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Original article

Screening of rare actinomycetes isolated from natural wetland ecosystem (Fetzara Lake, northeastern Algeria) for hydrolytic enzymes and antimicrobial activities

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ABSTRACT

Actinomycetes from unexplored habitats are considered as a promising source for novel bioactive compounds with a broad range of biological activities. A study was carried out to isolate and identify rare actinomycetes producing antimicrobial from a natural wetland. Water samples from Fetzara Lake (North eastern-Algeria) were collected and subjected to rare actinomycetes isolation using different rich media. Eight selected actinomycetes were screened *in vitro* for hydrolytic enzymes, antibacterial and anticandidal activities. Based on the 16S rRNA sequencing, the eight actinomycetes isolates were categorized into four different rare genera *Actinomadura, Nocardia, Nonomuraea* and *Micromonospora*. Interestingly, significant anticandidal and antibacterial activities against both Gram-positive and Gram-negative bacteria were observed. Furthermore, the actinomycetes isolates were able to produce different hydrolytic enzymes with potential industrial and food processing applications such as amylase, cellulase, protease, and lipase. Overall, the study revealed that the selected aquatic rare actinomycetes recovered from Fetzara Lake presented good candidates to be explored as new sources of bioactive compounds.

1. Introduction

Actinomycetes are present in various ecological habitats and are particularly abundant in soil (Takizawa et al., 1993; Kinkel et al., 2012), as well in fresh water and other marine environments (Lam, 2006; Valli et al., 2012). They are considered highly valuable as they produce various secondary metabolites and other biologically useful compounds such as antibiotics, antitumor agents, immunosuppressive agents, vitamins, nutritional materials, herbicides, pesticides, antiparasitic agents and enzymes (Bérdy, 2012; Mohseni et al., 2013; Abdelmohsen et al., 2015). Actinomycetes have been for decades major storehouse microorganisms for the discovery of natural products (Choi et al., 2015) and *Streptomyces*, the best-characterized genus of actinomycetes, is considered one of the most important types of industrial bacteria due to its superior capabilities in producing valuable secondary metabolites and bioactive compounds (Pak and Elliot, 2010). They are able to metabolize various compounds of complex structures such as polysaccharides, alcohols, amino acids and aromatic compounds by the production of extracellular enzymes such as amylase, chitinase, cellulase, glucanase, and protease (Antonopoulos et al., 2001).

In recent years, antimicrobial resistance is spreading faster than the introduction of new compounds into clinical practice, causing a public health crisis (Laallam et al., 2015; Ling et al., 2015; Menasria et al., 2015). The research for novel source of a potent bioactive compound for multidisciplinary uses as yet be needed (Rungroch and Nakaew, 2015) and the development of new technologies to find and produce such compounds have again attracted interest in this field (Tilmann et al., 2015).

The rare actinomycetes are considered as a promising source for novel bioactive compounds and hydrolytic enzymes with a broad range of biological activities and pharmacological properties (Arul et al., 2014; Benhadj and Gacemi-Kirane, 2016). Rare actinomycetes are defined as genera in which the isolation frequency by conventional methods is lower than the *Streptomyces* abundance such as *Actinomadura, Actinoplanes, Amycolatopsis, Actinokineospora, Acrocarpospora, Actinosynnema, Catenuloplanes,*

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Cryptosporangium, Dactylosporangium, Kibdelosporangium, Kineosporia, Kutzneria, Microbiospora, Microtetraspora, Nocardia, Nonomuraea, Planomonospora, Planobispora, Pseudonocardia, Saccharomonospora, Saccharopolyspora, Saccharothrix, Streptosporangium, Spirilliplanes, Thermomonospora, Thermobifida and *Virgosporangium* (Lazzarini et al., 2000; Tiwari and Gupta, 2013).

Because of the exhaustion of the usual terrestrial sources, the discovery of new compounds from marine ecosystems has subsequently increased and relatively few efforts with aquatic rare actinomycetes have been attempted. It has been shown that marine actinomycetes are phylogenetically and physiologically distinct from their terrestrial relatives and were found to represent a rich source for diverse and interest bioactive secondary metabolites and enzymes with potential industrial and clinic applications (Maldonado et al., 2005). Algeria harbors several wetlands and hypersaline lakes, with rare typology and ecology in the world, and of which 50 are classified as being of international importance as Ramsar sites (Menasria et al., 2018). From sub-tropical in the coastal northeast part of the country to semi-arid in the Hauts Plateaux and an arid climate across the Sahara, the Algerian wetlands constitute an important habitat in terms of biodiversity and functional role. However, all aspects related to microbiota (diversity and bioactivity) are poorly investigated and remains unidentified. In response, and for the first time, the aim of this work is to characterize and to study of hydrolytic enzymes, antibacterial and anticandidal activities of rare actinomycetes isolated from Fetzara Lake (northeastern Algeria), a natural representative wetland in the Mediterranean region.

2. Material and methods

2.1. Study area

Located in north-eastern Algeria, the Lake Fetzara (Lat 36°-43′and 36°50′N, Long 7°24′ and 7°39′E) is one of the most important coastal wetlands within the Western Mediterranean Basin. In 2003, the lake was included as a wetland with international importance under the Ramsar Convention (Ramsar Convention Official Website, www.ramsar.org). The total site area covers 18600 ha, presenting an important natural reserve for migratory birds and wildfowl species.

2.2. Actinomycetes isolation and their maintenance

The rare actinomycetes were isolated from Fetezara lake, using agar plating method in two different culture media, (i) International Streptomyces Project N°2 (ISP2) supplemented with (2.5 μg/ml of rifampicin, 10 μg/ml of amphotericin B and 75 μg/ml of fluconazole) and (ii) Emmerson agar supplemented with (10 μg/ml of streptomycin, 10 μg/ml amphotericin B and 75 μg/ml of fluconazole). Five water samples were heat treated at 50 °C and dilution series were prepared from samples with aliquots (0.1 ml) being spread plated onto the appropriate agar. The plates were incubated at three different temperatures 10 °C, 28 °C and 37 °C up to 4 weeks and the colonies of actinomycetes were recognized according to their macroscopic and microscopic characteristics. Suspected actinomycetes were subcultured and maintained on ISP2 agar at 4 °C and at -80 °C on the 20% of glycerol as mycelia suspension.

2.3. Phenotypic and physiological characterization

Pure isolates were characterized based on their microscopic, morphological and biochemical characters using standard methods (Shirling and Gottlieb, 1966; Gordon et al., 1974). For the evaluation of growth characteristics, physiological and biochemical characteristics, the actinomycetes isolates were incubated for 15 to 28 days.

The morphological characterization of the isolates was carried out according to the International Streptomyces Project (ISP) using ISP1, ISP2, ISP3, ISP4 and ISP5 media. The observations of growth characteristics were assessed after 3, 7, 14 and 21 days of culture at 30 °C. The ISP6 and ISP7 were used for detection of production of melanoid pigments. Growth was tested at pH 5.0–10.0 (at intervals of 2.0 pH units) and at 4, 25, 30, 37, and 44 °C on nutrient agar. NaCl tolerance was studied on nutrient agar containing NaCl at final concentrations of 0–10% (w/v) (at intervals of 2.5%). The minimal basal medium (ISP9) was used to determine the capacity of the isolates to use different carbon source at 1% of final concentration.

2.4. Molecular identification

2.4.1. Genomic DNA extraction

For DNA extraction, biomasses were obtained by growing the strains in Hickey-Tresner (HT) liquid medium and the genomic DNA was extracted as described by Kieser et al. (2000).

2.4.2. PCR amplification and phylogenetic analysis

The 16S rRNA gene was amplified using universal primers Fd1 and rP2 primers FD1 (5′-AGAGTTTGATCCTGGCTCAG) and rP2 (5′-AAGGAGGTGATCCAGCC) as described by Weisburg et al. (1991) and the purified PCR products were sequenced with the same primers for PCR reaction. The homology search was performed by comparing the sequence with thus present in the public database (NCBI) using the standard Basic Local Alignment Search Tool (BLAST) program as well as with the EzTaxon-server (http://eztaxon-e.ezbiocloud.net/). Phylogenetic analyses were conducted using MEGA software version 6 and the 16S rRNA genes of rare actinomycetes were aligned against neighboring nucleotide sequences using CLUSTALW Larkin et al. (2007). The phylogenetic tree was reconstructed by using the neighbour-joining (NJ) method (Saitou and Nei, 1987) and the topologies were evaluated by bootstrap sampling expressed as a percentage of 1000 replicates (Felsenstein, 1985).

2.5. Extracellular enzyme production

The actinomycetes isolates were screened for amylase, gelatinase, protease, lipase, urease, nitrate reductase and the hemolytic activity according to Larpent and Larpent Gourgaud (1997) using starch, gelatin, casein and tween 80 as substrates.

2.6. Antimicrobial essay

2.6.1. Microorganisms

Fifteen bacterial tests were used for the antibacterial bioassay with seven references strains, *S. aureus* ATCC25293, *S. aureus* ATCC43300, *Bacillus subtillis* ATCC 6633, *Micrococcus luteus* DSM 1790, *Escherichia coli* DH5α, *E. coli* ATCC25422, *Pseudomonas aeruginosa* ATCC 27853 and eight other clinical isolates. For antifungal activity, five strains of *Candida albicans* were used. All clinical strains were recovered from hospitalized patients at the Hospital Center of Tebessa (Northeastern Algeria).

2.6.2. Agar diffusion method

Antimicrobial activity of isolated actinomycetes was evaluated using cultures on ISP2, Bennet, and Glucose Yeast Extract Agar (GYEA) by the agar diffusion method (Badji et al., 2007; Kitouni et al., 2005). Actinomycetes were inoculated using spore suspension in three different media (ISP2, Bennet, and GYEA) and incubated for one week at 35 °C. After incubation, plugs of different actinomycete cultures were taken and deposited on the surface

of the Luria Bertani soft media (0.7%) (For bacteria) and Sabouraud dextrose agar (For *Candida*) which had previously been seeded with the indicator strains. The plates were kept at 4 °C for 2 h and then incubated at 37 °C for 24 h. The antimicrobial activities were determined by measuring the diameter of the inhibition zone.

3. Results and discussion

3.1. Isolation and phenotypic characterization of actinomycetes

Based on phenotypic characteristics (macro- and microscopic), eight suspected rare actinomycete strains were isolated from Fetzara Leke. As reported, for successful cultivation of rare actinomycetes groups, major requirement, and appropriate isolation methods are recommended (Bredholdt et al., 2007). In particular, the utilization of enriched selective media supplemented with different antimicrobial agents (antibacterial and antifungal antibiotics) (Shirling and Gottlieb, 1966; Qin et al., 2011).

The morphological and cultural characteristics of the actinomycete isolates were examined using different culture media. Colors of aerial and substrate mycelia were determined using the ISCC-NBS centroid color chart (Kelly and Judd, 1955). The eight actinomycetes isolates showed good growth on ISP1 and ISP2 medium (Fig. 1) and low growth on ISP6 and ISP7 associated with the production of diffusible pigments, after five to 10 days of incubation. The strains were moderately halo tolerates, with a NaCl concentration range for growth of 0– 7%. Growth occurs at 25 to 40 °C (optimum, 37 °C) and pH 6.0–10.0 (optimum, pH 7.0) except for the isolate E3N418 which present a growth at 44 °C (Table 1).

As presented in Table 1, different physiological characteristics and carbon substrates utilization were observed. Both of the two isolates EN418 and E3N419 presented potential assimilation profile comparing to other isolates.

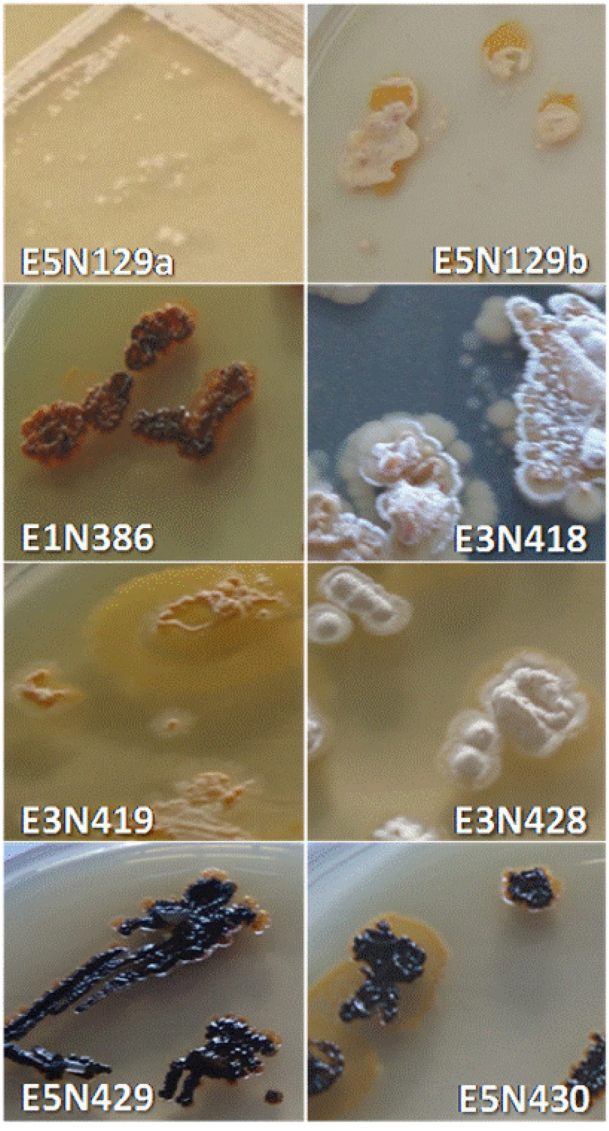

Fig. 1. Colony morphology of the eight strains grown on ISP2 at 28 °C for 2 weeks.

3.2. Molecular identification

The obtained sequences of the eight isolates E1N386, E3N418, E3N419, E5N129a, E5N129b, E5N428, E5N429 and E5N430 were subjected to alignment with the homologous closed sequences. The BLAST search of 16S rDNA sequences of the isolated actinomycetes showed highest similarity between (99 and 100%) with four different rare actinomycetes genera as well as *Actinomadura, Micromonospora, Nocardia,* and *Nonomuraea.* The constructed phylogenetic tree for partial 16S rRNA (>1400pb) sequences revealed that actinomycetes strains form four distinct phyletic lines within the four described genera species (Fig. 2). In addition, these results are supported and can be easily separated using the combination of physiological properties and the phenotypic characterization.

The three isolates E1N386, E5N430 and E5N429 belonged to the genus *Micromonospora,* of which the E1N386 is closely related (99.86%) to the newly *Micromonosporasa vinacea* GUI63(T) isolated from *Pisum sativum* nodules (Carro et al., 2016) and both isolates E5N430 and E5N429 shared 100% of similarity with *Micromonospora tulbaghiae* DSM45142 (Fig. 1). The isolates E5N129a, E5N129b and E3N419 are belonged to the genus *Nocardia* and closely related to the *Nocardia abscessus* NBR1003774 and *Nocardia rhamnosiphila* 202GMO, respectively. The only isolate E3N418 belonged to the genus *Nonumeraea* was identified as *Nonomuraea kuesteri* GW 14-1925 with 100% of similarity. The isolate E5N428 belonged to the genus *Actinomadura* and closely related to *Actinomadura geliboluensis* A8036.

3.3. Extracellular enzyme production

The metabolic characterization revealed other actinomycetes potency to produce different extracellular hydrolytic enzymes (Table 1). Furthermore, seven isolates produced at least three different enzymes and the majority produced the lipase in the first place followed by caseinase, gelatinase, cellulose, and amylase. These enzymes represent the largest groups of industrial enzymes (Kirk et al., 2002), which are extensively exploited commercially, in food, pharmaceutical, and detergent industry. The actinomycetes have a great capacity for biodegradation of different and complex substrate present in their natural habitats (McCarthy and Stanley, 1992; Tuomela et al., 2000) indicating the variety for their complex metabolites and genomic organization (Bentley et al., 2002) (in particular *Streptomyces* genus) (Narayana et al., 2007).

The scarcity of reports on industrially relevant enzymatic activities from the identified rare actinomycetes indicated their potential for the production of various hydrolytic enzymes with a promising prospect for industrial application.

3.4. Antimicrobial activity

The antimicrobial activity of the rare actinomycetes isolates is presented in Tables 2 and 3. The primary screening on plates showed that the actinomycetes strains exhibited a significant and

Table 1
Phenotypic characteristics and extracellular enzyme production of the isolated actinomycetes (+ Growth or positive reaction of the test strain, – no growth or negative reaction, ± average growth).

Characteristics		Isolates							
		E5N129a	E5N129b	E1N386	E3N418	E3N419	E5N428	E5N429	E5N430
Temperature	−4 °C	–	–	–	–	–	–	–	–
	25 °C	+	+	+	+	±	±	±	±
	30 °C	+	+	+	+	+	+	+	+
	37 °C	+	±	+	±	±	+	+	+
	44 °C	±	±	±	+	–	–	–	±
pH	3	–	–	–	–	–	–	–	–
	5	–	–	–	–	–	–	–	–
	7	+	+	+	+	+	+	+	+
	9	+	+	+	+	+	+	+	+
	10	+	+	±	±	+	+	+	+
NaCl%	0	+	+	+	+	+	+	+	+
	2.5	±	±	±	±	+	–	+	+
	5	±	±	–	±	+	–	+	+
	7	–	±	–	±	+	–	±	±
	10	–	–	–	±	–	–	–	–
Carbon source	Arabinose	+	–	–	+	–	–	+	+
	Fructose	+	–	+	–	–	–	–	+
	Galactose	–	–	–	–	–	–	–	+
	Glucose	+	+	+	+	+	+	+	–
	Melibiose	+	–	–	+	–	–	+	–
	Rhamnose	–	–	–	+	–	–	+	–
	Ribose	+	+	–	–	+	–	–	–
	Saccharose	–	–	–	–	–	–	+	–
	Xylose	–	–	–	–	+	+	–	+
	Inositol	+	–	–	±	–	–	+	–
	Mannitol	+	–	–	+	–	–	+	+
	Sorbitol	–	–	–	±	–	–	+	–
	Citrate	–	–	–	–	+	–	–	+
Decarboxylation and other	ADH	–	–	–	–	–	–	–	–
	LDH	–	–	–	–	–	–	–	–
	ODC	–	–	–	–	–	–	–	–
	TDA	–	+	–	+	–	+	+	–
	IND	–	–	–	–	–	–	–	–
	VP	–	–	–	–	–	–	–	–
	ONPG	–	+	–	–	–	–	–	–
	H_2S	–	–	–	–	–	–	–	–
Extracellular enzyme	Amylase	–	–	+	+	+	+	–	–
	Gelatinase	+	+	+	–	+	–	+	+
	Cellulase	+	+	+	–	–	–	–	–
	Lipase	+	+	–	+	+	–	+	+
	Protease	–	–	–	+	+	–	+	+
	Urease	–	+	–	–	–	–	–	–
	Hemolysis	γ	γ	β	γ	γ	α	β	β

variable antibacterial activity against both Gram-negative and Gram-positive bacteria (more frequent) and against at least one indicator organism except for the isolate E5N429 from which no activity was recorded. Such differences in susceptibility are in concordance with other studies by the fact that the Gram-negative strains were highly resistant to many antibiotics (Lucet and Birgand, 2011). In addition, different antibacterial activities were obtained using the three different media (ISP2, Bennet, and GYEA). These results were confirmed by Vijayakumar et al. (2012) reported the influence the culture conditions and the medium composition on the production of antimicrobial molecules. Furthermore, many actinomycetes presented a relatively different spectrum and antimicrobial activities due to different bioactive substances secreted rather than a single inhibitory compound (Benhadj et al., 2018; Mitra et al., 2008).

Antifungal activity of rare actinomycetes has been highlighted during the tests carried out on the clinical isolates. Among the tested actinomycetes, the isolate E5N129a (*Nocardia*) and the two *Micromonospora* strains (E1N386 and E3N418) were significantly effective against the tested pathogenic yeasts (10.5 ± 1.5 to 33.5 ± 1.5 mm) (Table 3). In contrast, weak or no activity was recorded using the *Nonomuraea* isolate (E5N428), *Nocardia* sp. E5N428 and the two *Micromonospora* strains (E5N429, E5N430). The anticandidal activity of a rare actinomycete has been reported recently (Tanvir et al., 2016). Further, the two genera *Micromonospora* and *Nocardia* were previously reported for the production of antimicrobial compounds which showed broad-spectrum against both bacterial and fungal pathogens (Bredholdt et al., 2007; Kavitha et al., 2010).

Actinomycetes from several unexplored environments have been studied intensively in last few decades and more than 50 rare actinomycete taxa are reported to be producing more than 2000 bioactive compounds (Mitra et al., 2008; Subramani and Aalbersberg, 2013). Several studies reported the production of various antimicrobial compounds by *Actinomadura, Nocardia* and *Nonumeraea* strains (Jalali et al., 2016; Kodani et al., 2016). Similar studies indicated the antagonistic activity of actinomycetes isolates such as *Micromonospora*, against human pathogens (Lee et al., 2012; Talukdar et al., 2012). However, no previous reports on antibacterial activity were found for *Micromonosporasa vinacea, Nocardia abscessus*, *Nocardia rhamnosiphila*, *Nonomuraea kuesteri* or *Actinomadura geliboluensis* strains.

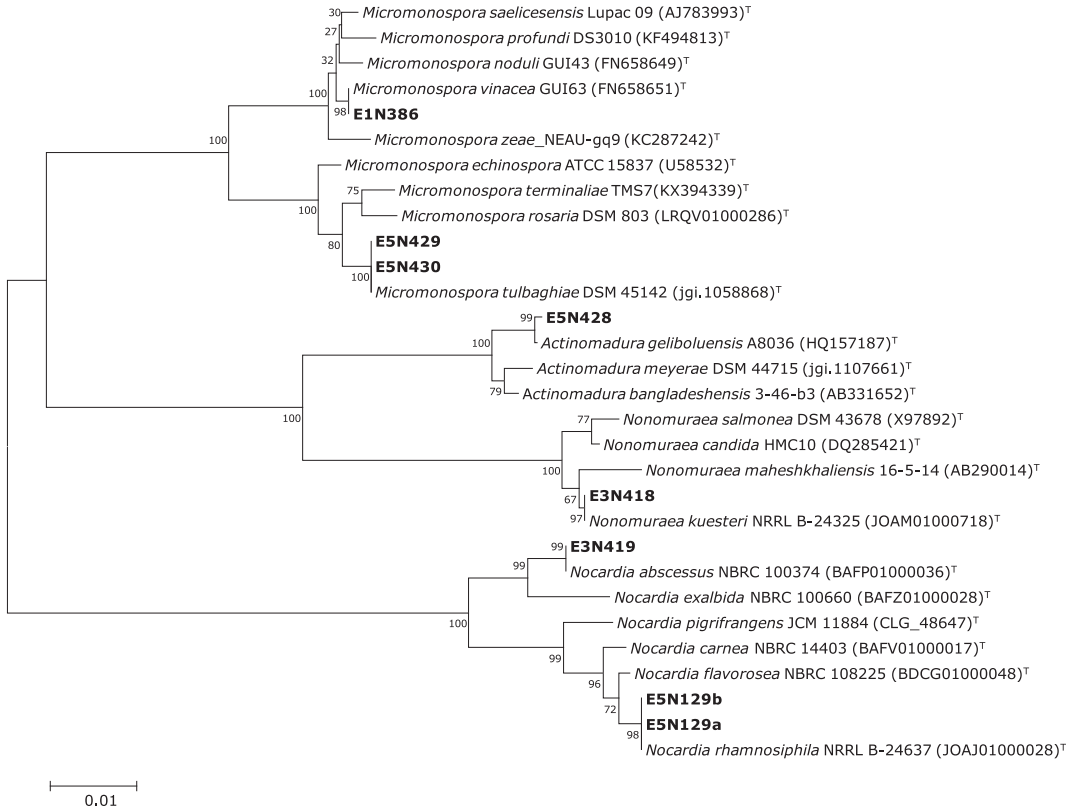

Fig. 2. Neighbor-Joining tree based on 16S rRNA sequence. The phylogenetic tree shows the relationships between isolated actinomycetes and related type strains. Percentage bootstrap values based on 1000 resampled data sets are shown at the nodes; only values above 50% are given. The scale bar indicates 0.01 nucleotide substitution per nucleotide position.

Table 2
Antibacterial activity of the isolated actinomycetes (I: ISP2; B: Bennet; G: GYEA).

Tested bacteria	Actinomyctes isolates																							
	E5N129a			E5N129b			E1N386			E3N418			E3N419			E5N428			E5N429			E5N430		
	I	B	G	I	B	G	I	B	G	I	B	G	I	B	G	I	B	G	I	B	G	I	B	G
E. coli ATCC25422	–	–	–	–	–	–	–	–	–	++[a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
E. coli DH5α	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P. aeruginosa ATCC27853	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
S. aureus ATCC25293	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	–	–	–	–	–	–	++	–	–
S. aureus ATCC43300	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
M. luteus DSM1970	–	–	–	–	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B. subtilis ATCC6633	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Staphylococcus sp.IC13	+++	+++	–	–	–	+++	+++	–	–	+++	–	–	–	–	+++	–	–	–	–	–	–	–	–	++
Citrobacter koseri IC8	–	–	–	–	–	–	–	++	++	–	–	++	–	–	–	++	–	–	–	–	–	–	–	–
Enterobacter sakazakii IC11	++	–	–	–	–	++	++	–	–	++	–	–	–	–	–	–	–	–	–	–	–	–	–	++
Klebsiella sp.IC10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
Morganella sp.IC1	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	–	–	–	–	–	–	++	–	–
Porteus mirabilis IC2	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	–	–	–	–	–	–	+	–	–
Serratia sp.IC4	–	–	–	–	–	–	–	–	–	–	–	–	–	++	–	–	–	–	–	–	–	+	–	–
Serratia sp.IC7	–	–	–	–	+++	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] Inhibition zone expressed as (+ < 20 mm; 20> ++ >30 mm; 30> +++ >40 mm).

Table 3
Anticandidal activity of the eight selected actinomycetes. The strains were grown at 30 °C on ISP2 agar medium during 14 days. Activity (diameter of inhibition, in mm) is given as the largest inhibition zone observed (D: day at which the activity was the most important). NA: no activity.

Isolates	Anticandidal activity (mm)									
	Candida albicans ICF 18		*Candida albicans* ICF 19		*Candida albicans* ICF 22		*Candida albicans* ICF 23		*Candida albicans* ICF 24	
E5N129a	NA		33.5 ± 1.5	(D10)	31.5 ± 1.5	(D10)	25 ± 1	(D10)	15 ± 2	(D10)
E5N129b	NA		NA		NA		NA		NA	
E1N386	21 ± 4	(D3)	25.5 ± 4.5	(D3)	24.5 ± 2.5	(D10)	23.5 ± 0.5	(D10)	10.5 ± 1.5	(D7)
E3N418	NA		31 ± 3	(D10)	22 ± 3	(D10)	23 ± 2	(D10)	12 ± 1	(D10)
E3N419	NA		NA		NA		NA		NA	
E5N428	NA		9 ± 0	(D7)	NA		10 ± 0	(D7)	9 ± 0	(D7)
E5N429	NA		NA		NA		NA		NA	
E5N430	NA		NA		NA		NA		NA	

Overall, the actinomycetes are one of the most attractive sources of new enzymes and bioactive metabolites. Recently, rare actinomycetes have been shown to be an important source of novel secondary metabolites and useful antibiotics. In spite of the limited number of isolates tested, this work constitutes a primary investigation on rare actinomycetes isolated from underexploited habitat (Fetzara Lake) which is a very specific ecosystem with regard to the occurrence of novel micro-flora that hold promising sources of extracellular enzymes and antibacterial compounds.

Conflict of interest

The authors declare that they have no conflict of interest

Authors' contributions

MB and DGK conceived and designed the study. MB, KG, and ZA conducted the experiment and laboratory work. MB and TM analysed data and drafted the manuscript. All authors read and approved the manuscript.

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