

Original Research Article

Identification and Characterization of Two Novel Thermostable and Thermoresistant Esterases Isolated from Rice Rhizosphere by Activity-Based on Metagenomic Screening

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ABSTRACT

A 72,000 recombinant phages metagenomic library was constructed from rice rhizosphere. An esterase screening was performed and resulted in the identification of 6 positive esterase clones. Two of them, Ela1 and Ela2, were selected for a further characterization. Sequence analysis revealed that Ela1 exhibits a high homology with proteins annotated as acetyl xylan esterase (AXE) and Ela2 with SGNH hydrolases. Both enzymes are carboxylic ester hydrolases, with a high stability, an alkaline optimum pH 8-9 and active at high temperatures (75°C). Additionally, a 16S rRNA library was performed in order to characterize the biodiversity and biological diversity of the ecosystem source of this gene. It confirmed the predominance of thermophilic groups of bacteria matching with the esterases Ela1 and Ela2 annotation results and biochemical characterization. Thus, rice rhizosphere, which is a high-pressure selective ecosystem, arises as a very appropriate source of novel enzymes with a great potential for biotechnological and industrial applications.

Keywords

Metagenomic, Esterases, Rhizosphere, Lipolytic enzymes, Biotechnology

Introduction

Lipolytic enzymes attracting an enormous attention because of their biotechnological potential (Benjamin and Pandey 1998). They constitute one of the most important group of biocatalysts for biotechnological applications. Furthermore, novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers and biodiesel, the

production of enantiopure pharmaceuticals, agrochemicals, and flavour compounds (Jaeger and Eggert 2002).

Carboxylic ester hydrolases (EC 3.1.1.X) generally known as lipolytic enzymes are a diverse group of hydrolases that catalyze the cleavage and formation of ester bonds

(Bornscheuer 2002). They include two groups of enzymes, namely non-specific esterases or carboxylesterases (EC 3.1.1.1) and lipases (EC 3.1.1.3), which have been early, differentiated on the basis of their substrate specificity (Arpigny and Jaeger 1999; Chahinian and Sarda 2009).

Lipolytic enzymes are widely distributed in animals, plants and microorganisms. Many of them show a wide range of possible substrates and they also exhibit high stereospecificity. These make them some of the most versatile and important biocatalysts in industrial biotechnology applications as the synthesis and hydrolysis of stereospecific compounds, including the metabolic processing of drugs and antimicrobial agents (Bornscheuer 2002; Jaeger and Eggert 2002; Panda and Gowrishankar 2005; Choi *et al.* 2013).

Lipases isolated from different sources have a wide range of properties depending on their sources with respect to positional specificity, fatty acid specificity, thermostability, pH optimum, etc. (Huang 1984). One could probably find a lipase from nature that would be suitable for desired application.

Esterases and lipases within the microbial communities have particularly received considerable attention, because they are widely distributed operating in most of environments where they have important physiological functions (McQueen and Schottel 1987; Martínez-Martínez *et al.* 2013). Metagenomic approach based on expression is proposed as the most advantageous compared to methods based on independent culture for several reasons, such as sequencing errors (Gomez-Alvarez *et al.* 2009) and the erroneous assignment of substrate specificity (Fernández-Arrojo *et al.* 2010). In contrast, the activity-directed

techniques have been shown to provide a direct view of known or new protein families and functionalities (Martínez-Martínez *et al.* 2013). In this sense there is an increasingly greater number of studies but to date only a few have used the rhizosphere as a source of esterases as is the case of Lee *et al.* (2010) and none have specifically used the rhizosphere of rice.

The rhizosphere is a selective environment, source of specialized microorganisms. Besides, agronomic characteristics of rice crop provide an environment with an increased selective pressure. All this makes the rice rhizosphere potentially an excellent source of new genes of interest.

Metagenomics will enable us to study the unknown ecological functions of microbial communities, trying to unravel the biological functions that are present in a given environment or the type of organisms present therein. Additionally, by comparing data from different environments, the fundamental rules of microbial ecology, the mechanisms by which microorganisms adapt to environmental conditions or horizontal transfer phenomena may be studied.

Moreover, identification of new genes and proteins (new families of proteins) will be possible and analysis of diversity within known families may be carried out as basic knowledge studies. Identification of new genes and proteins is a relevant issue for biotechnological applications. Functional metagenomics, which involves the heterologous expression of metagenomic DNA in a surrogate host and activity-based screening, provides a means of discovering genes, the function of which may not be obvious from their sequence (Torres-Cortés *et al.* 2011).

In view of the above, the aim of this work

was to carry out a metagenomic analysis in an ecosystem such as the rhizosphere to characterize genetic and functionally the rhizosphere microbial communities and on the other hand, to discover new genes with interest in agriculture and industry.

This study presents a metagenomic approach in rice cropping rhizosphere with two main objectives: 1) Characterization of microbial communities in rice rhizosphere through a structural analysis with 16S rRNA gene library and 2) Search of new lipolytic enzymes: a function-based screening on a 10kb λ phage library for esterases was carried out; a biochemical characterization of two esterases was performed in order to evaluate their potential for biotechnological applications.

Materials and Methods

Soil Description and Sampling

The soil used to construct the metagenomic library was a loamy soil from an agricultural field with a long history of rice cropping (10 years) located in Vegas Altas Badajoz (Spain) (38° 57' 0'' N, 5° 51' 0'' W) (CASAT, www.casat.es). Sampling was conducted in July 2009 in tillering stage. Rice tiller is a specialized grain-bearing branch that is formed on the non-elongated basal internode and grows independently of the mother stem (culm) by means of its own adventitious roots.

One hectare was marked within the production area. Then it was surface gridded, grids were numbered from 1 to 100 and three grids (3 replicates) were selected through a random number program. Three plants (3 repetitions) of rice (*Oryza sativa* var. Thaibonnet) were sampled in each grid. The soil closely adhered to roots (rhizosphere soil) from the 3 plants of each

grid were united. At the end, 3 samples were obtained. Rhizosphere soils were transported to the laboratory at 4°C and sieved (2mm pore) before DNA was extracted within the following 48 hours.

DNA Extraction

DNA was extracted from 5 g soil samples, using a PowerMax® Soil DNA Isolation Kit (MoBio) and subsequently cleaned and concentrated on Amicon® Ultra Centrifugal Filter Units (Millipore, Billerica, MA, USA). DNA from the 3 replicates was pooled together. This DNA was used for 16S rRNA and metagenomic libraries construction.

16S rRNA Gene Library Construction

Extraction of DNA was done as indicated above. Amplifications of 16S rRNA gene fragments were carried out using the primers 16sF (AGA GTT TGA TCC TGG CTC AG) and 16sR (AAG GAG GTG ATC CAG CCG CA [52]). The 25 μ l PCR mixtures consisted of: Ultratools PCR buffer 10X with MgCl₂ (1X), dNTP Mix Ultratools (each dNTP 250 μ M), Ultratools DNA polymerase (1,4 units), primer concentration (0.6 μ M, each). PCR products were purified using the QIAquick PCR purification kit (Qiagen) and then ligated into the pCR 4-TOPO vector (Invitrogen). *Escherichia coli* strain DH5 α (Invitrogen) was then transformed with the ligation products. One hundred clones were randomly picked and suspended in tubes containing 6 ml LB and grown for 24 hours at 37°C. Plasmid DNA extraction was performed with the QIAprep Spin Miniprep kit (Qiagen) and fragment inserts were sequenced on a Applied Biosystems 3730xl DNA Analyzer.

Sequences were visualized with Sequence Scanner software v1.0. and editing was

performed using the software Clone Manager Professional Suite v6.0. Sequence alignment was carried out on the server MAFFT v6.0 (<http://mafft.cbrc.jp/alignment/software/>) and annotated with BLASTN 2.2.29. (Basic Local Alignment Search Tool, Altschul *et al.*(1997)in the National Center for Biotechnology Information (NCBI: <http://www.ncbi.nlm.nih.gov/>) and Ribosomal Database Project Release 10 (RDP: <http://rdp.cme.msu.edu/>) databases.

Biodiversity and richness estimation (biological diversity) were carried out with DOTUR-1.53, which provided estimation of biodiversity using the Shannon-Wiener index and of richness using Chao index. Distance considered between OTUs was 0.03 in both cases. A phylogenetic tree with the 16S rRNA soil bacteria partial sequences of all the clones was inferred with MEGA5(Tamura *et al.* 2011)from aligned sequences in MAFFT v6.0.

The sequences reported in this work are available in the NCBI database under the accession numbers, JQ30089-JQ300438.

Metagenomic Library Construction and Activity-Based Screening

Extraction of DNA was done as indicated above. *Escherichia coli* strain XL1-Blue MRF' was used for library construction and strain XL0LR was used for expression of the lipolytic activity from phagemids (Stratagene). The pBK-CMV and the ZAP Express vector digested with BamHI (Stratagene) were used to construct the soil metagenomic library.

Escherichia coli cells were routinely cultured in LB medium at 37°C, and plated in LB medium contained 1.5% Agar. When appropriate, media were amended with 10

mg L⁻¹ tetracycline and 50 mg L⁻¹ kanamycin for strain and plasmid maintenance.

The extracted metagenomic DNA was partially digested with Sau3A and subjected to electrophoresis. DNA fragments of 9–10 kb in size were isolated from the gel and concentrated on Amicon® Ultra Centrifugal Filter Units (Millipore, Billerica, MA, USA). DNA (150 ng) was ligated to lambda vector, according to the recommended protocols. Library titre was determined by mixing various dilutions of the packaged ligation product with *E. coli* XL1-Blue cells, according to the manufacturer's instructions, and counted the plaques formed.

The ZAP Express vector is designed to allow simple, efficient in vivo excision and recircularization of any cloned insert contained within the lambda phage vector, to form a phagemid (pBCK-CMV plasmid). Thus, it is possible to convert a lambda phage (Ferrer *et al.* 2005).

The screening for lipolytic enzymes in this metagenomic library was performed directly on the lysis plaques produced by the phages. A total number of 15,000 phages were plated to be screened. The esterase activity was revealed by the addition naphthyl acetate and fast-blue (SIGMA). In this test, a 0.2 mg ml⁻¹ naphthyl acetate in acetone and a 0.8 mg ml⁻¹fast-blue in dimethylsulfoxide solution were prepared. These two are added to 0.5 % agarose (800 µl of substrate per 45 ml of agarose) and poured into the plates as an overlay on the colony or colonies whose activity is to be tested. When hydrolyzed, the naphthyl acetate reacts with the fast-blue, resulting in a brown precipitate which indicates the phage lysis plaque where esterase activity is occurring (Ferrer *et al.* 2004).

Sequencing and Gene Annotation of Positive Clones with Esterase Activity

pBK-CMV phagemids were excised from the two esterase positive selected phages using the coinfection with helper phage according to Stratagene protocols. Then, pBK-CMV phagemids DNAs clones pBK-CMV $ela1$ and pBK-CMV $ela2$ were extracted and purified by using a Speedtools plasmid DNA purification kit (Biotools) according to the manufacturer's instructions. The insert DNA was sequenced using universal primers and primer walking procedure as custom service by SECUGEN S.L. (Spain). ORFs in the assembled sequences were predicted by Vector NTI Suite 10 (Invitrogen, USA) and a BLASTX search performed against the NR database to access annotation information. Translated protein sequences of ORFs coding for hydrolases and their nearest neighbors were determined by BLASTP searches against the NCBI-NR database. Structural classification of protein domain were predicted by SUPERFAMILY database located at <http://supfam.org/SUPERFAMILY/hmm.html> (Hasan et al. 2006). The signal peptide predictions were conducted using SignalP 4.1 Server located at <http://www.cbs.dtu.dk/services/SignalP/> (Petersen et al. 2011).

Sequences of DNA inserts encoding for $Elal$ and $Elal2$ genes and several hypothetical proteins were deposited in GenBank with the following accession numbers, KC977252 and KF700947, respectively.

General Recombinant DNA Techniques, Gene Cloning and Expression

DNA manipulations were performed by standard methods. Digestion of DNA with restriction endonucleases (New England Biolabs, USA), dephosphorylation with alkaline phosphatase (Roche, Germany) and

ligation with T4 DNA-ligase (Fermentas, Germany) were performed in accordance to the manufacturer's instructions. Plasmid Mini Kit from Qiagen (Germany) was used to prepare plasmid DNA. Plasmids and DNA fragments were purified by Qiagen DNA purification kits (Qiagen, Germany). pET26b(+) (Novagen) was used to subclone the gene encoding for esterase activities and *E. coli* BL21 Gold (DE3) (Stratagene) was used for heterologous expression. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth or on LB agar supplemented with the appropriate antibiotics (kanamycin, 40-50 $\mu\text{g ml}^{-1}$; tetracycline, 12.5 $\mu\text{g ml}^{-1}$).

The following primer pairs were used to amplify $ela1$ and $ela2$ genes from the corresponding phagemids: $Elal.fw$ (5'-ATATACATATGGCTCTCTTTGACAAA CC ACTGGA -3') / $Elal.rev$ (5'-GCCGC AAGCTTCCCCCAGAGATCGCCCAG-3') and $Elal2.fw$ (5'-ATATACATA TGACCCGCACTCGACG-3') / $Elal2.rev$ (5'-ATATA CATATGG CCGCGCAGC ATACC-3'). Primers were designed to introduce *NdeI* and *HindIII* sites (underlined) in both cases and to remove $ela2$ signal peptide sequence. The PCR was performed in 30 cycles, with the corresponding phagemid DNA as template, 0.5 μM of each primer, 0.2 mM dNTP's, 5 units HotStarTaq® DNA Polymerase (Quiagen) and 1× reaction buffer provided by the supplier. *NdeI* and *HindIII* digested PCR-products were purified and cloned into the expression vector pET26b(+) (Novagen) introducing a C-terminal His•Tag® sequence. Expression vectors harbouring esterase genes were transformed into *E. coli* BL21-Gold (DE3) (Novagen). The nucleotide sequence was confirmed by DNA sequencing (LGC genomics).

The expression was done in shaking flasks. Plasmids pET26b(+) harbouring $ela1$ and $ela2$ respectively, were freshly transformed

in *E. coli* BL21 and transformants were used to inoculate 30 ml LB broth supplemented with 40 $\mu\text{g ml}^{-1}$ kanamycin. Cultures were grown overnight at 37 °C on a rotary shaker (130 rpm). Then, cultures composed of 200 ml LB supplemented with 40 $\mu\text{g ml}^{-1}$ kanamycin were inoculated to obtain an optical density (600 nm) of 0.1. The cultures were grown at 37 °C and 130 rpm until an optical density of 0.6 was reached. Then the cultures were incubated at 20, 25, 28 and 37 °C, respectively and induced with IPTG at a final concentration of 0.05 mM. The induced cultures were incubated with shaking at 130 rpm for 22 h and samples at 0, 3, 6, 8 and 22 hours were collected during the induction time. Finally, protein expression was analyzed by SDS-PAGE, in 12% polyacrylamide gels, and visualized by Coomassie Brilliant Blue staining.

Protein Purification

Induced cells were harvested by centrifugation at 2150 g and 4 °C for 30 min. Cell pellet was disrupted by sonication under ice cooling (cell disruptor, BRANSON Ultrasonics) and cell debris was removed by centrifugation at 26300 g for 30 min and 4 °C. Purification of the 6xHistidine-tag enzymes from soluble fraction was performed with Ni-NTA Sepharose Column (IBA BioTAGnology, Germany). The elution buffer was exchanged with 100 mM Tris HCl pH 7 by the use of PD-10 desalting columns (GE Healthcare). The protein concentration was measured using the Bradford protein assay (Bradford 1976).

Enzyme Characterization

The purified Ela1 and Ela2 were subjected to a series of biochemical analyses, including specific activity, optimum pH, optimum ionic strength, optimum temperature and thermostability. To measure

enzyme activity of the purified protein, p-nitrophenyl (p-NP) esters of acetate (p-NPA) and butyrate (p-NPB) and α -naphthyl acetate (Sigma-Aldrich) were employed as substrates. The amount of p-nitrophenol or 1-Naphthol released by esterase-catalyzed hydrolysis were used to determine the catalytic activity. The reaction mixture for p-NP esters was done as follows. Two hundred μL of the substrate solution prepared in 50 mM Tris HCl buffer pH 7 and 10% DMSO was mixed with 20 μL of enzyme solution. The product (p-nitrophenol) was measured at 405 nm at 25°C for 5 min. The reaction mixture for α -naphthyl acetate consisted of 0.6 mg ml^{-1} Fast Blue RR (Sigma-Aldrich) dissolved in 50 mM Tris HCl buffer, pH 7, 2% DMSO and 1% Triton X-100. Two hundred μL of the substrate solution was mixed with 50 μL of enzyme solution and the product (1-naphthol) was measured at 450 nm at 25°C for 10 min. A blank was measured using buffer instead of enzyme solution. One unit of enzyme activity was defined as the amount of enzyme needed to release 1 μmol p-NP or 1-Naphthol per min under the above-mentioned conditions. A Spectromax Plus 384 plate reader (Molecular Devices) was used to follow the reaction unless otherwise indicated. All measurements were performed in triplicate and repeated three times. The values were the mean of the data.

Kinetic Parameters and Thermostability

Kinetic parameters were determined under the standard conditions described above. Esterase activity was determined with different substrate concentrations ranging from 0.05 mM to 8 mM for p-NP esters and from 0.15 mM to 3.3 mM for α -Naphthyl acetate. Values of K_m , V_{max} , k_{cat} and k_{cat}/K_m were calculated from Hanes-Woolf plots in conditions described above using the Enzyme kinetics SigmaPlot 11.0 software.

Thermostability was investigated as follows. Purified enzymes were incubated in 100 mM Tris HCl buffer pH 7 at 4, 25, 37 and 50 °C at different time intervals to determine remaining enzyme activity by the standard assay with p-NPA. Enzyme activity was monitored until a sharp decrease was detected. The Kinetic parameters K_m , V_{max} , k_{cat} and k_{cat}/K_m were also calculated.

Optima for Temperature, Ionic Strength and pH

The effect of temperature on enzyme activity and the optimum ionic strength was investigated using 5.1 mM p-NPA as substrate under the standard conditions described before with the following modifications. The final volume reaction was 1 ml and the substrate solution was previously conditioned at different temperatures by incubating 4 min, then the reaction was conducted at the different temperatures ranging from 10 to 75 °C. A Cary Series UV-Vis Spectrophotometer (Agilent Technologies) was used to follow the reaction. The optimum ionic strength was studied under the standard conditions by adding different concentrations of NaCl (0-0.9 M) into the standard reaction mixture.

The effect of pH on enzyme activity was determined using 3.3 mM α -Naphthyl acetate as substrate at 25 °C in the following buffers: 50 mM citrate buffer (pH 4-6), 50 mM Tris-HCl (pH 7-8), 50 mM Glycine-NaOH buffer (pH 9-10). The molar extinction coefficients of 1-Naphthol were corrected for pH variation.

Results and Discussion

Structural Characterization of Microbial Communities with 16S rRNA Gene Library

Analyses performed with the sequences

from the structural study indicated that the ecosystem had a high biodiversity and high biological diversity (Shannon-Wiener = 4.36 bits). The taxonomic classification of the sequences (Figure 1) showed a predominance of Gram-negative bacteria, being the *Proteobacteria* group the most abundant, which is frequent in soil. It is striking the relatively high presence of *Acidobacteria* phylum bacteria. Although this group is frequently found in soils, little is known about them since they are non-culturable bacteria. Among the Gram positive bacteria, the *Firmicutes* phylum was present but not very numerous and the *Actinobacteria* phylum was abundant.

There was also presence of the phylum *Chloroflexi* (represented almost exclusively by the family *Anaerolineaceae*), the phylum *Verrucomicrobia* and the family *Planctomycetaceae*.

Construction of a Metagenomic Library and Esterase Activity Screening

A metagenomic library (10 kb inserts) was constructed with DNA extracted from rice rizosphere (Badajoz, Spain) in tillering. A total of 72,000 recombinant phages were obtained using the lambda ZAP-expressing phagemid system and subsequently amplified. The amplified library was subjected to functional screening for novel esterase enzymes (Reyes-Duarte *et al.* 2012). Approximately 15,000 phages were screened and 6 esterase positive clones were identified, purified and excised into pBK-CMV phagemids (Mølgaard *et al.* 2000; Ferrer *et al.* 2009).

Identification of Esterase Genes and Sequence Analysis

Two esterase positive clones were randomly selected for a further characterization. Inserts of the selected clones called pBK-

CMVela1 and pBK-CMVela2 were completely sequenced by primer walking using universal primers. Both inserts were lower than 10 kb in size (7.5 and 8.6 kb, respectively). Subsequent BLASTX analysis using the NCBI non-redundant protein database revealed 3 and 4 open reading frames (ORF) in the 7.5 and 8.6 kb inserts, respectively. In both cases one of the identified ORF showed similarity with a carboxylic ester hydrolase. In the case of the 7.5 kb DNA fragment, the ORF consisted of 987 nucleotides and encoded a protein of 328 amino acids (named Ela1). The 8.6 kb DNA fragment contained an ORF of 783 nucleotides and encoded a protein of 260 amino acids (named Ela2), moreover, a Signal P4.1 analysis showed up a cleavage site between amino acid positions 24 and 25.

Analysis of the deduced amino acid sequence revealed that Ela1 belongs to alpha/beta-hydrolases superfamily and to the acetyl xylan esterase-like protein family. The common sequence Gly-X-Ser-X-Gly found in many esterases, lipases, and serine proteases (Brenner 1988) was located as Gly-Gly-Ser-Gln-Gly in the sequence from positions 186 to 190. Moreover a peptidase S9 domain was detected. A phylogenetic tree was constructed with ten retrieved protein sequences from the NCBI database that showed high similarity with Ela1 (Figure 2). This analysis showed that Ela1 exhibited the higher similarity with several proteins annotated as acetyl xylan esterase-like protein from bacteria of the phylum *Chloroflexi* as *Caldilinea aerophila* (accession no. YP_005442439; identity 73%); *Roseiflexus* sp (accession no. YP_001275160; identity 66%); *Anaerolinea thermophile* (accession no. YP_004175118; identity 64%); *Herpetosiphon aurantiacus* (accession no. YP_001543662; identity 63%). Ela1 also showed high homology with an acetyl xylan esterase from *Thermobaculum terrenum* (accession no.

YP_003321945; identity 63%) being a thermophile bacteria closely related to the phyla *Chloroflexi* and *Thermomicrobia* (Botero *et al.* 2004). Moreover, Ela1 exhibited similarity with a thermostable acetyl esterase from *Thermotoga maritima* (accession no. 3M81_A; identity 60%) (Martínez-Martínez *et al.* 2013) and acetyl xylan esterases from other thermophile and/or thermo-resistant bacteria as *Dictyoglomus thermophilum* (accession no. YP_002251796; identity 61%); *Thermobispora bispora* DSM 43833 (accession no. YP_003652570; identity 55%); and bacterium with xylan degrading enzymes such as *Acidothermus cellulolyticus* 11B (accession no. YP_873803; identity 56%) and *Cellulomonas flavigena* DSM 20109 (accession no. YP_003636033; identity 55%) (Morris *et al.* 1998; Barabote *et al.* 2009; Abt *et al.* 2010; Liolios *et al.* 2010). Alignment between Ela1 and Ela2 showed a 14% of matching (according to Clone Manager 6.0, Align Plus 4.10. Method: FastScan- Max Qual).

On the other hand, the analysis of the deduced amino acid sequence of Ela2 revealed that this protein belongs to SGNH hydrolase superfamily and specifically to the platelet-activating factor-acetylhydrolase (PAF-AH) like subfamily. Moreover an active site similar to the typical Ser-His-Asp (Glu) triad from other serine hydrolases (Ser84-Asp233-His236) and the oxyanion hole that characterizes these enzymes were predicted. Members of the SGNH-hydrolase superfamily facilitate the hydrolysis of a broad range of substrates, including polysaccharides, lysophospholipids, and other ester containing compounds (Mølgaard *et al.* 2000; Lo *et al.* 2003), giving SGNH hydrolase great potential in both basic research and industrial applications (Bae *et al.* 2013).

A phylogenetic tree was also constructed with protein sequences that showed high similarity with Ela2 (Figure 3). In this case, the analysis revealed that Ela2 showed similarity with different classes of proteins, showing the highest similarity with several proteins annotated as GDSL family lipolytic protein as the case of *Pirellula staleyi* DSM 6068 (accession no. YP_003370957.1; identity 72%), *Rhodopirellula maiorica* (accession no. WP_008697892.1; identity 59%), *Pedosphaera parvula* (accession no. WP_007416179.1; identity 44%), *Candidatus Nitrospira defluvii* (accession no. YP_003799892.1; identity 50%), *Marinomonas sp. MWYL1* (accession no. YP_001339539.1; identity 47%), and *Pseudoalteromonas atlantica T6c* (accession no. YP_663334.1; identity 45%). Also proteins annotated as 1-alkyl-2-acetylglycerophosphocholine esterase showed a high similarity with Ela2, as the case of *Planctomyces limnophilus* DSM 3776 (accession no. YP_003630933.1; identity 65%), *Glaciecola mesophila* (accession no. WP_006991740.1; identity 46%), and with N-acetylglucosamine-6-sulfatases from *Blastopirellula marina* and *Verrucomicrobium spinosum* (accession no. WP_002649964.1, identity 48%; accession no. WP_009962309.1; identity 48%, respectively). All these proteins showed a SGNH hydrolase-type esterase domain but the molecular functions of these homologous proteins were inferred from electronic annotation and there is no more information in the literature about its demonstrated structure or functions.

Cloning and Heterologous Expression

Once the encoding esterases genes were identified and their possible roles predicted, we proceeded to the subcloning of both genes and its heterologous expression to finally perform a biochemical characterization of the purified enzymes. So

couples of primers for subcloning Ela1 and Ela2 into pET26b (+) were designed and ligation products were transformed into *E.coli* BL21 for expression assays. The expression was strongest at 25 °C for both enzymes. In figure 4 the monitoring of the expression of Ela1 and Ela2 in the soluble fraction at 0, 3, 6, 8, 22 hours after induction with IPTG and purification result is shown. For Ela1, high expression was obtained at 6 hours after induction and this expression was maintained up to 22 hours. However, in the case of Ela2, the expression was very low throughout the trial.

The molecular mass of Ela1 and Ela2 were predicted to be 37.9 and 27.8 kDa, respectively; on the basis of the amino acid sequence using ProtParam tool (Swiss Institute of Bioinformatics) and the mobility on the SDS polyacrylamide gels (Fig. 4), consistent with the molecular masses obtained for an acetyl xylan esterase from *Bacillus subtilis* (Tian *et al.* 2014) and for the recombinant protein FNE, classified as SGNH hydrolase, from *Fervidobacterium nodosum* (Yu *et al.* 2010).

Kinetic Parameters

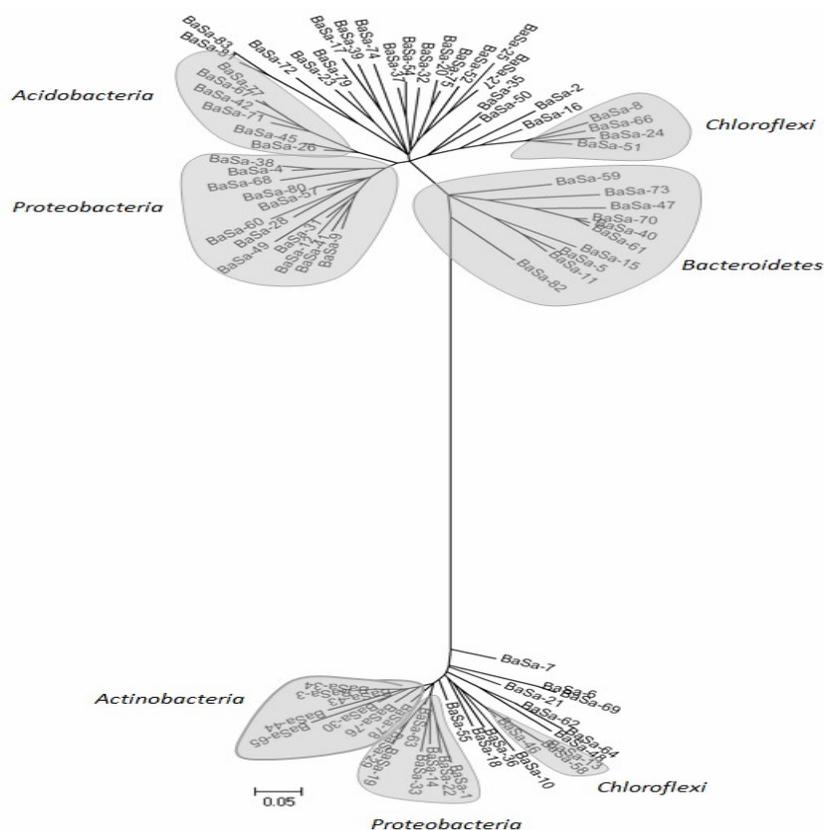
Enzymes Ela1 and Ela2 hydrolyzed acyl esters. However, with p-NPB as substrate, neither enzyme showed any activity (Table 1). Ela1 exhibited specific activities of 110.3 and 72.9 $\mu\text{molmin}^{-1}\text{mg}^{-1}$ protein for p-NPA and α -Naphthyl acetate, respectively. Conversely, Ela2 exhibited a higher specific activity for α -Naphthyl acetate (86.5 U mg^{-1} protein) than p-NPA (40.7 U mg^{-1} protein). The K_m and V_{max} values were also determined from the Hanes-Woolf plot and subsequently k_{cat} and k_{cat}/K_m parameters were calculated (Table 1) supporting a high affinity toward p-NPA and α -Naphthyl acetate for Ela1 and Ela2, respectively.

Table.1 Specific Activities and kinetic Parameters of Ela1 and Ela2^a

	Specific activity [$\mu\text{mol min}^{-1}$ mg^{-1}]	K_m [mM]	V_{max} [$\mu\text{mol min}^{-1}$]	k_{cat} [1 s^{-1}]	k_{cat}/K_m [$\text{s}^{-1}\text{mM}^{-1}$]
Ela1					
p-NPA	110.3 ± 3.3	0.4 ± 0.04	93.3 ± 2.8	69.4	173.4
p-NPB	0	0	0	0	0
α -Naphthyl acetate	72.9 ± 2.4	0.7 ± 0.06	61.8 ± 2.0	45.9	65.6
Ela 2					
p-NPA	40.7 ± 1.1	0.3 ± 0.01	4.1 ± 0.05	19.0	62.6
p-NPB	0	0	0	0	0
α -Naphthyl acetate	86.5 ± 1.8	0.2 ± 0.01	8.7 ± 0.1	40.3	201.5

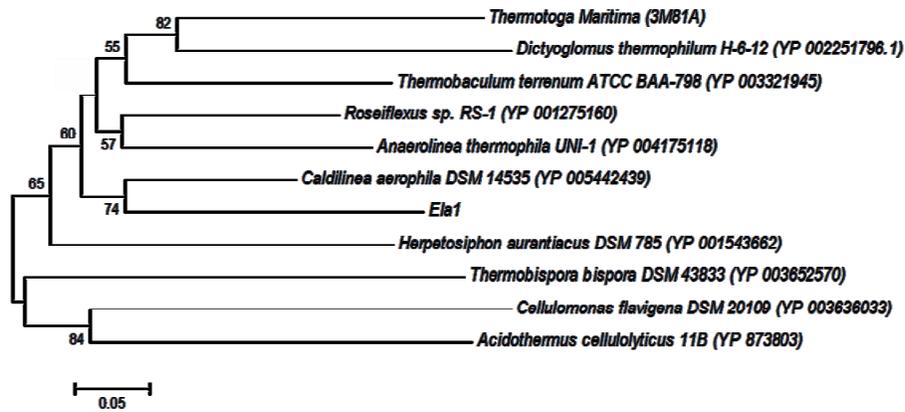
a. Measurements were taken under standard conditions (pH 7 and 25° C). Ela1 data correspond with data taken after 24 hours of incubation at 25 °C. Kinetic measurements for each substrate were repeated three times and the values are given ± SE for specific activity, K_m and V_{max} . K_{cat} and K_{cat}/K_m parameters were calculated by using the previous averages

Figure.1



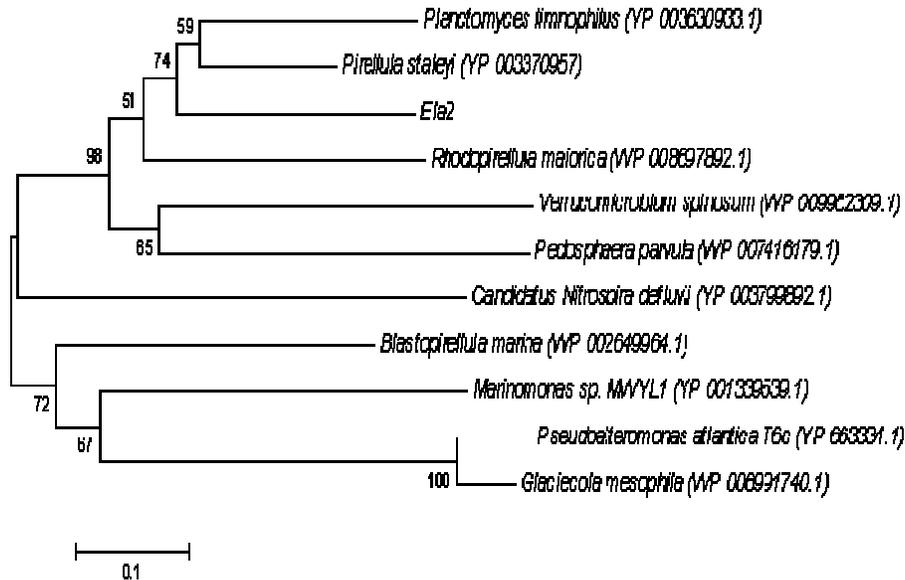
Phylogenetic tree performed with the 16S rRNA sequences. The evolutionary distances were inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500. The tree was conducted with MEGA5 [48]. Phylogenetic classification (taxonomic levels) was obtained in Ribosomal Database Project (<http://rdp.cme.msu.edu>).

Figure.2



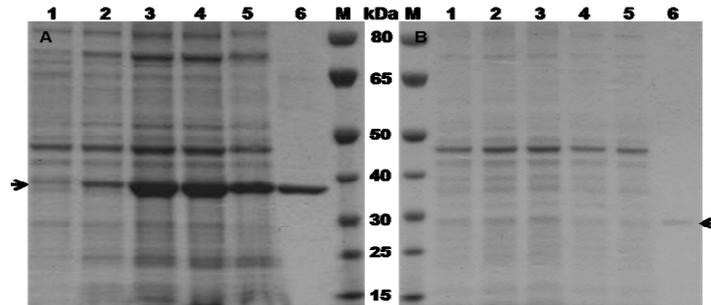
Evolutionary relationships of *Ela1* and homologous proteins obtained from NCBI database. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The tree was conducted with MEGA5 [48]

Figure.3



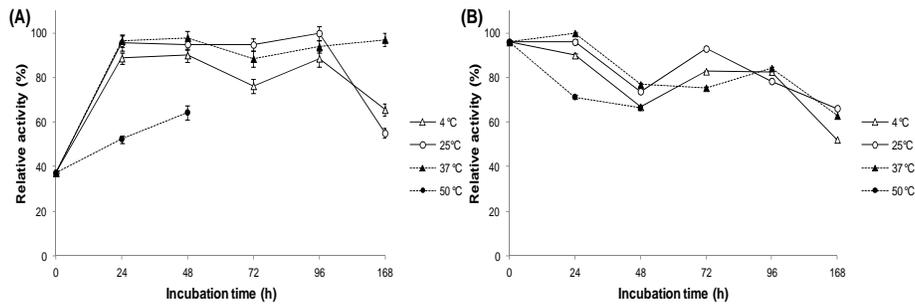
Evolutionary relationships of *Ela2* and homologous proteins obtained from NCBI database. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The tree was conducted with MEGA5 [48]

Figure.4



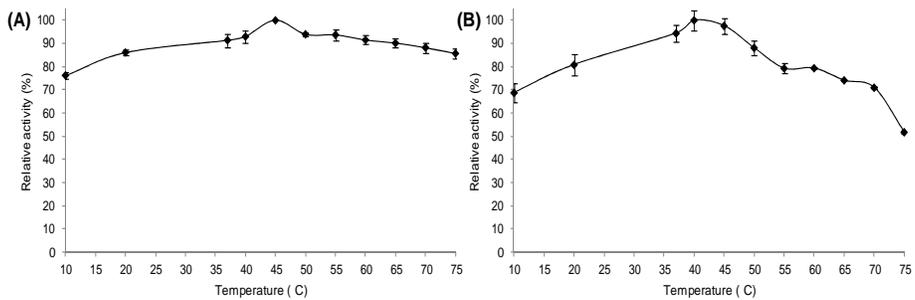
SDS-PAGE analysis (4-12%) of esterases Ela1 (A) and Ela2 (B) expressed in *E. coli* BL21-Gold(DE3) and purified by affinity chromatography. Key: M, pre-stained protein molecular weight marker SM0671 (Fermentas); Lanes 1-5: cleared lysates from induced cells after 0 h, 3 h, 6 h, 8 h and 22 h; Lane 6: purified esterase

Figure.5



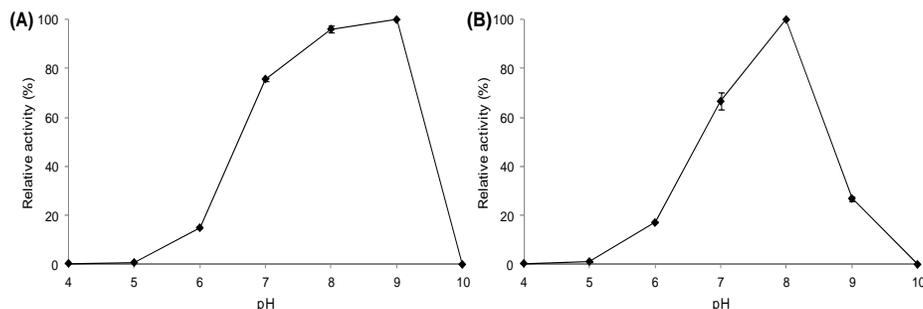
Stability of (A) Ela1 and (B) Ela2. Purified enzyme was first incubated for 0-168 hours under 4, 25 and 37 °C and for 0-48 hours at 50 °C. Then, the residual activity was measured using p-NPA as the substrate under standard conditions (pH 7 and 25° C) and repeated three times. The highest activity was set as 100%. Values are given \pm SE

Figure.6



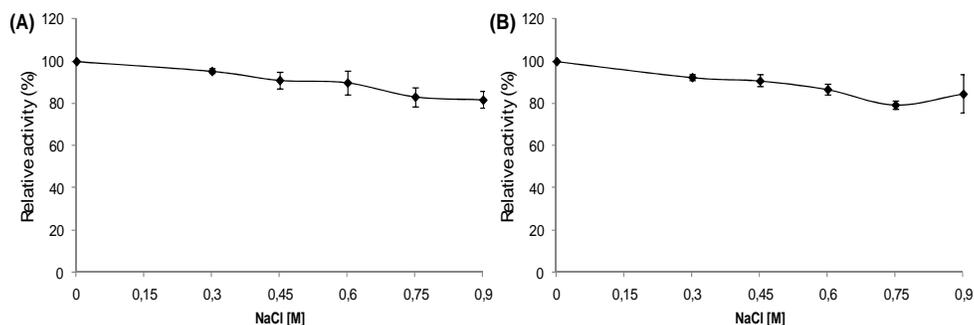
Optimum temperature of (A) Ela1 and (B) Ela2. Enzyme activity was measured under various temperatures (10-75°C). Measurements were taken in 50 mM Tris HCl buffer pH 7 using p-NPA as substrate and repeated three times. The highest activity was set as 100%. Values are given \pm SE

Figure.7



Optimum pH of (A) Ela1 and (B) Ela2. Enzyme activity was measured under various at pH (4-10). Measurements were taken at 25 °C using α -Naphthyl acetate as substrate and repeated three times. The highest activity was set as 100%. Values are given \pm SE

Figure.8



Optimum ionic strength of (A) Ela1 and (B) Ela2. Esterase activity was measured under standard conditions (pH 7 and 25 °C) with 0-1 M NaCl using p-NPA as the substrate and repeated three times. The highest activity was set as 100%. Values are given \pm SE

Influence of Temperature, pH and Ionic Strength on Esterase Activity

The stability at 4, 25 and 37 °C during 1 week and at 50 °C for 48 hours were also assayed. Results are summarized in figure 5 where the remaining specific activity during the experiment is shown. Interestingly, Ela1 displayed a higher activity after 24 hours of incubation in the case of all temperatures assayed suggesting that after purification a period of time for the correct folding is required. Both enzymes showed a high stability but Ela1 showed greater stability than Ela2. Ela1 was highly stable at 37 °C as 97% of its activity is retained at 168 hours.

On the other hand, at 4 and 25 °C was also very stable presenting the 88 and 100% of the activity up to 96 hours, respectively. Furthermore, after 48 hours at 50 degrees, it still showed 64% activity. On the other hand, Ela2 also showed high stability but more moderate than Ela1. In this case, the enzyme retained 90, 96, 100 and 71% of its activity at 4, 25, 37 and 50 °C, respectively at 24 hours, and showed no other changes along the experiment.

Both enzymes were active between 10 °C and 75°C (Figure 6) and showed similar optimal temperatures (40-45 °C). In the case of Ela1, the optimal temperature for its

activity was 45°C (Figure 6 a) and it was 40°C for Ela2 (Figure 6 b). By contrast, the lowest activity for Ela1 was at 10°C and for Ela2 at 75°C. Ela1 retained values of up to 76% while Ela2 retained only 51% of the activity in the most unfavorable temperature conditions.

Concerning optimum pH, purified Ela1 and Ela2 also displayed activity between pH 6 and 9, with an optimal activity at pH 9 and 8, respectively (Figure 7). Ela1 showed higher tolerance to pH than Ela2. The enzyme activity was found to be over 65%, at pH 7 and 8 for Ela2 while Ela1 maintained it at pH 9.

Finally, the optimum ionic strength was assayed and, again, similar results were obtained for both enzymes. They were active from 0 to 0.9 M NaCl being the enzyme activity over 80% up to 0.6 M in all cases (Figure 8).

Structural Characterization of Microbial Communities with 16S rRNA Gene Library

Besides the presence of bacterial groups typically found in soil, it is also interesting to note the presence of some other bacterial groups which, although are not highly abundant they are directly related to the annotations found for the two esterase genes isolated and studied in this work. This is the case of the phylum *Chloroflexi* (represented almost exclusively by the family *Anaerolineaceae*), the phylum *Verrucomicrobia* and the family *Planctomycetaceae*. Many species from *Chloroflexi* and *Planctomycetes* phyla are thermophilic (Sekiguchi *et al.* 1998) which is completely in accordance with the annotations found for esterases Ela1 and Ela2 and with the thermostability shown by these two esterases. These data are consistent with other studies that also report

presence of microorganisms belonging to the phylum *Planctomycetes* such as *Pirulella*, *Planctomyces* and *Verrucomicrobium* in thermophilic granular sludges (Sekiguchi *et al.* 1998) and the presence of lipolytic enzymes showing homology with proteins from *Planctomyces*, *Rhodopirulella*, *Blastopirulella* and *Pirulella* in activated sludge of a swine wastewater (Liaw *et al.* 2010).

Identification of Esterase Genes and Sequence Analysis

Analysis of the deduced amino acid sequence revealed that Ela1 belongs to alpha/beta-hydrolases superfamily and to the acetyl xylan esterase-like protein family. Moreover, a phylogenetic tree was constructed with ten retrieved protein sequences from the NCBI database that showed high similarity with Ela1 (Figure 2).

This analysis showed that Ela1 exhibited the higher similarity with several proteins annotated as acetyl xylan esterase-like protein from bacteria of the phylum *Chloroflexi* as *Caldilinea aerophila*, *Roseiflexus* sp, *Anaerolinea thermophile* *Herpetosiphon aurantiacus* and *Thermobaculum terrenum*. Moreover, Ela1 exhibited similarity with a thermostable acetyl esterase from *Thermotoga maritima* and acetyl xylan esterases from others thermophile and/or thermo-resistant bacteria as *Dictyoglomus thermophilum*, *Thermobispora bispora* and bacterium with xylan degrading enzymes such as *Acidothermus cellulolyticus* and *Cellulomonas flavigena*, both included in the Class *Actinobacteria* which was highly represented in this rhizosphere according to the microbial community structural analysis (Figure 1).

These results suggest that Ela1 is a novel acetyl xylan esterase since all the

homologous proteins are annotated as such protein (Figure 2) and because some of them belong to microorganisms with demonstrated xylanolytic activity (Barabote *et al.* 2009; Abt *et al.* 2010). Acetyl xylan esterases are enzymes that hydrolyse the ester linkages of the acetyl groups of the xylose moieties of natural acetylated xylan from hardwood. These enzymes are one of the accessory enzymes, which are part of the xylanolytic system, together with xylanases, beta-xylosidases, alpha-arabinofuranosidases and methylglucuronidases; these are all required for the complete hydrolysis of xylan. Xylan, next to cellulose, is the most abundant renewable polysaccharide in nature. It is a major hemicellulose component of plants and is located predominantly in the secondary cell walls of angiosperms and gymnosperms. The esterases involved in xylan breakdown could be an important research tool in studying and resolving cell wall structure and mechanisms. Furthermore, these enzymes could be applied to the pulp and paper industry (McCleary and Matheson 1987; Christov and Prior 1993) as reported for xylanases from *Dictyoglomus* (Morris *et al.* 1998) or *Cellulomonas* (Abt *et al.* 2010). However, in all cases the acetyl xylan esterase function is based in a sequence prediction.

The fact that Ela1 presents homology with the enzymes of the microorganisms described above makes us think that the rhizosphere is also an ecosystem with a high potential for the discovery of new glycosyl hydrolases.

The analysis of the deduced amino acid sequence of Ela2 revealed that this protein belongs to SGNH hydrolase superfamily and specifically to the platelet-activating factor-acetylhydrolase (PAF-AH) like subfamily. Members of the SGNH-hydrolase

superfamily facilitate the hydrolysis of a broad range of substrates, including polysaccharides, lysophospholipids, and other ester containing compounds (Mølgaard *et al.* 2000; Lo *et al.* 2003), giving SGNH hydrolase great potential in both basic research and industrial applications (Bae *et al.* 2013).

The phylogenetic tree constructed with protein sequences that showed high similarity with Ela2 (Figure 3), revealed that Ela2 showed similarity with different classes of proteins, showing the highest similarity with several proteins annotated as GDSL family lipolytic protein as the case of *Pirellula staleyi*, *Rhodopirellula maiorica*, *Pedosphaera parvula*, *Candidatus Nitrospira defluvii*, *Marinomonas sp.* *Pseudoalteromonas atlantica*. Also proteins annotated as 1-alkyl-2-acetyl-glycerophosphocholine esterase showed a high similarity with Ela 2, as the case of *Planctomyces limnophilus*; *Glaciecola mesophila* and with N-acetylglucosamine-6-sulfatases from *Blastopirellula marina* and *Verrucomicrobium spinosum*.

All this proteins showed a SGNH hydrolase-type esterase domain but the molecular functions of these homologous proteins were inferred from electronic annotation and there is no more information in the literature about its demonstrated structure or functions. However, there is information in the literature, which relates Ela2 with a phospholipase A2 activity because the PAF-AH domain predicted for Ela2 is related with a phospholipase A2 function. This possible function is very interesting because the discovery of the prokaryotic phospholipase A2 domain is relatively recent (Matoba *et al.* 2002). Phospholipase A2 hydrolyzes the 2-acyl ester bonds of 1,2-diacylglycero-3-phospholipids (Matoba *et al.* 2002; Sugiyama *et al.* 2002). Due to the

versatility of this class of enzymes the potential functions of Ela2 are very diverse, in a previous study (Sugiyama *et al.* 2002) an interesting possibility was proposed that is how *Streptomyces* cells might produce the secreted PLA2 to take ecological priority among microorganisms living in a soil so PLA2 might function as a toxin to kill microorganisms having phosphatidylcholine as a membrane component (Sugiyama *et al.* 2002). Moreover, the potential of these esterase production genes is twofold, firstly new esterase genes may have application in the industry, and also, an environmental application using these enzymes to break the AHLs lactone ring and therefore disrupting the quorum sensing of pathogenic bacteria plant, and therefore, playing a key role in plant fitness in natural environments and plant resistance to pathogens.

Cloning and Heterologous Expression

Monitoring of the expression of Ela1 and Ela2 in the soluble fraction at 0, 3, 6, 8, 22 hours after induction with IPTG and purification showed that, for Ela1, high expression was obtained at 6 hours after induction with IPTG and this expression was maintained up to 22 hours. However, in the case of Ela2, the expression was very low throughout the trial (Figure 4). In both cases the purification of the enzymes was satisfactory because although the concentration was lower for Ela2 this was enough for the further biochemical characterization.

The results obtained for Ela2 may be because it is a secreted protein and therefore, although the signal peptide was eliminated in the subcloning process, the intracellular expression may not be the most convenient. This assumption agrees with the recent work of Takemori *et al.* (2012) where periplasmic production of phospholipase A2 from *Streptomyces violaceoruber* is optimized by

removing the native signal peptide and fusing with pelB signal sequence.

Biochemical Characterization: Kinetic Parameters

Enzymes Ela1 and Ela2 hydrolyzed acyl esters. However, with p-NPB as substrate, neither enzyme showed any activity (Table 1). k_{cat} and k_{cat}/K_m parameters calculated (Table 1) showed a high affinity toward p-NPA and α -Naphthyl acetate for Ela1 and Ela2, respectively. These findings again are consistent with the results obtained by Yu *et al.* (2010) and by Koseki *et al.* (2006) for a SGNH hydrolase and an AXE, respectively, in both cases, purified esterases displayed the greatest hydrolytic activity toward short chain esters (C2).

Influence of Temperature, pH and Ionic Strength on Esterase Activity

Both enzymes were active between 10 °C and 75 °C (Figure 6) and showed similar optimal temperatures (40-45 °C). In the case of Ela1, the optimal temperature for its activity was 45°C (Figure 6 a) and it was 40°C for Ela2 (Figure 6 b). By contrast, the lowest activity for Ela1 was at 10°C and for Ela2 at 75°C. Results correlate well with those obtained in stability experiments (Figure 5), again, Ela1 showed higher activity than Ela2 at different temperatures other than optimal temperature. Ela1 retained values of up to 76% while Ela2 retained only 51% of the activity in the most unfavorable temperature conditions. All these results together are very promising since heat resistance and thermostability are shown. Moreover, both enzymes, and especially Ela1, might be adapted to a wide temperature range which is comparable to other esterases such as the QsdH from *Pseudoalteromonas byunsanensis* that exhibits activity ranging from 20°C to 60°C, reaching its optimum activity at 40°C

(Huang *et al.* 2012). These results are also in agreement with the results of phylogenetic analysis, which demonstrated that Ela1 and Ela2 showed homology with enzymes from heat-resistant microorganisms or identified from thermophilic granular sludges.

Concerning optimum pH, purified Ela1 and Ela2 also displayed activity between pH 6 and 9, with an optimal activity at pH 9 and 8, respectively (Figure 7). These results suggest that Ela1 and Ela2 are alkaline enzymes as it was found for others esterases isolated from plant rhizosphere soil metagenome (Lee *et al.* 2010). Furthermore, alkalophilic thermophiles have great potential in detergent and leather industries (Hasan *et al.* 2006).

Finally, the optimum ionic strength was assayed and both enzymes were active from 0 to 0.9 M NaCl being the enzyme activity over 80% up to 0.6 M in all cases (Figure 8). Opposite results were found by Selvin *et al.* (2012) where the recombinant lipase Lpc53E1 from the metagenome of a marine sponge displayed increased levels of activity upon exposure to increased concentrations of NaCl, probably associated to the higher environmental pressure in the marine system.

All this findings explain that microbial lipases are currently receiving considerable attention due to their diversity in catalytic activity, high yield and low cost production, and relative ease of genetic manipulation (Fan *et al.* 2011). Also, they have a great potential for agriculture since communication by quorum sensing (QS) between species, either synergistically or competitively, may play an important role in the dynamics of these microbial communities. In addition to the positive interaction between the species, numerous reports have demonstrated that interference

with QS-mediated signal molecules also frequently occurs not only between bacteria but also between bacteria and higher plants (Braeken *et al.* 2008). Enzymatic AHL-degrading activities seem to be widespread and have been described in many species (Park *et al.* 2003; d'Angelo-Picardet *al.* 2005).

In summary, enzymes Ela1 and Ela2 show an excellent biotechnological application potential because they are very stable, have heat resistance and are alkaline enzymes. Both could be used for many applications with high interest in different industries after a more comprehensive biochemical characterization. Our results also show that rice rhizosphere, which is a high pressure ecosystem and therefore can be considered a great selective ecosystem, is very useful to isolate novel enzymes by functional metagenomics approaches.

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