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# Biodegradative potential and characterization of a novel aromatic-degrading bacterium isolated from a geothermal oil field under saline and thermophilic conditions

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ABSTRACT

An aerobic, thermophilic, halotolerant, Gram-positive and sporulated bacterium, designated strain VP3, was isolated from a geothermal oil field, located in Sfax, Tunisia, after enrichment on vanillic acid. The temperature range for growth was 37–65 °C, with optimal growth occurring at 55 °C. The NaCl concentration range for growth was 0–80 g $l^{-1}$, with an optimum at 10 g $l^{-1}$ NaCl. Strain VP3 was able to degrade completely 5 mM vanillic acid after 9 h of incubation, at 55 °C and in the presence of 30 g $l^{-1}$ NaCl. Strain VP3 was also found to degrade, under saline and thermophilic conditions, a wide range of other aromatic compounds, including benzoic, *p*-hydroxybenzoic, protocatechuic, *p*-hydroxyphenylacetic, cinnamic, *p*-coumaric, caffeic and ferulic acids, phenol and *m*-cresol. In addition, the bacterium was grown on crude oil and diesel as sole carbon and energy sources. Phenotypic characteristics and phylogenetic analysis of the 16S rRNA gene sequence of isolate VP3 revealed that it was very closely related to *Aeribacillus pallidus*, with sequence similarity of 99%.



## 1. Introduction

Aromatic compounds are a widespread class of natural compounds comprising low molecular weight monoaromatics such as, *p*-hydroxybenzoic, vanillic, gallic, ferulic, caffeic, *p*-coumaric, and aromatic amino acids, and high-molecular weight polyaromatics such as, tannins, lignin and humic acids. Aromatics are also important components in petroleum-based fuels, agricultural, chemicals, and consumer products (Villemur, 1995). Consequently, they are important components of domestic and industrial wastewaters (Çinar and Grady, 2001). Most aromatic compounds, particularly those containing single benzene rings, are subject to microbial degradation under both aerobic and anaerobic degradations (Heider and Fuch, 1997). Bacteria, yeast and fungi are capable of utilizing phenolic compounds. Several bacterial strains belonging to the species of *Pseudomonas*, *Bacillus*, *Klebsiella*, *Ochrobactrum*, *Rhodococcus*, etc. were reported for phenol degradation (Arutchelvan et al., 2005; Chamkha et al., 2011; Zídková et al., 2013).

Produced water from the oil industry is a salty wastewater that is produced in conjunction with oil or natural gas. Its organic composition is very complex and varies widely between reservoirs. These constituents include salt, aromatics, saturated hydrocarbons, and in some cases heavy metals and trace elements (Cuadros-Orellana et al., 2006). The oil fields present an unusual combination of extreme environmental conditions, including temperature, salinity and pressure (Fardeau et al., 2004). It is also known that petroleum reservoirs are anaerobic environments dominated by anaerobic microorganisms (Magot et al., 2000). They include fermentative and sulphate-reducing bacteria and methanogenic *Archaea* (Magot et al., 2000; Fardeau et al., 2004; Nazina et al., 2005b), whereas predominantly aerobic hydrocarbon degraders have been isolated from hydrocarbon-contaminated ecosystems, even in extreme habitats (Magot et al., 2000; Röling et al., 2003; Chaillan et al., 2004; Mnif et al., 2009, 2011; Ferhat et al., 2011). Aerobic culturable bacteria belonging to the genera *Gordonia*, *Brevibacterium*, *Aeromicrobium*, *Dietzia*, *Burkholderia* and *Mycobacterium* were isolated from petroleum-polluted soils and cyanobacterial mats. They were cultivated with crude oil as sole carbon and energy source (Chaillan et al., 2004). Many studies showed that aerobic microorganisms can also be found in oil fields, into which they come with injection waters, drilling solutions or, probably, due to the natural hydrodynamics of subsurface waters (Nazina et al., 2005b; Chamkha et al., 2011; Mnif et al., 2011). A wide variety of bacteria has been isolated from, or has been detected in oil field

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samples by molecular techniques. Aerobic, facultatively anaerobic and microaerophilic microorganisms have been detected (Nazina et al., 1993; Voordouw et al., 1996; Telang et al., 1997; Magot et al., 2000; Wen and Hong-Bo, 2011; Mnif et al., 2013).

Temperature and salinity were important environmental parameters that influence the degradation process of petroleum compounds (Röling et al., 2003; Zheng et al., 2011). These parameters influenced the structure and physiology of microbial communities and changed the physical and chemical properties of the pollutants (Abed et al., 2006). The diversity and metabolic potential of degrading bacteria decreased with the extreme environmental conditions (Margesin and Schinner, 2001; Margesin et al., 2013). Bacterial strains capable of degrading hydrocarbons at high salt concentrations were isolated (Oren et al., 1992; Margesin and Schinner, 2001).

To our knowledge, a little work was done on the aerobic biodegradation of aromatic compounds, under both thermophilic and saline conditions, by microorganisms that inhabit high-temperature oil fields. In the present study, we report the isolation and the characterization of a novel aerobic, thermophilic and halotolerant bacterium from a Tunisian geothermal oil field that degraded a wide variety of aromatic substrates and was able to grow on some complex hydrocarbons, under thermophilic and saline conditions.

## 2. Materials and methods

### 2.1. Origin of strains

Strains were isolated from the production water (an oil/water mixture) of the oil field TPS "Thyna Petroleum Services", located at 11 km north-west of Sfax city, Tunisia. The formation water was withdrawn from the oil-bearing horizons from depths of 1300 m, with a temperature of 78 °C, a salinity of 100 g $l^{-1}$ and a pH 7.6, after passing through a pipeline of about 20 km. Samples were directly collected in sterile bottles and stored in dark at 4 °C until use. Ten strains were isolated from enrichment cultures inoculated with the production water. These strains were routinely cultured and maintained in the basal medium supplemented with 5 mM vanillic acid, as described below. Then one of them, named strain VP3, was chosen for further investigation.

### 2.2. Culture media

The basal medium composition was as follows (g $l^{-1}$): NaCl (30), $KH_2PO_4$ (0.5), $NH_4Cl$ (0.4), $MgCl_2$ $6H_2O$ (0.33), $CaCl_2$ (0.05), yeast extract (0.5) and 1 ml trace-element solution (Widdel and Pfennig, 1981). The pH was adjusted to 7 with 10 M KOH solution. Aliquots of 30 ml were dispensed into flasks and sterilized by autoclaving at 121 °C for 20 min. Substrates were injected from concentrated sterile stock solutions to obtain the desired final concentration. Strain VP3 was routinely grown on basal medium containing 5 mM vanillic acid.

### 2.3. Enrichment and isolation procedure

The production water was used to inoculate, at 10% (v/v), into 30 ml of basal medium containing 5 mM vanillic acid as carbon and energy source. Vanillic acid was sterilized using a sterile filter 0.22 μm. The culture was then incubated at 55 °C under aerobic conditions (150 r.p.m). The enrichment culture was subcultured several times under the same conditions prior to isolation, until the substrate was completely metabolized. Non-inoculated tubes were run in the same conditions to verify that vanillic acid was not partially transformed by abiotic ways in the aerobic medium. Cell growth was monitored by measuring the optical density at 600 nm. The disappearance of vanillic acid was confirmed by HPLC analysis. Aliquots (100 μl) of $10^{-1}$–$10^{-10}$ dilutions were plated onto vanillic acid (5 mM) agar basal medium. The plates were incubated at 55 °C under aerobic conditions for 1–2 days, until colony formation. Single colonies were picked up and serially diluted in the fresh basal media containing 5 mM vanillic acid. This procedure was repeated until only one type of colony was observed. The purity of the isolates was checked microscopically.

### 2.4. Physiological studies

For all experiments, basal medium containing 5 mM vanillic acid was used. Different amounts of NaCl were directly weighed in flasks prior to dispensing 30 ml medium to obtain the desired NaCl concentration (range 0–150 g $l^{-1}$). The temperature range for growth was determined between 4 and 80 °C. For heat resistance, cells grown in basal medium containing 5 mM vanillic acid were exposed to temperatures of 80, 90 and 100 °C for 10 min. The cultures were then cooled quickly to ambient temperature, inoculated into fresh Luria-Bertani (LB) medium (g $l^{-1}$): tryptone (10), yeast extract (5) and NaCl (10). The growth was recorded after 24 h incubation at 55 °C under agitation (150 r.p.m). Gram reaction was determined using the BioMérieux Gram stain Kit according to the manufacturer's instructions. Catalase activity was determined by bubble production in 3% (v/v) hydrogen-peroxide solution. Oxidase activity was determined by oxidation of 1% *p*-aminodimethylaniline oxalate. Experiments were performed in duplicate with an inoculum subcultured at least once under the same test conditions. The substrates tested for utilization were injected from pre-sterilized and concentrated stock solutions into flasks containing 30 ml pre-sterilized basal medium. The following aromatic compounds were used at a concentration of 5 mM: benzoic acids (benzoic acid, gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid, and syringic acid), cinnamic acids (cinnamic acid, *p*-coumaric acid, ferulic acid, and caffeic acid), phenolics (phenol, catechol, *m*-cresol, *p*-chlorophenol, and tyrosol), and *p*-hydroxyphenylacetic acid. The aromatic compound stock solutions were prepared, neutralized if necessary and sterilized by filtration (pore size 0.2 μm; Millipore). In addition, the strain VP3 was tested for its ability to metabolize many hydrocarbons including, crude oil (1% (v/v)), diesel (1% (v/v)), toluene (3 mM), naphthalene (300 mg $l^{-1}$) and phenanthrene (200 mg $l^{-1}$). An increase in OD (600 nm) in substrate-containing cultures, compared with control tubes lacking substrates, was considered as positive growth.

### 2.5. Analytical methods

A phase contrast microscope OLYMPUS BX50 equipped with an OLYMPUS DP 70 digital camera was used to determine the morphology at exponential-phase cultures. Bacterial growth was assessed by measuring the optical densities at 600 nm in a Shimadzu model UV-110-01 spectrophotometer. Aromatic compound analyses were carried out by High-Performance Liquid Chromatography (HPLC). There were performed on a Shimadzu apparatus composed of an LC-10ATvp pump and an SPD-10Avp detector set at 280 nm. Separation was achieved using a $C_{18}$ column (4.6 × 250 mm, 5 μm particle size, Knauer GmbH) maintained at 40 °C. The mobile phase used was 0.1% phosphoric acid in water (A) versus 70% acetonitrile in water (B) for a total running time of 50 min. The elution conditions applied were as follows: 0–25 min, 15–25% B; 25–35 min, 25–80% B; 35–37 min, 80–100% B; 37–40 min, 100% B. Finally, washing and reconditioning steps of the column were included (40–50 min) with a linear gradient of 100–15% B. The flow rate was 0.8 ml $min^{-1}$, and