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# Galactooligosaccharides production by β-galactosidase immobilized onto magnetic polysiloxane–polyaniline particles

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ABSTRACT

Magnetized polysiloxane coated with polyaniline (mPOS–PANI) was used as a support for β-galactosidase immobilization via glutaraldehyde. The galactooligosaccharides (GOS) production by this derivative was investigated under different initial lactose concentrations (5–50%) and temperatures (30–60 °C). The initial lactose concentration in the reaction media affected the total amounts of produced GOS and their time course production was described as a "bell-shaped" curve as a result of the balance between transgalactosylation and hydrolysis. No significative difference was observed for the free and immobilized enzymes. The reaction rates for lactose hydrolysis and GOS formation increased with increasing temperature from 30 °C to 60 °C, but GOS production at all lactose conversion levels was almost unchanged with changing temperature. The mPOS–PANI matrix was also characterized by scanning electronic microscopy (SEM), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR), vibrating sample magnetometry (VSM), thermomagnetization, differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA).



## 1. Introduction

Galactooligosaccharides (GOS) are produced by the β-galactosidase catalytic transfer of one or more D-galactosyl units into the D-galactose moiety of lactose [1]. The properties of the final product depend on the source of the enzyme and conditions used in the reactions [2]. GOS are non digestible oligosaccharides acting as growth-promoting substrate for bifidobacteria in the human intestine [3,4].

β-Galactosidase is present in a variety of sources, including plants, animals and micro-organisms [5]. They are versatile biocatalysts used for lactose hydrolysis [6] facilitating milk digestibility and improving the functional properties of dairy products and for GOS formation. GOS synthesis has been studied by free [7] and immobilized enzymes [8–10], using whole cells [11] and by fermentation [12].

Immobilization is an important step in commercial and fundamental enzymology allowing the repetitive and economic utilization of enzymes [13]. Compared with free enzyme in solution, enzyme immobilized on a solid support provides many advantages, including enzyme reusability, continuous operation, controlled product formation, and simplified and efficient processing [8]. Recently, the synthesis of GOS by the action of *Aspergillus oryzae* β-galactosidase immobilized on magnetic polysiloxane–polyvinyl alcohol (mPOS–PVA) was studied, using glutaraldehyde as activating agent [10].

Polyaniline (PANI), a conducting polymer, has been used for immobilization of antigen from *Yersinia pestis* [14] and different enzymes, such as, lipase [15], horseradish peroxidase [12], glucose oxidase [16,17], xanthine oxidase [18].

In this study, magnetic particles of polysiloxane (POS) coated with PANI (mPOS–PANI) were synthesized and characterized. β-galactosidase was then covalent immobilized via glutaraldehyde on mPOS–PANI and used for lactose hydrolysis and GOS production.

## 2. Experimental

### 2.1. Synthesis, magnetization and polyaniline coating of support

POS particles were synthesized as follows: 5 ml of tetraethylorthosilicate (Fluka, Germany) and ethanol (Riedel-de Haën, Germany) were mixed in a beaker. After raising the temperature

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to 70 °C, under stirring, 100 μl of concentrated HCl was added and incubated for 50 min. The solution was distributed into ELISA microplates (100 ml/well) and allowed to solidify for about 72 h at 25 °C. The resulting beads were smashed using a mortar and pestle; the powder (2 g) was suspended in de-ionized water (100 ml) and 10 ml of a solution containing 0.6 M $FeCl_2$ and 1.1 M $FeCl_3$ (1:1) were added drop wise under magnetic stirring, pH was adjusted to 11.0 (using 33%, w/v, $NH_4OH$) and incubated for 30 min at 100 °C. The resulting magnetized particles were thoroughly washed with de-ionized water until pH 7.0, were dried at 105 °C overnight and finally sieved (<100 μm). The mPOS particles were treated with 0.1 M $KMnO_4$ solution at 50 °C overnight, washed with distilled water and immersed into 0.5 M aniline solution prepared in 1.0 M $HNO_3$. Polymerization was allowed to occur for 2 h at 25 °C and after that the mPOS–PANI were successively washed with distilled water, 0.1 M citric acid and distilled water.

### 2.2. β-Galactosidase immobilization

mPOS–PANI particles (100 mg) were incubated with 2.5% w/v glutaraldehyde (1 ml) and 20 mM citrate–phosphate buffer, pH 4.5 (9 ml) for 2 h under stirring at 25 °C. Activated mPOS–PANI were successively washed with distilled water and incubated overnight with 10 ml of β-galactosidase (4 mg solid/ml) from *A. oryzae* (SIGMA, Japan). The amounts of free and immobilized enzyme in the experiments were 0.149 mg and 3.41 mg/g of support, respectively.

### 2.3. β-Galactosidase activity and protein determinations

The β-galactosidase activity assays were carried out using lactose (50 g/l) with substrate prepared in citrate–phosphate buffer solution. The product released in the assay, glucose, was determined by HPLC analyze. One β-galactosidase unit (U) was defined as the amount of enzyme which liberated 1 μmol of glucose per min per mg of protein at 40 °C and pH 4.5. The protein concentration was determined according to the method described by Smith et al. [19] using bovine serum albumin as standard. The amount of immobilized protein was calculated by the difference between the amount of protein offered to the support for immobilization and that found in the supernatant and the washing buffers. $K_m$ and $V_m$ were calculated from Lineweaver Burk plots.

### 2.4. GOS production

GOSs formation kinetics with free and immobilized enzyme on mPOS–PANI was studied using lactose as substrate that was prepared by dissolving lactose in citrate–phosphate buffer solution. Samples were taken at appropriate time intervals and analyzed for sugar content by high performance liquid chromatography (HPLC). The reaction kinetics was studied at six different initial lactose concentrations (50, 100, 200, 300, 400 and 500 g/l) and four different temperatures (30 °C, 40 °C, 50 °C, and 60 °C).

### 2.5. HPLC analysis

An HPLC (Jasco AS-2057 Plus), employing a column of MetaCarb 67H at 60 °C, a refractive index detector (Jasco RI-2031 Plus) and a mobile phase 0,001 N $H_2SO_4$ at a flow rate of 0.5 ml $min^{-1}$ (Jasco PU-2080 Plus) was used for the determination of concentration of all sugars present in the assay solution (GOSs, lactose, glucose, and galactose). The concentration (w/v) of these sugars [in the retention time for lactose (9.66 min), glucose (11.32 min), galactose (12.03 min), and oligosaccharides including tri- (8.68 min), and tetra-saccharides (8.16 min)] are proportional to their peak areas. Thus, normalized sugar concentrations as weight percentages of total sugars or initial lactose were determined from peak areas and are reported in this work. It should be noted that the accuracy of this approximation was verified by checking the material balance.

### 2.6. Support characterization

The samples of uncoated magnetic particles and coated with PANI were mounted on stubs, coated with gold, and examined in a Leica Cambridge S360 scanning electronic microscope (SEM) and micrographs were taken. The X-ray diffraction patterns of the samples were collected on a Philips-PW1710 diffractometer with Cu Kα radiation ($\lambda = 1.54056$ Å). Infrared spectra were recorded on an ABB FTLA2000 spectrometer in the range of 500–4000 $cm^{-1}$ using KBr pellets. Thermal analyses (thermogravimetrical analysis (TGA) and differential scanning calorimetry (DSC)) of mPOS and mPOS–PANI were done using a TGA-50 and a DSC-50 both from Shimadzu (Izasa, S.A., Portugal). Acquisition of the results was done by TA-50WS software (version 1.14). Samples were weighed (10 mg; the exact mass was recorded) into aluminium pans (Izasa, S.A., Portugal) and heated over the temperature range 25–500 °C, at a scanning rate of 5 °C/min, under 20 ml/min nitrogen flow. The magnetic properties of sample mPOS–PANI were investigated by measuring its hysteresis loop at room temperature using an Oxford instrument Vibrating Sample Magnetometer (VSM) and the thermomagnetization from room temperature to 1000 K using a Cahn 2000 Curie–Faraday balance.

## 3. Results and discussion

The magnetic-β-galactosidase-POS-PANI composite presented 3.41 mg of protein per g of preparation (47.6% of offered protein under the experimental conditions). This water insoluble enzymatic derivative presented, respectively, apparent $K_m$ and maximum velocity of 50.7 M of lactose and 73 U/mg of protein that represents almost the double $K_m$ and 69.5% values of those estimated for the free enzyme (105 U/mg of protein and 25.8 M of lactose, respectively). The higher value found for the apparent $K_m$ of the immobilized enzyme compared to the free enzyme can be attributed to several microenvironment-events such partitioning, diffusional or mass transfer, conformational and steric effects whereas the decrease in maximum velocity value as a result of inactive immobilized enzyme molecules besides these events. Similar behavior was reported by Bayramoglu et al. [20] studying immobilized β-galactosidase onto magnetic poly(GMA-MMA) beads. They observed an increase of about 2.6-fold of apparent $K_m$ and decrease about 2.3-fold of maximum velocity, compared to the free enzyme. Gaur et al. [21] immobilized *A. oryzae* β-galactosidase on tree different methods of immobilization and also observed in all methods, as a result of immobilization, increased apparent $K_m$. It is worthwhile to register that in our lab an immobilized β-galactosidase onto a polysiloxane–polyvinyl alcohol magnetic composite did not show statistical difference between its apparent $K_m$ and the free enzyme $K_m$ [22].

### 3.1. Effects of lactose concentration and temperature on GOS production by free and immobilized enzyme

The initial lactose concentration is by far the most significant factor affecting GOS formation [2]. This is caused by the reduced water activity of the reactive solutions as substrate concentration increases [23]. Higher substrate concentrations and consequently reduced water availability make the transfer reactions of galactose to water molecules less likely to occur leading to a greater degree of polymerisation of oligosaccharides being formed [24]. Fig. 1A

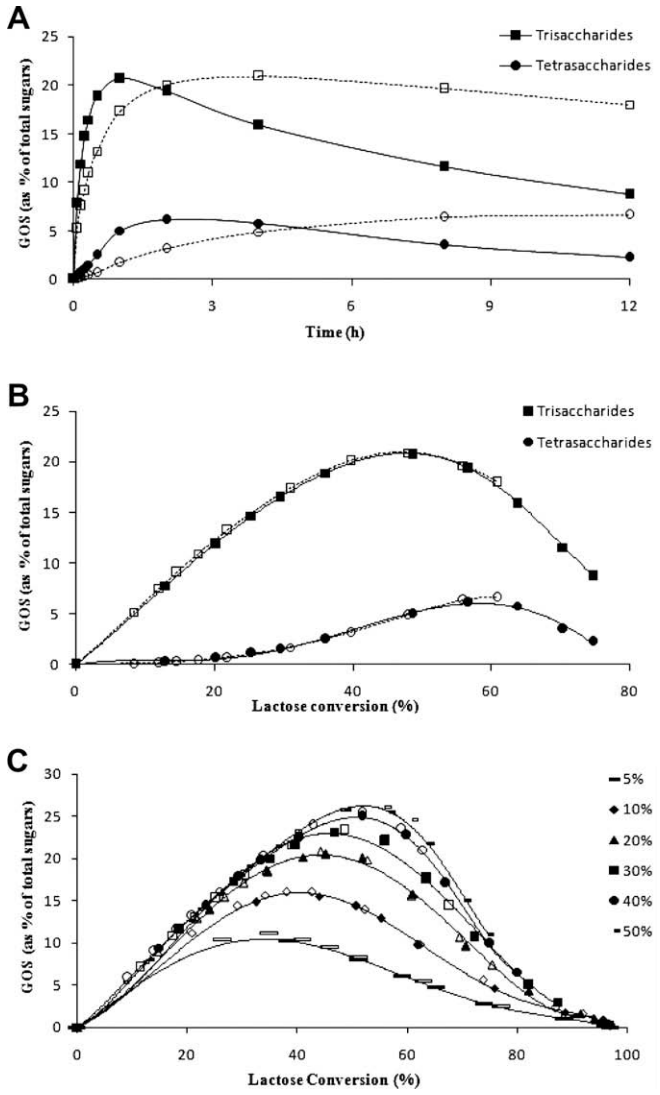


**Fig. 1.** GOS (tri- and tetra-saccharides) production by free (open symbols) and immobilized (closed symbols) *A. oryzae* β-galactosidase onto mPOS–PANI at pH 4.5 and 40 °C. Production using 50% (w/v) lactose in terms of time course (A); related to percent of lactose conversion (B) and using other lactose concentrations (C).

shows a typical tri- and tetra-saccharides time course production for 50%, w/v initial lactose concentration by the action of free and immobilized *A. oryzae* β–galactosidase. More detailed information on the catalytic behavior of free and immobilized enzymes can be obtained from the plots in Fig. 1B and C. Oligosaccharide concentration increased initially to a maximum and subsequently decreased when transgalactosylation activity became less pronounced than the hydrolytic activity [2]. Fig. 1B shows that the GOS (tri- and tetra-saccharides) production kinetics at 50% w/v initial lactose concentration is closely related to lactose conversion. Identical experiments were performed for the other lactose initial concentrations (5–40%, w/v) and similar results were obtained as can be seen in Fig. 1C representing the total mass of GOS (expressed as percent of total sugars) against the percent of hydrolyzed lactose.

Fig. 1C shows that GOS production increased with increasing lactose concentration. In Fig. 1B, it can noticed that the trisaccharide (80.5% of GOS total) production is higher, about 20.7% of total sugars for 50%, w/v of lactose, than the tetrasaccharide (24.2% of GOS total), about 6.2% of total sugars for 50% w/v of lactose.

No significative difference was observed for the free and immobilized enzymes. As the initial lactose concentration increased from 5% to 50%, the maximum GOS content increased from 11.2% (at 35% conversion) to 26.1% (at 56% conversion) for the free enzyme and from 10.3% (at 38% conversion) to 25.7% (at 49% conversion) for the immobilized enzyme. The maximum amount of tri-, tetra-saccharides and total GOS obtained for the immobilized enzyme were 103.5 g/l, 30.9 g/l and 128.6 g/l for a lactose conversion of about 49%, 57%, and 49%, respectively, in 500 g/l of lactose. For the free enzyme, 104.5, 33.2 and 130.3 g/l were obtained for about 48%, 61% and 56% lactose conversion, respectively in 500 g/l of lactose. These results suggest that enzyme immobilization on mPOS–PANI does not impose any limitation or changes on GOS formation

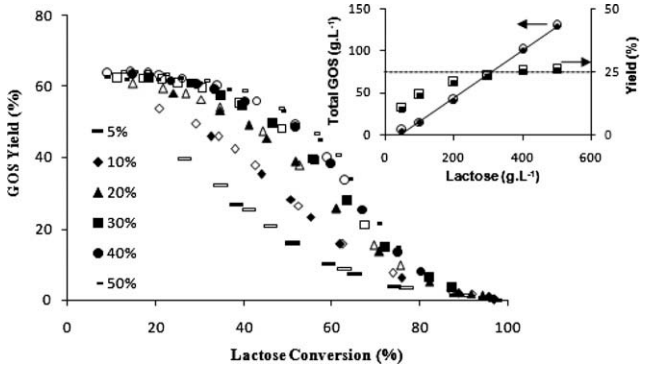


**Fig. 2.** GOS yield *versus* percent of lactose conversion by free (open symbols) and immobilized (closed symbols) *A. oryzae* β-galactosidase onto mPOS–PANI at pH 4.0 and 40 °C. The insert presents the same relationship expressed in terms of total GOS (g/l) and maximum GOS yield. The initial concentrations of lactose were 5% —; 10% (♦); 20% (▲); 30% (■); 40% (●) and 50% (■), w/v.

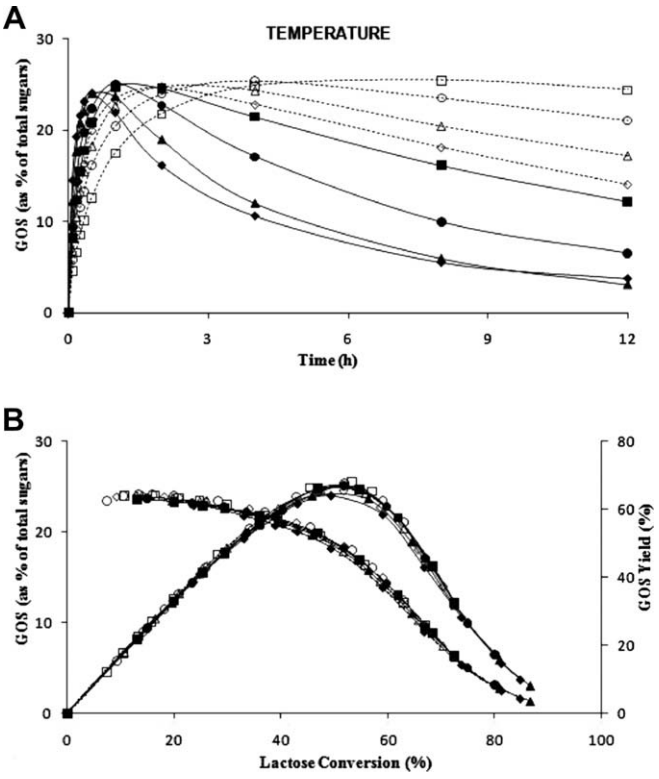


**Fig. 3.** Effect of temperature on the GOS production during the hydrolysis of 40% (w/v) lactose catalyzed by the free (open symbols) and immobilized (closed symbols) *A. oryzae* β-galactosidase onto mPOS–PANI. A: time course of GOS production; B: GOS production *versus* lactose consumption. The temperatures were 30 °C (■); 40 °C (●); 50 °C (▲); 60 °C (♦).

from lactose. Thus, the GOS formation ability of the enzyme was not affected by the immobilization of the enzyme onto mPOS–PANI.

β-Galactosidase immobilized on mPOS–PANI acting on 20% (w/v) lactose was successively reutilized for 10 cycles at 25 °C and the obtained activity was 87% of the initial one (data not shown). This performance of the mPOS–PANI β-galactosidase derivative is an additional advantage besides that from its easy of separation from the reaction medium by a magnetic field. It is worthwhile to draw attention that the free and immobilized *A. oryzae* β–galacotsidase were also capable to hydrolyze lactose into glucose and galactose but these results are not presented because the aim of this contribution was to investigate the transgalactosylation action of the enzyme.

GOS yields during lactose conversion catalyzed by the free and the immobilized β-galactosidase on mPOS–PVA are shown in Fig. 2. The maximum GOS yield increased with the initial lactose concentration by the catalysis of both enzyme preparations. The maximal values ranged from 39.5% (5%, w/v, lactose) to 64.1% (higher lactose concentrations) for the free enzyme and from 26.9% (5%, w/v, lactose) to 62.1% (higher lactose concentrations) for the immobilized one. It was generally observed that the hydrolysis and transgalactosylation reactions occurred simultaneously and that 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 [25]. The insert of Fig. 2 presents these same data under other perspective clearly showing that increases in lactose concentration lead to increases in GOS production. However, in terms of GOS yield, no increase was observed for lactose concentrations higher than 300 g/l, the yield value remaing constant at 23.3–26% for both free and immobilized enzyme. Neri et al. [10] with β-galactosidase from *A. oryzae* and Park et al. [26] with a thermostable β-galactosidase from *Sulfolobus solfataricus* observed the same behavior for a high lactose concentration.

Fig. 3 shows the effect of temperature on GOS production rate and yield during lactose hydrolysis catalyzed by free and immobilized enzyme preparations at pH 4.5 and 40% (w/v) initial lactose concentration. The reaction rates for lactose hydrolysis and GOS

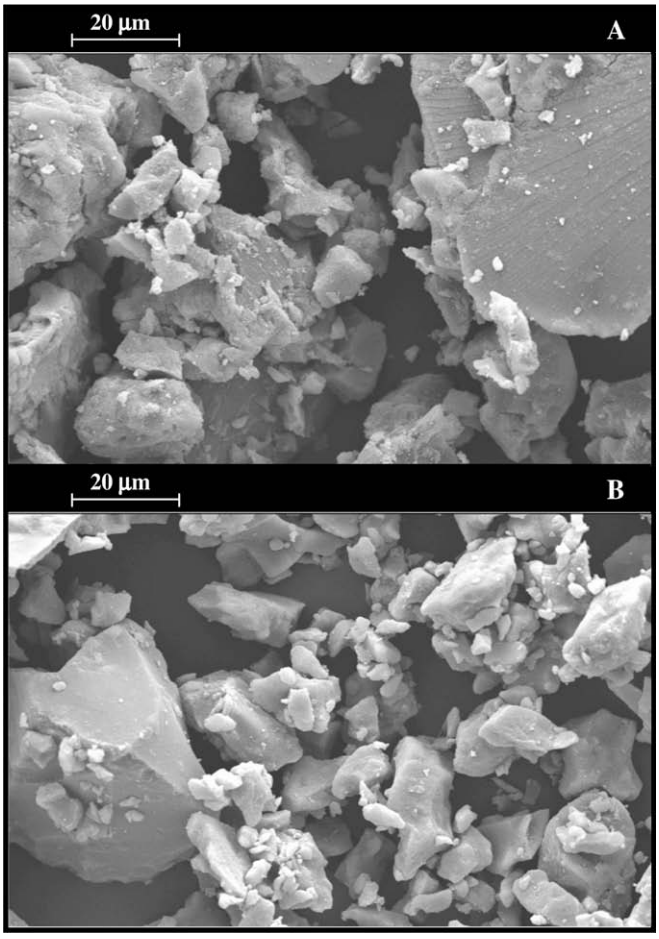


**Fig. 4.** SEM image of the mPOS (A) and mPOS–PANI (B).

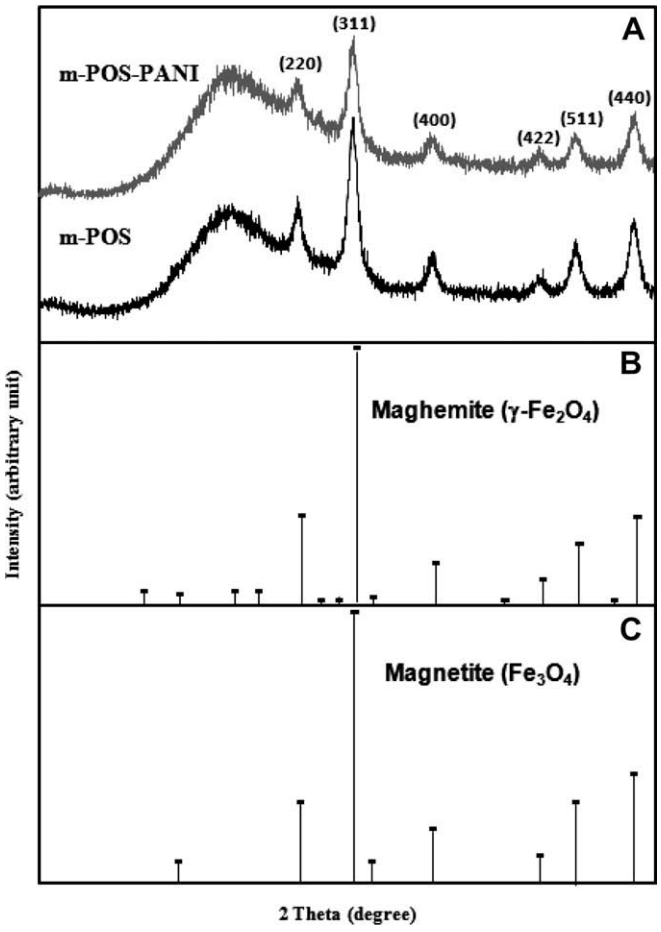


**Fig. 5.** XRD spectra of the mPOS and mPOS–PANI support (A) and patterns of iron oxides maghemite (B) and magnetite (C) according to the JCPDS database.

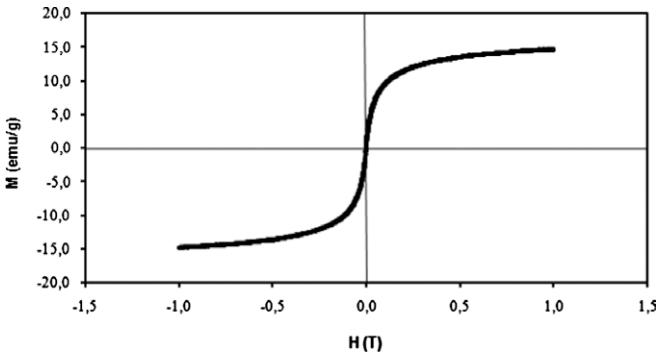


**Fig. 6.** Hysteretic loop of the mPOS–PANI at 295 K.

formation increased with increasing temperature from 30 °C to 60 °C, but GOS production at all lactose conversion levels was almost unchanged with changing temperature. Similar results were reported with *A. oryzae* β-galactosidase immobilized on Cotton Cloth by Albayrak and Yang [8] and on mPOS–PVA by Neri et al. [10].

### 3.2. Characterization

SEM analysis of the mPOS and mPOS–PANI revealed different sized 15–100 μm rhombohedra particles and no difference in the structure after coating with PANI was observed (Fig. 4).

Fig. 5 shows the results of X-ray diffraction analysis of uncoated magnetic composite of polysiloxane (mPOS) and coated with polyaniline (mPOS–PANI). Patterns of iron oxides of the Joint Committee on Powder Diffraction-International Centre for Diffraction Data database (JCPDS-ICDD) were included for comparison. Both magnetic composites presented broad peaks that can be ascribed to the spinel structure. Magnetite and maghemite are two iron oxides that crystallize in the spinel structure. They presented similar spectrum and it is difficult to distinguish one from another by X-ray diffraction pattern based on these features.

Magnetization measurements were performed and as can be observed in Fig. 6 the saturation magnetization was measured to be approximately 15 emu/g, lower than 60–70 emu/g reported for the small particles of magnetite [27,28]. The presence of cations vacancy in maghemite can be responsible for the decrease in the saturation of magnetization compared to magnetite [29].

The curves of the magnetic moment of the mPOS–PANI samples against temperature show a tendency to linear temperature dependence from 295 K until near the transition temperature $T_c$ = 860 K (Fig. 7). This was observed for 2 distinct applied fields $H$ = 0.51 T and 0.071 T (see Fig. 7A and 7B). It should be noted that the behaviour of the temperature dependences, as well as the Curie temperature remains unchanged after heating–cooling cycles.

As follows from literature, maghemite has a ferromagnetic structure with ferromagnetic ordering of ions in octahedral and tetrahedral positions. The sublattices are coupled through antiferromagnetic exchange [30–32]. The model accounting for the change in the spin of iron ions in tetrahedral positions offers a satisfactory explanation for the linear dependence of the magnetization against temperature [33] as observed in our data.

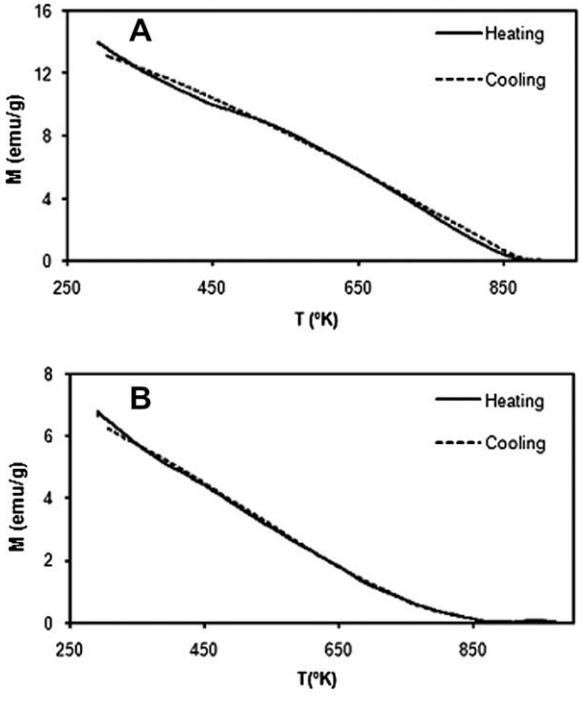


**Fig. 7.** Thermomagnetization of mPOS–PANI from 295 K to 1000 K for $H$ = 0.51 T (A) and 0.071 T (B).

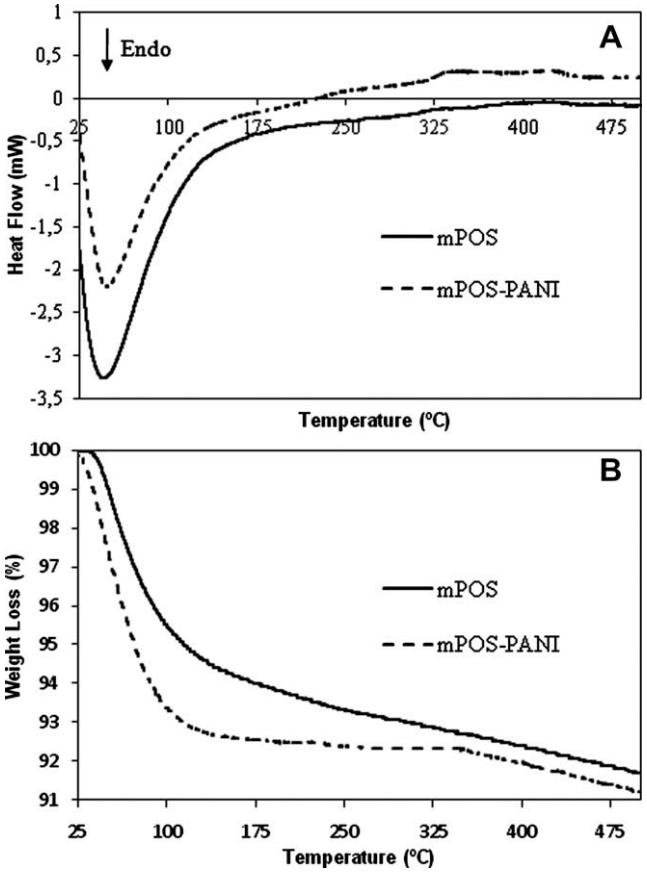


**Fig. 8.** DSC heat flow (A) and thermogravimetry curves (B) for mPOS and mPOS–PANI.

Thermoanalytical techniques, such as DSC and TGA, have been used to characterize polymers and POS has been receiving an increasing attention, due to its thermal resistance and endurance at high temperatures [34]. Fig. 8 shows the results from the thermal analysis relative to mPOS and mPOS–PANI. The DSC curves (Fig. 8A) show a clear endothermal peak, up to 175 °C, associated mostly to the loss of residual moisture. This is also evidenced by the greater weight loss in Fig. 8B. The remaining weight loss in mPOS and mPOS–PANI maybe mostly associated with the removal of retained solvent (ethanol) from the xerogel, as described by Retuert et al. [35]. PANI may also endure thermal degradation (starting near 325 °C). A similar degradation was reported by Fryczkowski et al. [36] and Basavaraja et al. [37] for PANI complexes, though at lower temperatures. The degradation of PANI in mPOS–PANI does not, however, entail significant enthalpic changes (Fig. 8A).

## 4. Conclusion

From the above displayed results one can conclude that immobilized β-galactosidase onto mPOS–PANI derivative is capable to hydrolyze lactose and to produce GOS as the free enzyme. The initial lactose concentration in the reaction media affected the total amounts of produced GOS and their time course production was described as a "bell-shaped" curve as a result of the balance

between transgalactosylation and hydrolysis. This water insoluble enzymatic derivative can be reused and collected by a magnetic field easily. The reaction rates for lactose hydrolysis and GOS formation increased with increasing temperature from 30 to 60 °C, but GOS production at all lactose conversion levels was almost unchanged with changing temperature. The magnetic particles have rhombohedra shape sizing 15–100 μm (SEM analysis) and are mostly maghemite (Magnetization measurements and X-ray diffraction analysis).

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## References

[1] R.R. Mahoney, Food Chem. 63 (1998) 147.
[2] M.A. Boon, A.E.M. Janssen, A. van der Padt, Enzyme Microb. Technol. 26 (2000) 271.
[3] K. Ohtsuka, Y. Benno, K. Endo, O. Ozawa, H. Ueda, T. Uchida, T. Mitsouka, Bifidus 2 (1989) 143.
[4] C. Kunz, S. Rudloff, Acta Paediatr. 82 (1993) 903.
[5] J.E. Prenosil, E. Stuker, J.R. Bourne, Biotechnol. Bioeng. 30 (1987) 1019.
[6] A.P. Bakken, C.G. Hill, C.H. Amundson, Biotechnol. Bioeng. 39 (1992) 408.
[7] S. Reuter, A.R. Nygaard, W. Zimmermann, Enzyme Microb. Technol. 25 (1999) 509.
[8] N. Albayrak, S.T. Yang, Biotechnol. Bioeng. 77 (2002) 8.
[9] C.W. Chen, C.C. Ou-Yang, C.W. Yeh, Enzyme Microb. Technol. 33 (2003) 497.
[10] D.F.M. Neri, V.M. Balcão, R.S. Costa, I.C.A.P. Rocha, E.M.F.C. Ferreira, D.P.M. Torres, L.R.M. Rodrigues, L.B. Carvalho Jr., J.A. Teixeira, Food Chem. (2008), doi:10.1016/j.foodchem.2008.11.068.
[11] G. Tzortzis, A.K. Goulas, G.R. Gibson, Appl. Microbiol. Biot. 68 (2005) 412.
[12] Z. Li, M. Xiao, L. Lu, Y. Li, Process Biochem. 43 (2008) 896.
[13] G.B. Oliveira, J.L. Lima-Filho, M.E.C. Chaves, W.M. Azevedo, L.B. Carvalho Jr., React. Funct. Polym. 68 (2008) 27.
[14] R.A.L. Coêlho, G.M.P. Santos, P.H.S. Azevêdo, G.A. Jaques, W.M. Azevedo, L.B. Carvalho Jr., J. Biomedic. Mat. Res. 56 (2001) 257.
[15] G. Lee, J. Kim, J. Lee, Enzyme Microb. Technol. 42 (2008) 466.
[16] V. Leite, V.L. Silva, W.M. Azevedo, E.H.M. Melo, J.L. Lima-Filho, Biotechnol. 87 (1994) 133.
[17] A.H. Parente, E.T. Marques Jr., W.M. Azevedo, F.B. Diniz, E.H. Melo, J.L. Lima-Filho, Appl. Biochem. Biotechnol. 37 (1992) 267.
[18] W. Nadruz Jr., E.T.A. Marques, W.M. Azevedo, J.L. Lima-Filho, L.B. Carvalho Jr., Braz. J. Med. Biol. Res. 29 (1996) 347.
[19] P.K. Smith, R.I. Khron, G.F. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, et al., Anal. Biochem. 150 (1985) 76.
[20] G. Bayramoglu, Y. Tunali, M.Y. Arica, Catal. Commun. 8 (2007) 1094.
[21] R. Gaur, H. Pant, R. Jain, S.K. Khare, Food Chem. 97 (2006) 426.
[22] D.F.M. Neri, V.M. Balcão, M.G. Carneiro-da-Cunha, L.B. Carvalho Jr., J.A. Teixeira, Catal. Commun. 9 (2008) 2334.
[23] R. Cruz, V.A. Cruz, J.G. Belote, M.O. Khenayfes, C. Dorta, L.H.S. Oliveira, E. Ardiles, A. Galli, Bioresource Technol. 70 (1999) 165.
[24] T. Hansson, P. Adlercreutz, Food Biotechnol. 15 (2001) 79.
[25] K. Iwasaki, M. Nakajima, S. Nakao, Process Biochem. 31 (1996) 69.
[26] H.Y. Park, H.J. Kim, J.K. Lee, D. Kim, D.K. Oh, World J. Microbiol. Biot. 24 (2007) 1553.
[27] S.F. Chin, M. Makha, C.L. Raston, M. Saunders, Chem. Commun. (2007) 1948.
[28] X. Liu, M.D. Kaminski, J.S. Riffle, H. Chen, M. Torno, M.R. Finck, L. Taylor, A.J. Rosengart, J. Magn. Magn. Mater. 311 (2007) 84.
[29] M.E. Mendoza, F. Donato, R. Silva, M.A. Pérez, J.L. Carrillo, J. Phys. Chem. Solids 66 (2005) 927.
[30] R. Janot, D. Guerard, J. Alloy Compd. 333 (2002) 302.
[31] G.M. Costa, E. De Grave, R.E. Vandenberghe, Hyperfine Interact. 117 (1998) 207.
[32] J. Restrepo, Y. Labaye, J.M. Greneche, Physica B 384 (2006) 221.
[33] G.I. Barinov, S.S. Aplesnin, Phys. Solid State 48 (2006) 84.
[34] H. Zhixiong, X. Wenfeng, X. Dongcai, J. Wuhan Univ. Technol. 21 (2006) 47.
[35] J. Retuert, A. Nuiiez, F. Martinez, Macromol. Rapid Comm. 18 (1997) 163.
[36] R. Fryczkowski, C. Slusarczyk, J. Fabia Synth. Met. 156 (2006) 310.
[37] C. Basavaraja, R. Pierson, T.K. Vishnuvardhan, Do Sung Huh, Eur. Polym. J. 44 (2008) 1556.