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Immobilization of *Candida rugosa* lipase on eco-friendly supports by spouted-bed technology: Use in the synthesis of isoamyl caprylate

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Abstract

Candida rugosa lipase (LCR) was immobilized on low-cost supports (by-products) and dried using a spouted-bed system. The yields of immobilized derivatives were in the range 61.5–78.7%. Lipase immobilized on rice husk showed the best results, presenting 94.1% of the original activity, followed by sugarcane bagasse (90.3%) and green coconut fiber (87.3%). Moisture content in the obtained powders varied between 4.7 and 5.6% and the water activities were in the range 0.21–0.35. Among all the tested biocatalysts for aroma production the lipase immobilized on rice husk showed the highest activity towards the formation of isoamyl caprylate (62.40 g.L^{-1}).

Keywords: *Spouted bed dryer; Enzyme dehydration; Enzyme immobilization; Enzyme stabilization; Aroma production.*

1. Introduction

Lipases (triacylglycerol acylhydrolase - EC 3.1.1.3) are serine hydrolases formerly characterized by the ability to reacting with a wide range of substrate with a high enantio and regio selectivity.[1] However, the commercialization of enzymes with potential industrial applications, including lipases, depends on their stability during enzymatic reaction and/or storage period.[2] In fact, the water presence in enzyme formulations is the mainly drawbacks for protein stabilization and consequently, for enzyme application.[3] Drying technologies can be utilized to obtain dehydrated and stable thermosensitive products like microbial enzymes.[4] On the other hand, enzyme immobilization are other mechanisms used to improve enzymes properties (like stability, activity, inhibition by reaction products and selectivity toward non-natural substrates), and allows recovery and reuse of the biocatalyst.[2] In this work, we associated the benefits of drying and immobilizing processes and performed them in a single step employing a spouted bed system. Furthermore, we used eco-friendly supports like coconut fibers, rice husk and sugarcane bagasse to prevent emergence of environment ethical issues and cut down the production costs of immobilization processes. Additional information on the catalytic activity was obtained by testing the immobilized derivatives obtained in synthetic applications, that is, in the esterification reaction of isoamyl alcohol with caprylic acid.

2. Materials and Methods

2.1 Enzymatic activity of the free and immobilized lipase

Candida rugosa lipase from Sigma-Aldrich was used in the immobilization assays. Lipase activity assay was performed using ρ-nitrophenyl palmitate (p-NPP) as substrate according to Mayordomo et al. (2000).[5] The mixture was incubated at 40°C for 30 min and then 0.5 mL of 2% trizma base was added.[6] The optical density was measured at 410 nm. Enzymatic activity is given as μmol of *p*-NPP produced per minute per mg of enzyme (IU).

2.2 Lipase Immobilization on agricultural byproducts

"In natura" Agricultural byproducts supplied by local farmers, were oven dried, ground and sieved to obtain particles with sizes between 50 and 150 mesh - Figura 1.

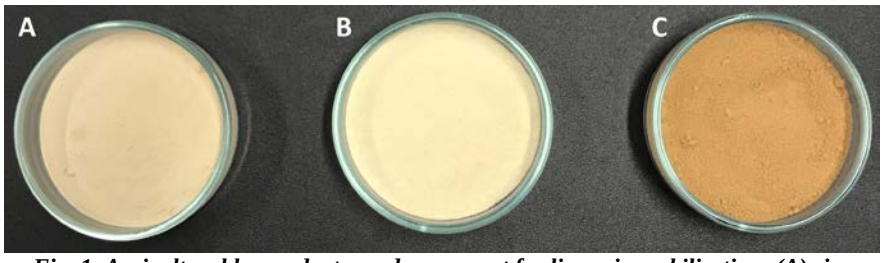

Fig. 1. Agricultural by-products used as support for lipase immobilization: (A) rice husk, (B) sugarcane bagasse, (C) green coconut fiber.

The supports were activated using glutaraldehyde prior to the covalent immobilization method. *Candida rugosa* lipase was immobilized in the by-products previously activated by covalent binding in the presence of polyethylene glycol as a stabilizing agent.[8]

2.3 Spouted Bed Drying of Immobilized Lipase

Immobilized *C. rugosa* lipase was dried using a homemade conical–cylindrical spouted bed, with an internal angle of the conical base of 40° and inlet orifice diameter of 15 mm. A cylindrical column with a diameter of 85mm and height of 300mm was connected to the conical base. Concave cylindrical Teflon particles with a mean diameter of 5.45mm and density of 2,160 kg/m^3 were used as inert material. Table 1 shows the operating parameters of the spouted bed dryer.

Table 1. Spouted bed parameters set for drying of enzyme-support system.

Spouted bed drying parameters	
Inlet gas temperature (T_{gi}), °C	100.0
Drying gas flow rate (Q), m^3/min	0.660
Feed system position	top spray
Mass feed flow rate (Ws), g/min	5.5
Static bed height (H_0), cm	5.5
Mass of inert material (M_i), g	255.0

2.4 Dryer Performance and Product Properties

The spouted bed drying performance and physicochemical product properties were assessed by the following assays: **A. Enzymatic activity:** The lipase activity assay was performed using *p*-NPP as the substrate according to Mayordomo et al.[5] using an immobilized derivative (10 mg) in 50mM of phosphate buffer, pH 6.5. **B. Product stability:** The stability of the immobilized derivatives was assessed by determination of the enzyme activity of dried powder during 3 months of storage at 5 °C. **C. Reuse cycles:** Residual enzymatic activity determination for immobilized lipase after each batch of reaction was determined. The immobilized derivative was recovered by centrifugation and washed with buffer (sodium phosphate buffer 50mM, pH 6.5) for the next reaction test. **D. Product moisture content (Xp):** The moisture content of the spouted bed–dried product was determined by the oven drying method at 105 °C up to a constant weight and was calculated from triplicate analyses. **E. Water activity (aw):** Water activity was determined using an AQUALAB 4TEV-Decagon according to the method of Norenã et al.[9] **F. Efficiency of powder production:** The product recovery (R_E) was defined as the ratio between the total mass of the product recovered to the mass of immoblilized enzyme composition fed to the system (dry basis).

2.5 Synthesis of Isoamyl Caprylate using the immobilized *Candida rugosa* lipase

Reaction systems consisted of isooctane (20 mL), isoamyl alcohol (0.30 mol/L), caprylic acid (0.30 mol/L) and immobilized lipase derivatives (30 units activity/mL of substrate). The mixture was incubated at 40 °C for 48 h with continuous shaking at 150 rpm. The consumed isoamyl alcohol and the formed product were determined by gas chromatography using a 5% DEGS CHR-WHP 80-100 mesh 6 ft 2.0 mm ID and 1/8" OD column (Restek, Frankel Commerce of Analytic Instruments Ltd, SP, Brazil) and octanol as an internal standard.[10] Caprylic acid concentrations were titrated with 0.02 mol.L^{-1} potassium hydroxide solution using phenolphthalein as an indicator. The alcohol molar conversion (X, %), the productivity (P, g.L^{-1} isoamyl caprylate h^{-1}) and initial reaction rates (A, μM isoamyl caprylate $min^{-1}.g^{-1}$ catalyst) were calculated based on Perez et al. (2007).[11]

3. Results and Discussion

Table 2 shows the effect of different supports and glutaraldehyde concentration on outlet drying gas temperature (T_{go}), temperature inside the spouted bed dryer (T_{in}), process yield (R_E), and residual enzyme activity (R_{EA}) of the product.

Table 2. Effect of support and glutaraldehyde concentration on outlet drying gas temperature, bed temperature, process yield and residual lipase activity.

Support	GLU (%)	T_{go} (°C)	T_{in} (°C)	R_E (%)	R_{EA} (%)
Rice husk	0.5	81.5±0.6	59.1±0.6	61.5	48.5±0.7
	1.5	78.1±0.4	58.5±1.1	76.2	94.1±0.4
	2.5	79.9±1.2	60.1±0.2	73.0	71.9±1.2
Sugarcane bagasse	0.5	80.8±0.5	59.4±1.3	66.4	53.6±1.1
	1.5	79.3±0.9	58.1±0.9	78.7	90.3±1.4
	2.5	81.8±0.3	60.8±0.2	73.5	62.0±0.9
Green coconut fiber	0.5	82.1±0.5	61.4±1.3	65.1	44.9±1.3
	1.5	79.3±0.9	59.1±0.9	74.6	87.3±0.9
	2.5	81.8±0.3	60.8±0.2	70.8	51.0±1.2

The residual enzymatic activity of all drying formulations used in this study was in the range 44.9-94.1%. Among all preparations evaluated, those containing 1.5% of glutaraldehyde showed the best result because they exhibited the highest retention of enzyme activity after spouted bed drying in all assays. *Candida rugoda* lipase immobilized in rice husk actived with 1.5% of glutaraldehyde showed the best result maintaining 94.1 % of intitial enzyme activity. The inlet gas temperature used during the drying process was 100 °C, a temperature

that theoretically could provoke enzyme denaturation. However, the spouted bed dryer mechanism and the cooling effect caused by water evaporation prevents the loss of enzymatic activity. Indeed, the average temperature inside the spouted bed (Tin) was 59.7 °C, a condition more appropriate for termosensitive biomaterials comparatively to the inlet temperature applied. The immobilized devivatives recovery rate was compatible with the expected production when a home-made spouted bed is used. Efficiency of powder productions were in the range 61.5–78.7%, depending on the support used. Table 2 shows the moisture content, water activity and residual lipase activity after storage period and five reuse cycles for the lipase immobilized on byproducts actived with 1.5 % of glutaraldehyde. The moisture content in the obtained powders ranged from 2.3 to 5.7% while the water activities of the immobilized derivatives were in the range 0.18–0.33. These values are considered safe to avoid microorganisms growth.[12] Stability tests were performed for all spouted bed dried samples which were stored at 5°C for up to 3 months. The immobilized derivatives obtained had decreased enzyme activity with an average of only 12.4%, whereas the free enzyme form lost 51.7% of its initial activity in the same period. It can be observed that the biocatalysts prepared retained an average of 67.3% of the initial activity after five reuse cycles. Lipase immobilized in rice husk showed the best result maintaining 71.4% of initial enzyme activity. The enzyme activity retention after reuse cycles is very important parameter related to the feasibility of applying on industrial scale.

Table 2. Moisture content, water activity and residual lipase activity after storage period and five reuse cycles for the lipase immobilized on byproducts actived with 1.5 % of glutaraldehyde.

Support	Xp (%, d.b.)	A_w (-)	Storage R_{EA} (%)	Reuse R_{EA} (%)
Sugarcane bagasse	5.0 ± 0.93	0.25 ± 0.09	88.5 ± 1.13	68.6 ± 1.12
Green coconut fiber	5.6 ± 1.20	0.35 ± 0.05	82.4 ± 0.79	62.1 ± 0.93
Rice husk	4.7 ± 0.85	0.21 ± 0.03	91.7 ± 0.58	71.4± 1.03

To verify the behavior of the biocatalysts in non-aqueous media, additional information on the catalytic activity was obtained by testing the immobilized derivatives in synthetic applications, that is, in the esterification reaction of isoamyl alcohol with caprylic acid. This reaction has been suscesfully used to screen the best source of lipase to mediate the synthesis of aroma ester using fusel oil and caprylic acid, being the *Candida rugosa* selected as the most suitable lipase. The results for the tested immobilized lipases are shown regarding consumption of the starting materials and ester formation as a function of time (Figure 2), being the results summarized on Table 3. For all tested lipase immobilized derivatives, the reaction was driven towards to completion (molar conversion higher than 80%) at 48 h. For all runs, no reverse reaction was observed and both starting material were simultaneously consumed with the correspondent ester formation. Under these conditions, productivities

(1.12 - 1.30 g.L^{-1} isoamyl caprylate h^{-1}) were similar for all immobilized derivatives though different initial reaction rates were found (192 to 617 μM min^{-1}.g^{-1}) depending on the support used to immobilize the *Candida rugosa* lipase (LCR). The highest intial rate was found for the LCR immobilized on rice husk and the lowest for LCR immobilized on green coconut fiber.

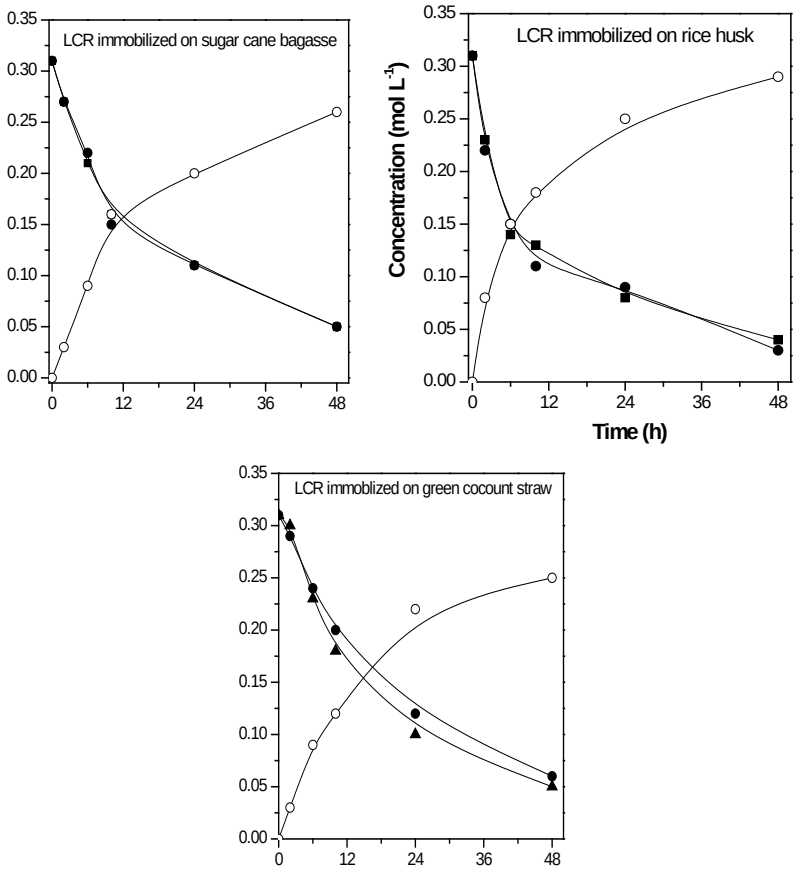

Fig. 2. Performance of Candida rugosa lipase immobilized on different matrixes in the synthesis of isoamyl caprilate (○) from isoamyl alcohol (●) and caprylic acid (▲) at equimolar ratio (40°C, 150 rpm, 0.30 mol.L^{-1} of each starting material in the presence of isooctane as solvent).

Among all the tested biocatalysts the LCR immobilized on rice rusk showed the highest activity towards the formation of isoamyl caprylate (62.40 g.L^{-1}). This is an expected behavior compared with published data, since rice husk is considered among several matrixes derived from lignocellulosic materials the most suitable support for immobilizing lipases due to its high silica contents.[8]

Table 3. Values for initial reaction rate, productivity (P) and molar conversion in the synthesis of isoamyl caprylate by C. rugosa lipase immobilized on different matrixes at 40 °C.

Immobilized Lipase	Reaction rate ($\mu M.min^{-1}g^{-1}$)	P[a] ($g\ L^{-1}\ h^{-1}$)	Molar conversion[a] (%)
Sugar cane bagasse	263	1.16	83.87 ± 0.56
Rice husk	617	1.30	88.71 ± 2.20
Green coconut straw	192	1.12	82.25 ± 2.28

[a] Calculated at 48 h reaction

4. Conclusions

The high values of enzyme activity retention and low water content of immobilized derivatives obtained showed that the use of agricultural by-products (eco-friendly supports) combined with the spouted-bed system is a promising technology to be applied for immobilization and stabilization of enzymes of commercial appeal. The tested immobilized lipases showed potential to catalyze the esterification reactions although at different rates. The highest performance was attained by the LCR immobilized on rice husk.

5. Nomenclature

A_w	Water activity	(-)
H_0	Static bed height	(cm)
M_i,	Mass of inert material	(g)
P	Productivity	($g\ L^{-1}\ h^{-1}$)
RE	Process yield	(%)
R_{EA}	Residual enzyme activity	(%)
Tgi	Inlet gas temperature	(°C)
Tgo	Outlet gas temperature	(°C)
Tin	Temperature inside the spouted bed	(°C)
Q	Volumetric flowrate of the spouting gas	(m^3/min)
Ws	Enzyme composition feed flowrate	(g/min)
Xp	Product moisture content	(%, db)
GLU	Glutaraldehyde	-

6. Acknowledgements

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