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

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Electronic Supplementary Information (ESI)

Method S1. Assay of laccase activity

0.100 mL of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) solution (0.02 M), 0.850 mL of citrate buffer and 0.050 mL of enzyme solution 0.125 mg mL^{-1} were added and the absorbance at 420 nm was monitored for 3 minutes. All the experiments were repeated 5 times. A solution containing 0.100 mL of ABTS, 0.02 M and 0.900 mL of citrate buffer were used as a reference. The laccase activity value (U/mL) was calculated using the following equation:

$$Activity\ {}^{U}/_{mL} = \frac{{}^{\Delta A}/_{min}}{36} \times \frac{V_{tot}}{V_{enz}}$$

Method S2. Recyclability of laccase from *Trametes* sp. in the oxidation of rice husk

The first oxidation cycle was carried out at 70°C in 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 minutes every 8 hours to increase the exposure of the enzyme to oxygen. At the end of the reaction, the recyclability of the laccase was evaluated by removing the oxidized product and then adding fresh RH and TEMPO in the same amount as in the first cycle. The reaction was conducted as described above. Samples were withdrawn and analyzed after 24 and 48h, so that the same laccase solution was employed for a total of 96 h.

Method S3. Determination of Carbonyl Groups Content

The content of carbonyl groups was determined by reaction with hydroxylamine chlorhydrate as previously described by Cespugli et al.[5] To 200 mg of rice husk, 25 mL of 0.25 M solution of hydroxylamine and chlorhydrate was added and the reactions performed for 2h, 25°C. The resulted hydrochloric acid was titrated with sodium hydroxide 0.1 M NaOH to bring the pH to 3.20. After titration the rice husk was dried for 6 h in an oven at 120 °C. To remove any interferences, a blank consisting of a non-oxidized rice husk was treated with the same method, which was previously washed 6 times with a water/ethanol mixture (50:50). Each measurement was performed in triplicate. The content of carbonyl groups was calculated using the following equation:

$$\frac{mmol_{\ aldehyde}}{g_{carrier}} = \frac{(V_{NaOH} \times C_{NaOH})}{m_{dry\ carrier}}$$

where V_{NaOH} is the volume in mL necessary to adjust the pH of the mixture to 3.20, C_{NaOH} is the concentration of NaOH (0.1 mmol mL^{-1}) and $m_{dry\ carrier}$ is the mass in grams of the anhydrous samples.

Method S4. Determination of the Carboxylic Groups

The content of carboxyl groups was determined as previously described by Cespugli et al. (Cespugli et al 2018). About 100 mg of sample was suspended in a beaker containing about 70 mL of 0.01 M HCl. The suspension was magnetically stirred for 30 min and subsequently titrated with 0.1 M NaOH. At the end of the titration, the suspension was filtered, and the sample was dried for 6 h in an oven at 120 °C to determine the exact weight. As reference, a solution consisting of 70 mL of 0.01 M HCl was titrated. The content of carboxylic groups ($mmol_{COOH}/g_{sample}$) was determined with the following formula:

$$Carboxilic\ groups = \frac{C_{NaOH} \times (V_1 - V_2)}{m_{sample}}$$

where C_{NaOH} is the concentration of NaOH (0.1 mmol mL^{-1}), V_1 and V_2 are the volumes (mL) of NaOH necessary to control the weak acid present in the mixture (see ESI) and m_{sample} is the mass (g) of the anhydrous sample. The value of the carboxyl group content was calculated as the average of two measurements, and it is reported with the standard deviation.

Method S5. 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 performed using 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, a timing filter blowing. The process was performed by adapting a procedure previously described by Trastullo et al. (2015).

100 g RH powder was introduced in the internal chamber of the granulator and the powder was fluidized using a constant airstream (0.08-0.13 bar). The binders were dissolved in 50 mL distilled water and mixed with 50 g of native TLL as provided by the supplier. The final solution was introduced in the granulator using a peristaltic pump and sprayed onto the fluidized particles (nebulization pressure of 1.15 bar). The immobilized lipases prepared using Kollidon®25 binder contained a 15% w/w Kollidon®25. When maltodextrin was used as binder only 54% of the final solution was sprayed onto the fluidized particles due to the high adhesion of the material on the bottom of the granulator. The final concentration of the binder was 8% w/w maltodextrin. For hydroxyethyl cellulose, the solution was diluted to 100 mL due to the high viscosity and the final immobilized biocatalyst contained about 3% w/w hydroxyethyl cellulose, so that the loading per g of RH was 50% respect to the other two formulations. During the process, the temperature in the granulation chamber was maintained in the range of 33-44°C. Due to the high adhesion of the material on the lateral walls of the granulation, the spaying was stopped every 3-4 minutes, followed by an intermediate drying step using a warm airstream. The formulated lipases have been dried for 5 minutes using a warm airflow at 40°C.

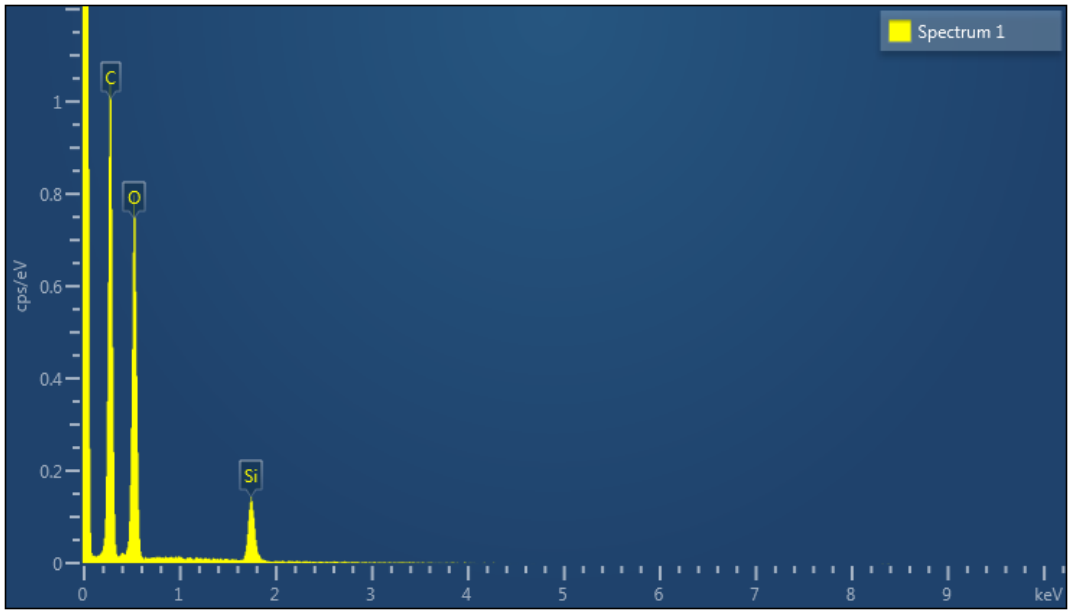

Figure S1. Spectrum of energy dispersive X-ray spectroscopy (EDS) analysis acquired with a beam energy of 5 keV

Method S6. Monitoring of the synthesis of butyl butyrate catalyzed by F-TLL in hexane and toluene.

Butyric acid was determined by HPLC using ODS-Hypersil (C18) column (250 x 4.6 mm, 5 µm, P/N 30105 – 254630) thermostated at 30°C using a Shimadzu system equipped with UV detector and autosampler. The compounds were eluted isocratic, using a mixture of 50% acetonitrile - 50% milli Q water, each component containing 0.05% (v/v) trifluoroacetic acid, with a flow rate of 1 mL/min and the sample injection volume of 20 µL. The eluting components were detected at 220 nm and the run time was 10 min (samples in hexane) or 15 min (samples in toluene). The retention time for butyric acid was RT = 3.44 min.

Method S7. Monitoring of the synthesis of butyl butyrate catalyzed by F-TLL in isooctane: 1-butanol (0.25 mL) was added to butyric acid (0.25 mL) at 1:1 molar ratio together with 9.5 ml of iso-octane. The reaction was initiated by the addition of 130 mg immobilized F-TLL. The reaction mixture was incubated at 45°C and stirred in an orbital shaker (250 rpm) for 21 h and monitored as reported in supplementary materials.

The reaction was monitored by determining the residual acid content by titration of 0.2 mL of sample with NaOH (0.05 N) using phenolphthalein as indicator and 4 mL of EtOH/ethyl acetate (1:1) as quenching agent. The amount of ester was calculated from the equivalents of consumed acid. A calibration curve was constructed by using butyric acid, 1-butanol, and commercial butyl butyrate as standards.

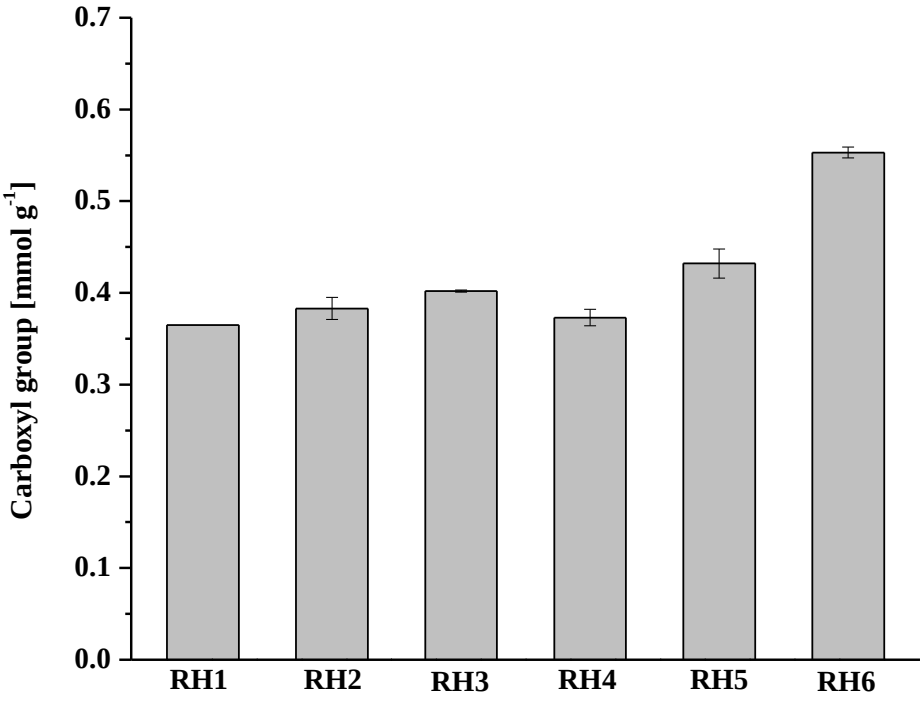

Figure S2. Concentration of carboxyl groups on milled rice husk (RH) upon oxidation in the presence of different laccases and different enzymatic concentrations (Laccase-Mediator

System – LMS). RH1: untreated RH; RH2: laccase Novozyme 51003 8 Uml^{-1} and TEMPO mediator; RH3: Laccase C 8 U ml^{-1} and TEMPO; RH4: Laccase C 40 U/ml NO TEMPO; RH5: Laccase C 40 U ml^{-1} with TEMPO; RH6: chemical oxidation by NaIO4.confirmed that the oxidation of aldehydes to carboxylic groups is not laccase mediated. Data confirm that , since there is no significant influence neither of the enzymatic units employed, nor of the type of laccase. Not even the presence of TEMPO affects the formation of carboxylic groups, and the variation of carboxylic groups, both with chemical and laccase treatments, appears limited.

Method S5. Construction of tri-dimensional models of lipases

The models of the were constructed in their open conformation using the crystal structure of CALB (pdb code 1LBS) and TLL (pdb code: 1DTE). Since the crystal structure of *Rhizopus oryzae* is not available and the model was constructed using as templates the crystal structures of lipases from *Rhizomucor miehei* and *Rhizopus niveus,* (pdb code: 1LGY), which differ by only 2 amino acids (Ferrario et al. 2011) Protein structure visualizations were performed by using VMD software. Lysine residues on the surface are highlighted in red. The catalytic triad is represented in sphere mode and blue colour. The arrow indicates the opening of the active site.

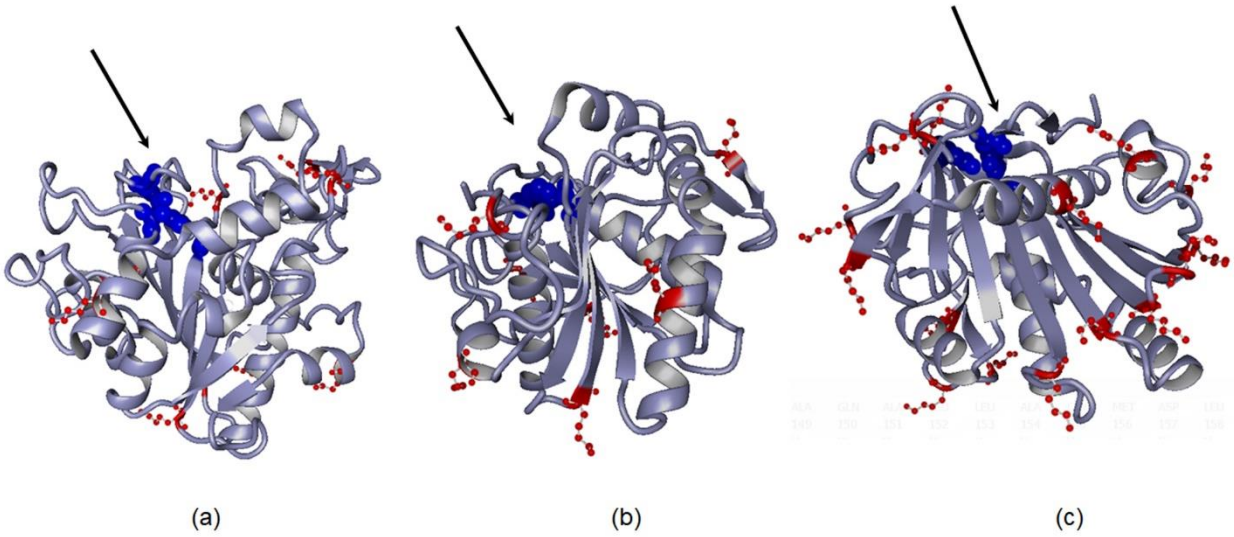

Figure S 3. Tridimensional models of the lipases in open conformations used in the study: a) CALB; b) TLL; c) ROL. Lysine residues on the surface are highlighted in red. The catalytic triad is represented in sphere mode and blue color. The arrow indicates the opening of the active site.

Method S7. HPLC analysis of butyric acid from the synthesis of butyl butyrate catalyzed by F-TLL

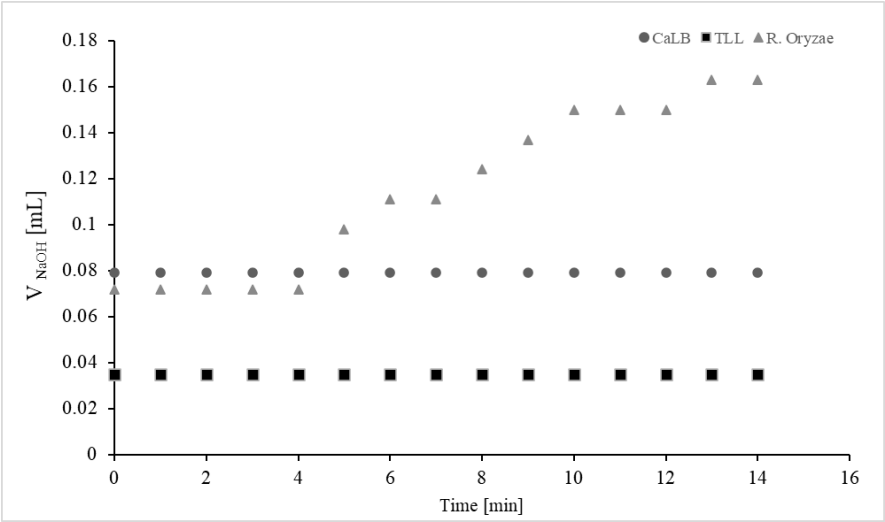

Figure S4. Study of the leaching of the three lipases from the functionalized RH carrier after immobilization. The presence of the native enzyme in the aqueous solution was evaluated by measuring the residual activity present in the aqueous solution after filtration of the immobilized biocatalysts employed for the hydrolysis of tributyrin (see material and methods, assay of hydrolytic activity of lipases). The procedure was repeated for ten cycles.

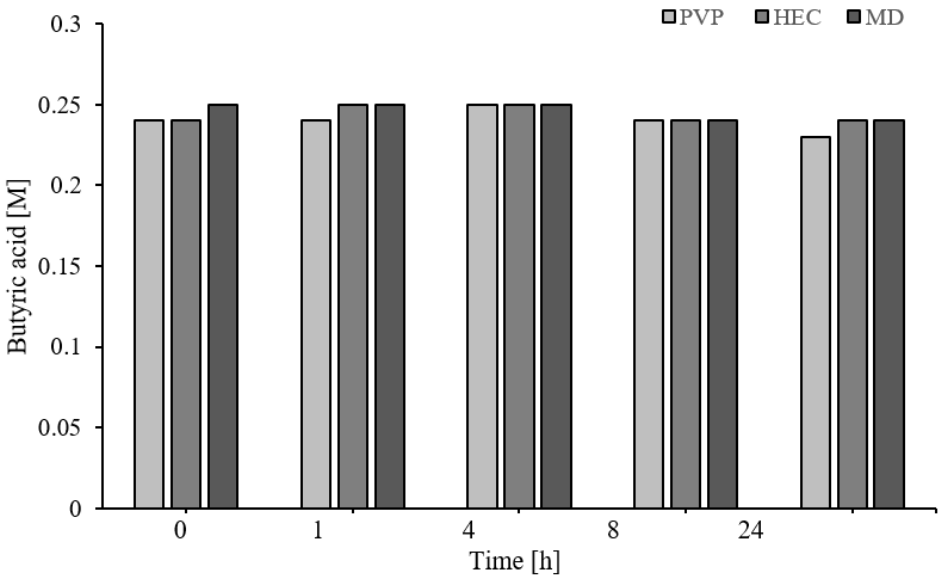

Figure S5. Time course evaluation of non-selective adsorption of butyric acid on RH granules free of lipase. Concentration of butyric acid in control reactions. Reaction conditions: 0.3 M butyric acid, 0.9 M butanol, toluene, 80-85% rice husk-Kollidon matrix (without enzyme), 48°C; Abbreviations: PVP- polyvinylpyrolidone (Kollidon); HEC-hydroxyethylcellulose; MD-maltodextrine

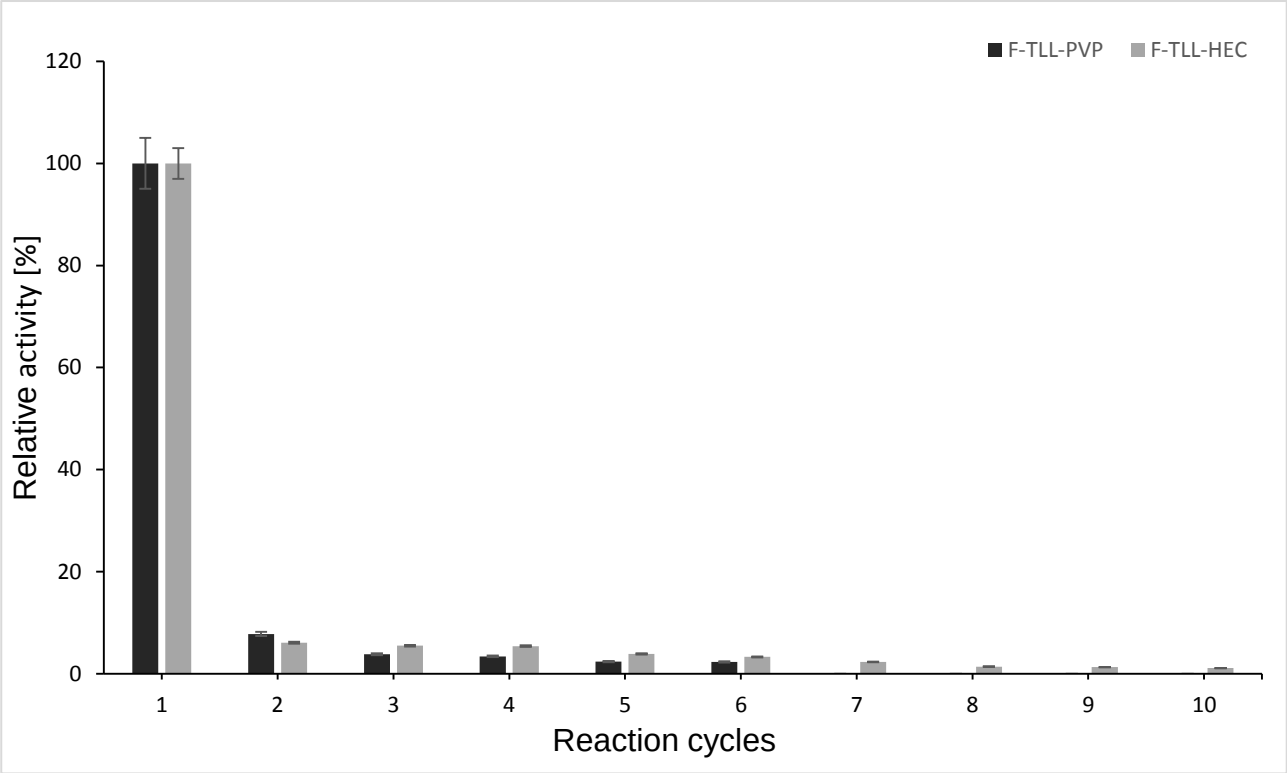

Figure S6. Decrease of residual activity of the two formulations obtained by adsorbing TLL on rice husk after five cycles of tributyrin hydrolysis reaction.

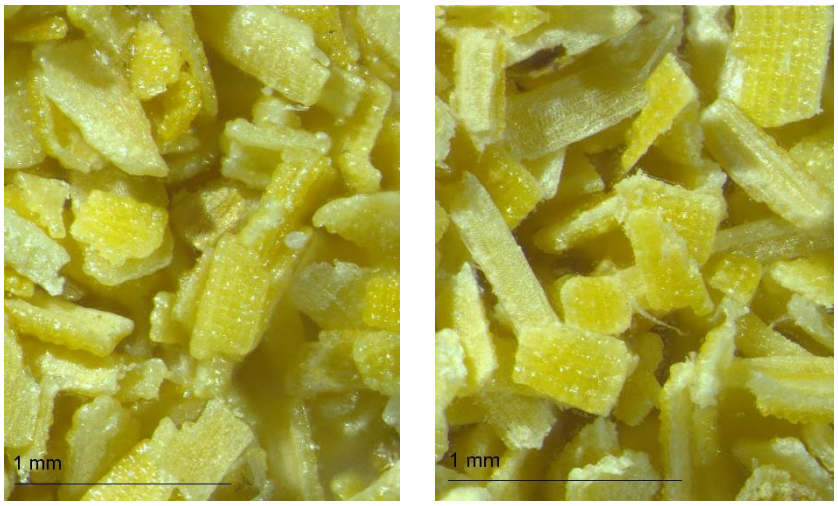

Figure S7 Comparison of the microscope images (60x) of the F-TLL-PVP immobilized lipase before (left) and after (right) 10 reaction cycles of tributyrin hydrolysis.

References

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