Supporting Information for

Candida rugosa lipase bio-conjugation to cellulose nanocrystals with high immobilization efficiency: comparison with nonspecific approach

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Gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 12% polyacrylamide matrix. Before the analysis, protein samples were suspended in a water solution of DTT-SDS (1% m/V). 20 µg sample portions were loaded for each gel well. After the electrophoretic run, protein bands were stained with Coomassie brilliant blue. A calibration curve was prepared plotting the migration of each reference band from the starting well expressed in cm vs log(MW).

The *CRL* batch used in the present work appeared as a single and pure band corresponding to a MW ~65 kDa. [1] The outcome of this test is shown in Figure S1. Wells 1 and 2 referred to two different packages of the same product, which have the same characteristics and were both used for the self-assembly experiments with reproducible results. For these two batches, the band migration distance presents a good agreement with the molecular weight of the protein.

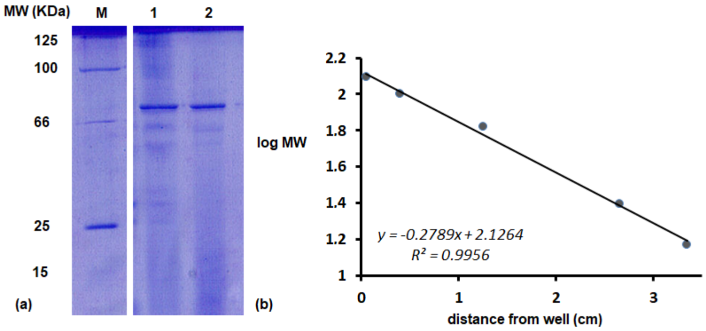


Figure S1. (a) SDS-PAGE electrophoretic run performed on M (reference marker) and the two *CRL* batches (1 and 2); (b) calibration line extrapolated from the reference marker.

Synthesis of *p*NPL

Anhydrous dichloromethane was stored and dispensed in a nitrogen filled glovebox. All other reagents were purchased at the highest commercial quality and used without further purification with the exception of pyridine, that was distilled over molecular sieves (4 Å) under a nitrogen atmosphere and stored and dispensed in a nitrogen filled glovebox. Preparative column chromatography was performed using silica gel 60, particle size 40 ÷ 63 µm from Merck. Merck silica gel 60 F_{254} aluminium sheets were used for TLC analyses. ^{1}H-NMR spectra were recorded at 500 MHz on a Varian spectrometer, using the residual proton peak of $CDCl_3$ at 7.26 ppm as reference. Coupling constants values are given in hertz. Melting points (uncorrected) were determined on a Stuart Scientific Melting point apparatus SMP3.

***p*-nitrophenyl laurate (*p*NPL)** A 10 mL two necked round bottomed flask was charged under a nitrogen flux with 500 mg of *p*-nitrophenol, 3 mL of freshly distilled anhydrous pyridine and 1.25 mL of lauroyl chloride. The mixture was heated at 85°C for 7 hours. After cooling to room temperature, the mixture was poured in a glass becher filled with ice and 20 mL of HCl 3N. After that, the mixture was transferred to a 100 mL extraction funnel and extracted with ethyl acetate (3x25 mL). The organic extracts were collected and washed with 20 mL of a saturated solution of $NaHCO_3$ and with brine (2x20 mL). Then the organic extracts were dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel, using 20 g of silica gel (0.04- 0.063 mm) and eluting with hexane:ethyl acetate in volumetric ratio 95:5. 866 mg of *p*-nitrophenyl laurate (white solid) were isolated. Mp 46.5-47 °C (Lit.[2] 44-45 °C). ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.25 (d, J= 9.0 Hz, 2H), 7.27 (d, J= 9.0 Hz, 2H), 1.75 (quintuplet, J = 7.5 Hz, 2H), 1.62 (quintuplet, J = 7.5 Hz, 2H), 1.41 (quintuplet, J = 7.5 Hz, 2H), 1.20-1.36 (m, 12 H), 0.87 (t, J = 6.7 Hz, 3H) ppm.

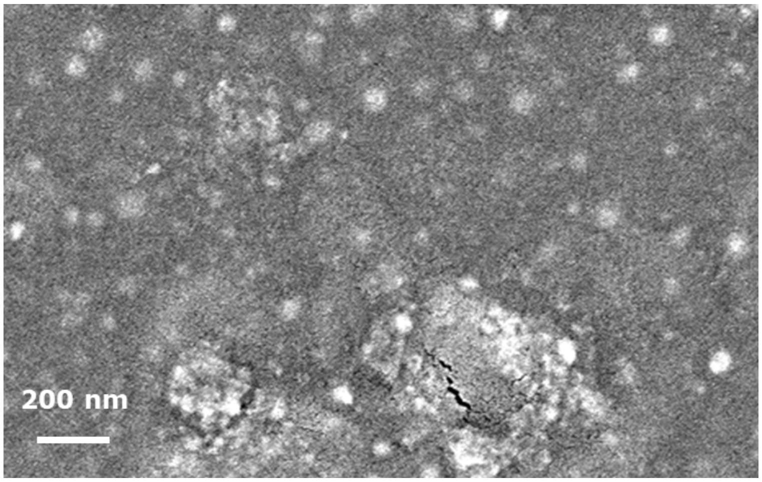


Figure S2. SEM micrograph of pristine lipase from *Candida rugosa* deposited from water.

ATR-FTIR spectra enlargements

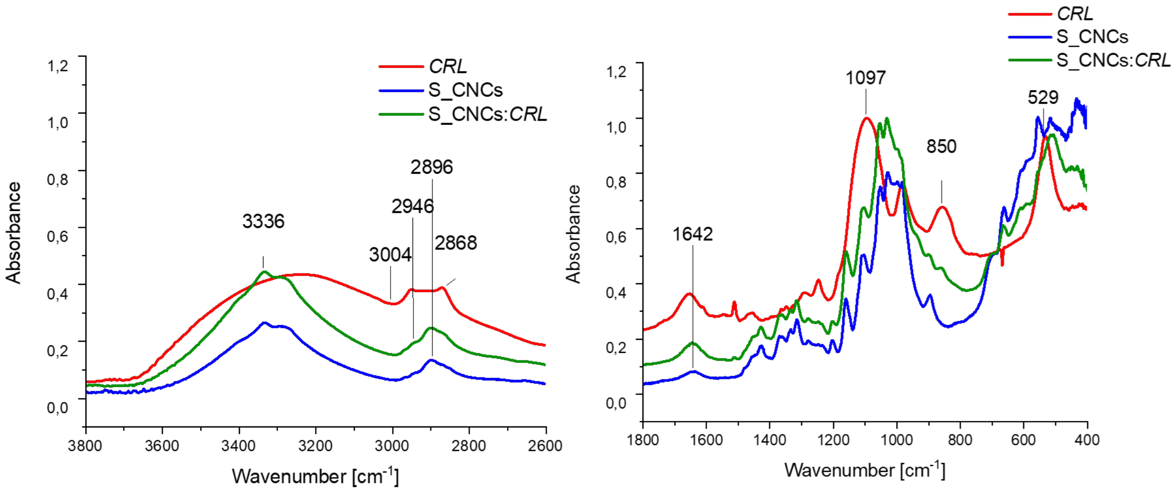


Figure S3. ATR-FT-IR spectra recorded in the region 3800-2500 cm^{-1} (left panel) and in the region 2000-400 cm^{-1} (right panel) for pure lipase (red line), dried S_CNCs (blue line) and immobilized biocatalyst on S_CNCs (green line).

Lipase activity at pH 7.0

Figure S4 shows the kinetics of lipase in pH 7.0 buffer. Lipase takes about 50 seconds to become active, and the calculated activity was 26 enzyme units per mg of lyophilized protein. The theoretical value (declared by the seller) is ~20 enzymatic units (measured at 37°C).

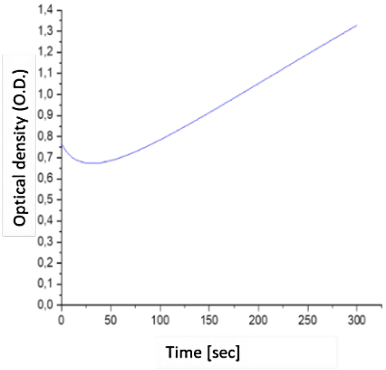
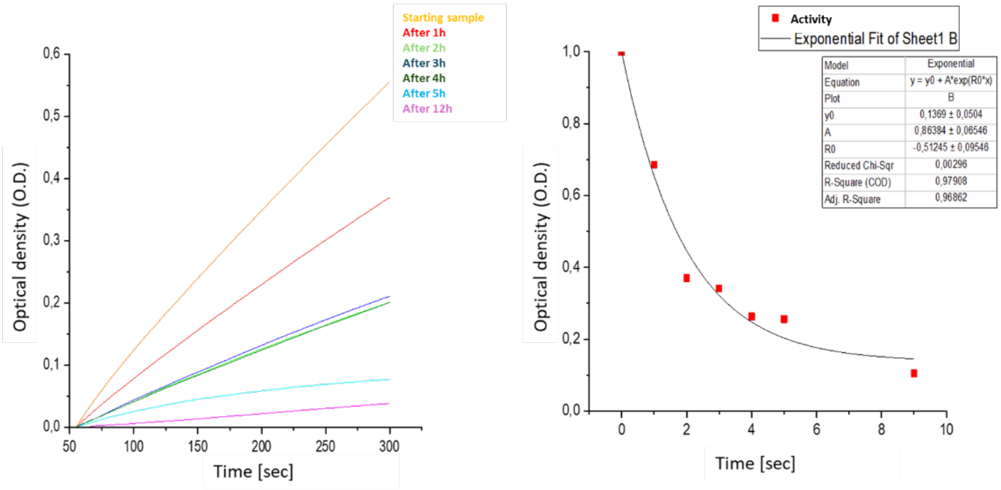


Figure S4. Recorded kinetic of lipase in 100 mM phosphate buffer at pH 7.0.

Thermal stability of lipase at pH 7.0

To evaluate the thermal stability of *CRL*, a 1 mg/mL solution of it in pH 7.0 buffer was subjected to aging at 65°C for a time ranging from 0 to 10 hours. The activity of *CRL* was measured immediately after the aging process, producing the results shown in Figure 6(a). The obtained activity was related to the activity of the unaged lipase, obtaining the graph presented in Figure 6(b). Thanks to these experiments, it was noticed that lipase activity decreases exponentially by increasing the aging time at 65°C. It, therefore more than halves its activity after just 2 hours of treatment at 65°C.



Model	Exponential
Equation	y = y0 + A*exp(R0*x)
Plot	B
y0	0,1369 ± 0,0504
A	0,86384 ± 0,06546
R0	-0,51245 ± 0,09546
Reduced Chi-Sqr	0,00296
R-Square (COD)	0,97908
Adj. R-Square	0,96862

Figure S5. Kinetics recorded on lipase 1 mg/mL solutions aged at 65°C (a) and decrease of lipase activity with aging at 65 °C (b).

pH stability of lipase

To evaluate the stability at various pHs of *CRL*, we prepared a 1 mg/10 mL solution of it in different phosphate buffer systems at pH 2, 4, 6, 8, 10. The activity was measured after aging the solution for 3 hours at room temperature. The results are shown in Figure 7(a). The activity obtained was related to the activity of the unaged lipase, resulting in the graph presented in Figure 7(b). Thanks to these experiments, it was noticed that the activity of lipase decreases at pH higher than 8 and lower than 4 while we can see that its activity is maximum at pH 8, while between pH 4 and 8 it is maintained at an acceptable level (higher 80%). *CRL* is a robust and stable enzyme. Therefore, we decided to carry out the nonspecific immobilization experiments at pH 7.0, value at which a higher fraction of lysins is found in their protonated form.

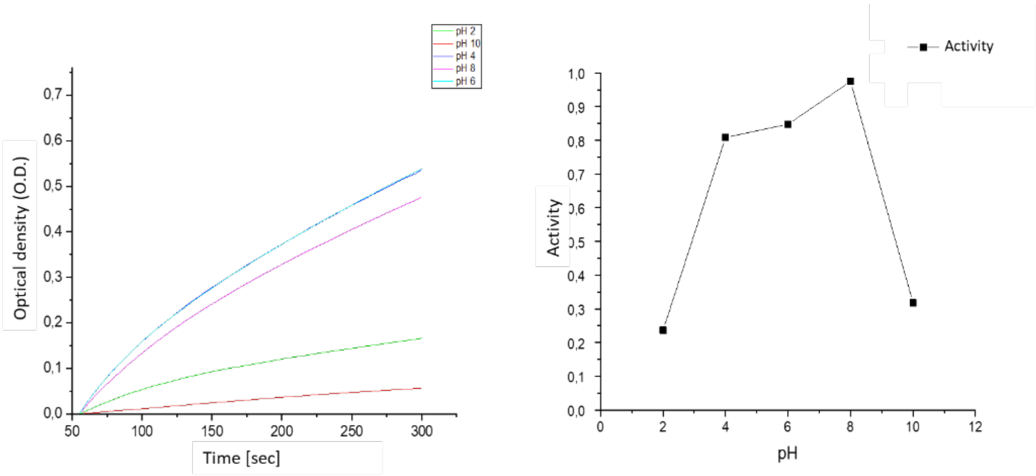


Figure S6. Kinetics recorded on lipase 1 mg/10 mL solutions aged at different pHs for 3 hours (a) and decrease of lipase activity with aging at different pH for 3 hours (b).

Calibration curve obtained from BCA test

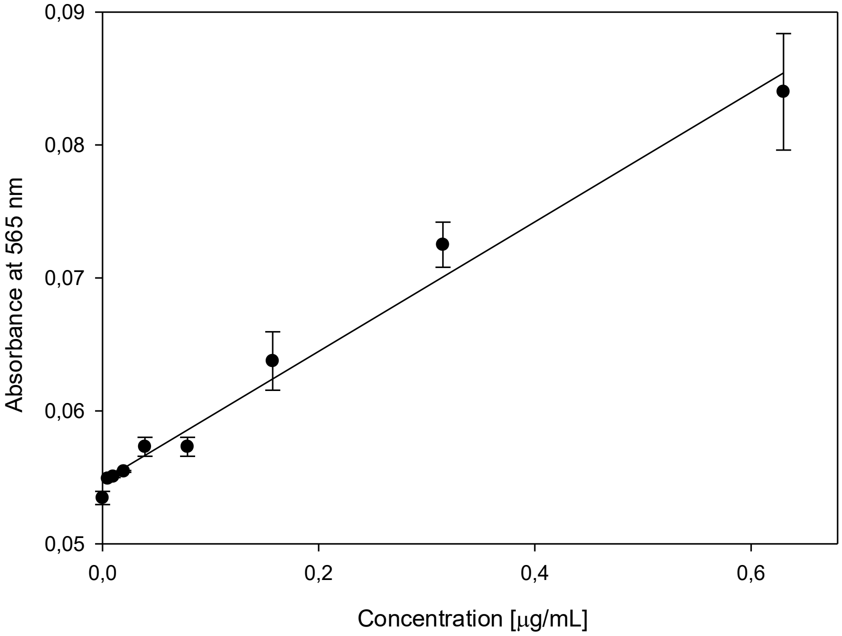


Figure S7. Calibration curve for lipase obtained by BCA method recording the absorbance at 565 nm and used for the quantification of lipase immobilized on cellulose nanocrystals.

Conductimetric Titration Curve for TO_CNCs

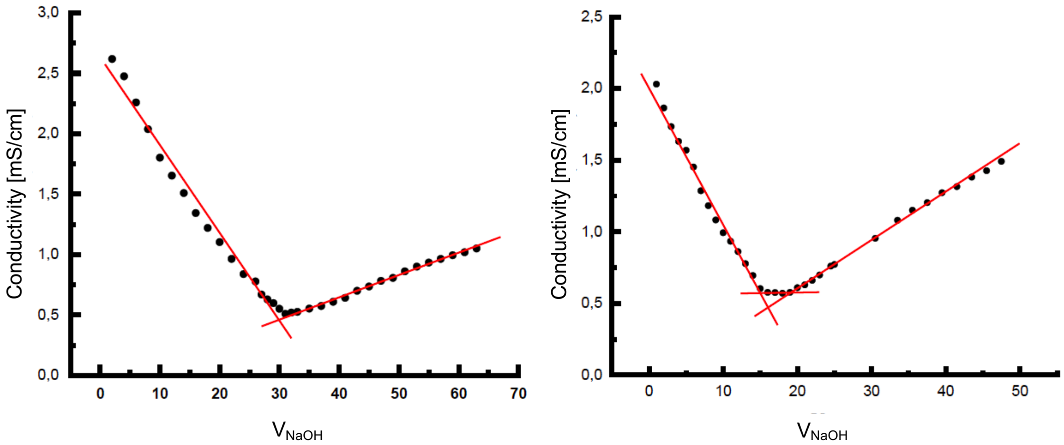


Figure S8. Titration curves obtained for N_CNCs (left) and TO_CNCs (right). Volume expressed in mL. The quasi-constant section in the right plot represents the neutralization of weak acidic carboxyl groups.

Bibliography

1. Chang, S.-W. , Lee, G.-C., Shaw, J.-F., Efficient production of active recombinant Candida rugosa LIP3 lipase in Pichia pastoris and biochemical characterization of the purified enzyme. *J. Agr. Food Chem.*, **2006**, *54* ,5831-8.
2. O'Neil, M. J. The Merck Index – An Encyclopedia of Chemicals, Drugs, and Biologicals, Whitehouse Station, NJ: Merck & Co., Inc, **2006**.