Environmental Engineering and Management Journal March 2015, Vol.14, No. 3, 631-638
http://omicron.ch.tuiasi.ro/EEMJ/

"Gheorghe Asachi" Technical University of Iasi, Romania

SIMPLE ECO-FRIENDLY *β*-GALACTOSIDASE IMMOBILIZATION ON FUNCTIONALIZED MAGNETIC PARTICLES FOR LACTOSE HYDROLYSIS

Kalim Belhacene[1], Elena Florentina Grosu[2], Alexandra Cristina Blaga[2], Pascal Dhulster[1], Mariana Pinteala[3], Renato Froidevaux[1*]

[1]*Charles VIOLLETTE Institute– Equipe Laboratoire des Procédés Biologiques, Génie Enzymatique et Microbien (ProBioGEM) – EA 1026, Université Lille 1 – Sciences et Technologies, Villeneuve d'Ascq, France*
[2]*"Gheorghe Asachi" Technical University of Iasi, Faculty of Chemical Engineering and Environmental Protection, Department of Organic, Biochemical and Food Engineering, 73 Prof. Dr. docent Dimitrie Mangeron Str., 700050 Iasi, Romania*
[3]*"Petru Poni" Institute of Macromolecular Chemistry of Romanian Academy, Center of Advanced Research in Bionanoconjugates and Biopolymers, 41A Aleea Grigore Ghica Voda, 700487 Iasi, Romania*

Abstract

β-galactosidase from *Aspergillus oryzae* was strongly immobilized on magnetic particles functionalized with amino groups. By simple incubation without any activating agents, electrostatic interactions between amino groups and enzymes allowed obtaining a strong linkage. The immobilization efficiency was studied with the quantification of amino groups of the particles and of immobilized *β*-galactosidase. Kinetic parameters, especially the maximal velocity *Vmax* and the affinity *Km*, were determined with two substrates, o-NPG and lactose, and compared with free enzyme values in order to evaluate the influence of our immobilization methodology on the kinetic behavior of the enzyme. Therefore, magnetic capacity of the functionalized particles allows recovering and reusing the support. Results show efficient immobilization of *β*-galactosidase (58 μg/mg of support), able to hydrolyze substrates during multiple cycles of use. Thus, magnetic particles functionalized with amino groups represent an attractive support for simple and efficient *β*-galactosidase immobilization process.

Key words: β-galactosidase, immobilization, lactose, magnetic, particles

Received: November, 2014; Revised final: March, 2015; Accepted: March, 2015

1. Introduction

β-galactosidase is an important enzyme biocatalyst used in food industry for the hydrolysis of lactose (Kim et al., 2001) necessary for lactose-intolerant people (Carpio et al., 2000). In addition, this enzyme catalyzes the formation of galacto-oligosaccharides, which are prebiotic additives for the so-called "healthy foods" (Matsumoto et al., 1989). However, since the price of *β*-galactosidase is quite high, the direct addition of the enzymes to the substrate is economically unacceptable (Mahoney, 1997). Immobilised *β*-galactosidase biocatalyst can be reused several times, which decrease the costs of the process (Genari et al., 2003). Several studies have been carried out to immobilize this enzyme to enhance the biotechnological conversion of food lactose in bioreactors (Roy and Gupta, 2003). The performance of an immobilized enzyme is mainly governed by the properties of supporting materials, the technique used to immobilize the enzyme, and the nature of reactor used (packed bed, fluidized bed or membrane reactor) (Marangoni, 2005; Roy and Gupta, 2003).

[*] Author to whom all correspondence should be addressed: e-mail: renato.froidevaux@univ-lille1.fr; Phone: +33(0)320417566; Fax: +33(0)328767356

A great number of methodologies (adsorption, covalent bonding, direct cross-linking, and entrapment) have been described to attach *β*-galactosidase to supports such as polymeric films, beads and fibrous matrices. However, most of the traditional methods require toxic reagents, surfactants, silanes or cross-linking agents such as glutaraldehyde, which may have detrimental effects on food and human health (Tanaka and Kawamoto, 1999).

Recent interest in nanotechnology has provided a wealth of diverse scaffolds (Xu et al., 2014) that could potentially support enzyme immobilization due to their potential applications in biotechnology (Sun et al., 2012), immunosensing and biomedical areas (Li et al., 2014). Immobilization of enzymes is advantageous for commercial application due to convenience in handling, ease of separation of enzymes from the reaction mixture and reuse, low product cost and a possible increase in thermal and pH stability (Husain, 2010; Lei et al., 2002; Tischer and Wedekind, 1999; Wang, 2006). An important requirement for protein immobilization is that the matrix should provide a biocompatible and inert environment (Krug, 2009), i.e. it should not interfere with the native structure of the protein, which thereby could compromise its biological activity (Mitchell et al., 2002).

Magnetic fields have been utilized in support systems to study enzyme immobilization (Bayramoglu et al., 2008; Gupta and Gupta, 2005; Kuroiwa et al., 2008; Pimentel et al., 2007; Selim et al., 2007). Several magnetic particles and magnetic supports such as microspheres of various biomaterials, encapsulating the magnetic particles and copolymers with magnetic particles have been used with good results (Dyal et al., 2003; Koneracka et al., 2002; Kouassi et al., 2005; Saiyed et al., 2003). The high surface to volume ratio provided by the magnetic particles favors high binding capacity and high catalytic specificity of the enzyme (Johnson et al., 2008; Konwarh et al., 2009). In addition, magnetic field susceptibility revealed a mechanism for efficient recovery of the enzyme complex thereby preventing the enzyme contamination of the final product.

A novel and efficient immobilization of *β*-galactosidase from *Aspergillus oryzae* was recently developed by using magnetic Fe_3O_4-chitosan particles as support (Pan et al., 2009). The magnetic Fe_3O_4-chitosan particles were prepared by electrostatic adsorption of chitosan onto the surface of Fe_3O_4 particles made through co-precipitation of Fe^{2+} and Fe^{3+}. *β*-galactosidase was covalently immobilized onto the composites using glutaraldehyde as activating agent. The immobilization process was optimized by examining immobilized time, crosslinking time, enzyme concentration, glutaraldehyde concentration, and initial pH values of glutaraldehyde and enzyme solution. As a result, the immobilized enzyme presented a higher storage, pH and thermal stability than the soluble enzyme. Galactooligosaccharides were formed with lactose as substrate by using the immobilized enzyme as biocatalyst with a maximum yield of 15.5% (w/v) obtained when 50% lactose was hydrolyzed (Pan et al., 2009). In the same way, Neri et al. (2008) immobilized *β*-galactosidase also on polysiloxane-polyvinyl alcohol magnetic composite for lactose hydrolysis using glutaraldehyde as activating agent for immobilization (Neri et al., 2008).

All these cases of immobilization implied the preparation of support with an activating agent, like glutaraldehyde necessary for immobilization process. The utilization of this kind of agent entails risk for the user and for the environment. In this work, we used magnetic particles functionalized with amino groups to immobilize *β*-galactosidase only by promoting the electrostatic interactions between the amino groups and the enzyme. With this methodology, we avoided the utilization of chemicals and toxics and thus proposed a support able to retain enzyme and maintain its activity for substrate hydrolysis.

The kinetic of the hydrolysis of synthetic (o-NPG) and natural (lactose) substrate was studied, both with the *β*-galactosidase in solution and immobilized on functionalized magnetic particles. Firstly, some preliminary research was carried out to determine the number of amine functions on the magnetic particle surface and the immobilization capacity. Secondly, kinetic studies have been performed in a range of concentrations of substrates. With the kinetic data obtained, a comparison was performed using Lineweaver-Burk representations in order to determine the effect of immobilization on the activity of the enzyme.

2. Materials and methods

2.1. Materials

β-galactosidase (*β*-D-galactoside galactohydrolase, E.C. 3.2.1.23, 10.4U/mg, MW: 105kDa) from *Aspergillus oryzae* was purchased by Sigma-Aldrich Chemical Co. Synthetic *β*-galactosidase substrate purchased from Sigma-Aldrich Chemical Co. *ortho*-nitrophenyl-*β*-D-galactopyranoside (o-NPG, C12H15NC, MW 301.3g/mol) was prepared in 0.02M sodium acetate buffer for UV-visible measurements. In order to inactivate the enzyme in experiments where the feasibility of the process was tested, 1M Na_2CO_3 solution was prepared. All aqueous solutions were prepared in distilled water.

The immobilization support was hydrophilic magnetite particles functionalized with amine groups gifted by the Institute of Macromolecular Chemistry “Petru Poni”, Iasi, Romania. This particles were constituted by a magnetic ferric core and hydrophobic shell formed by an oleic acid-oleyamine complex which modified by aminosilane monomer (Durdureanu-Angheluta et al., 2012).

2.2. Determination of the number of amine functions on the magnetic particles

In order to characterize the immobilization potential of the functionalized magnetic particles, the number of amino groups was determined by chemical tag using the 4-nitrobenzaldehyde (4-NBA). This compound reacts with the primary amine functions to form a Schiff base (Fig. 1) (Abbas, 2009). A 4-NBA 2mM solution was prepared in anhydrous methanol and incubated with a quantity of particles during 4h at 50°C. Then, the particles were washed twice 5 minutes by ethanol and methanol and dried. Finally, the particles were re-suspended in 3mL of distilled water containing 20µL of acetic acid during 15h at 50°C to hydrolyze the Schiff base formed. After hydrolysis, the solution was recovered and the quantity of 4-NBA was determined by spectrophotometry at 267nm and compared with a range of different concentrations of 4-NBA.

2.3. β-galactosidase immobilization on functionalized magnetic particles

One milligram of particles were weighed on microbalance (Sartorius ISO 9001 microbalance) and transferred on Eppendorf tube, washed with distilled water and sonicated fastly in order to homogenate the mix. After, the water was removed and the particles were dried and conserved at room temperature before utilization. Then, the dried particles were re-suspended in 1mL of acetate sodium buffer 0.02M pH4.5 containing 1mg of *β*-galactosidase. The particle and enzyme solutions were incubated together during one night at 4°C under agitation. After incubation, the mix was centrifuged and *β*-galactosidase solution was removed. Particles were washed several times with buffer solution to eliminate the non-fixed enzymes. The buffer used for the wash was conserved for released enzyme determination. Particles were stored dry at 4°C before enzymatic tests.

2.4. Determination of the immobilized β-galactosidase quantity

The determination of the quantity of immobilized enzyme was necessary to establish the immobilization capacity of the functionalized magnetic particles and also to determine the specific activity after carrying out of our immobilization procedure in comparison with free *β*-galactosidase. Then, the non-immobilized enzyme was quantified after incubation and also after particle wash, and was subtracted from the initial quantity of enzyme. For this, different quantities of particles (0.250 mg, 0.500 mg, 0.750 mg and 1mg) were incubated with 1mg of *β*-galactosidase on buffer, under agitation at 4°C during a night. After incubation, the mix was centrifuged at 13400 rpm, and the supernatant was removed and conserved for the enzyme quantification. Particles were washed by 1mL of buffer solution under agitation at 4°C, centrifuged and the supernatant was removed. 6 cycles of wash were done, and the buffer was conserved for enzyme quantification. The quantity of *β*-galactosidase was determined by enzyme test with the synthetic substrate o-NPG. 100µL of buffer used for the wash were introduced in 2 mL of o-NPG 10mM. After 10 minutes, the reaction was stopped by addition of 1mL of Na_2CO_3 1M. The absorbance of the sample was measured by spectrophotometer at 415nm. Using range of o-NP, the reaction rate of the sample was calculated and compared with a range of reaction rate determined in function of enzyme concentrations (0.01, 0.05, 0.1, 0.5 and 1mg/mL), allowing the determination of enzyme quantity (Fig. 2).

2.5. Determination of immobilized β-galactosidase activity

Two substrates were used to determine the catalytic efficiency of enzyme free and immobilized on functionalized magnetic particles: o-NPG and lactose.

2.5.1. With synthetic o-NPG substrate

The determination of *β*-galactosidase activity was investigated with o-NPG (o-nitrophenyl B-d-galactopyranoside) prepared in sodium acetate buffer 0.02M pH4.5. Hydrolysis gave a colored product (o-NP) easy to analyze by absorbance. Enzyme was introduced in 10mL of o-NPG, under agitation. 500µL were collected at different times (each minute until 10 minutes and after 15, 20, 30, 45 and 60 minutes) and mixed with 250µL of Na_2CO_3 1M to stop the reaction. Collected samples were analyzed at 415nm (UV/visible spectrophotometer, Ultraspec 1100 pro, Amersham Biosciences).

For immobilized *β*-galactosidase on functionalized magnetic particles, substrate solution was carried out with the particles and enzymatic activity was determined with the same protocol that for free *β*-galactosidase. After reaction, particles were collected and washed 3 times to eliminate the potential fixation of substrate or product.

For each condition (free and immobilized *β*-galactosidase), in order to determine the kinetic parameters of the enzyme, different concentrations of substrate were tested (0.5, 1, 2, 5 and 10mM), the concentration of product was quantified with a range of o-NP and parameters were calculated using Michaelis-Menten and Lineweaver-Burk representations.

2.5.2. With lactose substrate

Lactose was used in order to evaluate the variation of the catalytic performance with a substrate commonly encountered in industrial process. 1mg of enzyme was introduced in 10mL of different concentration of lactose (10mM, 25mM, 50mM and 100mM prepared in acetate buffer 0.02M pH 4.5, under agitation. 300µL were collected at different times (each minute until 10 minutes and

after 15, 20, 30, 45 and 60 minutes) and mixed with 150µL of Na_2CO_3 1M to stop the reaction. 10µL of the mix were added on 1mL of glucose enzymatic kit and incubated at 37°C during 10 minutes.

The glucose produced during the hydrolysis reaction reacted with the glucose oxydase to produce hydrogen peroxide which reacted with peroxydase to obtain a colored product (quinoneimine). Then, the sample was analyzed at 505nm to determine the quantity of glucose produced and compared with a range of glucose (Fig. 3). For immobilized *β*-galactosidase, 1mg of functionalized magnetic particles were mixed with different concentrations of lactose (80, 100, 150 and 200mM) and the reaction was studied with the same protocol that for free enzyme. Kinetic parameters were also determined with Michaelis-Menten and Lineweaver-Burk representations.

2.6. Performance of immobilized β-galactosidase on functionalized magnetic particles

In order to prove the efficiency of the immobilization procedure, the performance of the immobilized enzymes was evaluated by several activity tests with the same particles. An amount of 1mg of functionalized magnetic particles was introduced in 10mL of o-NPG 10mM. Each minute until 10 minutes then after 15, 20, 30, 45 and 60 minutes, 300µL were collected and mixed with 150µL of Na_2CO_3 1M to stop the reaction.

Samples collected were analyzed at 415nm. The same experiment was reproduced 10 times. The reaction rate of immobilized enzyme, for each experiment, was calculated with the Michaelis-Menten equation.

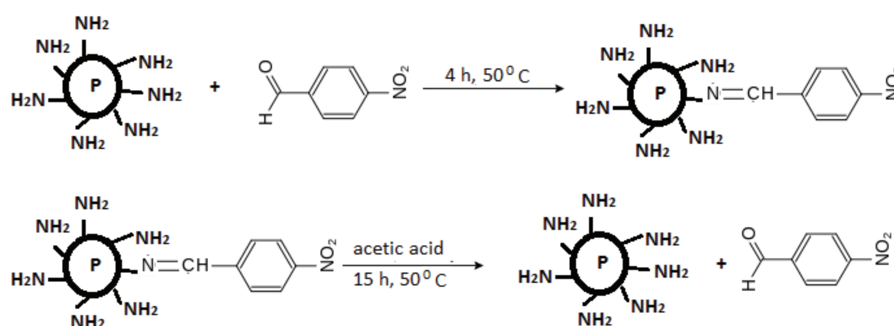


Fig. 1. Principle of reaction between amino groups and NBA; NBA is complexed with amino groups to form a Schiff base which is hydrolyzed in acidic conditions to release NBA analyzable at 267nm

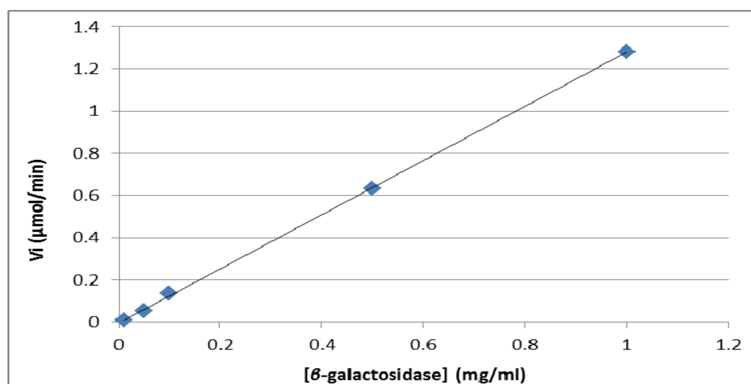


Fig. 2. Range of enzyme reaction rate in function of *β*-galactosidase concentration

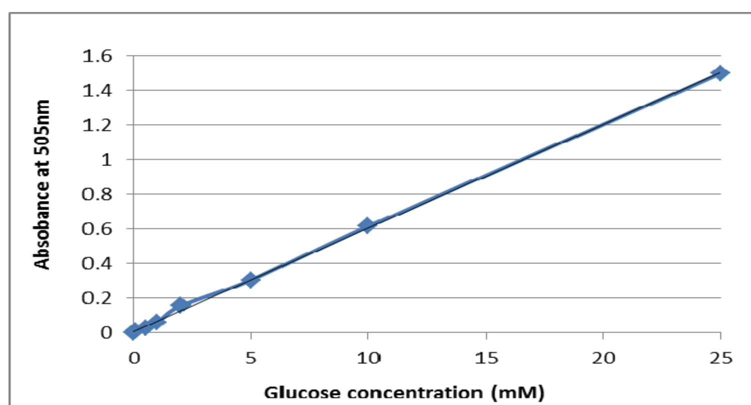


Fig. 3. Determination of glucose concentration with Glucose RTU

3. Results and discussion

3.1. Chemical characterization of magnetic particle surface

The magnetic particles used in our study were constituted by a ferric core covered by an oleic acid shell and functionalized with 3-aminopropyltriethoxysilane, leading to amino groups on the surface of the particles (Durdureanu-Angheluta et al., 2012).

In order to determine the potential of immobilization, the number of amino groups was calculated. For this, the labeling method using 4-NitroBenzAldehyde (4-NBA) as tag was used (Abbas, 2009) with four different quantities of particles (Table 1). Results allowed characterizing the chemical surface of the magnetic particles and we observed a good proportionality between the number of amine functions and the quantity of particles. Therefore, 1 mg of particles contains about $4.1.10^{17}$ amine functions.

3.2. Immobilization capacity of functionalized magnetic particles

In order to determine the quantity of immobilized *β*-galactosidase, the quantity of non-adsorbed enzyme and the mass balance were calculated after each wash of functionalized magnetic particles, and compared with the initial quantity carried out.These quantities were determined with a range of reaction rate in function of the concentration of enzyme (Fig. 2). The quantity of enzyme was resumed in the Table 1. After incubation of particles with 1mg of enzyme, the solution contained between 92 and 97% of the initial quantity of enzyme (Fig. 4). Results showed a very good proportionality of the quantity of enzyme immobilized in function of particles quantity (Fig. 5). Therefore, one milligram of functionalized magnetic particles was able to immobilize 58µg of *β*-galactosidase.

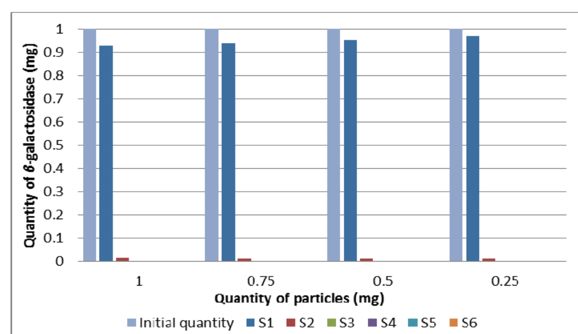


Fig. 4. Quantification of *β*-galactosidase after each step of particle treatment

3.3. Determination of kinetic parameters

The activity of free and immobilized *β*-galactosidase was determined according to the hydrolysis of synthetic substrate o-NPG.

In the study with free *β*-galactosidase, 100µg of enzyme were introduced with 10 milliliters of different concentrations of o-NPG (0.5, 1, 2, 5 and 10mM) under agitation. Each minute, 500µL were collected, mixed with 250µL of Na_2CO_3 1M and analyzed at 415nm. The kinetic parameters (*Km* and *Vmax*) were determined from the Lineweaver-Burk representation.

Concerning the study with immobilized *β*-galactosidase, one milligram of functionalized magnetic particles was introduced in 10 milliliters of o-NPG solutions (0.5, 1, 2, 5 and 10mM) under agitation. The kinetic parameters for the immobilized *β*-galactosidase were determined with the same procedure than with free enzyme. The values were resumed in the Table 2.

Table 1. Quantification of the number of amine functions in function of the quantity of functionalized magnetic particles

	0.250mg	*0.500mg*	*0.750mg*	*1mg*
Concentration of NBA (mM)	0.052	0.115	0.173	0.235
Number of amine functions	$0.94.10^{17}$	$2.09.10^{17}$	$3.09.10^{17}$	$4.15.10^{17}$

Table 2. Kinetic parameters of free and immobilized *β*-galactosidase determined with o-NPG

	Km (mM)	*Vmax (µmol/ min)*	*Specific Vmax (µmol/ min/mg)*	*Kcat (s^{-1})*	*Kcat/Km ($mM^{-1}.s^{-1}$)*
Free *β*-galacto sidase	1.899	1.044	10.44	17.85	9.39
Immobilized *β*-galacto sidase	5.82	0.62	10.68	18.72	3.21

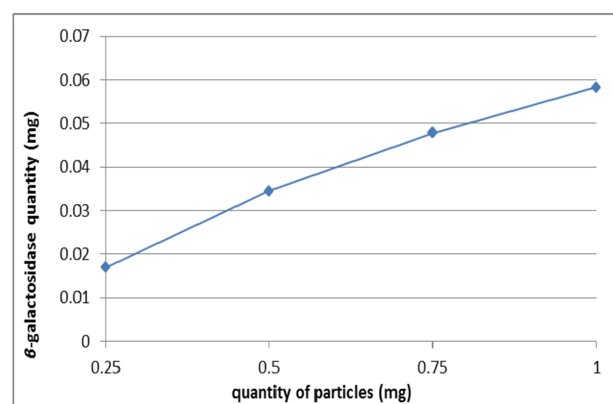


Fig. 5. Quantity of *β*-galactosidase immobilized in function of the quantity of functionalized magnetic particles

Results show a decrease of the maximal velocity *Vmax* and an important increase of the *Km*, translating a high decrease of the affinity of immobilized *β*-galactosidase for the substrate. Indeed, in the immobilized case, *β*-galactosidase adsorbed on the magnetic particles causes diffusional

limitations and consequently limited access to the active sites. Then, this phenomenon could be logically responsible of the kinetic parameter differences between free and immobilized β-galactosidase. However, when the values of *Vmax* were calculated in function of the quantity of enzyme, we observed the same specific *Vmax* values, indicating that our immobilization procedure did not affect the reaction rate of enzyme.

These results were confirmed with the values of the catalytic constant *Kcat* that was the same for free and immobilized β-galactosidase (~95% conserved in immobilized form compared with free form). Finally, catalytic efficiency values were different (3 times higher for free than for immobilized enzymes), translating a decrease of the global specificity of enzyme for this substrate, probably caused by the immobilization conditions of enzyme on the particles.

To conclude, the hydrolysis of o-NPG by β-galactosidase immobilized on functionalized magnetic particles showed a good conservation of the catalytic efficiency but the process slightly affected the affinity of enzyme for its substrate.

3.4. Efficiency of the immobilization procedure

The more significant advantage when enzyme immobilization is investigated is the ability to reuse the materials with adsorbed enzyme and its influence on the enzyme activity. Then, in order to evaluate the efficiency of our immobilization procedure, 11 cycles of o-NPG hydrolysis were carried out with the same functionalized magnetic particles with immobilized β-galactosidase. The residual activity was then calculated after each cycle and resumed in Fig. 6.

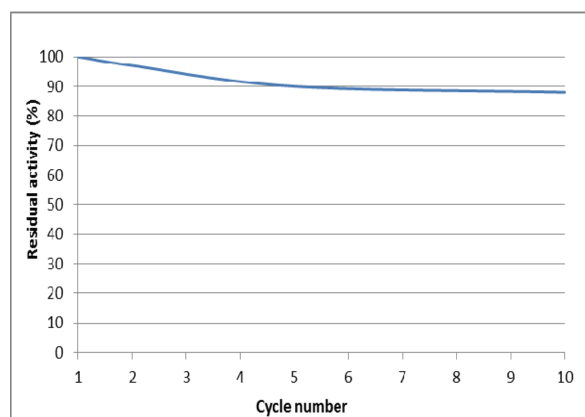


Fig. 6. Residual activity of immobilized β-galactosidase obtained after several cycles of o-NPG hydrolysis

Results show that the residual activity of immobilized β-galactosidase decreases of only 10% after 5 wash cycles. Then, the enzyme activity is always maintained after 10 cycles. This result demonstrates the efficiency of our immobilization procedure on functionalized magnetic particles and the possibility of immobilized β-galactosidase reuse while conserving its activity.

3.5. Lactose hydrolysis by β-galactosidase immobilized on functionalized magnetic particles

Hydrolysis of lactose was carried out with β-galactosidase immobilized on functionalized magnetic particles and kinetic was compared with those realized with free enzyme. Concerning the study with free β-galactosidase, one milligram of enzyme was introduced in 10mL of different concentrations of lactose (10, 25, 50 and 100mM) under agitation. Each minute, 300µL were collected and analyzed in order to determine the quantity of glucose produced.

Concerning the study with β-galactosidase in immobilized state, one milligram of particles was introduced in 3mL of different concentrations of lactose (80, 100, 150 and 200mM) under agitation. For each study, the kinetic of lactose hydrolysis was represented using Michaelis-Menten and Linewaever-Burk representations (Figs. 7 and 8) and kinetic parameters were then determined in the Table 3. Results show a very important increase of *Km* (45 folds) associated with a decrease of *Vmax* (6 folds).

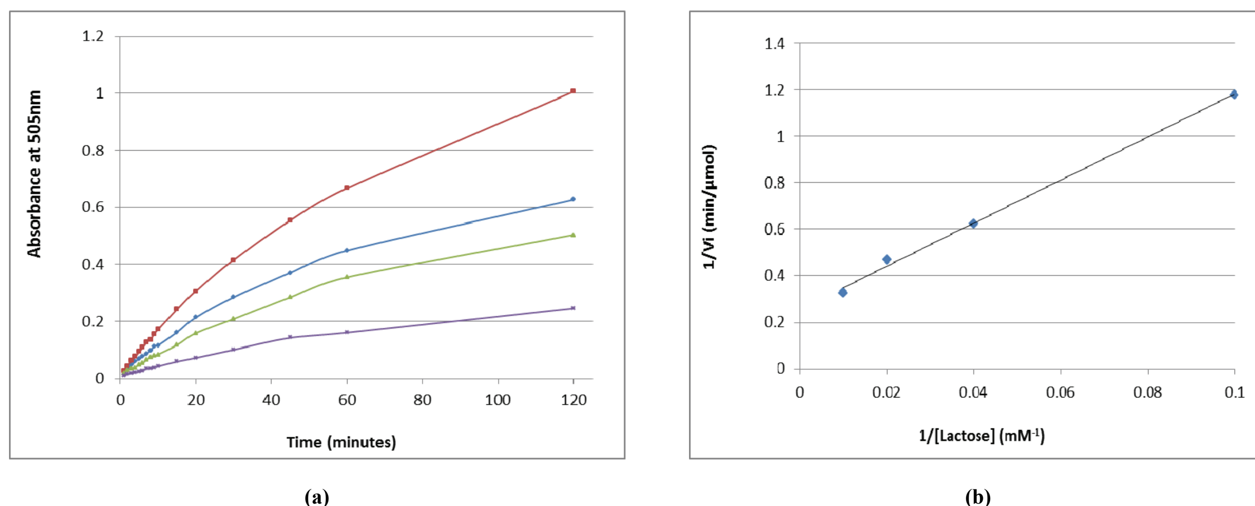
The specific enzyme activity and the catalytic constant were then affected, translating a strong impact of the immobilization process on the apparent enzyme activity. Therefore, the catalytic efficiency value was very low when the β-galactosidase was immobilized (15 folds lower than in free state), caused by the high value of *Km*. By comparing the results of kinetic parameters between the two substrates (Tables 2 and 3), we observe a strong impact of our immobilization procedure on lactose hydrolysis comparing to o-NPG hydrolysis.

It seems that the diffusional effects further influence the hydrolysis kinetic, probably due to the strong quantity of enzyme molecules immobilized on the magnetic particle surface. Indeed, the surface characterization showed an important quantity of amino groups allowing the immobilization of a high amount of β-galactosidase.

However, this high quantity would strongly limit the lactose accessibility to the active sites of the enzyme, also affecting its kinetic behavior. Solution could be to decrease the quantity of β-galactosidase immobilized on the functionalized magnetic particles in order to favor its catalytic efficiency.

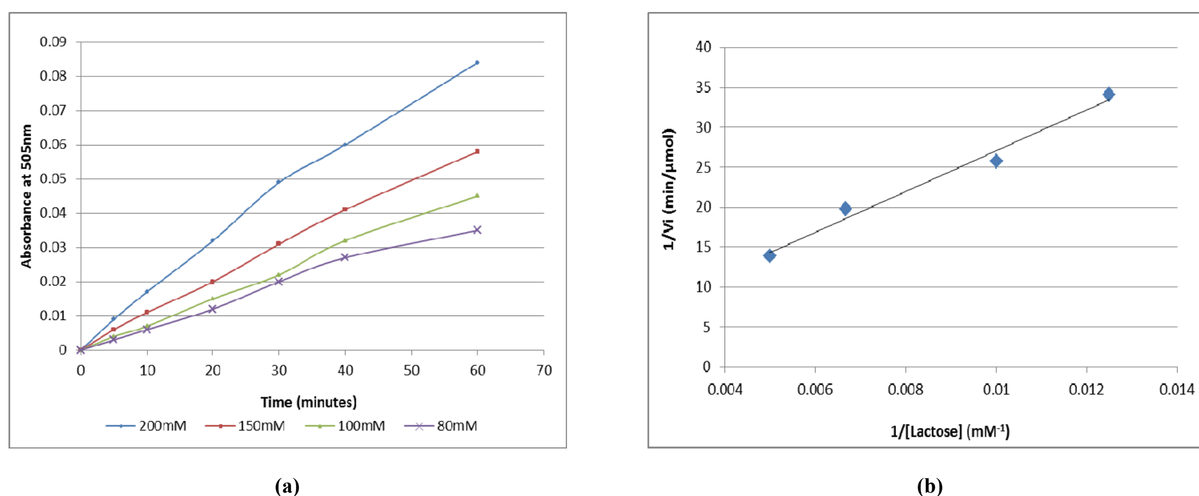
4. Conclusions

Utilization of magnetic particles functionalized with amino groups allowed immobilizing β-galactosidase for obtaining biofunctional support able to hydrolyze synthetic (oNPG) and natural (lactose) substrate. This support retains its activity after several assays, demonstrating the efficiency of our immobilization procedure. Without use of chemical products for immobilization process, such as glutaraldehyde commonly used, this kind of magnetic particles constitutes an interesting alternative for green process.



(a) **(b)**

Fig. 7. Determination of kinetic parameters of free *β*-galactosidase for lactose hydrolysis with different concentrations of substrate (10mM, 25mM, 50mM and 100mM) with 1mg of enzyme and using Michaelis-Menten (a) and Lineweaver-Burk (b) representations



(a) **(b)**

Fig. 8. Determination of kinetic parameters of *β*-galactosidase immobilized on one milligram of functionalized magnetic particles. Reaction carried out was lactose hydrolysis for different concentrations (80, 100, 150 and 200mM) using Michaelis-Menten (a) and Lineweaver-Burk (b) representations

Table 3. Kinetic parameters of free and immobilized *β*-galactosidase determined with lactose

	Km (mM)	*Vmax (µmol/min)*	*Specific Vmax (µmol/min/mg)*	*Kcat (s^{-1})*	*Kcat/Km ($mM^{-1}.s^{-1}$)*
Free *β*-galactosidase	36	3.89	38.9	6.80	0.189
Immobilized *β*-galactosidase	1639	0.64	11.03	19.38	0.012

Moreover, the magnetic property of the particles facilitates the manipulation during the hydrolysis application and allows a fast recovery after utilization. Therefore, a continuous process for lactose hydrolysis could be envisaged.

References

Abbas A., Vivien C., Bocquet B., Guillochon D., Supiot P., (2009), Preparation and multicharacterization of plasma polymerized allylamine films, *Plasma Processes and Polymers*, **6**, 593-604.

Bayramoğlu G., Kiralp S., Yilmaz M., Toppare L., Arica M.Y., (2008), Covalent immobilization of chloroperoxidase onto magnetic beads: Catalytic properties and Stability, *Biochemical Engineering Journal*, **38**, 180-188.

Carpio C., Gonzalez P., Ruales J., Batista-Viera F, (2000), Bone-bound enzymes for food industry application, *Food Chemistry*, **68**, 403–409.

Durdureanu-Angheluta A., Dascalu A., Fifere A., Coroaba A., Pricop L., Chiriac H., Tura V., Pinteala M., Simionescu B.C., (2012), Progress in the synthesis and characterization of magnetite nanoparticles with amino

groups on the surface, *Journal of Magnetism and Magnetic Materials*, **324**, 1679-1689.

Dyal A., Loos K., Noto M., Chang S.W., Spagnoli C., Shafi K., Ulman A., Cowman M., Gross R.A., (2003), Activity of Candida rugosa lipase immobilized on γ-Fe_2O_3 magnetic nanoparticles, *Journal of American Chemical Society*, **125**, 1684-1685.

Genari A.N., Passos F.V., Passos F.M.L., (2003), Configuration of bioreactor for milk lactose hydrolysis, *Journal of Dairy Science*, **86**, 2783–2789.

Gupta A.K., Gupta M., (2005), Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications, *Biomaterials*, **26**, 3995–4021.

Husain Q., (2010), β Galactosidase and their potential applications, *Critical Review in Biotechnology*, **30**, 41–62.

Johnson A.K., Zawadzka A.M., Deobald L.A., Crawford R.L., Paszczynski A.J., (2008), Novel method for immobilization of enzymes to magnetic nanoparticles, *Journal of Nanoparticle Research*, **10**, 1009–1025.

Kim J.H., Lee D.H., Lee J.S., (2001), Production of galactooligosaccharide by galactosidase from *Kluyveromyces maxianus* var lactis OE-20, *Biotechnology and Bioprocess Engineering*, **6**, 337–340.

Koneracka M., Kopčanský P., Timko M., Ramchand C.N., De Sequeira A., Trevan M., (2002), Direct binding procedure of proteins and enzymes to fine magnetic particles, *Journal of Molecular Catalisys B: Enzymatic*, **18**, 13-18.

Konwarh R., Karak N., Rai S.K., Mukherjee A.K., (2009), Polymer-assisted iron oxide magnetic nanoparticle immobilized keratinase, *Nanotechnology*, **20**, 25–35.

Kouassi G.K., Irudayaraj J., McCarty G., (2005), Examination of cholesterol oxidase attachment to magnetic nanoparticles, *Journal of Nanobiotechnology*, **3**, 1–9.

Krug H.F., (2009), *Nanotechnology*, Vol. 2, *Environmental Aspects*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Kuroiwa T., Noguchi Y., Nakajima M., Sato S., Mukataka S., Ichikawa S., (2008), Production of chitosan oligosaccharides using chitosanase immobilized on amylase coated magnetic nanoparticles, *Process Biochemistry*, **43**, 62–69.

Lei C., Shin Y., Liu J., Ackerman E.J., (2002), Entrapping enzyme in a functionalized nanoporous support, *Journal of American Chemical Society*, **124**, 11242–11243.

Mahoney R.R., (1997), *Lactose: Enzymatic Modification*, P. F. Fox Chapman and Hall, New York.

Marangoni A.G., (2005), *Enzyme Kinetics: a Modern Approach*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Matsumoto K., Kobayashi Y., Tamura N., Watanabe Y., Kan T., (1989), Production of galactooligosaccharides with β-galactosidase, *Denpun Kagaku*, **36**, 123–130.

Mitchell D.T., Lee S.B., Trofin L., Li N., Nevanen T.K., Soderlund H., martin C.R., (2002), Smart nanotubes for bioseparations and biocatalysis, *Journal of American Chemical Society*, **124**, 11864–11865.

Neri D.F.M., Balcao V.M., Carneiro-da-Cunha M.G., Carvalho L.G., Teixeira Jr J.R., (2008), Immobilization of β-galactosidase from *Kluyveromyces lactis* onto a polysiloxane-polyvinyl alcohol mangnetic (mPOS-PVA) composite for lactose hydrolysis, *Catalysis Communications*, **9**, 2334-2339.

Pan C., Hu B., Li W., Sun Y., Ye H., Zeng X., (2009), Novel and efficient method for immobilization and stabilization of β-D-galactosidase by covalent attachment onto magnetic Fe_3O_4-chitosan nanoparticles, *Journal of Molecular Catalisys B: Enzymatic*, **6**, 208-215.

Pimentel M.C.B., Leao A.B.F., Melo E.H.M., Ledingham W.M., Lima-Filho J.L., Sivewright M., (2007), Immobilization of *Candida rugosa* lipase on magnetized dacron: kinetic study, *Artificial Cells Blood Substitutes and Biotechnology*, **35**, 21–35.

Roy I., Gupta M.N., (2003), Lactose hydrolysis by LactozymTM immobilized on cellulose beads in batch and fluidized bed modes, *Process Biochemistry*, **39**, 325–332.

Saiyed Z.M., Telang S.D., Ramchand C.N., (2003), Application of magnetic techniques in the field of drug discovery and biomedicine, *Biomagnetic Research Technolology*, **1**, 2–8.

Selim K.M.K., Ha Y.S., Kim S.J., Chang Y., Kim T.J., Lee G.H., et al., (2007), Surface modification of magnetite nanoparticles using lactobionic acid and their interaction with hepatocytes, *Biomaterials*, **28**, 710–716.

Sun L., Yihan Li Y., Yang P., Zhu G., Dovichi N.J., (2012), High efficiency and quantitatively reproducible protein digestion by trypsin-immobilized magnetic microspheres, *Journal of Chromatography A*, **1220**, 68-74.

Tanaka A., Kawamoto T., (1999), Cell and Enzyme Immobilization, *American Society for Microbiology*, Washington, **94**, 504-513.

Tischer W., Wedekind F., (1999), Immobilized enzymes: methods and applications, *Topics in Current Chemistry*, **200**, 95-126.

Wang P., (2006), Nanoscale biocatalyst systems, *Current Opinion in Biotechnology*, **17**, 574-579.

Xu Y., Li C., Zhu X., Huang W.E., Zhang D., (2014), Application of magnetic nanoparticles in drinking water purification, *Environmental Engineering and Management Journal*, **13**, 2023-2029.