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# Production and characterization of novel hydrocarbon degrading enzymes from *Alcanivorax borkumensis*

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ABSTRACT

This study investigates the production of alkane hydroxylase, lipase and esterase by the marine hydrocarbon degrading bacteria *Alcanivorax borkumensis*. The focus of this study is the remediation of petroleum hydrocarbons, hexane, hexadecane and motor oil as model substrates. *A. borkumensis* showed an incremental growth on these substrates with a high cell count. Growth on motor oil showed highest alkane hydroxylase and lipase production of 2.62 U/ml and 71 U/ml, respectively, while growth on hexadecane showed the highest esterase production of 57.5 U/ml. The percentage of hexane, hexadecane, and motor oil degradation during *A. borkumensis* growth after 72 h, was around 80%, 81.5% and 75%, respectively. Zymogram showed two different bands with a molecular weight of approx. 52 and 40 kDa, respectively with lipase and esterase activity. Alkane hydroxylase reached optimum activity at pH 8.0 and 70 ± 1 °C for hexane and hexadecane and 75 ± 1 °C for motor oil. Lipase and esterase showed optimum activity at 35 ± 1 °C and 40 ± 1 °C, respectively and pH 7.0. The crude enzymes showed higher stability in a wide range of pH, but they were not thermostable at higher temperatures.


## 1. Introduction

Petroleum hydrocarbons are an important energy resource for the industry and the population. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. The release of hydrocarbons into the environment accidentally or due to human activities is the principal cause of water and soil contamination. The removal of petroleum-hydrocarbons by of mechanical and physical methods is used as a primary response during the oil spill. However, the efficiency by mechanical removal is limited and the total and ultimate oil removal completely rely on bioremediation process carried out by indigenous microorganisms inhabiting the affected areas. Among related phylogenetic groups able to degrade hydrocarbon pollutants, hydrocarbonoclastic bacteria (HCB) are key players [1–3]. Importantly, *Alcanivorax borkumensis*, a rod-shaped marine γ-proteobacterium is able to grow on a highly restricted spectrum of substrates, predominantly alkanes. This Gram-negative, aerobic, halophilic bacteria was first to bloom in the oil-polluted open ocean and coastal waters, reaching 80–90% of the whole microbial community [4,5]. Moreover, many studies on hydrocarbons biodegradation have demonstrated the pivotal role that *A. borkumensis* play in oil bioremediation [5–7].

As many other hydrocarbonoclastic bacteria, *A. borkumensis* was able to produce a glycolipid biosurfactant to access hydrocarbons within an emulsified droplet [8]. Its genome has been completely sequenced and hence leading to a better understanding of the cellular biology of hydrocarbons metabolism [9], and 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 autochtonous hydrocarbonoclastic bacteria in oil spill remediation [13]. Therefore, understanding the biochemical pathway is a key feature in environmental bioremediation. With this aim, we investigated the production of alkane hydroxylase, lipase and esterase. 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 wider 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 show generous enzymatic activity that is up to two orders of magnitude higher than common esterases, have a large

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substrate spectrum, exceptional enantioselectivity and chemical resistance, which provides them a competitive advantageous over other esterases from other microorganisms and other enzymes for the resolution of chiral mixtures in biocatalysis [9]. Other than its extensive production by *A. borkumensis*, lipase demonstrates an important role in oily hydrocarbons biodegradation. In fact, lipase activity has been used as a biochemical and biological parameter for testing hydrocarbon degradation and it is an excellent indicator to monitor the decontamination of a hydrocarbon polluted site [14]. The strain was grown with hexane, hexadecane or motor oil, as a unique carbon source, with a view to establishing a biochemical approach adopted in response to petroleum hydrocarbon exposure during the remediation. The choice of substrates is not arbitrary since they are found in oil spills. Thus, a thorough characterization of these enzymes in terms of their enzymatic properties and their efficiency to degrade cited substrates has been investigated.

## 2. Materials and methods

All chemical reagents of the highest purity, such as pyruvic acid, hexane, hexadecane, Bradford reagent, 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, *A. borkumensis* was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ (Braunschweig, Germany). The composition of motor oil used in this study is (in mg/l) is: 69.8 of C10–C50, 1.83 of naphthalene, <44 of benzene, <30 of toluene, <44 of ethyl-benzene and <84 of xylene.

### 2.1. Bacterial strain

*Alcanivorax borkumensis* strain SK2 (DSM 11573) was used in this study. *A. borkumensis* was sub-cultured and streaked on agar plates, incubated for 72 h at 30 ± 1 °C and then preserved at 4.0 ± 1 °C for future use. Standard growth media consisted of (per liter of distilled water): 23 g NaCl, 0.75 g KCl, 1.47 g $CaCl_2 \cdot 2H_2O$, 5.08 g $MgCl_2 \cdot 6H_2O$, 6.16 g $MgSO_4 \cdot 7H_2O$, 0.89 g $Na_2HPO_4 \cdot 2H_2O$, 5.0 g $NaNO_3$ and 0.03 g $FeSO_4 \cdot 7H_2O$ [15]. The media was supplied with either hexane, hexadecane or motor oil at a concentration of 3% (v/v) as the carbon and energy source and the growth was monitored at 30 ± 1 °C, 150 rpm for 72 h. Agar plates were prepared with the same media and 18 g/l agar was added to them. Experiments were conducted in replicates. Cell growth was monitored by measuring the Colony Forming Units per ml (CFU/ml).

### 2.2. Inoculum preparation

For inoculum preparation, a loopful of *A. borkumensis* from the agar plates was employed to inoculate a 250 ml Erlenmeyer flask containing 50 ml of sterilized medium. The flask was incubated on an incubator-shaker at 150 rpm and 30 ± 1 °C for 24 h and the actively growing cells from these flasks were used as 3% (v/v) inoculum for the production of *A. borkumensis*.

### 2.3. Polyacrylamide gel electrophoresis (PAGE) and zymography

A native PAGE composed of 12% resolving and 4% stacking gel was performed according to the method described by Laemmli [16] to identify the enzymes with lipase/esterase activities by activity staining (zymogram). About 50 μl of the crude enzyme produced by *A. borkumensis* was loaded on the native PAGE gel without denaturing the sample. The electrophoresis was performed at constant voltage of 85 V in Tris-glycine buffer (pH -8.3) at 25 °C.

Activity staining for putative lipase/esterase was performed according to the method described by Prim et al. [17]. In brief, the native PAGE gel after electrophoresis was washed in distilled water and soaked in 2.5% Triton X-100 at room temperature followed by a wash in 50 mM Tris-HCl buffer (pH -8.0). The washed gel was immersed in 100 μM 4-methylumbelliferone butyrate (substrate) solution in the same buffer. A visible activity band was observed after 10 min by exposing the gel to UV light.

Zymogram for alkane hydroxylase was performed as described by Flores-Flores et al. [18]. The enzyme activity was tested using the crude extract and by submerging the gel in a reaction mixture composed of 10 ml of 50 mM tris buffer pH 8.5 added with 0.4 μm/ml of NADH, 6.25 ml of o-dianisidine reagent ((20 mg 3,3′-dimetoxibenzidine dissolved in 3 ml 0.025 M hydrochloride acid, added with agitation to 50 ml of 50 mM tris buffer pH 8.5 and brought up to 100 ml with the same buffer)) and 1 ml of substrate (hexadecane) and incubating the gel at room temperature with gentle agitation, until the activity bands appeared. The protein loaded was 63.5 μg per lane.

### 2.4. Protein and enzymes assays

Cells of each sampling were centrifuged at 4 °C for 10 min at 5000 ×*g*. The supernatant was used for total protein estimation and enzymatic assays. Total protein concentration was determined according to the standard Bradford method [19].

#### 2.4.1. Alkane hydroxylase assay

For cell disruption, *A. borkumensis* cell pellet (1 g), 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 13000 ×*g* for 20 min. The supernatant was used as a crude intracellular enzyme extract.

Alkane hydroxylase activity was measured as described by Glieder et al. [20]. Briefly, the crude enzyme assay was carried out in sodium phosphate buffer (0.1 M, pH 8.0) with either hexane, hexadecane or motor oil as a substrate (0.5–1 mM) and dimethyl sulfoxide (DMSO; 1%, v/v). The reaction was initiated by addition of NADPH (200 μM), and the oxidation of NADPH was monitored at 340 nm.

The enzymatic assay was performed on the crude enzyme produced by *A. borkumensis* grown in three different substrates: hexane, hexadecane and motor oil. One unit is defined as the amount of enzyme required for consumption of 1 μmol of NADPH per min.

#### 2.4.2. Lipase assay

Extracellular lipase activity was evaluated by the titrimetric method according to Lopes et al. [21] by using an olive oil emulsion composed of 25 ml of olive oil and 75 ml of 7% Arabic gum solution was emulsified in a liquefier for 2 min. About 5 ml of olive oil emulsion was then added to 0.1 M phosphate buffer (pH 7.0) and 1 ml of the enzymatic suspension (10 mg/ml) and incubated at 37 °C for 30 min 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 0.05 M NaOH. One unit of lipase activity was defined as the amount of enzyme which liberated 1 μmol of fatty acids per minute.

#### 2.4.3. Esterase assay

Extracellular esterase activity was measured by the titrimetric method according to Lopes et al. [21] by using olive oil as a substrate. The reaction mixture is composed of: 5 ml of olive oil, 2 ml of 0.1 M phosphate buffer (pH 7.0) and 1 ml of the enzymatic extract (10 mg/ml). The mixture was incubated at 37 °C for 30 min 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 0.05 M NaOH. One unit of esterase activity was defined as the amount of enzyme which liberated 1 μmol of fatty acids per minute.