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Recycling by-design of plastic through formulation with thermally protected enzymes in layered double hydroxide structures

Angela Romano, Antonella Rosato, Grazia Totaro *, Giulio Zanaroli, Annamaria Celli, Laura Sisti

Dipartimento di Ingegneria Civile, Chimica, Ambientale e dei Materiali, Università di Bologna, Via Terracini 28, 40131, Bologna, Italy

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ABSTRACT

The use of polymer-degrading enzymes in polymer formulation is a very attractive strategy for the life-cycle of plastics. The design of a material able to degrade on-demand could help reach the ambitious targets pursued by recent European policies. Only 32% of the resulting waste from the 50 million tonnes of plastics consumed each year in Europe is actually recycled. Since packaging accounts for more than 40% of all plastics produced every year, the improvement of its circularity from origin to subsequent life cycles is now a priority. The present research suggests a solution to improve the recyclability of plastics via a life cycle approach employing thermally stable enzymes as innovative materials providing a new potential for plastic and its end life. More specifically, cutinase, selected as a highly degrading polyester hydrolytic enzyme, was thermally protected by immobilization in Mg/Al layered double hydroxide structures. The cutinase immobilization efficiency was found to be high, as well as its release ability in an appropriate medium. The thermal stability of cutinase was strongly improved after immobilization, as highlighted by a 6-times increase of its half-life at 90 °C, compared to the free enzyme, and by a high activity retention (>60%) after short exposure to temperatures up to 200 °C. Moreover, it was demonstrated that a film of poly (butylene succinate-*co*-adipate) formulated with 5 wt% of immobilized cutinase, completely degraded within 24 h.

1. Introduction

The use of plastic-degrading enzymes is considered as an attractive and effective method for the management of plastic waste. Only 32% of the resulting waste from the 50 million tonnes of plastics consumed each year in Europe is actually recycled (EEA European Environment Agency, 2021), therefore, efforts must be made to design adequate plastic formulations able to degrade on-demand. This could help reach the ambitious targets pursued by recent European policies as well as improve the recyclability of plastics. Indeed, the enzymatic degradation of polymers is reported in many studies. Recent reviews summarize the state-of-the-art about such topic, with respect to the end-of-life of recalcitrant petroleum-based plastics and biodegradable polymers (Satti and Shah, 2020; Wei and Zimmermann, 2017). For example, synthetic polyesters such as poly(ethylene terephthalate) (PET) or polyurethane (PUR) have been proven to be susceptible to enzymatic degradation by microbial hydrolases, as well as biopolyesters such as poly(lactic acid) (PLA), poly(butylene succinate) (PBS), poly(butylene succinate-*co*-adipate) (PBSA), and polycaprolactone (PCL) (Satti and Shah, 2020; Wei and Zimmermann, 2017). Different hydrolytic enzymes, such as lipases, esterases, ureases and proteases are responsible for the hydrolysis of various types of polymers, as they break specific chemical bonds in the presence of water. Such hydrolases show different features in substrate preferences and interfacial activation. For example, esterases are best fitted for short-chain fatty acids, while lipases prefer to hydrolyse long-chain substrates (Satti and Shah, 2020). However, raw enzymes are not used in polymer formulations because the high processing temperatures would denature them, by destroying their three-dimensional structure and catalytic center. Recent studies report about the possibility to increase the thermal stability of enzymes through the genetic construction of new variants (Andre et al., 2020; David et al., 2020; Duquesne et al., 2020; Marty et al., 2018; Tournier et al., 2018). Other authors describe the use of enzymes in polymer formulation through complex multi-step processes at different temperatures: the enzyme is first embedded with a carrier (i.e. the arabic gum) in an aqueous formulation with a low melting polymer as PCL, then such

* Corresponding author.
E-mail address: grazia.totaro@unibo.it (G. Totaro).

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melt is mixed with a higher melting polymer, such as PLA (Dalibey et al., 2019; Guemard et al., 2016; Guemard and Dalibey, 2019).

A simpler strategy to thermally protect enzymes, as compared to the above-mentioned approaches, is to immobilize them on layered double hydroxide (LDH) type structures. LDHs are inorganic, versatile and multifunctional hosts, thanks to their easy one pot synthesis, tunable composition and functionalization according to the desired final properties. Moreover, LDHs are bio- and food-compatible and can be employed in many different fields as catalysts, anion exchangers, sorbents and scavengers for pollutants, additives and/or stabilizers in polymer formulations (Mishra et al., 2018). Structurally, LDHs are natural/synthetic clays whose structure is derived from brucite Mg $(OH)_2$ with layers built from octahedra in which the centre Mg^{2+} is surrounded by OH groups. The isomorphous substitution of some divalent cations by trivalent cations leads an excess of positive charges, counterbalanced by some interlayer anions and water resulting in a neutral structure. The general formula of such so-called hydrotalcite-like compounds is $[M^{II}_{1-x}\ M^{III}_{x}\ (OH)_2]^{x+}(A^{n-})_{x/n}\ mH_2O$, wherein M^{II}, M^{III}, and A^{n-} denote divalent cations (Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, etc.), trivalent cations (Al^{3+}, Fe^{3+}, Ga^{3+}, and Cr^{3+}) and the interlayer anion (Cl^-, CO_3^{2-}, NO_3^-, etc.), respectively. A large variety of organic anions have been used to intercalate the space between the lamellae to organo-modify the hydroxyl platelets and to adapt them to specific end-uses (Marek et al., 2018, 2019, 2020; Sisti et al., 2019a, 2019b, 2020; Totaro et al., 2018). Recently, LDHs were used to intercalate cumbersome molecules making them an inorganic vessel for drug release and/or as polymer filler (Taviot-Guého et al., 2018). Similarly, the LDH was used as cargo of corrosive inhibitors, dormant inside the LDH host structure dispersed into a polymer coating and released under stress (Stimpfling et al., 2016). LDHs are also suitable for the immobilization of enzymes. Some enzyme/LDH systems recently reported include lipase with the aim to increase its catalytic properties (Dong et al., 2014), papain and cellulase as potential drug delivery or biosensor systems (Zou and Plank, 2012, 2015), lysozyme as potential disinfection agents (Bouaziz et al., 2017; Yang et al., 2013), nitroreduttase as contaminants detectors (Bruna et al., 2019), lactate dehydrogenase as potential biosensors or biofuel cells (Djebbi et al., 2016), dextranase as potential biosensors or biocatalysts (Ding et al., 2018), tyrosinase as biosensors (Soussou et al., 2017).

In the present research study, cutinase was selected to be protected within LDH, since it is reported to hydrolyse several polyesters such as PBS, PBSA and PCL (Ferrario et al., 2016; Rosato et al., 2022). To our knowledge, cutinase was never immobilized in LDH systems. Cutinases are serine esterases belonging to the α/β hydrolase superfamily and containing the typical Ser–His–Asp catalytic triad. Unlike lipases, they lack the hydrophobic lid, making the active site of cutinase large enough to accommodate a large substrate as the cutin, which is the major constituent of cuticle, a protective layer (of waxes and lipids) covering the epidermis of leaves, shoots, and other tender parts of terrestrial plants. Cutin is an insoluble lipid polyester, made of hydroxylated C16 and C18 fatty acids, linked together via ester bonds. Microorganisms like fungi and bacteria can hydrolyse this polymer by secreting cutinases, which are multifunctional enzymes able to catalyse hydrolysis reactions, esterifications and transesterifications. They show good stability at basic pH (8–11), in the 20–50 °C temperature range, and also in the presence of other enzymes. As a result, they could potentially be widely used in food products (i.e. to produce dehydrated fruits), chemicals (i.e. for the production of biodiesel), detergents (i.e. in laundry and dishwashing detergent formulations), environmental (i.e. biodegradation of pesticide wastes) and textile industries (i.e. to improve the wettability of cotton fibers) (Chen et al., 2013).

The aim of the current study is to increase the stability and thermal resistance of cutinase, allowing its use in polymeric formulation. At the end-of-life of the plastic item, by means of a specific trigger, the enzyme can be released to exert its degrading activity with consequent acceleration of the biodegradation rate. The possibility of a direct use of such enzyme in a polymer formulation through an adequate protective system, preserving the activity of the enzyme and increasing its thermal stability, could lead to new "on-demand" degradable materials. Therefore, significant progress could be made in terms of circularity. Since packaging represents 40% of the production of plastics in EU, including petro-derived plastics, and requires the consumption of more than 19 million tons of oil and gas, improving its circularity from origin to subsequent life cycles is quite urgent. As a matter of fact, the strategy here proposed is a recycling strategy by-design, perfectly fitting a circular economy system.

In detail, cutinase is here immobilized on LDH with Mg and Al as cations. Mg was selected as it proved more appropriate in terms of sustainability and biocompatibility. Indeed, the natural LDH, commonly used as antacid in commercial drugs, is based on Mg/Al (Sisti et al., 2020). The effect of different enzyme amounts is evaluated in terms of immobilization efficiency and release ability. Infra-red spectroscopy, X-ray diffraction, thermogravimetry and thermal resistance experiments were carried out. The half-life of free and immobilized enzyme is also evaluated. The LDH/cutinase sample containing the highest enzyme amount is dispersed by compression moulding in a PBSA matrix and its biodegradation is also tested.

2. Experimental

2.1. Materials

Cutinase from *Humicola insolens* (product Novozym©51032) was provided by ChiralVision as a solution (concentration of 20 g L^{-1}). Sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), 4-nitrophenyl butyrate (4-NPB, ≥98%), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), copper sulfate ($CuSO_4$), bovine serum albumin (BSA), sodium tartrate ($C_4H_4Na_2O_6$), 2N Folin & Ciocalteu's phenol reagent, aluminum nitrate $Al(NO_3)_3{\bullet}9H_2O$ and magnesium nitrate $Mg(NO_3)_2{\bullet}6H_2O$ were purchased from Sigma-Aldrich (now known as Merck, St. Louis, Missouri, USA). A commercial co-polyester, namely poly (butylene succinate-*co*-adipate) (PBSA, commercial name bioPBS FD92PM), having the following composition $(PBS)_{0.7}$-$(PBA)_{0.3}$ was supplied by PTT MCC Biochem Company. Pural Mg61 from Sasol, supplied by Nachmann Srl (Italy), was used as commercial carbonate reference. All the materials were used as received.

2.2. Immobilization of cutinase on LDH

All the LDHs were prepared by a slow coprecipitation. The molar ratio enzyme/Al^{3+} was assessed by considering the average molecular weight of the amino acidic sequence (122 g mol^{-1}) of cutinase. Briefly, $Mg(NO_3)_2{\cdot}6H_2O$ (0.209 g, 8.16×10^{-4} mol) and $Al(NO_3)_3{\cdot}9H_2O$, (0.153 g, 4.08×10^{-4} mol) in molar ratio 2/1, were mixed with deionized and decarbonated water (50 mL) and added dropwise to a water solution (100 mL) containing cutinase in an appropriate molar ratio with respect to Al^{3+} (for example 5.6 mL, 20 g L^{-1}, for 2.2/1 M ratio). The coprecipitation lasted 3 h, with stirring, under nitrogen flow in order to avoid the presence of carbonate as a competing ion. The pH was kept constant (10 ± 0.4) with the addition of NaOH solution (0.049 mol L^{-1}). After the coprecipitation, the reaction mixture was aged for 3 h at room temperature (TA). The solid product was filtrated on Büchner, washed with 300 mL of deionized and decarbonated water (in small aliquots of 50 mL), vacuum-oven dried overnight at TA. The products were light yellow. The final reaction medium and the washing solution were collected to measure the amount and activity of the residual enzyme. LDHs are labeled LDH/Cut-X, where X is the enzyme ratio respect to Al^{3+}.

Details of all syntheses are reported in Table 1. The best composition was prepared and characterized twice, providing reproducible results but, for clarity, just one sample of such composition is here reported.

Table 1
LDH samples: enzyme loading, immobilization efficiency, protein and activity recovery after release in Na_2SO_4 solution.

Code	Experimental enzyme loading (g)[a)]	Enzyme loading (mol)[b)]	Enzyme/Al (molar ratio)	Immobilization efficiency (%)[c)]	After 12 h release in Na_2SO_4 solution	
					Protein amount (%)	Total activity recovery (%)
LDH/Cut-3.4/1	0.168	1.38×10^{-3}	3.4/1	41.4 ± 0.6	41.6 ± 0.4	41.1 ± 0.9
LDH/Cut-2.8/1	0.139	1.14×10^{-3}	2.8/1	49.1 ± 2.3	50.5 ± 1.8	49.6 ± 0.6
LDH/Cut-2.2/1	0.111	9.10×10^{-4}	2.2/1	80.2 ± 2.4	67.2 ± 0.5	66.7 ± 2.6
LDH/Cut-1.0/1	0.052	4.26×10^{-4}	1.0/1	84.2 ± 5.0	31.4 ± 0.6	23.7 ± 1.3
LDH/Cut-0.5/1	0.027	2.21×10^{-4}	0.5/1	81.4 ± 6.4	28.6 ± 1.4	7.6 ± 0.8

a) Evaluated through the Lowry method.
b) Enzyme average molecular weight calculated considering the amino acidic sequence.
c) Evaluated on water medium and washing solutions.

2.3. Determination of enzyme immobilization efficiency and activity

The enzyme amount is determined by measuring the initial and final enzyme concentration in the medium, by using the Lowry method (Lowry et al., 1951). The enzyme in the washing waters is also taken into consideration. More in detail, each sample (0.5 mL) was boiled for 1 min, rapidly cooled on ice and supplemented with 2.5 mL of a reagent mixture consisting in 2% Na_2CO_3 (in NaOH 0.1 mol L^{-1}), 1% $CuSO_4$ (in dionex water) and 2% $C_4H_4Na_2O_6$ (in dionex water) in 100:1:1 ratio. After the reagent addition, the samples were immediately well mixed and incubated for exactly 10 min. A volume of 0.25 mL of Folin and Ciocalteu's phenol reagent (1 eq L^{-1}) was then added to each sample, which was immediately vortexed. After 30 min of incubation in the dark at TA, the absorbance of each sample was then measured at 540 nm by means of a UV–Vis spectrophotometer (Varian Cary 100 bio, Dual Beam). The enzyme concentration was calculated through a calibration curve prepared using bovine serum albumin (BSA) dilution series (1–50 mg L^{-1} of BSA) as standards; the calibration curve was constantly verified.

The immobilization efficiency of the enzyme was calculated according to Equation (1):

$$\textit{Immobilization efficiency}\ (\%) = \frac{A - B}{A} * 100 \quad (1)$$

A = total protein amount in the initial solution;
B = protein amount in final and washing solutions.

The enzyme activity was simultaneously checked by a continuous spectrophotometric assay using 4-nitrophenyl butyrate (4-NPB) as substrate. More in detail, a volume of 0.1 mL of each sample was added to 3 mL of sodium phosphate buffer (0.1 mol L^{-1}, pH 7.8) containing 1 mmol L^{-1} of 4-NPB. In the presence of the enzyme, this substrate is rapidly hydrolyzed into butyric acid and 4-nitrophenol, whose formation can be spectrophotometrically measured at 420 nm over time. In particular, the absorbance (Abs_{420nm}) was continuously monitored until constant using a UV–Vis spectrophotometer thermostated at 25 °C, and the absorbance per minute was obtained using the maximum linear rate. One unit of enzyme is defined as the amount of enzyme that hydrolyses 1 μmol of 4-nitrophenyl butyrate to butyric acid and 4-nitrophenol per minute under the assay conditions.

The activity of the LDH-immobilized enzyme was evaluated by adding a certain amount of LDH/Cut-2.2/1 powder to 3 mL of sodium phosphate buffer solution containing the 4-NPB substrate (1 mmol L^{-1}); the absorbance at 420 nm was continuously measured using the spectrophotometer thermostated at 25 °C and the enzymatic activity defined as above.

For better explanation, Fig. 1 shows the flowchart of the procedure described.

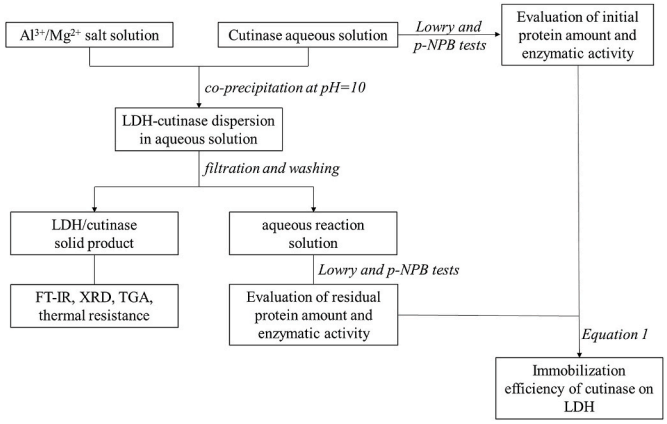

Fig. 1. Flow-chart of experimental design.

2.4. Kinetic release experiments

A preliminary test was carried out on sample LDH/Cut-2.2/1 in both water and Na_2SO_4 solution (1.2 wt%). Briefly, a known amount of LDH/Cut-2.2/1 (approximately 50 mg) was incubated into 10 mL of water, and 10 mL of sulfate solution. At specific times, both suspensions were centrifuged (2655 rcf, TA, 5min) and a small aliquot of the supernatants was recovered to measure the enzyme amount and activity. The total time was 12 h.

Release tests of all the other samples were performed by incubating approximately 50 mg of LDH/enzyme just into 10 mL of Na_2SO_4 solution (1.2 wt%). After 12 h, the suspensions were centrifuged (2655 rcf, TA, 5 min) and the supernatants were recovered to measure the protein amount and activity recovery using the Lowry and the 4-NPB method, respectively, as described above.

2.5. Half-life and thermal resistance

To investigate the free enzyme half-life, small aliquots of cutinase were exposed to 90 °C over different times, quickly cooled on ice and assay for their residual activity. Tests were performed in a thermostatic bath: 0.1 mL of enzyme solutions were added to screw-cap glass vials containing 0.1 mol L^{-1} sodium phosphate buffer (5 mL). The residual activity [A] was measured by p-NPB assay and compared to the initial one [A^0]. The enzyme half-life was calculated following a second order kinetic ($r^2 = 0.99$) according to Equation (2):

$$t_{1/2} = \frac{1}{(k \times [A^0])} \quad (2)$$

where k is the kinetic constant, i.e., the slope obtained by plotting 1/[A] versus the time; A^0 is the initial enzyme activity (Zanaroli et al., 2011).

To evaluate the thermal stability of the LDH-immobilized enzyme, small aliquots of LDH/Cut-2.2/1 powder were subjected to different thermal treatments: 70 °C for 35 s; 90 °C for several minutes; 150 °C (3 min) and 200 °C (10 s); 105 °C (3 min) and 200 °C (10 s). 90 °C was chosen to evaluate the immobilized enzyme half-life, while the others were chosen to simulate industrial processes (such as dry/wet lamination or film extrusion), in order to have an idea of the performance on an industrial scale. After the thermal treatment, the residual enzymatic activity was evaluated by p-NPB assay after release in 1 mL of Na_2SO_4 solution (1.2 wt%) and compared to a control sample, used as reference. Tests were performed in an oven: around 5 mg of sample were put in oven paper in order to favour an immediate heating of the powder.

2.6. Biodegradation of a formulated polyester/LDH-immobilized enzyme

A film consisting in poly(butylene succinate-*co*-adipate) with 5 wt% of immobilized cutinase (LDH/Cut-3.4/1) was prepared by compression moulding (120 °C, 1 min, 10000 Psi). As reference, a film of PBSA with a commercial LDH (Pural Mg61) was similarly prepared. The resulting film thickness was 150 (±30) μm. Both types of film were cut into small pieces and incubated in sodium phosphate buffer 0.1 mol L^{-1} (9 mL) under the optimal reaction condition of cutinase (pH 8, 40 °C). Similarly, both types of film were incubated under vacuum at room temperature. The enzymatic degradation was investigated via weight loss test, by measuring the initial weight of the films and their weight after 24 h. The test was performed in duplicate. In addition, the release of cutinase in the liquid fraction was assessed in terms of both protein concentration and enzymatic activity, by the Lowry test and the p-NPB assay, respectively.

2.7. Characterization

Infrared spectra of sample powders were recorded using an ATR FT-IR over the wavenumber range 650–4000 cm^{-1} using a PerkinElmer Spectrum One FT-IR spectrometer equipped with a Universal ATR sampling accessory. Sixteen scans were taken for each spectrum at a resolution of 2 cm^{-1}.

Thermogravimetric analysis (TGA) was performed under air atmosphere for all samples, using a PerkinElmer TGA7 apparatus (gas flow 30 mL/min) at a 10 °C min^{-1} heating rate from 50 to 800 °C. The 20% mass loss temperatures (T^{D}_{20}) were measured. A skim milk solution freeze-dryed lyophilized sample was used to test cutinase.

The X-ray diffraction (XRD) analysis was carried out at room temperature by means of a Philips X-Pert Pro diffractometer. Data were acquired by exposing the samples to Cu-Kα X-ray radiation. The patterns were collected with a step size of 0.03°, over 2θ range of 2.0–70°, with an accumulation time of 10 s per step.

3. Results and discussion

3.1. Immobilization efficiency

The immobilization of cutinase on Mg/Al LDHs through a classical coprecipitation procedure (Figs. 2–3) is investigated here. Among all LDH synthetic procedures (exchange, hydrothermal treatments, reconstruction, etc.) the coprecipitation route is preferably used, as it is a "soft chemistry" tunable process that allows to select variable conditions (pH, temperature, buffer, solvent, reagents concentration) which avoid the structural change of the enzyme and loss of its catalytic activity (Charradi et al., 2010). The isoelectric point (pH at which the protein has a net charge of zero) of cutinase is 7.8, thus at pH 10, carboxylate COO^{-} groups should be present and able to electrostatically interact with the cationic layers of LDH. Different enzyme loadings were tested: from 3.4/1 to 0.5/1, in terms of molar ratio respect to Al, calculated by considering a molecular weight of 122 g mol^{-1} for cutinase, averaged from the amino acidic sequence (Uniprot ID A0A075B5G4). This was

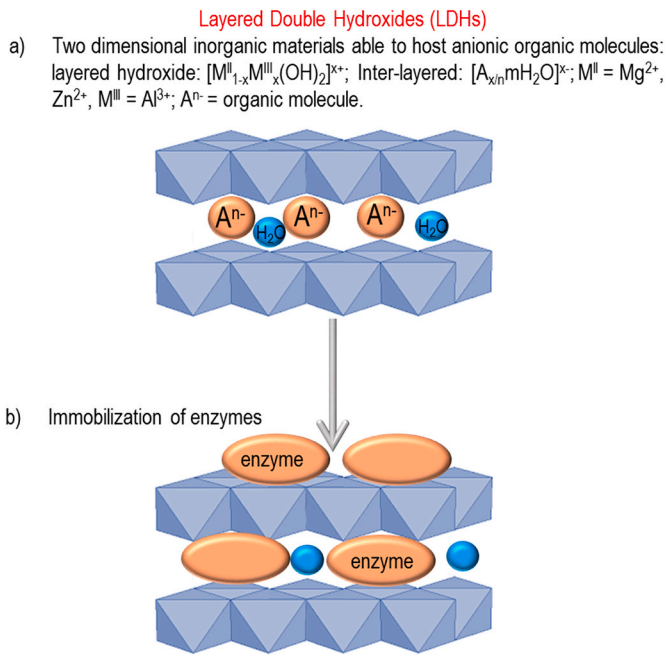

Fig. 2. a) Schematic representation of LDH and b) immobilization of enzymes for their thermal stabilisation.

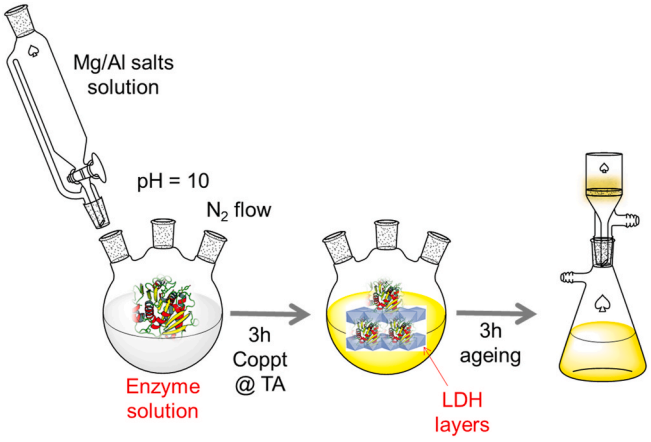

Fig. 3. Scheme of enzyme immobilization protocol.

done, in order to evaluate the optimal loading, with the highest immobilization efficiency. The ratio Mg/Al was 2/1, which is reported to maximize the charge density of the layers, favouring the enzyme intercalation (Bruna et al., 2019).

The amount of the immobilized enzyme, or immobilization efficiency, was indirectly determined by measuring the initial and final enzyme concentration in the reaction medium and in the washing solutions using the Lowry method. The results are reported in Table 1. As it can be observed, the immobilization efficiency is very high and quite constant, around 80%, for the samples with an Al/enzyme ratio in the range 2.2/1–0.5/1, while higher enzyme loadings seem to inhibit in part an effective immobilization. This is a good result compared for example to the more complex system prepared by Dong et al., who reached an immobilization efficiency of 56% (Dong et al., 2014). The initial and final activity of the enzyme in the reaction medium and the activity in the washing solutions was simultaneously checked by the p-NPB assay (data not shown) and it agrees with the protein content. More in detail, a very low enzymatic activity (less than 10% compared to the initial one) occurred in the final and washing solutions of the samples

LDH/Cut-2.2/1, 1.0/1 and 0.5/1, which exhibited higher immobilization efficiency. Conversely, it was found that the samples with a lower enzyme loading efficiency, i.e., LDH/Cut-2.8/1 (49%, Table 1) and 3.4/1 (41%, Table 1) showed approximately 47 and 69% of enzymatic activity, respectively (data not shown).

The LDH/Cut samples were characterized by Infra-red spectroscopy to check the presence of cutinase and the formation of the LDH structure: the profiles of all samples prepared, compared to a commercial reference containing carbonate, are shown in Fig. 4. All LDH spectra show the characteristic lattice vibration bands at low region ($\nu_{M\text{-}O}$ and $\delta_{O\text{-}M\text{-}O}$). A wide absorption band appears at 3300 cm^{-1}, which is due to NH and OH stretching of hydroxyl group and water molecules present in the interlayer space of LDHs. A sharp amide I peak appears at 1640 cm^{-1}, which is mainly due to the C═O stretching vibrations, together with a sharp amide II band at 1530 cm^{-1}, due to the bending modes of the N–H group and the stretching mode of the C–N group. The positions of the amide I and II bands in the FT-IR spectra of proteins are a sensitive indicator of conformational changes in the protein secondary structure. Yang et al. (2013), reported 1650 and 1540 for amide I and amide II of lysozyme intercalated LDH. The carbonate stretching at 1360 cm^{-1} is visible in all profiles. However, the intensity of carbonate stretching is lower with respect to amide I and amide II, thus confirming the fact that carbonate is not predominant when the enzyme ratio is 2.2/1. Higher and lower loadings seem to contain more carbonate with respect to cutinase, by observing the relative intensities of such bands. In case of LDH/Cut-3.4/1, amide II is less intense with respect to the other samples, but this is probably due to a different hydration. Grdadolnik actually reported that stronger hydration causes slight conformational changes in protein structure, causing changes in intensity, frequency and/or band shape visible in the Amide A (3300 cm^{-1}), Amide I, Amide II and Amide III (1230-1300 cm^{-1}) band regions (Grdadolnik, 2003). Therefore, cutinase is successfully immobilized on LDH, as well as carbonate ion in varying amounts.

X-ray diffraction analysis confirms the results obtained by FT-IR. Some diffraction patterns of the LDHs are visible in Fig. 5 and the crystallographic data are reported in Table 2. The reference sample profile exhibits the typical reflections of LDH with a series of (00*l*) peaks appearing as narrow, symmetric, strong lines at 2θ-low angle, corresponding to the basal reflections, while at higher 2θ the reflection (110) indicates the intra-layer structural ordering. All the LDHs contain carbonate but large halos are visible and the maxima of (003) and (006) reflections are shifted to slightly lower 2θ values: the system is somehow disturbed by cutinase, which is most likely adsorbed on the LDH surface, rather than intercalated between the lamellae. Lower enzyme loadings (1.0/1 or 0.5/1) feature better defined profiles, according to the fact that more carbonate should be present. In general, it can be concluded that immobilized systems were here obtained.

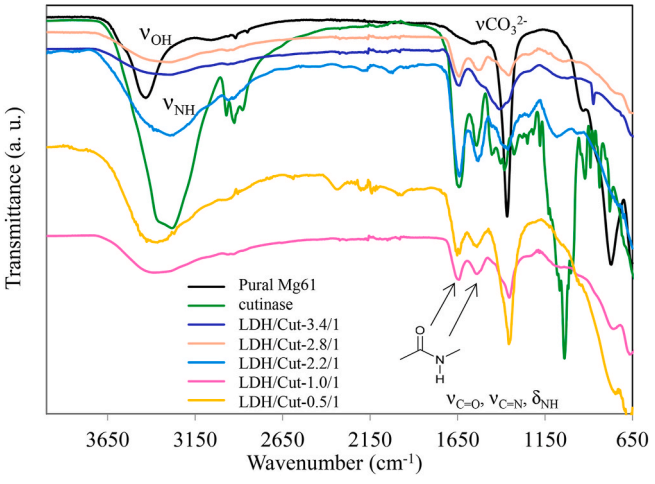

Fig. 4. ATR FT-IR curves of LDHs with Cutinase compared with a commercial reference sample containing carbonate (Pural Mg61).

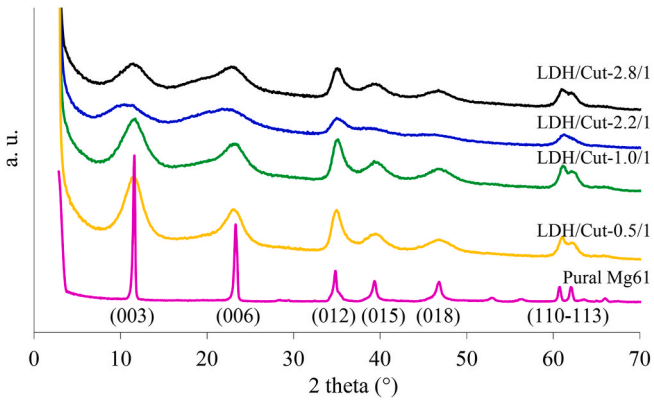

Fig. 5. X-ray diffraction patterns of LDHs with Cutinase compared to a commercial sample containing carbonate (Miller indices indicated).

Table 2
Cell parameters and thermal stability of LDH samples.

Code	$c' = d_{003}$ (Å)[a]	$c = 3c'$ (Å)[b]	d_{110} (Å)[c]	$a = 2d_{110}$ (Å)[d]	T_{20}^{D} (°C)[e]	Residue (%)[e]
Pural Mg61	7.6	22.8	1.52	3.04	343	57
LDH/Cut-3.4/1	7.5	22.5	1.51	3.02	352	39
LDH/Cut-2.8/1	7.6	22.8	1.51	3.02	323	34
LDH/Cut-2.2/1	8.2	24.6	1.51	3.02	322	27
LDH/Cut-1.0/1	7.6	22.8	1.51	3.02	304	37
LDH/Cut-0.5/1	7.6	22.8	1.51	3.02	357	52

[a] c' is the interlayer distance and it is determined by the 003 reflection in XRD analysis.
[b] c is the total thickness of the brucite-like layers and the interlayer distance.
[c] Determined by the 110 reflection in XRD analysis.
[d] a (lattice parameter) is related to the cation-cation distance.
[e] TGA under air flow (30 mL/min), samples were kept overnight under vacuum at TA before analysis; residue@800 °C.

As a matter of fact, on the basis of the literature on LDH/enzymes, it seems simpler to obtain immobilized systems, where the proteins are mostly adsorbed on the surface of layers, with respect to the intercalated systems. This could probably be due to the steric hindrance of enzymes, whose intercalation would involve a compression between the lamellae (Zou and Plank, 2012, 2015). However, the immobilization should be quite strong, as suggested by the fact that the "saw-toothed" reflections (110) and (113) tend to merge in all compositions, especially in 2/1. Such merging is due to a shift to lower 2θ of (113), which is strongly *l*-dependent and generally caused by intercalation (Totaro et al., 2018).

Fig. 6 and Tables 2 and 3 report profiles and data related to the thermogravimetric experiments. The commercial LDH commonly loses weight in the following 4 steps: 1) interlayer water (70–190 °C); 2) Al-Hydroxides (190–280 °C); 3) Mg-Hydroxides (280–405 °C); 4) CO_2 (405–580 °C) (Yang et al., 2002). The remaining residue is a solid solution of MgO and Al_2O_3. Cutinase loses weight almost immediately, because of the residual moisture. The enzyme loss should occur in the range 200–300 °C. The higher temperature weight losses could be due to the stabilizers employed by the manufacturer, residual glycerol or possibly some ingredients used during the lyophilisation process (Almazrouei et al., 2019). Of course, the improved thermal stability is evident in all LDH prepared (Fig. 6). Such improved thermal stability should be due to a more rigid structure, less sensitive to the deactivation induced by high temperature (Dong et al., 2014). In general, the LDH samples show 3/4 wt losses. Zou et al. (Zou and Plank, 2015) reported that an intercalated cellulase loses weight in the range 400–530 °C,

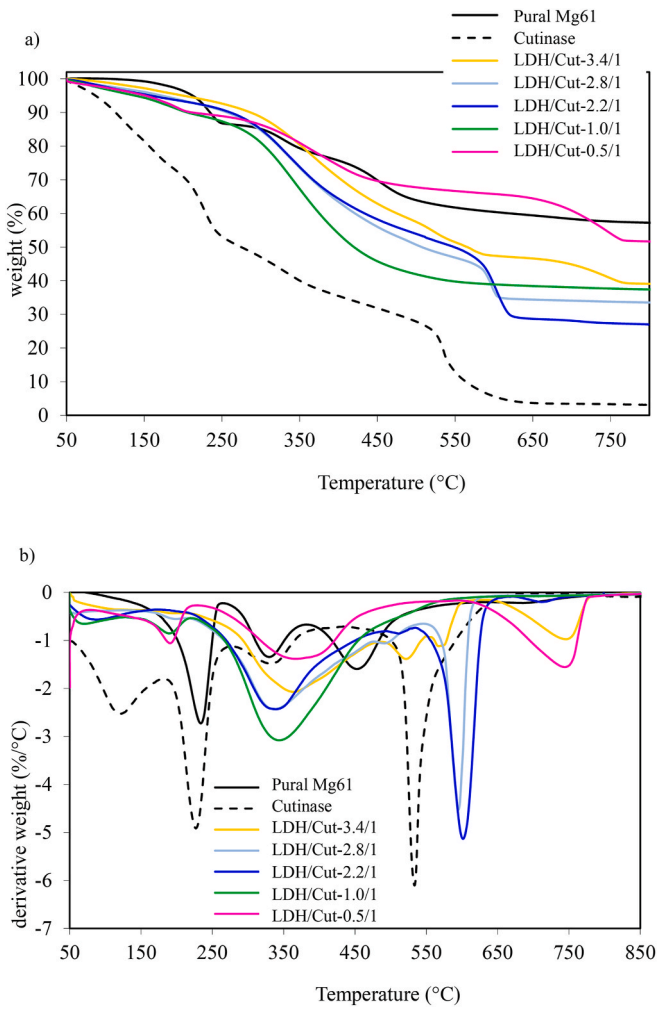

Fig. 6. TGA a) and b) dTGA profiles of LDH with cutinase compared with cutinase.

Table 3
Weight loss step of the materials and corresponding temperature range.

Sample	Step			
	1	2	3	4
Cutinase	28% 50–190 °C	22% 190–280 °C	18% 280–450 °C	29% 450–850 °C
Pural Mg61	13.3% 50–250 °C	7.7% 250–350 °C	14% 350–490 °C	7% 490–850 °C
LDH/Cut-3.4/1	7% 50–240 °C	34% 240–490 °C	12% 490–620 °C	8% 620–850 °C
LDH/Cut-2.8/1	8% 50–230 °C	41% 230–500 °C	16% 500–620 °C	2% 620–850 °C
LDH/Cut-2.2/1	6% 50–190 °C	42% 190–520 °C	23% 520–640 °C	2% 640–850 °C
LDH/Cut-1.0/1	11% 50–230 °C	50% 230–580 °C		2% 580–850 °C
LDH/Cut-0.5/1	10% 50–220 °C	24% 220–580 °C		14% 580–850 °C

while Dong et al. (2014) reported that immobilized candida lipase loses weight from 200 to 300 °C. Being cutinase mostly immobilized on the surface of LDH, it seems reasonable to consider a similar T range as appropriate for the samples in the current study. However, by observing the weight losses reported in Table 3, it is clear that the enzyme degrades together with hydroxyls, therefore it is quite impossible to estimate the LDH formulae by TGA. However, the sample with the lower residue is LDH/Cut-2.2/1, coherent with a higher organic loading. The difference between the LDH profiles could be ascribable to the hydration level (or the moisture content), which is higher in LDH/Cut-1/1 and LDH/Cut-0.5/1. Chen et al. indeed reported that the moisture content is critical to protein stability (Chen and Oakley, 1995). Moreover, it must be underlined that the LDHs prepared are mixed systems, containing different amounts of water, enzyme (intercalated and immobilized on the surface) and carbonate. Therefore, some interactions affecting the thermal behaviour seem reasonable.

3.2. *Retaining activity into the inorganic host layers*

The association of enzymes with inorganic materials mainly involves non-covalent interactions, such as hydrogen bonds, van der Waals forces, hydrophobic affinity and ionic exchanges (Charradi et al., 2010). The inorganic layers should act as a shield for the enzyme, protecting and preserving its activity, that should be activated just after release from the inorganic structure. In this sense, the activity was tested on LDH/Cut-2.2/1 powder by a continuous spectrophotometric assay using 4-nitrophenyl butyrate (4-NPB) as a substrate, finding a negligible activity recovery around 0.3%, confirming the fact that the enzyme is dormant inside the LDH. This suggests that the interactions between cutinase and LDH layers may have induced some alterations in the three-dimensional structure of the enzyme molecules, with their catalytic triad becoming less easily available to the substrate (Frey et al., 2010). Such "activity loss" compared to free enzymes has also been reported by previous studies on LDH/enzymes assembly (Bruna et al., 2015, 2019; Charradi et al., 2010).

Therefore, the inorganic LDH structure preserves the enzyme, which remains dormant during the protection.

3.3. *Release properties*

The triggering occurs through an ion exchange mechanism in salt solutions: the anions from the salt interact with the positive charged lamellae of the host structure and replace the anionic protein, which can be released and can recover its degrading activity. Kinetic release studies were conducted to evaluate the enzyme residual activity. A preliminary study is carried out on LDH/Cut-2.2/1, in water and Na_2SO_4 aqueous solution (1.2 wt%). The salt and its concentration were chosen from literature (Zou and Plank, 2015). The recovery after release was evaluated in terms of protein amount and activity and the results are shown in Fig. 7. It can be observed that, as expected, the release is faster in salt solution rather than just water because the presence of sulfate anions favour the exchange with the enzyme and/or carbonate anions. Most of the activity is recovered in the first 15 min (around 37% in Na_2SO_4 aqueous solution) and the plateau is reached in 3–4 h approximately. After 12 h, the total activity recovery is 67% in sulfate solution, 14% in water solution. Moreover, as can be seen by comparing the protein with the activity curve in Fig. 7, the data are in almost perfect agreement and there is no significant loss of enzyme activity (less than 2% with respect to the released protein amount) indicating that the enzyme mostly returns to its original conformation and activity after the release from the LDH host structure.

Similar tests were conducted for all the other samples (in sulfate solution) and the data are reported in Table 1. As already mentioned, the immobilization efficiency is high and constant up to an enzyme loading = 2.2/1, but as to the protein released, the values obtained decrease above and below such enzyme loading ratio. It seems that such loading is the optimum balance in terms of immobilization efficiency and higher protein released. Moreover, a loss of activity of the released enzyme was observed in the samples LDH/Cut-1.0/1 and LDH/Cut-0.5/1. For example, in LDH/Cut-0.5/1 the protein released is 29% while the total activity recovery is 8%. This might be explained by the lower enzyme loading during the synthesis, with consequent higher loading of the competitive carbonate ion, which inhibits the enzyme in terms of

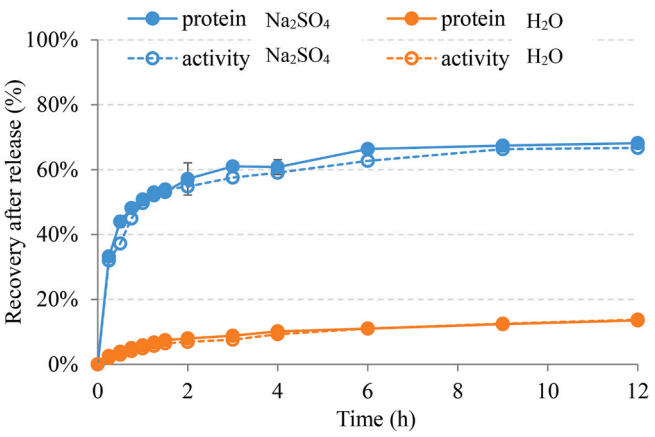

Fig. 7. Enzyme release kinetics from LDH/Cut-2.2/1 in Na_2SO_4 solution or H_2O.

activity. It is reported that many anion species, such as halides, cyanide, bicarbonate, carbonate, nitrate, nitrite, hydrosulfide, bisulfite, and sulfate, etc., could determine an inhibition of microbial enzymes (Innocenti et al., 2008).

FT-IR analysis was performed after release in water and Na_2SO_4 aqueous solution. FT-IR curves are shown in Fig. 8. The band of sulfate is located around 1110 cm^{-1} and is confirmed by literature. Such band increases in LDH/Cut-2.2/1 after release in sulfate solution (red profile), confirming the exchange of sulfate anion in LDH. The band due to carbonate increases as well after release.

3.4. Thermal resistance

The native cutinase has a good thermal stability, exhibiting an optimal reaction temperature between 35 and 70 °C (Lipases for biocatalysis, 2021). In order to investigate the thermal resistance of the free enzyme, the half-life (time at which the enzyme activity is reduced to a half of the original one) of the native cutinase was calculated at 90 °C, i.e., a temperature higher than that included in its optimal reaction range and, at the same time, below the boiling point of the buffer solution. The result is expressed as residual activity over time and is shown in Fig. 9: a half-life of 22 min (±0.54) at 90 °C was estimated for the free cutinase by fitting the residual activity data with a second order kinetics (r^2 = 0.99). These thermostability results are fairly comparable to those of other cutinases. In particular, previous studies reported that the thermal-denaturing half-lives at 80 °C of cutinases from *Malbranchea cinnamomea, Thielavia terrestris* and *Humicola insolens* were around 92, 64 and 15 min, respectively (Duan et al., 2017, 2019; Hong et al., 2019). On the other hand, the half-life of the LDH-immobilized cutinase at 90 °C reached up to 136 min. Therefore, the thermal resistance of the cutinase increases 6 times thanks to its immobilization on MgAl/LDH support, thus playing an important role in the protection of the enzyme molecules from the heat. Other studies also reported increased thermal resistance of the enzymes when immobilized in LDH systems, although less significant than those observed in this study (Dong et al., 2014; Rahman et al., 2004).

Simultaneously, additional thermal stress experiments were carried out on LDH/Cut-2.2/1 powder by subjecting it to high temperatures (i.e. 35 s at 70 °C; 3 min at 150 and 105 °C, followed by 10 s at 200 °C) in order to simulate some industrial processes such as dry/wet lamination or film extrusion. The residual activity with respect to the control sample (not subjected to thermal stress, assumed as 100%) resulted very high. In particular, a negligible activity loss occurred after incubation of the LDH-immobilized cutinase at 70 °C, while more than 60% of enzyme residual activity was measured after the treatment at higher temperatures (Table 4).

Therefore, the protecting role of the inorganic LDH structure is confirmed, also in extreme conditions, namely after thermal treatments at high temperatures.

3.5. Preliminary evaluation of enzymatic degradation of a polyester (poly (butylene succinate-co-adipate) (PBSA) formulated with 5 wt% of LDH/cutinase

An application of the immobilized cutinase can be to accelerate the polymer biodegradation, avoiding the problem of materials accumulation in composting facilities. To prove this concept, the enzymatic degradation of a PBSA film formulated with the LDH-protected cutinase containing the highest enzyme loading (LDH/Cut-3.4/1) was here investigated via weight loss tests. A low amount, 5 wt%, of immobilized cutinase was employed. A negligible weigh loss occurred in the pristine PBSA and that formulated with a commercial LDH (PBSA-LDH/Pural Mg61), while PBSA-LDH/Cut-3.4/1 was completely degraded after 24 h of incubation in sodium phosphate buffer used as triggering agent (Table 5). This suggests that the enzyme can be released from LDH system and can rapidly degrade the polymer. Approximately 0.12 mg mL^{-1} and 75.5 U mL^{-1} of cutinase was found indeed in the liquid fraction. These values corresponded to a release from LDH system of approximately 34% and 16% of protein amount and enzymatic activity, respectively (Table 6). Moreover, the films kept at room temperature under vacuum for 24 h, did not decrease their initial weight, highlighting the fact that the polyester is not degraded until the enzyme is triggered.

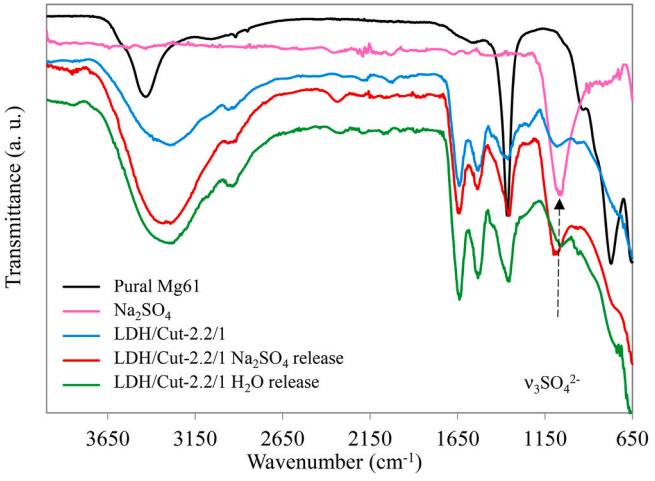

Fig. 8. ATR FT-IR curves of LDH/Cut-2.2/1 after release in water and Na_2SO_4 solution.

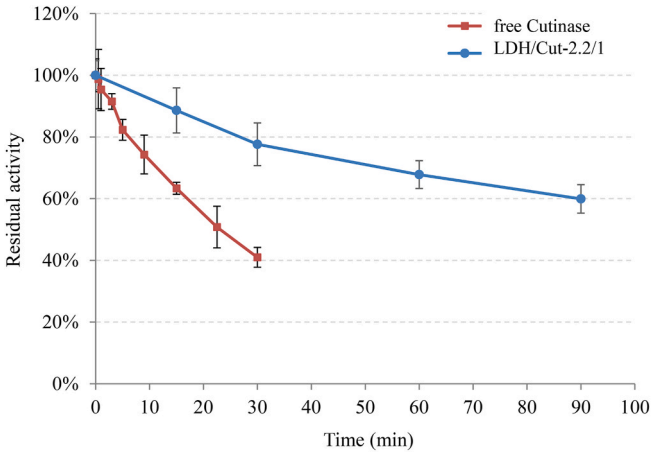

Fig. 9. Thermal stability of free and LDH-immobilized Cutinase at 90 °C.

Table 4
Test conditions and enzyme residual activity results of thermal resistance experiments.

Sample	Conditions: Temperature (°C) and Time (sec)	Residual activity upon 4 h (respect to the control) (%)
LDH/Cut-2.2/1	ambient	100 ± 12
	70 °C (35 s)	96 ± 16
	105 °C (180 s) + 200 °C (10 s)	67 ± 13
	150 °C (180 s) + 200 °C (10 s)	63 ± 14

Table 5
Degradation (%) of PBSA-LDH/Cut-3.4/1 and PBSA-LDH/Pural Mg61 after 24 h of incubation under vacuum or in sodium phosphate buffer 0.1 M.

Sample film	Degradation after 24 h at TA, under vacuum (%)	Degradation after 24 h at 40 °C in phosphate buffer (%)
PBSA	0.0 ± 0.0	0.5 ± 0.0
PBSA-LDH/Pural Mg61	0.0 ± 0.0	4.3 ± 0.3
PBSA-LDH/Cut-3.4/1	0.0 ± 0.0	100.0 ± 0.0

The kinetics of degradation and the optimization of the lower amount of LDH/cutinase required, will be reported in a next paper. However, such results are really promising in terms of employment of such systems in plastic biodegradation or recycling.

4. Conclusions

The enzyme cutinase is here successfully immobilized in MgAl/LDH structures. The results proved that the best composition in terms of higher immobilization efficiency and higher release is the one containing an enzyme molar ratio 2.2/1 with respect to Al. It is notable that cutinase results dormant in LDH structure and becomes active only after release in Na_2SO_4 aqueous solution. The thermal stability of cutinase is strongly improved after the immobilization, since the immobilized enzyme half-life increases 6 times with respect to the free cutinase at 90 °C and the residual activity after some thermal stress experiments is very high. Moreover, the immobilized cutinase, embedded in a PBSA film, can be released in an appropriate medium and the enzyme is able to completely degrade the PBSA in 24 h. All such findings are very promising in view of a potential use in polymeric formulations for plastic biodegradation or recycling, fostering the eco-design of new materials degradable "on-demand" at the end-of-life. In fact, a low amount of the additive (5 wt%) guarantees a fast biodegradation of the polymer. The systems prepared could help improve the overall economy efficiency: they would enhance the effectiveness of current composting facilities, implement the reduction of landfilling rates for packaging materials, and imply an overall decrease in CO_2 emission. Moreover, it must be underlined that after the degradation the resulting oligomers and monomers could be recovered and chemically upcycled into value added performance materials contributing to the goal of realizing a sustainable plastic economy.

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Table 6
Protein concentration (mg mL^{-1}) and enzymatic activity (U mL^{-1}) measured in buffer after 24 h incubation of PBSA-LDH/Cut-3.4/1, and the respective release (%) of protein and activity.

Protein concentration (mg mL^{-1})	0.12 ± 0.01
Protein release (%)	33.8 ± 2.8
Enzymatic activity (U mL^{-1})	75.5 ± 10.7
Enzymatic activity release (%)	15.8 ± 2.2

CRediT authorship contribution statement

Angela Romano: Investigation, Data curation, Visualization. **Antonella Rosato:** Investigation, Data curation, Visualization. **Grazia Totaro:** Investigation, Data curation, Writing – original draft, preparation, Writing – review & editing, Visualization. **Giulio Zanaroli:** Conceptualization, Methodology. **Annamaria Celli:** Supervision. **Laura Sisti:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Dataset Available on Zenodo repository (https://zenodo.org/): DOI: 10.5281/zenodo.5006115.

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