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β-Galactosidase from *Aspergillus oryzae* immobilized onto different magnetic supports: A comparative experimental and modelling study of the galactooligosaccharides production

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Topic: Multi-scale and/or multi-disciplinary approach to process-product innovation.

Abstract

β-Galactosidase from *Aspergillus oryzae* is an enzyme with a wide industrial application, mostly in the hydrolysis of lactose and, more recently, in the synthesis of oligosaccharides. Several advantages are associated with the application of immobilized enzymes on magnetic supports. In this work, β-galactosidase was covalently immobilised onto a Polysiloxane-Polyvinyl Alcohol Magnetic Composite (mPOS-PVA), Magnetic Polysiloxane with Polyaniline (mPOS-PANI), Magnetized Dacron (DACRON) and Magnetite with Polyaniline (MAG-PANI) using glutaraldehyde as activating agent being the synthesis of GOS evaluated and compared at different temperatures (30, 40, 50 and 60 ^{o}C) and various initial lactose concentration (50, 100, 200, 300, 400 and 500 g/L). The kinetic parameters obtained by fitting the experimental data were compared in order to determine the effect of the immobilization process with different supports on the synthesis of oligosaccharides. These results clearly demonstrate that all supports may be used for β-galactosidase immobilization as, besides improving the enzyme hydrolytic and GOS synthesis properties, its separation from the obtained reaction products is easy to accomplish.

1 Introduction

During the lactose hydrolysis catalyzed by β-galactosidase GOS are produced by transgalactosylation activity and the production increases by increasing the initial concentrations of lactose in the reaction mixture (Albayrak and Yang, 2002). GOS are nondigestible oligosaccharides, which are prebiotics acting as growth-promoting substrates for bifidobacteria in the human intestine. Enzymes have been utilized in a large number of practical applications, particularly in biomedical and biotechnological fields, through immobilization on a variety of supports (Bayramoglu *et al*., 2008). The applications of immobilized enzymes may be challenged by difficulties that arise in enzyme recovery and recycling, which are the most important processes that can decrease the overall cost of the enzyme immobilization process (Lee *et al*., 2007). The use of magnetic micro-beads as supports for enzyme immobilization is mainly based on the magnetic feature of the solid-phase that enables to achieve a rapid separation in a magnetic field, as well as decrease of operation cost. In addition, the nano-scaled immobilized enzyme can greatly alleviate the transfer barrier, and thus improve the catalytic efficiency (Hong *et al*., 2008). Immobilization of enzyme or biomolecules to the magnetic microbeads is usually accomplished through reactive groups existing on their surface. Nowadays, these functional composites cold be produced in many ways, i.e. emulsion polymerization (Csetneki *et al*., 2004), in situ formation (Li *et al*., 2006) and sol-gel (Luo *et al*., 2006), but usually involve the coating of magnetically susceptible particles with synthetic polymers or biomacromolecules (Hong *et al*., 2008). Actually, the use of magnetic particles in enzyme technology is constantly increasing.

Permanent magnetization could cause the particles to aggregate even if the supports are removed from the magnetic field (Neri *et al*., 2008 and Bayramoglu *et al*., 2008). In this study, β-galactosidase from *Aspergillus oryzae* was covalently immobilized onto different magnetic supports (mPOS-PANI, DACRON, mPOS-PVA and MAG-PANI) for GOS production. The effects of the temperature, initial lactose concentration and reutilization were investigated. In addition, the kinetic parameters were determined and compared between the four supports using experimental data (10-50% initial lactose concentration).

2 Materials and methods

Supports synthesis: mPOS-PVA – beads were synthesized according to the procedure described by Barros *et al.* (2002) and the magnetized particles were obtained by Carneiro-Leão *et al*. (1991); MAG-PANI – magnetite (MAG) was obtained by co-precipitation (Carneiro-Leão *et al*., 1991) and after coated with polyaniline (PANI) as was done by Oliveira *et al*. (2008); mPOS-PANI – POS was prepared polymerizing the tetraethylorthosilicate and after was coated with polyaniline as described in Oliveira *et al*. (2008); DACRON – was converted to magnetic DACRON according to Pinheiro *et al*. (1999). The immobilization of β-galactosidase was done using glutaraldehyde as activating agent. The supports activated (100 mg) were incubated with 10 mL β-galactosidase solution (4 mg solid/mL) in 20 mM citrate-phosphate buffer, pH 4.5, for 18 h at 4 °C and 20 rpm. Production of GOSs from lactose was studied with immobilized enzyme on various supports in different conditions. The reaction kinetics was studied at six different initial lactose concentrations (5, 10, 20, 30, 40, and 50 %, w/v and four different temperatures (30, 40, 50, and 60ºC). GOSs, lactose, glucose and galactose concentration were determined using HPLC. As the concentration (w/v) of these sugars is proportional to their peak areas, normalized sugar concentrations as weight percentages of total sugars or initial lactose were determined from peak areas and are reported in this work.

Trisaccharides synthesis and lactose hydrolysis mechanisms including glucose and galactose competitive inhibition was mathematically modelled following four ordinary differential equations:

The lactose hydrolysis (*L*) is given by,

$$\frac{dL}{dt}=\frac{n_{1L}[L][H_2O]+n_{2L}[Tri][H_2O]+n_{3L}[L]^2}{d_1} \quad \text{where,} \quad n_{1L}=\frac{-k_1k_2}{k_4};n_{2L}=k_2;n_{3L}=\frac{-2k_1k_3}{k_4} \qquad \text{Equation 1}$$

the glucose (*G*) formation by,

$$\frac{dG}{dt}=\frac{n_{1G}[L]^2+n_{2G}[L][H_2O]}{d_1} \quad \text{where,} \quad n_{1G}=\frac{k_1k_3}{k_4};n_{2G}=\frac{k_1k_2}{k_4} \qquad \text{Equation 2}$$

and the galactose (*Gal*) by,

$$\frac{dGal}{dt}=\frac{n_{1Gal}[H_2O][L]+n_{2Gal}[Tri][H_2O]}{d_1} \quad \text{where,} \quad n_{1Gal}=\frac{k_1k_2}{k_4};n_{2Gal}=k_2 \qquad \text{Equation 3}$$

Finally, the trisaccharides production (*Tri*) is described by the equation 4

$$\frac{dTri}{dt}=\frac{n_{1Tri}[Tri][H_2O]+n_{2Tri}[L]^2}{d_1} \quad \text{with,} \quad n_{1Tri}=-k_2;n_{2Tri}=\frac{k_1k_3}{k_4} \qquad \text{Equation 4}$$

and $$d_1=\frac{k_2}{k_4}[H_2O]+\left(\frac{k_3}{k_4}+\frac{k_1}{k_4}\right)[L]+[Tri]+\frac{k_2k_5}{k_4k_6}[G][H_2O]+\frac{k_3k_7}{k_4k_8}[Gal][L]+\frac{k_2k_7}{k_4k_8}[Gal][H_2O]+\frac{k_3k_5}{k_4k_6}[G][L]$$

The kinetic model was adapted from the work of Boon *et al.* (1999) and the rate expressions were derived with the King-Altman method (King and Altman, 1956; BioKin, Lda). The model used only lactose as substrate and temperature effect was not included. The parameters were grouped into five parameters (k_1,is the overall reaction rate constant; k_4, describes the reaction of water with the galactosyl-enzyme complex; k_3/k_4, ratio between rate constants oligosaccharides synthesis and hydrolysis of the formed oligosaccharides; k_5/k_6 and k_7/k_8 ,account for glucose and galactose inhibition, respectively) that were estimated and one fixed

parameter (k_2, describes the reaction of water with the galactosyl-enzyme complex), which was assumed constant. The estimation of the model parameters was performed by the simulated annealing algorithm of the Systems Biology toolbox for *Matlab* (Mathworks) software (Schmidt and Jirstrand, 2006) using the results of the independent batch experiments, with the sets of experimental data from different initial lactose concentration (10%-50%; w/v) as substrate. All experimental data points were fitted simultaneously for each concentration and support.

3 Results and Discursion

Effects of lactose concentration on GOS production by free and immobilized enzyme on different magnetic supports

The percent of total GOS plotted against the percent of lactose conversion at 5% and 50% of initial lactose concentration, 40 ºC and pH 4.5 by different supports and free enzym, is shown in Figure 1A. Similar experiments were performed for different initial lactose concentrations (10 to 40%, w/v) and the tri- and the tetra-saccharides were quantified (data not show). GOS (tri- and tetra-saccharides) production increased with increasing lactose concentration. However, for each lactose concentration the GOS production decreased after a certain degree of lactose conversion. This can be attributed to a preferential hydrolysis (formation of glucose and galactose) rather than GOS synthesis. Furthermore, there was no marked difference between the free and immbilized enzyme performances. As the initial lactose concentration increased from 5% to 50%, the maximum GOS content in the product increased from 11.2% (at 35% conversion) to 26.1% (at 56% conversion) for the free enzyme, from 10.3% (at 38% conversion) to 25.7% (at 49% conversion) for the immobilized enzyme on mPOS-PANI, from 10.1% (at 34% conversion) to 26.2% (at 54% conversion) for the immobilized enzyme on DACRON, from 10.4% (at 30% conversion) to 26.0% (at 55% conversion) for the immobilized enzyme on mPOS-PVA, and from 10.8% (at 33% conversion) to 26.0% (at 52% conversion) for the immobilized enzyme on MAG-PANI. The maximum amount of tri-, tetra-saccharides and total GOS obtained for the free enzyme were 104.5 g L^{-1}, 33.2 g L^{-1} and 130.3 g L^{-1} for a lactose conversion of about 48%, 61% and 56%, respectively, in 500 g L^{-1} of lactose. For the immobilized enzyme, the corresponding values of 103.7 g L^{-1} on DACRON, 31.9 g L^{-1} on MAG-PANI and 131.0 g L^{-1} on DACRON were obtained for about 46%, 59%, and 54% lactose conversion, respectively in 500 g L^{-1} of lactose. These results suggest that enzyme immobilization on different supports does not impose any limitation or changes on GOS formation from lactose. Similar results were obtained with β-galactosidase from *A. oryzae* immobilized on cotton cloth (Albayrak and Yang, 2002). Thus, the GOS formation ability of the enzyme was not affected by the immobilization of the enzyme onto different supports, what is a clear demontration of the absence of mass transfer limitations using mPOS-PANI, DACRON, mPOS-PVA and MAG-PANI as supports.

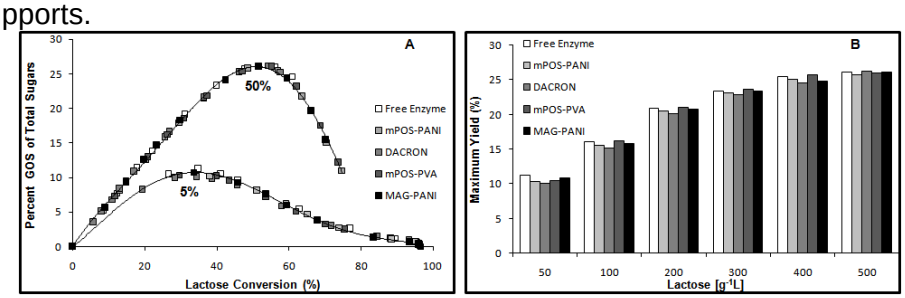


Figure 1. Total GOS (A) formation by free and immobilized (closed symbols) *A. oryzae* β-galactosidase on different supports at pH 4.5 and 40° C catalysing the hydrolysis of initial lactose concentrations (5 and 50%, w/v). GOS Yield (B) *versus* lactose concentration by free and immobilized *Aspergillus oryzae* β-galactosidase on different suports.

GOS yields during lactose hydrolysis catalyzed by the free and the immobilized β-galactosidase on different supports are shown in Figure 1B. The maximum GOS yield

increased with the initial lactose concentration for both enzyme preparations. It was observed that the hydrolysis and transgalactosylation reactions occurred simultaneously and that the product profile of the reaction is largely dependent on lactose concentration. The hydrolysis reaction dominates at low lactose concentration while GOS formation dominates at high lactose concentrations. β-Galactosyl groups should have a higher probability of attaching to lactose than water at increasing lactose concentrations (Iwasaki et al., 1996).An increase was observed up to 300 g L^{-1} of initial lactose concentarion, while for higher lactose concentrations the GOS yield was almost constant at 22.8–26.2% for free and immobilized enzyme on different suports. Park et al (2007) observed the same behavior for high lactose concentration with a thermostable β-galactosidase from *Sulfolobus solfataricus*. It is possible that some disaccharides such as allolactose and galactobiose are formed by the enzymatic transfer of galactose to glucose and galactose, respectively. The absence of this side reactions, was confirmed by checking the molar balances on glucose and galactose over the course of the reaction, assuming that all GOS only contained one unit of glucose with galactose as the remaining sugar. It is also possible that different trisaccharides were also formed, but were not detected by HPLC.

Higher operational and thermal stability, making possible its reuse, has been demonstrated to be one of the advantages of this system for enzyme immobilization (Neri *et al*, 2008). An immobilized β-galactosidase on different supports preparation acting on 20% (w/v) lactose was successively reutilized for 10 cycles at 25 ºC and at the end the enzymatic derivative retained approximately 87% for mPOS-PANI, 90% for DACRON, 84% for mPOS-PVA, and 85% for MAG-PANI of its initial activity (see Figure 2), confirming the advantages of the magnetic supports β-galactosidase derivative.

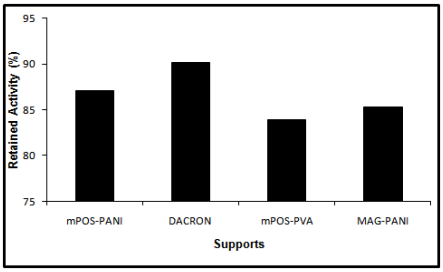


Figure 2. Effect on the activity of β-galactosidase immobilized on different supports after 10 reutilization.

Effects of temperature

Temperature normally has a pronounced effect on the enzyme reaction rates but showed to have a minimal effect, if any, on GOS production (Iwasaki et al., 1996; Monsan and Paul, 1995; Albayrak and Yang, 2002). As shown in Figure 3A and B, GOS production was almost unchanged despite temperatures varying from 30° C to 60° C (Figure 3B) for the conversion of 40% (w/v) lactose, for both the free and immobilized enzyme preparations. Albayrak and Yang (2002) reported similar results for β-galactosidase from *Aspergillus oryzae* immobilized on cotton cloth. It is well known that enzymes do not affect equilibrium constant of the reactions.

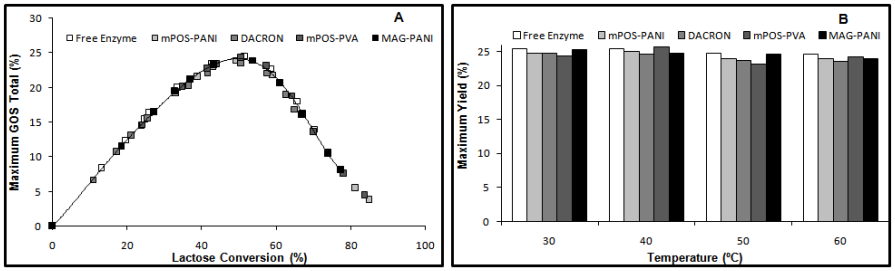


Figure 3. Effects of temperature on the GOS production from the hydrolysis of 40% (w/v) lactose catalyzed by the free and immobilized *A .oryzae* β-galactosidase on different supports at 60 ºC (A). GOS Yield (B) on different supports at various temperature.

Kinetic analysis β-galactosidase free and immobilized on different supports

To verify the precondition that the model parameters are independent of the initial lactose concentrations and in this way to use the parameters by taking the average values, the 95% confidence interval of the slope of the linear dependence of the kinetic parameters on initial lactose concentration was estimated (data not shown). For all the simulations runs the average parameters from each initial lactose concentration were used (Table 1).
To determine the effect of the different supports on the kinetic parameters, experiments were carried out at various initial lactose concentrations ranging from 10-50% (w/v). Figure 4 shows a comparison of the experimental and calculated concentrations profiles with the averaged parameters obtained for the supports at 20% initial lactose concentration. As can be seen, the good quality of the model is remarkable. The model describes well the time course of lactose conversion, glucose and galactose formation and overpredicts slightly the trisaccharides concentration for all the runs.

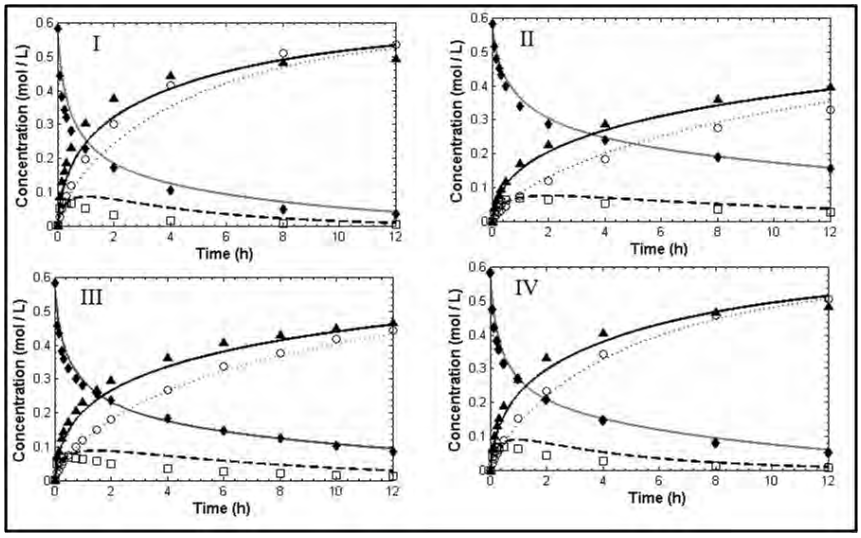


Figure 4. Comparison between experimental (symbols) and simulated data (descending gray solid line: lactose; ascending black solid line: glucose; depicted line; galactose and dashed line: trisaccharide) of (♦) lactose, (▲) glucose, (○) galactose and (□) trisaccharides concentration by immobilized β-galactosidase on Support I (mPOS-PANI), II (DACRON), III (mPOS-PVA) and IV (MAG-PANI) at pH 4.5, 40ºC for 20% initial lactose concentration. Lines represent the simulation using the fitted parameters.

The parameter k_3, that regulates the oligosaccharides synthesis and k_4, which regulates the hydrolysis of the formed oligosaccharides are assumed to be key parameters in the overall process. Not surpringly, as can be observed in Table I, the ratio between k_3 and k_4 is of the same order of magnitude for free and immobilized enzyme on different supports, indicating that the relative extent of the synthesis and hydrolysis reactions is not affected by the immobilization procedure. This is in accordance with the obtained experimental data (see Figure 1).

Table I. Estimated average values and 95% confidence intervals of parameters for the immobilized at different supports and free β-galactosidase obtained by fitting the experimental data for *A. oryzae* at different initial lactose concentration, pH 4.5 and 40ºC.

Parameter (units)	mPOS-PANI	DACRON	mPOS-PVA	MAG-PANI	Free
k_1 (h^{-1})	1.93 ± 0.93	1.09 ± 0.78	1.87 ± 0.70	1.59 ± 0.76	3.06 ± 2.90
Log(k_3/k_4) (-)	1.50 ± 0.043	1.52 ± 0.029	1.49 ± 0.013	1.50 ± 0.017	1.55 ± 0.22
k_4 (h^{-1})	3.69 ± 2.82	4.83 ± 1.613	4.59 ± 1.37	6.15 ± 2.50	3.45 ± 5.06
Log(k_5/k_6) (-)	1.023 ± 0.22	1.12 ± 0.24	1.015 ± 0.17	1.012 ± 0.46	1.50 ± 0.33
Log(k_7/k_8) (-)	0.75 ± 0.35	1.480 ± 0.13	1.45 ± 0.026	0.85 ± 0.41	1.51 ± 0.21

4 Conclusions

All supports proved to be an adequate support for *Aspergillus oryzae* **β**-galactosidase immobilization and its use on GOS production using lactose as substrate. The galacto-oligosaccharides formation ability of the enzyme was not affected by its immobilization onto the magnetic supports that can be easily recovered by applying a magnetic field. The galacto-oligosaccharides production was almost unchanged despite temperatures varying from 30° C to 60° C for the free and immobilized enzyme preparations on different supports. The model describes the experimental data well in the range of the initial lactose concentration used. Such kinetic model may be used bio-process engineering studies to increase knowledge of catalytic studies and the most appropriate support for immobilization process can be selected.

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