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

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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 5 L 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 5 L 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.

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 (Wang et al., 2010a,b; 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,b).

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

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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 5 L 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 72 h. 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 1 mL 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 24 h. 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 24 h. 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 5 L 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 5 L stirred tank fermenter (Biostat B plus, Sartorius Stedim Biotech, Germany). The working volume was 3 L. 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