Isolation and Characterization of Thermophilic Bacteria from the Mexican Thermal Pool El Carrizal, Veracruz.

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Abstract

Extremophiles are microorganisms that posses application possibilities on several industrial fields including agricultural, chemical, laundry, pharmaceutical, food, petroleum and in bioremediation. This work reports the isolation of nineteen thermophilic, alkalitolerant and halotolerant bacterial strains from two thermal sites in Veracruz, México: El Carrizal thermal pool and Los Baños hot spring, that belong to the *Geobacillus, Anoxybacillus* and *Aeribacillus* genera. The strains produce lipases, proteases, and amylases under thermophilic conditions. They may have good potential for application in microbial enhanced oil recovery, since they are thermophilic, halotolerant, and produce exopolymers, acids, surfactants, and are able to grow in kerosene as the sole carbon source; these strains may be used in biodesulfurization since they can grow in dibenzothiophene producing 2-HBP under thermophilic conditions.

Keywords: thermophiles; extremoenzymes; microbial enhanced oil recovery; biodesulfurization

Introduction

Extremophiles are a group of microorganisms that thrives in environments previously thought to be hostile to life [1]. These environments may include extremes in physical parameters (temperature, radiation, pressure), geochemical parameters (desiccation, salinity, pH, oxygen species or redox potential), or even biological extremes (nutritional extremes, population density, parasites, prey) [2]. Discovery and research on extremophiles and their enzymes have provided invaluable data and application possibilities on molecular

biology, evolutionary biology, and occupy an important place in the environmental biotechnology industry, with applications spanning agricultural, biomedical and industrial sectors such as food, laundry, pharmaceutical, petroleum and bioremediation [3-5]. Thermophiles, microorganisms that grow in temperatures greater than 45ºC, are among the best studied of the extremophiles. They have been isolated from hot springs, solfataras, geothermally heated soils, oil reservoirs, and from some mesobiotic environments like soils, composting vegetation, and river-, lake-, and seawater [1, 2, 6]. The enzymes produced by these microorganisms deserve special industrial interest. These biocatalysts are extremely thermostable and usually resistant to chemical denaturants such as detergents, chaotropic agents, organic solvents and extremes of pH [7]. Performing biotechnological processes at elevated temperature has many advantages: the elevation of temperature is accompanied by a reduced risk of contamination, a decreased viscosity and an increased diffusion coefficient of organic compounds, hence the bioavailability, solubility of organic compounds and reaction rates are improved [7, 8]. In consequence, there is a continuous interest in isolating and characterizing thermophilic bacteria and their enzymes in order to increase the possibilities for their application in industry.

Of great potential could be the application of thermophiles in petroleum industry, since the bioavailability of less soluble hydrophobic substrates such as polyaromatic and aliphatic hydrocarbons could also be improved dramatically at elevated temperatures. In that respect two processes have attracted attention recently: the removal of sulphur from petroleum fractions by microorganisms, biodesulfurization (BDS), and the use of microbes down oil wells in order to enhance oil production after primary and secondary recovery procedures (Microbial Enhanced Oil Recovery, MEOR). The application of thermophiles in both

processes will provide crucial advantages compared to mesophilic microorganisms due to the increase in bioavailability and reaction rates, and because thermophiles could survive the harsh environment of the oil reservoir [9]. Nowadays the extremophiles' research has resulted in the isolation of thermophilic bacteria from a great variety of terrestrial and hot-water environments. In particular, aquatic ecosystems possess an enormous microbial biodiversity which can be explored in order to isolate and discover new microorganisms and biocatalysts [10]. In this work we report the isolation and characterization of thermophilic bacteria from two Mexican geothermal locations in the state of Veracruz, México, and the analysis of some of their potential biotechnological applications.

Materials and Methods

Sampling sites. Samples were obtained from the thermal pool "El Carrizal" and from the hot spring "Los Baños". Both sites belong to the hydrothermal system formed by the Citlaltépetl-Cofre de Perote Vocalnic Range (CCPVR) in the central part of the state of Veracruz, México, situated in the eastern Trans-Mexican Volcanic Belt, a continental mostly calc-alkaline province of Quaternary age [11]. El Carrizal thermal pool is located at Northern Latitude 19°19'00'' Western Longitude 96°38'40'', 250 m above msl, next to the river Pescados-La Antigua. Los Baños hot spring is located near to the river Actopan, Northern Latitude 19°37'42'' Western Longitude 96°27'28'', 78 m above msl.

Sampling. Soil and water samples (250 ml) mixed with biofilms deposited in submerged rocks were collected in sterile flasks and immediately transported to the lab for

enrichment and cultivation. El Carrizal water composition was analyzed by the Water National Commission (CNA), México; Los Baños water was analyzed at the Geosciences Center (Juriquilla, Qro.) from the National Autonomous University of México (UNAM) (Table 1).

Cultivation and isolation. Samples were inoculated at 3% (v/v) in Luria Bertani (LB) medium at $pH^{60ºC}$ 7 (10 g/L bactotryptone, 5 g/L NaCl, and 5 g/L yeast extract) [12], and incubated aerobically in an orbital shaker at 60ºC and 150 rpm, until growth was noticed. Culture aliquots were plated out in LB agar medium containing 2 % (w/v) agar (Bioxon), and incubated at 60ºC for 18 h. Individual colonies were isolated, and streaked onto the agar medium until single, uniform colonies were obtained. The pure isolates were ultimately grown in LB medium until a DO_{600nm} 0.6-0.8 was reached, and 80% (v/v) glycerol stocks were prepared and stored at –70ºC.

Characterization of the isolates. Isolates were characterized with respect to cell and colonial morphology, Gram reaction, metabolic products (Microscan kit), spore formation, and to the effect of salinity, pH and temperature on growth. Temperature and pH growth limits of each strain were established by performing experiments at 25-75°C and pH 5-11. Then, optimum temperatures and pHs were found using a 2^3 factorial design (T= 40, 55 and 70°C, pH= 7, 7.5 and 8) analyzed by the modified Gompertz Bacterium model [13]. Media pHs were adjusted at the incubation temperature. The influence of salinity on growth was determined by growing cells on LB medium $pH^{60ºC}$ 7 containing 0, 0.3, 0.5, 1, 1.5, and 2 M NaCl, at 60ºC, 150 rpm for 72 h. Cell growth was determined by optical density at 670 nm. Growth on a single carbon source was tested on liquid medium containing: carbohydrate 0.5 % (w/v), yeast extracts 5 g/L and NaCl 5 g/L, pH 6.5. The different carbon sources

were: D- glucose, D-manose, D-arabinose, D- xilose, lactose, manitol, sucrose, ramnose and starch [14]. All growth tests were done at 55 °C for 48 h.

16S rRNA sequencing and phylogenetic analysis. Phylogenetic analysis was based on 16S rRNA sequencing [15]. The sequences were aligned with those previously deposited in the EMBL/GenBank database (www.ncbi.nlm.gov) and at the EzTaxon server (http://www.eztaxon.org) [16]. The nucleotide sequences data were submitted to GenBank nucleotide sequence database.

Screening for enzyme activity. Isolates were evaluated for lipolytic, proteolytic and amilolytic activity. Isolates were screened for lipase activity on agar plates containing Rhodamine B 0.001% (w/v), nutrient broth 0.8% (w/v), NaCl 0.4% (w/v), agar 1% (w/v), and olive oil 3%, in distilled water, $pH^{55°C}$ 6.5 [17]. Plates were incubated at 55ºC for 18 h, and lipase production was identified as an orange halo around colonies under UV light at 350 nm. Screening for proteolytic activity was performed by plating the isolates on agar medium containing casein 1% (w/v), glucose 1% (w/v), KH_2PO_4 1 g/l, $MgSO_4$ 0.2 g/l, $pH^{55°C}$ 7 at 55ºC for 24 h. Formation of a clear halo around the colony was an indication of proteolytic activity. Screening for starch hydrolysis activity was performed by plating the isolates on agar medium containing beef extract 3 g/l and soluble starch 10 g/l, $pH^{55°C}$ 7 and incubating at 55ºC for 24 h. Staining of the plates with iodine reagent was carried out to reveal clear halos of starch hydrolysis [18].

Screening for Microbial Enhanced Oil Recovery (MEOR) activities. Isolates were evaluated for oil recovery potential with respect to their capability to produce surfactants, gases, acids, and exopolymers.

a.- Growth on kerosene and biosurfactant production.- Isolates were grown on a medium containing kerosene as the sole carbon source [19]: in g/L Na_2HPO_4 2.2;

KH_2PO_4 1.4; $MgSO_4.7H_2O$ 0.6; $(NH_4)_2SO_4$ 3.0; yeast extract 1.0; NaCl 0.05; $CaCl_2$ 0.02; $FeSO_4.7H_2O$ 0.01; kerosene 20ml/L; $pH^{55ºC}$ 6.8, at 55ºC. Growth was monitored by light microscopy. Biosurfactant production was assessed by the emulsification index (E-24) measurement [20] as follows: 2 ml of cell-free growth medium (or 2 ml of non-cultivated medium as negative control) were added to 3 ml of kerosene and vortexed at high speed for 2 min. After 24 h, emulsion index (E-24) was calculated as the height of the emulsion layer, divided by the total height, multiplied by 100.

b.- Production of gases and acids.- Isolates were grown on Durham tubes containing LB medium and phenol red (18 mg/L) at 55ºC for 24 h. Gas production test was considered positive when a gas bubble was observed at the top of the inverted tube. Acid production was considered positive when the medium color changed to yellow.

c.- Production of exopolymers.- Isolates were grown on a production medium [21] containing (in g/L): yeast extract 2.5; glucose 20; NaCl 1.0; K_2HPO_4 5.0; $MgSO_4.7H_2O$ 0.2; $(NH_4)_2SO_4$ 0.6; $pH^{55ºC}$ 6.5 at 55ºC and 150 rpm for 3 days. Cell broth was centrifuged at 15,000xg for 20 min at 4ºC, and cells were washed with distilled water and dried at 105ºC until constant weight was reached to determine biomass. The supernatant (or non-cultivated medium as negative control) was mixed with 2 volumes of 95% ethanol and incubated at 4ºC for 24 h to precipitate the crude products, then centrifuged at 15,000xg for 30 min at 4ºC, and repeatedly washed with acetone-ether-distilled water (1:1:1). The pellet was dried, and the exopolymer yield was determined by the ratio exopolymer weight/biomass.

Screening for biodesulfurization activity. Isolates were grown on 5 mL of a medium containing (in g/L): yeast extract 0.25; $FeSO_4.7H_2O$ 0.001; $CaCl_2.2H_2O$ 0.001;

$MgSO_4$ 0.2; $(NH_4)_2SO_4$ 2.0; K_2HPO_4 4.0; NaH_2PO_4 4.0; and 0.05% dibenzothiophene (DBT) (dissolved in N,N'-dimethylformamide), $pH^{55ºC}$ 7 at 55ºC and 150 rpm for 12 days. Cell growth was measured by monitoring the optical density at 660 nm. The cultures were centrifuged and the supernatants (or non-cultivated medium as negative control) were subjected to Gibb's assay to detect 2-hydroxybiphenyl (2-HBP) produced by microbial degradation of DBT [22].

Gibb's assay.- The production of 2-HBP was monitored as follows: 0.150 mL of culture's supernatant was mixed with 0.03 mL of 1M $NaHCO_3$ (pH 8). Twenty microliters of Gibb's reagent (1 mg of 2,6-dichloroquinone-4-chloroimide per mL in ethanol) was then added, and the reaction mixture was agitated at room temperature for 15 to 45 min for full color development. The absorbance of the reaction mixture was determined at 595 nm and converted to mg/L by interpolation on a 2-HBP standard curve (1-10 mg/L).

Results

Isolation and characterization of bacterial strains. Inoculation of samples from thermal environments in LB medium at $pH^{60ºC}$ 7, 60ºC and 150 rpm resulted in abundant growth after 12 h of culture. Eleven different colonies from El Carrizal and eight from Los Baños were obtained after repeated streaking on LB agar, differing mainly in colony size and cellular shape. Isolates were designated as strains CCR1 to CCR11 (El Carrizal) and DR01-DR08 (Los Baños). Cultures grown on liquid and agar media for 24 h at 55ºC and $pH^{55ºC}$ 7 were used for characterization. Colonies were circular, punctiform, or irregular,

smooth with entire margins, varying in diameter between 1.5 and 8 mm. The isolates were either positive or negative for Gram staining, positive for oxidase test, endospore-forming, and thin rod-shaped varying in cell length (Table 2). As it has been reported for other thermophilic bacteria [23], changes in cell morphology were observed: depending on culture temperature and incubation time strains CCR1, CCR7, CCR8, CCR9, CCR10 and CCR11 occurred as single motile long rods, as aggregates, or as long filaments that formed filamentous cell masses. The isolates were aerobes capable to oxidize carbohydrates. The strains were thermophilic, neutrophilic, and alkalitolerant, growing in a pH 5-11, and a temperature of 27ºC to 70ºC, with the optimum pH between 6.5 and 7.5, and the optimum temperature between 55ºC and 70ºC (Tables 2 and 3). Growth rates were fairly rapid, showing doubling times as short as 20 min (CCR5) and 22 min (CCR4). Most of the strains were halotolerant, since they could grow in the presence of up to 3% NaCl (0.5 M).

Phylogenetic Analysis. The phylogenetic analysis was performed on the most promising isolates (for strains CCR1, CCR2, CCR4, CCR7 and CCR10 we obtained the partial 16S rRNA sequence, and for strains CCR3, CCR11, DR01, DR02, DR03 and DR04, we obtained the complete 16S rRNA sequence). The 16S rRNA sequences obtained were aligned to those available in the EMBL/GenBank database and at the EzTaxon server. Results are shown in Table 4. The alignment placed strains CCR1, CCR2, CCR7, CCR10 and CCR11 in *Geobacillus* genus; strains CCR3, DR01 DR02 and DR04 showed a 99.5–99.6% similarity with *Anoxybacillus kamchatkensis*, a thermophilic facultative aerobic bacterium isolated from the Geyser valley, Kamchatka [24]. Strain CCR4 showed identical 16S rRNA sequence as the type strain of *Anoxybacillus rupiensis*, a thermophilic bacterium isolated from Rupi basin (Bulgaria) [25]. The particular phylogenetic location of strain

DR03 let us re-classify it into a new genus in the family *Bacillaceae* as *Aeribacillus pallidus* gen nov., comb. nov. [26].

Enzymatic Activities. Isolates were tested for enzymatic activities on agar plates (Table 2 and 3). Eleven strains showed hydrolytic halos around colonies when grown on casein medium at 55°C, indicating proteases production. Nine strains produced lipases, since an orange halo was observed around colonies under UV light when grown on olive oil and Rhodamine B medium. Eight strains were capable to produce amylases, showing clear halos of starch hydrolysis after staining starch plates with iodine reagent.

Screening for activities required for Microbial Enhanced Oil Recovery. Isolated strains from El Carrizal thermal pool were analyzed for their capability to produce exopolymers, gases, acids, and biosurfactants, and to grow in a medium containing kerosene as the sole carbon source. Results are shown in Table 4. All the strains produced exopolymers after 72 h at 55ºC (0.3-11.8 mg/mg biomass); nine out of eleven strains produced biosurfactants when grown in kerosene as the sole carbon source (E24= 1.6-7.5%); most of them produced acids, but none produced gas. The chemical composition of the acids, exopolymers and biosurfactants remains to be determined.

Screening for biodesulfurization activity. All the isolates were capable of growth on a DBT containing medium, especially strains CCR1, CCR7 and CCR10. After 12 days at 55ºC, all the microbial cultures were positive to the Gibb´s assay, with the highest production of 2-HBP by strains CCR1, CCR2, CCR3, and CCR9, which showed 2-HBP levels up to 2.8 mg/l (Table 5).

Discussion

Enrichment of thermal water from both El Carrizal thermal pool and Los Baños hot spring resulted in the isolation of nineteen thermophilic, alkalitolerant and halotolerant bacterial strains. Phylogenetic analysis placed strains CCR1, CCR2, CCR7, CCR10 and CCR11 as belonging to different species of the *Geobacillus* genus which includes Gram-positive spore-forming rods, neutrophilic, moderately thermophilic and aerobic or facultatively anaerobic species with a temperature range for growth from 37-75ºC, with an optimum at 55-65ºC [27]. Isolates CCR3, CCR4, DR01, DR02 and DR04 were placed in the *Anoxybacillus* genus, which includes Gram-positive spore-forming rods, alkaliphilic or alkalitolerant, thermophilic and aerotolerant or facultative anaerobes, with a temperature range of growth of 35-70°C [28]. The strains showed high similarities (> 98%) with various species of the *Anoxybacillus* genus (*A. kamchatkensis, A. rupiensis, A. ayderensis, A. gonensis, A. flavithermus, A. pushchinoensis*) so it should be necessary to perform further molecular analyses, such as DNA-DNA hybridizations, in order to establish the exact phylogenetic location of the strains. Based on the 16S rRNA gene sequence strain DR03 was identified as *Geobacillus pallidus*. However, *G. pallidus* (DR03 and type strains) showed low 16S rRNA gene sequence similarities with respect to *Anoxybacillus* (92.5–95.1%) and *Geobacillus* (92.8–94.5%) species, as well as to *Bacillus subtilis* (92.2–92.4%). In addition, *G. pallidus* could be differentiated from *Anoxybacillus* and *Geobacillus* on the basis of DNA G+C content, 16S rRNA gene sequence analyses and fatty acid and polar lipid profiles. From these results, it was proposed that *G. pallidus* (DR03) should be reclassified to *Aeribacillus pallidus* gen nov., comb. nov. [26].

Isolated strains produced hydrolytic enzymes (proteases, lipases and amylases) under thermophilic conditions (55ºC). Thermophilic enzymes are attractive because they are stable and active under conditions comparable to those prevailing in various industrial processes, such as in food processing, laundry, textile and pharmaceutical industries. A thermoalkalophilic lipase produced by strain *Geobacillus thermoleovorans* CCR11 has been already purified and characterized by the research group [29].

The successful application of in situ MEOR depends largely on the capability of microorganisms to grow and produce metabolite(s), such as biosurfactants, exopolymers, gas and acids, under the extreme conditions similar to those existing in the oil reservoirs [30]. Although the ideal microorganisms would be extremophiles, since they can grow optimally in extreme temperatures, pressures, pH conditions and salt concentrations, there are few reports on the application of extremophilic organisms in MEOR [9, 19, 30-36]. In this work we report bacterial strains that may be applied in MEOR, since they are thermophilic and halotolerant and hence could probably withstand reservoir conditions. Additionally, isolated strains showed a good yield in exopolymers production after 72 h at 55ºC (up to 11.8 mg/mg dry weight of cells), in comparison with other mesophilic fungal and bacterial species, which have shown variable production yields (from 0.34 mg/mg dry weight of cells [20] to 700 mg/mg dry weight of cells [32]). With respect to biosurfactant production, emulsification indexes (E24) found (1.6-7.5 %) were low in comparison to those found with *Bacillus subtilis* under thermophilic conditions (33-90%) [9] or with the thermophilic *Bacillus sp*. AB-2 (80-90%) [19]. However, they could be improved by the optimization of some important parameters, such as culture temperature, initial pH of the medium, oxygen supply, nitrogen concentration, and carbon source.

In order to find out if the isolated strains could cleave heterocyclic organosulfur compounds refractory to the desulfurization process in a C-S-bond-targeted fashion at high temperatures, we cultured strains in a medium containing dibenzothiophene (DBT) and analyzed the production of 2-HBP at 55ºC. The carbon-sulfur (C-S) bond targeted reaction is preferable and ideal for desulfurization because it keeps the remaining hydrocarbon molecules fully active as energy sources without any loss of their thermal units [22]. All the isolates were capable to generate 2-HBP from DBT under thermophilic conditions.

Many mesophilic bacteria capable to degrade DBT in a C-S targeted fashion have been isolated (*Rhodococcus erythropolis*, *Nocardia* spp., *Agrobacterium* sp MC501, *Gordona* sp., CYKS1, *Klebsiella* spp, *Xanthomonas* spp., *Microbacterium* and *Pseudomonas* [36, 37]), but few thermophilic bacteria have been reported to do so (*Paenibacillus sp* [21, 37], *Mycobacterium phlei* [39-41], and *Mycobacterium godii* X7B [42]). Biodesulfurization of fossil fuels is attracting more and more attention because such a bioprocess is environmentally friendly. Performing biodesulfurization at high temperatures has many advantages since both the bioavailability of hydrophobic compounds and the reaction rates increases with temperature, and because the process costs could be diminished by avoiding the cooling of petroleum fractions prior to the biodesulfurization reaction. Although work remains to be done regarding the exact mechanisms and the optimal conditions for desulfurization, the CCR strains may be useful candidates for thermophilic desulfurization.

In conclusion, the thermal environments analyzed in Veracruz, México, are a good source of extremophilic microorganisms with promising biotechnological potential since we isolated nineteen thermophilic and alkalitolerant bacterial strains that produce hydrolytic enzymes under thermophilic conditions; may have good potential for application in microbial enhanced oil recovery, since they are thermophilic, halotolerant, and produce

exopolymers, acids, surfactants, and are able to grow in kerosene as the sole carbon source; and may be used in biodesulfurization since they can grow in dibenzothiophene producing 2-HBP under thermophilic conditions.

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Table 1.- Characteristics of El Carrizal thermal pool* and Los Baños hot spring water**.

Compound	Concentration (mg/L)	
	El Carrizal	Los Baños
$CaCO_3$	232.2	40.6
$CaSO_4$	502	nd
$MgSO_4$	325.6	379.01
Na_2SO_4	66	23.4
LiCl	9.16	1.12
KCl	8.2	8.32
NaCl	61.8	36.8
$Na_2B_4O_7$	12.2	283.6
SiO_2	39.4	nd
Al_2O_3	2.4	nd
Parameter		
Temperature (ºC)	39	58
pH	7	6

* analyzed by the Water National Commission (CNA), Mexico. **Analyzed by Geosciences Center (Juriquilla, Qro.) from the National Autonomous University of México (UNAM).

Table 2.- Morphological and physiological characteristics of El Carrizal thermal pool isolates.

CHARACTER	CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7	CCR8	CCR9	CCR10	CCR11
Cell size (μm)	6	4-6	6-10	10	6-12	5-9	4-14	6-12	7-13	4-8	8-14
Motility	+	+	+	-	+	+	+	+	+	-	+
Temperature (ºC):											
Range	40-65	40-70	40-70	40-70	40-70	40-70	40-70	40-70	40-70	40-70	40-70
Optimum	nd	nd	63-70	62-68	55-60	nd	65-70	65-70	nd	55-60	55-60
pH:											
Range	7-8	7-8	5-9	5-8	7-9	5-9	7-9	7-9	7-8	7-9	7-8
Optimum	nd	nd	7-7.5	7-7.5	7-7.5	nd	7-7.5	7-7.5	nd	7-7.5	6.5-7
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Doubling time (min) at opt. conditions	nd	nd	69	22	20	nd	41	39	nd	41	78
NaCl:											
0.3 M	+	+	+	+	+	-	+	+	+	+	+
0.5 M	+	+	-	+	-	-	-	+	+	+	+
1-2 M	-	-	-	-	-	-	-	-	-	-	-
Production of:											
Proteases	-	+	+	-	+	+	+	+	+	+	+
Lipases	-	-	-	-	+	+	-	-	+	-	+
Amylases	-	-	+	-	+	+	-	-	+	-	+

Table 3.- Morphological and physiological characteristics of Los Baños hot spring isolates.

CHARACTER	DR01	DR02	DR03	DR04	DR05	DR06	DR07	DR08
Cell size (μm)	3-5	2-3	2-5	5-6	3-4	3-4	2-3	3-2
Motility	-	-	-	-	+	+	+	-
Spores	+	+	+	+	+	-	+	+
Temperature (ºC):								
Range	37-60	37-60	37-60	37-60	40-70	25-60	25-70	25-70
pH:								
Range	6-11	6-11	6-11	7-11	6-10	7-9	6-10	6-10
Oxidase	+	+	+	+	+	+	+	+
Production of:								
Proteases	-	-	-	-	-	-	+	+
Lipases	+	-	+	-	+	+	-	+
Amylases	+	-	-	-	-	-	+	+

Table 4.- Phylogenetic analysis of the thermophilic isolates from El Carrizal thermal pool and Los Baños hot spring.

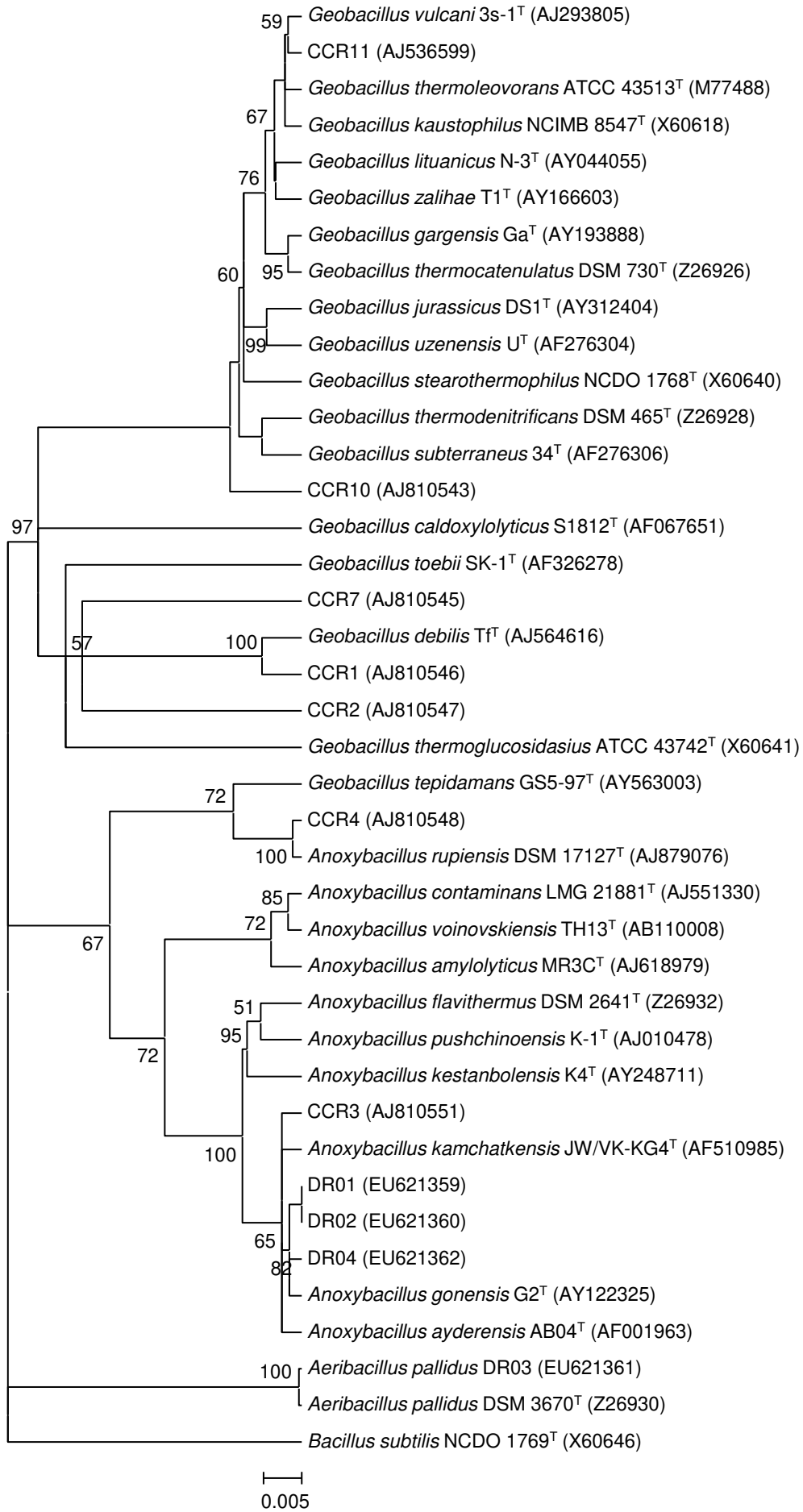
STRAIN	CLOSEST MATCH	% SIMILARITY	EMBL ACCESSION NUMBER
CCR1	*Geobacillus debilis*	99.4	AJ810546
CCR2	*Geobacillus thermoglucosidasius*	100	AJ810547
CCR3	*Anoxybacillus kamchatkensis*	99.6	AJ810551
CCR4	*Anoxybacillus rupiensis*	100	AJ810548
CCR7	*Geobacillus toebii*	99.6	AJ810545
CCR10	*Geobacillus subterraneus*	98.9	AJ810543
CCR11	*Geobacillus thermoleovorans*	99.9	AJ536599
DR01	*Anoxybacillus kamchatkensis*	99.5	EU621359
DR02	*Anoxybacillus kamchatkensis*	99.5	EU621360
DR03	*Aeribacillus pallidus*	99.9	EU621361
DR04	*Anoxybacillus kamchatkensis*	99.5	EU621362

Table 5.- Analysis of isolated strains for microbial enhanced oil recovery activities and biodesulfurization.

STRAIN	GROWTH ON	PRODUCTION OF				DBT DEGRADATION
	Kerosene	Gas	Acids	Exopolymers (mg/mg biomas)	Surfactants (E_{24}, %)	2-HBP (mg/L)
CCR1	+	-	-	11.8	5	2.8
CCR2	+	-	+	1.5	7.5	2.7
CCR3	+	-	+	2.2	1.8	2.9
CCR4	+	-	+	0.31	5	0.7
CCR5	+	-	+	4.1	1.6	1.3
CCR6	+	-	+	5	5.3	0.9
CCR7	+	-	+	5.7	5	1.8
CCR8	+	-	+	2.2	5	1.3
CCR9	-	-	+	3.4	0	2.7
CCR10	+	-	-	3.9	0	1.9
CCR11	+	-	+	5	3.7	2.1

E_{24} : Emulsification index DBT: dibenzothiophene 2-HBP: 2-hydroxibiphenyl

Figure 1. Consensus neighbour-joining phylogenetic unrooted tree (Jules-Cantor model and pairwise deletion option) from sequences of the 16S rRNA gene, encompassing all *Aeribacillus*, *Anoxybacillus* and *Geobacillus* species. The number of GenBank accession sequences is indicated in brackets. The bar represents distance values calcuated by MEGA. Bootstrap values (>50%) after 1000 replicates are shown.


59
Geobacillus vulcani 3s-1T (AJ293805)
CCR11 (AJ536599)
Geobacillus thermoleovorans ATCC 43513T (M77488)
67
Geobacillus kaustophilus NCIMB 8547T (X60618)
Geobacillus lituanicus N-3T (AY044055)
76
Geobacillus zalihae T1T (AY166603)
Geobacillus gargensis GaT (AY193888)
60
95
Geobacillus thermocatenulatus DSM 730T (Z26926)
Geobacillus jurassicus DS1T (AY312404)
99
Geobacillus uzenensis UT (AF276304)
Geobacillus stearothermophilus NCDO 1768T (X60640)
Geobacillus thermodenitrificans DSM 465T (Z26928)
Geobacillus subterraneus 34T (AF276306)
CCR10 (AJ810543)
97
Geobacillus caldoxylolyticus S1812T (AF067651)
Geobacillus toebii SK-1T (AF326278)
CCR7 (AJ810545)
57
100
Geobacillus debilis TfT (AJ564616)
CCR1 (AJ810546)
CCR2 (AJ810547)
Geobacillus thermoglucosidasius ATCC 43742T (X60641)
72
Geobacillus tepidamans GS5-97T (AY563003)
CCR4 (AJ810548)
100
Anoxybacillus rupiensis DSM 17127T (AJ879076)
85
Anoxybacillus contaminans LMG 21881T (AJ551330)
72
Anoxybacillus voinovskiensis TH13T (AB110008)
67
Anoxybacillus amylolyticus MR3CT (AJ618979)
51
Anoxybacillus flavithermus DSM 2641T (Z26932)
95
Anoxybacillus pushchinoensis K-1T (AJ010478)
72
Anoxybacillus kestanbolensis K4T (AY248711)
CCR3 (AJ810551)
100
Anoxybacillus kamchatkensis JW/VK-KG4T (AF510985)
DR01 (EU621359)
DR02 (EU621360)
65
DR04 (EU621362)
82
Anoxybacillus gonensis G2T (AY122325)
Anoxybacillus ayderensis AB04T (AF001963)
100
Aeribacillus pallidus DR03 (EU621361)
Aeribacillus pallidus DSM 3670T (Z26930)
Bacillus subtilis NCDO 1769T (X60646)
0.005