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Bench-scale production of enzymes from the hydrocarbonoclastic bacteria *Alcanivorax borkumensis* and biodegradation tests

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Highlights

- Scaling-up enzymatic production by *Alcanivorax borkumensis* in controlled conditions in a 5L fermenter.
- High production of alkane hydroxylase and lipase.
- Using motor oil at 3% and 5% (v v^{-1}) as a substrate for growth showed better enzyme production than the standard media.
- Enzymatic biodegradation in two different PAHs contaminated groundwater showed high removal efficiency.

Abstract

This study investigates motor oil (3, 5, 7.5 and 10% (v v^{-1})) as a sole carbon source for the production of *Alcanivorax borkumensis* in shake flasks and a 5L bench-scale fermenter in comparison to the standard media. Shake flask studies showed a significant and higher cell growth (p=0.000038), lipase (p=0.006900) and alkane hydroxylase production (p=0.000921) by *Alcanivorax borkumensis* when motor oil was used as the substrate. Based on Tukey post-hoc tests, 5% motor oil concentration was selected as the optimal substrate concentration. The 5L fermenter experiments conducted using motor oil at 5% (v v^{-1}) concentration, under controlled conditions exhibited significant and higher alkane hydroxylase and lipase activities (55.6 U mL^{-1} (p=0.018418) and 208.30 U mL^{-1} (p= 0.020087), respectively) as compared with those of motor oil at 3% (v v^{-1}) and n-

hexadecane at 3% (v v^{-1}) concentration which was used as control. Cell growth was significantly higher when motor oil (3 or 5%) was used as a substrate (p= 0.024705).

Enzymatic degradation tested on two different polycyclic aromatic hydrocarbons (PAHs) contaminated groundwaters showed 37.4% removal after 5 days with a degradation rate of 196.6 ppb day^{-1} and 82.8% removal after 10 days with a degradation rate of 217.54 ppb day^{-1} for the 1st site and an almost complete biodegradation with 95% removal and 499.02 ppb day^{-1} removal rate after only 5 days for the 2nd site.

Keywords: Fermentation; *Alcanivorax borkumensis*; Alkane hydroxylase; Lipase; PAHs.

1. Introduction

Petroleum is the most cost-effective energy source in the current global economy. At the same time, this energy source is considered as the major pollutant in the marine environment. Its widespread and extensive use has caused serious ecological problems. Ecological effects involve the release of insoluble hydrocarbons that can cause severe disruption of the ecological balance and their ultimate residence on the water surface (Marchut-Mikolajczyk et al., 2015; Kadri et al., 2017). Therefore, these oil spills stimulate the growth of hydrocarbonoclastic bacteria that can use hydrocarbons as their preferred source of carbon and energy (Naing et al., 2013). In optimal conditions, these indigenous hydrocarbonoclastic bacteria are key players that consume considerable amounts of petroleum, resulting in a decline in the amount of crude oil in the affected environments (L. Wang et al., 2010; W. Wang et al., 2010; Yakimov et al., 2007).

Of particular importance, *Alcanivorax borkumensis*, a Gram-negative, aerobic, halophilic bacterium was first isolated from enriched mixed cultures, obtained from

seawater sediment samples collected near the Isle of Borkum (North Sea) (Bookstaver et al., 2015; Naing et al., 2013; Yakimov et al., 1998). This strain is an archetypal member of the hydrocarbonoclastic bacterial family that has been shown to effectively assimilate various hydrocarbons as carbon and energy source which makes it a promising bacterium for remediation (Kasai et al., 2002; Sabirova et al., 2011; Wang and Shao, 2014). In this regard, *A. borkumensis* became a potential bacterium that can be used as a reference in studies involving hydrocarbon degradation in the marine environment (Bookstaver et al., 2015; Hassanshahian et al., 2014; Naether et al., 2013; Scoma et al., 2016a, 2016b).

The use of enzymes produced from this strain is advantageous because they can perform moderate conditions (neutral pH and moderate temperature) (Ruggaber and Talley, 2006). Moreover, enzymatic treatment of soil and groundwater contaminated with hydrocarbons can be a replacement to conventional bioremediation (Gianfreda and Rao, 2004; Ruggaber and Talley, 2006). In fact, enzymes play a major role in the microbial removal of oil, fuel additives, and other recalcitrants (Van Beilen and Funhoff, 2007). Their advantage over microbial treatment includes their important reaction activity, lower sensitivity towards the concentrations of recalcitrant, coverage of a wide range of physicochemical gradients in the environmental matrix and simple management of field application. Moreover, enzymes are biodegradable, which results in small amounts or non-generation of by-products. Enzymes have the capability to mineralize a recalcitrant compound and also transform it into a state in which it is more biodegradable (Gianfreda and Rao, 2004; Wu et al., 2008).

In particular, the *A. borkumensis* alkane hydroxylase system is able to degrade a large range of alkanes up to C32 and branched aliphatics, as well as isoprenoid

hydrocarbons, alkylarenes and alkylcycloalkanes. This spectrum is much larger based on the knowledge about alkane hydroxylase complexes. Hence, studying alkane hydroxylase is of key importance (Schneiker et al., 2006). Furthermore, lipases played an important role in oily hydrocarbons biodegradation and the lipase activity has been always used as a biochemical and biological parameter for testing hydrocarbons degradation and it is also an excellent indicator to monitor the decontamination of a hydrocarbon polluted site (Mahmoud et al., 2015).

The production of enzymes at large-scale is mostly applied in batch fermentation in stirred tank bioreactors. The challenge of scaling-up is a consequence of the difficulty in estimating the different variables that influence the scale-up process during cultivation. In contrast, when factors, such as pH, temperature, dissolved oxygen (DO) and composition of raw materials are controlled, microorganisms will be more responsive which is a characteristic of scaling up (Hsu and Wu, 2002). Enzymes production is the best example because they are largely affected by media composition, substrate concentration, physical factors, such as aeration, agitation, dissolved oxygen, temperature; inoculum density and incubation time (Gupta et al., 2002). To the best of our knowledge the production of *Alcanivorax borkumensis* in controlled conditions of a bioreactor and using motor oil, which is a mixture of different hydrocarbons, as a carbon source has not been done before, and also studying the enzymes produced by this hydrocarbonoclastic bacteria in bench scale and their application on a contaminated water has not been investigated.

Hence, the principal objective of this research was to explore the crude enzymes production and measuring the alkane hydroxylase and lipase from *Alkanivorax borkumensis* in a 5L bioreactor using either n-hexadecane or motor oil as sole carbon

source with different concentrations. K_La, OUR, OTR, cell count, protein concentration, enzymes activity, were measured. Degradation tests on real contaminated waters were carried out to investigate the efficiency of the produced enzymes.

2. Materials and methods

All chemical reagents of the highest purity, such as n-hexadecane, NADPH (nicotinamide adenine dinucleotide phosphate), DMSO (Dimethyl sulfoxide) and p-nitrophenyl palmitate among others, were procured from Sigma-Aldrich, Fisher Scientific or VWR (Mississauga, Ontario, Canada). The strain, *Alcanivorax borkumensis* was ordered from DSMZ (Braunschweig, Germany).

2.1.Bacterial strain

The strain used in this study was *Alcanivorax borkumensis* SK2 (DSM 11573). *A. borkumensis* was sub-cultured and streaked on agar plates with an agar concentration of 18 g L^{-1}, incubated for 72 h at 30±1 °C and then kept at 4.0±1 °C for future use. The composition of the culture media (per liter of distilled water) is as follows: 23 g NaCl, 0.75 g KCl, 1.47 g $CaCl_2$. $2H_2O$, 5.08 g $MgCl_2$. $6H_2O$, 6.16 g $MgSO_4$. 7H2O, 0.89 g Na_2HPO_4. $2H_2O$, 5.0 g $NaNO_3$, and 0.03 g $FeSO_4$. $7H_2O$ (Yakimov et al., 1998). 3% (v v^{-1}) n-hexadecane was used as the sole carbon and energy source and considered as a control in this study. The pH value of medium was adjusted to 7.5 with 10% solution of NaOH, and the growth was conducted in a shaking incubator at 30±1 °C, 150 rpm for 72h. Motor oil at different concentrations (3%, 5%, 7.5% and 10% v v^{-1}) was also studied as a carbon and energy source.

Motor oil was characterized using Agilent 7890B gas chromatograph (GC) on a VF-5MS-FS column (0.25 mm diameter, 30 m long and 0.25 μm film thickness) coupled to an Agilent, model 5977 A. The mass spectrometer detector operated with a mass range between m/z 60 and 130. The GC column temperature was first kept at 40 °C for 4 min and then heated at a rate of 1°C min^{-1} up to 52 °C and maintained at this temperature for 18 min. The injection temperature was 40 °C. Helium was used as the carrier gas with a column flow rate of 1mL min^{-1}. The composition (in mg L^{-1}) is as follows: 69.8 of C10-C50, 1.83 of naphthalene, ≤ 44 of benzene, ≤30 of toluene, ≤ 44 of ethyl-benzene and ≤ 84 of xylene.

2.2.Inoculum preparation

A loopful of *A. borkumensis* from the agar plates was utilized to inoculate a 500 mL Erlenmeyer flask with a working volume of 100 mL. The flask was then incubated at 150 rpm and 30±1°C for 24h. A 3% (v v-1) inoculum from this flask (first stage) was then used to inoculate 500 mL Erlenmeyer flasks with 100 mL of media containing different motor oil concentrations (3%, 5%, 7.5%, 10% v v^{-1}) and 3% (v v^{-1}) n-hexadecane. The flasks were shaken and incubated for 24h. The cells from these flasks were used as inoculum (second stage or pre-culture) for the production of *A. borkumensis* in shake flasks or in the 5L fermenter.

2.3.Fermentation in Erlenmeyer flasks

Erlenmeyer flasks containing 100 mL of sterilized media with different motor oil concentrations (3%, 5%, 7.5%, 10% v v^{-1}) and with 3% (v v^{-1}) n-hexadecane and inoculated with 3% (v v^{-1}) of pre-culture were prepared, as given earlier. The flasks were incubated

in a shaker-incubator for 72 h at 30 ± 1 °C, 150 rpm. The colony forming units per mL (CFU mL^{-1}), alkane hydroxylase and lipase activity was determined as described in the following sections. Experiments were performed in triplicates. From the results obtained, the optimum motor oil concentrations with the best *A. borkumensis* growth were selected for further fermenter tests.

2.4.Fermentation procedure in 5 L computer-controlled bioreactor

In order to have an evaluation of the impacts of Carbon source on the growth of *A.bokumenss* and on the production of enzymes, the fermentation was performed in a 5L stirred tank fermenter (Biostat B plus, Sartorius Stedim Biotech, Germany). The working volume was 3L. Among other accessories, this tank fermenter is equipped with a programmable logic control (PLC) board to monitor dissolved oxygen (DO), anti-foam, pH, impeller speed, temperature, and aeration rate. The calibration of the polarographic pH-electrode (Mettler Toledo, USA) was done using two buffers of pH 4 and pH7 (VWR, Canada). Sodium thiosulfate water was used to calibrate the oxygen probe to zero while air saturated water was used to calibrate it to 100%. The calibration was done before the sterilization cycle. The anti-foaming component that was used in this study is propylene glycol (Sigma-Canada). Then, the fermenter and the medium were sterilized and cooled down. N2 gas and air were sparged to recalibrate the DO probe to zero and 100%, respectively. The agitation rate was varied from 250 to 400 rpm. The temperature was kept at 30 ± 1°C during fermentation. This was accomplished by circulating water inside the fermenter jacket. The pH value was also maintained at 7.5 ± 0.1 by pumping 4 M NaOH and 3 M H_2SO_4. During this step, the dissolved oxygen and the pH were continuously controlled by a polarographic dissolved oxygen probe and a pH sensor, respectively. Three

media were used: standard medium with 3% (v v-1) of n-hexadecane, motor oil at 3% (v v-1) and motor oil at 5% (v v-1). All the three fermenters were then inoculated with 3% (v v-1) inoculum. Sampling was carried out every 6 hours to determine the colony forming units per mL. A part of each sample, a cell-free supernatant obtained by centrifugation at 8000 rpm for 10 min and 4 °C was used to determine protein concentration. Extracellular enzymes activity of lipase was calculated using the supernatant extracted by centrifuging the culture at 8000 rpm for 10 min at a temperature of 4 °C. The pellet with the biomass was resuspended in phosphate buffer (pH 8.0) and then ultrasonicated to recover intracellular alkane hydroxylase crude enzymes as described in the following sections.

2.5.Analysis parameters

2.5.1. Determination of volumetric oxygen transfer coefficient (K_La), oxygen transfer rate (OTR) and oxygen uptake rate (OUR)

The volumetric oxygen transfer coefficient, (k_La), measurement was based on the dynamic method (Hatch, 1974; Stanbury et al., 2013), in which the air input was interrupted and, then reinstated. When the aeration was off, the dissolved oxygen decreased and when the aeration was on, the dissolved oxygen increased. All these values were recorded. The k_La was calculated from the mass balance on DO, immediately after each sampling of the fermentation broth. During the batch fermentation, the mass balance for the DO concentrations can be expressed as follows:

$$\frac{dC_L}{dt} = OTR - OUR \quad (1)$$

Where dC_L/dt is the accumulation oxygen rate in the liquid phase

OTR is the oxygen transfer rate from the gas phase to the liquid phase:

$$OTR = k_L a(C^* - C_L) \quad (2)$$

OUR is the oxygen uptake rate by the microorganism:

$$OUR = Q_{O_2} X \quad (3)$$

$K_L a$ is the volumetric oxygen transfer coefficient

C* is the oxygen concentration at saturation

C_L is the dissolved oxygen concentration in the medium

Q_{O2} is the specific oxygen uptake rate of the microorganism employed

X is the biomass concentration

The DO electrode in the medium was calibrated at 30±1 °C and then shifted to air-saturated distilled water at a known temperature and ambient pressure to transform the concentration of oxygen in the broth from percentage air saturation to mmol O_2 L^{-1}. This reading was used with the known saturation concentration of oxygen at saturation in distilled water (0.07559 mmol L^{-1}) (100%), to determine the saturation concentration of oxygen in the media at 30 °C.

2.6.Total cell count

The viable cells count was determined by counting colonies grown on agar medium with different dilutions. For all counts, the average of triplicate plates was used for each tested dilution and the results were expressed as colony forming units per mL (CFU mL^{-1}).

2.7.Total protein and enzyme assays

2.7.1. Total protein assay

Total protein concentration was determined according to the Bradford method (Bradford, 1976).

2.7.2. Sonication

A. borkumensis cell pellet was suspended in 1 mL of 0.1 M of phosphate buffer, pH 8.0. Two different ultrasound frequencies of 22 kHz and 30 kHz were used to sonicate the mixture for 6 min (the 30s off and 30s on) at 4±1 °C. The mixture was then centrifuged at 13 000 × g for 20 min to extract the intracellular crude enzyme.

2.7.3. Alkane hydroxylase assay

Alkane hydroxylase activity was measured by determining NADPH depletion. The assay mixture was composed of the supernatant containing the enzyme, the phosphate buffer (0.1 M, pH 8), the alkane substrate (0.5-1 mM), and the dimethyl sulfoxide (DMSO; 1%, vol/vol). Alkanes were added to the buffer using alkane stock solutions in DMSO. The reaction starts by adding NADPH (200 µM), and the oxidation of NADPH was estimated spectrophotometrically at 340 nm (Glieder et al., 2002). One unit of enzyme activity is defined as the amount of enzyme required for consumption of 1 µmol of NADPH per min. The alkane hydroxylase substrate used was n-hexadecane.

2.7.4. Exolipase Assay

Lipase activity was evaluated by spectrophotometer using p-nitrophenyl palmitate as the substrate (M. Thoner, Diploma-Thesis, University of Bochum, Bochum, Germany, 1973). The substrate solution was prepared as follows: 30 mg of p-nitrophenyl palmitate diluted in 10 mL of isopropanol was mixed with a solution composed of 90 mL of 0.05 M Sorensen phosphate buffer, pH 8.0, 207 mg of sodium deoxycholate and 100 mg of gum Arabic. 2.4 –mL of this pre-warmed substrate solution (37 °C) was mixed with 0.1 mL of the enzyme. After 15 min of incubation at 37 °C, the enzyme activity was evaluated at 410

nm against an enzyme-free control. One unit of enzyme activity is defined as the amount of enzyme needed to liberate 1 nmol of p-nitrophenol per minute.

2.8.Evaluation of PAHs biodegradation capacity and kinetics

2.8.1. Biodegradation

To test the efficiency of the crude enzyme produced by *Alcanivorax borkumensis* grown in the 5L fermenter using 5% (v v^{-1}) of motor oil as a substrate, the lyophilized extracellular and intracellular crude enzyme (which was stored at -20 °C) was used at a concentration of 1 mg mL^{-1} (this concentration is a 50-50 mixture of the extracellular and intracellular enzyme) for the degradation of groundwater (GW) contaminated with petroleum hydrocarbons which was procured from TechnoRem Inc. from two different confidential sites located near to Montreal city (Quebec, Canada). The initial composition of the contaminated water is presented in Table 2. Degradation tests were performed in hermetically sealed 1L glass bottles, in order to avoid evaporation, and they were incubated in an incubator shaker at 30°C and 100 rpm. Samples were taken after 5 and 10 days for degradation analyses. GC-MS was used to quantify the PAH compounds before and after enzymatic degradation using the method MA. 400 – HAP 1.1.

2.8.2. Kinetic study

The rate of the total PAHs degradation was determined using first-order kinetic model (Eq (1)). This choice was based on some previous kinetic studies on biodegradation of petroleum hydrocarbons (Abbassi and Shquirat, 2008).

$$S = S_0\, e^{-kt} \qquad Eq\ (1)$$

Applying the natural logarithm of Eq (1):

$$Ln\frac{S}{S_0} = -kt \qquad Eq\ (2)$$

Where:

S_0: initial PAHs concentration,

S: final PAHs concentration,

k: specific degradation rate constant,

t: degradation time.

2.9.Statistical analysis

All the experiments were performed in triplicates. Data presented are the mean values with standard deviation (±SD). To evaluate the statistical significance of the measured values of cell growth and enzymes production in shake flask and in the fermenter, ANOVA test has been carried out with 95% confidence level using Statistica, version 7.0 (StatSoft, USA) and the results which have $p < 0.05$ were considered as significant. Also, Tukey HSD test has been implemented for the multiple comparisons to emphasize the results obtained using ANOVA.

3. Results and Discussion

3.1.Shake flask optimization

The fermentation experiment at different motor oil concentrations (3%, 5%, 7.5% and 10% (v v^{-1}) was conducted in shake flasks using an inoculum volume of pre-culture at 3% (v v^{-1}) and the results of cell count, alkane hydroxylase activity and lipase activity at the end of fermentation are presented in Figure 1. The control in this experiment was the standard medium. The results showed a significant increase in the total cell count (p=0.000038) and in the production of lipase (p=0.006900) and alkane hydroxylase

(p=0.000921) when motor oil is used as a substrate in comparison to the standard medium. In contrast, total protein and protease production were not affected by the increase in motor oil concentration (p=0.115 and p=0.2128, respectively).

For the cell count, the increase was approximately uniform with approximately 1.5 times more colony forming units per mL than the control when the concentration of motor oil increased from 3% to 5% (v v^{-1}). Further increase in motor oil concentrations (from 7.5% to 10% v v^{-1}) generated a flat growth with 1.05 and 1.02 increase in total cell count when increasing the concentration of motor oil from 5% to 7.5% (v v^{-1}) and from 7.5% to 10% (v v^{-1}), respectively.

Furthermore, Tukey post-hoc analysis showed that the highest significant cell growth was observed with 5, 7.5 and 10% motor oil and that no significant difference was observed between the three different motor oil concentrations.

Mishra and Singh, (2012) studied the growth of three different hydrocarbonoclastic bacteria (*P. aeruginosa* PSA5, *O. intermedium* P2 and *Rhodococcus* sp. NJ2) using 1% (v v^{-1}) n-hexadecane and found that the duplication started immediately after incubation with an exponential phase achieved between day 2 and day 4. The authors also observed that the degradation of n-hexadecane was lower when growing *Rhodococcus* sp. NJ2 in comparison with *P. aeruginosa* PSA5 and *O. intermedium* P2, despite a higher bacterial growth,.

Chebbi et al., (2017) demonstrated that the newly isolated *Pseudomonas* sp. strain from motor oil-contaminated soil was able to use both motor oil and n-hexadecane, among other hydrocarbons as a sole carbon and energy source.

As a result of the significant increase in cell growth, considerable increases in enzymes activities were noticed when increasing the concentration of motor oil. For example, when the concentration of the added motor oil increased, alkane hydroxylase activity increased in parallel from 10.3±0.5 U mL^{-1} (3% motor oil) to 21.7±1.2 U mL^{-1} (5% motor oil). For lipase, the enzymatic activity increased from 47±2.6 U mL^{-1} to 59±3.4 U mL^{-1}, when the motor oil concentration increased from 3 to 5 %, respectively.. When the motor oil concentration increased to 7.5 and 10%, the lipase activity increased to 65 and 66.7 U mL^{-1}, respectively. Tukey post-hoc analysis showed that the highest significant alkane hydroxylase and lipase production was observed with 5, 7.5 and 10% motor oil and that no significant difference was observed between the three different motor oil concentrations.

These results can be explained by the increase in viscosity and a relative increase in the degree of heterogeneity at a higher motor oil concentration which may cause limitation of oxygen transfer, especially in shake flasks, causing a slowdown in *Alcanivorax borkumensis* growth, and later enzymes production. Increase in osmotic pressure across the bacterial membrane due to increased motor oil concentration could also contribute towards inhibition of growth, and enzymatic production (Dang Vu et al., 2009).

Consequently, motor oil concentration in the range of 3% to 5% (v v^{-1}) is the most advantageous for *A. borkumenis* growth and synthesis of enzymes. At higher concentration, the media became less advantageous for microbial growth and enzymes synthesis. This result will be taken into account for the next work when choosing substrates to study the fermentation of *A. borkumensis* in a 5L bioreactor.

3.2.Fermentation parameters

Profiles of fermentation parameters, namely, dissolved oxygen concentration DO (% saturation), agitation (rpm), aeration (L min^{-1}), volumetric oxygen transfer coefficient (k_La), oxygen uptake rate (OUR) and oxygen transfer rate (OTR) for the three different media: standard media, motor oil 3% v v^{-1} and motor oil 5% v v^{-1} (which is the optimal concentration obtained from flask experiments based on cell growth, alkane hydroxylase and lipase production). Results are presented in Figure 2 and the maximum values of these studied parameters are presented in Table 1, with constant values of pH and temperature (7.5 and 30 °C, respectively). Determination of these environmental variables is important in scale-up during fermentation (Hsu and Wu, 2002). Agitation rate between 250-500 rpm and air flow rate between 2.5-3.5 L min^{-1} were varied to control the DO value and maintain it above the critical required level (>25% saturation based on previously reported works). During the first stage of fermentation (from 0–42 h depending on the media), a decrease in the DO values was detected. Therefore, a higher agitation from 250 to 500 rpm and a higher aeration rate reaching 3.5 L min^{-1} was adopted to maintain high dissolved oxygen greater than 60%. This decrease was followed by an increase, until the end of the fermentation. Thus, the agitation and the aeration were preserved at the same level. The values of oxygen uptake rate (OUR), oxygen transfer rate (OTR) and oxygen transfer coefficient (k_La) are presented in Figure 2.

The decrease in DO observed is in coincidence with the exponential growth phase of *A. borkumensis* which required higher OUR. On the other side, the increase in DO and decrease in OUR is the consequence of the stationary growth phase reached by *A. borkumensis* at a lower oxygen rate.

The maximum values of k_La observed for different media were in the following order: 187.07 h^{-1} (Motor oil 5% v v^{-1}) > 187 h^{-1} (n-hexadecane 3% v v^{-1}) > 167.82 h^{-1} (motor oil 3% v v^{-1}) (Table 1). These variations in k_La values depend on several factors, namely aeration and agitation degree, rheological properties of the media and the concentration of antifoam (Stanbury et al., 2013).

Figure 2 shows that agitation speed, in the case of 3% v v^{-1} n-hexadecane and 3% v v^{-1} motor oil were almost the same with a slight difference and less than that of 5% v v^{-1} motor oil. Nevertheless, k_La values for the three different media were very close. The possible reason for this could be attributed to different rheological characteristics of the three media (Dang Vu et al., 2009).

3.3.*Alcanivorax borkumensis* growth and enzyme production during fermentation

Scale-up studies are important to establish the enzymes production ability to be used later for bioremediation before considering it for deployment in actual contaminated sites (Priya et al., 2015). Moreover, the role of enzymes as biocatalysts in degrading and detoxifying contaminants is substantial (Sharma et al., 2014). Therefore, determining enzyme activity is crucial and can be used as an indicator of the activity and metabolism in a petroleum-contaminated soil. Thus, through this study, the enzyme activity of the intracellular alkane hydroxylase which is an enzyme that oxidizes alkanes and enable microorganisms to use hydrocarbons as a source of carbon and energy (Glieder et al., 2002) and also the extracellular lipase, which degrades lipids in glycerol and fatty acid (Jaeger et al., 1994) was established.

Profiles of colonies forming units per mL (CFU mL^{-1}), during growth of *A. borkumensis* in different media are presented in Figure 3 and the maximum values of

growth parameters (maximum cell counts and maximum specific growth rates) are mentioned in Table 1. And, protein concentration, alkane hydroxylase activity, and lipase activity during the fermentation of the three media were determined and their profiles, as well as their maximum values, are presented in Figure 4 and Table 1, respectively.

Results showed that with 3 and 5% (v v^{-1}) of motor oil concentration a significant increase in the cell growth (p=0.024705) was observed as compared to the standard media with increasing turbidity. At the end of fermentation (starting from 48h), total cells reached the same values for both concentrations of motor oil. The cell growth started immediately after inoculation of the bioreactor reaching its maximum value at 60 h for both media with motor oil (2.9 $x10^{10}$ and $3x10^{10}$ CFU mL^{-1}) and at 72 h for the media with n-hexadecane ($7.1x10^{9}$). These results were confirmed with Tukey post-hoc analysis where the media containing 3% and 5% (v v^{-1}) of motor oil were statistically comparable in term of cell growth and the values were significantly higher than the standard media (p 0.05).

The total cells of the media with motor oil were followed by a decelerating growth phase and subsequently, the culture entered stationary phase. Nevertheless, maximum alkane hydroxylase and moreover maximum lipase activities were secreted during the decelerating growth phase and subsequent stationary phase signifying non-growth associated production phase. Liu et al., (2014) studied the growth of *Acinetobacter* sp. LS-1 on hexadecane and found that the utilization of hexadecane was closely related to the strain's growth with the increasing cell turbidity. The growth of this strain was very slow until 12 h incubation with only 5% hexadecane degradation, and then the log phase continued until 24 h, the stationary phase until 30 h with eventual decline phase. The same

strain LS-1 was grown with 1% crude oil for one week reaching a cell turbidity of 0.35 OD_{600}.

For the lipase and alkane hydroxylase enzymes, the increase in concentration of motor oil from 3% (v v^{-1}) to 5% (v v^{-1}) caused an increase in the amount of available nutrients, which induced synthesis of the enzymes to convert these substrates into easily consumable nutrients for *A. borkumensis* growth and synthesis and for degradation of petroleum hydrocarbons. The lipase and alkane hydroxylase production increased significantly with the use of motor oil (p=0.018418 and 0.020087) for lipase and alkane hydroxylase, respectively). The secretion of lipase and alkane hydroxylase was observed only after 3 h for both motor oil and standard medium. This relative delay might be due to a requirement of a critical cell mass level for starting enzymes synthesis. From Figure 4, It can be clearly observed that alkane hydroxylase and lipase activities increased with an increase in cell mass and seemed to be growth associated. In fact, Sharma et al., (2014) have claimed that if the changes in bacterial population had the same pattern as the soil enzyme activities over the experience, this reflects the role of these microbiological properties in the bioremediation of diesel-contaminated soils.

Tukey post-hoc analysis showed that lipase production in 3 % and 5% (v v^{-1}) of motor oil were statistically comparable (*p=0.588790)*. For the alkane hydroxylase production, the highest significant production was achieved with media containing 5% motor oil in comparison to 3% motor oil (p=0.036746) and the standard media (p=0.018821*)*.

A maximum of 347 µg mL^{-1} of protein was observed with 3% (v v^{-1}) of motor oil after 72h. Mishra and Singh, (2012) observed similar protein concentration range between

0.11 and 0.65 mg mL^{-1} with the strains, *Pseudomonas aeruginosa* PSA5, *Ochrobactrum intermedium* P2 and *Rhodococcus* sp. NJ2 when using n-hexadecane as a carbon source. The protein concentration started to decrease after 72h from 295.8 to 272.8 µg mL^{-1} in the case of 5% motor oil, but continued increasing when using the standard media and 3% motor oil. Similar behavior was also noticed in the case of bacterial strain, *O. intermedium* P2 which showed a continuous increase in protein content till 8th day of incubation and it was not growth associated (Mishra and Singh, 2012). The protease production was not affected by the increase in motor oil concentration (p=0.0648) as was observed in shake flasks experiments.

For hydrocarbons degradation using *Alcanivorax borkumensis*, two degradative enzymes are mainly involved viz. alkane hydroxylase and lipase. Therefore, their production profile during *A. borkumensis* growth was investigated to understand their role in the biodegradation. In this study alkane hydroxylase and lipase activities exhibited different pattern during the incubation period and this can be explained by the simple reason that not all enzymes are produced by a cell in the same amounts and some are produced more than the others. When these enzymes will be applied for soil decontamination, it will be difficult to distinguish since both intracellular and extracellular enzyme contributes to the overall process (Andreoni et al., 2004).

The alkane hydroxylase was induced during both n-hexadecane and motor oil degradation, but at different incubation periods reaching the maximum between 60 and 72h. The highest alkane hydroxylase production was observed with 5% motor oil (55.6 U mL^{-1}). Several other strains have been reported in the literature to produce alkane hydroxylase with a genetically well characterized enzymatic complex, such as

Rhodococcus sp., *Pseudomonas* sp and *Acinetobacter* sp. (Wentzel et al., 2007). Mishra and Singh, (2012) demonstrated the induction of alkane hydroxylase production by *P. aeruginosa* PSA5, *O. intermedium* P2 and *Rhodococcus* sp. NJ2 when using n-hexadecane but at different incubation times ranging from 2 to 8 days.

The highest lipase production was induced by the usage of motor oil rather than n-hexadecane with 208 U mL^{-1} obtained fro 3% motor oil and 236 U mL^{-1} with 5% motor oil after 72h incubation. Comparable results were found by Mishra and Singh, (2012), who reported the least induction of lipase enzyme compared to other degradative enzymes during the degradation of n-hexadecane with three different bacterial strains (*O. intermedium P2, P. aeruginosa PSA5, Rhodococcus sp. NJ2*). Kanwar et al., (2002) used different n-alkane substrates as a carbon source for the growth of *Pseudomonas* sp. G6 and reported a maximum of only 25 U mL^{-1} of lipase using 2 % (v v^{-1}) n-hexadecane.

3.4.Biodegradation tests

To evaluate the efficiency of the produced enzymes, two different groundwaters contaminated with PAHs were used and the PAHs composition was analyzed before and after enzymatic degradation in 5 and 10 days. Results presented in Table 2 showed that for the 1st site 37.4% of total PAHs were removed after 5 days with a degradation rate of 196.6 ppb day^{-1} and that 82.8% were removed after 10 days with a degradation rate of 217.54 ppb day^{-1}. Removal of anthracene, naphthalene, phenanthrene, and pyrene were 77.55%, 86.58%, 81.78% and 98.52%, respectively after 10 days of enzymatic degradation. The removal of PAHs was also determined for the 2nd site contaminated groundwater. Enzymatic biodegradation was almost complete with 95% removal and 499.02 ppb day^{-1} removal rate after only 5 days and a slight further removal after 10 days with a slower

degradation rate (95.1% and 249.81 ppb day^{-1}, respectively). Results presented in Table 3 showed the first-order kinetic model using the linear regression. For the 1st site, about 10 days biodegradation had the highest k value (0.176 day^{-1}) compared to the 5 days treatment (0.093 day^{-1}). Moreover, for the 2nd site k values were higher (0.598 and 0.301 $days^{-1}$ for the treatment of 5 and 10 days, respectively). These observations demonstrate that 10 days treatment is more efficient than 5 days treatment.

Comparable results were also reported by previous studies. Malik and Ahmed, (2012) studied the removal of petroleum hydrocarbons using a bacterial consortium and found a 51 to 68% degradation efficiency on polyaromatic fractions (anthracene, naphthalene, phenanthrene and pyrene) with a total concentration of 14784 ppb after 24 days (Malik and Ahmed, 2012). Also, Patel et al., (2016) used the strain *Anabaena fertilissima* for 16 days and found a removal of 46% for anthracene and 33% for pyrene, at concentrations of 5 mg L^{-1} and 3 mg L^{-1}, respectively (Patel et al., 2016). Obayori et al., (2014) studied the degradation of two different grades of engine oil namely SAE 40W and SAE 20W 50 by *Pseudomonas aeruginosa* LP5 and observed higher degradation rate in the first 12 days than the last 9 days. The values for fresh SAE 40W, used SAE 40W, fresh SAE 20W-50 and used SAE 20W-50 were 177.42, 75.23, 207.14 and 74.37 (mg L^{-1} d^{-1}) respectively in the first 12 days. The percentage of removal in this period of time ranged between 92% and 96% (Obayori et al., 2014). Basuki et al., (2015) reported the removal of 35 components out of 47 components of oil by *Acinetobacter junii* TBC 1.2 (Basuki et al., 2015).

Studies on other similar strains, such as *Pseudomonas* sp. have shown that the presence of mono- and dioxygenases enzymes allows the oxidative degradation of some aromatic

hydrocarbons, such as phenanthrene through two possible degradation pathways: salicylate or protocatechuate (Chebbi et al., 2017). These pathways share the same common upper route and are initiated by the double hydroxylation of the aromatic ring. However, further studies are needed to explore the mechanisms and modes of action of this promising bacterial strain and the produced enzymes.

4. Conclusion

Scaling-up enzymes production in optimized conditions and inexpensive media is crucial to commercializing the process. The present study was an attempt in this direction. Alkane hydroxylase and lipase production pattern varied with the type of the carbon source as well as its concentration and diverse petroleum sources could be used by the organism as a sole carbon source for enzyme production. The enzymes production was scaled-up to 5 L fermenter using 3% and 5% (v v^{-1}) motor oil and 3% (v v^{-1}) n-hexadecane as control. The agitation speed, as well as the aeration, influenced the extent of mixing in the bioreactor and the nutrients availability and resulted in high yields of alkane hydroxylase and lipase activities. Enzymatic degradation tested on two different PAHs contaminated groundwaters showed 82.8% removal after only 10 days with a degradation rate of 217.54 ppb day^{-1} for the 1^{st} site and 95% removal was observed after only 5 days for the 2^{nd} site. Thus *Alcanivorax borkumensis* showed the potential for use in petroleum-contaminated areas. Further studies on the formulation of these produced enzymes need to be made for further applied technique.

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Figure captions

Figure 1. Effects of different motor oil concentration (% v v^{-1}) on the growth of *Alcanivorax borkumensis* and production of alkane hydroxylase and lipase.
Figure 2. Fermentation parameters of (a) 3% (v v^{-1}) n-Hexadecane, (b) 3% (v v^{-1}) Motor oil and (c) 5% (v v^{-1}) Motor oil.
Figure 3. Profiles of total cell count (CFU mL^{-1}) during fermentation of *A. borkumensis* using 3% n-hexadecane and 3% motor oil and 5% motor oil.
Figure 4. Profiles of protein concentration, alkane hydroxylase activity and lipase activity during fermentation using (a) 3% (v v^{-1}) n-Hexadecane, (b) 3% (v v^{-1}) Motor oil and; (c) 5% (v v^{-1}) Motor oil.

Parameters	3% (v v^{-1}) n-Hexadecane	3% (v v^{-1}) Motor Oil	5% (v v^{-1}) Motor Oil
Max. k_La (h^{-1})*	187±4.40 (40h)	167.82±7.35 (62h)	187.09±10.93 (55h)
Max. OTR (mmol O_2 L^{-1} h^{-1})*	0.87±0.06	0.78±0.06	0.87±0.09
Max. OUR (mmol O_2 L^{-1} h^{-1})*	0.16±0.01	0.43±0.05	0.21±0.01
Max. specific growth rate (μmax h^{-1})*	0.29±0.02	0.1236±0.01	1.4746±0.09
Max. total cell count (× 10^{10} CFU mL^{-1})**	0.71±0.08(72h)	2.90±0.39(60h)	3±0.47(60h)
Max. protein concentration (μg mL^{-1})**	154.60±6.30	347.60±12.30	295.80±12.20
Max. alkane hydroxylase activity (U mL^{-1})**	30.10±2.10	42.95±3.20	55.6±3.80
Max. lipase activity (U mL^{-1})**	107.33±4.50	236.48±21.50	208.30±14.00

Table 1. Maximum values of fermentation process parameters in different media.

*The presented values are the mean values obtained from two separate experiments conducted for each fermentation condition.

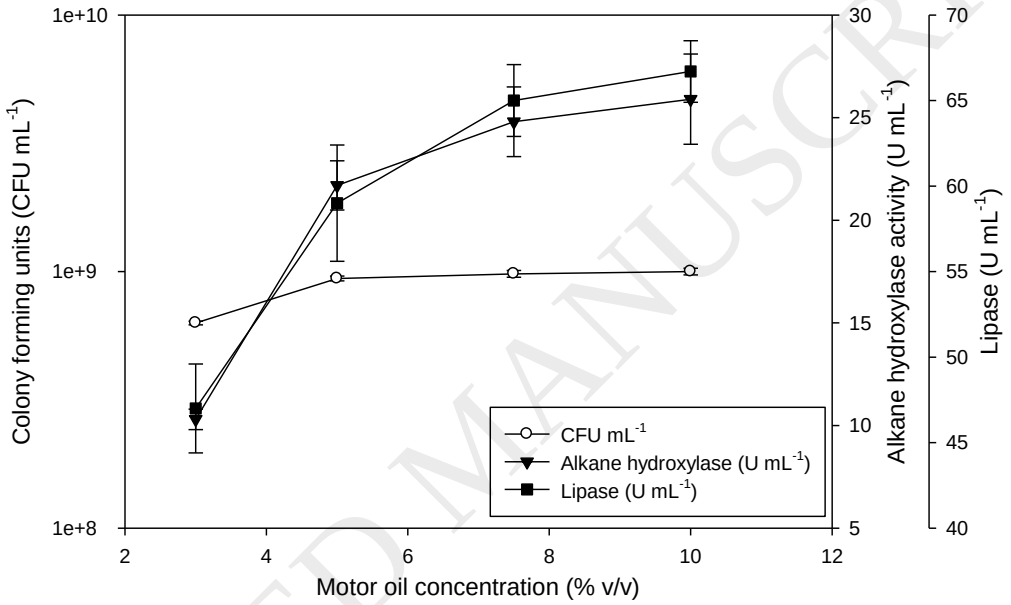
**The values are the mean of three determinations of two separate experiments conducted for each fermentation condition. The presented values are the mean ± SD. Different letters within the same row indicate the significant differences among these values determined by one-factor analysis of variance (Turkey HSD test, p ≤D0.05).

Table 2. Biodegradation of Contaminated Ground Water with produced enzymes.

Compounds	**Initial concentration 1st site (ppb)**	**Enzymatic treatment**		**Initial concentration 2nd site (ppb)**	**Enzymatic treatment**	
		After 5 days (ppb)	*After 10 days (ppb)*		*After 5 days (ppb)*	*After 10 days (ppb)*
Acenaphtene	76.0	30.2	11.8	32.9	18.5	5.9
Acenaphthylene	---	---	37.4	---	---	8.0
Anthracene	29.4	13.7	6.6	7.96	7.5	7.6
Benzo(a)anthracene	13.0	11.1	10.5	<7.50	1.30	4.0
Benzo (b) fluoranthene	<22.4	<19.8	<22.0	<22.40	<3.70	<10.10
Benzo(j)fluoranthene	<10.7	<10.7	<10.0	<10.70	<0.60	<3.90
Benzo [k] fluoranthene	<8.2	<8.0	<8.4	<8.20	<2.60	<7.0
Benzo (c) phenanthrene	---	14.4	7.1	---	<0.40	<0.80
Benzo (g,h,i) peylene	<8.9	4.6	4.4	<8.90	0.70	2.7
Benzo(a)pyrene	<7.15	6.5	4.3	<7.15	<1.0	6.0
Benzo(e)pyrene	<6.4	6.7	5.9	<6.40	<0.90	<7.30
Chrysene	11.2	8.3	5.3	<8.55	1.10	3.7
Dibenzo (a,h)anthracene	<8.9	<8.0	4.9	1.61	<0.65	0.8
Dibenzo (a,h) pyrene	---	---	1.0	---	<0.25	<0.30
Dibenzo (a,i) pyrene	<18.5	8.9	3.8	<18.15	<0.90	<1.05
Dibenzo (a,l) pyrene	---	---	<1.9	---	<0.55	<0.60
Dimethyl-1,3 naphtalene	---	---	---	---	---	---
Dimethyl-7,12benzo(a)anthracene	---	---	---	---	<0.30	---
Fluoranthene	64.9	45.1	5.0	52.2	8.8	14.0
Fluorene	93.2	40.4	17.2	20.3	25.0	6.7
Indeno(1,2,3-cd)pyrene	<7.15	6.4	5.4	<7.15	<0.75	3.0
Naphtalene	2170	1320	291	786	37.6	49.4
Phenanthrene	146	76.1	26.6	38.6	25.9	3.1
Pyrene	33.4	31.9	3.5	<8.55	5.6	14.1
Trimethyl-2,3,5 naphtalene	---	---	---	---	---	---
Methyl-1 naphtalene	---	---	---	---	---	---
Methyl-2 naphtalene	---	---	---	---	---	---
Methyl-3 chloranthrene	---	---	---	---	---	<0.30
Σ PAHs	**2627.1**	**1644.1**	**451.7**	**939.57**	**132**	**129**
Removal percentage PAHs (%)		**37.4**	**82.8**	**64.2**	**95.0**	**95.1**

Table 3. Specific degradation rate constant (k) and correlation coefficient (R^2) during enzymatic degradation of PAHs.

Enzymatic treatment 1st site	k (day^{-1})	R^2	Enzymatic treatment 2nd site	k (day^{-1})	R^2
5 days	0.093	0.932	5 days	0.598	0.949
10 days	0.176	0.981	10 days	0.301	0.915



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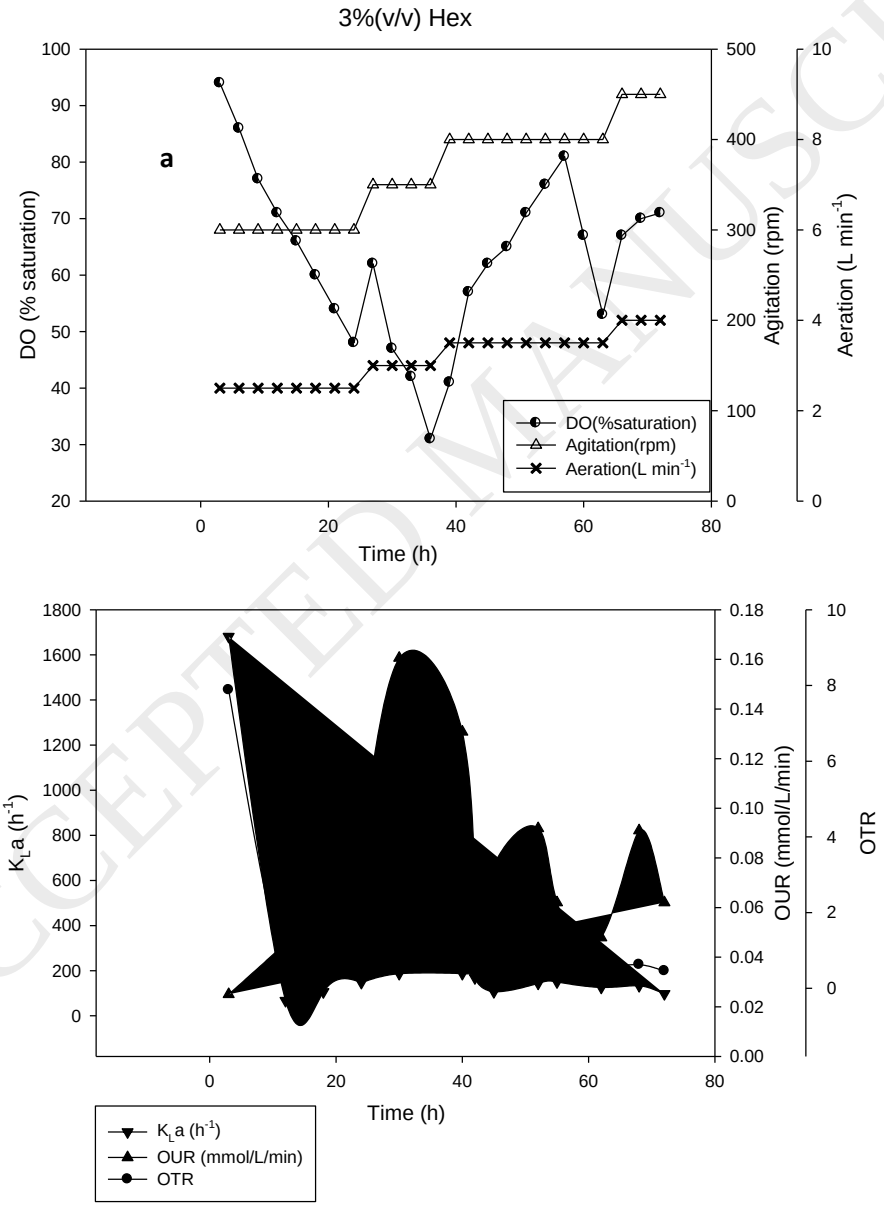

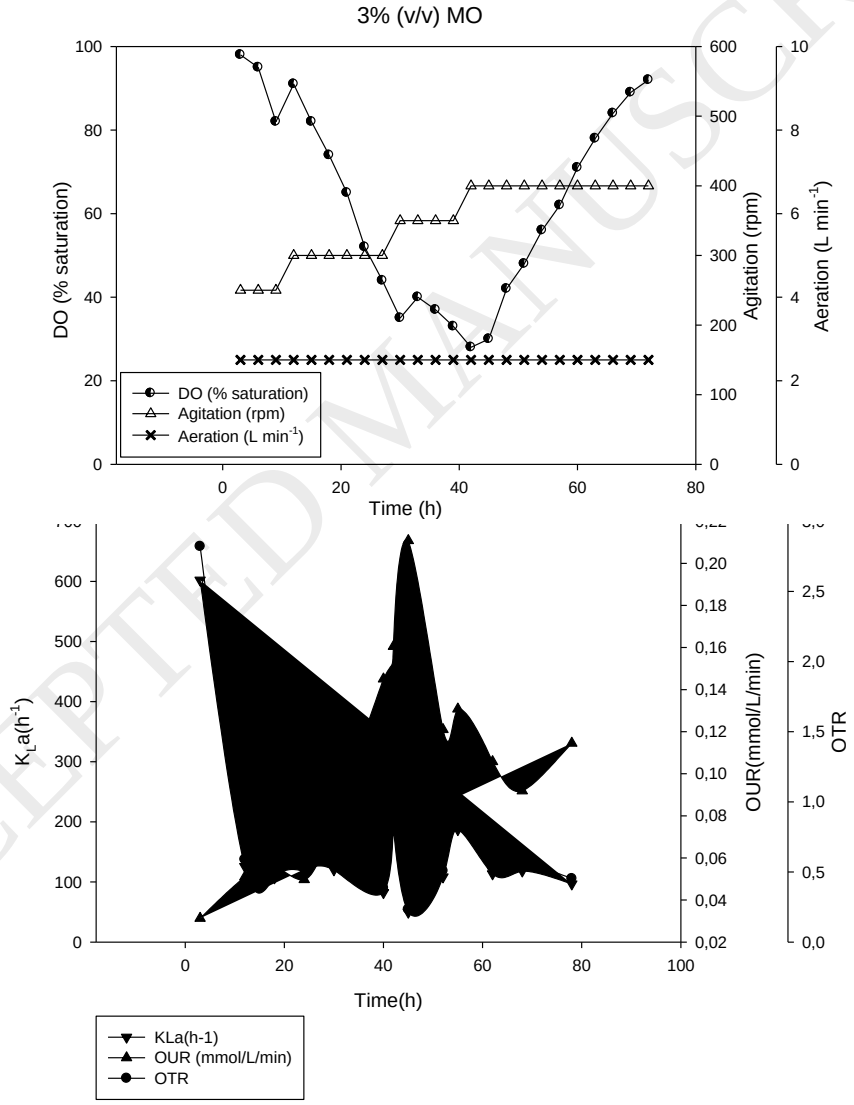

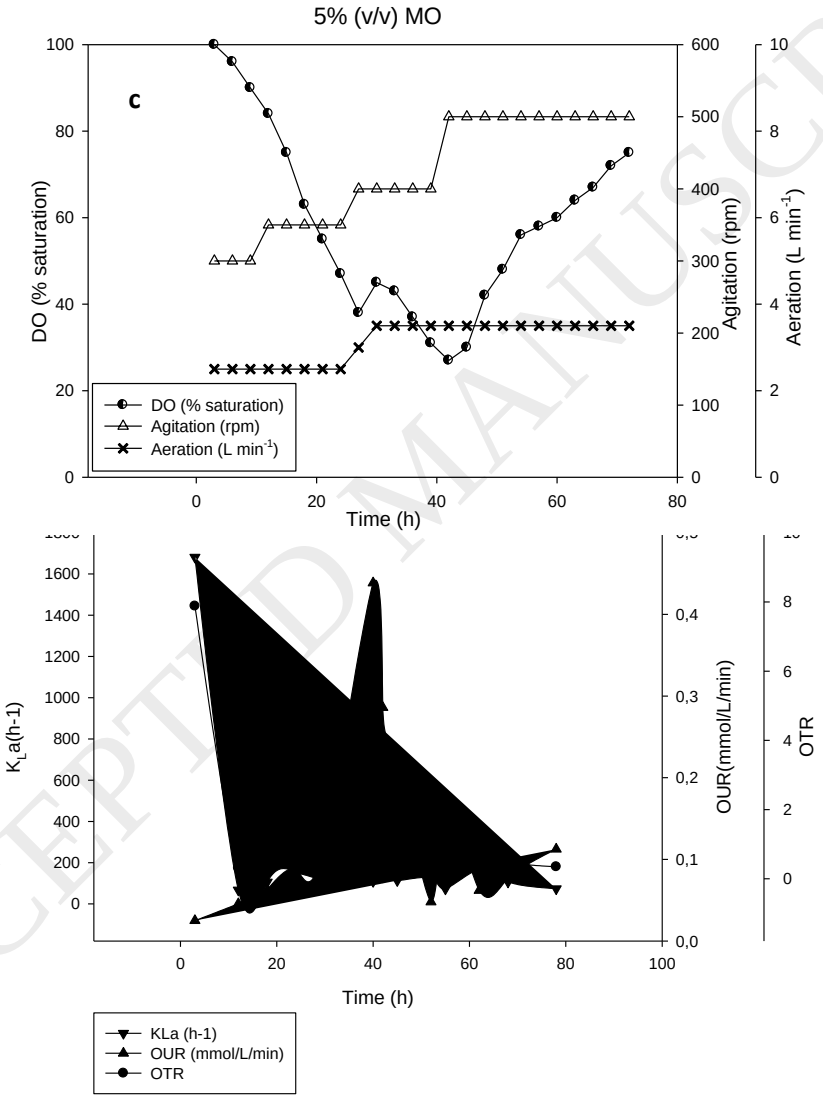

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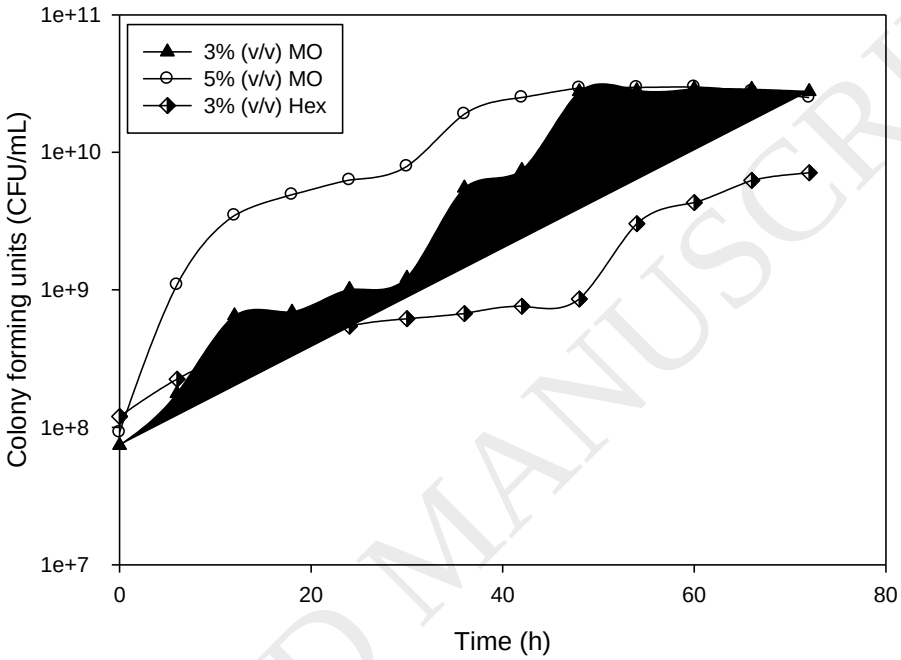

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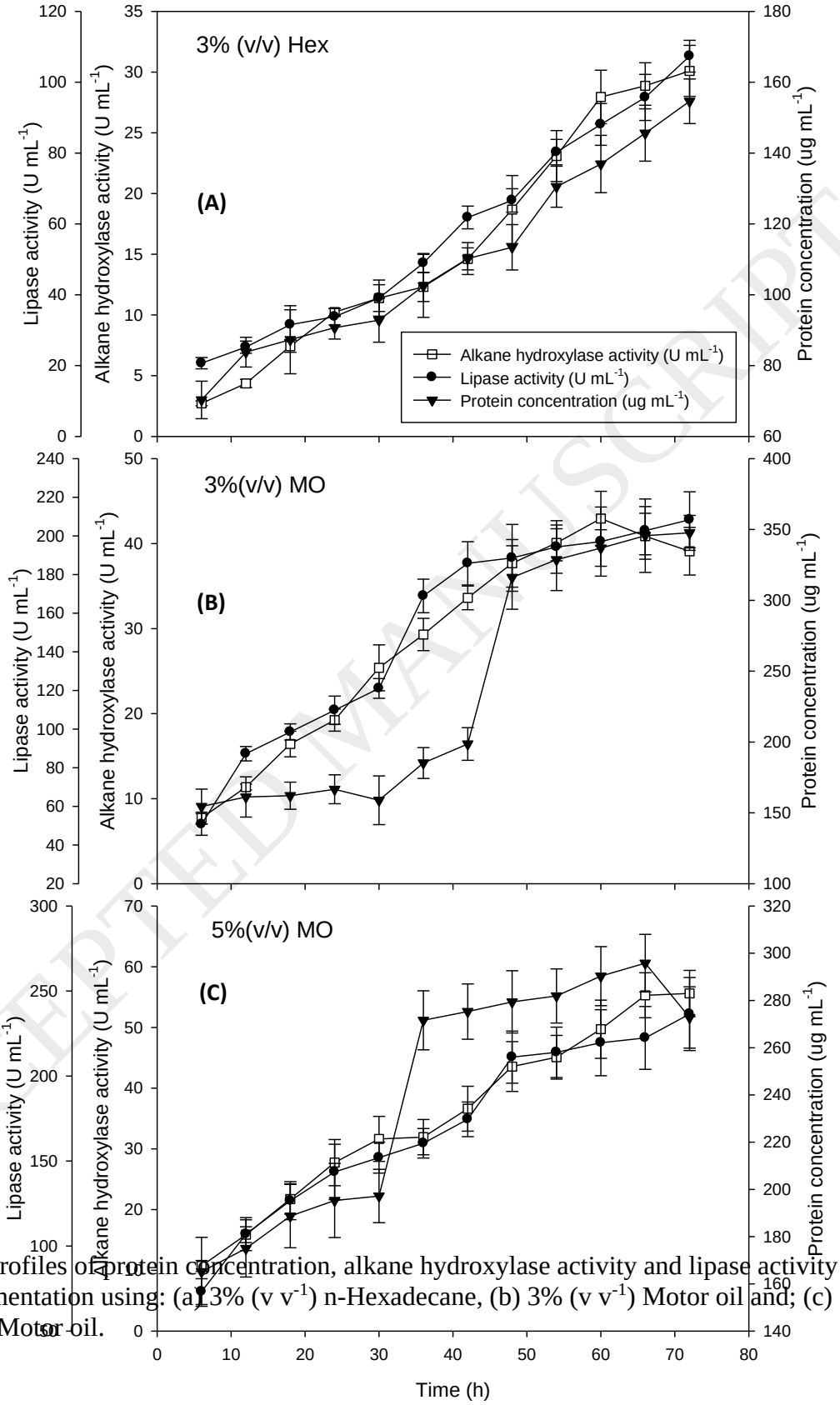

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