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Immobilization of Arylmalonate Decarboxylase

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Abstract: Arylmalonate decarboxylase (AMD) is a monomeric enzyme of only 26 kDa. A recombinant AMDase from *Bordetella bronchiseptica* was expressed in *Escherichia coli* and the enzyme was immobilized using different techniques: entrapment in polyvinyl alcohol (PVA) gel (LentiKats®), covalent binding onto magnetic microparticles (MMP, PERLOZA s.r.o., Lovosice, Czech Republic) and double-immobilization (MMP-LentiKats®) using the previous two methods. The double-immobilized AMDase was stable in 8 repeated biocatalytic reactions. This combined immobilization technique has the potential to be applied to different small proteins.

Keywords: arylmalonate decarboxylase; immobilization; LentiKats; magnetic microparticles

1. Introduction

Arylmalonate decarboxylase [EC 4.1.1.76, AMDase] is a biotechnologically important enzyme, recently reviewed by Myiamoto and Kourist [1], which is able to catalyse an enantioselective decarboxylation of α-aryl-α-methylmalonates forming corresponding α-arylalkanoates and carbon dioxide, such as decarboxylation of α-phenylmalonic acid forming α-phenylacetic acid (Figure 1) [2]. This enzyme has the unique ability to produce optically active α-arylpropionates that are part of an important class of nonsteroidal anti-inflammatory drugs (NSAIDs) [3], widely used against pain, fever and inflammation due to their analgesic, antipyretic and anti-inflammatory effects [4]. The AMDase shows high enantioselectivity and has been used for the preparation of optically pure aryl, alkenyl, aliphatic and hydroxycarboxylic acids, such as flurbiprofen or naproxen [5–7]. Arylmalonate decarboxylase from *Bordetella bronchiseptica* has been modified by protein engineering to increase the racemase activity [8]. The mechanism of asymmetric decarboxylation of AMDase has also been described; it was proposed that the Cys 188 in AMDase can act as a proton donor to form the asymmetric center of the product [9].

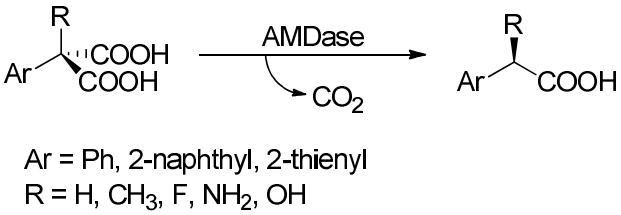

Figure 1. Reaction catalyzed by AMDase [7].

The application of enzymes is often complicated due to the lack of long-term stability or the difficulty in enzyme recovery and recycling [10]. To improve enzyme activity, and increase thermodynamic and kinetic stability, many immobilization techniques are available and have been described in the literature [11–13]. One of the methods used for the entrapment of enzymes is the LentiKats® system of polyvinyl alcohol (PVA) gel-based lens-shaped particles, which has successfully been applied to the immobilization of many enzymes [14]. Another immobilization method is enzyme adsorption on various carriers, as described in [15,16]. As a novel approach, immobilization is now also undertaken on the surface of magnetic particles, a method that has so far mostly been tested in the area of bioscience and medicine, especially for drug targeting and bioseparation, including cell sorting [17]. Moreover, the method has been applied in diagnostics, molecular biology, bioinorganic chemistry and catalysis [18]. The immobilization of enzymes via direct binding on magnetic particles has also been described [19]. Various biotechnologically important proteins and enzymes have been immobilized on fine magnetic particles, such as bovine serum albumin, glucose oxidase, streptokinase, chymotrypsin and dispase [20].

So far, there has been only two reports for AMDase immobilization: on polystyrene nanoparticles, without a significant loss of enzyme activity after four repeated conversions [21], and on amino C2 acrylate, with about 40% loss of the initial activity after the fourth conversion [22].

The novel approach to immobilization presented in this paper combines two immobilization techniques. The crude enzyme extract (CEE) of recombinant arylmalonate decarboxylase was first covalently bonded onto magnetic microparticles (MMP) and subsequently entrapped in PVA gel (LentiKats®). The characterization of the immobilized enzyme and its repeated use was tested and compared with the free enzyme.

2. Results and Discussion

2.1. AMDase production

The enzyme was prepared according to a routinely used protocol using IPTG (isopropyl β-D-1-thiogalactopyranoside) for induction [23]. The presence of the target protein was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Mini PROTEAN® Tetra Cell, Bio-Rad, Hercules, CA, USA), observing the band of 26 kDa. The whole-cell biomass was inactive (data not shown). This was ascribed to the phenol structure of α-phenylmalonic acid, which hampers the penetration of the cell membrane. For this reason, AMDase CEE was used with activity of 82.8 U/mL_{CEE} (66.03 U/mg_{CEE}). The enzyme was tested for its stability. At 4 °C, the enzyme had activity 0.18 U/mL after two weeks and 0.03 U/mL after three weeks. Storage of the enzyme in the form of CEE (without addition of any cryoprotectants) at −80 °C resulted in almost 100% of original activity after three weeks.

2.2. Immobilization and Characterization of AMDase in LentiKats®

To improve the enzyme stability and enzyme properties, AMDase CEE was immobilized in polyvinyl alcohol (PVA) gel (LentiKats®). It was previously reported that immobilized cell lysate is more stable than immobilized purified enzyme [22]. The activity obtained was 24.2 U/mL (calculated per mL of immobilized CEE), approx. 1.64 U/g_{LK}, which means that the enzyme lost about 70.8% of its activity as a free enzyme. The possible explanation for this major drop might be due to the fact

that AMDase is a small monomeric enzyme and was not entirely entrapped. Also the interaction between the PVA monomers and the enzyme may decrease the activity of the enzyme. Compared with another small enzyme, hydroxynitrile lyase (28–30 kDa), also immobilized using LentiKats® technology, displayed a 13% residual activity after entrapment [24].

The pH, temperature profiles and kinetic parameters were compared with the free form of the enzyme. The pH profile (Figure 2) was only slightly affected by immobilization. The immobilized enzyme was equally active in the range of 7.5–8.5, whereas the free enzyme at pH 7.5 retained only 45% relative activity. This confirms that PVA gel has a protective effect on the biocatalyst, even though at pH 7 the enzyme was completely inactive in both forms. The shift in pH activity to the acidic region was observed as well with the native AMDase from *A. bronchisepticus* KU 1201 [2].

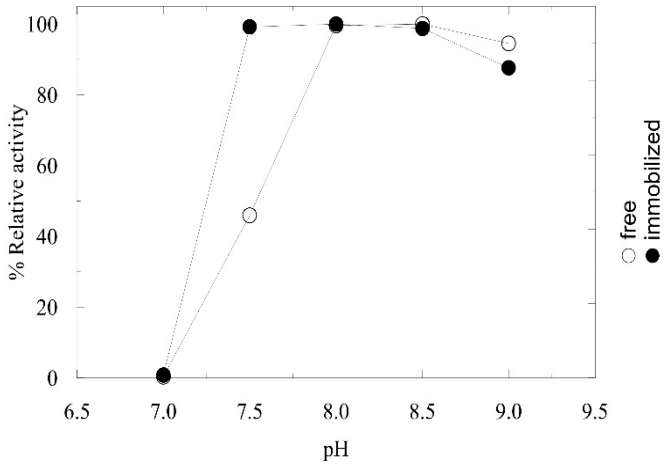

Figure 2. pH profiles of free and immobilized AMDase crude enzyme extract. Reaction conditions: 5 mL TrisHCl (Tris(hydroxymethyl)aminomethane hydrochloride) buffer (100 mM), α-phenylmalonic acid 1.8 g/L, 0.5 g of LentiKats® (immobilized form) or 0.013 mg of enzyme (free form); 37 °C and 200 rpm.

The temperature profiles (Figure 3) also showed some slight differences between the free and immobilized AMDase CEE. The optimum temperature for the immobilized biocatalyst was 37 °C, the same as in [2]; at 25 °C the activity decreased to 70% of relative activity for immobilized CEE and to 50% for the free enzyme. Higher temperatures were not tested due to the low stability of the PVA particles [25].

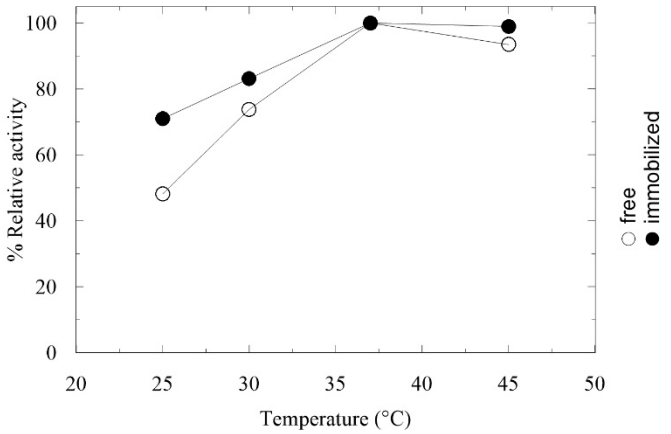

Figure 3. Temperature profiles of free and immobilized AMDase crude enzyme extract. Reaction conditions: 5 mL TrisHCl buffer (100 mM, pH = 8.5), α-phenylmalonic acid 1.8 g/L, 0.5 g of LentiKats® (immobilized form) or 0.013 mg of enzyme (free form); 200 rpm.

The kinetic parameters were calculated according to the Michaelis–Menten linearized equation [26] with different concentrations of α-phenylmalonic acid as a substrate. The comparison of the Michaelis constant (K_m) and maximum initial velocity (V_{max}) values are shown in Table 1. The value of K_m was higher for both free and immobilized enzymes than the values described elsewhere (0.3–13.9 mM, [2,27–29]). The possible explanation could be that the reactions were performed with CEE, whereas other enzymes or proteins can interfere with the activity of AMDase or with the substrate. Also the turnover rate is approx. 3.5 times lower than the one described in [29]. The immobilized AMDase had 1.5 times higher K_m than the free enzyme, which was the case also with other enzymes immobilized in LentiKats® [30,31]. It is difficult to say whether the higher K_m was due to the limitations caused by immobilization, or because of the enzyme. The turnover rate of CEE LentiKats® is approximately 10 times lower than the one of AMDase immobilised on polystyrene nanoparticles [21].

Table 1. Kinetic parameters of AMDase in free and immobilized form.

Kinetic Parameters	CEE Free	CEE LentiKats®	CEE MMP-LentiKats®
K_m [mM]	25.2	38.6	67.2
V_{max} [U/mg$_{CEE}$]	204.5	80	1.85
k_{cat} [s^{-1}]	88.62	34.67	0.8
k_{cat}/K_m [$s^{-1}.mM^{-1}$]	3.52	0.9	0.01

A comparative biocatalytic reaction was performed with free and immobilized AMDase (Figure 4) with 10 g/L of the substrate to see the ability of the biocatalyst to perform a complete substrate conversion. The reactions were performed using 0.82 U of both immobilized and free CEE (0.5 g of LentiKats® or 0.013 mg of AMDase CEE). Using the immobilized enzyme, the conversion reached 100% after 20 h, while with the free enzyme the conversion was not completed even after 120 h. This clearly demonstrates that the enzyme was stabilized by the PVA gel, as also observed in [32].

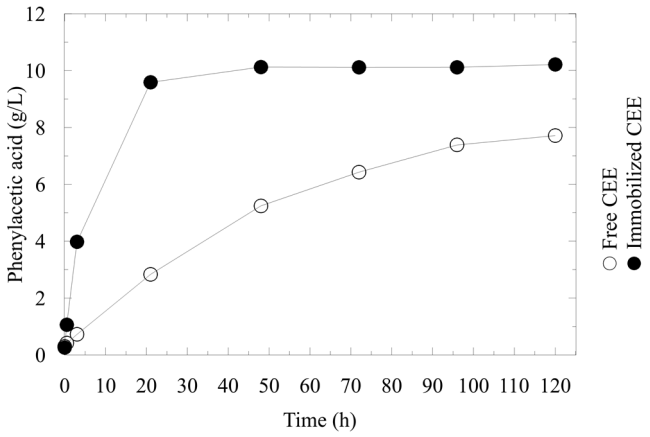

Figure 4. Comparison of biocatalytic reaction by free and immobilized AMDase crude enzyme extract. Reaction conditions: 5 mL TrisHCl buffer (100 mM, pH = 8.5), α-phenylmalonic acid 10 g/L, 0.5 g of LentiKats® (immobilized form) or 0.013 mg of enzyme (free form); 37 °C and 200 rpm.

2.3. Repeated Use of LentiKats® -Immobilized AMDase CEE

The activity of immobilized AMDase was tested in 20 consecutive biocatalytic reactions (Figure 5). Each reaction took 1.5 h, after which the particles were washed with Tris-HCl buffer (100 mM, pH 8.5, approx. 15 mL) and used for the next reaction. The activity of immobilized AMDase CEE decreased rapidly after each biotransfomation reaction, with 90.1% loss of the initial activity (in the first reaction) after the 20th cycle.

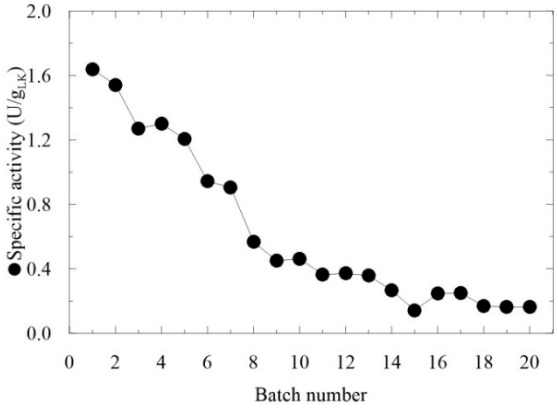

Figure 5. Repeated biocatalytic reactions with immobilized AMDase crude enzyme extract in LentiKats®. Reaction conditions: 5 mL TrisHCl buffer (100 mM, pH = 8.5), α-phenylmalonic acid 1.8 g/L, 0.5 g of LentiKats®; 37 °C and 200 rpm.

A possible explanation for this loss could be the fact that AMDase is a very small monomeric enzyme of 26 kDa. The size of the enzyme prevents it being entrapped in the pores of the LentiKats® particles created by PEG. Due to this problem, the enzyme is probably washed out after each repeated conversion (Figure 4). Therefore, various modifications of the LentiKats® preparation using different concentrations of PEG were tested to diminish the pores, but with no significant improvement in enzyme stability (See Supplementary Material 1; Figures S1,S2). Also the combination of cross-linked enzyme aggregate (CLEA) immobilization and subsequent entrapment of AMDase CLEA in LentiKats® was tested, as reported in Wilson et al. [33] and in Torello et al. [24]. However, no positive results in terms of enzyme stabilization or possibility of repeated use were obtained (See Supplementary Material 1; Figure S3).

2.4. Immobilization of AMDase on MMP and MMP-LentiKats®

To improve the stability of the immobilized enzyme, a novel approach of combined immobilization was tested. First, AMDase CEE was immobilized on magnetic cellulose microparticles. Before immobilization, the particles were oxidized using sodium periodate to create dialdehyde cellulose [34] to which the enzyme was covalently bonded through amino groups. The concentration of protein was tested before (11.47 mg/mL) and after (0.66 mg/mL) the incubation of the activated magnetic microparticles (MMP) with the CEE, showing that only 5.8% of the total protein was not bonded on the MMP. AMDase immobilized on MMP was used for three consecutive reactions (Figure 6). Even after the third conversion, the enzyme was still active and able to convert 100% of the substrate in 20 min. The activity recovery was 3.24%, and the specific activity dropped from 66.03 U/mg (free enzyme) to 2.14 U/mg (covalently bonded enzyme on MMP). It has been reported that immobilization using covalent binding can cause low recovery of the enzyme activity. Probable reasons for this could be the destruction of the enzyme's active conformation during the immobilization reaction, the multipoint attachment to the support, steric hindrance of the enzyme or the strong strength of the covalent binding [35].

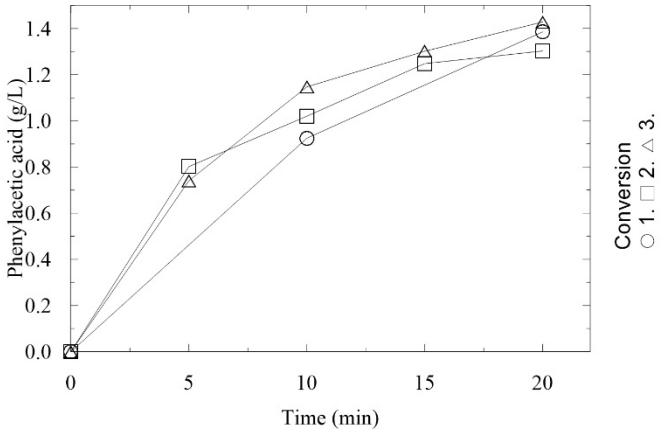

Figure 6. Three consecutive biocatalytic reactions with AMDase covalently bonded on magnetic microparticles. Reaction conditions: 5 mL TrisHCl buffer (100 mM, pH = 8.5), α-phenylmalonic acid 1.8 g/L, 10 mg of MMP; 37 °C and 200 rpm.

The washing and manipulation of the microparticles is difficult due to their size. For this reason a new immobilization strategy was tested. To further improve the manipulation of the particles, the AMDase CEE immobilized on MMP was then entrapped in PVA gel. Thanks to the size of the LentiKats® particles, the manipulation and washing after each reaction was significantly eased. The specific activity of the double-immobilized AMDase was 0.45 U/mg. The immobilization yield of immobilized AMDase on MMP-LentiKats® was 21% (comparing to the activity of AMDase immobilized only on MMP). To verify the stability of the immobilized enzyme, eight biocatalytic reactions were performed in succession (Figure 7), without any significant loss of the activity. This demonstrates that immobilization significantly stabilized the enzyme compared to free CEE (described in Section 3.1). This proposed immobilization strategy also showed an improvement in repetitive use compared to the strategies described so far [21,22]. Double-immobilized enzyme also retained 64% of its activity after three months of storage at 4 °C (compared to 0.04% retained activity for the free enzyme after three weeks storage at the same conditions as described in Section 2.1). This also demonstrates the significant enzyme stabilization achieved by this immobilization method.

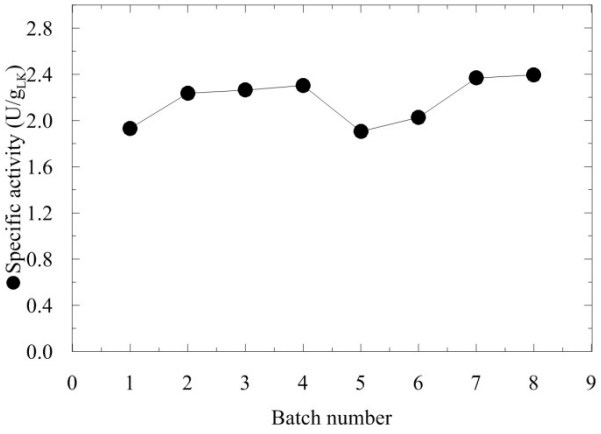

Figure 7. Repeated biocatalytic reactions with AMDase crude enzyme extract immobilized in MMP-LentiKats®. Reaction conditions: 5 mL TrisHCl buffer (100 mM, pH = 8.5), α-phenylmalonic acid 1.8 g/L, 0.5 g of MMP-LentiKats®; 37 °C and 200 rpm.

The kinetic parameters of double-immobilized AMDase (Table 1) were worse than those of AMDase only immobilized in LentiKats®. That means that the covalent bonds and PVA entrapment are somehow slowing the enzyme activity and the ability to bind the substrate. However, these results do not affect the repetitive use or the stability of the enzyme as a double-immobilized catalyst.

3. Materials and Methods

3.1. Microorganism and Media

Escherichia coli BL21 (DE3) + pET28b carrying the *amd* gene was cultivated in Lysogeny broth (LB) media: 5% (*w/v*) yeast extract, 10% (*w/v*) tryptone, 10% (*w/v*) NaCl. For the solid media 2% (*w/v*) agar was added. After autoclaving, 20 µg/mL of filter sterilized kanamycin (Sigma Aldrich, St. Louis, MO, USA) was added to all LB media.

3.2. Cultivation, Induction and Crude Enzyme Preparation

The inoculum was prepared overnight, inoculating 3 mL of LB media with a single colony and cultivated at 37 °C and 200 rpm for approximately 12 h. 1% inoculum was transported to 200 mL of LB media in a 500 mL cultivation flask and cultivated at 37 °C and 200 rpm until the optical density at 600 nm (OD_{600}) reached 0.5–0.6. Then, 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the induction was performed for 20 h at 30 °C and 200 rpm. The biomass was later centrifuged in 50 mL Falcon tubes at 4 °C, 7190 g for 5 min. The pellet was resuspended in 40 mL of Tris(hydroxymethyl)aminomethane hydrochloride buffer (Tris HCl, 100 mM, pH = 8.5). The cells were disrupted by continual cell disruptor (Constant cell disruption systems, Constant Systems LTD, Daventry, UK) in two cycles at 4 °C and 20000 PSI (Pounds per square inch; 20000 PSI= 1378,95 bar) and 50 µL of protease inhibitor (Complete EDTA-free, Roche Diagnostics, Penzberg, Germany) was added. The CEE was ultracentrifuged (Avanti® centrifuge 1-30I, Beckman Coulter, Indianapolis, IN, USA) at 10 °C and 50,000 *g* for 30 min. The supernatant was used for activity assay and immobilization.

3.3. Immobilization in LentiKats®

The prepared CEE was immobilized by entrapment in PVA gel using the LentiKats® system (www.lentikats.eu). 10 g of PVA and 6 g of polyethylene glycol (PEG, 1000 g/mol) were mixed together in 79 mL of distilled water. The mixture was heated to 94 °C for approx. 30 min at constant mixing, after which it was cooled to 40 °C and 5 mL (6.25 mg) of prepared CEE was added. The gel drops were printed on plastic carriers using laboratory equipment for immobilization (LentiPrinter®). The immobilized enzyme particles were dried at 40 °C for approx. 45 min and then hardened in 0.1 M Na_2SO_4 solution for an additional 45 min. The immobilized enzyme was used for biocatalytic reactions or stored in Tris HCl (100 mM, pH = 8.5) buffer at 4 °C.

3.4. Double-Immobilization on Microparticles and Entrapment in LentiKats®

The CEE was immobilized on magnetic PERLOZA MG 100 microparticles (MMP, PERLOZA s.r.o., Lovosice, Czech Republic) via covalent binding. These magnetic particles (diameter 80–100 µm) consist of 85% cellulose beads (approx. 30% of which is magnetite) and 15% demineralized water. The MMP (100 mg) were incubated with 2 mL of 0.05 M $NaIO_4$ for 21 h at 4 °C. Then washed 5 times with potassium phosphate buffer (0.1 M, pH 8). CEE (800 µL, 14 mg/mL) and potassium phosphate buffer (800 µL, 0.2 M, pH 8) was added to the washed MMP. After 20 h of incubation at 4 °C and moderate shaking, the MMP were washed 10 times with 10 mL of Tris-HCl buffer (0.1 M, pH 8). 50 µL samples were taken before and after incubation with MMP for immobilized capacity calculations (how much enzyme bound to the MMP) using protein analysis. MMP with AMDase (90 mg) were mixed with 2.9 mL PVA gel, as described in Section 3.3.

3.5. Biocatalytic Reactions with AMDase

CEE of AMDase in the free and immobilized form were used for decarboxylation of α-phenylmalonic acid (Sigma Aldrich, St. Louis, MO, USA). The reaction mixture with the free enzyme contained 10 µL (0.013 mg) of the enzyme, 1.8 g/L of the substrate and 4.99 mL of Tris-HCl buffer (100 mM, pH 8.5). The reaction mixture with AMDase immobilized in LentiKats® contained

0.5/1 g of LentiKats® particles, 1.8 g/L of the substrate and 5 mL of Tris-HCl buffer (100 mM, pH 8.5). The reaction mixture with AMDase immobilized on MMP contained 10 mg of MMP particles, 1.8 g/L of the substrate and 5 mL of Tris-HCl buffer (100 mM, pH 8.5). The reaction mixture with AMDase immobilized on MMP entrapped in LentiKats® (MMP-LentiKats®) contained 0.5 g of MMP-LentiKats® particles, 1.8 g/L of the substrate and 5 mL of Tris-HCl buffer (100 mM, pH 8.5). The reactions for kinetic measurements were performed using 0.45; 0.9; 1.8; 2.7; 3.6; 5.4 and 6.3 g/L of the substrate. After each repeated biocatalytic reaction with immobilized enzyme the particles were washed with Tris-HCl buffer (100 mM, pH 8.5, approx. 15 mL) before using them for another reaction.

The reactions were performed in a 50 mL Falcon™ conical centrifuge tube at 30 °C and at 200 rpm (Incubator shaker series I26, New Brunswick Scientific, Eppendorf, Berzdorf, Germany). One unit (U) of enzyme activity was defined as the amount of enzyme able to catalyse the formation of 1 µmol of α-phenylacetic acid in 1 min at 30 °C and pH 8.5.

All experiments were performed in 2 parallels and the errors were under 5%.

3.6. Activity Calculations

The initial specific activity of free and immobilized enzyme was calculated as in Zajkoska et al., from the initial linear increase of α-phenylacetic acid [36]. The activity of immobilized AMDase in MMP-LentiKats® was related to gram of LentiKats® used for the reaction. Detailed activity calculations are described in Supplementary Material 1.

3.7. Analytical Methods

The biomass concentration was measured spectrophotometrically (BioSpectrophotometer, Eppendorf, Hamburg, Germany) at 600 nm and the protein concentration was measured using the Bradford protein assay [37].

Processing of samples taken during biocatalytic reactions: 200 µL of the sample was mixed with 100 µL of 2 M HCl and centrifuged for 1 min at 13300 g; the supernatant was used for HPLC analysis. The concentrations of α-phenylmalonic and α-phenylacetic acid were measured by HPLC with Agilent Technologies 1220 Infinity LC apparatus. The column was a Supelco Waters Spherisorb S5ODS2, 25 cm × 4.6 mm (5 µm) (guarded by column with same filling - 3.2 cm × 4.6 mm); in the mobile phase, the flow rate was 0.8 mL/min with acetonitrile, distilled water and trifluoroacetic acid (60/40/0.05), with UV detection at 245 nm. The retention times were 3.2 min for α-phenylmalonic acid and 3.9 min for α-phenylacetic acid.

4. Conclusions

The work presented here demonstrates a novel approach to the immobilization of small enzymes and proteins. AMDase, which is a monomeric enzyme of only 26 kDa, was successfully covalently bonded to magnetic microparticles and entrapped in PVA gel. Covalent binding attached the enzyme to the magnetic microparticles and thus the enzyme did not leak from the LentiKats® particles, and the PVA gel protected the enzyme from unfavorable reaction conditions. Even though the kinetic parameters of double-immobilized AMDase were worse than the free AMDase or immobilized in PVA gel, this new method has stabilized the enzyme, which retained stable activity for eight consecutive conversions. In addition, the enzyme immobilized by the method described had significantly improved storage stability. Furthermore, the presence of a magnet in the particles might simplify the separation of the biocatalyst from reaction mixture by employing a magnetic field. The double-immobilization method developed has not previously been described and is suitable for various other small proteins or enzymes, significantly stabilizing them for repeated use.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/8/12/603/s1, Figure S1: Repeated biocatalytic reactions with immobilized AMDase crude enzyme extract in LentiKats® with modified PVA gel-3 g of PEG. Reaction conditions: 5 mL TrisHCl buffer (100 mM, pH = 8.5), α-phenylmalonic acid 1.8 g/L, 0.5 g of LentiKats®; 37 °C and 200 rpm. Figure S2: Repeated biocatalytic reactions with immobilized

AMDase crude enzyme extract in LentiKats® with modified PVA gel-1 g of PEG. Reaction conditions: 5 mL TrisHCl buffer (100 mM, pH = 8.5), α-phenylmalonic acid 1.8 g/L, 0.5 g of LentiKats®; 37 °C and 200 rpm. Figure S3: Repeated biocatalytic reactions with double-immobilized AMDase crude enzyme extract in CLEA and LentiKats®. Reaction conditions: 5 mL TrisHCl buffer (100 mM, pH = 8.5), α-phenylmalonic acid 1.8 g/L, 0.5 g of LentiKats®; 37 °C and 200 rpm.

Author Contributions: K.M., J.H. and M.H. performed the experiments; M.R. designed the experimental setup; R.K. provided plasmid carrying AMD gene; R.S. and L.Z. provided microparticles for immobilization and operational manual; K.M. and M.R. analysed the data and wrote the manuscript; M.R. and M.R. contributed funding of the research.

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Conflicts of Interest: The authors declare no conflict of interest.

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