SCREENING OF LIPASES FROM MANGROVE SEDIMENTS BY METAGENOMIC AND CULTIVATION

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ABSTRACT – *Mangroves are costal ecosystems where extreme environmental conditions are found, being considered valuable source for the discovery of novel microbial enzymes. Lipases constitute the most important group of biocatalysts being used in various biotechnological applications. In this context, the current work aimed to screening bacterial isolates and metagenomic clones from mangrove sediments for lipase/esterase production. A total of 90 isolates and 1152 clones were tested in LB agar supplemented with 1% tributyrin. A number of 58 isolates and 3 clones were lipase positives, being the clones considered potential for novel lipases. The isolates were identified by 16S rRNA partial sequences as belonging to the genera Lysobacter, Shewanella, Acinetobacter, Bacillus, Variovorax, Stappia, Rhodobacter, Staphylococcus, Pseudomonas and Luteimonas. These results are very encouraging and the next steps involve further study for enzymes characterization.*

1. INTRODUCTION

Mangroves are costal ecosystems located in estuaries shorelines where harsh environmental conditions are found, such as periodic flooding by tides and variations in salinity, oxygen, temperature and nutritional availability (Holguin *et al.*, 2006). Those environments have intense biological activities and many of theirs valuable processes are directly related to their sediment microorganism's activity. The high adaptation of these organisms to these environments makes these areas a valuable source for the discovery of novel microorganisms and/or genes that can be used for various biotechnological applications.

Despite this potential, the study of microbial diversity and function of mangrove sediments are scarce. Traditionally, microbial enzymes and other compounds with biological activity have been accessed by isolation and cultivation of microorganisms from environmental samples. However, it has been estimated that less than 1% of the total diversity of soil microorganisms are cultivated on the existing laboratory conditions (Amann *et al.*, 1995). Therefore, metagenomic is a useful tool to access

all the genetic potential present in an environmental sample. It consists of extracting DNA directly from the environment, with the use of cloning vectors as an optional step, allowing to access genes and/or functions of uncultured microorganisms (Handelsman, 2004).

Enzymes are used in several industrial sectors such as textile, food and pharmaceutical, representing a global trade estimated to be 4 billion dollars (Abiam, 2015). However, the expansion of commercial biocatalysis is often hampered due to lack of enzymes that are specific or have optimal performance under the extreme industrial conditions, leading to an increase demand for novel enzymes.

Several studies have demonstrated the importance of metagenomic library in the discovery of novel enzymes such as chitinases (Cottrell; Moore; Kirchman, 1999), lipases and esterases (Rondon *et al.*, 2000), amylases (Rondon *et al.*, 2000) and proteases (Gupta *et al.*, 2002). Among these, lipolytic enzymes have attracted enormous attention due to their wide biotechnological applications. Those can be classified according to their substrate preferences as lipases that hydrolyse water-insoluble long-chain acylglycerols (C>10) or esterases that hydrolyse water-soluble short-chain acylglycerols (C≤10) (Ruiz; Pastor; Diaz, 2003).

Taking this into account, the aim of this work was to evaluate the potential of bacterial isolates and a metagenomic library obtained from the sediment of Jaguaribe River mangrove (Ceará, Brazil) for the production of lipolytic enzymes.

2. MATERIAL AND METHODS

2.1 Bacterial strains isolation from mangrove sediment

25 g of Jaguaribe River mangrove's sediment were used to isolate bacterial strains. The sample was homogenized with 225 ml of sterile saline for 30 minutes at room temperature on an orbital shaker at 150 rpm. Then, serial decimal dilutions were prepared (10^{-1} - 10^{-8}) and 0.1 ml of these dilutions were plated by spreading on ATGE medium surface (15.0 g/L agar, 5.0 g/L tryptone, 5.0 g/L glucose and 2.5 g/L yeast extract) supplemented with 20 g/L NaCl (saline-ATGE). The plates were incubated at room temperature for 48 h. Colonies morphology were analyzed with on the stereomicroscope and the colonies with distinct morphological characteristics were re-isolated until obtaining pure colonies. The obtained isolates are stored in TGE (5.0 g/L tryptone, 5.0 g/L glucose and 2.5 g/L yeast extract) supplemented with 15% glycerol at -80 °C

2.2 Screening for bacterial isolates lipase and esterase

The obtained isolates were screened for the production of lipolityc enzymes. Each isolate was grown in saline-ATGE medium supplemented with tributyrin (1% v/v) for detection of lipolityc activity and triolein (1% v/v) for lipase activity. Assays were conduced in duplicate and the activities

were detected by the appearance of halo around the colonies, which was indicative of substrate hydrolysis (Rondon *et al.*, 2000).

2.3 Molecular identification of the selected isolates

The isolates with positive activities for lipolytic enzymes were subjected to extraction of genomic DNA (Foster and Twell, 1996) for molecular identification. The obtained DNA was used as a template for amplification of the 16S rRNA gene using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAGGAGGTGATCCAGCC-3') (Lane, 1991). All the PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA) and subjected to sequencing at Macrogen (Seoul, Korea) using the primers 27F, 1525R, 518F (5'-GCA CCA GGC GCC GTA ATA CG-3') and 800R (5'-TAA TAC CAG GGT ATC TCC-3'). The partial sequences of high quality (Phred> 20) were used to generate consensus sequences using the Codon Code Aligner 5.1 package (Ewing and Green, 1998). The obtained sequences were compared to sequences deposited in GenBank using the BLAST tool (Altschul *et al.*, 1990).

2.4 Metagenomic library construction and screening for lipolytic activity

For the construction of the metagenomic library, microbial DNA from the Jaguaribe River mangrove's sediment was extracted using the PowerMax Soil DNA Extraction kit (MoBIO, USA). Afterwards, the obtained DNA was fragmented with *EcoRV* (Fermentas) and the inserts ranging from 1 to 8 Kb were ligated in the vector pJET1.2/*blunt* (Fermentas) and used to transform *Escherichia coli* TOP 10F'. The transformed clones were stored at LB broth (10 g/L Peptone, 5 g/L yeast extract and 5 g/L NaCl) supplemented with 10% v/v glycerol at -80 °C until further use. For lipolytic activity screening, clones were initially reactivated in 96-well plates containing 1.0 mL of LB broth with ampicillin (100 µg/ml) and incubated at 37 °C for 24 hours under 150 rpm. After that, clones were replicated with 96-pin array onto LB agar medium containing 1% v/v tributyrin. The plates were incubated at 37 °C for 48 hours. Positive activity was analyzed by the detection of a hydrolysis halo around the colonies.

2.5 Insert DNA sequencing of positive clones for lipolytic activity

The positive clones for the hydrolysis of tributyrin were grown in LB broth with ampicillin (100 µg/mL) and incubated at 37 °C for 24 hours under 150 rpm. Then, 3 mL of each culture were subjected to plasmid extraction. The quality of the obtained plasmid was analyzed by agarose gel 1% w/v electrophoresis and quantification was performed by spectrophotometry. Then, plasmids were subjected to sequencing at Macrogen (Seoul, Korea) using the primers shown in Table 1. The sequences were analyzed in Codon Code Aligner 5.1 package and compared to sequences deposited in GenBank using the BLASTx tool (Altschul *et al.*, 1990).

Table 1 – Primers used for sequencing of the DNA inserts of the 3 positive clones.

Clone	Primers	
	Forward	Reverse
G6	5'-CGACTCACTATAGGGAGAGCGGC	5'-AAGAACATCGATTTTCCATGGCAG
	5'-ATAACAGATTCAAACACCGGC	5'-TTGGAACCATCATCATACTCAGC
	5'-GTTGACGAAACTATGATATGCCG	5'-ATTGGACGATTATCACCAGTGG
G7	5'-CGACTCACTATAGGGAGAGCGGC	5'-AAGAACATCGATTTTCCATGGCAG
	5'-GCCATCACACATAGAACCAGAGG	5'-TAGCGATGGGATGGAACTGCTCC
H7	5'-CGACTCACTATAGGGAGAGCGGC	5'-AAGAACATCGATTTTCCATGGCAG

3 RESULTS AND DISCUSSION

3.1 Screening for lipases/esterases and molecular identification of bacterial strains

The mangrove sediments of the Jaguaribe River allowed the isolation of 90 strains with distinct colony morphology (Figure 1). The isolates were screened using tributyrin and triolein substrates. A number of 58 isolates were positive for the tributyrin (C4), indicating the presence of esterase. And 3 isolates (JA21, JA22 E JA31) were positive for the triolein (C18), indicating the presence of lipase.

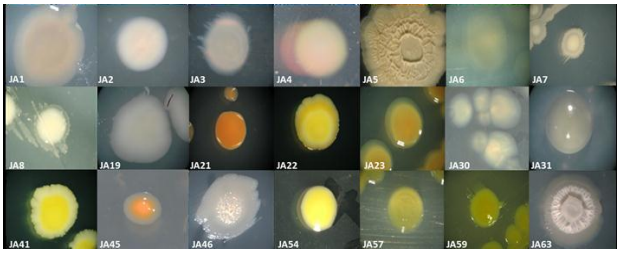

Figure 1 - Colony morphology diversity of some of the isolates.


Figure 2 – Bacterial isolate showing positive lipolytic activity. The arrow shows the clear zone related to the tributyrin degradation.

The isolates that showed best lypolitic activity were selected for molecular identification. Based on the 16S rRNA sequence analysis, isolates were identified as belonging to the following genera: *Lysobacter*, *Shewanella*, *Acinetobacter*, *Bacillus*, *Variovorax*, *Stappia*, *Rhodobacter*, *Staphylococcus*, *Pseudomonas* and *Luteimonas* (Table 1). Most of the isolates could not be clearly identified at specie level, apart from 3 isolates that were assigned as belonging to *Rhodococcus capsulatus* (JA2 and JA30) and *Shewanella algae* (JA19).

Table 1 - Molecular identification of the selected isolates based on the analysis of the 16S rRNA.

Isolate	Specie	Identity (%)	Tributyrin	Triolein
JA1	*Lysobacter sp.*	97	+	-
JA2	*Rhodobacter capsulatus*	100	+	-
JA3	*Shewanella sp.*	99	+	-
JA4	*Acinetobacter sp.*	100	+	-
JA5	*Bacillus sp.*	100	+	-
JA6	*Lysobacter sp.*	97	+	-
JA7	*Bacillus sp.*	99	+	-
JA8	*Acinetobacter sp.*	99	+	-
JA19	*Shewanella algae*	100	+	-
JA21	*Variovorax sp.*	96	+	+
JA22	*Stappia sp.*	99	+	+
JA23	*Lysobacter sp.*	97	+	-
JA30	*Rhodobacter capsulatus*	100	+	-
JA31	*Lysobacter sp.*	97	+	+
JA41	*Staphylococcus sp.*	99	+	-
JA45	*Pseudomonas sp.*	96	+	-
JA54	*Staphylococcus sp.*	99	+	-
JA59	*Luteimonas sp.*	99	+	-
JA63	*Bacillus sp.*	99	+	-

The 3 lipase positive isolates were identified as belonging to *Variovorax* sp. (JA21), *Stappia* sp. (JA22) and *Lysobacter* sp. (JA31). *Variovorax* spp. have been isolated from various sources, being mainly isolated from soil (Yoon *et al.*, 2006), however the current work is the first report of the isolation of this gender from mangrove. Some strains of this genus are known as esterase producers, without description for lipase activity (Kim *et al.*, 2006). Members of the genera *Lysobacter* and *Stappia*, positives for lipase and esterase, have been isolated from marine environment (Pujalte *et al.*, 2005; Park *et al.*, 2008).

3.2 Screening for lipolytic activity in metagenomic library

A total of 1152 clones screened for lipolytic activity using 1% tributyrin as substrate led to

discovery of 3 positive clones identified as LipG6, LipG7 and LipH7 (Figure 3).

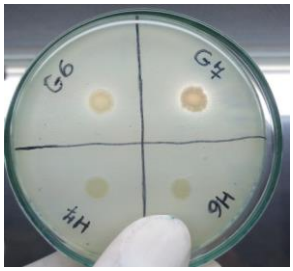

Figure 3 – Positive clones for esterase retrieved from a mangrove sediments metagenomic library.

In general, thousands of clones are screened to obtain few positive clones. In the current work, a total of 1152 clones with an average insert DNA size of 3 kb, representing a total size of 3.4 Mb of total metagenomic DNA, led to 3 positive clones for esterase, which corresponded to aproximately one lipolytic gene per 1.2 Mb DNA (one positive clone per 384 tested clones). Henne *et al.* (2000) found one positive clone for lipase after screening 73,000 clones with an average insert DNA size of 6.5 kb, corresponding to one positive gene per 474 Mb DNA. Pereira *et al.* (2015) achieved one lipolytic gene per 4.9 Mb after screening 4224 clones with average insert DNA size of 30-40 kb.

3.3 DNA Sequencing and gene annotation of the positive clones

The plasmidial DNAs of LipG6, LipG7 and LipH7 clones were extracted presenting an insert DNA size of approximately 4.0, 2.9 and 0.8 Kb, respectively. To evaluate their phylogenetic relationship with known lypolytic enzymes, the sequences were analyzed by performing Blastx against the non-redundant database from NCBI. Partial sequence of the clone LipG7 (1179 bp) reached 80% identity with 1.4-butanediol diacrylate esterase from *Portiococcus hydrocarbonoclasticus* followed by around 70% identity with esterases from *Methylibium sp. Geobacillus thermoglucosidasius* and *Alicyclobacillus pomorum* (Table 3). This enzyme is a carboxylesterase responsible for the conversion of 1,4-butanediol diacrylate to 4-hydroxybutyl acrylate (Gutierrez *et al.*, 2012). We believe that the low amino acid sequence identity with the so far reported esterases, indicates that LipG7 is a novel 1,4-butanediol diacrylate esterase, supporting the idea that mangroves are promising source for the discovery of novel genes that can be used for various biotechnological applications.

Table 3 – Sequence similarity of clone LipG7 with 1.4-butanediol diacrylate esterase retrieved from NCBI.

Description	Species	Query cover	E value	Identity	Acession number
1.4-butanediol diacrylate esterase	*Portiococcus hydrocarbonoclasticus*	99%	0.0	80%	WP036858555
1,4-butanediol diacrylate esterase	*Methylibium sp.*	98%	0.0	72%	WP036235362

1,4-butanediol diacrylate esterase	*Methylibium petroleiphilum*	98%	0.0	72%	WP011829040
1,4-butanediol diacrylate esterase	*Geobacillus thermoglucosidasius*	98%	0.0	71%	WP042383693
1,4-butanediol diacrylate esterase	*Alicyclobacillus pomorum*	98%	0.0	71%	WP026964421

Analysis of plasmidial DNA from the clones LipG6 and LipH7 revealed no homology to any known lipase or esterase, and the gene responsible for the lipolityc activity was not annotated so far. The main hits obtained after running Blastx showed homology to AraC family transcriptional regulator and to hypothetical proteins, respectively. Further studies are needed to better investigate the identity of lipolytic gene from LipG6 and LipH7.

4 CONCLUSION

Sediments from mangrove presented a cultured bacterial diversity with great potential for esterase and lipase production as well as promising novel lipase/esterase genes. Analysis of the insert DNA revealed a 1.4-butanediol acrylate esterase sharing only 80% amino acid sequence identity with a bacterial esterase previously described, suggesting that one could be a novel esterase. Further studies are needed to prove this hypothesis and reveal its biotechnological applications.

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