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ORIGINAL PAPER

Characterization of a novel immobilized biocatalyst obtained by matrix-assisted refolding of recombinant polyhydroxyoctanoate depolymerase from *Pseudomonas putida* KT2442 isolated from inclusion bodies

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Abstract Purification and matrix-assisted refolding of recombinant His-tagged polyhydroxyalkanoate (PhaZ) depolymerase from *Pseudomonas putida* KT2442 was carried out. His-tagged enzyme was overproduced as inclusion bodies in recombinant *E. coli* M15 (pREP4, pPAZ3), which were denatured by 8 M urea, immobilized on Ni^{2+}-nitrilotriacetate-agarose matrix, and refolded by gradual removal of the chaotropic agent. The refolded enzyme could not be eluted with 1 M imidazole buffer, leading to an immobilized biocatalyst where PhaZ depolymerase was homogeneously distributed in the agarose support as shown by confocal scanning microscopy. Polyhydroxyoctanoate could not be hydrolyzed by this novel immobilized biocatalyst, whereas the attached enzyme was active in the hydrolysis of *p*-nitrophenyl alkanoate esters, which differed in their alkyl chain length. Taking advantage of the observed esterase activity on *p*-nitrophenylacetate, functional characterization of immobilized PhaZ depolymerase was carried out. The immobilized enzyme was more stable than its soluble counterpart and showed optimal hydrolytic activity at 37°C and 50 mM phosphate buffer pH 8.0. Kinetic parameters were obtained with both *p*-nitrophenylacetate and *p*-nitrophenyloctanoate, which had not been described so far for the soluble enzyme, representing an attractive and alternative chromogenic assay for the study of this paradigmatic enzyme.

Keywords PHA depolymerase · Immobilization · Refolding · Polyhydroxyalkanoate

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Introduction

Polyhydroxyalkanoate depolymerases are microbial enzymes that degrade polyhydroxyalkanoates (PHAs), biopolyesters produced by a wide range of bacteria when the environmental conditions are not optimal for growth [1, 11, 18]. There is a great interest in the study of the PHAs hydrolysis by PHA depolymerases that lies not only in their potential use as bioplastics or biomaterials but also in the production of chiral (*R*)-hydroxyalkanoic acids (RHAs) [3, 27]. For instance, (*R*)-3-hydroxybutyric acid has proven to exhibit antimicrobial, insecticidal, and antiviral activities [2]. In addition, RHAs are valuable intermediates that can be used as starting materials for the synthesis of antibiotics, vitamins, flavors, and pheromones [4, 17, 20, 25].

PHA depolymerases can be broadly classified under intracellular and extracellular depolymerases [11, 15, 26]. Extracellular depolymerases degrade partially crystallized or denatured PHA, whereas intracellular depolymerase act on amorphous or native PHA. In addition, PHA depolymerases are specific for either short-chain length PHA (*scl*-PHA, three to five carbon atoms per monomer, EC 3.1.1.75) or for medium-chain length PHA (*mcl*-PHA, six or more carbon atoms per monomer, EC 3.1.1.76). In this sense, PhaZ depolymerase of the model strain *Pseudomonas putida* KT2442 is an intracellular depolymerase that is

located in PHA granules and hydrolyzes specifically *mcl*-PHAs containing aliphatic and aromatic monomers. This paradigmatic enzyme has been recently purified and biochemically characterized after its overexpression in *Escherichia coli* [5]. It is worth mentioning that most of the overproduced recombinant enzyme was deposited with the insoluble fraction of the crude extract, and only a very low amount of soluble enzyme was present in the supernatant of the crude extract after ultracentrifugation. In addition, the efficiency of the purification of the recombinant enzyme using Ni^{2+}-NTA agarose gel was rather low due to weak binding of the His-tagged PhaZ to the matrix and the purified enzyme showed poor stability. In this article, His-tagged PhaZ depolymerase of *P. putida* KT2442 was purified and "on-column" refolded from inclusion bodies by matrix-assisted procedures in order to directly prepare an immobilized biocatalyst. Likewise, kinetic and microstructural characterization of the immobilized biocatalyst have also been carried out.

Materials and methods

Overproduction of recombinant PhaZ depolymerase from *P. putida* KT2442

A preculture of *E. coli* M15 (pREP4, pPAZ3) was incubated overnight in LB medium plus ampicillin and kanamycin as described [5]. Culture was diluted until an optical density (OD) at 600 nm of 0.1. When OD reached 0.5, the culture was induced with 0.5 mM IPTG, and cells were further incubated for 4 h at 37°C before being pelleted [5].

Purification of recombinant PhaZ depolymerase inclusion bodies

His-tagged PhaZ depolymerase was produced as insoluble inclusion bodies, which were extracted from the cell pellet by using the B-PER bacterial protein extraction reagent (Pierce, Rockford, USA) according to the protocol described by the manufacturer.

Immobilization of the His-tagged PhaZ depolymerase

The inclusion bodies preparation was dissolved in 50 mM Tris–HCl buffer pH 8 with 150 mM NaCl and 8 M urea (buffer A) up to a 0.7 mg/ml protein concentration estimated by using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, USA). A total of 7.8 ml of this enzyme solution was loaded into a column filled with 2.6 ml of Ni^{2+}-NTA agarose gel (Qiagen, Hilden, Germany) equilibrated with buffer A. After washing the column with six column volumes of buffer A, the enzyme attached to the agarose gel was refolded by applying a gradient from 8 to 0 M urea. The total buffer volume was 15 column volumes, and the flow rate was 0.4 ml/min.

Electrophoretic and Western-blot techniques

About 20 µl of immobilized enzyme on Ni^{2+}-NTA agarose was boiled in the presence of 10 µl of a solution containing 4% (w/v) SDS (sodium dodecyl sulphate) and 10% (v/v) β-mercaptoethanol and, after centrifugation, 20 µl of the supernatants were analyzed by SDS-PAGE [16]. This treatment ensured the release of all the enzyme molecules attached to the support. Polyacrylamide gels at 12.5% concentration were used to carry out SDS-PAGE, and gels were stained with Coomassie Brilliant Blue G-250. Western blotting was carried out by employing a rabbit polyclonal antiserum against PhaZ as described [5].

Preparation of PHA latex

PHA was isolated from *P. putida* KT2442 grown in minimal medium with sodium octanoate (0.75% wt/vol) as the carbon source [5], and disrupting the cells by French press. PHA latex was provided by Biopolis, Spain, as previously described [23]. PHA latex was a heteropolymer (3-hydroxyoctanoate-*co*-hexanoate (92% Ho and 8% Hx). Radioactive PHA was isolated from *Pseudomonas putida* KT 2442 grown in minimal medium with 10 µCi of [^{14}C] octanoic acid as carbon source [5].

PHO depolymerase activity assay by pH-stat

PHA depolymerase activity of the immobilized His-tagged enzyme on Ni^{2+}-NTA agarose was assayed by the determination of the NaOH consumption rates necessary to keep at pH 8.0 a low-buffered suspension of PHA latex [7, 10]. A pH-stat apparatus equipped with Taimo 1.3 software was used (Titrando 842, Methrom, Switzerland). The reaction temperature was 37°C, and 0.02 N NaOH was used for the titration. The volume of the reaction mixture was 3 ml, which contained 5 mM Tris–HCl, 300 mM NaCl, 400 µl PHA latex, and 100 µl of the immobilized depolymerase after the refolding process. The minimum and maximum rates of NaOH ranged from 2 µl/min to 5 µl/min. Controls without latex, without immobilized biocatalyst, and with Ni^{2+}-NTA agarose were performed to check possible titration not due to enzymatic activity.

PHA depolymerase radioactive assay

The assay mixture of 250 µl contained 50 µl of radioactive PHA latex (specific activity of 284.11 cpm/µl), 0.2 M Tris–HCl pH 8.0, 0.3 M NaCl, and 125 µl of the refolded

enzyme. The mixture was incubated at 37°C for 30 min, and the reaction was stopped with 10 µl of formaldehyde. The samples were centrifuged at 16,000 × *g* for 25 min at 4°C, and the radioactivity in 200-µl portions of the supernatant was determined in a scintillation counter [5].

Esterase activity assay

Esterase activity of the immobilized depolymerase on Ni^{2+}-NTA agarose was measured at 37°C using *p*-nitrophenylacetate (*p*NPA) as substrate. The volume of the reaction mixture was 1 ml, which contained 50 mM Tris–HCl pH 8.0, 300 mM NaCl, 0.3 mM *p*NPA, and 20 µl of the immobilized depolymerase after the refolding process. All measurements for immobilized enzyme were carried out in triplicate and the maximum error was below 5%. One international activity unit (IU) was defined as the amount of enzyme producing 1 µmol/min of *p*-nitrophenol (*p*NP) under the assay conditions described above.

Effect of pH on immobilized depolymerase activity

The effect of pH on the immobilized PhaZ depolymerase activity was studied at different pH values ranging from 6.5 to 11 in 50 mM Tris/MOPS/borate polybuffer at a constant ionic strength (*I*) of 350 mM by addition of NaCl in amounts calculated using a Visual Basic program developed in our laboratory, which allows analysis of buffer systems with up to four tetraprotic species, and 37°C. The enzymatic activity of immobilized enzyme was determined using the standard esterase assay described above at each pH value.

Temperature studies

The effect of temperature on the immobilized PhaZ depolymerase activity was studied at different temperatures ranging from 25 to 60°C in 50 mM potassium Tris/HCl buffer pH 8.0. The enzymatic activity of immobilized enzyme was determined using *p*NPA as substrate as described above at each temperature. The effect of temperature on the stability of the immobilized derivatives was evaluated by incubating 20 µl of immobilized derivative in 970 µl of 50 mM Tris/HCl buffer, 150 mM NaCl pH 8.0 during 30 min at different temperatures ranging from 30 to 60°C. Then, the mixture was incubated at 4°C in bath ice for 5 min and the activity of the immobilized enzyme was determined after adding *p*NPA following the standard esterase assay.

Substrate specificity of immobilized PhaZ depolymerase

The kinetic parameter values for the immobilized His-tagged PhaZ depolymerase were determined using *p*-nitrophenylacetate and *p*-nitrophenyloctanoate (*p*NPO) as substrates. Enzymatic activity was measured using 20 µl of immobilized biocatalyst, which were added to a 980 µl of buffer solution with different substrate concentrations. Enzymatic reactions were performed in 50 mM Tris–HCl pH 8.0, 300 mM NaCl at 37°C.

Confocal scanning microscopy of immobilized PhaZ depolymerase

Immobilized enzyme (10 mg) was incubated with 5 ml of phosphate buffer saline (PBS: 137 mM NaCl, 26.8 mM KCl, 1.47 mM KH_2PO_4, 1.12 mM Na_2HPO_4 dihydrate, pH 7.4) containing 1% (w/v) BSA for 30 min. After that, immobilized derivative was washed three times with 5 ml of PBS containing 0.1% (w/v) BSA, and incubated for 2 h at 37°C with 5 ml of an antibody to PhaZ depolymerase solution prepared in PBS with 0.1% (w/v) BSA. Rabbit antibodies against PhaZ depolymerase were prepared as described [5]. The biocatalyst was washed again with 5 ml of PBS containing 0.1% (w/v) BSA, and incubated for 2 h at 25°C with 5 ml of Alexa Fluor 488 dye goat anti-rabbit whole antibody conjugate prepared in PBS with 0.1% (w/v) BSA at a final concentration of 5 µg/ml of antibody conjugate. A negative control experiment was carried out to check a specific binding of the secondary antibody to the support. Immobilized derivative was washed three times with 5 ml of PBS containing 0.01% (w/v) BSA for 10 min, followed by three washings with 5 ml of PBS for 10 min, and finally three times with 5 ml of deionized water for 5 min. The biocatalyst was then analyzed using a Confocal Radiance 2000 coupled to Axiovert S100 TV microscope (Carl Zeiss, Jena, Germany) with three passes with a Kalman filter and a 1024 × 1024 collection box. The images were analyzed by a Leica Confocal Software Lite Version (Leica Microsystems, Wetzlar, Germany).

Results

Immobilization of the His-tagged PhaZ depolymerase on Ni^{2+}-NTA agarose gel

Isolation and purification of the inclusion bodies of His-tagged PhaZ depolymerase produced by fermentation of *E. coli* M15 (pREP4, pPAZ3) was achieved by using B-PER bacterial protein extraction reagent from PIERCE following the protocol provided by manufacturer (Fig. 1, lane 2). Then, as described in the experimental section, the denatured inclusion bodies solution was loaded into a column filled with 2.6 ml of Ni^{2+}-NTA agarose gel equilibrated with buffer A containing 8 M urea, in order to allow His-tag to interact with the support. After washing the column with six column volumes of buffer

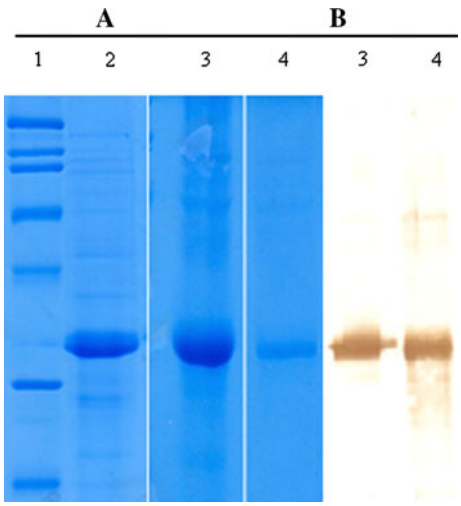

Fig. 1 SDS-PAGE (**a**) and Western blot (**b**) analysis of the refolded His-tagged PhaZ depolymerase on Ni^{2+}-NTA agarose gel. *Lane 1* broad range prestained molecular weight markers (BioRad); *lane 2* inclusion bodies preparation; *lane 3* sample of Ni^{2+}-NTA agarose with His-tagged PhaZ depolymerase before the urea gradient; *lane 4* sample of Ni^{2+}-NTA agarose with His-tagged PhaZ depolymerase after the urea gradient

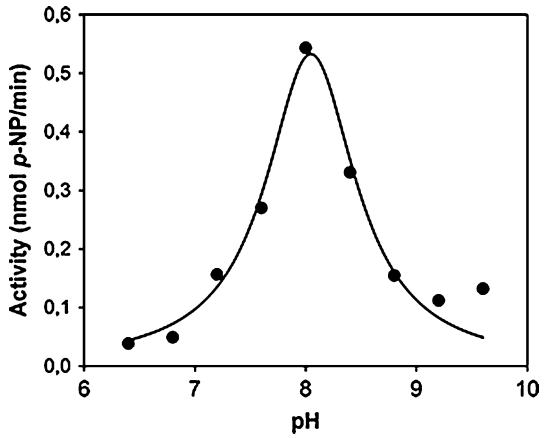

Fig. 2 Effect of pH on immobilized His-tagged PhaZ depolymerase activity. The assays were carried out with 20 µl of immobilized enzyme (650 µg protein/ml) in a final reaction volume of 1 ml of 50 mM Tris–HCl / MOPS / borate polybuffer system at different pH values ranging from 6.5 to 11 containing 3 mM *p*NPA as substrate for 30 min at 37°C. NaCl was added to adjust the ionic strength (*I*) to 350 mM

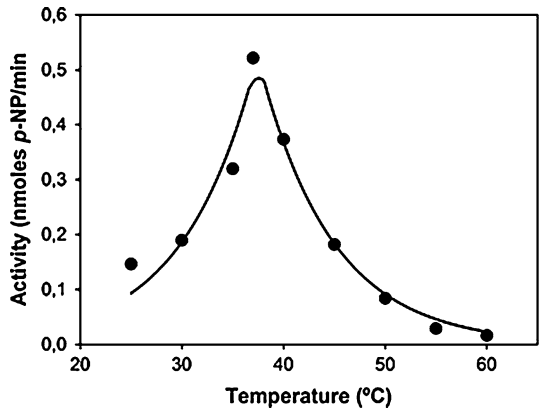

Fig. 3 Effect of temperature on immobilized His-tagged PhaZ depolymerase activity. The assays were carried out with 20 µl of immobilized enzyme (650 µg protein/ml) in a final reaction volume of 50 mM Tris/HCl pH 8.0 buffer containing 300 mM NaCl, and 3 mM *p*NPA as substrate for 30 min

A containing 8 M urea, the enzyme remained attached to the support as confirmed by SDS-PAGE and Western blot (Fig. 1, lanes A3 and B3). Finally, the enzyme bound to the agarose gel was subjected to a refolding process by applying an elution gradient of buffer A containing from 8 to 0 M urea. In such elution conditions the enzyme remains attached to the support (Fig. 1, lanes A4 and B4). An immobilized enzyme loading 650 µg protein/ml wet agarose was finally achieved. Interestingly, the enzyme cannot be eluted by increasing the imidazole concentration of the elution buffer up to 1 M, indicating that the His-tag could hide during the refolding process. This result might also explain the very weak binding of the soluble His-tagged enzyme during its purification using Ni^{2+}-NTA agarose [5], indicating that the His-tag could be buried into the protein structure and could not interact efficiently with the nickel attached to the support. On the other hand, it is worth mentioning that the refolding of the denatured inclusion bodies failed when the process was carried out in solution (data not shown), therefore, our method provides a simple one-step chromatographic procedure to generate a refolded immobilized enzyme ready to use. Finally, immobilized enzyme could only be eluted from the Ni^{2+}-NTA agarose by using 20 mM potassium phosphate buffer pH 8.0 containing 500 mM NaCl and 50 mM EDTA.

Characterization of immobilized PhaZ depolymerase in Ni^{2+}-NTA agarose

Hydrolysis of polyhydroxyoctanoate (PHO) catalyzed by immobilized His-tagged PhaZ depolymerase on Ni^{2+}-NTA agarose gel was measured by pHstat and radioactive assay, showing no enzymatic activity with PHO latex. In addition, the obtained immobilized biocatalyst on Ni^{2+}-NTA agarose gel was successfully assayed for enzymatic activity by spectrophotometric determination of *p*-nitrophenol (*p*NP) released during the hydrolysis of *p*-nitrophenylacetate at 37°C and pH 8.0. By using this chromogenic assay, the characterization of immobilized biocatalyst was further performed to establish the optimal conditions for catalytic activity. The effect of pH and temperature on immobilized PhaZ depolymerase activity is described on Figs. 2 and 3. As observed, the maximum enzyme activity was achieved at pH 8.0 and 37°C. In addition, the effect of temperature on stability of immobilized recombinant PhaZ

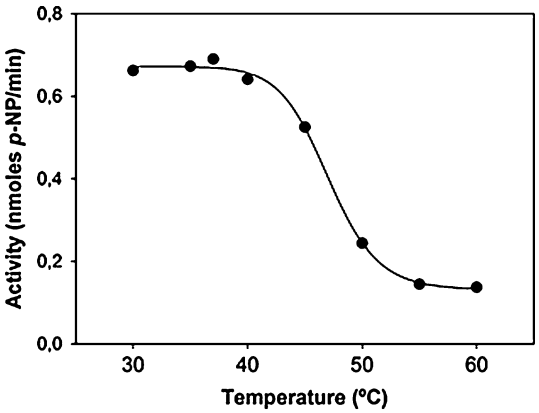

Fig. 4 Effect of temperature on immobilized His-tagged PhaZ depolymerase stability. Immobilized enzyme was incubated for 30 min at the indicated temperature in 50 mM Tris–HCl pH 8.0 buffer containing 150 mM NaCl. Residual activity was assayed at 37°C in standard conditions described in experimental section

depolymerase was also studied. Immobilized enzyme maintained 100% activity up to 40°C, but the activity quickly diminished at higher temperatures (Fig. 4). Interestingly, the soluble enzyme is very unstable, becoming inactive after 30 min of incubation at 37°C [5], whereas the immobilized derivative is more thermostable, and furthermore, it retains its full activity after storage for several weeks at 4°C. Finally, kinetic parameters of immobilized His-tagged PhaZ depolymerase were determined by using two different chromogenic substrates such as *p*-nitrophenylacetate and *p*-nitrophenyloctanoate (Table 1). Immobilized enzyme showed similar K_m values for both substrates, whereas V_{max} was higher with *p*-nitrophenylacetate (1.58 nmol/min) than the value obtained with *p*-nitrophenyloctanoate (0.98 nmol/min).

Immobilized PhaZ depolymerase distribution in Ni^{2+}-NTA agarose detected by confocal scanning microscopy

Distribution of immobilized PhaZ depolymerase molecules in Ni^{2+}-NTA agarose beads was investigated using confocal scanning microscopy, taking into account that agarose shows no autofluorescence. As shown in Fig. 5, green-colored areas represent the presence of immobilized enzyme labeled with Alexa Fluor-488 dye, which provides the maximal fluorescence emission at 519 nm. Once individually captured confocal xy sections at different *z*-positions (with a 1-μm depth increment) were collected, different three-dimensional volumes of the immobilized biocatalyst and their enzyme distribution could be reconstructed (Fig. 5). As observed, presence of a homogeneous enzyme distribution either inside the carrier or in the support surface was confirmed.

Table 1 Kinetic parameters of immobilized His-tagged PhaZ depolymerase on Ni^{2+}-NTA agarose

	*p*NP-acetate	*p*NP-octanoate
V_{max} (nmol/min)[a]	1.58 ± 0.14	0.98 ± 0.08
K_m (mM)	0.97 ± 0.20	0.93 ± 0.16

[a] See conditions in experimental section

Discussion

His-tagged PhaZ depolymerase of *P. putida* KT2442 overproduced by *E. coli* have been purified from inclusion bodies, and further refolded and immobilized on Ni^{2+}-NTA agarose gel by using a simple one-step metal affinity chromatography. To obtain active enzyme, the attached enzyme was refolded by applying an inverse gradient of urea ("on-column refolding"). A similar approach was successfully applied to the refolding of PHA synthase from *Pseudomonas aeruginosa* [19] and other recombinant membrane proteins [22], which were produced by *E. coli* and isolated from inclusion bodies. In contrast to these reports, PhaZ depolymerase remained attached to Ni^{2+}-NTA agarose after washing with 1 M imidazole buffer, and only the presence of EDTA allowed the elution of both enzyme molecules and nickel atoms from the support. These data would support that the His-tag of the recombinant enzyme might be hidden during the refolding process, and only quelation of nickel by EDTA could break the enzyme-support interaction leading to enzyme elution. Thus, matrix-assisted refolding of His-tagged PhaZ depolymerase allowed the direct preparation of an immobilized biocatalyst in which the recombinant enzyme is strongly attached to Ni^{2+}-NTA agarose beads. Unfortunately, hydrolysis of polyhydroxyoctanoate (PHO) catalyzed by immobilized enzyme failed, likely due to the substrate hindrance to enter into the active site of the immobilized depolymerase, which is linked inside the support and/or near to the agarose surface as observed by confocal scanning microscopy of immobilized enzyme, which was labeled with Alexa Fluor-488 dye antibody conjugates, a very useful technique for the determination of enzyme distribution in activated carriers [8, 9]. Such catalytic behavior was not observed in PHO depolymerase from *Pseudomonas fluorescens* GK13 adsorbed onto Accurel MP-1000, which was successfully used in the production of (*R*)-3-hydroxyoctanoic acid from PHO [6]. In this latter case, the authors indicated a favorable substrate partitioning in the microenvironment of the immobilized enzyme due to the hydrophobic nature of both substrate and polypropylene support as one of the reason for efficient PHA hydrolysis. Taking into account this fact, such partitioning

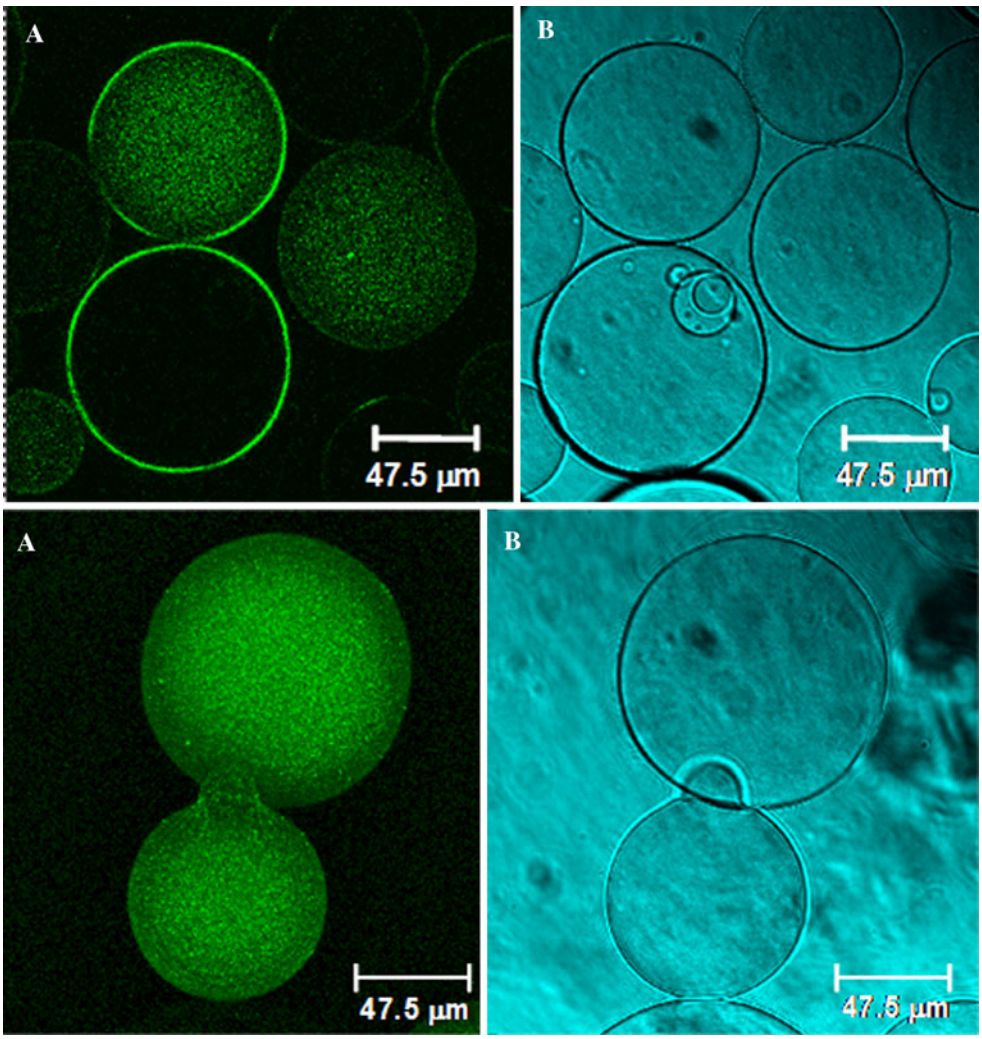

Fig. 5 Microscopy of immobilized PhaZ depolymerase in Ni^{2+}-NTA agarose: **a** confocal scanning microscopy; **b** optical microscopy. The immobilized enzyme labeled with Alexa Fluor-488 dye antibody conjugates is perfectly attached and homogeneously distributed on the carrier

could barely occur in our immobilized *P. putida* depolymerase on hydrophilic agarose, and this might also explain the nil activity on PHA latex. On other hand, kinetic parameters of immobilized His-tagged enzyme using two *p*-nitrophenyl ester derivatives have been determined, being the first time that esterase activity is described for this enzyme since the hydrolysis of such substrates has been not detected with the soluble form [5]. This hydrolytic activity has demonstrated to be very useful to carry out the functional characterization of the immobilized biocatalyst. In this sense, our results indicate that the immobilized enzyme is much more stable than its soluble counterpart, which is quickly deactivated at 37°C [5]. On other hand, the immobilized derivative shows an optimal activity at 37°C and pH 8.0 using *p*NPA as substrate. Regarding to its substrate specificity, catalytic efficiency (V_{max}/K_M) of immobilized PhaZ depolymerase was surprisingly higher with *p*NPA than with the medium-chain length *p*NPO (Table 1). Such behavior could be related to diffusional problems of hydrophobic *p*NPO to cross the hydrophilic agarose matrix and reach the catalytic active site of the immobilized enzyme. In this sense, soluble *mcl*-PHA depolymerases from *Pseudomonas alcaligenes* LB19 [12], *Pseudomonas luteola* M13-4 [21], *P. fluorescens* GK13 [6, 24], *Streptomyces* sp. KJ-72 [14], and *Xanthomonas* sp. JS02 [13] commonly hydrolyze *p*NPO better than short-chain length *p*NP-alkanoates but, up-to-date, none of these enzymes has been immobilized to agarose in order to assess a possible change in their substrate specificity, as it occurs with our immobilized *mcl*-PHA depolymerase.

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