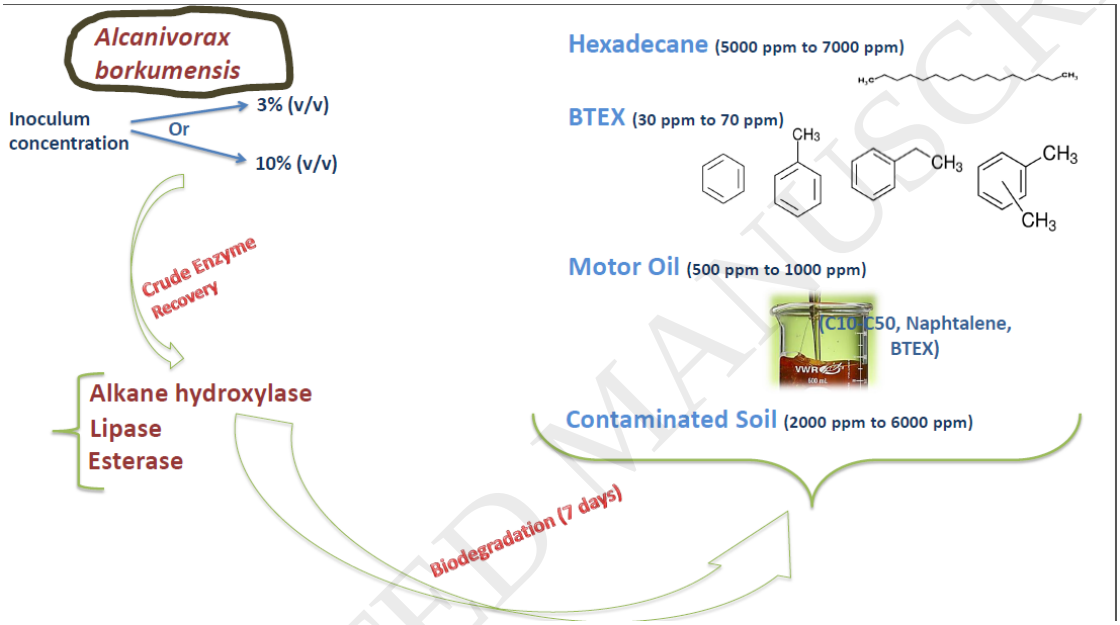
Ex-situ Biodegradation of petroleum hydrocarbons using *Alcanivorax borkumensis* enzymes

Tayssir Kadri[1], Sara Magdouli[1], Tarek Rouissi[1] and Satinder Kaur Brar[1*]

[1]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9.

*Corresponding author: Tel: + 418 654 3116; Fax: + 418 654 2600; *E-mail*: satinder.brar@ete.inrs.ca

Graphical abstract



Highlights

- Production of enzymes by *Alcanivorax borkumensis* is studied.
- Biodegradation of different petroleum hydrocarbon substrates is investigated.
- Enzymes efficiency depends on the inoculum and hydrocarbons concentration.

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Abstract

Bioremediation for degradation of hydrocarbons is a widely used alternative for the recovery of contaminated sites. The current study aimed to use *Alcanivorax borkumensis* crude enzyme preparation as an agent for enhanced microbial hydrocarbons biodegradation in contaminated water and soil. The inoculum and hydrocarbons concentration have a remarkable effect on the biodegradation with the crude enzymes. The high enzymatic production reaching 145.71 U/mg for alkane hydroxylase, 3628.57 U/mg for lipase and 2200 U/mg for esterase led to a significant degradation efficiency of the different concentrations of petroleum hydrocarbon substrates reaching 73.75% for 5000 ppm of hexadecane, 82.80% for 1000 ppm of motor oil, 64.70% for 70 ppm of BTEX and 88.52% for 6000 ppm of contaminated soil. The study suggested that *Alcanivorax borkumensis* is a potential hydrocarbon-degrading bacterium with higher enzymatic capacities for bioremediation of hydrocarbon-polluted environment.

Keywords: Biodegradation; Petroleum Hydrocarbons; *Alcanivorax borkumensis*; Enzymes Production

1. Introduction

Petroleum is the most cost-effective energy source in the current global economy. Its widespread and extensive use has led to serious ecological problems and severe disruption of the ecological balance [1].

The removal of petroleum-hydrocarbons by means of mechanical and physical methods is used as a primary response during oil spill. However, the efficiency of mechanical removal is limited. Bioremediation has been proven to be successful and eco-friendly in the mineralization and removal of petroleum hydrocarbon contaminants in different habitats, such as water and soil [2–4]. Some recently characterized bacterial species are highly specialized for hydrocarbon degradation. These species are called hydrocarbonoclastic bacteria, and they play a key role in the removal of hydrocarbons from polluted environments [5–8]

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. Importantly, *Alcanivorax borkumensis*, a marine bacterium that can assimilate various hydrocarbons [6,9,10]. *Alcanivorax borkumensis*is present in non-polluted seawater in low numbers; however, their number can increase as a result of an oil spill, and they are believed to play an important role in the natural bioremediation of oil spills worldwide [13–15]. Moreover, *Alcanivorax borkumensis* is able to produce a glucolipid biosurfactant [14,15] which may enhance the bioavailability of hydrocarbons by either increasing apparent hydrocarbon solubility in the aqueous phase or by expanding the contact surface area due to emulsification [16].

A. borkumenis genome has been completely sequenced and hence leading to a better understanding of the cellular biology of hydrocarbons metabolism [17]. Many genes encoding for enzymes initiating the degradation of these hydrocarbons have been detected [10–12]. However, very few biochemical studies have been carried out on the enzymatic effectiveness of this autochthonous hydrocarbonoclastic bacteria in oil spill remediation [20]. Therefore, understanding the biochemical pathway is a key feature in environmental bioremediation. With this aim, the production of alkane hydroxylase (EC: 1.14.15.3), lipase (EC: 1.3.3.-) and esterase (EC: 1.3.3.-) was investigated. The *A. borkumensis* alkane hydroxylase system is able to degrade a large range of alkanes up to C32 and branched aliphatic, as well as isoprenoid hydrocarbons, alkylarenes and alkylcycloalkanes. This spectrum is much larger based on knowledge about alkane hydroxylase complexes. This makes the choice of alkane hydroxylase of a unique importance. Furthermore, *A. borkumensis* genome includes 11 genes coding for different lipases/esterases of unknown specificity. Two of these esterases were purified and functionally characterized. They showed generous enzymatic activity that is up to two orders of magnitude better than common esterases, have a large substrate spectrum, exceptional enatioselectivity and chemical resistance, which provides them a competitive advantageous over other esterases from other microorganisms and other enzymes for the

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resolution of chiral mixtures in biocatalysis [17]. Other than the extensive production by *A. borkumensis*, lipase played an important role in oily hydrocarbons biodegradation. In fact, lipase activity has been used as biochemical and biological parameter for testing hydrocarbon degradation and it is an excellent indicator to monitor the decontamination of a hydrocarbon polluted site [21].

The present work aims to study the capacity of crude enzymes produced by *Alcanivorax borkumensis*, to degrade petroleum hydrocarbons represented by hexadecane with a concentration varying from 5000 mg/L to 7000 mg/L, BTEX with a concentration of the six compounds varying from 30 mg/L to 70 mg/L (1:1:1:1:1:1), motor oil with a concentration varying from 500 mg/L to 1000 mg/L and contaminated soil with a concentration of total petroleum hydrocarbons varying from 2000 mg/L to 6000 mg/L. these concentrations were chosen depending on soil characterization and PAHs, BTEX and C10-C50 content. Also we varied the inoculum concentration from 3% (v/v) to 10% (v/v) for higher enzyme production and measured alkane hydroxylase, lipase and esterase specific activity during the degradation process when using the highest petroleum hydrocarbon substrate concentration (7000 ppm for hexadecane, 70 ppm for BTEX, 1000 ppm for motor oil and 6000 ppm for the contaminated soil). The biodegradation was carried out during 3 days for BTEX and 7 days for the remaining substrates.

2. Materials and methods

All chemical reagents of highest purity, such as hexadecane, BTEX (Benzene, Toluene, Ethylene, Xylene), NADPH (nicotinamide adenine dinucleotide phosphate) and DMSO (Dimethyl sulfoxide) among others, were procured from Sigma-Aldrich, Fisher scientific or VWR (Mississauga, Ontario, Canada). The strain, *Alcanivorax borkumensis* was purchased from DSMZ (Braunschweig, Germany).

2.1 Soil and motor oil characteristics

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The contaminated soil used in this study was provided by TechnoRem Inc from a confidential site in Quebec. This soil was chosen due to its intense contamination with total petroleum hydrocarbons. The soil comprised 59% of 1 mm sized particles, 38% of particles with a size range between 250 µm and 500 µm and 3% of very fine particles with a size less than 250 µm.

The chemical characteristics of the heavily contaminated soil used during this study are presented in Tables 1, 2 and 3.

Total solids (TS) and moisture content were measured using the protocol 2540B of Federation et al. [22] which consists of heating the clean dish to 103±1 to 105±1 °C for 1 h. Later, it was stored and cooled in the desiccator. And finally, it was weighed immediately before use. To determine the elemental concentration of carbon and nitrogen, the sample was first dried at 60±1°C for 8 h and placed into a glass vial. Two sub-samples (2-3 mg each) were analyzed using a Leco-932 CHNS Analyzer in CHN mode. In CHN mode, samples are combusted in the presence of pure O_2 and the combustion gases are measured to determine initial elemental concentrations of C, H and N.

Oil content was determined by adding n-hexane to the soil (1:1 w/v) and then centrifuging at *10,000 x g* for 30 minutes to recover the pellet. Once again, n-hexane was added to the pellet (1:1 w/v) and then centrifuged at the same speed and time to recover the pellet. Finally, n-hexane was dried at room temperature and the oil content (supernatant) was weighed. Metals content was determined using inductively coupled plasma optical emission spectrometry (ICP-OES). Samples digestion was fulfilled following the method MENVIQ.89.12/213.Mét 1.3. All the analyses were performed in triplicates.

The method used to determine the PAHs is MA. 400 - HAP 1.1, which consists of: extracting PAHs using dichloromethane or hexane after adding recovery standards ("surrogates"). Later, the extract was concentrated to a small volume under a nitrogen stream and purified on a

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silica gel-alumina column. The final volume of purified extract was concentrated to meet the target detection limits. Finally, the extract was concentrated and then analyzed by chromatography gas phase coupled to a mass spectrometer (GC-MS) operating in the mode selective ion acquisition ("SIM") was used. The method used to determine the C10-C50 was MA. 400 – HYD. 1.1, which consists of extracting aqueous samples with hexane using a mechanical stirrer. Solid samples are first dried with acetone, and then extracted with hexane with the aid of an extraction system "mixer painting". As for organic liquids, they are directly diluted in hexane. Thereafter, silica gel is added to the extract to adsorb polar substances and then the supernatant was analyzed by gas chromatography coupled to flame ionization detector.

The composition of motor oil in this study comprised: 69.8 mg/L of C10-C50, 1.83 mg/L of naphthalene, ≤ 44 mg/L of benzene, ≤30 mg/L of toluene, ≤ 44 mg/L of ethyl-benzene and ≤ 84 mg/L of xylene.

2.2 Bacterial strain

Aerobic bacterial strain, *Alcanivorax borkumensis* SK2 (DSM 11573) which has the capability to degrade petroleum hydrocarbons was used in all the experiments in this study. The strain was stored at 4 °C on agar plates coated with a film of hexadecane.

2.3 Culture conditions

Initial culture of *A. borkumensis* strain was grown aerobically in batch culture in 250-mL Erlenmeyer flasks for 72h at 30±1 °C and 150 rpm in synthetic sea water medium SM1 in order to mimic the conditions of an oil spill in the environment (high carbon concentration and nitrogen limitation). This medium was supplemented each time with a different substrate used as a sole carbon and energy source: 3% hexadecane, 3% motor oil and 3% BTEX.. SM1 contained (per liter of distilled water): 23 g NaC1, 0.75 g KCl, 1.47 g $CaCl_2$. $2H_2O$, 5.08 g $MgCl_2$. $6H_2O$, 6.16 g $MgSO_4$. $7H_2O$, 0.89 g Na_2HPO_4. $2H_2O$, 5.0 g $NaNO_3$ and 0.03 g $FeSO_4$.

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$7H_2O$. To prevent precipitation, four separate solutions were prepared and later mixed together after autoclaving when the solutions had cooled to room temperature; the first solution contained Na_2HPO_4 and $NaNO_3$ (the pH value of medium was adjusted to 7.5 by the addition of a 10% solution of NaOH), the second solution contained NaCl, KCl and $CaCl_2$, the third solution contained $MgCl_2$ and $MgSO_4$, and the fourth solution contained $FeSO_4$. Bacto agar (Difco, Fisher Scientific, Mississauga, Ontario) (15 g/L) was added to the first solution for the preparation of solid media [15]. About 3% (v/v) and 10% of sub-cultures were used to inoculate the different media which contained the same carbon source (3% (v/v) of hexadecane, 3% (v/v) of motor oil or 3% (v/v) of BTEX) as a sole source of carbon and energy. For Colony Forming Units per mL (CFU/mL), the solution was serially diluted and plated on agar medium plates which was incubated for 72 h at 30°C [23].

For enzymes recovery, the bacterial culture was centrifuged at 12,000 x g for 30 minutes at 4 °C, and the supernatant containing the extracellular enzymes was recovered and stored at -20 °C for further biodegradation experiments. *A. borkumensis* cell pellet (1 g) frozen at -20 °C was re-suspended in phosphate buffer (1 mL, 0.1 M, pH 8.0). The mixture was sonicated by using two frequencies of ultrasounds (22 kHz and 30 kHz) for 6 min at 4 °C and centrifuged at 12 000 × g for 30 min. The supernatant was used as a crude intracellular enzyme extract.

2.4 Biodegradation using the crude enzymes mixture

The performance of the extracellular and intracellular crude enzymes from *A. borkumensis* was evaluated in batch tests in Milli-Q water. The test solutions contained 50 mL Milli-Q water, 10 mg/mL of an equal mixture of intracellular and extracellular crude enzyme and the different petroleum hydrocarbons concentrations: 5000, 6000 and 7000 mg/L of hexadecane, 30 mg/L, 50 mg/L and 70 mg/L of BTEX compounds mixture (1:1:1:1:1:1), 500, 750 and 1000 mg/L of motor oil, and 2000, 4000 and 6000 mg/mL of contaminated soil. The control tests are only composed of Milli-Q water at different concentrations of the different substrates

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without the enzyme mixture. The solutions were incubated at 30 °C for 7 days with shaking at 100 rpm in an incubator shaker. All experiments were carried out in triplicates.

2.5 Sampling strategy and parameters assayed

At the beginning (T_0) and at the end (T_7) of the experimental period, sub-samples of enzymatic degradation were taken. Measures of enzymes activities (alkane hydroxylase, lipase and esterase) and concentrations of different petroleum hydrocarbons (hexadecane, BTEX and motor oil) were carried out. All experiments were performed in triplicates.

2.6 Proteins and enzymatic assays

2.6.1 Total protein assay

Both intracellular and extracellular protein concentration were determined according to the Bradford method [24], and the specific activity (units per mg of proteins) for both the intracellular and the extracellular enzymes was determined. .

2.6.2 Alkane hydroxylase assay

Alkane hydroxylase activity was measured at 25°C using a cofactor (NADPH) depletion assay to determine relative activities. The intracellular enzyme was diluted into phosphate buffer (0.1 M, pH 8), substrate (0.5-1 mM), and dimethyl sulfoxide (DMSO; 1%, vol/vol). Substrates were added to the buffer using stock solutions in DMSO. The reaction was initiated by addition of NADPH (200 µM), and the oxidation of NADPH was monitored at 340 nm (Glieder et al., 2002). One unit is defined as the amount of enzyme required for consumption of 1 µmol of NADPH per min. Hexadecane, BTEX and motor oil were used as substrates.

2.6.3 Lipaseassay

Extracellular lipase activity was performed by titrimetric method according to Lopes et al. [25] by using an olive oil emulsion composed of 25 mL of olive oil and 75 mL of 7% Arabic gum solution which was emulsified in liquefier for 2 minutes. About 5 ml of olive oil emulsion was then added to 0.1M phosphate buffer (pH 7.0) and 1 mL of the enzymatic

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suspension (10 mg/mL) and incubated at 37°C for 30 minutes under shaking. Subsequently, the emulsion was immediately disrupted by the addition of 15 mL of a mixture of acetone-ethanol (1:1 v/v). The released fatty acids were titrated with 0.05M NaOH. One unit of lipase activity was defined as the amount of enzyme which liberated 1 μmol of fatty acids per minute.

2.6.4 Esterase assay

Extracellular esterase activity was measured by titrimetric method according to Lopes et al. [25] by using olive oil as a substrate. The reaction mixture is composed of: 5 mL of olive oil, 2 mL of 0.1M phosphate buffer (pH 7.0) and 1 mL of the enzymatic extract (10 mg/mL). The mixture was incubated at 37 °C for 30 minutes under shaking and it was immediately disrupted by adding 15 mL of acetone-ethanol mixture (1:1 v/v). The released fatty acids were titrated with 0.05M NaOH. One unit of esterase activity was defined as the amount of enzyme which liberated 1 μmol of fatty acids per minute.

2.7 Gas chromatography

GC analysis of petroleum hydrocarbons biodegradation was performed using Hewlett-Packard 6890/5973 with flame ionization detector (FID). Analyses were carried out with helium as the carrier gas at a flow rate of 2 mL/min on a DB-1 column (30 m, 0.53 mm i.d., 1.0 mm film thickness). Oven temperature was programmed from 60 °C 260 °C at a rate of 4 °C/min. Split/Splitless injector and detector (FID) temperatures were 260 °C and 260 °C, respectively, and 1 mL of the sample was injected [26].

2.8 Statistical analysis

All the experiments were performed in replicates and an average of 3 replicates was calculated along with the standard deviation.

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3. Results and discussion

3.1 Dynamics of petroleum hydrocarbons degradation with crude enzymes

The present study deals with the enzymatic degradation of *A. borkumensis* which was grown in the presence of xenobiotic petroleum hydrocarbons being reported to be toxic substances, such as hexadecane, motor oil and BTEX. Overall, degrading capacities of crude enzymes produced were also tested in the presence of contaminated soil which contains a range of contaminants as described in Table 1.The bacterial strain was able to use the various tested substrates as sole carbon source and energy for its growth and proliferation which was confirmed by cell count. Fig. 1 shows that the cell number reached 4.2 x 10^8 CFU/mL and 8.4 x 10^8 CFU/mL in the case of hexadecane with 3% and 10% (v/v) inoculum, respectively. In the case of BTEX, the cell count reached 3.7 x 10^8 CFU/mL and 8.9 x10^8 CFU/mL with 3% and 10% (v/v) inoculum, respectively. And finally in the case of motor oil, the cell count was 7.1x 10^8 CFU/mL and 13 x10^8 CFU/mL with 3% and 10% (v/v) inoculum, respectively. The crude enzyme was isolated after 72 hours of bacterial growth.

Enzymatic degradation can be affected by many parameters, such as the hydrocarbons present in the contaminated site, the concentration of the pollutant, the environmental compartment in which the process is being carried out and the enzymes adaptation [27,28].. In the current study the influence of substrate concentration on the degradation rate of *A. borkumensis*, was very representative. Herein, the percentage of removal was correlated with the concentration of hydrocarbons and with the specific activity of each of the studied enzymes over time period.

Hexadecane used in this study is part of the aliphatic fraction of crude oil and it is one of the most important components of diesel [29]. This compound is present at many oil-contaminated sites and its biodegradability has been well characterized [30]. For these reasons, hexadecane is used in this study as a model molecule to study aliphatic hydrocarbon

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biodegradation since it has been always considered as a model [31]. The concentrations adopted in our research (5000 mg/L, 6000 mg/L and 7000 mg/L) were based on the concentration of C10-C50 that has been found in the characterized contaminated soil (Table S3).

The removal percentage of $C_{16}H_{34}$ decreased from 73.75% to 59.74% after 7 days of enzymatic degradation, while increasing the concentration of hexadecane from 5000 to 7000 mg/L when using 10% (v/v) of inoculum concentration. As shown in Fig. 2, the specific activity of alkane hydroxylase, lipase and esterase was quite stable after 7 days of degradation using 7000 ppm hexadecane and 10% (v/v) inoculum. This specific activity decreased from 73.80, 2000, 2320 U/mg, respectively after extraction of the crude enzymes (initial activity) to 28.57, 1628, 1781.48 U/mg, respectively after 7 days of degradation. These findings are in agreement with Maletić et al. [32] who reported that hydrocarbon degradation is ultimately dependent on their concentration. Most of the previous studies on hexadecane biodegradation have used an initial concentration lower than 1 g/L and have been carried out for up to 45 days [33,34]. Setti et al. [35] reported 86.4% of hexadecane mineralization at an initial concentration of 12 g/L by *Pseudomonas sp.* after 31 days of biodegradation. Colombo et al. [36] reported that several fungal strains were able to biodegrade up to 80% of aliphatic hydrocarbons after 90 days, using a contaminated soil with a concentration of 10% crude oil which contain 16.5 mg of aliphatic hydrocarbons per g of soil. Moreover, Volke-Sepulveda et al. [37] found that an initial concentration of 45 g/L of hexadecane was totally mineralized after 31 days of culture using a solid state fermentation.

Moreover as noticed from Fig. 2, the enzyme specific activity considerably increased while increasing the inoculum, from 48, 1642.85, and 1666.66 U/mg to 73.80, 2000 and 2320 U/mg for alkane hydroxylase, lipase and esterase, respectively. This increase in enzymes activity had a significant effect on hexadecane degradation. In fact, as observed in Fig. 2, the

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degradation percentage increased almost twice (from 37.70 to 73.75) when using 5000 mg/L of hexadecane and from 32.40 to 38.50 when using 6000 mg/L of hexadecane and also almost two time degradation increase was observed when using 7000 mg/L of hexadecane (from 28.31 to 59.74), after 7 days of degradation with enzymes produced by the inoculated *A. borkumensis*.

Peng et al. [38] has stated that BTEX mixture is the most toxic TPH component for living cells. The BTEX degradation abilities of the enzymes produced by *A. borkumensis* inoculated with 3% (v/v) and 10% (v/v), were evaluated during 3 days using a BTEX mixture containing 30 mg/L, 50 mg/L and 70 mg/L of each of the six compounds (benzene, toluene, ethylbenzene, o-xylene, m-xylene and p-xylene (1:1:1:1:1:1)) in the enzymatic preparation.

As shown in Fig. 2, the initial enzymes activity obtained after extraction from the culture broth with 3% (v/v) inoculum measured for the mixture of 70 mg/L of BTEX were 59.45, 2432.43 and 2928 U/mg for alkane hydroxylase, lipase and esterase, respectively. The enzyme activity increased to 75, 2785.71 and 3243.24 U/mg for alkane hydroxylase, lipase and esterase, respectively while increasing the inoculum concentration to 10% (v/v). Also, as shown in Fig. 3the removal percentage of benzene, ethylbenzene and o-xylene decreased from 72.22%, 74.8 %, 79.3% to 69.5%, 50.25% and 63.71%, respectively while increasing substrate concentration from 50 mg/L to 70 mg/L using 3% (v/v) inoculum concentration. However, in the presence of higher inoculum concentration (10% (v/v)), the degradation decreased from 76.48%, 71.69%, 81.2% to 71.22%, 65.45%, 74.8% respectively.. These observations were in agreement with Li et al. [39] who showed an inhibitory effect of higher benzene concentration (more than 80 mg/L) in the presence of *Planococcus* sp. strain ZD22. Similarly, Hamed et al. [40] reported that specific growth rate of *P. putida* in batch systems has been set up to be a decreasing function of benzene and toluene concentrations. BTEX

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compounds upon reaching certain concentrations can inhibit the microbes and their enzymatic activity due to complex micro- and macro-level interactions [41]. Furthermore, Mathur and Majumber [42] claimed that at higher initial concentrations (>150 mg/L benzene and >200 mg/L toluene), degradation rate was lower. The removal efficiency could also be attributed to the simple structure and the molecular composition of BTEX [43,44]. In the case of toluene and as shown in Fig. 3, about 84.22% of removal percentage was obtained at an initial concentration of 70 mg/L on day 3. Thus, toluene had been claimed as the most easily biodegradable among the six compounds of BTEX. In the current study, the mixture of different BTEX compounds (benzene, toluene, ethylbenzene and xylene) together is seen to affect one another. For example, they can interact synergistically or antagonistically as reviewed by Dou et al. [45]. Herein, the interaction between BTEX components in this study seems to be synergistic since a high removal percentage (up to 60%) is reached while using the mixture of the above contaminants in a time period of 3 days.

Other xenobiotic components were tested in the current study for their removal efficiency, such as motor oil which showed a potential degradation (Fig. 2). As mentioned earlier in this study, this compound was characterized and it is composed of (in mg/L): 69.8 C10-C50, 1.83 naphthalene, ≤ 44 benzene, ≤30 toluene, ≤44 ethyl-benzene and ≤84 xylene. Thus, *A. burkumensis* grew well on engine oil producing high crude enzymes activities which was confirmed in Fig.2 by the specific activity of the enzymes. This activity increased from 131.81 to 145.71 U/mg for alkane hydroxylase and from 3434.78 to 3628.57 U/mg for lipase and from 2043.47 to 2200 U/mg for esterase when using 1000 ppm motor oil as substrate and increasing the inoculum concentration. This increase in enzymes activity led to an increase in removal from around 74% to around 83% after 7 days of enzymatic degradation. . As discussed earlier, the biodegradation carried out by *A. burkumensis* seemed to be concentration dependent as observed with BTEX. These results are advantageous compared

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with *Pseudomonas aeruginosa* which was able to utilize 81% of used engine oil within 4 weeks compared to 7 days in the current study [46]. Likewise, Basuki et al. [47] reported the removal of 35 out of 47 components of used oil by *Acinetobacter junii* TBC 1.2. Besides, *Pseudomonas aeruginosa* LP5 degraded more than 90% of all oil types within 21 days. The incomplete degradation obtained (between 59-83%) can be further improved by extending the fermentation time to more than 7 days.

In the current study the degradation efficiency varied largely between the different compounds, hexadecane, BTEX or motor oil reflecting a complexity in the structure and chain-length of the different studied substrates. These observations substantiated the findings of Das and Chandran [48] who stated that all the mechanisms of biodegradation and the degradative enzymes produced are dependent on physical and chemical properties of hydrocarbons.

These results are advantageous compared to similar strains that presented higher degradation capacities. *Acinetobacter baumannii* isolated from crude oil exhibited 62.8% of TPH biodegradation after 7 days [49]. *Cellulosimicrobium cellulans* exhibited hydrocarbon degradability of 18.86% after 15 days [50]. Similarly, Ijah [51] reported more than 52% obtained in 16 days.

3.2 Biodegradation kinetics of contaminated soil

The crude enzyme used for the degradation of the contaminated soil was produced with *A. borkumensis* using hexadecane as sole carbon source (since it is the standard carbon source). The specific activity obtained during the degradation of 6000 ppm of petroleum hydrocarbons in soil was studied when using both 3% and 10% (v/v) inoculum. In the case of 10% (v/v) inoculum and 7 days degradation period, this specific activity decreased from 73.8 to 17.47 U/mg for alkane hydroxylase, from 2000 to 994.26 U/mg for lipase and from 2320 to 1286 U/mg for esterase and it was associated with a high removal percentage reaching 88.52%. The

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higher removal percentage of contaminated soil was observed after 7 days of culture with 64.23%, 79.59% and 88.52% of degradation calculated with an initial concentration of contaminated soil of 2000 mg/L, 4000 mg/L and 6000 mg/L, respectively. In the 3rd day, 40.67%, 54.43% and 67.29% were observed with 10% (v/v) inoculum in the case of 2000 mg/L, 4000 mg/L and 6000 mg/L of contaminated soil, respectively (Fig. 4). This rapid degradation rate on the 3rd day is likely due to the consumption of the easily degradable compounds of low molecular weight found in the soil.

Based on a first order model, degradation constant (k) and half-life ($t_{1/2}$) were determined. Table 2 presents the kinetic parameters calculated for the removal of TPH in the soil as well as the hydrocarbons consumption data (i.e. global consumption rate; maximum consumption rate). The half-life was around 5.12 days. This indicates that it would take about 10 days to achieve complete biodegradation of the carbon source by applying the tested inoculum and enzymes. According to the analysis, the estimated biodegradation in the soil reached around 64% of TPH removal. The calculated global consumption rate (GCR) was about 566.14±42.1 mg kg^{-1} d^{-1} for contaminated soil with 6170.7 mg^{-1} kg^{-1} of TPH while the maximum consumption was 363.4 mg^{-1} kg^{-1}.

Herein, the degradation rate using enzymes from *A. borkumensis* was very important compared to other reported strains. In fact, Diaz-Ramirez [52] evaluated the biodegradation of hydrocarbons using a bacterial consortium and has found around 62% of removal within 30 days. In the contaminated soil, the biotransformation of hydrocarbons was probably due to degradation of short chain compounds (low molecular weight) and medium sized alkanes. Herein, the higher content of petroleum hydrocarbons C10-C50 (6020 mg kg^{-1}) activated the capacity of biodegradation in the selected microorganism. The biodegradation pathway initially involved the degradation of short to medium chain aliphatics (C10) presented by methyl naphthalene up to 25 mg kg^{-1}. Similarly, Marquez-Rocha et al. (2001), reported the

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degradation of medium sized hydrocarbons (> C12) contained in the diesel, together with the short-chain compounds. To make the process wholesome, ecotoxicological studies should be carried out and special attention should be put on the effects of hydrocarbons on the physicochemical properties of the soil.

4.Conclusion

Crude enzyme extracted from *Alacanivorax borkumensis* showed high efficiency in terms of removal of hydrocarbons. This study confirms the alternative of using bacterial enzymes for the bioremediation of hydrocarbons. High enzymatic activity reaching 145.71 U/mg for alkane hydroxylase, 3628.57 U/mg for lipase and 2200 U/mg for esterase was obtained leading to more than 80% removal of different compounds with different concentrations namely BTEX, motor oil, hexadecane and contaminated soil, in a short period of time (3 days for BTEX and 7 days for the remaining compounds). Thus, *Alacanivorax borkumesis* derived enzymes may be used as a powerful approach for the clean-up of environments polluted with petroleum compounds in both aquatic and terrestrial ecosystem.

Acknowledgments

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Figures

Figure Captions:

Fig. 1: Colony forming units per mL of Alcanivorax borkumensis using different substrates and inoculated with (a) 3% (v/v) and (b) 10% (v/v).

Fig. 2: Specific enzyme activity and removal percentage of different petroleum hydrocarbons using the crude enzyme produced by *A. borkumensis* at different inoculum size 3% (v/v) (not scratched histograms) and 10% (v/v) (scratched histograms).

Fig. 3: Removal percentage of different BTEX compounds during 3 days using the crude enzyme produced by *A. borkumensis* at different inoculum size 3% (v/v) (not scratched histograms) and 10% (v/v) (scratched histograms).

Fig. 4: Specific enzyme activity and removal percentage of petroleum hydrocarbons in the soil using the crude enzyme produced by *A. borkumensis* at different inoculum size 3% (v/v) (not scratched histograms) and 10% (v/v) (scratched histograms).

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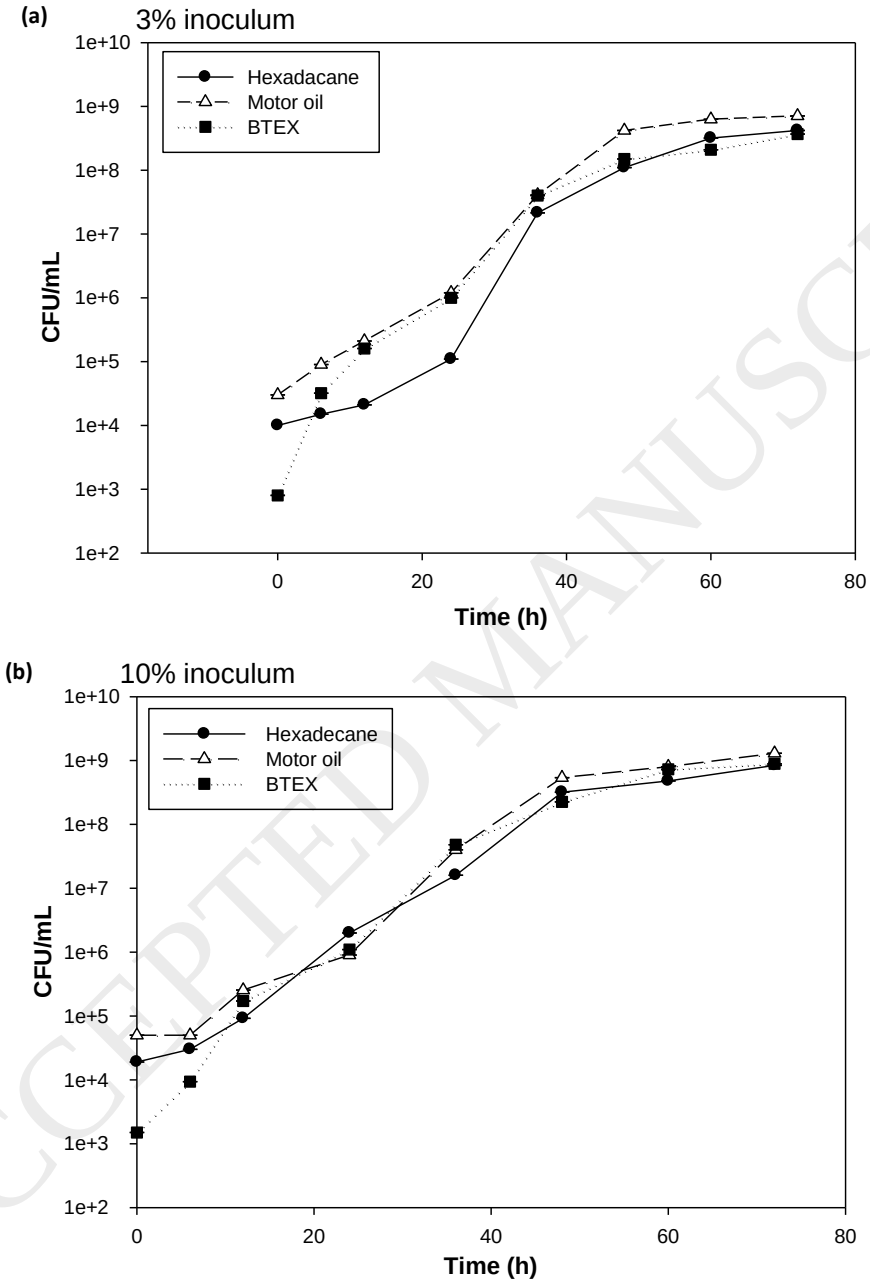

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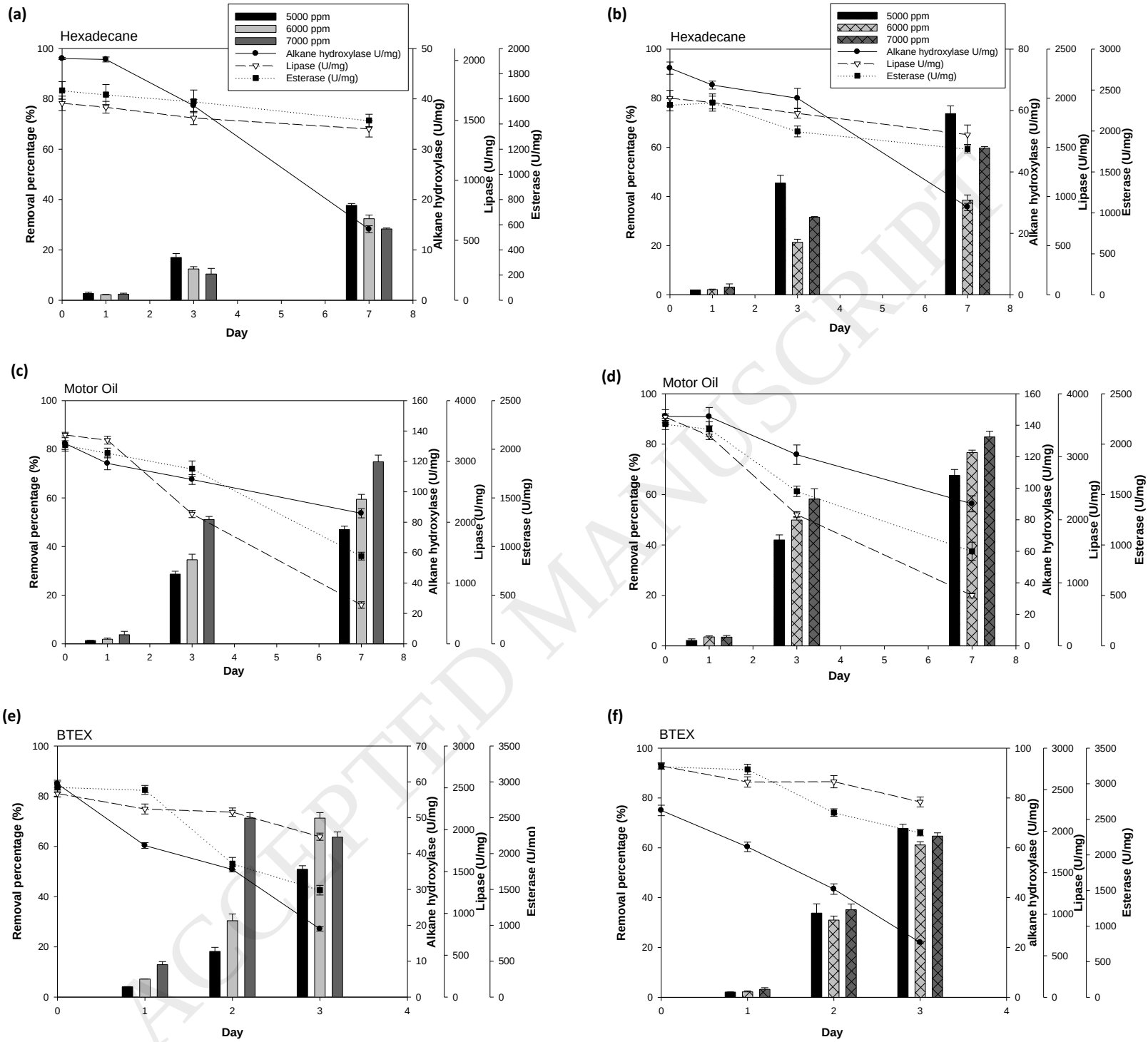

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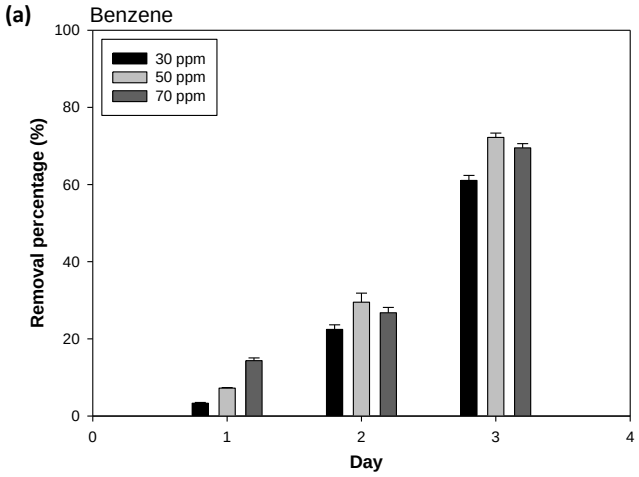

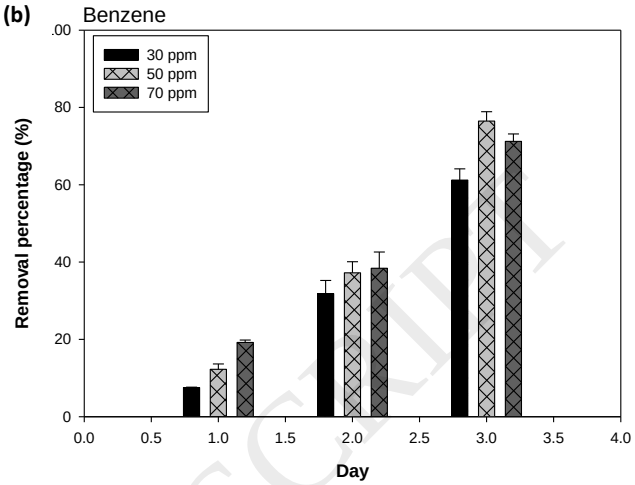

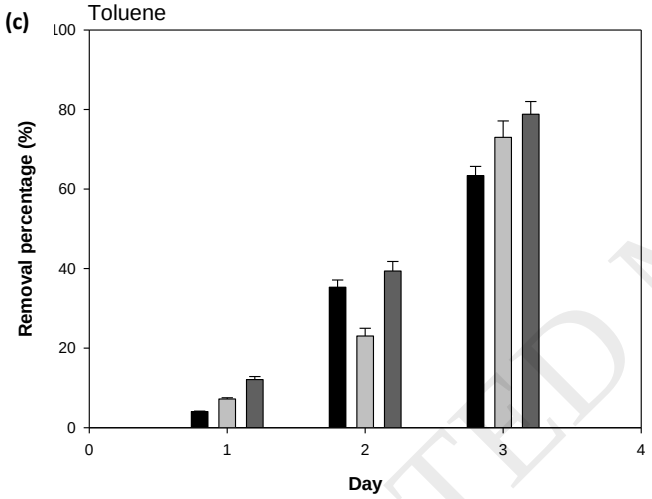

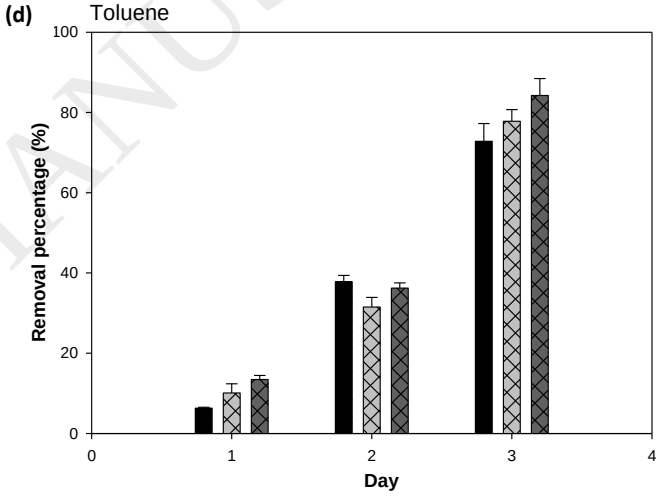

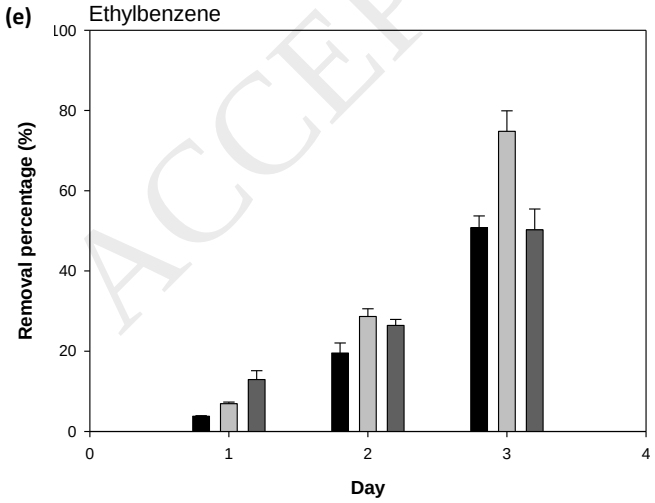

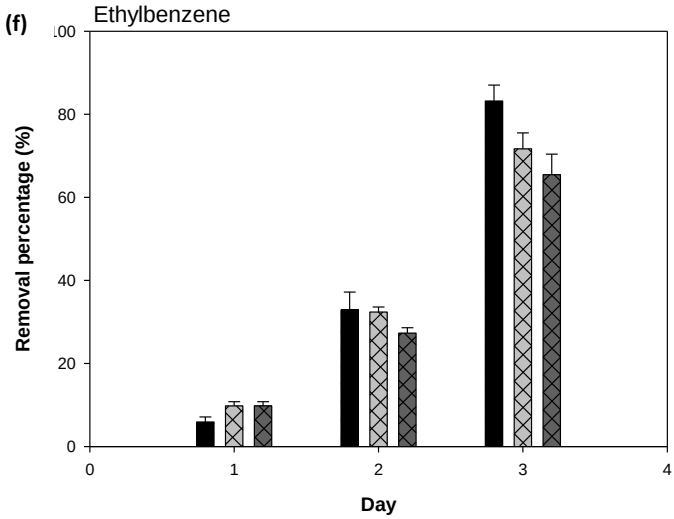

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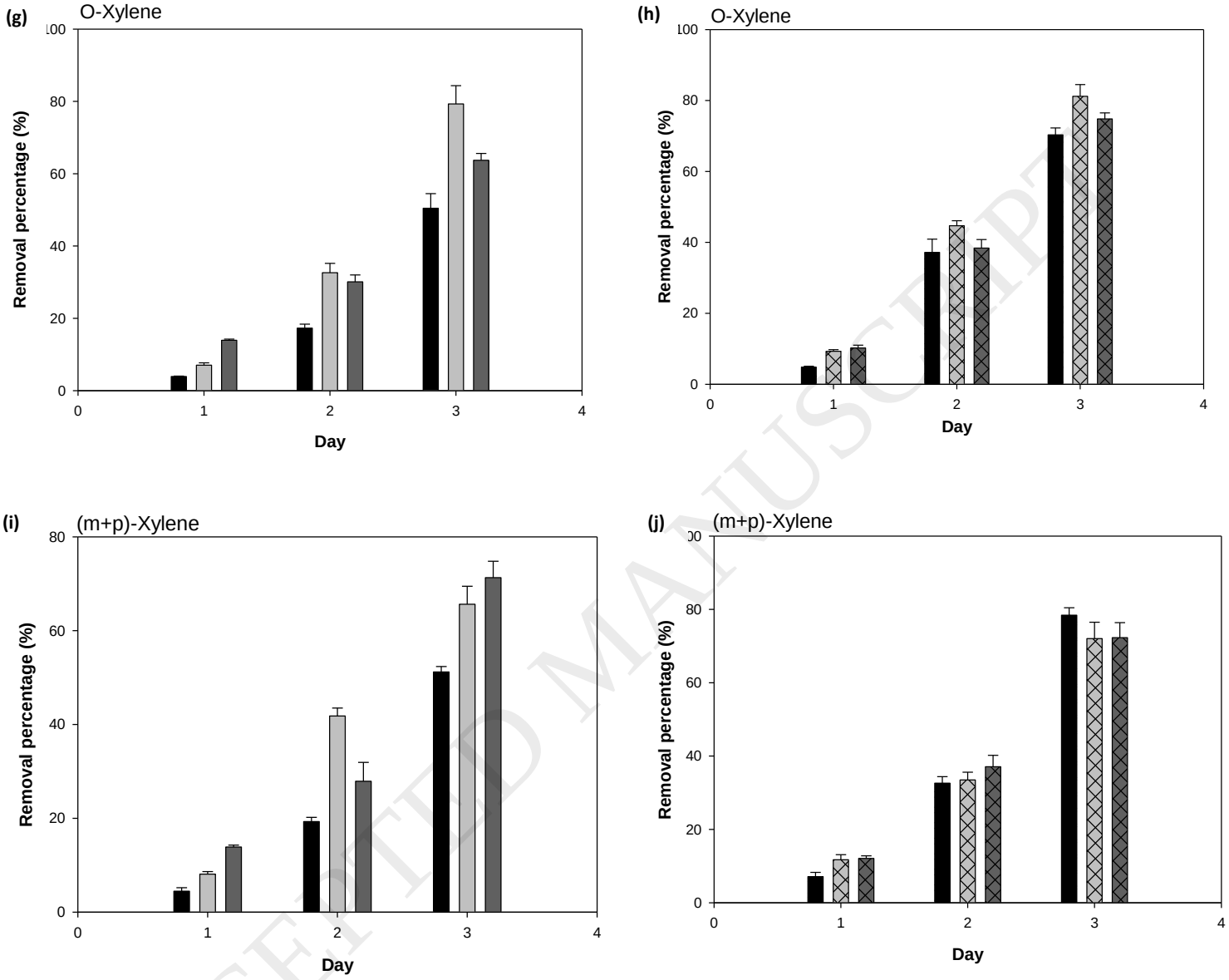

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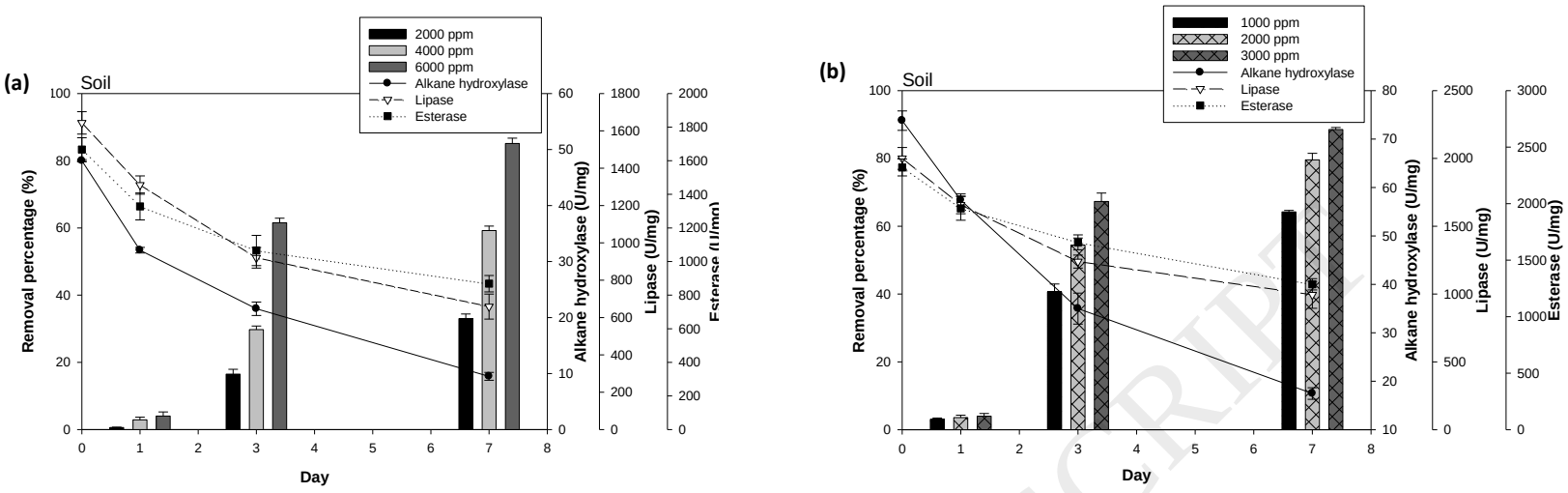

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Tables

Parameters	Units	Soil	Soluble fraction
Total solids	%	75.36±0.93	-
Moisture content	%	24.17±0.45	
pH	-	7.80±0.10	-
Density	$g.cm^{-3}$	1.15±0.02	-
Total carbon (C)	gC/100g	0.38±0.02	98.00 mg/L
Total nitrogen (N)	gN/100g	0.06±0.002	1.61 mg/L
C/N ratio	-	6.33	-
Oil content	%	13.87±0.81	-

Table 1: Soil characteristics.

Table 2: Biodegradation constant and half-life for total petroleum hydrocarbons removal in soil, after 7 days assay.

	Biodegradation (%)	**GCR [a] ($mg\ kg^{-1}\ d^{-1}$)**	**MCR [b] ($mg\ kg^{-1}\ d^{-1}$)**	**k [c] (d^{-1})**	**Half life time (day)**
Contaminated soil	64.23±0.45	566.14±42.10	363.40±32.50	0.16	5.12

[a] Global Consumption Rate (GCR) was calculated considering the initial and residual hydrocarbon content after 7 days.

[b] Maximum Consumption Rate (MCR).

[c] Biodegradation constant was calculated as:

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$$C = C_0 e^{-kt} \quad (1)$$

Where:

C= Total petroleum hydrocarbon concentration at "*t*" time

C_0= Initial concentration of petroleum hydrocarbon

k = Biodegradation constant represented as the slope of the *Ln C/C₀ vs time* (days).

[a] Global Consumption Rate (GCR) calculated as:

$$GCR = \frac{[TPH_{final}] - [TPH_{initial}]}{t_0 - t_{final}} \quad (2)$$

Where:

GCR: Global consumption rate

TPH_{final} = Total petroleum hydrocarbon concentration at the end of the assay

TPH_{inital} = Initial total petroleum hydrocarbon concentration

t_0= initial time

t_{final} = final experimental time

[b] Maximum Consumption Rate (MCR) calculated as:

$$MCR = \frac{[TPHr]_n - [TPHr]_{n-1}}{t_{n-1} - t_n} \quad (3)$$

Where:

MCR: Maximum consumption rate determined between each sampling interval

$[TPH_r]_n$= Residual total petroleum hydrocarbon concentration at *tn*

$[TPH_r]_{n-1}$= = Residual total petroleum hydrocarbon concentration at *tn*-1

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