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Design of a Biocatalytic Flow Reactor Based on Hierarchically Structured Monolithic Silica for Producing Galactooligosaccharides (GOSs)

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Abstract: Climate change mitigation requires the development of greener chemical processes. In this context, biocatalysis is a pivotal key enabling technology. The advantages of biocatalysis include lower energy consumption levels, reduced hazardous waste production and safer processes. The possibility to carry out biocatalytic reactions under flow conditions provides the additional advantage to retain the biocatalyst and to reduce costly downstream processes. Herein, we report a method to produce galactooligosaccharides (GOSs) from a largely available feedstock (*i.e.* lactose from dairy production) using a flow reactor based on hierarchically structured monolithic silica. This reactor enables fast and efficient biotransformation reactions under flow conditions.

Keywords: Flow biocatalysis · Galactooligosaccharides · Galactosidase · Immobilized enzyme · Monolithic silica

Emilio Cutrona and ***Andrea Buscemi*** both studied Industrial Biotechnology at the University of Palermo, Italy. After his MSc research project carried out at the University of Applied Sciences and Arts Northwestern Switzerland (FHNW), Emilio took a position as scientific lab associate at INOFEA, a Swiss biotech company developing immobilized enzymes solutions. Andrea is currently working as research assistant at FHNW. ***Riccardo Dejoma*** is also studying Biotechnology at the University of Palermo; he is currently performing his master research training at the FHNW. ***Patrick Shahgaldian*** is professor of Molecular Nanotechnology at the FHNW and a senior member of the Swiss Nanoscience Institute. The Shahgaldian group is working on the development of synthetic nanosystems capable of molecular recognition and biocatalysis.

1. Introduction

The design of inorganic materials with hierarchical architectures is an active field of research in material sciences.[1–5] A large variety of hierarchically structured materials has been produced for a range of applications including catalysis,[3] energy conversion and storage devices,[6,7] sensors,[6,8] gas adsorption and storage,[9] and separation[10] to name but a few. Silica has been extensively studied as a material for producing hierarchical architectures. Apart from a variety of hierarchically ordered mesoporous silica nanoparticles,[11–14] monolithic silica-based materials[15] are attracting substantial interest, primarily for separation science applications.[16–18] Such materials have also been used as solid supports to design chemical[19–21] and biochemical[22–27] flow reactor systems. For example, Ma *et al.* have developed a monolithic biocatalytic reactor using trypsin, a proteolytic enzyme widely used in proteomics.[22] They demonstrated that the degradation of a model peptide was 600 times faster when compared to the same reaction carried out in solution. Szymańska *et al.*, working with the same protease enzyme, optimized the monolithic silica structure, with hierarchical bi- and tri-modal pore structures.[23] Recently, the group of Walde developed a novel enzyme bioconjugation method of silica monoliths.[24,25] It is based on the electrostatic adsorption, at the surface of negatively charged silica, of a polycationic dendronized polymer conjugated with an enzyme (*i.e.* horseradish peroxidase, HRP).[24] While only maintained through electrostatic interactions, the system showed good stability under operational conditions. A similar electrostatic bioconjugation approach was achieved using an α-poly(D-Lys) polymer conjugate.[25] Wu *et al.* developed a method to produce, using a one-pot strategy, a monolithic silica-based material that can be directly modified using a click reaction to attach an HRP enzyme.[28]

In our group, we focus our efforts to supramolecularly engineer enzymes within designer organosilica shields.[29–34] While this method allows reaching markedly enhanced enzyme stability, their implementation in flow reactors is often tedious owing to the nanoparticulate nature of the material produced. In the present manuscript, we report a facile method to produce a flow reactor based on monolithic silica to transform lactose into galactooligosaccharides (GOSs). GOSs are typically produced by transgalactosylation reactions biocatalyzed by lactase enzymes (β-galactosidase, β-Gal, EC 3.2.1.23). The transgalactosylation reaction is known to be favored *vs.* hydrolysis at high lactose concentrations.[35] GOSs (more specially tri- and tetra-saccharides) find applications in human nutrition as they are non-digestible carbohydrates acting as prebiotic substances modulating the colonic microbiota.[35,36]

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2. Results and Discussion

2.1 *Synthesis*

The silica monolith used to construct the biocatalytic flow reactor was produced as previously described.[23] Briefly, the polymeric phase separation agent [poly(ethylene glycol) 35000] was dissolved in water containing nitric acid; after the silica precursor (tetraethylorthosilicate, TEOS) was added to this solution dropwise, the cationic surfactant (cetyltrimethylammonium bromide, CTAB) was dissolved in this mixture, which was then allowed to react for 10 days at 40 °C. After calcination at 500 °C, the monoliths were characterized by scanning electron microscopy (SEM, Fig. 1). The SEM micrographs confirmed the porous structure of the monolith, with pore size ranging from 1.5 to 3.0 µm. At high magnification, SEM investigations revealed that the overall architecture is formed by small nanoparticles (29±4 nm), formed during the sol-gel process. The produced monolith (221 mg, 2,9 cm in length, 7 mm in diameter) was sealed in low-density polyethylene tubes and used to immobilize the selected enzyme.

The general strategy for enzyme immobilization is depicted in Fig. 2. We selected a β-Gal from *Aspergillus Oryzae* as model enzyme. β-Gal enzymes have been previously used in continuous reactors for GOS production using packed bed reactor systems.[37,38] The monolith was first reacted with aminopropyl-triethoxysilane (APTES) in order to introduce amino functions at the silica surface. To that end, a volume of 8 mL of APTES was flowed in the reactor at a rate of 200 µL min^{-1}; the flow was then stopped, and the silane was allowed to react for 90 min. After washing with nanopure water, the system was allowed to cure for 16 hours at 20 °C. We used glutaraldehyde as crosslinker allowing the covalent attachment of the target enzyme on the amino functions introduced at the surface of the silica support. The amino-modified monolith was reacted with glutaraldehyde (40 mM) under flow conditions (200 µL min^{-1}) for 90 min, thoroughly washed with MES buffer [2-(*N*-morpholino)ethanesulfonic acid, 10 mM, $MgCl_2$ 5 mM, pH 6.2]. For protein immobilization, β-Gal (1.2 mg mL^{-1}) in acetate buffer (100 mM, $MgCl_2$ 5 mM, pH 4.4) was flowed in the reactor. In order to determine the amount of immobilized β-Gal, samples were collected every minute and assayed for their protein content using the established bicinchoninic acid (BCA) protein assay. Consequently, the reactor was washed with the same acetate buffer and washing fractions were assayed (Fig. 3). During the enzyme immobilization, carried out with an enzyme concentration of 1.2 mg mL^{-1}, the concentration of enzyme measured in the eluate remained constant, with a value averaging 230 µg mL^{-1}. This can be explained by the fact that the reaction was performed under flow conditions and that the residence time of the enzyme was not sufficient to reach full immobilization. Additionally, the large capacity of the silica monolith explains the fact that saturation was not reached. The amount of enzyme retained in the reactor was evaluated to be 19 mg. During the washing phase, a peak of eluted protein was measured after 4 min with a concentration of enzyme of 1 mg mL^{-1}; this loss of enzyme can be safely attributed to a first wash away of non-specifically adsorbed enzyme explaining the first elution peak. The protein concentration in the eluate decreased rapidly to reach 130 µg mL^{-1} after 20 min and 50 µg mL^{-1} after 60 min. Overall, this loss represents 26% of the initially retained enzyme; as much as 13.8 mg of enzyme remained immobilized in the reactor; this corresponds to 61 µg of enzyme per mg of silica. This represents an immobilization yield of 67%.

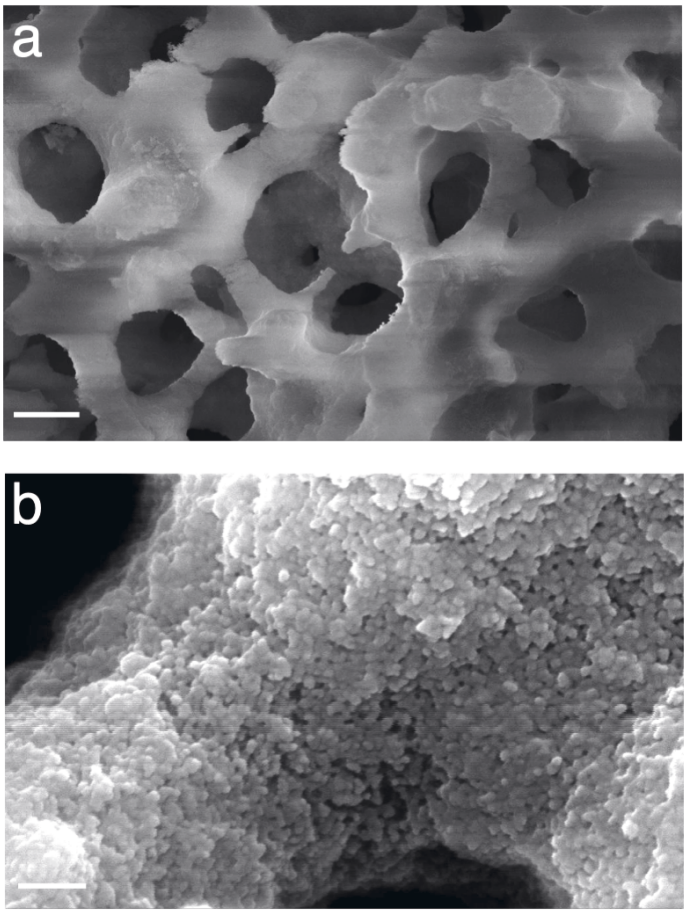

Fig. 1. Scanning electron micrographs of the silica monoliths produced. Scale bars represent 2 µm (a) and 200 nm (b).

The biocatalytic activity in the reactor was first assayed spectrophotometrically using an artificial substrate, namely *o*-nitrophenol-β-D-galactoside (ONPG). A solution of ONPG in acetate buffer was flowed in the reactor at a flow rate of 600 µL min^{-1} (Fig. 4). The results showed that after a first phase of dilution of the reaction product in the running buffer (*ca.* 2 mL), the concentration of the product reaches a plateau at 8 mM, similar to the maximum conversion measured in those conditions in batch conditions. This shows that even with a contact time shorter than 1 min, the enzymatic reaction proceeded with high efficiency.

Next, the bioreactor efficiency was tested using lactose as substrate, at a high concentration of 200 g L^{-1} known to favor GOS formation;[35] the transformation products were analyzed by HPLC (Fig. 5). The results showed that at a flow rate as low as 20 µL min^{-1}, the hydrolysis reaction was highly favored, the eluate mainly contained glucose and galactose. Only a marginal amount of DP3 can be measured. This can be explained by an excessive contact time with the biocatalytic reactor, as GOS formed in the reactor can be hydrolyzed by the immobilized enzyme. Increasing the flow rate resulted in a higher amount of lactose substrate in the hydrolysate, along with higher amounts of GOSs (degree of polymerization DP3 and DP4). This represents a GOS production of *ca.* 40 g L^{-1}, in fairly good agreement with yields measured in batch conditions for the same enzyme.[35] The system proved stable, without any significant loss of activity, for at least 14 hours. Additional experimental optimization (*i.e.* reaction pH, enzyme coupling stability, temperature) can certainly allow reaching even higher conversion rates.

3. Conclusion

In summary, we have designed a simple and effective flow reactor using a hierarchically structured silica monolith, in which an hydrolytic enzyme, namely β-Gal, was immobilized. The biocatalytic efficiency of this system was demonstrated with the production of GOS from lactose. The straightforward production of the silica monolith, along with an inexpensive bioconjugation method and simple reactor design can be exploited for a large number of biocatalytic bio-transformations.

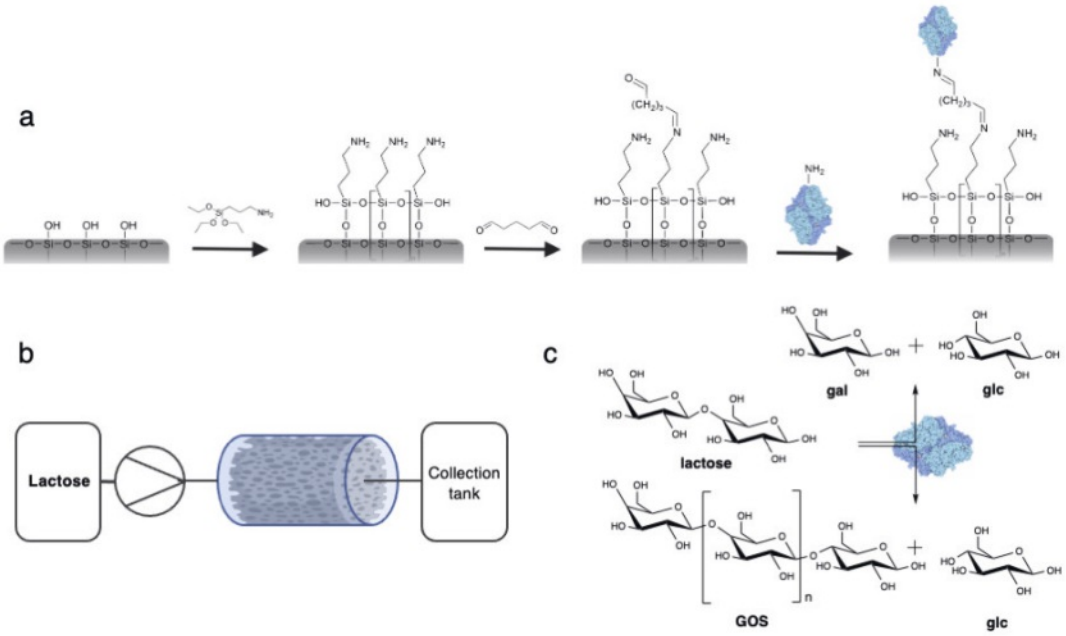

Fig. 2. Chemical strategy of enzyme immobilization (a), schematic representation of the reactor system (b) and biocatalytic reaction of lactose hydrolysis in galactose (gal) and glucose (glc) and GOS formation (c).

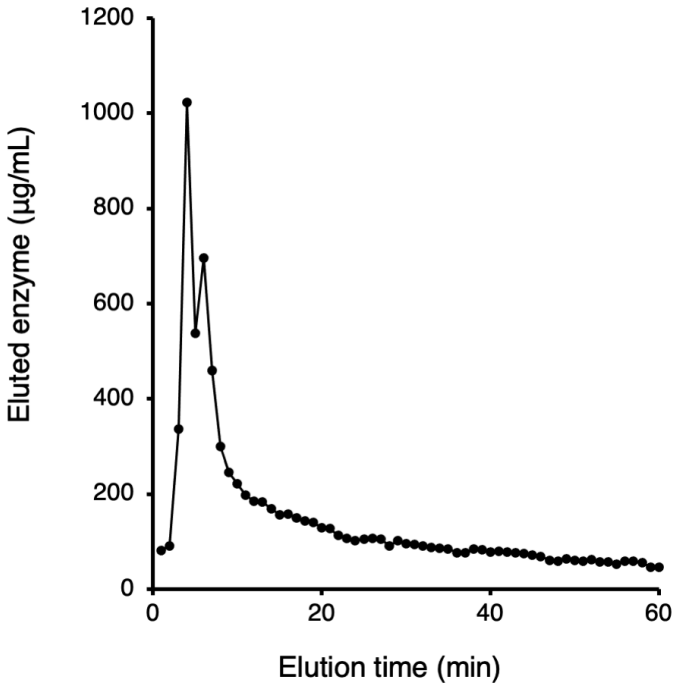

Fig. 3. Enzyme concentration measured in the eluate during the washing phase after enzyme immobilization, as measured using a BCA protein assay.

4. Experimental

4.1 *General*

All solvents and chemicals were purchased from Merck (Switzerland) and used without further purification. A Synergy H1 (BioTek, Switzerland) microplate reader into 96-well plate (Greiner ®Bio-One, PS, f-bottom) was used for spectrophotometric measurements of ONPG hydrolysis. All buffers were prepared with nanopure water (resistivity ≥18 MΩ cm) produced with a Millipore ®Synergy purification system (Merck, Switzerland). BCA assays were carried out using a Pierce™ (ThermoFisher, Switzerland) BCA Protein assay kit.

4.2 *Synthesis of Silica Monoliths*

Cylindrical silica monoliths with a diameter of 7 mm were synthetized from tetraethyl orthosilicate (TEOS), polyethylene glycol 35000 (PEG), water, nitric acid and cetyltrimethylammoium bromide (CTAB) at a molar ratio of 1:0.52:14.25:0.26:0.027.[23] The synthesis procedure was as follows: a volume of 840 µL of HNO_3 was diluted in 12.5 mL of water at 4 °C before addition of PEG (1.6 g, 35000). After complete dissolution of the polymer was a volume of TEOS (11 mL) slowly added. Consequently, 500 mg of CTAB were dissolved in the reaction mixture. Volumes of 2 mL of the reaction mixture were placed into 2 mL syringes and cured 10 days at 40 °C. The solid produced was immersed in

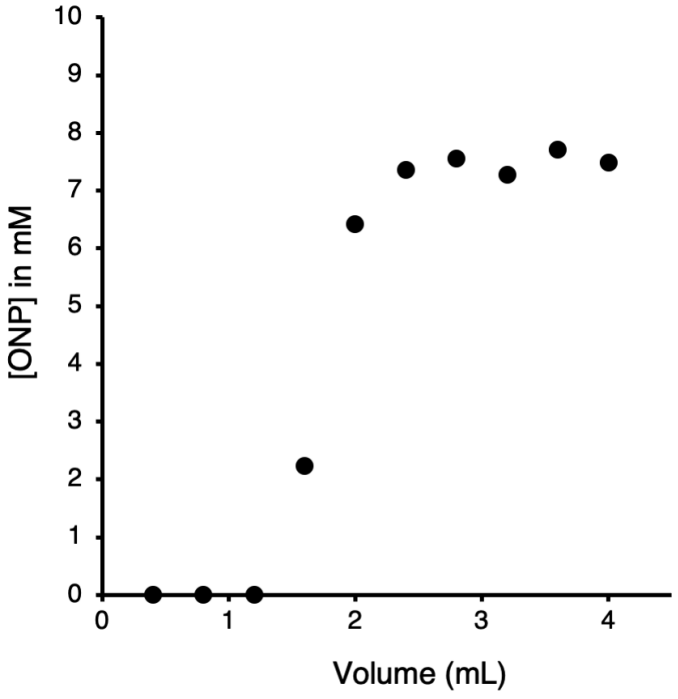

Fig. 4. Biocatalytic activity of the biocatalytic monolithic silica measured with an artificial substrate, namely ONPG (22 mM) in acetate buffer pH 4.4. Every data point represents a single measurement.

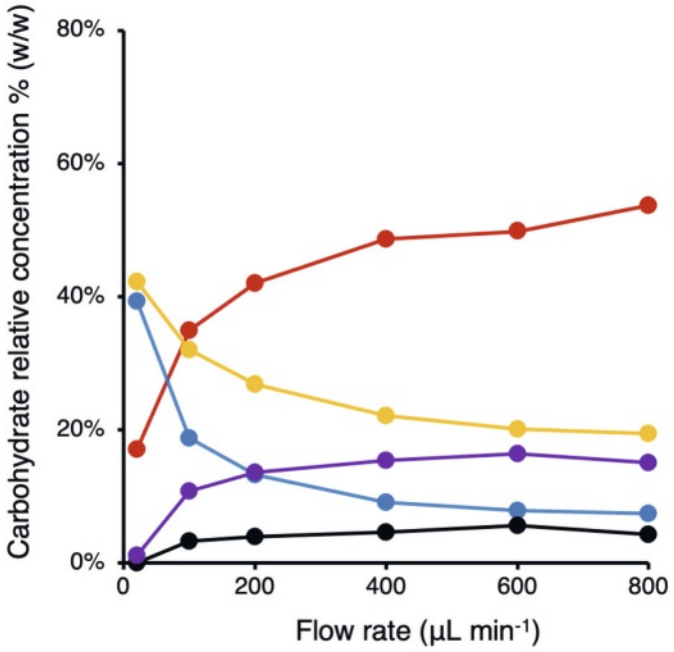

Fig. 5. Biocatalytic transformation of lactose (red) in glucose (orange) and galactose (blue), GOS with degree of polymerization 3 (purple) and 4 (black). Every data point represents a single measurement.

an ammonia solution (1M) at 100 °C for 9 hours, dried at 60 °C and eventually calcinated at 500 °C for 16 hours.

4.3 *Enzyme Immobilization and Activity Testing*

The enzyme (β-Gal from *Aspergillus oryzae,* A50 Optiferm, 50000 U g^{-1}) was dialyzed (SnakeSkin, 10 kDa MWCO) against the activity buffer (acetate 100mM, $MgCl_2$ 5mM, pH 4.4). The enzyme concentration was determined by a BCA protein quantification carried out according to the manufacturer's protocol. The silica monolith was functionalized by pumping 8 mL of neat APTES at a flow rate of 200 µL min^{-1}. The flow was stopped and APTES was left to react in the monolith for 90 min, thoroughly washed with water and left to cure for 16 h at 20 °C. Further, an aqueous solution of glutaraldehyde (40 mM) was flowed at a rate of 200 µL min^{-1} for 90 min. Subsequently the silica monolith was washed with 36 mL of immobilization buffer (10 mM MES, 10 mM $MgCl_2$, pH 6.2) at a flow rate of 600 µL min^{-1}. Immobilization of β-Gal was performed by pumping 17 mL of enzyme solution (1.2 mg mL^{-1} in acetate buffer) at a flow rate of 200 µL min^{-1} and collecting aliquots of the eluate every minute for protein quantification. After immobilization, the silica monolith was washed by pumping 36 mL of acetate buffer at a flow rate of 600 µL min^{-1}. The activity of the immobilized β-Gal was tested by flowing a solution of ONPG (22 mM in activity buffer) at 600 µL min^{-1} and analyzing the eluate spectrophotometrically, as described elsewhere.[31]

4.4 *GOS Production*

For the production of GOS, lactose (200 g L^{-1}) in sodium phosphate buffer (50 mM, pH 7.0) was used and aliquots of the eluate were collected at increasing flow rates (20, 100, 200, 400, 600 and 800 µL min^{-1}); the collected eluate was diluted (1:5 in water) and analyzed by means of HPLC (1260 Infinity system, Agilent) equipped with a refractive index detector and a HiPlex column (Agilent). Elutions were carried out with neat water at a flow of 0.3 mL min^{-1} at 80 °C. The enzymatic activity was measured by evaluation of both substrate (lactose) consumption and product (glucose, galactose and GOS) formation. Identification of oligosaccharides was done by comparison with standard compounds (glucose and galactose as monosaccharide standards, lactose as disaccharide standard, raffinose as a trisaccharide standard and maltotetraose as tetrasaccharide standard). Quantification of oligosaccharides was done by integration of peak areas and the use of calibration curves obtained with corresponding standards.

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