

Innovative Carrier Materials for Advancing Enzyme Immobilization in Industrial Biocatalysis.

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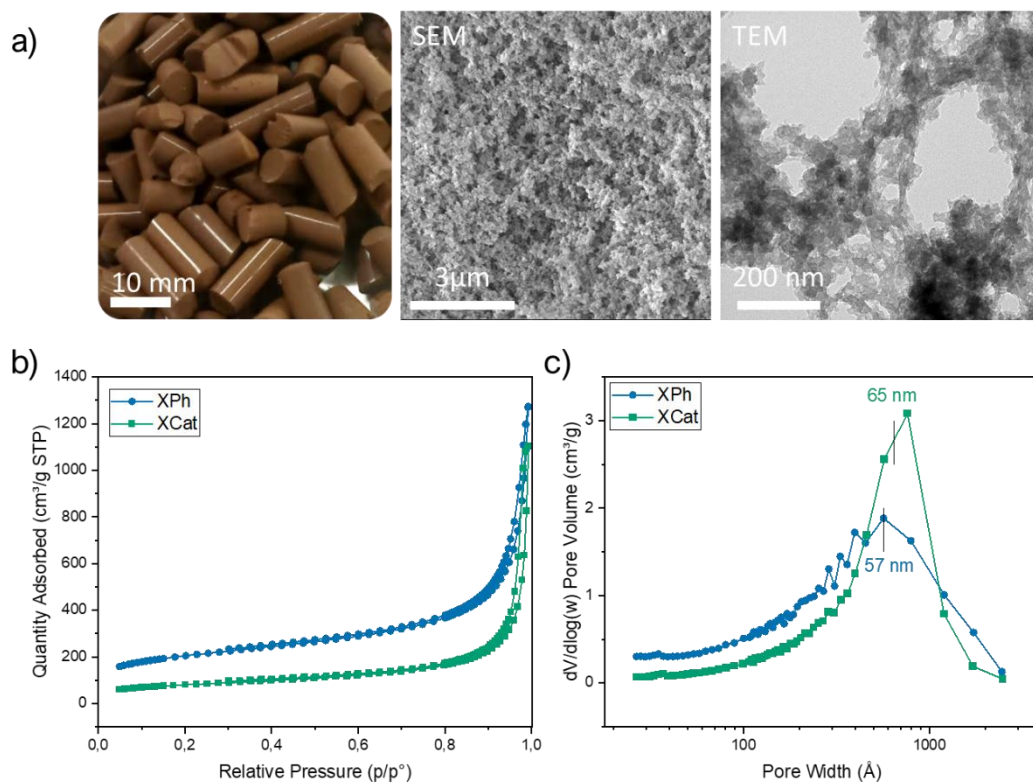


Figure S1-a) XPh CarboZym support pictures at macroscopic & microscopic (SEM & TEM) level; **b)** Adsorption-desorption isotherms of nitrogen on XPh & XCat materials; **c)** BJH pore distribution from XPh & XCat materials

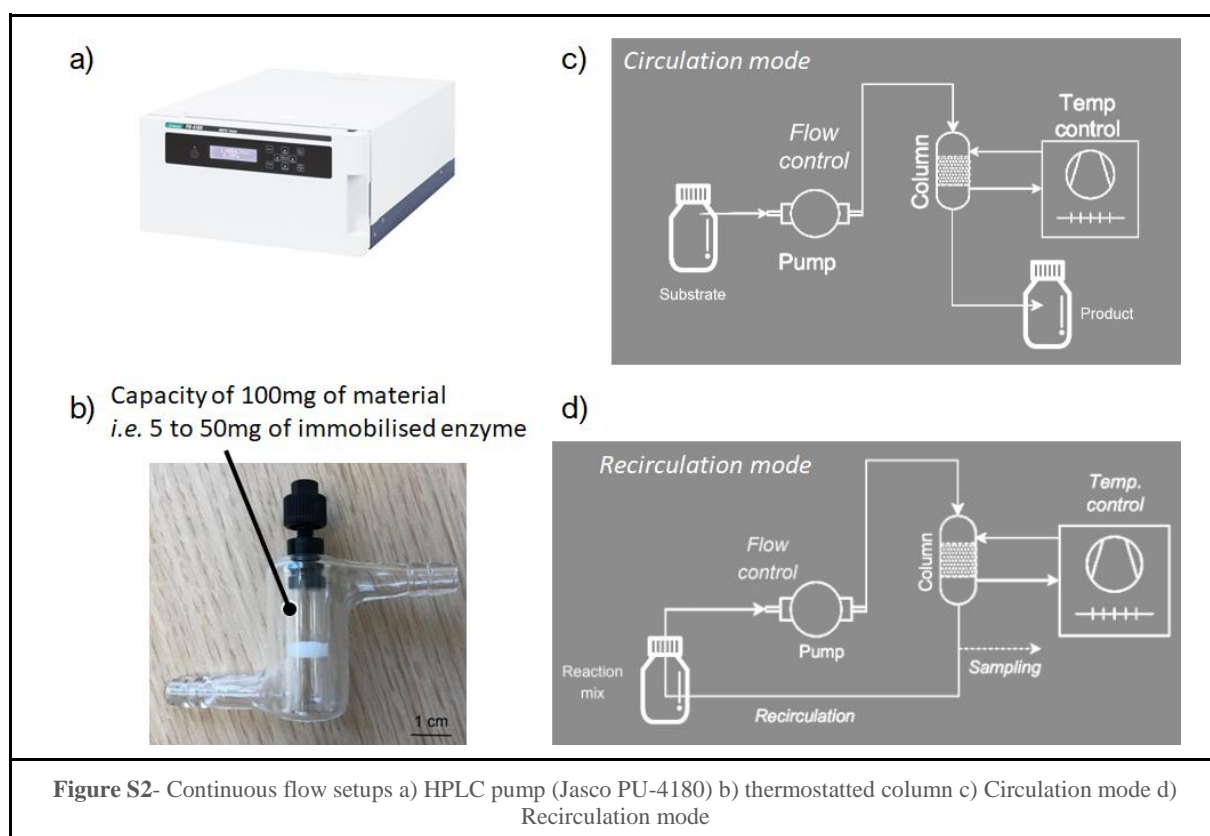


Figure S2- Continuous flow setups **a)** HPLC pump (Jasco PU-4180) **b)** thermostatted column **c)** Circulation mode **d)** Recirculation mode

Experimental section

Cryogel preparation:

Gels were synthesized using xylose and phloroglucinol or catechin according to protocol described in patents WO2024153593A1 & WO2024153594A1.

Case studies:

CalB experimentation:

Enzyme Immobilization

25 mg of cryogel were weighed and added to 1 mL of a Tris buffer solution (20 mM, pH 7) containing 2.5 mg of desalted commercial CalB enzyme. The suspension was stirred at 800 rpm for 2 hours at 4°C. Samples were taken at 10, 20, 30, 60, 90, and 120 minutes for kinetic monitoring. After each sampling, the suspension was centrifuged at 4000–6000 rpm for 2–5 minutes. After the final sample, the supernatant was discarded, and the solid was washed twice with 1 mL of Tris buffer, with each wash involving stirring at 4°C for 3 minutes followed by centrifugation.

Enzyme Quantification by Residual Activity

The enzyme content in each supernatant sample was quantified through residual activity assays. For each sample, 20 µL of the supernatant was added to a reaction mixture containing 1.5 mM p-nitrophenyl butyrate (pNPB), 1% (wt) Genapol, and 20 mM Tris-HCl buffer (pH 7). The mixture was stirred for 5 minutes at 25°C, and the formation of p-nitrophenol was monitored at 410 nm. The resulting absorbance values were compared to a standard curve to determine the protein concentration in the supernatant, which was then used to calculate the amount of protein leached from the cryogel during immobilization.

Activity of Immobilized Enzyme

The hydrolytic activity of the immobilized CalB was assessed by measuring the conversion of pNPB to p-nitrophenol. The reaction was carried out in a 1 mL solution containing 15 mM pNPB in 20 mM Tris-HCl (pH 7) with 1% (wt) Genapol. The reaction was initiated by adding 25 mg of the enzyme-loaded cryogel (10% wt enzyme). The mixture was stirred at 800 rpm for 1 hour at 25°C. Enzyme activity was determined over five reaction cycles, with each cycle involving the same procedure followed by centrifugation, washing with buffer, and re-initiation of the reaction.

Continuous Use of CalB Enzyme

In continuous operation (see figure S2 for continuous flow set-up), approximately 65 mg of cryogel, pre-loaded with immobilized CalB, was placed in a thermostatted glass column maintained at 4°C using a cryostat. The enzyme solution, prepared in Tris buffer (20 mM, pH 7), was percolated through the column at a flow rate of 0.1 mL/min using an HPLC pump. Following enzyme immobilization, the column was flushed with 5 mL of Tris buffer at the same flow rate to remove unbound proteins.

Enzymatic Activity in Continuous Flow

The continuous enzymatic reaction was performed by circulating a reaction mixture containing 5 mM p-nitrophenyl butyrate (pNPB) in Tris-HCl buffer (20 mM, pH 7) with 1% (wt) Genapol through the column. In recirculation mode, 13.3 mL of the reaction mixture was cycled through the column at 0.5 mL/min, with 100 µL samples taken hourly. The formation of p-nitrophenol was monitored at 410 nm,

and the enzyme activity was calculated based on the quantity of p-nitrophenol produced, normalized to the mass of immobilized enzyme.

GsOYE experimentation:

Enzyme Immobilization (Batch Mode)

In batch mode, 10 mg of cryogel was weighed and added to a 1 mL solution of Tris-HCl buffer (50 mM NaCl, pH 7.5) containing 1 mg of GsOYE enzyme, resulting in a 10% enzyme loading on the cryogel. The suspension was stirred at 800 rpm for 2 hours at 4°C to facilitate enzyme immobilization. Samples were taken at intervals (10, 20, 30, 60, 90, and 120 minutes), followed by centrifugation at 4000–6000 rpm for 2 to 5 minutes to separate the solid cryogel from the supernatant. After the final sampling, the supernatant was discarded, and the solid cryogel was washed twice with the buffer solution to remove unbound enzyme. Each wash involved stirring the suspension for 3 minutes at 4°C, followed by centrifugation.

Enzyme Quantification by Bradford Assay

The amount of enzyme immobilized on the cryogel was determined using the Bradford protein assay. A standard curve was prepared using BSA (0.1 g/L) in the same Tris-HCl buffer (50 mM NaCl, pH 7.5). Bradford reagent was added to each sample, including the immobilized enzyme supernatant samples, and incubated for 15 minutes in the dark. The absorbance was measured at 595 nm. The protein content in the samples was calculated by comparison with the standard curve, and the amount of enzyme immobilized was determined based on the amount of protein remaining in the supernatant after immobilization.

Enzyme Activity of Immobilized GsOYE

The catalytic activity of immobilized GsOYE was measured by following the reduction of cyclohexenone to cyclohexanone. The reaction mixture contained 50 mM cyclohexenone, 75 mM NADH, and 2% DMSO in Tris-HCl buffer (50 mM NaCl, pH 7.5). The reaction was initiated by adding 10 mg of enzyme-loaded cryogel to the mixture and stirred at 30°C for 60 minutes at 800 rpm. NADH consumption was monitored at 340 nm using UV spectrophotometry, and enzyme activity was calculated based on the amount of NADH consumed. The activity of the immobilized enzyme was evaluated over five reaction cycles, with each cycle involving the same process followed by washing the cryogel and re-initiating the next reaction.

Continuous Use of GsOYE Enzyme

In continuous mode (see figure S2 for continuous flow set-up), 50 mg of cryogel was placed in a thermostatted glass column maintained at 4°C using a cryostat. The enzyme solution, prepared in Tris-HCl buffer (50 mM NaCl, pH 7.5), was percolated through the column at a flow rate of 0.1 mL/min using an HPLC pump in order to result in a 10% enzyme loading. Following enzyme immobilization, the column was flushed with 5 mL of Tris buffer at the same flow rate to remove unbound proteins. The continuous enzymatic reaction was performed by circulating a reaction mixture containing 50 mM 1-cyclohexenone in 200 mM Tris-HCl-NaCl buffer (50 mM NaCl, 2% DMSO, pH 7.5) through the column. In recirculation mode, 6 mL of the reaction mixture was cycled through the column at 1 mL/min for up to 5 hours, with 100 µL samples taken hourly. The consumption of NADH was monitored at 340 nm to determine conversion ratio.

B9L0N2 experimentation:

Transformation (Under Sterile Conditions)

Plasmid Preparation

Dehydrated plasmids were re-suspended in 5 mM Tris-HCl buffer, pH 8.5, as recommended by TwistBioscience (the plasmid supplier), to achieve a final concentration of 10 ng/ μ L. The suspension was gently mixed by tapping and incubated at 30°C for 15 minutes without agitation.

Simultaneously, BL21(DE3) and DH5 α competent cells were thawed on ice.

Plasmid Dilution and Transformation

The plasmid suspension was further diluted 1:10 with the same buffer to obtain a final DNA concentration of 1 ng/ μ L. Subsequently, 5 μ L of this diluted plasmid solution was added to 50 μ L of competent cells. The mixture was gently mixed by tapping and incubated on ice for 30 minutes.

Heat Shock

For heat shock transformation, the following conditions were applied:

- **BL21(DE3) cells:** Incubated at 42°C for 30 seconds followed by 2 minutes on ice.
- **DH5 α cells:** Incubated at 42°C for 20 seconds followed by 2 minutes on ice.

After heat shock, SOC medium was added: 950 μ L for DH5 α cells and 250 μ L for BL21(DE3) cells. The suspensions were incubated at 37°C for 1 hour at 225 rpm. In parallel, pre-poured LB agar plates containing 50 μ g/mL kanamycin were removed from cold storage and placed in the incubator to equilibrate to room temperature, avoiding thermal shock.

Plating

The transformed cells were then plated on the LB agar kanamycin plates and incubated overnight at 37°C.

Culture (Under Sterile Conditions)

Pre-culture

For pre-culture, three isolated colonies from the transformation plates were selected and inoculated into a Falcon tube containing 5 mL of LB medium supplemented with 50 μ g/mL kanamycin. The cultures were incubated overnight at 37°C with shaking at 180 rpm. After incubation, 800 μ L of the pre-culture was mixed with 800 μ L of sterile 50% glycerol to prepare glycerol stocks, which were stored at -80°C (N2A03 room).

Main Culture

For the main culture, 200 mL of LB medium with 50 μ g/mL kanamycin in a non-baffled 1L Erlenmeyer flask was inoculated with 2 mL of pre-culture and incubated at 37°C with shaking at 180 rpm until the optical density at 600 nm (OD₆₀₀) reached between 0.4 and 0.6 (approximately 3 hours).

Induction

To induce protein expression, 200 μ L of 1 M IPTG was added to the culture, yielding a final IPTG concentration of 1 mM. The flask was then incubated at 25°C with shaking at 180 rpm for 5 hours.

Purification

Cell Lysis

The cell pellet from 100 mL of culture was re-suspended in lysis buffer (see buffer composition table below) to achieve a suspension with an OD of 50. Cells were lysed using sonication with a 10 mm probe at 40% amplitude for 10 minutes, alternating between 15 seconds ON and 15 seconds OFF. The samples were kept on ice throughout the sonication process to prevent overheating and protein degradation.

Centrifugation

The lysate was centrifuged at 14,000 rpm for 30 minutes at 4°C, and 20 µL of the supernatant was collected for further analysis.

Affinity Chromatography

The supernatant was loaded onto a 1 mL HisTrap column using the ÄKTA system with a flow rate of 1 mL/min. The following elution steps described in table S1:

Step	Solution	Volume (mL)	Fraction Recovered
Ni Recharging	0.1 M NiSO ₄ solution	5	/
Equilibration	Lysis buffer	15	/
Sample Loading	Supernatant	X	Unbound fraction X mL
Washing	Wash buffer	15	Wash fraction 15 mL
Elution	Elution buffer	10	Eluted fraction 10 x 1 mL

Table S1: Elution steps and corresponding solution

The most concentrated elution fractions were pooled and subjected to desalting using a 5 mL Desalting column on the ÄKTA system at a flow rate of 3 mL/min. The desalting steps are described in table S2 & S3:

Step	Solution	Volume (mL)	Fraction Recovered
Equilibration	Desalting buffer	100	/
Sample Loading	Eluted fractions	X	/
Elution	Desalting buffer	10	Desalted fraction 10 x 1 mL

Table S2: Desalting steps and corresponding solution

Buffer	Composition
Lysis buffer	50 mM KPi, 300 mM NaCl, 10 mM Imidazole, pH 8
Wash buffer	50 mM KPi, 300 mM NaCl, 20 mM Imidazole, pH 8
Elution buffer	50 mM KPi, 300 mM NaCl, 250 mM Imidazole, pH 8
Desalting buffer	50 mM KPi, 300 mM NaCl, 10% (w/v) glycerol, pH 7.5

Table S3: Buffer Composition

Enzyme Immobilization (Batch Mode)

In batch mode, 10 mg of cryogel was weighed and added to a 1 mL solution of 50 mM potassium phosphate buffer (KPB) (300 mM de NaCl, 10 % glycerol pH 7.5) containing 1 mg of B9L0N2 enzyme, resulting in a 10% enzyme loading on the cryogel. The suspension was stirred at 800 rpm for 2 hours at 4°C to facilitate enzyme immobilization. Samples were taken at intervals (10, 20, 30, 60, 90, and 120 minutes), followed by centrifugation at 4000–6000 rpm for 2 to 5 minutes to separate the solid cryogel from the supernatant. After the final sampling, the supernatant was discarded, and the solid cryogel was washed twice with the buffer solution to remove unbound enzyme. Each wash involved stirring the suspension for 3 minutes at 4°C, followed by centrifugation.

Enzyme Quantification by Bradford Assay

The amount of enzyme immobilized on the cryogel was determined using the Bradford protein assay. A standard curve was prepared using BSA (0.1 g/L) in the same 50 mM KPB (300 mM de NaCl, 10 % glycerol pH 7.5). Bradford reagent was added to each sample, including the immobilized enzyme supernatant samples, and incubated for 15 minutes in the dark. The absorbance was measured at 595 nm. The protein content in the samples was calculated by comparison with the standard curve, and the amount of enzyme immobilized was determined based on the amount of protein remaining in the supernatant after immobilization.

Enzyme Activity of Immobilized B9L0N2

The catalytic activity of immobilized B9L0N2 was measured by following the transamination of pyruvate in presence of racemic α -methylbenzylamine to alanine and acetophenone. Transaminase uses the cofactor pyridoxal phosphate (PLP) to carry out this reaction. The reaction mixture contained 25 mM pyruvate, 25 mM racemic α -methylbenzylamine, 0.1 mM PLP, and 1% DMSO in 50 mM KPB (300 mM NaCl, pH 7.5). The initial enzyme/substrate ratio is fixed at 0.025. The reaction was initiated by adding 10 mg of enzyme-loaded cryogel to the mixture and stirred at 25°C for 10 minutes at 800 rpm. Acetophenone formation was monitored at 245 nm using UV spectrophotometry. The activity of the immobilized enzyme was evaluated over five reaction cycles, with each cycle involving the same process followed by washing the cryogel and re-initiating the next reaction.

Continuous Use of B9L0N2 Enzyme

In continuous mode (see figure S2 for continuous flow set-up), 70 mg of cryogel was placed in a thermostatted glass column maintained at 4°C using a cryostat. The enzyme solution, prepared in 50mM KPB (300 mM NaCl, 10 % glycerol, pH 7.5), was percolated through the column at a flow rate of 0.1 mL/min using an HPLC pump in order to result in a 5% enzyme loading. Following enzyme immobilization, the column was flushed with 5 mL of Tris buffer at the same flow rate to remove unbound proteins. The continuous enzymatic reaction was performed by circulating a reaction mixture containing 25 mM pyruvate, 25 mM racemic α -methylbenzylamine, 0.1 mM PLP in 50 mM KPB (300 mM NaCl, 1% DMSO, pH 7.5) through the column. In circulation mode, 10 mL of the reaction mixture was passed through the column at 0.1 mL/min for up to 5 hours, with 100 μ L samples taken hourly. The acetophenone formation was monitored at 245 nm to determine conversion ratio.