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Molecular Detection and Characterization of Novel Lipase Genes of the Lipolytic Yeast *Candida palmioleophila*

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In this study we analyzed the genetic variability of lipase gene sequences from eight oil and grease-degrading strains of *Candida palmioleophila* and to relate it to their degrading ability. The genetic variability of lipase genes was analyzed by PCR-restriction fragment length polymorphism (PCR-RFLP) and low-stringency single specific primer- PCR (LSSP-PCR), in order to obtain specific DNA fingerprints from each strain, which were subsequently compared by bioinformatic programs. DNA fingerprints were contrasted to the ability of each strain to remove palm oil in liquid culture. The results showed that at least three genes encoding lipases are present in *C. palmioleophila*, two of them resembling the *LIP*2 and *LIP*6 genes of *C. albicans.* DNA fingerprints obtained by LSSP-PCR revealed differences in the sequences of *C. palmioleophila* lipase genes, which allowed to group the strains according to their degrading activity. C. palmioleophila strains SACL05, SACL08 and SACL11, which showed the highest removal of palm oil after 72 h (77 to 79 % removal), were grouped in a single clade in dendrograms. Similarly, strains SACL01, SACL03, SACL06 and SACL09, which showed intermediate removal activity of palm oil (54 to 76%) grouped in a different clade. This suggests the genetic variability in lipase genes is directly related to the differences found in the efficiency of degradation of oils. On the other hand, DNA fingerprints obtained by PCR-RFLP did not allow to differentiate the strains and did not generate changes in the bands patterns between the analyzed strains. In conclusion, this study reported for the first time the detection and characterization of lipase genes from the lipolytic yeast *Candida palmioleophila*, and their association to the degradation of oils.

1. Introduction

Lipases are an important group of enzymes produced by a wide range of microorganisms, useful in different fields such as the pharmaceutical, industrial, chemical and environmental due to their regioselectivity, enantioselectivity and selectivity in the chain length of the substrate in which they act. For these reasons, there is a growing demand for identifying new lipolytic enzymes for the development and optimization of bioprocesses (Sharma et al. 2011). Lipases can be used in environmental applications, specifically in wastewater treatment processes from oil refineries, as toxic wastes are generated during all processing processes causing serious contamination in soils and water. These enzymes are produced by animals, plants, and microorganisms in general, which catalyze the ester-chain hydrolysis of triacylglycerols and release fatty acids and glycerol (Guncheva and Zhiryakova 2011). The most representative genera of microorganisms producing lipolytic enzymes are *Candida*, *Pseudomonas*, *Burkholderia*, *Thermomyces*, *Rhizopus*, *Bacillus*, *Staphylococcus*, *Geobacillus*, *Acinetobacter*, *Rasltonia* and *Yarrowia* (Bell et al. 2002).
The lack of knowledge about new microorganisms exhibiting lipase activity has led to new investigations about the prospection of new lipolytic strains and to identify new lipases with biotechnological potential, especially for the mitigation of the environmental impact. Previous studies of our group have shown that different strains of the lipolytic yeast *C. palmioleophila* present different removal efficiencies of fats and oils under the same conditions, even though they show 100% similarity in their ribosomal sequences (Rodriguez-Mateus et al. 2016). These microorganisms could have a high potential for their use as bioremediation agents of effluents

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contaminated with fats and oils. The microbial degradation of fats and oils in these microorganisms is mainly modulated by extracellular lipases; however, to date, there are no studies about *C. palmioleophila lipases*, or their coding genes. The variability in these genes could significantly influence the activity and efficiency of these enzymes. Thus, the objective of this study was to detect and characterize genes encoding lipases in *C. palmioleophila* in order to correlate their genetic variations to their reported ability to metabolize grease and oils.

2. Materials and methods

2.1 Native degrading microorganisms

Eight strains of *Candida palmioleophila* (SACL01, SACL03, SACL04, SACL05, SACL06, SACL08, SACL09, SACL11) and two strains of *Bacillus* sp. (SACB02 and SACB10), previously isolated from a grease trap of a palm oil refining process (Rodriguez-Mateus et al. 2016), were used in this study. All 10 strains showed lipolytic activity and the ability to remove grease, oils and organic matter present in wastewaters from palm oil extraction, either single or mixed (Agualimpia et al. 2016). In addition, genomic DNA from *Candida albicans* strain CA was used as a positive control during the amplification of *LIP*1, *LIP*2 and *LIP*6 genes.

2.2 Amplification and detection of lipase genes

Yeast and bacterial genomic DNA extraction was performed by salting out (Miller et al. 1988) using a lysis buffer (10 mM Tris-Cl, 10 mM EDTA, 50 mM NaCl) containing 20 mg/ml proteinase K (Sigma-Aldrich, USA), 20 mg/ml lysozyme for bacteria (Merck, USA) or 67 mg/ml lyticase for yeast (Sigma-Aldrich, USA) to break down cell walls and membranes. Extracted DNA preparations were quantified and quality checked using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, USA). PCR amplifications of four different lipase targets were carried out using the primers described in table 1. Due to the lack of available sequences for the lipase genes of *C. palmioleophila* in sequence repositories, primers targeting the open reading frame of the *LIP*1, *LIP*2 and *LIP*6 genes from the related yeast *Candida albicans* (Hube et al. 2000), as well as highly degenerate primers targeting the oxyanion hole and active-site regions of putative lipase genes (Bell et al. 2002), were used. *LIP*1, *LIP*2 and *LIP*6 sequences were amplified using a unique amplification protocol, consisting in a reaction mix composed of 1X PCR buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 1 U Taq DNA polymerase (Vivantis, Malaysia) and 1.5 µM of each primer. Amplification program consisted of 35 cycles of 94 °C (30 s), 52 °C (30 s) and 72 °C (1.5 min) and a final extension at 72 °C for 5 min. Oxyanion hole and active-site regions of putative lipase genes were amplified in a reaction mix composed of 1X PCR buffer, 2 mM $MgCl_2$, 200 µM dNTPs, 1.5 U Taq DNA polymerase (Vivantis, Malaysia) and 4 µM of each primer (either OXF1/ACR1 or OXF1/ACR3 primer combination). Amplification program consisted of 45 cycles of 94 °C (30 s), 52 °C (30 s) and 72 °C (1.5 min) and a final extension at 72 °C for 7 min. PCR products were analyzed by 1 % agarose gel electrophoresis and visualized under UV light.

Table 1. PCR primers used for the amplification of lipase sequences in C. palmioleophila

Primer	Sequence	Target	Expected Size of PCR product	Reference
OXF1 ACR1	CCYGTKGTSYTNGTNCAYGG AGGCCNCCCAKNGARTGNSC	Oxyanion hole and active-site regions of putative lipase genes	250 bp	Bell et al. (2002)
OXF1 ACR3	CCYGTKGTSYTNGTNCAYGG AGGCCRCCNTGNGARTGNSC		250 bp	Bell et al. (2002)
LIP1a LIP1b	ACAAATTCACTGGGATCAAGAG ATAAGTGACATGGACGTTACTG	Open reading frame of *C. albicans LIP*1 gene	545 bp	Hube et al. (2000)
LIP2a LIP2b	TTTCCGACTTTGCTGTTCCAG ATAATACTGCTTACAAGACCAAG	Open reading frame of *C. albicans LIP*2 gene	589 bp	Hube et al. (2000)
LIP6a LIP6b	TTAAACCTGGTGCCAAAGCTG TCGATGCCCTGGTGGTGAAC	Open reading frame of *C. albicans LIP*6 gene	441 bp	Hube et al. (2000)

2.3 LSSP-PCR and PCR-RFLP characterization of lipase genes

Low-stringency single specific primer PCR (LSSP-PCR) reactions were carried out by purifying the 250 bp amplicons obtained with primer sets OXF1/ACR1 and OXF1/ACR3 and then performing a second round of amplification by using only one primer specific for one end of the fragment, under conditions of low stringency (Barreto et al. 1996). The reaction mix contained 1X PCR buffer, 1.5 U of Taq DNA polymerase (Vivantis, Malaysia), 200 µM dNTPs, 2 mM $MgCl_2$ and 4 µM of primer OXF1 or ACR1. Amplification was achieved by using an initial denaturation at 94 °C for 5 min, followed by 35 cycles with denaturation at 94 °C for 30 s, annealing at 32 °C for 30 s and extension at 72 °C for 1 min. Ten microliters of each reaction were analyzed on 8 % nondenaturing polyacrylamide gels in TBE 0.5X buffer for 6 h at 110 V, being afterwards silver stained as described by Sanguinetti et al. (1994). Gels were photographed and analyzed using the Pyelph v4.0 program (Pavel and Vasile 2012) to create a binary data matrix of 0 and 1 representing the absence or presence of bands, respectively. Dendrograms were created using the UPGMA clustering method (Michener and Sokal 1957). PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) reactions were carried out by purifying amplicons obtained with the primer set LIP6a/LIP6b using the QIAquick PCR purification kit (QIAgen), and then performing a digestion with 2 units of *Alu*I restriction enzyme (Vivantis, Malaysia) for 16 h at 37 °C. Fragment separation was performed on 2.5 % agarose gels stained with SYBR gold nucleic acid gel stain (Invitrogen, USA), and visualized under UV light.

3. Results and discussion

3.1 Detection of lipase genes in *C. palmioleophila*

As shown in figure 1, the use of PCR primers flanking the oxyanion hole and active-site regions of putative lipase genes allowed to detect lipase gene sequences in all eight *C. palmioleophila* native strains. The OXF1/ACR1 primer combination produced the best results regarding band intensity and relative absence of unspecific bands, producing the expected amplicons of approximately 250 bp (Fig 1A). Using the same primer combination, Bell et al. (2002) obtained 250 bp amplicons which after being sequenced, showed high homology with lipases and phospholipase sequences; Terahara et al. (2010) reported also the detection of putative esterase genes from environmental DNA using primers OXF1-ACR1, whose sequences showed 32-80% identity lipases and esterases of the α/β hydrolase family. In contrast, the use of OXF1/ACR3 primer combination did not produce unique bands, but rather a combination of PCR products ranging from 250 to 400 bp (Fig 1B). Such heterogeneity in PCR products was not unexpected, due to the degenerate nature of the primers and the stringency conditions used for PCR amplification. In spite of the above, it must be noted that this primer combination also allowed to detect lipase sequences in all *C. palmioleophila* strains, as well as in lipolytic *Bacillus* sp. strains.

On the other hand, the use of specific primers designed to amplify lipase genes in *C. albicans* produced diverse results when tested on *C. palmioleophila*. The *LIP*1 gene was not detected in any of the *C. palmioleophila* strains, while *LIP*2 gene was detected only in strains SACL04, SACL09 and SACL11, but produced a shorter PCR amplicon of approximately 500 bp (Fig C, D). The *LIP*6 gene was instead detected in all eight *C. palmioleophila* strains, but amplifications also presented differences in PCR band number and size. Two different *LIP*6 bands of about 600 bp and 1,400 bp were detected, both of them being larger than the expected amplicon of 441 bp. In addition to suggesting differences in the *LIP*6 gene length between *C. albicans* and *C. palmioleophila*, this was also an indicative of clear differences among gene sequences. In *C. albicans*, five of the ten known lipase genes are located on the chromosome 1 (Hube et al. 2000; van het Hoog et al. 2007). *LIP*1, *LIP*2 and *LIP*6, the genes evaluated in this study, are also located on this chromosome. Together, the above results suggest that at least three genes encoding lipases are present in *C. palmioleophila*, two of them resembling the *LIP*2 and *LIP*6 genes of *C. albicans*.

3.2 Variability of lipase genes in *C. palmioleophila* and relation with palm oil removal

LSSP-PCR and PCR-RFLP were used to detect variations within sequences from lipase genes of oil and grease degrading microorganisms. As shown in figure 2A, banding profiles obtained by LSSP-PCR allowed to detect small variations among the eight *C. palmioleophila* and two lipolytic *Bacillus* sp. strains when OXF1 primer was used. Overall, clustering analysis showed a correlation between LSSP-PCR profiles and the degradation activities reported previously for each of these strains (Rodriguez-Mateus et al. 2016). Specifically, dendrograms based on UPGMA cluster analysis separated banding profiles into two major clusters: one grouping most of the *C. palmioleophila* strains which showed the higher palm oil degradation efficiency (strains SACL11, SACL05 and SACL08) and the other grouping most of the *C. palmioleophila* strains with an average palm oil degradation efficiency (strains SACL01, SACL03, SACL06 and SACL09). This suggests that small differences in this gene could explain, at least in part, some of the differences

observed regarding oil degradation by these lipolytic strains as observed previously in hydrocarbon-degrading microorganisms (Zafra et al. 2016).

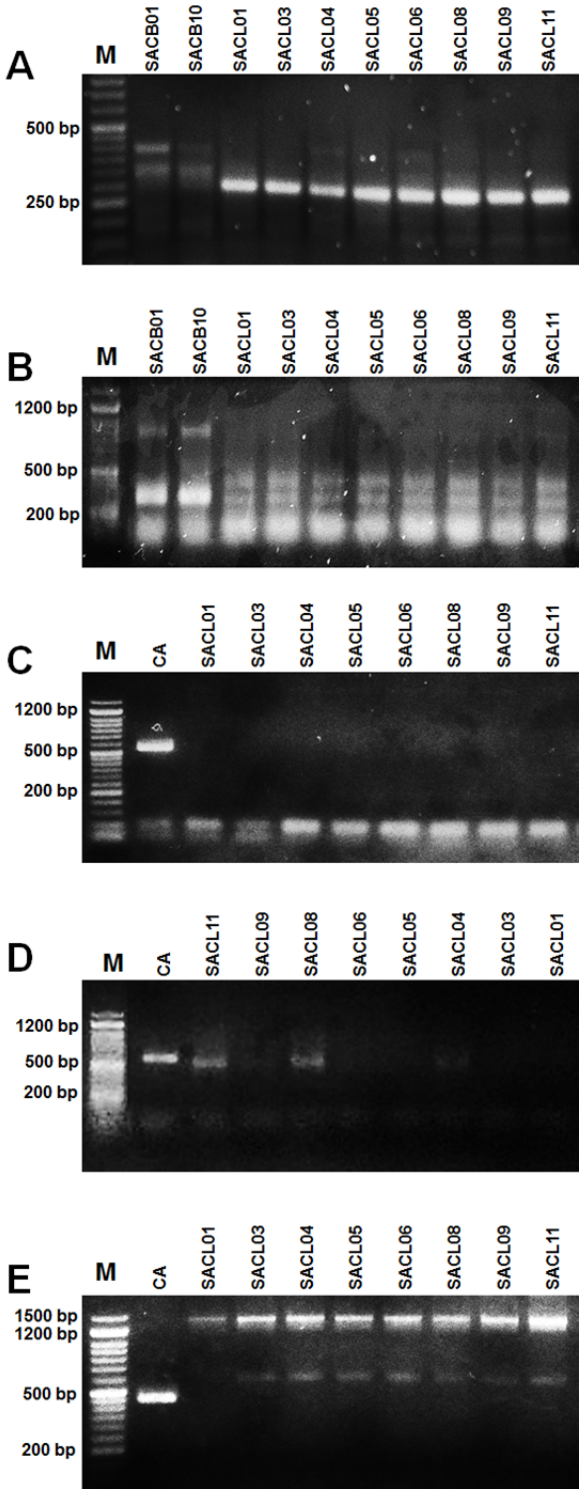

Figure 1. PCR detection of lipase genes in strains of C. palmioleophila using different primer combinations. (A, B) Amplification of putative lipase genes with OXF1/ACR1 and OXF1/ACR3 primer combination, respectively. (C) Amplification of C. albicans LIP1 gene with primers LIP1a/LIP1b. (D) Amplification of C. albicans LIP2 gene with primers LIP2a/LIP2b. (E) Amplification of C. albicans LIP6 gene with primers LIP6a/LIP6b. M: 100pb opti-DNA weight marker (abm, Canada); CA: C. albicans strain CA.

Band profiles from lipolytic *Bacillus* sp. strains grouped into a separated cluster, even though they shared about more than 80 % of bands with those of *C. palmioleophila*. Only one strain (SACL04), which presented the lowest palm oil-removal activity (37 %), did not grouped into its corresponding cluster and instead showed similarity to the band profiles of strains with the higher removal percentages of palm oil (77 to 79 %). Since in this case the variability detected in the analyzed gene does not correlated with the palm oil removal efficiency, we hypothesize that oil removal efficiency in strain SACL04 could be more closely related to a different lipase gene, since up to ten lipase genes have been reported in *Candida* species and most of them are sensitive to different substrates (Guncheva and Zhiryakova 2011; Hube et al. 2000). On the contrary, LSSP signatures obtained with the ACR1 primer did not allowed to separate *C. palmioleophila* strains into different clusters, but as expected, these band profiles were notoriously different than those of *Bacillus* strains (Fig 2B). This could be an indicative of the marked differences between lipase genes from yeast and bacteria, as reported previously (Bell et al. 2002). Previously, the differences between the band profiles obtained with the OXF1 and ACR1 primers have also been reported to be prominent (Orozco et al. 2012). Band profiles obtained with primer ACR1 were 100% similar between the strains corresponding to each of the genus (*Bacillus* or *Candida*). On the other hand, due to the overall low amplicon length obtained for most of the PCR assays, PCR-RFLP typing was performed only on the purified 1,400 bp products from *LIP*6 gene amplifications in order to maximize the probability of finding restriction sites. The selection of *Alu*I restriction endonuclease for RFLP-assays was based on the described *LIP*6 gene sequence of *C. albicans* deposited in GenBank (NCBI Reference Sequence NC_032089.1, region 2,114,091 to 2,115,482), in which we detected restriction sites for *Alu*I restriction enzyme *in silico* but not for other common restriction enzymes (BamHI, EcoRI, HindIII, NotI). However, no restriction sites were detected in any of the *C. palmioleophila* strains by using this enzyme in PCR-RFLP assays (data not shown).

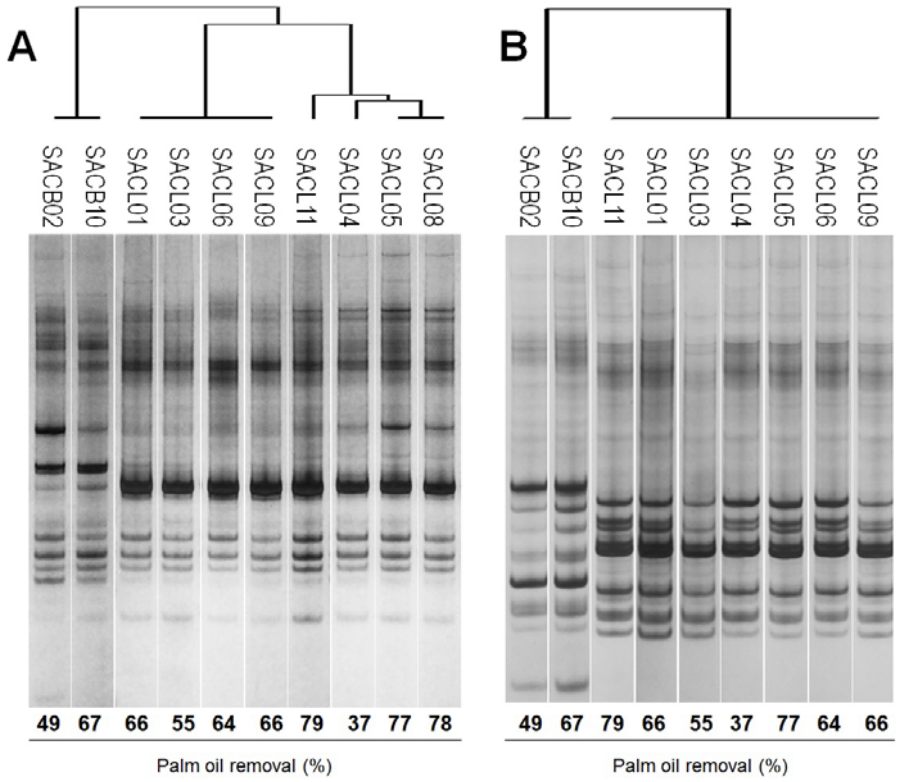

Figure 2. LSSP-PCR signatures of putative lipase gene fragments from lipolytic strains of C. palmioleophila and Bacillus sp. Clustering was performed using the UPGMA method. (A) Banding patterns obtained with OXF1 primer. (B) Banding patterns obtained with ACR1 primer. Removal percentages of palm oil by each of the strains as described by Rodriguez-Mateus et al. (2016) are indicated.

4. Conclusions

In conclusion, the results suggest that at least three genes encoding lipases are present in *C. palmioleophila*, two of them resembling the *LIP*2 and *LIP*6 genes of *C. albicans*. Moreover, we found that genetic variations in the oxyanion hole and active-site regions of a putative lipase gene were related to the ability to metabolize more or less efficiently crude palm oil by *C. palmioleophila*, which confirmed a key role of these enzymes on the differences in oil degradation by this lipolytic yeast. The above findings summed to the reported potential of this yeast to remediate POMEs and complex oil and grease-polluted matrices, makes the study of these new biocatalyst from *C. palmioleophila* especially interesting for environmental and pharmacological purposes, among others, and opens the possibility of finding native enzymes presenting naturally high lipolytic activity. To the best of our knowledge, this is the first description of the lipase genes of the lipolytic yeast *C. palmioleophila* and their role in palm oil degradation.

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