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EXPRESSION AND PURIFICATION OF A NEW ESTERASE FROM METAGENOMIC CLONE SIMILAR TO BACTERIAL ESTERASE OF THE *Porticoccus hydrocarbonoclasticus*

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ABSTRACT – *Esterases are lipolytic enzymes widely used in industrial applications. This work aimed the overexpression and purification of the esterase LipG7 identified from a metagenomic library constructed from mangrove sediments, which gene sequence shares 80% amino acid identity to 1,4-butanediol diacrylate esterase from the bacterium Porticoccus hydrocarbonoclasticus. LipG7 was expressed in three commercial Escherichia coli strains, Rosetta-gami, ArcticExpress and BL21 and the activity evaluated against 4-nitrophenyl butyrate substract. Recombinante esterase was obtained in soluble form only in Rosetta-gami after treatment with guanidine hydrochloride. It was active against 4-nitrophenyl butyrate at 30 °C with specific activity of 216.3 ± 16.4 U/mg that was significantly enhanced in presence of* Mg^{2+} *ion.*

1. INTRODUTION

The global market of the hydrolases currently generates circa of 4 billion dollars per year (ABIAM, 2015). Esterases are characterized by the hydrolysis of the fatty acid short-chain ester bonds (C<10). These enzymes can be applied in several industrial segments such as pharmaceutical, cosmetics, food, detergent, tannery, textile, paper and biodiesel (GUNCHEVA; ZHIRYAKOVA, 2011).

Taking into account that less than 0.1% of microorganisms from soil are cultivable, metagenomic techniques can be applied in order to enable uncultured access to all genetic potential in an environmental sample (GAO et al., 2016). The interest in the discovery of novel enzymes and/or enzymes with improved characteristics has guided research through the metagenomics of different ecosystems.

The present work aimed the expression, purification and activity evaluation of LipG7, a recombinant esterase identified in a metagenomic library from mangrove sediments. This enzyme is 80% identical to 1,4-butanediol diacrylate esterase of *P. hydrocarbonoclasticus*, therefore it represents a new candidate for hydrocarbon biodegradation.

2. MATERIALS AND METHODS

2.1. Production of LipG7

In previous work, the LipG7 esterase gene was isolated from a metagenomic library and subcloned under the control of the pET302 / NT expression vector (Thermo Fisher, USA) into 3 different commercial strains of Escherichia coli (BL21, Rosetta Gami and Arctic Express). From these results it was found that only in the Rosetta gami strain the enzyme was obtained in its active form.

For the production of the enzyme, the strain *E. coli* Rosetta-gami loaded with the esterase gene (*LipG7*) was induced using a 2 L Erlenmeyer flask containing 400 mL of LB broth and ampicillin (100 μg /mL). The strain was grown at 37 °C under 200 rpm agitation until an optical density of 0.6 - 0.8 at 600 nm. Induction of the enzyme expression was accomplished by the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30 °C for 3 h. After induction, cells were lysed by sonication (Qsonica Q125, USA) using 2 cycles of 3 sec *on* and 3 sec *off*, for 5 min at 30% amplitude and centrifuged at 15,000 x g, 4 °C for 45 min.

Two denaturing agents, guanidine hydrochloride and N-lauryl sarcosine detergent, were tested for the treatment of the insoluble fraction. For the guanidine hydrochloride test, the pellet containing the insoluble protein was resolubilized in 100 mM Tris-HCl pH 8.0 buffer, 5 mM EDTA, 5 mM dithiothreitol (DTT), 2 M Urea and 2% TritonX-100 and centrifuged at 15,000 x g, 4 °C for 30 min. This process was repeated 3 times and aliquots were withdrawn in each wash cycle. After that, the pellet was resuspended in 50 mM Tris-HCl buffer, pH 8.0 and 3 M guanidine hydrochloride and was left at room temperature for 30 min and then centrifuged at 8,000 x g, 4 °C for 30 min (PALMER; WINGFIELD, 2004). For the N-lauryl sarcosine, the pellet was solubilized in 100 mM Tris-HCl buffer pH 8.0 with 0.2% N-lauryl sarcosine and incubated in low rotation at 13 °C for 16 h (PETERNEL et al., 2008).

2.2. LipG7 purification

The protein purification was performed by affinity chromatography using the Ni-Sepharose 6 Fast Flow column (GE Healthcare Life Sciences, USA). The column was first equilibrated with 50 mM Tris-HCl pH 8.0 buffer and 100 mM NaCl and subsequently loaded with the protein extract containing the enzyme in the soluble form. The column was then washed with 50 mM Tris-HCl pH 8.0 buffer, 100 mM NaCl and 6 mM imidazole for the removal of low-affinity proteins. Elution of the enzyme was evaluated by the addition of 50, 100, 150, 200 and 250 mM imidazole. The performance of induction, treatment of inclusion and purification bodies were evaluated by Laemmli SDS-PAGE electrophoresis. The enzyme concentration at the end of the procedure was determined using spectrophotometry at 280 nm with the aid of the enzyme extinction coefficient (1.367 ml/mg/cm) which was deduced from the amino acid sequence.

Lipolytic activity was measured by the release of 4-nitrophenol during the hydrolysis of the substrate 4-nitrophenyl butyrate (C4) (Sigma-Aldrich, USA). The hydrolysis reaction was done in 96-well microplates in a total volume of 260 μl, using 1mM of the 4-nitrophenyl butyrate substrate and 50 mM phosphate buffer pH 7.2 at 30 °C for 30 min. The amount of 4-

nitrophenol resulting from the enzymatic activity was observed by spectrophotometry at the wavelength of 410 nm whose distinguishing coefficient of 123.45 was calculated by constructing a standard curve of that compound. All experiments were analyzed in triplicates in this way the results are expressed as mean values and the standard deviation is calculated. A unit of enzyme activity was defined as the amount required to release 1 μmol of 4-nitrophenol per min.

2.3. Effect of the addition of divalent ions and detergents on LipG7 activity

The catalytic activity of LipG7 was evaluated regarding influence of metal ions, nonionic surfactants (TritonX-100 and Tween 80) and EDTA. For this, enzyme activity reaction containing 1 mM of 4-nitrophenyl butyrate in 0.5 mM phosphate buffer pH 7.2 was measured in the presence of 0.5 mM of the Mg^{2+}, Mn^{2+} or Ca^{2+}, 1% (v/v) of Triton X-100, 1% (v/v) of Tween 80 and 0.5 mM EDTA. The reaction was incubated at 30 °C for 30 min and then analyzed in a spectrophotometer at 410 nm. For greater reliability of the results, controls of the reactions were made for each condition. All experiments were analyzed in triplicates and the data were statistically analyzed using the ANOVA method and the multiple comparisons method (Tukey test) using the program GraphPad Prism version 6.01.

3. RESULTS AND DISCUSION

3.3. Resolubilization and refolding of LipG7 from inclusion bodies

In order to obtain LipG7 from inclusion bodies a chaotropic agent and a weak detergent were tested. The treatment using the N-lauryl sarcosine detergent was able to solubilize the enzyme, but it did not show catalytic activity. On the other hand, the solubilization of the inclusion bodies using 3M of guanidine hydrochloride proved effective in the denaturation and solubilization of the protein, presenting catalytic activity after renaturation to its native form.

3.4. LipG7 purification

To evaluate the ideal concentration of imidazole, solutions of 50, 100, 150, 200 and 250 mM were used for elution. As a result, Laemmli SDS-PAGE showed that the concentration of 150 mM imidazole is ideal to elute a large part of the enzyme without compromising its catalytic activity, presenting a specific activity of 216.3 ± 16.4 U/mg after dialysis of the imidazole (Figure1). This value is higher than those found in metagenomic libraries of other samples from other soils such as Jiménez et al. (2012) and Kim et al. (2006) who prospected for new extremophilic esterases with a specific activity of 0.142 U / mg and 63.7 U / mg, respectively.

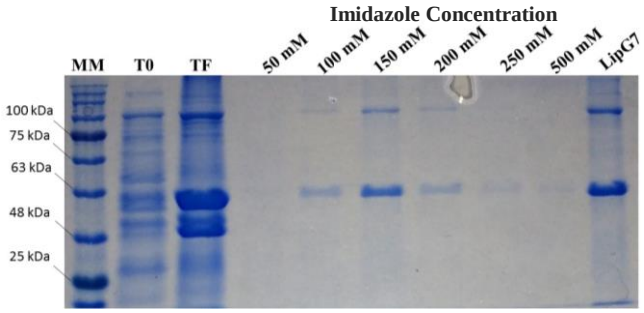

Figure 1 – Eletrophoresis gel of the purification of the recombinant esterase (LipG7) under gradient of imidazole. Legend: MM, molecular marker; T0, initial protein profile; TF, final protein profile.

3.5. Effect of the addition of divalent ions and detergents on LipG7 activity

The activity of esterases is frequently affected by factors such as divalent metal ions and nonionic surfactants (CHOI et al., 2004). The addition of 0.5 mM of the Mg^{2+}, Mn^{2+} or Ca^{2+} ions promoted a significant increase in the catalytic activity of LipG7, highlighting the Mg^{2+}. The addition of 0.5 mM EDTA did not present a significant difference in relation to the control. And the presence of Tween 80 and Triton X-100 detergents decreased the catalytic activity as shown in figure 2. In our study, the increase in activity in the presence of $MgCl^{2+}$ indicates that the catalytic site of LipG7 can be stimulated by the action of Mg^{2+}.

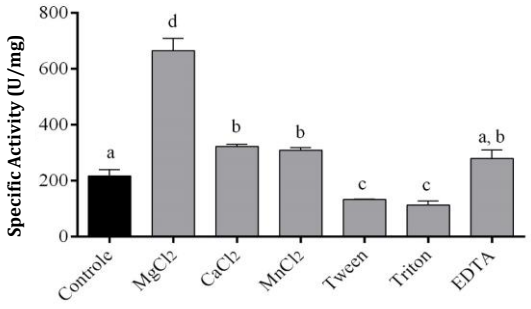

Figure 2 – Esterase activity of LipG7 under the influence of bivalent ions, detergents and EDTA. Different letters indicate mean differences ($p < 0.05$), according to ANOVA.

4. CONCLUSION

LipG7 recombinant esterase was overexpressed in *E. coli* Rosetta-Gami, partially purified for affinity chromatography using 150 mM imidazole and its activity against 4-nitrophenyl butyrate at 30 °C was enhanced in presence of Mg^{2+}. Accessing the metagenomic pool of lipases and esterases from mangrove sediments can be an immediate source of novel biocatalyst.

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