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Turning biomass into functional composite materials: Rice husk for fully renewable immobilized biocatalysts

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ABSTRACT

Rice husk is an underexploited, low density and highly robust composite material, massively available from rice processing. Here we report two new procedures for the formulation of immobilized lipases applicable in fats and oils transformations. The enzymes were covalently anchored on aldehyde groups introduced on rice husk by laccase-catalysed oxidation of the cellulose component. The method avoids the use of toxic glutaraldehyde while allows for the application and recycling of the biocatalysts in aqueous media. The second method used a fluidized bed granulator for the coating of the particles of rice husk (200–400 μm) in the presence of water-soluble binders. The formulations are mechanically stable and suitable for applications in different hydrophobic media. Both methods allow for the recovery and reuse of the rice husk at the end of the life cycle of the biocatalysts.

1. Introduction

When considering the largest scale applications of enzymes in industry, they include glucose isomerase for production of high fructose syrup (10^7 ton/y), β-galactosidases for lactose hydrolysis in milk (10^5 ton/y), and lipase for transesterification of food oil (10^5 ton/y). All these processes are embedded in bioeconomy value chains and utilize immobilized enzymes (DiCosimo et al., 2013). An immobilized insoluble enzymatic formulation not only enables their reusability, thus lowering production costs, but also reduces waste stream generation (Girelli et al., 2020). In the last years, we have tackled the challenge of making biocatalysis more sustainable and suitable for the large-scale processes of bioeconomy by developing immobilized biocatalysts that exploit residual biomass from agricultural products. Achieving both the environmental and economic sustainability of immobilized enzymes is quite challenging, since the "ready to use" carriers, such as fossil-based methacrylic resins, are quite expensive and also responsible of greenhouse gas emission (Kim et al., 2009). Finally, the stability of organic fossil-based carriers was reported unsatisfactory under chemical and mechanical stress (Hilterhaus et al., 2008; Pellis et al., 2015; Korupp et al., 2010).

Considering that for bulk products the allowable cost contribution of immobilized enzymes should be around 0.05 € $kg_{product}^{-1}$ (Tufvesson et al., 2011), rice husks (RH) attracted our attention as a potential renewable carrier for enzyme immobilization. This lignocellulosic material is the second most abundant biomass globally, produced in around 120 Mt per year, of which only 20 Mt are used (Tuck et al., 2012). RH is an extremely robust composite material made of SiO_2, lignin, hemicellulose and cellulose, (Corici et al., 2016) which provides a versatile chemical platform for introducing functionalities (Raynaud 2014). It also meets circularity criteria because it can be re-utilized at the end of its proposed industrial application (Contreras et al., 2012). Finally, as natural material, it has the subsidiary advantage that it is subjected to less stringent legislative constraints even after chemical modification (Raynaud 2014). The tubular structure of tracheids confers to RH a very low density (< 0.4 g mL^{-1}), while SiO_2 is responsible for its remarkable mechanical robustness.

Our previous studies reported the chemical oxidative cleavage ($NaIO_4$) of the cellulose present in RH for the introduction of carbonyl functionalities, which were exploited for the binding of enzymes in the presence of glutaraldehyde as crosslinking agent (Corici et al., 2016; Cespugli et al., 2018; Pellis et al., 2017).

Applications of lipases in an aqueous environment require the covalent anchoring of the enzyme on the carriers for preventing the detachment of the protein and its partition in the medium (Biermann et al. 2000, Schmid and Verger 1998). Despite the wide number of studies present in the literature (Hanefeld et al., 2009), very few examples of co-

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valent binding on solid supports with satisfactory and clearly stated immobilization yields have been reported so far because of their structural and conformational features (Hilterhaus et al., 2008; Cantone et al., 2013). We have previously reported (Cespugli et al., 2018) that CaLB can be immobilized covalently on chemically oxidized RH after the introduction of a hexamethylenediamine spacer (HMDA) and employing glutaraldehyde as bi-functional agent able to form imine bonds with the spacer and the amine groups of lysine residues present on the surface of the lipase. The toxicity of GA is widely documented although it is still used in food industry according to strictly defined official regulations. The adverse health effects on humans include sensitization of skin and respiratory organs and closed-systems are recommended to prevent hazards from GA exposure in industrial plants (Takigawa and Endo 2006; Toxicological Profile, 2015; EFSA, 2011)

The present study illustrates two sustainable and scalable methods that demonstrate how it is possible to exploit RH as immobilization carrier while overcoming the use of toxic reagents, such as $NaIO_4$ and glutaraldehyde. The first approach exploits laccase enzymes for the oxidation of the cellulose component of RH and the introduction of aldehyde groups usable for the direct covalent anchoring of proteins. The method was validated with lipase B from *Candida antarctica* (CaLB) and lipase from *Thermomyces lanuginosus* (TLL), applied in the hydrolysis of triglycerides in aqueous media. The second immobilization method employs a fluidized bed granulator for the coating of RH with TLL in the presence of water-soluble binders and allows for the inexpensive immobilization of lipases to be used in low-water media.

2. Materials and methods

2.1. Chemicals

Lipase B from *Candida antarctica* (EC 3.1.1.3, CAS number 9001–62–1, liquid solution, activity: 1372 TBU g^{-1} (TBU = enzymatic Units calculated with tributyrin hydrolysis), protein content = 84 mg mL^{-1}) and Laccase Novozym 51,003 (EC 1.10.3.2., CAS number 80,498–15–3) from *Myceliophthora thermophila* expressed in *Aspergillus sp*. (activity: 64 U_{ABTS} mL^{-1}) were from Novozymes (Denmark). Lipase from *Thermomyces lanuginosus* (specific activity: 3176 TBU g^{-1}, liquid solution, protein content = 38 mg mL^{-1}) maltodextrin, Kollidon®25, 2,2,6,6-tetramethylpiperidin-1-yl)oxy (TEMPO), NaOH, $NaIO_4$, Bradford reagent, tributyrin and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were from Sigma-Aldrich. Lipase from *Ryzopus oryzae* (powder, activity: 29,496 U g^{-1}) was kindly donated by Amano Corporation (Japan). Laccase C (EC 1.10.3.2) from *Trametes versicolor* (2176 U_{ABTS} g^{-1}) was from ASA Spezialenzyme GmbH (Germany). PEG 3000 was from Merck. Arabic gum was from Fluka™. Hydroxyethylcellulose was from Esperis S.p.A. (Italy). The RH was kindly donated by Riseria Cusaro, Binasco (Italy), milled and sieved as previously reported (Cespugli et al., 2018). All enzymatic specific activities and protein content reported above were determined independently.

2.2. Chemical oxidation of rice husk

The chemical oxidation of RH was performed as previously described by Cespugli et al. (2018)

2.3. Assays for enzymes activity

Activity of laccases was evaluated using the ABTS method reported by Piscitelli et al. (2005) (see supplementary materials method S1).

The activity of lipases (hydrolysis of tributyrin) was assayed as previously described by Cespugli et al. (2018), Martins et al. (2013b).

2.4. Enzymatic oxidation of RH

Protocol at 20 °C: a 10 mM solution of TEMPO was added to 0.2 g of RH previously washed with 10 mL sodium citrate buffer 0.1 M pH 5. The laccase and the phosphate buffer were added to obtain a final concentration of 8 or 40 U mL^{-1} in 25 mL total. The protocol at 70 °C employed sodium citrate buffer 0.1 M pH 5 using a final laccase concentration of 8 U mL^{-1}. The reaction mixtures were magnetically stirred (350 rpm) and air was insufflated for 2 min every 8 h to increase the exposure of the enzyme to oxygen. At the end of the reaction, the mixture was filtered and washed with distilled water (15 mL, 4 times). The recyclability of the laccase was demonstrated as reported in ESI (Electronic Supplementary Information, method S2).

2.5. Immobilization of lipases on oxidized RH

The lipases were immobilized according to the protocol of Cespugli et al. (2018) modified as follows: on 0.2 g of oxidized RH by adding 1 mL of solution containing 2000 U (TBU) of each commercial lipase, diluted in phosphate buffer (0.5 M at pH 8) and adding PEG-3000 (0.5 mL of a 2 mg mL^{-1} solution in phosphate buffer 0.5 M at pH 8) as stabilizer.

2.6. Determination of carbonyl and carboxylic groups content

The content of carbonyl and carboxylic groups were determined as previously described by Cespugli et al. (2018). The details are included in the ESI material as method S3 and S4

2.7. Physical immobilization of lipase TLL on RH

The immobilization of TLL on 100 g of RH in the presence of polyvinylpyrrolidone (Kollidon®25), maltodextrin and hydroxyethyl cellulose was carried out in a fluid bed granulator Mini-Glatt fluidized bed (Glatt GMbH, Binzen, Germany) equipped with a conical vessel (volume of 0.75 L), three metallic filters and a timing filter blowing. The immobilization was performed by adapting a procedure previously described by Trastullo et al. (2015) and details are available in ESI as method S5.

2.8. Energy dispersive X-ray spectroscopy (EDS)

Non-metallized samples were investigated with Zeiss Supra 40 high-resolution Field Emission Gun (FEG) Scanning Electron Microscope (SEM). Images were obtained by collecting secondary electrons with the electron high tension (EHT) equal to 10 keV thanks to an Everhart-Thornley detector. The microscope was equipped with an Oxford Aztec energy dispersive X-ray spectroscopy (EDS) system and an X-act 10 mm silicon drift detector (SDD) for compositional analysis. The same energy of 10 keV was exploited to collect the silicon signal on the sample (Figure S1).

2.9. SEM microscopy

Samples were metallized with the S150A Sputter Coater instrument (Edwards High Vacuum, Crawley, West Sussex, UK) before being observed with the Leica Stereoscan 430i scanning electron microscope (Leica Cambridge Ltd., Cambridge, UK) integrated with an Si detector (Li) PENTAFET PLUS TM, with an ATW TM window (Oxford Instruments, Oxfordshire, England) for microanalysis.

2.10. Stereoscopic microscopy

The immobilized biocatalysts were characterized by using a stereomicroscope (Leica MZ 16, Leica Microsystems, Wetzlar, Germany), equipped with a Kiralux CS505CU camera (Thorlabs Inc., Newton, NJ, USA).

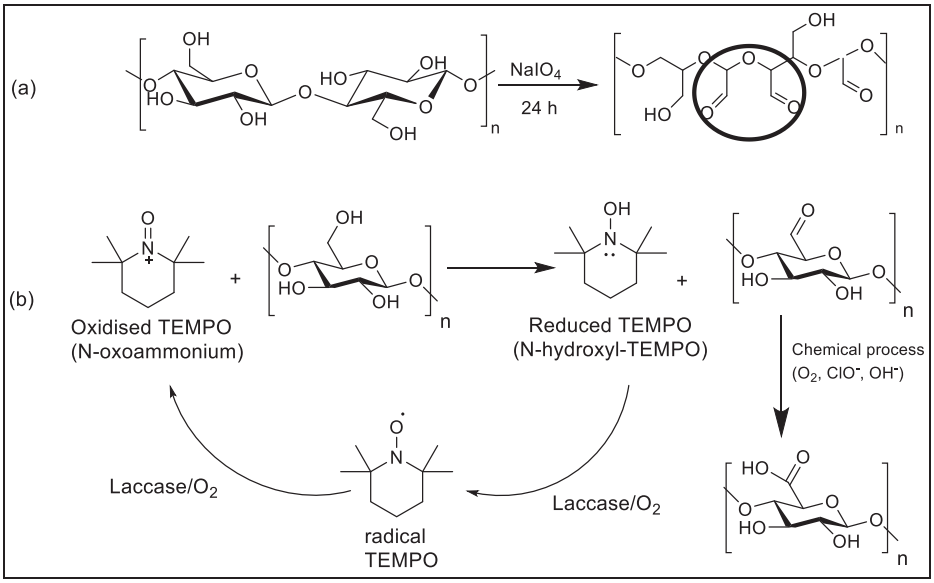

Fig. 1. Schematic comparison of the chemical and enzymatic mediated oxidation of cellulose. The $NaIO_4$ causes the oxidative cleavage of the glucose with the formation of two aldehyde groups. The laccase/TEMPO system oxidizes the primary hydroxyl group to an aldehyde, which undergoes spontaneous oxidation to carboxyl group.

2.11. Synthesis of butyl butyrate catalyzed by immobilized TLL in hexane and toluene

The immobilized TLL (160 mg) were incubated for 1 h at 48 °C in hexane or toluene (6.676 mL). The reaction was initiated by the addition of 1-butanol (0,618 mL) and butyric acid (0,206 mL) at 3:1 molar ratio. The reactions were carried out at 48 °C, in an orbital shaker (250 rpm) for 24 h and monitored by HPLC as reported in ESI (method S6). Control reactions without enzyme were performed under identical conditions. All the experiments were made in duplicate and the mean values were considered.

2.12. Synthesis of butyl butyrate catalyzed by physically immobilized TLL in isooctane

1-butanol (0.25 mL) and butyric acid (0.25 mL) (1:1 molar ratio) were added to 9.5 ml of isooctane and 130 mg immobilized TLL. The reaction was incubated at 45 °C in an orbital shaker (250 rpm) for 21 h and monitored as reported in ESI (method S7). All the experiments were made in duplicate and the mean values were considered.

3. Results and discussion

3.1. Enzymatic functionalization of RH

The covalent binding of proteins on any carrier requires the introduction of functional groups able to form suitable bonds with the side chains of amino acids present on the protein surface. We have previously reported the functionalization of RH through an oxidative cleavage of the cellulosic component by means of $NaIO_4$ (Corici et al., 2016; Cespugli et al., 2018). To improve the sustainability and scalability of the oxidative process, a protocol was developed in which the toxic $NaIO_4$ was replaced by laccase enzymes, copper-oxidases present in various organisms like bacteria, plants, insects and fungi (Claus 2004). Big molecules and non-phenolic compounds are oxidized with difficulty by laccases due to the reduced accessibility of the active site and also because of their low redox potential (Bourbonnais et al. 1995). This drawback can be overcome with the use of a *laccase-mediator system* (LMS), namely by adding a small molecule able to generate stable radical species (Cañas and Camarero 2010). In our work we have taken inspiration from the work of Patel and co-workers that describes the oxidation of the C_6 hydroxyl of glucose in the cellulose chain catalyzed by laccase in the presence of TEMPO-radical (Patel et al., 2011) (Fig. 1b)

In the present study, milled RH particles (0.2–0.4 mm) were oxidized by laccase from *Trametes* sp. and laccase from *Myceliophthora thermophile* and the content of carbonyl groups was assessed using the hydroxylamine hydrochloride assay (Cespugli et al., 2018; Guigo et al., 2014). Data in Fig. 2a indicate that laccase from *Trametes* sp. was more effective in oxidizing the RH and that the TEMPO mediator was necessary. The amount of the resulting carbonyl groups depends on the laccase units, with the highest concentration achieved in the presence of 40 U mL^{-1} of laccase from *Trametes* sp.

To decrease the amount of laccase in the oxidation process, a larger amount of RH was used in the reaction mixture while maintaining the same concentration of enzyme (Fig. 2b). The temperature was also increased to 70 °C and the recyclability of the laccase for further oxidative cycles was demonstrated. After 48 h of enzymatic treatment, the oxidized RH was removed by filtration and some fresh rice-husk was added in the enzymatic solution. Fig. 2b shows that without the addition of TEMPO the concentration of carbonyl groups still increased after 24 h, indicating a residual presence of unreacted TEMPO deriving from the first cycle. When TEMPO was added to the recycled enzyme, the carbonyl groups increased by 166% in 24 h and 177% in 48 h. Overall, the data suggest that the TEMPO concentration can be reduced in both cycles and that the oxidation occurs much faster in the second cycle (terminated in 24 h), thus demonstrating the recyclability of the laccase. The observed higher activity in the second cycle could be ascribed to activation phenomena occurring upon exposure to high temperature or denaturing factors, as reported in our study on laccase from *Steccherinum ochraceum* 1833. By means of molecular dynamics simulations we disclosed minor superficial conformational modifications of the laccase that improve its activity without causing severe unfolding nor the disruption of the electron transfer pathway (ETP) (Ferrario et al., 2015).

Current data on the preservation of the activity of the laccase from *Trametes* sp. during 96 h of reaction at 70 °C suggest that the enzymatic oxidative functionalization of RH can be further optimized in order to maximize the number of cycles and minimize of economic impact of the treatment. The TEMPO mediator seems to play a major role and although its concentration can be reduced, its environmental and economic impact deserves for further attention. Future studies will be focused on the replacement of TEMPO with renewable and inexpensive mediator molecules, which have been proved effective in previous studies (Cañas and Camarero 2010; Medina et al., 2013)

In all cases, the content of carbonyl groups measured after the enzymatic LMS method (Fig. 2a) resulted to be considerably lower than the 1 mmol g^{-1} obtained with 0.2 M $NaIO_4$. This difference can be partially ascribed to the formation of two carbonyl groups per each glucose

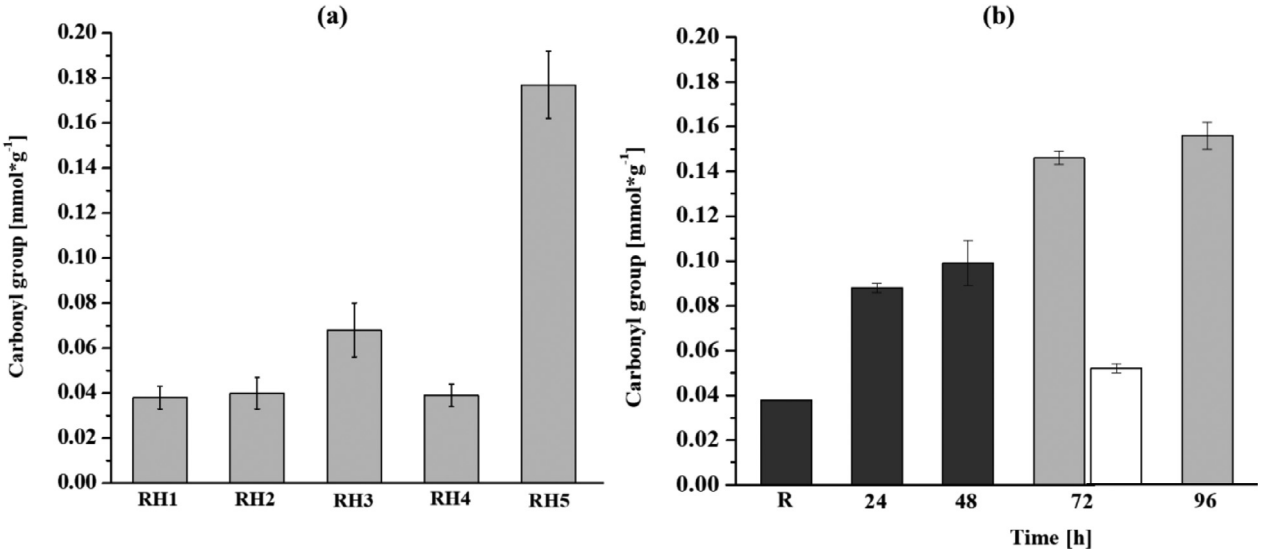

Fig. 2. (a) Concentration of carbonyl groups upon different oxidation treatments at 20 °C. RH1: untreated rise husk; RH2: Novozyme 51,003 20 U mL^{-1}; RH3: Laccase C 8 U mL^{-1}; RH4: Laccase C 40 U mL^{-1} without TEMPO; RH5: Laccase C 40 U mL^{-1} with TEMPO. (b) Oxidation at 70 °C and recycling of laccase: first 48 h cycle of oxidation (black bars); laccase reused for a second oxidation cycle on fresh RH (gray bars); recycled laccase but without addition of TEMPO.

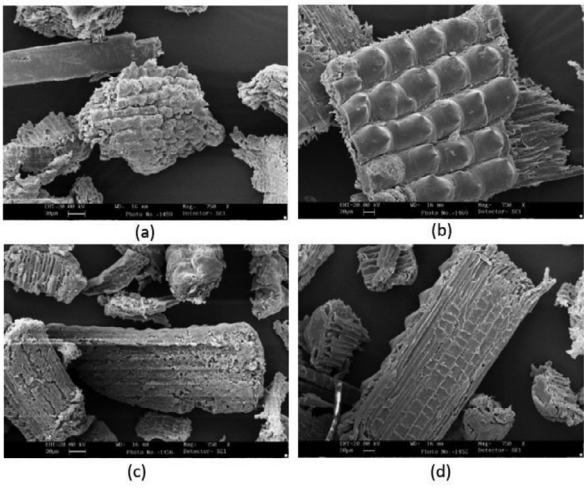

Fig. 3. SEM images illustrating the effect of $NaIO_4$ (a, c) vs enzymatic oxidation (b, d) on the morphology of RH. Corrosive effect of the $NaIO_4$ on the surface(a) and on the inner cellulosic matrix of tracheid (d).

unit, whereas in the case of LMS oxidation one single carbonyl group per glucose unit is formed. Moreover, SEM microscopy highlighted the corrosive effect of $NaIO_4$, which leads to marked covalent modification of the polysaccharide because of the oxidative cleavage, opening the access to deeper layers of the material (Fig. 3), both inside the tracheid structures (Fig. 3c) and on the outer surface (Fig. 3a).

Fig. 3 indicates the poor selectivity of the $NaIO_4$ towards the various components of the lignocellulosic matrix of the RH. On the contrary, the enzymatic treatment with laccase causes negligible morphological modification on the external surface, while leads to a modest increase of the roughness of the internal surface of the tracheids.

Morphological studies making use of energy dispersive X-ray spectroscopy (EDS) were of aid in understanding the robustness of the outer surface of the husk and, more precisely, the localization of SiO_2 (Park et al., 2003; Coletta et al., 2013). Using the EDS (Fig. 4 right) the Kα1 signal of the silicon (ESI Figure S1) was collected (green dots) to appreciate the presence SiO_2 on the surface of the RH and to confirm its role in protecting the rice grain from mechanical and chemical agents.

The formation of carboxylic groups during both the chemical and enzymatic oxidation processes were also evaluated (ESI Figure S2) and they confirmed that the oxidation of aldehydes to carboxylic groups is not laccase mediated.

3.2. Glutaraldehyde free covalent immobilization of lipases on RH

In the present study, we aimed at simplifying the immobilization protocol, by reducing the synthetic steps while avoiding the use of glutaraldehyde (GA). We have explored the possibility to form the imine bond directly between lysine residues and the carbonyl group on C_6 formed *via* laccase oxidation, which appears more accessible than the di-aldehyde groups obtained using $NaIO_4$. Two more lipases were tested in the study: lipase from *Thermomyces lanuginosus* (TLL) and lipase from *Rhizopus oryzae* (ROL), which are widely employed in the food sector for the transesterification of oils and fats (Higashiyama and Sumida 2004). Therefore, avoiding the use of glutaraldehyde in their formulations would be desirable (Zeiger et al., 2005).

The tri-dimensional models of the lipases are reported in ESI (Figure S 3). TLL and ROL undergo interfacial activation (Ferrario et al. 2011) when approaching a hydrophobic phase, whereas CaLB does not undergo significant conformational changes and its active site is permanently accessible (Ferrario et al. 2011, Basso et al., 2007). The analysis of the number and the position of lysine residues potentially involved in the covalent binding with the carrier indicates that CaLB and TLL have 9 and 7 lysines respectively, whereas ROL has 15 lysines, one of them located in the proximity of the active site. That feature makes the immobilization of ROL quite challenging, since the formation of the covalent bond can occlude the active site but also prevent the necessary conformational changes connected to the interfacial activation. Indeed, previous data collected in our group showed that the hydrolytic activity is completely lost upon covalent immobilization of ROL in aqueous buffer on methacrylic epoxy resins (Gardossi et al., 2012).

Data reported in Table 1 demonstrate that TLL and CaLB can be anchored directly on the enzymatically oxidized RH, while avoiding the use of glutaraldehyde and spacers. Higher loading was obtained in the case of RH oxidized enzymatically, indicating a regio-preference in the formation of imine bonds with the more accessible aldehyde groups on C_6.

The use of glutaraldehyde causes a significant reduction of the specific activity of CaLB formulations, probably ascribable to the forma-

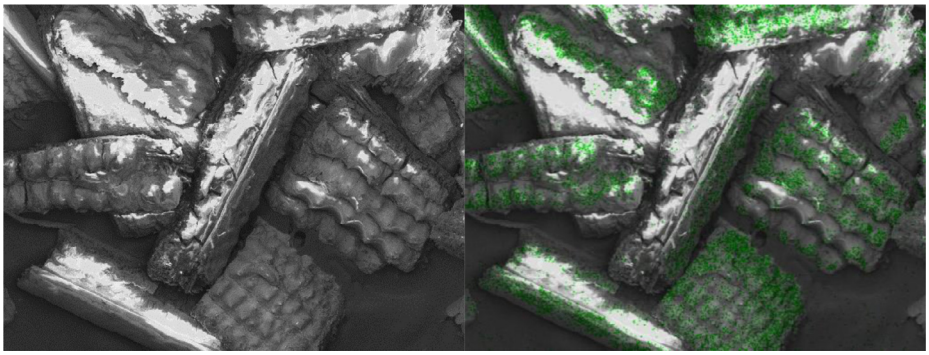

Fig. 4. Left: SEM image of fragments of milled RH, showing the linear ripples with conical shape on the external surface. Right: 2D map the distribution of SiO_2.

Table 1
Formulations of lipases covalently immobilized on RH. In all cases 10.000 U of lipase per g of RH were employed. The activity is expressed as the average of three measurements. LMS=laccase from *Trametes* sp. + Tempo mediator. Last three entries were published in ref. (Cespugli et al., 2018) and are reported for comparison.

Lipase	Oxidation method	Other functionalization	Protein loaded (%)	hydrolytic activity (Ug^{-1}_{dry})	immobilization yield (%)
CALB	LMS	no	65	590±2	5.9
TLL	LMS	no	72	974± 65	9.74
ROL	LMS	no	53	328± 42	3.28
CALB	$NaIO_4$	no	33	290±45	2.90
TLL	$NaIO_4$	no	56	643 ± 67	6.43
ROL	$NaIO_4$	no	32	171 ± 14	1.71
CALB	LMS	GA	55	155±5	1.55
CALB	$NaIO_4$	GA	65	<50	<0,5
CaLB	LMS	HMDA+GA§	17	56 ± 25	0.56
CaLB	$NaIO_4$	HMDA+GA§	72	316	3.16
CaLB	n.a.	Epoxy (on methacrylic resin)§	95	709	7.09

§ previously published.

tion of unspecific covalent bonds and crosslinks. When compared to our previously published data of immobilization of CaLB on RH chemically oxidized and functionalized with diamino spacers with GA, the specific activity with the simplified protocol is almost doubled. The immobilization yield is comparable to that observed for CaLB immobilized on epoxy methacrylic carrier (Cespugli et al., 2018)

The immobilization yields are in line with previous experimental data obtained in the covalent immobilization of different lipases on epoxy methacrylic resins using aqueous buffer as immobilization medium, which have been always below 10% (Pellis et al., 2016; Gardossi et al., 2012). The difficulties encountered in the covalent immobilization of lipases have been discussed previously and they are ascribable both to their conformational and superficial structural features (Cantone et al., 2012; Ferrario et al., 2011). Conversely, very few studies report the actual immobilization yield accompanied by the evaluation of the protein leaching from the support, as a proof of the robust anchorage of the enzyme on the carrier. At the best of our knowledge, no covalently immobilized lipase is commercialized for industrial applications, although the hydrolytic applications of these enzymes request this type of formulation for enabling an efficient recycling of the biocatalyst and the reduction of the costs. As a matter of fact, an analysis of Tufvesson and co-workers (Tufvesson et al., 2011) indicated that in the case of biocatalysts immobilized by adsorption 63% of the material cost is ascribable to the carrier and only 37% to the enzyme. Taking into account that the cost of carriers for covalent immobilization is about 2–6 times higher, it is evident that the economic viability of these biocatalysts is mainly linked to the price of the carrier and the preservation of the enzyme activity across multiple reaction cycles (i.e. productivity). Therefore, there are several potential advantages deriving from the covalent immobilization of lipases on oxidized rice husk. Firstly the cost of the carrier is reduced; secondly the enzyme can be separated by the hydrolysis product and recycled, thus further decreasing the economic impact of the biocatalyst; finally, the re-use of the non-fossil and biodegradable carrier at the end of the biocatalyst life cycle would integrate environmental and economic sustainability.

3.3. Stability and recyclability of covalently immobilized lipases in aqueous media

The stability of the immobilized lipases was evaluated in the hydrolysis of tributyrin, under vigorous mechanical stirring. The results in Fig. 5 indicate an excellent stability of CaLB on rice husk and TLL on rice husk after 10 reaction cycles, with about 80% and 70% of recovered activity, whereas ROL retains only 15% of activity, which was ascribed to the leaching of ROL from RH, as experimentally observed (ESI, Figure S4). Notably, all biocatalysts reported in Table 1 were tested for protein leaching and only ROL formulations showed the detachment of the protein from the carrier.

Despite the presence of 15 lysine residues, ROL forms covalent bonds with difficulty, as also evident from the lower percentage of protein loaded (Table 1). This behavior cannot be ascribed to a unique factor, but rather to the combination of structural and superficial features (e.g. glycosylation) that prevent an effective adsorption of the enzyme on the carrier and the nucleophilic attack of the amino groups of the lysines (Basso et al., 2007). The observed loss of activity is most probably connected to conformational modifications occurring during the immobilization in aqueous systems, (Ferrario et al., 2011) as previously illustrated by some molecular dynamics simulations. The exposure to aqueous media induces unfavorable conformational modifications in some lipases, by causing the occlusion of the active site but also a rearrangement of the catalytic residues responsible for the catalytic mechanism (Ferrario et al., 2011). This observation would explain why the covalent immobilization of ROL is much more efficient when carrier out in a hydrophobic media (Gardossi et al., 2012).

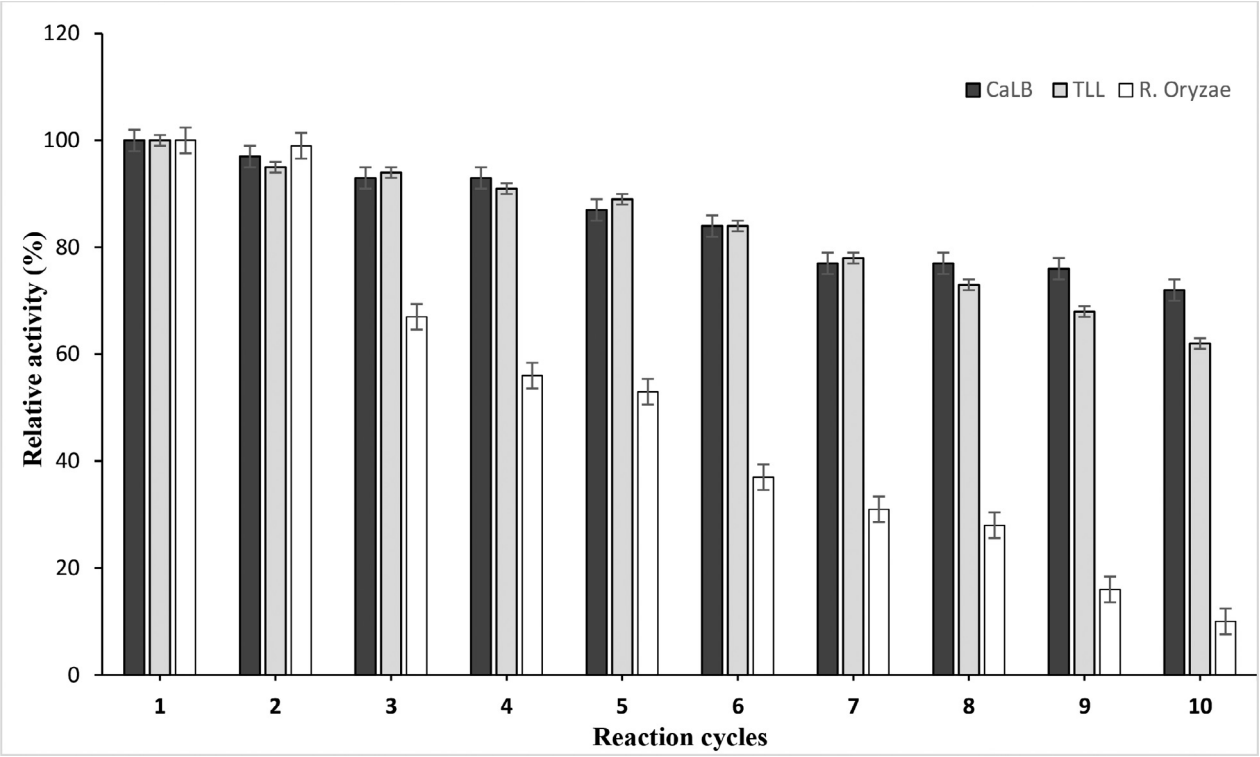

Fig. 5. Operational stability of CaLB on rice husk (black bar) TLL on rice husk (gray bar) and ROL on rice husk (white bar) in multiple hydrolytic cycles expressed as the percentage of retained activity after each cycle. Measurements were carried out in duplicate and reported as the average value.

3.4. Physical immobilization of TLL in a fluidized bed

The second part of the study was aimed by developing scalable procedures for the immobilization of lipases to be applied in low-water hydrophobic media, while ensuring environmental and economic sustainability. Generally, the application of immobilized enzymes in hydrophobic media does not require the covalent binding of the protein to the carrier, since the enzyme has a lower affinity for the hydrophobic phase (Pellis et al., 2015).

Lipase TLL was selected for the study and it was immobilized on milled RH (200–400 μm) in a fluidized bed in the presence of an aqueous solution of different binders approved for pharmaceuticals and food applications: Kollidon 25 (polyvinylpyrrolidone=PVP) (Reddy and Sharma, 2020), hydroxyethyl cellulose (HEC) (Di Giuseppe 2018) and maltodextrin (MLDX) (Velásquez-Cock et al., 2018). A uniform layer of enzyme coated the RH and the immobilized biocatalysts appear as distinct particles, without any example of agglomerates (Fig. 6).

3.5. Synthesis of butyl butyrate catalyzed by TLL physically immobilized on rice husk particles

The efficiency of the TLL formulations obtained through physical immobilization was evaluated in the synthesis of butyl butyrate starting from butyric acid and 1-butanol (Martins et al., 2013a). This short chain fruity ester, present in pineapple flavor (Martins et al., 2013), is used in perfumes and fragrances, waxes, washing, cleaning, cosmetics and personal care products (Xin et al., 2016). Three organic solvents were tested: toluene (log P 2.5), hexane (log P 3.5) and isooctane (log P 4.6). Figs. 7 illustrates the time-course of the reactions in hexane and toluene, indicating striking differences of performance of the biocatalysts. In hexane,PVP-TLL leads to complete conversion in less than 5 h, whereas the profiles of the other two reactions suggest some lag-time before the starting of the reactions, possibly ascribable to some mass transfer limitation. Conversions > 93% were achieved in all cases and it was noticed that the absence of agglomerates confers high mechanical stability to the formulations, preventing disaggregation and production of fines upon stirring.

All reactions carried out in toluene were considerably slower: PVP-TLL was the best performing formulation with > 80% conversion after 8 h of reaction, while MLDX-TLL stopped working after 10% of conversion.

Control reactions were performed in the absence of the biocatalyst, using lipase free particles of RH treated in the fluidized bed only with the binders. Moreover, blank experiments demonstrated the absence of any aspecific adsorption of the substrate on the biocatalyst when incubated in toluene (ESI Figure S5).

When the synthesis was conducted in isooctane (Batistella et al., 2012), the substrates were employed in equimolar amounts, resulting in a lower concentration of 1-butanol as compared to the previous synthesis. In this way we wanted to verify the possibility to achieve quantitative conversion even without using an excess of the polar alcohol, which is known to affect negatively the stability of enzymes.

Interestingly, HEC-TLL and MLDX-TLL performed significantly better as compared to what was previously observed in toluene and with similar conversions and reaction rate, whereas the reaction catalyzed by PVP-TLL stopped after 35% of conversion due to the formation of aggregates. However, figure 9b shows how the addition of 0.1% v/v of water improved the behavior of PVP-TLL, by preventing the aggregation. These data suggest that the performance of PVP-TLL improves in the presence of hydrophilic components in the reaction mixture, and explain the higher observed efficiency of this formulation in the presence of a higher concentration of the hydrophilic 1-butanol (Fig. 8). Therefore, the operational conditions must be optimized according to the LogP of the medium, the nature of the substrates and also the water content (Basso et al., 2001) Previous studies demonstrated that TLL can work efficiently in a wide range of water activity values, going from 0.33 to 0.97 (Persson et al., 2002; Adlercreutz 2013). Therefore, the hydration appears to exert only a minor effect on the intrin-

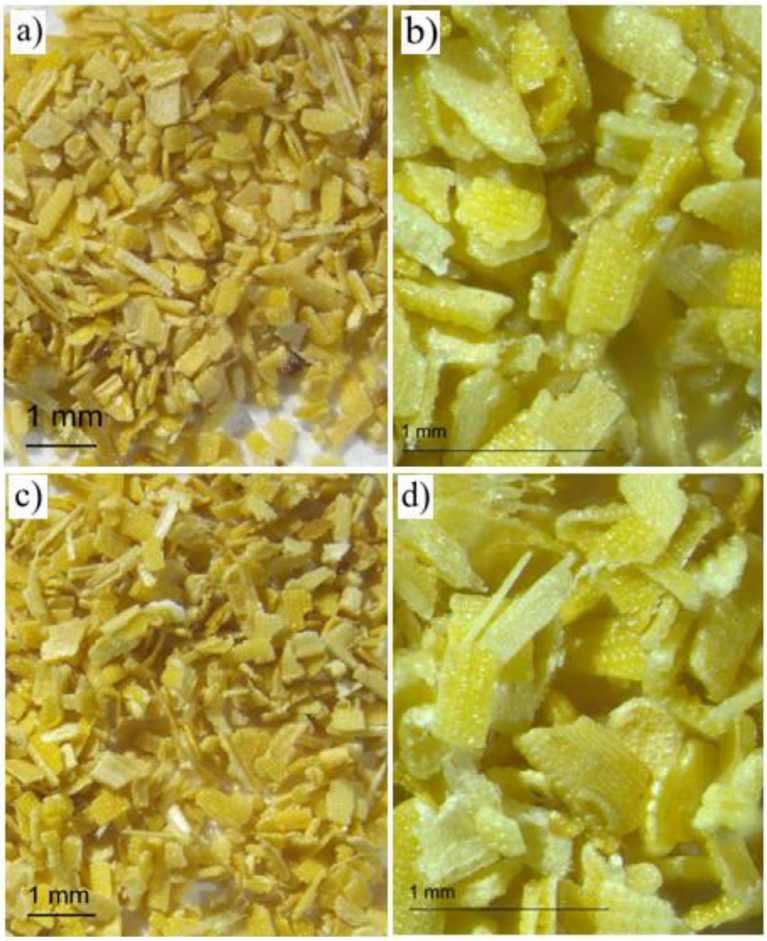

Fig. 6. TLL physically immobilized on rice husk particles and prepared using a fluidized bed reactor. Images obtained by means of stereoscopic microscopy at different magnification values: PVP (a)–10x (b)–60x and HEC (a)–10x (b)–60x.

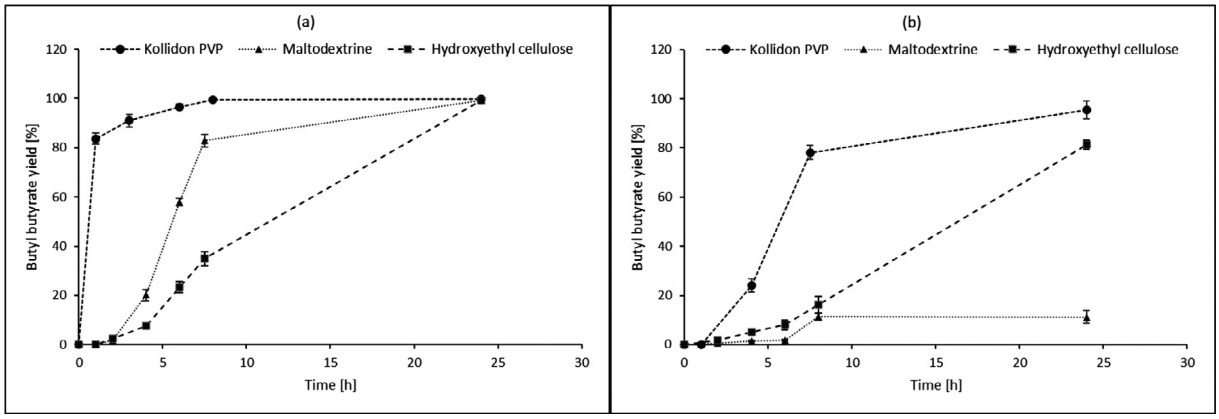

Fig. 7. Reaction profiles of the synthesis of butyl butyrate synthesis catalyzed by TLL physically immobilized on rice husk particles in hexane (a) and toluene (b).

sic activity of the lipase, whereas the partition phenomena appear to predominate.

3.6. Stability of physically immobilized TLL and reusability of rice husk

PVP-TLL and HEC-TLL were tested under hydrolysis conditions and vigorous stirring in order to evaluate the mechanical stability of the formulations but also to monitor the detachment of the enzyme from the RH. The determined hydrolytic activities (TBU) were 4459 Ug^{-1} for HEC-TLL and 6609 U g^{-1} for PVP-TLL. Compared to the previously immobilized TLL onto silica granules, the hydrolytic activities obtained in this study were at least 2 times higher (Ferrer et al., 2002). Microscope images (ESI Figure S6) demonstrate that the integrity of the RH is fully preserved after 10 cycles of reaction under vigorous mechanical stirring. It must be noted, that after 4 years of storage at 4 °C PVP-TLL and HEC-TLL preserved 96% and 82% of hydrolytic activity respectively. No microbial contamination was observed, indicating the long-term stability of the two formulations.

Notably, the most widely employed commercial formulation of TLL is Lipozyme TL IM (Sven et al., 1996), which consists in agglomerates having a size of about 600 μm, made by the enzyme adsorbed on silica particles (< 100 μm) with the aid of a binder. They are mechanically stable for both batch and fixed bed column operation but only in low water media. RH confers to the biocatalyst the additional advantage of

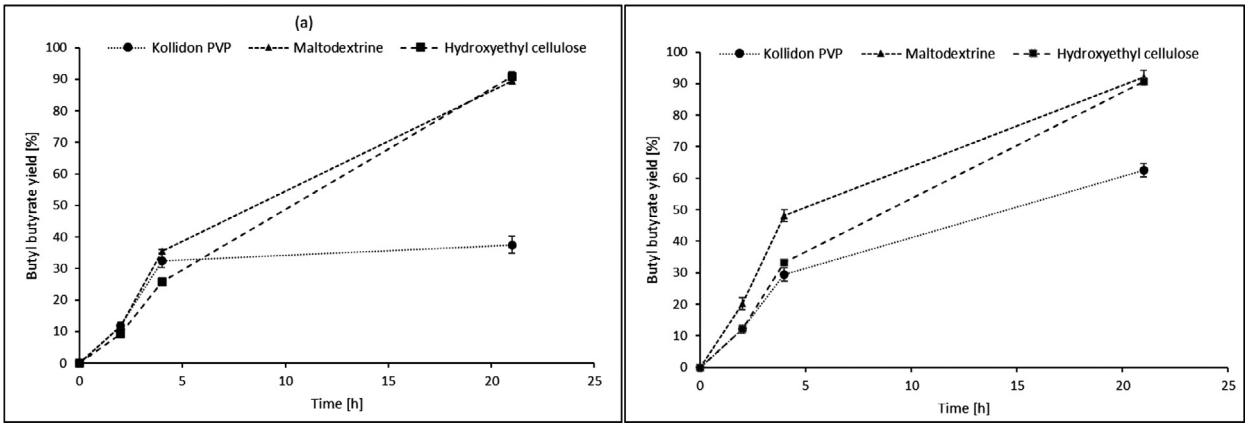

Fig. 8. Synthesis of butyl butyrate catalyzed by the three different formulations of immobilized TLL in isooctane (a) and isooctane with addition of 0.1% (v/v) of H_2O (b).

being made by a single robust fragment of composite material, which does not undergo disaggregation or the formation of fines under physical stress.

Concerning the reusability of RH, data confirm that the enzyme detaches from RH (>90%) after one cycle in aqueous solution (Figure S6) but the carrier remains intact (Figure S7). Therefore, at the end of the biocatalyst life cycle, the RH can be reutilized either as immobilization carrier or for secondary uses (Contreras et al., 2012).

In order to perform a very preliminary analysis of the economic sustainability of new value chains for the valorization of rice, we considered the Italian context. Rice mills sell only a fraction of rice husk for a maximum price of 90€ ton^{-1} (CCIAA, 2021). That rice husk is used by florists, farmers for animal litter and poultry farms. Some of the rice husk is employed by mills also for the internal production of energy and steam required for parboiled rice production. However, the ash content of burnt rice husk is very high (about 20% by weight) compared to other biomass such as poplar (1.0%) and dealing with the large amount of remaining ash is extremely difficult. Therefore, rice husks can be sustainably used as a fuel for energy recovery only when their ash is used as a resource. When burnt at 700 °C, rice husk yields ash contains 97% silica in amorphous form, which has many applications such as in constructional works, in the production of porcelain or as amendant in paddy fields. (Sekifuji and Tateda 2019).

Notably, the costs of commercial organic carriers, are around 50 € kg^{-1} for adsorption immobilization, which means that they have no chemical functionalities on their surface, but they simply interact with the protein by weak non-covalent interactions (Tufvesson et al., 2011). Carriers price reaches 100–300 € kg^{-1} when they are functionalized for covalent immobilization, and these figures make evident that there is potential for new uses of rice husk and that rice mills would find more advantageous to sell larger amounts of rice husk to industries able to transform it in added value products, which, at the end of their life cycle, could be transformed in energy or eventually return to the soil. The latter use would be particularly feasible for biocatalysts applied in the food sector, where nontoxic substrates are processed.

Conclusions

Data here reported demonstrate that RH is a versatile natural composite material that can be used both for the covalent and physical immobilization of lipases. The covalent immobilization was performed by anchoring the protein directly on the functionalized carrier, without the need of spacers or glutaraldehyde, leading to stable formulations of TLL and CaLB, retaining > 70% of activity after 10 cycles of hydrolysis. These biocatalysts, because of their stability and robustness, are applicable in various reaction media and under mechanical stress. The functionalization of RH was achieved by means of laccases in the presence of TEMPO mediator and the laccase was reused for different oxidative cycles. The next optimization steps will be focused on the replacement of TEMPO with renewable mediator molecules.

The inexpensive and easily scalable immobilization of lipase TLL was achieved using a fluid-bed granulator and water-soluble binders. Because of the low toxicity of the materials and the possibility of reusing the RH at the end of the biocatalyst life cycle, this method appears appropriate for industrial applications in low-aqueous media requesting low-toxic, inexpensive and sustainable solutions.

Overall, the methods here presented intend to provide a contribution to the development of a new wave of renewable industrial carriers for enzyme immobilization, able to replace petrol-based materials and to overcome their natural capital cost (Raynaud J., 2014).

Authors contribution

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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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bioeco.2021.100008.

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