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Full Length Research Paper

Metabolic Diversity of Thermophilic Bacteria from Hot Springs in Algeria

Wahiba Yakhlef *, **Abdelhak Darbouche**

Institute of Biology, Department of Microbiology, University Khenchela (Algeria)

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ABSTRACT

Hydrothermal samples have been collected from hot springs in the northeast of Algeria (57–98°C). The samples in question are considered as extreme ecosystems. The first spring (57°C) have the highest thermophiles isolation rates. Three thermophilic aerobic strains have been purified. The isolates have an optimum growth temperature of about 45 to 70 °C and pH (6.5 – 8.0).The isolates exhibited extracellular amylase, protease and nitrate-reductase activities at high temperature and showed an antibacterial activity against at least one of the test-bacteria studied using the agar cylinder method. The study of their morphological, physiological and biochemical characteristics suggests that these isolates belong to the genera *Pseudomonas sp., Thermus sp.* and *Geobacillus sp*. Such studies are needed to understand the microbial communities that are native to the hot springs and their interest in biotechnology.

Keywords: Hot springs, thermophily, bacteria, enzymatic activity, antibacterial activity

INTRODUCTION

Many terrestrial hot springs exist on Earth. Thermophilic microorganisms associated with these hot ecosystems have received considerable interest in recent years (Brock, 1978; Kristjansson & Stetter, 1992; Vieille & Zeikus, 2001). Enzymes from these microorganisms also got special attention from the scientist from all over the world since this enzymes resistant to chemical reagents and extreme pH and temperature values in comparison to their mesophilic homologues (Akmar *et al.*, 2011). There are few studies on thermophilic bacteria inhabiting the numerous terrestrial hot springs located in Algeria. *Pyrococcus*, hyperthermophilic *Archaea* and *Caldicoprobacter algeriensis*, xylanolytic thermophilic bacteria, were recently isolated from the North of Algeria (Kesha *et al*., 2007; Bouannane *et al*., 2011).

However, to our knowledge, no thermophilic aerobic species had been already described so far. The main objective of this study was to isolate thermophilic bacteria from hot springs in Guelma, Algeria, and then test their ability to produce thermostable enzymes and antimicrobial agents.

MATERIALS AND METHODS

Sample collection and isolation procedure

Samples were collected from three geothermal hot springs (57–98°C) located in Guelma, northeast of Algeria. Water samples were transported without temperature control and filtered the same

*Corresponding author: wyakhlef@hotmail.fr

day through membrane filters (Gelman type GN-6, pore size 0.45 µm, diameter 47 mm); the filters were placed on the surface of *Thermus* medium (Williams & da Costa, 1992). The plates containing the filters were wrapped in plastic bags and incubated at 55°C for up to 4 days. Cultures were purified by subculturing on *Thermus* medium and were maintained at -70 °C in the same medium with 15% (v/v) glycerol.

Morphological studies

The cellular morphology of strains was examined using light microscopy on cells grown on *Thermus* agar plates for 18h at 55°C. Each agar coated wet mount used for motility observations was prepared by placing 10 ml culture under a cover-glass on a glass slide that had been previously coated with a film consisting of 0.5% (w/v) agarose (Cambrex). Gram staining of strains was determined using the bioMérieux Gram stain kit according to the manufacturer's instructions. Micrographs were taken with a Nikon optishot microscope equipped with a Nikon FX-I1 camera system.

Physiological tests

Determination of the optimum growth temperature

All physiological tests were performed as described previously (Santos *et al*., 1989, Nunes *et al*., 1992). The growth temperature ranges of the strains were examined by measuring the turbidity (610 nm) of cultures incubated at different temperatures (15 to 85°C) in 300 ml Erlenmeyer flasks, containing 100 ml of *Thermus* medium, in a reciprocal water-bath shaker 200 (rpm).

Determination of the optimum pH of growth

The pH range for growth was examined by measuring the turbidity (610 nm) of cultures incubated at 55 °C in the same medium using 20 mM MES for pH values between 5.0 and 6.5, 20 mM Tris for pH values between 7.0 and 8.5, and 20 mM CAPSO [3-(cyclohexylamino)-2-hydroxy-1 propanesulf-onic acid] for pH values upper than 9.0; the pH of each buffer was adjusted with HCl or NaOH. The pH values of the cultures were determined at room temperature. Control media, containing each buffer adjusted to pH 7.5, were used to assess possible inhibitory effects of the buffering agents.

Biochemical tests

The isolates were identified by the use of conventional methods for the presumptive identification by biochemical tests. Oxidase activity was tested using a Bactident Oxidase strip (Merck), whereas catalase activity was determined by bubble production in a 3% (v/v) hydrogen peroxide solution. Hydrolysis of starch, casein, and urea was determined as described by Lanyi (1987). Acid production, by the isolate, from various carbohydrates was characterized using the API kit (bioMérieux) according to the manufacturer's instructions. Denitrification reactions were performed in 5ml nitrate broth tubes includes (gl^{-1}), 25, Brain heart infusion and 10, $NaNO_3$ at pH 7.2. After, inoculation the tubes were sealed and incubated under aerobic conditions at 55°C. All the biochemical tests are listed in Table 2.

Antibacterial activity

Thermophilic isolates were grown on *Thermus* agar plates for 48h at 55 °C. Agar cylinders (3 mm in diameter) were then taken with hollow punch and deposited on the surface of the Mueller–Hinton media (Merck), which had previously been seeded with each test-

bacteria. These last were obtained from the American Type Culture Collection (ATCC); they are two Gram-positive bacteria (*Staphylococcus aureus* ATCC *25923* and *Staphylococcus aureus ATCC 43300*) and two Gram-negative bacteria (*Escherichia coli* ATCC *25922* and *Pseudomonas aeruginosa ATCC 27853*). Plates were kept at 4°C for 4h, then incubated at 37 °C for 18–24 h and the activity of each isolate was estimates by measuring the inhibition zones in (mm). Inhibition zone 2 mm or more were considered as positive result (Aktypis *et al*., 1998; Lemriss *et al.,* 2003).

RESULTS AND DISCUSSION

Hot springs and samples

Three hot springs located in the northeast of Algeria were investigated by collection of three samples of water. Their temperature, pH and chemical composition (data provided by the direction of tourism Guelma; Lahlou *et al.*, 1998; Bouannane *et al*., 2011) determined at the sample site are reported in Table 1.

Generally, the pH of spring waters is neutral, but two of the springs tested were slightly alkaline (spring 1 at pH 7.8 and spring 2 at pH 7.7). Temperatures ranged from 57°C (spring 1) to 98°C (spring 3), which has the highest recorded temperature in Algeria. In this spring; we noted the presence of hydrogen sulfide (H_2S) and arsenic (As).

Isolation of thermophilic bacteria

Sample collected from the spring 1 (57°C) showed the higher rates of thermophiles isolation compared to others springs. However, Spring 3 (98°C) showed the lower isolate rate (Fig. 1). This is not surprising given the fact that oxygen has a low solubility at high temperature and reducing gases are widely present. However, this favors the predominance of anaerobic organisms in these environments (Stetter, 1996; Huber & Stetter, 1998; Adams & Kelly, 1998). Otherwise, many recent molecular studies have shown that hot springs with moderate temperature (55-70°C) have the highest microbial diversity (Lau *et al*., 2006).

Thermophilic bacteria were isolated according to techniques of Williams and da Costa. *Thermus* agar used in this study appeared to adequately support the growth of microbe of hot springs when incubated at high temperature. The isolates grew chemoheterotrophically under aerobic conditions, but no growth was observed under anaerobic conditions.

Characterization and identification of the isolates

The three strains selected were designated STG, SB1 and SDJ. Their morphological, physiological and biochemical characteristics are summarized in Table 2.

Strain STG, isolated from spring 1 (Hammam Ouled Ali, 57°C), formed very short, avoid, Gram-negative, non-motile, rod-shaped cells (Fig. 2). Colonies were smooth and brown. It had an optimum growth temperature between 45 and 55 °C and did not grow at 65 °C (Fig. 3-4). The optimum pH for growth of strain STG was between pH 7 and 7.5.

Strain SB1, isolated from spring 2 (Hammam Belhachani, 72°C), formed long, rod-shaped, non-motile cells that stain Gram-negative. Endospores are not formed. Colonies were yellow pigmented with optimum pH values between 7.5 and 8.0 and an optimum temperature between 60 and 70°C (Fig. 3-4).

Strain SDJ, isolated from spring 3 (Hammam Dbegh, 98°C), formed very long, rod-shaped, mobile, Gram- positive cells. Produce terminally located ellipsoidal spores.

Table 1 Physico-chemical data of the three hot springs.

	Samples		
	1	**2**	**3**
	Hammam Ouled Ali[a]	Hammam Belhachani[b]	Hammam Dbegh[b]
Location	36°30'N / 07°27'E	36°30'N / 07°23'E	36°27'N / 07°16'E
T(°C)*	57	72	98
pH*	7.8	7.7	7.3
Débit (L/s)	20	11	1650
Ca	224	70.2	130
Mg^{2+}	19	33.5	37.4
K^{+}	05	1.3	46
Na^{+}	40	22.3	240
Cl^{-}	ND	31.2	370
SO_4^{2-}	300	171.6	385
HCO_3^{-}	397	nd	183
H_2S	-	-	6.80
As	-	-	0.45

- : absence
nd : not determinate
* : measured *in situ*
[a] : data provided by the direction of tourism Guelma.
[b] : data from (Lahlou M. *et al*., 1998; Bouannane *et al*., 2011)

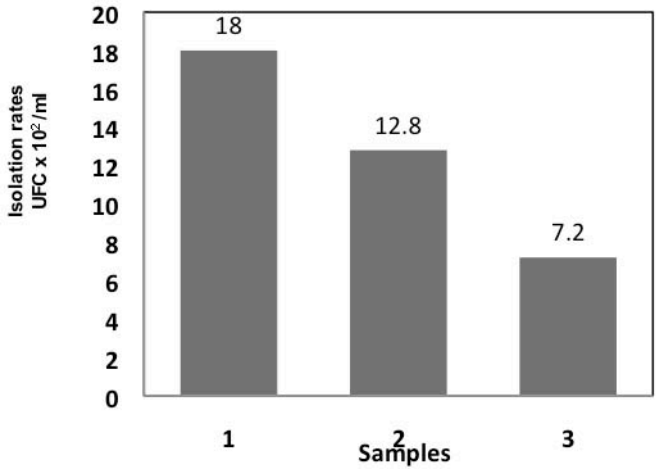

Fig. 1 Thermophiles isolation rates from the three hot springs. 1, from spring 1; 2, from spring 2; 3, from spring 3.

Colonies were mucous, flat, and non-pigmented with a filamentous shape. It had an optimum growth temperature between 50 and 60 °C and an optimum pH values between 6.5 and 7.0 (Fig. 3-4). Consequently, the isolates belong to thermophilic bacteria, because they grew optimally at temperatures up to 50°C, and they have a growth range from 45 to 70°C (Madigan *et al*., 2006). The three strains grow optimally at a pH range from 6.5 to 8.0 and no growth was detected at pH 5 or 9 (Fig. 3-4).

The fact that the Isolates are strictly aerobic microorganisms was further supported by the presence of catalase and oxidase activity for all the isolates.

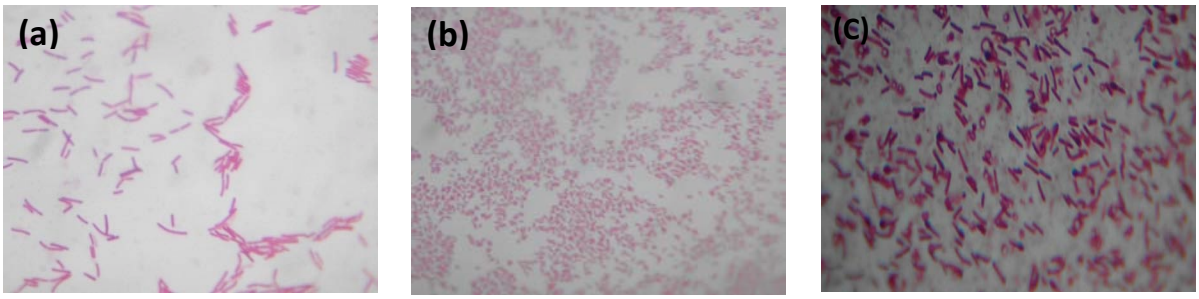

Fig. 2 Gram reaction of the thermophilic isolates. (a), strain SB1 Gram negative (long rods); (b), strain STG Gram negative (avoid rods); (c), strain SDJ Gram positive (long spore formers rods).

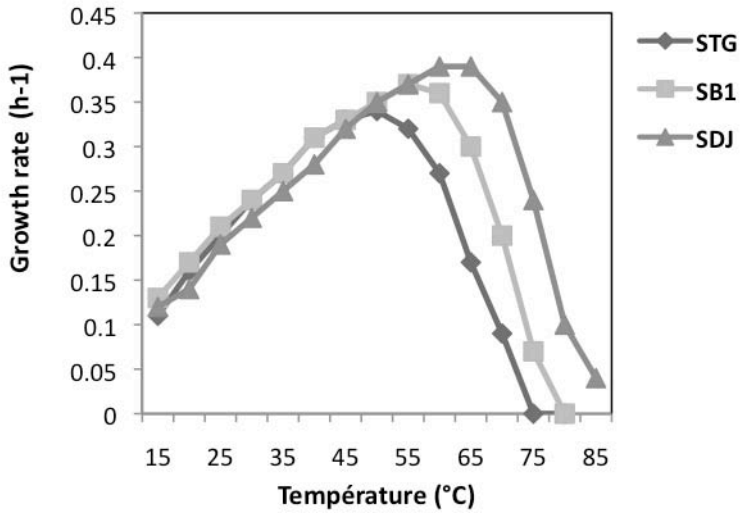

Fig. 3 Effect of temperature on the growth rates of the strains STG, SB1 and SDj.

They were unable to ferment the most of the carbohydrates or polyols examined.

The three thermophilic strains have a proteolytic enzyme that hydrolyzes casein into amino acids. They were also able to hydrolyse strach at high temperatures. Preliminary characterization of these enzymatic activities showed high thermostability, property which could be used in potential biotechnological applications.

Two strains (STG and SDJ) were able to reduce nitrate to nitrite. This ability to denitrify makes them good candidates to actively participate in the global nitrogen cycle within terrestrial hot springs which has been poorly studied so far (Khelifi *et al*., 2010).

The three strains were active against at least one of the tests used bacteria. These results confirm the results of several previous studies that affirm the ability of thermophilic microorganisms isolated from terrestrial hot springs to produce antibacterial substances active against pathogenic microorganisms (Muriana et al., 1991; *Novotny & Perry*, 1992; *Khalil et al.*, 2006). One such area of interest is the use of these bacteriocins in eliminating organisms that are responsible for food spoilage or food-related pathogenicity (*Novotny & Perry*, 1992).

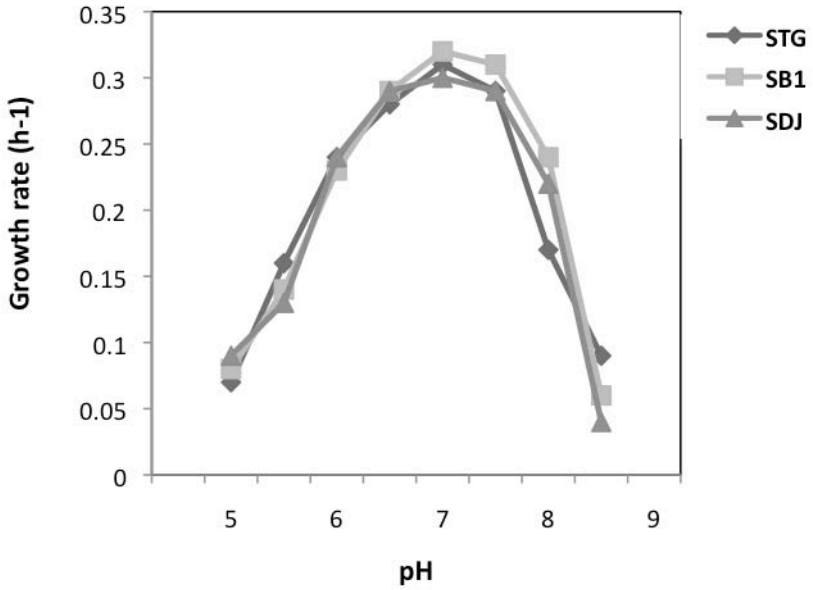

Fig. 4 Effect of pH on the growth rates of the strains SBA, SB1 and SDj.

The study of their morphological, physiological and biochemical characteristics suggest that thermophilic strains STG, SB1 and SDJ belong to the genera *Pseudomonas sp., Thermus sp. and Geobacillus sp.,* respectively (Table 2). These finding were on perfect concordance with the study of Sayeh *et al.*, who have demonstrated a large presence of *Firmicutes* in hot springs with very high temperatures (> 90 °C) However, at lower temperatures springs (50-70°C), members belonging to the *Gammaproteobacteria* subdivision were largely represented.

CONCLUSION

The research of thermophilic bacteria from Algerian hot springs with a temperature rang (57-98°) and pH rang (7.3-7.8) has led to the isolation of three strains that have similar characteristics to the genera *Pseudomonas sp., Thermus sp. and Geobacillus sp.* The isolates grew optimally at temperatures and pH range from 45 to 70°C and from 6.5 to 8.0, respectively. These thermophilic isolates were able to hydrolyze starch and casein at high temperatures, two of them exhibited denitrification reaction, on reducing nitrate to nitrite. The antibacterial activity was detected on the three strains. Finally, the isolation of such strains from Algerian hot springs extends our knowledge on the microbial diversity inhabiting such extreme ecosystems and their interest in biotechnology.

Table 2 Morphological, physiological and biochemical characteristics of the strains STG, SB1 and SDJ.

	STG	SB1	SDJ
Motility	-	-	+
Sporulation	-	-	+
Gram stain	-	-	+
Oxidase	+	+	+
Catalase	+	+	+
Anaerobic growth	-	-	-
Growth at temperature			
45 °C	+	+	+
55°C	+	+	+
65°C	-	+	+
75°C	-	+	+
Growth at pH			
5	-	-	-
7.5	+	+	+
9	+	+	-
Hydrolyse of			
Strach	+	+	+
Casein	+	+	+
Citrate	-	-	-
Denitrification			
NO_3^- ----> NO_2^-	+	-	+
Gas from nitrate	-	-	-
β-galactosidase	-	-	-
Arginine-dihydrolase	-	+	-
Lysine-décarboxylase	-	+	-
Ornithine-décarboxylase	-	+	-
Tryptophane-désaminase	-	-	-
H_2S	-	-	-
Uréase	-	+	-
Indole	+	-	-
Voges-Proskauer reaction	-	-	-
Methyl red test	-	-	-
Gas from glucose	-	-	-
Acid production from			
D-glucose	-	-	-
D-melibiose	-	-	-
D-mannose	-	-	+
L-arabinose	-	-	-
L-rhamnose	-	-	-
Lactose	-	-	-
Sucrose	-	-	+
Inositol	-	-	-
Sorbitol	-	-	-
Amygdaline	-	-	ND
Antibacterial activity			
S. aureus ATCC 25923	+	+	+
S. aureus ATCC 43300	+	+	+
E. coli ATCC 25922	-	-	+
P. aeruginosa ATCC 27853	-	-	+

+, positive result or growth; -, negative result or no growth; ND, not determined.

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