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ISOLATION OF LIPOLYTIC BACTERIA FROM COLOMBIAN ANDEAN SOILS: A TARGET FOR BIOPROSPECTING

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ABSTRACT

Microbial enrichments with a substrate of interest could enhance the possibility of finding certain desired metabolic activities. As lipases are one of the most important enzymes in industrial applications, the Colombian Andean soils were explored as a source of lipolytic microorganisms. Two Andean soils under low temperatures were sampled: paramo and glacier soils from "*Los Nevados*" National Natural Park. Both soils were enriched through a fed-batch fermentation using olive oil as the inductor substrate. Forty-three lipolytic isolates were obtained and their taxonomic assignments were performed on the basis of 16S rDNA gene sequencing. In both cases, the phylum Proteobacteria represented the majority of the isolates. Qualitative assays to measure the lipolytic activity were performed by using tributyrin, triolein or olive oil (1%). Two isolates identified as *Pseudomonas psychrophila* and *Stenotrophomonas rhizophila* produced the largest hydrolysis halos, with an optimal activity at pH 8 and 50°C. Only the *S. rhizophila* extracellular fraction hydrolyzed short and long chain pNP-esters, including pNP-palmitate. The broader substrate specificity of this isolate is probably due to the simultaneous presence of lipase and esterase activity in the crude extract. This is the first report of lipolytic activity in *S. rhizophila*, and the first preliminary characterization of the novel lipolytic activity in *S. rhizophila* and *P. psychrophila*. Further work is needed to purify and completely characterize the esterases and lipases produced by both species.

Keywords: 16S rDNA, Fed-batch fermentation, lipase, psychrotrophs

INTRODUCTION

Lipases are important biocatalysts with innumerable applications in food, dairy, detergent and pharmaceutical industries. The biological function of these enzymes is to catalyze the hydrolysis of triacylglycerols to release free fatty acids, diacylglycerols, mono-acylglycerols and glycerol **(Patil *et al.*, 2011)**. Lipolytic enzymes include esterases commonly defined as carboxyesterases (EC. 3.1.1.1) and lipases, originally called true lipases (**Sangeetha *et al.*, 2011**). Over the last few decades, lipases have gained special attention for their ability to act in micro-aqueous environments and catalyze esterification, trans-esterification, aminolysis and acidolysis reactions (**Joseph *et al.*, 2008**).
Bacterial lipases have valuable properties such as a wide variety of substrates, selectivity, stability in organic solvents, and activity in harsh conditions. Other advantages that promote research on these enzymes are their high yield, low production costs and the possibility of genetic manipulation **(Glogauer *et al.*, 2011; Meilleur *et al.*, 2009)**. Most of the lipases in industry are lipoproteins found in genera such as: *Achromobacter, Acinetobacter, Bacillus, Burkholderia, Pseudomonas, Staphylococcus, Chromobacterium,* among others **(Castilla *et al.*, 2017; Soliman *et al.*, 2007)**. These lipases can be used in different applications including production of fats and oils, detergents, fine chemicals, pharmaceutical drugs, pulp and paper, as well as use in bioremediation, waste treatment, oil biodegradation and medical applications **(Stergiou *et al.*, 2013).**
The low stability of well-characterized mesophilic enzymes under extreme conditions has prompted the search for new enzymes with better properties **(Tirawongsaroj *et al.*, 2008; Jiménez *et al.*, 2012; López *et al.*, 2014)**. Thus, the isolation of novel microorganisms capable of producing stable lipases for industrial applications is required **(Messiasa *et al.*, 2009; Abol *et al.*, 2016)**. Extreme environments with low temperatures are the most common places to find psychrophilic bacteria with lipolytic activity **(De Pascale *et al.*, 2012; De Santi *et al.*, 2016)**. Cold-adapted microorganisms produce lipases which function effectively at cold temperatures with high rates of catalysis, in comparison to those lipases synthesized by mesophiles or thermophiles that show low, or no activity at low temperatures **(Joseph *et al.*, 2008).** Some of the adaptations found in enzymes from extreme environments include structural flexibility, higher content of alpha helix compared to beta- sheets and temperature independent reaction rates **(Dalmaso et al., 2015).**
Previous studies have reported direct functional screening to isolate lipolytic microorganisms through enrichment cultures with lipid substrates including oils (olive oil, palm oil, oil mill), fatty acids and glycerol **(Yao *et al.*, 2013; Gombert *et al.*, 1999)**. Hence the enrichment cultivation in olive oil used in this study, was carried out based on the assumption that microorganisms able to survive on this substrate can synthesize lipases **(Gaoa *et al.*, 2000)**. One of the advantages of this technique is the accumulation of microorganisms with useful enzymes in growth-controlled conditions **(Ekkers *et al.*, 2012).**
The major aim of this study was to isolate lipolytic bacteria from low temperature environs found in the paramo and glacier soils from "*Los Nevados*" National Natural Park. The screening of lipolytic bacteria through the enrichment culture strategy, followed by the bacterial identification by 16s rDNA sequencing, and the preliminary enzyme characterization at different conditions (temperatures, pH and substrates) were the main steps that allowed us to bioprospect for putative novel lipases.

MATERIALS AND METHODS

Soil samples

Samples were taken from two cold environments in "*Los Nevados*" National Natural Park (Manizales – Colombia). The glacier soil sample (4°47´44.98" LN 75°21´52.33" LW at 5200 MAMSL) registered 4,1°C and pH 4,18. The sample from Paramo, rhizosphere-associated (4°51´51.1" LN 75°21´13.7" LW at 4322 MAMSL) registered 9°C and pH 4,88. Both samples were taken between 10 and 15 centimeters depth. The physico-chemical soil analysis was determined according to **Tedesco *et al.*, (1995)** including: pH, soil texture, electrical conductivity E.C. (ds/m), cation exchange capacity C.E.C (me/100), organic carbon percentage (%OC), phosphorus, potassium, sulfur, calcium, iron, and magnesium. All samples were stored at 4°C until they were used.

Enrichment cultures

The inoculum preparation started with the samples being washed in mineral salt medium (MSM), supplemented with tween 20 (0.5%) and olive oil 1%. This procedure was performed with eight grams of soil for each environment, followed by a 24 hours incubation at 140 rpm and at 22°C (**Wang *et al*., 2005).** After sedimentation, the supernatant was used as inoculum (10%) for the enrichment culture. This was performed with the same supplemented medium, in a fermenter batch adjusted to 1L bioreactor. The effective volume was 70%, and the cultures conditions were 22ºC, 140 rpm, pH 6.9 and 1.6 L min^{-1} of aeration during 3 weeks **(Lanser *et al*., 2002; Henne *et al*., 2000).** Every 5 days the bioreactor was fed with MSM ensuring the final effective volume. CFU mL^{-1} was estimated in nutrient agar (MoBio), **(Köhler, 2007; Wang *et al*., 2005).**

Isolation and morphological description

Functional screening was carried out at the end of the enrichment in MSM agar supplemented with olive oil or tributyrin at 22°C after three days **(Bunterngsook *et al*., 2010; Ertugrul *et al*., 2007)**. Colonies with hydrolysis halos were isolated and preserved in 15% glycerol solution at -80°C. The morphological description of colonies was performed and Gram staining was also included to verify cell shape and cell wall properties.

Lipase assay

Lipase production and secretion was evaluated in triplicate by agar diffusion assay. Briefly, MSM agar plates were supplemented with 1% of tributyrin, olive oil or triolein **(Chakravarthy & Narasu, 2012).** As preinoculum, one full loop of the isolated colonies was transferred in 10 ml LB broth at 20°C for 24 hours. After incubation, 5 µl of the inoculum was then transferred on filter paper disks and incubated on agar plates at 20°C for 8 days. The strains were examined daily for their ability to produce clarified zones **(Golani *et al*., 2016)**.

DNA extraction, amplification and sequencing of 16S rDNA gene

Total DNA was extracted from the isolates as previously described **(Spanevello *et al*., 2002).** Universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') **(Sarkar *et al*., 2014)** were used to obtain a PCR product of 1.5 Kb. A 100 µL reaction contained buffer 1X, MgCl 1.5 mM, dNTPs 0.2 mM, 0.3 µM of each primer, 0.5% of BSA and Taq Polymerase Promega 1U. PCR was carried out by an initial denaturation at 94ºC for 5 min; then 30 cycles of denaturation at 94ºC for 30 sec, annealing at 57ºC for 45 sec, extension at 72ºC for 1 min y 30 sec and finally an extension cycle at 72ºC for 7 min. PCR products were analyzed by electrophoresis 1.5% (wt/vol) agarose gels with SyBR-safe (InvitroGen™). The PCR products were sequenced via the Sanger method by Macrogen Inc. Korea.

Sequence alignments and phylogenetic analysis

The 16S rDNA sequences were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and the best hit was assigned to the query. Distance trees were constructed using the UPGMA algorithm, **(Bisht & Panda, 2011)** with the MEGA 7.0® package. The alignments were performed through CLUSTAL omega http://www.ebi.ac.uk/Tools/msa/clustalo0w2/). Bootstrap resampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies **(Felsenstein, 1985).** The sequences were submitted to the European Molecular Biology Laboratory (EMBL-ENA) Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/Submission/webin.html). The accession numbers of the nucleotide sequence data are listed in Table 1.

Growth curve experiments

The three strains that produced the largest hydrolysis halos, *Stenotrophomonas rhizophila* (strain USBA 843E), *Pseudomonas psychrophila* (strains USBA 844A, USBA 846E) were inoculated in Bioscreen® plates in triplicate with 200 µl as final volume and an OD_{540} adjusted to 0.02. Optical density was determined at 20°C each 6 h for 80 hours in order to obtain the their growth curves.

Lipase activity

USBA 843E, USBA 844A and USBA 846E strains were cultured in LB broth supplemented with olive oil 1% as inductor substrate at 20°C for 72 hours and 140 rpm. Then, they were centrifuged at 10.000 rpm for 7 minutes to recover the supernatant. The protein content was determined using the Bradford method **(Bradford, 1976)** and the enzymatic reaction was measured in triplicate in the Bioscreen®. For this reaction, 20 µl of supernatant were added to 180 µl of buffer Tris-HCl 0.1 M and 0.1 mM of lipid substrate emulsified in isopropanol **(Rashid *et al*., 2001).** The pNP-esters evaluated were: acetate, butyrate, decanoate and palmytate at 5ºC, 10ºC, 20ºC, 30ºC, 40ºC, 50ºC and 60ºC. The optimum pH of lipolytic activity of the strains was determined between 3 and 9, using 0.1M Glycine-HCl pH 3; 0.1M Acetate pH 4 and 5; 0.1 M phosphate pH 6; 0.1M Tris-HCl pH 7.8 and 9. This assay was carried out for 1 h at 30°C including biological triplicates. The activity was evaluated measuring the liberated p-nitrophenol at 405 nm. One unit of activity was defined as the amount of enzyme needed to release 1 µmol of p-nitrophenol per min.

Statistical analyses

The results obtained from the lipase activity assay were analyzed through the SPSS software (version 12.0). The analyses were performed using the Shapiro-Wilk Normality Test, and the pairwise comparison Kruskal-Wallis (Non-parametric) test to establish differences between the three substrates. The homogeneous subgroups test was used to select the strains with the best lipolytic activity.

RESULTS

Physico-chemical soil properties

The PNN glacier soil had special physicochemical properties due to its lack of vegetation, low temperature (4.1°), acidity (4.18), loamy texture, low percentage of organic Carbon (0,23%), high concentration of iron (385 ppm), phosphorus (94 ppm) and sulfate (88 ppm). Moreover, Paramo soil showed a similar pH (4.88), but a silt loam texture, with higher concentrations of potassium (85 ppm), calcium (333 ppm) magnesium (58 ppm), and organic carbon (5,7%), probably influenced by the presence of vegetation like *Espeletia* and *Calamagrostis*.

Enrichment culture and isolation of lipolytic bacteria

The initial pH was adjusted to 7 $\pm$ 0.2, and it decreased around 6.0 after 3 days. Regarding cell density, the paramo enrichment culture increased its initial cell count from10^5 CFU mL^{-1} to 10^9 CFU mL^{-1}, while the glacier enrichment increased from 10^3 CFU mL^{-1} to 10^8 CFU mL^{-1}. After the screening a total of 43 colonies were isolated, 30 from the paramo enrichment and 13 from the glacier.

Morphological description

The most common colony morphologies for the bacterial isolates were: wrinkled, mucoid, viscous, and translucent, with irregular and regular shapes, and with white, pale yellow or yellow tones. Exopolysaccharide production was observed in some isolates. Some colonies could change the morphotype after transferring to a new agar plate. Nevertheless, the microscopic observation showed the same Gram-negative, rod-shaped bacteria.

Lipase assay

After 8 days of incubation at 20ºC, six of the 43 isolates presented significant hydrolysis halos (statistical analysis). Isolates USBA 851A (*Pseudomonas fragi*), USBA 852C (*Ralstonia pickettii*), USBA 843E (*Stenotrophomonas rhizophila*), and USBA 844A (*Pseudomonas psychrophila*) produced the major hydrolysis halos in the tributyrin agar plates with diameters between 7 mm to 11 mm (Excluding the colony diameter). In triolein, the isolates USBA 844A and USBA 846E (*Pseudomonas psychrophila*) showed halos between 8 mm and 10 mm. In olive oil, the isolates USBA 851A (*Pseudomonas fragi*), USBA 851D (*Pseudomonas fragi*) and USBA 846E (*Pseudomonas psychrophila*) produced halos between 5 and 7 mm of diameter (Fig 1).

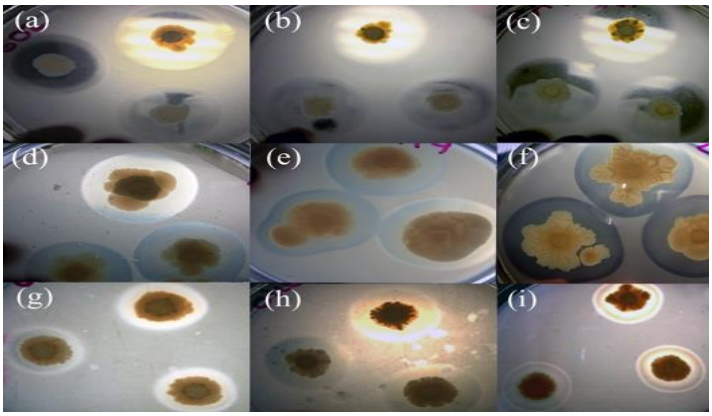

Figure 1 Hydrolysis halos obtained in tributyrin agar plates: a. USBA 851A b. USBA 852C c. USBA 844A. Triolein: d. USBA 851A e. USBA 844A f. USBA 846E. Olive oil: g. USBA 851A h. USBA 851D i. USBA 846E

As previously mentioned, *Stenotrophomonas rhizophila* and *Pseudomonas psychrophila*, among other isolates, produced the largest hydrolysis halos on the evaluated substrates. We note this fact because the lipolytic activity from *Stenotrophomonas rhizophila* has not been reported until now. This species of *Stenotrophomonas* is characterized by the absence of lipase and β-glucosidase production (**Wolf et al., 2002**). Additionally, the lipolytic activity of *Pseudomonas psychrophila* has been reported but it has not been evaluated. This finding led us to choose these isolates (USBA 843E, USBA 844A/USBA 846E) to perform the enzymatic activity assays on, in order to find novel lipases from psychrophilic bacteria.

Sequence alignments and phylogenetic analysis

The 16S rDNA gene sequences were compared with sequences reported in the GeneBank database using BLAST and the Ribosomal Database Project (RDPII), in order to determine the taxonomic assignment of the 43 isolates. The percent identities obtained were 99% and 100%, while the e-value was equal to zero (Tab 1).

Table 1 Taxonomic assignment of lipolytic bacterial isolates using BLASTN and accession numbers.

Code	Nearest BLAST (AC NCBI)	% Identity	Accession numbers
Glacier			
USBA 849A	*Pseudomonas mandelii* (LT629796.1)	99%	LT627086
USBA 843A	*Stenotrophomonas rhizophila* (KY474340.1)	99%	LT627087
USBA 850A	*Burkholderia fungorum* (LN868266.1)	100%	LT627088
USBA 852A	*Ralstonia pickettii* (KT354655.1)	100%	LT627089
USBA 843B	*Stenotrophomonas rhizophila* (KY474340.1)	99%	LT627090
USBA 852B	*Ralstonia pickettii* (KT354650.1)	100%	LT627091
USBA 849B	*Pseudomonas mandelii* (KU921563.1)	100%	LT627092
USBA 843C	*Stenotrophomonas rhizophila* (KR065721.1)	100%	LT627093
USBA 851A	*Pseudomonas fragi* (KX588592.1)	99%	LT627094
USBA 852C	*Ralstonia pickettii* (KY523570.1)	100%	LT627095
USBA 851B	*Pseudomonas fragi* (KX588591.1)	100%	LT627096
USBA 851C	*Pseudomonas fragi* (KX068625.1)	99%	LT627097
USBA 853A	*Dermacoccus nishinomiyaensis* (CP008889.1)	99%	LT627098
Paramo			
USBA 841A	*Aeromonas bivalvium* (KY124169.1)	99%	LT627099
USBA 842A	*Aeromonas veronii* (KY124169.1)	99%	LT627100
USBA 842B	*Aeromonas veronii* (KY124169.1)	99%	LT627101
USBA 843D	*Stenotrophomonas rhizophila* (KR065721.1)	99%	LT627102
USBA 843E	*Stenotrophomonas rhizophila (KM114951.1)*	99%	LT627103
USBA 843F	*Stenotrophomonas rhizophila (KM114951.1)*	99%	LT627104
USBA 844A	*Pseudomonas psychrophila (LT629795.1)*	100%	LT627105
USBA 843G	*Stenotrophomonas rhizophila (KR065721.1)*	99%	LT627106
USBA 843H	*Stenotrophomonas rhizophila (KY474340.1)*	99%	LT627107
USBA 849C	*Pseudomonas mandelii (LT629796.1)*	99%	LT627108
USBA 843I	*Stenotrophomonas rhizophila (KR065721.1)*	99%	LT627109
USBA 843J	*Stenotrophomonas rhizophila (KM114951.1)*	99%	LT627110
USBA 843K	*Stenotrophomonas rhizophila (KR065721.1)*	99%	LT627111
USBA 843L	*Stenotrophomonas rhizophila (KR259225.1)*	99%	LT627112
USBA 843M	*Stenotrophomonas rhizophila (KR259225.1)*	99%	LT627113
USBA 846A	*Pseudomonas psychrophila (KU173827.1)*	99%	LT627114
USBA 842C	*Aeromonas veronii (KY124169.1)*	99%	LT627115
USBA 846B	*Pseudomonas psychrophila (LT629795.1)*	100%	LT627116
USBA 845A	*Pseudomonas lini (KM114930.1)*	100%	LT627117
USBA 846C	*Pseudomonas psychrophila (LT629795.1)*	99%	LT627118
USBA 846D	*Pseudomonas psychrophila* (LT629795.1)	99%	LT627119
USBA 851D	*Pseudomonas fragi* (KX588592.1)	100%	LT627120
USBA 846E	*Pseudomonas psychrophila (LT629795.1)*	100%	LT627121
USBA 847A	*Pseudomonas jessenii (KU725946.1)*	99%	LT627122
USBA 843N	*Stenotrophomonas rhizophila (KR065721.1)*	99%	LT627123
USBA 843O	*Stenotrophomonas rhizophila (KR259225.1)*	99%	LT627124
USBA 843P	*Stenotrophomonas rhizophila (KR065721.1)*	99%	LT627125
USBA 848A	*Pseudomonas congelans (LN774224.1)*	99%	LT627126
USBA 843Q	*Stenotrophomonas rhizophila (KY474340.1)*	99%	LT627127
USBA 849D	*Pseudomonas mandelii (JX122162.1)*	99%	LT627128

The distance tree from glacier enrichment culture includes 4 clades from the *phylum* Proteobacteria, which are associated with the genera *Pseudomonas, Stenotrophomonas, Ralstonia* and *Burkholderia.* The fifth clade belongs to the *phylum* Actinobacteria, represented by the genus *Dermacoccus* (Fig 2a). The paramo enrichment culture tree showed only 3 clades. The isolates belong to the *phylum* Proteobacteria represented by the genera *Aeromonas*, *Pseudomonas* and *Stenotrophomonas* (Fig 2b).

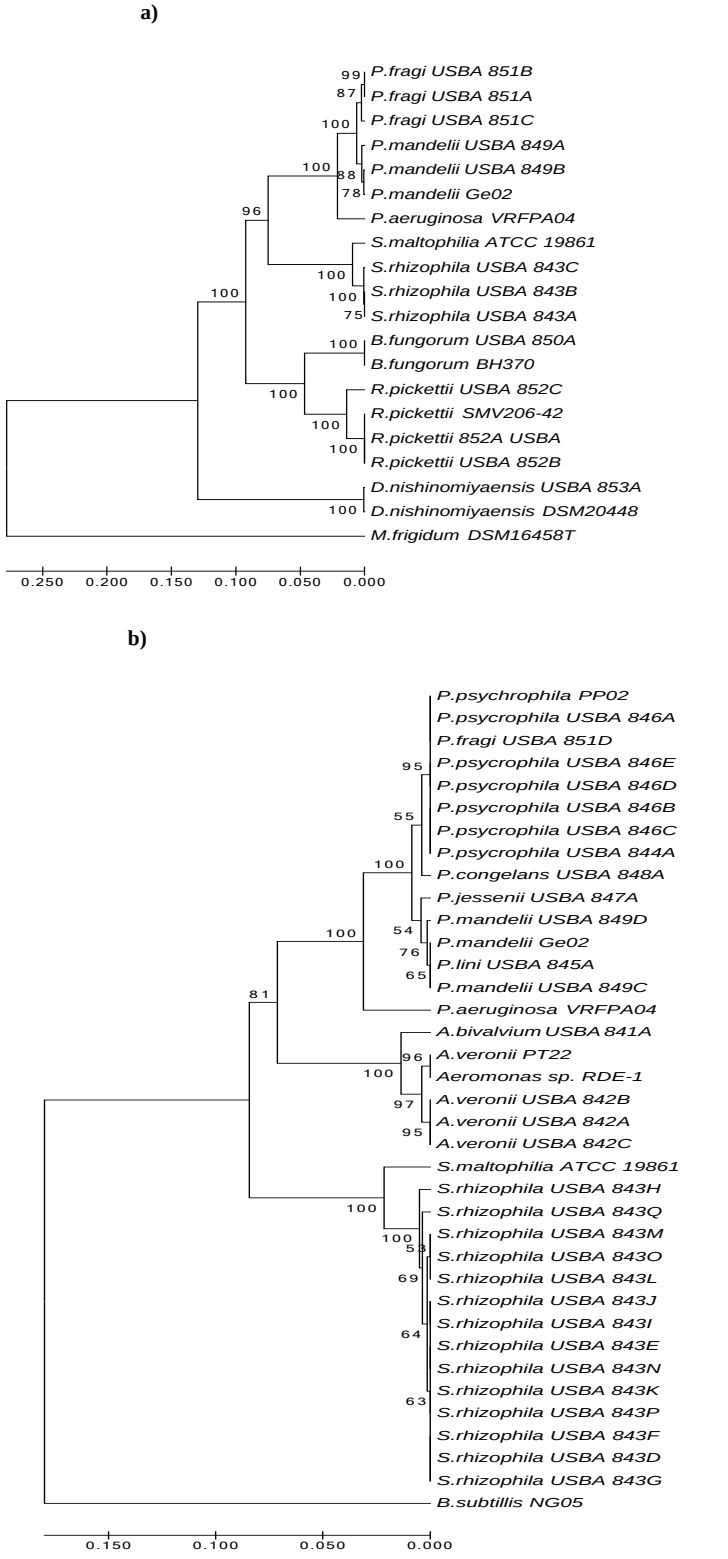

Figure 2 Phylogenetic trees generated with the 16S rDNA gene sequence from **a)** glacier enrichment culture **b)** paramo enrichment culture, compared with selected reference sequences: *P. mandelii* Ge02 (KR088369.1), *P. aeruginosa* VRFPA04 (CP008739.2), *S. maltophilia* ATCC 19861 (NR_040804.1), *B. fungorum* BH370 (LN868266.1), *R. pickettii* SMV206-42 (KT354655.1), *D. nishinomiyaensis* DSM20448 (NR_044872.1), *P. psychrophila* PP02 (KU173827.1), *A. veronii* PT22 (KY124169.1), and *Aeromonas sp.* RDE-1 (KY365513.1).The trees were generated using the UPGMA method and were rooted with *Methanogenium frigidum* (NR_104790.1) and *Bacillus subtillis* (JQ433875.1) as out groups. Scale bar indicates number of substitutions per site and the bootstrap analysis was performed on the basis of 1000 replicates

Growth curve experiments

The late stationary phase from the isolates USBA 843E (*Stenotrophomonas rhizophila*), USBA 844A and USBA 846E (*Pseudomonas psychrophila*) was reached after 72 hours of incubation. In this phase, the extracellular fraction was obtained to perform the p-nitrophenyl ester assays.

Lipase activity

The protein concentrations obtained from the extracellular fraction were: 0.108 mg mL^{-1} for the strain USBA 843E (*S. rhizophila*), 0.126 mg mL^{-1} for USBA 844A (*P. psychrophila*) and 0.092 mg mL^{-1} for USBA 846E (*P. psychrophila*). The lipase activity essay was carried out for 1 h at 30°C with four lipid substrates: pNP-acetate, pNP-butyrate, pNP-decanoate and pNP-palmitate. Short-chain pNP-esters (C2 and C4) were hydrolyzed for the three isolates. Only the USBA 843E (*S. rhizophila*) showed activity with the four substrates including pNP-palmitate. The broader substrate specificity of this isolate is probably due to the simultaneous presence of lipase and esterase activity in the crude extract (Fig 3).

Effect of temperature on enzyme activity

The USBA 843E (*S. rhizophila*) enzyme fraction showed maximal activity at 50°C in the four lipid substrates. Its highest activity was in short chain (C2 and C4) fatty acids. The extracellular fraction from USBA 844A (*P. psychrophila*) had high activity at 50°C in pNP-butyrate and at 40°C in pNP-acetate. Finally, the USBA 846E (*P. psychrophila*) showed its optimum activity at 50°C in pNP-acetate, pNP-butyrate and pNP-decanoate, and its highest activity was in short chain (C2 and C4) fatty acids (Fig 3). The pH used during the temperature assays was 7.

Effect of pH on lipase activity

The three isolates showed their optimal lipolytic activity at pH 8 in all the lipid substrates except for pNP-butyrate that was highly hydrolyzed at pH 9. For the isolate USBA 843E (*S. rhizophila*), the hydrolytic activity was observed at pH 7 in C2 and C4, pH 8 in all the lipid substrates, and pH 9 in C4 and C10 (Fig 3). The lipase from USBA 844A (*P. psychrophila*) showed activity at pH 7-8 in C2, and at pH 8-9 in C4 (Fig 3). For the isolate USBA 846E (*P. psychrophila*), the C2 hydrolysis was carried out at pH 6 to 9; of C4 at pH 8-9 and C10 at pH 8 (Fig 3). The temperature used during the pH assays was 20°C.

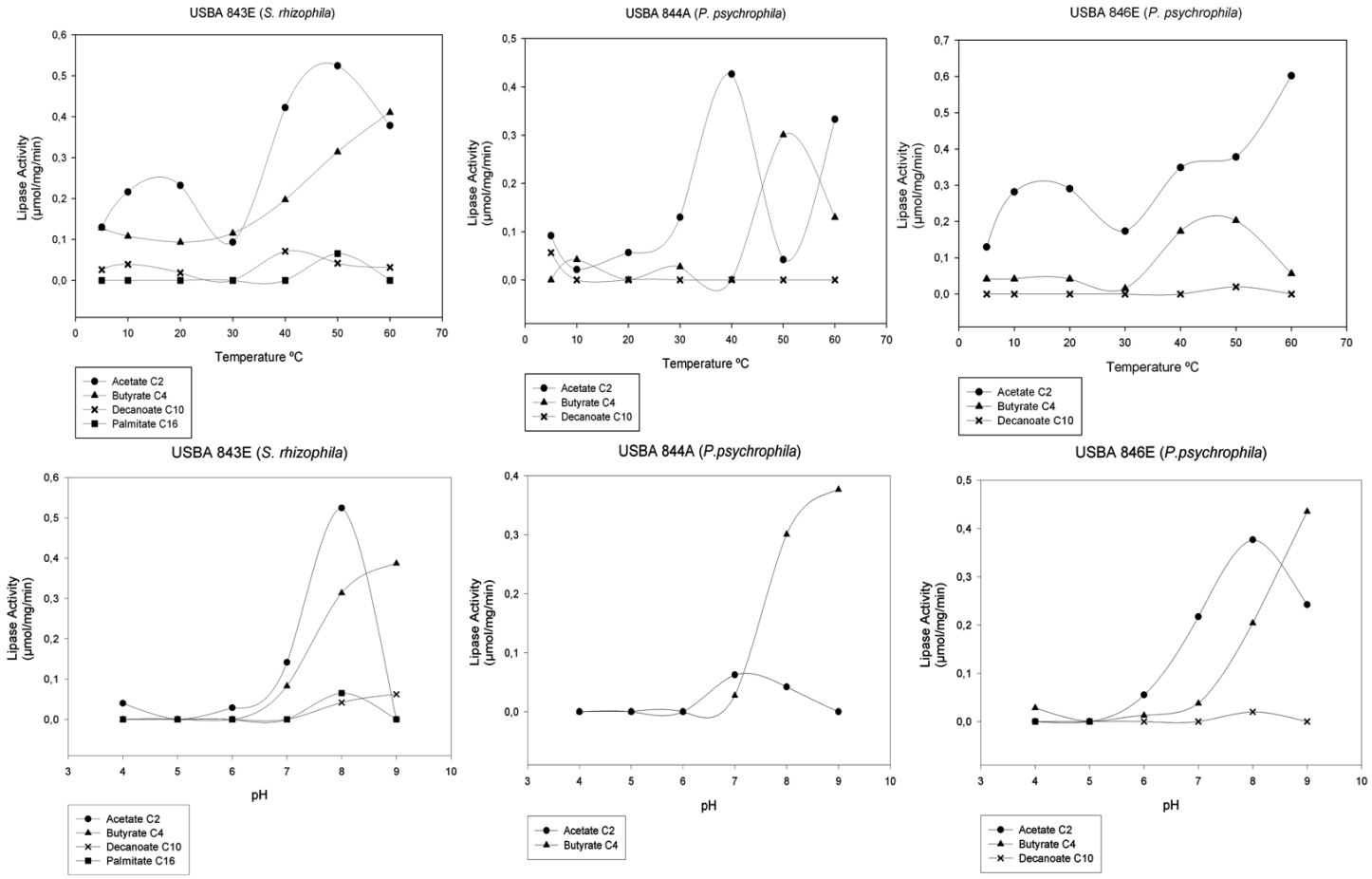

Figure 3 Lipolytic activities from the isolates USBA 843E, USBA 844A and USBA 846E at different temperatures and pH.

DISCUSSION

Enrichment of environmental samples with a substrate of interest aimed to expose microorganisms under a selection pressure. Here the "survival of the fittest" takes place under different physical, chemical and nutritional conditions **(Li *et al*., 2009).** The adaptation of natural and complex microbial communities to specific conditions (e.g. degradation of olive oil) can increase the prevalence of microbes carrying special metabolic capacities such as lipolytic activity **(DeAngelis *et al*., 2010).**

Pseudomonas and *Stenotrophomonas* were the most abundant genera recovered from the enrichments, and they also exhibited the largest hydrolysis halos. This shows how their ability to metabolize the given substrate allowed them to increase their population compared to other lipolytic bacteria. **Bardgett *et al.* (2005)** reported that the diversity tends to fall when the better-adapted microorganism dominates the community. In our case, the better-adapted microorganisms with the ability and required enzymes to degrade the non-conventional substrate, dominated the enrichment culture communities.

Furthermore, when we compared the lipolytic isolates from both soils, we found greater species diversity in the glacier enrichment culture than the one obtained from paramo. This is counter intuitive to expectation if we consider that the optimal environmental conditions, in terms of temperature, humidity and nutrient availability were present in the paramo soil **(Buytaert *et al*., 2006)**. In this case it is necessary to take into account that the enrichment method involved changes in the dynamics of the community. This could be caused predominantly by the microbial interactions within the bioreactor and the selection pressure in olive oil **(Bardgett *et al*., 2005)**.

The phyla Proteobacteria and Actinobacteria (with *Dermacoccus nishinomiyaensis* as the only isolate) identified in the glacier enrichment cultures have been reported from other cold environments **(Cheng & Foght, 2007; Steven *et al*., 2007; Neufeld *et al*., 2004; Shivaji *et al*., 2006)**. However, some studies also indicate the presence of different taxa such as Bacteroidetes and Firmicutes. They also report the presence of psychrophilic and lipolytic bacteria including *Pseudomonas*, *Aeromonas*, *Burkholderia* and *Ralstonia*, **(Yuan *et al*., 2010; Joseph *et al*., 2008; Hemachander & Puvanakrishnan, 2000)** which were also found in our enrichment cultures.

One particular finding in our study was the isolation of bacteria with different colony morphotypes. This polymorphism complicated the macroscopic description of some of the isolates, but after finishing the bacterial identification, this change was observed only for the *Pseudomonas* isolates. **Workentine *et al.* (2010)** described that *Pseudomonas* can change its colony morphotype depending on the culture conditions.

In terms of lipolytic activity, this potential for *Pseudomonas fragi* and *Ralstonia picketti* was confirmed by the large hydrolysis halos produced in the fatty substrates (Fig 1 and 3). These species have been previously reported for their lipolytic activity and stability at low temperatures **(Joseph *et al*., 2008; Hemachander & Puvanakrishnan, 2000)**. The other two isolates with interesting activity were *Pseudomonas psychrophila* and *Stenotrophomonas rhizophila*. They were chosen to perform enzymatic activity assays, not only for the production of hydrolysis halos, but also for their "novelty" value. **Bai & Rai Vittal. (2014)** studied the relationship between protease and lipase production with quorum sensing molecules in *P. psychrophila* PSPF19. Their objective was to inhibit the exoenzyme production. Therefore, they never characterized the lipases or proteases produced by the strain PSPF19. The closest report of the lipolytic activity in *P. psychrophila*, was performed by **Yumoto *et al.* (2001)** with *Pseudomonas fragi* lipase, which has a close phylogenetic association with *P. psychrophila.* In conclusion, our study is the first to date to perform the preliminary characterization of its lipolytic activity.

In 2002 **Wolf et al.** did the whole characterization of *Stenotrophomonas rhizophila*, a novel plant-associated bacterium. In their study they reported that the three strains evaluated showed a high antifungal activity and no lipase or β-glucosidase production, which differs from the characteristics observed from the 13 strains of *S. maltophila.* In addition, the growth rate of *S. rhizophila* at 4ºC was 6.25 times higher than the observed with *S. maltophila*, which lead us to predict an even higher potential for the purified enzyme of *S. rhizophila* at low temperatures. After this publication, **Hasan-Beikdashti *et al.* (2008)** optimized the culture conditions to produce the *S. maltophila* lipase to hydrolyze capsaicin and produce vanillylamine, which has antibacterial and anti-inflammatory properties. **Li *et al.*** in 2013 and 2016 cloned and characterized the lipases: CGMCC 4254 (novel cold-active and organic solvent-tolerant lipase), and the GS11 (cold-active, solvent-tolerant and alkaline) also from *S. maltophila*. Nevertheless, this is the first study that reports the lipolytic activity in *S. rhizophila*.

The optimal conditions for the three evaluated strains were 50ºC and pH 8 (Fig 8). Those temperatures and pH conditions have been already evaluated in *Pseudomonas aeruginosa* **(Dharmsthiti *et al*., 1998),** *Pseudomonas fluorescens* **(Kulkarni *et al*., 2002)**, *Pseudomonas sp. INK1* isolated in the Antarctic **(Park *et al*., 2012).** Our possible "novel" alkaline enzymes should be purified and tested

for a complete characterization in order to explore all the potential in terms of stability, substrate specificity and possible applications.

CONCLUSION

Exposing microorganisms to specific conditions in order to screen for capabilities that are not usually explored in non-conventional environments is a useful tool to discover biotechnological applications. Our study reports the first preliminary characterization of the lipolytic activity of *Stenotrophomonas rhizophila* and *Pseudomonas psychrophila*. Further studies to purify and completely characterize their lipolytic enzymes, will help us to understand all the potential offered by these "novel" lipases/esterases, that could be used for future applications. Our results demonstrate the vast opportunities offered by extreme environments for exploration and bioprospecting of different metabolic activities of interest.

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