JAP&T
TPA-Timişoara

Available online at http://journal-of-agroalimentary.ro

Journal of Agroalimentary Processes and Technologies 2015, 21(4), 315-324

Journal of Agroalimentary Processes and Technologies

Stability of β-D-galactosidase covalently immobilized onto chitosan microparticles

Emese Biró[1], Daniel Budugan[1], Agnes Sz. Nemeth[2], Francisc Péter[1*]

[1]Department of Applied Chemistry and Engineering of Organic and Natural Compounds, University Politehnica Timişoara, Carol Telbisz 6, 300001 - Timişoara, Romania,
[2]Research Institute of Chemical and Process Engineering, Faculty of Information Technology, University of Pannonia, Egyetem utca 10, H-8200 Veszprém, Hungary

Received: 06 October 2015; Accepted: 28 October 2015

Abstract

β-Galactosidase (β-gal), an important enzyme used in the food industry to hydrolyze milk and whey, was immobilized onto chitosan microparticles prepared by the emulsion crosslinking method. The immobilization conditions such as enzyme binding capacity of the microparticles, crosslinking agent concentration, and immobilization time were optimized. A 3% glutaraldehyde concentration was found as optimal and allowed an enzyme loading of 27 mg protein/g dry support. Reduction with sodium borohydride after the covalent attachment was beneficial for the catalytic activity, leading to a total activity yield of 23.5% related to the native *Kluyveromyces lactis* β-gal subjected to immobilization. The immobilized biocatalyst was characterized by pH dependence, thermostability and distribution of protein by FITC labeling.

Keywords: β-galactosidase, immobilization, chitosan, thermal stability, pH profile

1. Introduction

Enzymes are very specific and efficient catalytically active proteins, which can work without need of extreme temperatures, high pressures or corrosive conditions, as it is the case for the majority of chemical catalysts. Nowadays, one of the most important driving forces in science is to develop green, sustainable technologies. Beyond the mild reaction conditions, shorter synthetic routes, biodegradability of the catalyst and use of environmentally friendly solvents, biocatalytic processes are also characterized by high chemo-, regio-, and stereoselectivities, resulting in lower amount of generated waste.

Despite their excellent catalytic properties, some characteristics of enzymes are not adequate for industrial applications: difficult recovery for reutilization, unsatisfactory thermal, pH, storage and operational stability, possible inhibition by substrates and products, low selectivity against non-natural substrates. Therefore, enzyme properties need to be significantly improved before their use as industrial catalysts. There are several tools to achieve such an improvement, immobilization being one of the most significant. The most important characteristics of immobilized enzymes are simplicity, cost effectiveness and stability [1].

Enzymatic hydrolysis of lactose is one of the most important biotechnological processes in the food industry [2]. It can carried out by acid or enzymatic catalysis, but the most important processes of lactose hydrolysis from milk or whey utilize β-galactosidase (or lactase), an enzyme widely distributed in nature, that can be isolated from several microbial sources [3]. The main benefit of lactose-hydrolyzed products is that they represent one of the possible approaches

Corresponding author: *e-mail:* francisc.peter@upt.ro

to diminish lactose intolerance, present in more than half of population of the world [4].

In past years β-gal was immobilized by several methods, including biospecific adsorption [5], entrapment in alginate-chitosan core-shell microcapsules [6], or cross-linked enzyme aggregates (CLEA) [7], but covalent binding was certainly the most widely used technique, as allowed a large number of attachment possibilities between supports and enzymes. The immobilization of enzymes by covalent attachment to a solid carrier involves formation of a covalent bond between the amino acid side chain residues of the protein with reactive groups on the support surface [8]. Most of functional groups of proteins usually involved in covalent binding are nucleophilic amino (lysine, histidine and arginine), thiol (cysteine) and hydroxyl groups (serine, threonine and tyrosine), as well as electrophilic carboxylate groups (aspartate and glutamate). The ε-amino group of lysine is typically used for covalent attachment to the carrier. The advantages of lysine are that it is often located on the protein surface, can be found in enough high amounts in the structure of enzymes, exhibits higher reactivity than other functional groups, and provides good bond stability [9]. For efficient immobilization, reactive groups of enzymes should react in mild conditions with appropriate functional groups of supports. Usually, the available functional groups are not enough active and must be activated. Either the solid support or the enzyme may be activated, but to limit alteration of the tertiary structure of enzyme the functional groups of the support material are activated most often. The activation may occur prior to the coupling reaction, or a bi-functional linking reagent can be used to form the bond between enzyme and support. The major advantage of covalent binding is stabilization of the immobilized enzyme. Due to the stronger carrier-protein linkage, the obtained heterogeneous biocatalyst can be much more stable than in case of adsorption or entrapment [8]. However, it must be noted that harsh conditions employed during covalent binding can potentially alter the enzyme conformation, lowering the enzymatic activity. Moreover, binding of the active sites of enzyme to the support may result in total loss of activity [10].

Saito *et al.* used porous ceramic material for immobilization of a thermostable β-gal from *Escherichia coli*. From several activating organosilanes, 3-[2-(2-aminoethyleminoethylamino) propyl]trimethoxysilane led to the highest residual activity when β-gal was immobilized on the derivatized support by glutaraldehyde cross-linking. The amount of covalently immobilized β-gal was double, compared to the enzyme immobilized by physical adsorption on the same carrier [11].

The aim of this work was to develop immobilized biocatalysts for the hydrolysis of lactose, with enhanced stability. The emulsion crosslinking method was selected as immobilization technique and chitosan as support, based on the well-known advantages of this material and its wide utilization in biocatalysis [12]. Some of the most important characteristics influencing the stability of the immobilized β-gal were determined, compared to the native enzyme.

2. Materials and methods

2.1. Materials: Chitosan (from crab shells), Tween 80 and *o*-nitrophenyl β-D-galactopyranoside (ONPG) were obtained from Sigma Co. (St. Louis, MO, USA); n-hexadecane was from Fluka Chemie GmbH (Buchs, Switzerland). n-Hexane (97%), ethanol (96%), sunflower oil, glutaraldehyde (25% aqueous solution) were purchased from Reanal (Budapest, Hungary). Fluorescein isothiocyanate (FITC) used for protein labeling was purchased from Sigma Aldrich (Steinheim, Germany).

Concentration of the protein was determined using the Bradford method using Coomassie Brilliant Blue G-250 purchased from Bio-Rad, *o*-phosphoric acid 85% was from Merck and bovine serum albumin (BSA) was from Sigma Aldrich (Steinheim, Germany).

β-D-Galactosidase from *Kluyveromyces lactis* (Maxilact LX 5000) was a kind gift from DSM (Delft, The Netherlands).

All activity and protein determinations were made at least in triplicate. The data inserted in tables and figures were calculated as mean values, with the condition that the maximum absolute deviation of the individual data should not exceed 3%.

2.2. Enzyme activity assay:The activity of β-gal was determined based by using colorimetric method and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as artificial substrate according to the procedure proposed by Cavaille and Combes [13]. In presence of β-gal ONPG is hydrolyzed to D-galactose (colorless) and *o*-nitrophenol (ONP) (yellow), which permits a rapid quantification of the product.

Activity measurement of native β-gal. In a 4 mL glass cuvette, 2.6 mL potassium phosphate buffer 0.02 mol/L, pH 7.5, 100 µL enzyme (1:300 dilution) and 300 µL ONPG 30 mmol/L were mixed. The absorbance was measured at 420 nm after 1 minute with a JASCO V530 spectrophotometer (Jasco Analytical Instruments). One enzymatic unit U represents the amount of enzyme which transforms one µmol ONPG in one minute (µmol min^{-1} mL native $enzyme^{-1}$).

Activity measurement of covalently immobilized β-gal. 50 mg of immobilized enzyme was introduced in a 2 mL Eppendorf tube and 1 mL of 3 mmol/L ONPG solution was added. The mixture was shacked with Vortex mixer for 1 min and the developed colour was measured at 420 nm. One enzymatic unit U represents the amount of enzyme which transforms one µmol ONPG in one minute (µmol×min^{-1}×g^{-1} immobilized enzyme).

2.3. Protein content assay:The Bradford method [14] was used for determination of the protein concentration, with BSA as calibration standard, in the range of 0-0.9 mg/mL

2.4. β-gal immobilization onto chitosan microparticles by covalent-binding:Chitosan microparticles were prepared by the emulsion cross-linking method, adapted from the procedure proposed by to Denkbas and Odabasi [15]. Briefly, 2% aqueous chitosan solution (in 2%, v/v acetic acid) was emulsified by mechanical stirring (1000 rpm) in an oil phase composed of 40% sunflower oil and 60% n-hexadecane containing 1.5% (related to the chitosan content of the solution) Tween 80 surfactant, as previously described [16]. After 30 min mechanical stirring (EURO-ST B stirrer, IKA Labortechnik, Germany), the obtained microspheres were solidified by cross-linking with various glutaraldehyde concentrations (1-5%, v/v, related to the chitosan solution volume), and the mixture was stirred for 1 h more. Then the microspheres were filtered and washed with n-hexane, ethanol, distilled water and 0.02 mol/L potassium phosphate buffer pH 7.5, respectively.

Immobilization on chitosan microspheres has been carried out adding 1 g (wet weight) particles to a mixture of 3.75 mL 0.02 mol/L potassium phosphate buffer (pH 7.5) and 1.25 mL Maxilact LX 5000. The mixture was gently shaken at 10°C for 16 h (ILW 115 STD incubator, Pol-Eko-Aparatura, Poland, equipped with MIR-S100 orbital shaker, Sanyo, Japan), then the immobilized enzyme was filtered and washed with 0.02 mol/L potassium phosphate buffer (pH 7.5) until protein was no longer detected in the washing solution. Protein contents were measured by Bradford's method. The immobilized protein amount was calculated by subtracting the protein recovered in the supernatant from the protein subjected to immobilization. The immobilization yields were calculated as the ratio of immobilized protein to protein subjected to immobilization.

2.5. Influence of enzyme loading:The correlation between the catalytic activity and the protein amount subjected to immobilization was examined by immobilizing 600 µL native enzyme with protein quantities varying between 25-60 mg/mL, as described in section 2.4.

2.6. Influence of glutaraldehyde concentration:The interdependence between the activity of immobilized enzyme and the quantity of glutaraldehyde, which has double role as crosslinker in microparticle formation and linking agent in enzyme immobilization, was studied varying the glutaraldehyde concentration between 1-5%, related to the chitosan solution volume. The enzyme immobilization was performed as described section 2.4.

2.7. Stability studies: pH profile of native and immobilized enzyme. The pH profiles of the native and immobilized enzyme have been determined in the 5.5 to 11 pH range. 200 U native or 5 mg immobilized β-gal were incubated in 1 mL 0.02 mol/L universal buffer, and the activities following incubation were assayed, as described.

Thermal stability of native and immobilized enzyme. Thermal stability was evaluated by incubating the native (200 U) and immobilized (5 mg) enzyme in 0.02 mol/L potassium phosphate buffer pH 7.5 at 30°C, 40°C, 50°C and 60°C. Samples were taken every 30 min up to 4 h and 8 h respectively, for the activity assay.

2.8. Protein labeling with fluorescein isothiocyanate (FITC):β-Gal labeling with FITC was carried out in potassium phosphate buffer 0.02 mol/L pH 8.5. The glycerol has been removed by dialysis in potassium phosphate buffer for 24h and the resulted protein was concentrated by using a centrifugal filter device (Centricon PL-30, with a membrane nominal molecular weight limit (NMWL) of 30,000 Da). The coupling reaction was started by adding dropwise 600 µL of FITC (1mg/mL dissolved in dimethylformamide DMF) in the protein solution. The reaction mixture, protected from light, was incubated for 1 h at room temperature (based on PIERCE EZ-LabelTM FITC Labeling Kit).

The labeled β-gal was separated by unreacted FITC by several washes with potassium phosphate buffer 0.02 mol/L pH 8.5 using a centrifugal filter device (Centricon PL-30, with a membrane nominal molecular weight limit (NMWL) of 30,000 Da). UV-VIS spectra were collected after each washing until the absorbance at 493 nm, FITC absorption maximum decreased up to 0.1 absorbance units (A.U.). The protein concentration was determined by using Bradford assay and the FITC labelled protein was immobilized based on method described in section 2.4. Fluorescence micrographs were registered by Leica True Confocal Scanner (Leica TCS SPE) with 10 fold spot magnitude.

3. Results and discussion

3.1. Optimization of the immobilization parameters on chitosan microparticles: Chitosan microparticles were obtained by the emulsion cross-linking method. In this study, the optimization of the immobilization process was carried out with the parameters influencing the immobilization: enzyme loading, amount of glutaraldehyde and immobilization time. Glutaraldehyde has a double role in this process, cross-linker and activator, and the number of free aldehyde functions available for covalent binding is a key issue for efficient immobilization. In this respect, study of influence of glutaraldehyde concentration was imperious.

To characterize the overall efficiency of the immobilization process, the total activity yield was calculated as percentage of the total enzymatic activity recovered following immobilization (activity of the immobilized enzyme multiplied by the amount of the immobilized enzyme), related to the total β-D-galactosidase activity introduced in the immobilization process (activity of the free enzyme multiplied by the amount of free enzyme subjected to immobilization).

3.1.1. Influence of the loaded protein concentration: In order to maximize the amount of immobilized β-gal onto chitosan beads, the protein amount subjected to immobilization was varied between 0.37 mg and 18.67 mg, on 0.2 g wet chitosan beads (chitosan beads were kept in buffer solution). These amounts led to a protein/carrier ratio between 1.85 and 93.35 mg/g wet carrier (Table 1). The enzyme loading capacity was evaluated in relation with the immobilization yield of the protein, calculated as the difference between the initial protein subjected to immobilization and the protein remained in the supernatant after immobilization. The binding capacity of microparticles did not change significantly with increasing amounts of the available enzyme. The loaded protein values were, excepting the last entrance in Table 1, in a narrow interval, between 3.6-4.0 mg/g wet chitosan. As the enzyme has been immobilized on chitosan gel beads that contained a large amount of water, the enzyme/carrier ratio on these hydrated gel beads was obviously much lower than related to the dry carrier. 1 g chitosan microspheres obtained by emulsion cross-linking lost 86.4% of their weight by drying at room temperature until constant weight. Consequently, the enzyme loading values, related to the dry carrier, were much higher, between 27-29 mg/g. The results presented in Table 1 show that an increasing the amount of enzyme subjected to immobilization resulted in enhancement of loaded protein only at low protein/carrier ratios.

Table 1. Influence of loaded protein on the activity of immobilized β-galactosidase

Protein/carrier ratio [mg/g wet support]	Immobilization yield of protein [%]	Loaded protein [mg/g wet support]	Loaded protein [mg/g dry support]	Activity [U/g dry support]	Total activity yield [%]
93.35	4.10	3.82	28.1	236.7	0.89
37.35	9.42	3.52	25.9	151.4	1.43
18.65	21.30	3.97	29.2	135.3	2.56
7.45	48.70	3.63	24.7	148.5	3.51
3.70	99.19	3.67	27.0	277.9	13.06
1.85	100.00	1.85	13.6	152.9	14.42

The explanation is that the number of active groups of the support has a certain value and if the positions available for bounding are saturated, increase of the enzyme amount in the immobilization medium cannot raise the amount of loaded enzyme.

The loaded protein data were satisfactorily correlated with the activities of immobilized β-gal, which also exhibited close values. However, the activity differences were higher than observed for the loaded protein, probably because the activity of an immobilized enzyme depends not only on the attached protein but also on other factors, like steric hindrances and diffusional effects.

The efficiency of the immobilization process can be better evaluated using the total activity yield, which allows a global assessment of the obtained results. As it can be seen in Table 1, covalent immobilization on chitosan microspheres was accomplished with moderate efficiency, the total activity yield remaining below 15%. It must be pointed out that covalent binding can result in decrease of enzyme activity due to conformational changes [17], but this drawback is generally compensated by increased stability of the immobilized enzyme.

β-gal has tetramer structure composed of 4 identical subunits which form two identical dimmers with to active sites on each dimmer [18]. The reasons for decline of enzyme activity during or following the immobilization process could be numerous, and some of them were mentioned previously. Another explanation could be that native enzyme loses its activity when it is diluted or dialyzed because its tetramer structure is disturbed in aqueous medium. During a control experiment, the enzyme diluted 4 fold in 0.02 mol/L pH 7.5 potassium phosphate buffer was shacked during 16 h. After that interval, the diluted enzyme retained only 47.6% of the initial activity. The commercially available β-gal (MAXILACT LX 5000) is stabilized with 47% glycerol (stabilized with polyol) [19].

To check the behaviour of the non-stabilized enzyme, it was dialyzed for 24 h in 0.02 mol/L pH 7.5 potassium phosphate buffer. The dialyzed enzyme retained only 36.6% of its activity. Taking out the enzyme from the stabilized environment leads to decrease of its activity, probably due to conformational changes (dissociation of the tetramer structure in aqueous medium in absence of the polyol stabilizer of glycerol [20, 21]).

One of the possibilities of multimeric enzymes stabilization is immobilization by multi-subunit covalent attachment [22]. Because glutaraldehyde is also participating in the cross-linking of chitosan microspheres providing their formation, it is presumably that only few -CHO functional groups are remaining for covalent binding of the enzyme and they are not enough to implement multipoint attachment of the enzyme. Another explanation could be that the native enzyme is dissociated in aqueous medium before the covalent binding is fulfilled, its disintegration leading to activity lost because its monomer and trimer forms are inactive, only dimmer and tetramer forms are active [20, 21].

The results of this study showed that highest binding capacity for chitosan microparticles cross-linked with 3% glutaraldehyde was 27 mg protein/g dry support.

3.1.2. Influence of glutaraldehyde concentration: For optimization of the covalent binding process the influence of glutaraldehyde concentration was studied, keeping constant the other parameters which influence the process. Microparticles were

synthesized by emulsion cross-linking method in an oil phase composed of 40% sunflower oil and 60% n-hexadecane, with a stirring rate of 1500 rpm, 2.5% Tween 80 and 1.5% chitosan solution concentrations.

Different glutaraldehyde concentrations were applied for testing the binding capacity of microparticles. The glutaraldehyde amount was varied between 1% and 5%, related to the chitosan solution volume. The immobilization process was carried out at 10ºC, for 16 hours. The amount of native enzyme subjected to immobilization was 27 mg protein/g dry chitosan. Samples have been taken at 2, 4, and 16 hours and were analysed. The immobilization efficiency, expressed as total activity yield, was calculated for all samples and the obtained values are displayed in Fig. 1. The highest efficiency was reached with 2% and 3% glutaraldehyde, after 4 hours coupling reaction time. The activities were lower in case of low and high glutaraldehyde concentrations.

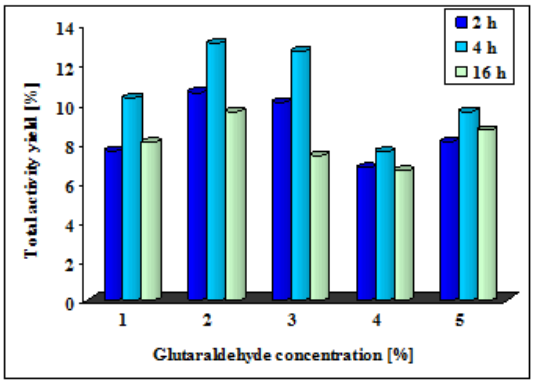

Figure 1. Effect of glutaraldehyde concentration on the catalytic efficiency of immobilized β-galactosidase

The glutaraldehyde being involved in the cross-linking of chitosan as well as in the enzyme binding process it is very difficult to evaluate how many free aldehyde groups are remaining for covalent binding of NH_2 groups of the enzyme. In case of 1% glutaraldehyde concentration, the cross-linking degree was lower, resulting in softer particles and only the remaining glutaraldehyde amount was implied in the binding of enzyme. Applying higher concentrations of glutaraldehyde resulted in harder microparticles with high better cross-linking degree; outside of glutaraldehyde being involved in formation of particles, several CHO groups remained for enzyme binding.

It can be also seen that activities after 16h reaction time decreased, regardless to the glutaraldehyde concentration.

Glutaraldehyde was introduced solely during the particle preparation stage, as experiments to add part of glutaraldehyde simultaneously with the enzyme solution resulted in very low activities. Glutaraldehyde is not only cross-linking reagent, but also could be a denaturing reagent. Therefore, higher glutaraldehyde concentrations can directly influence the activity of immobilized enzyme. As shown in Fig. 1, when the concentration of glutaraldehyde was lower than 3%, the activity of immobilized enzyme reached the maximum value. Increasing the concentration of glutaraldehyde at more than 3% resulted in decrease of activity. The reason could be that at high concentrations glutaraldehyde can undergo aldol condensation, which affects the construction of holes on the surface of beads, as it was demonstrated by other authors [23]. This effect not only makes the immobilization more difficult, but will also likely change the conformation of enzyme, leading to decline of enzyme activity. As a result, the optimal concentration of glutaraldehyde was set as 3%.

3.1.3. Influence of the coupling time on the support: Although the reactivity of the pending aldehyde groups on the surface of microparticles is enough high, the process takes several hours to be completed. To study the influence of immobilization time, the process has been carried out up to 10 h. Samples were taken at every 2h and the total activity yield was calculated as described above. The experiments were carried out with 3% glutaraldehyde concentration and the same enzyme/carrier ratio as in the previous experiments.

From Fig. 2 results that at up to 6 h immobilization time the activity of immobilized enzyme increased in time, but prolonged time led to activity decrease. These results are in accordance with the previous experiments, displayed in Fig. 1, where the same effect was noticed. Consequently, the optimal immobilizing time should be no more than 6 h.

3.1.4. Improvement of the catalytic efficiency by reduction with sodium borohydride: In order to increase the efficiency of the immobilization process, the Schiff bases obtained after covalent attachment of the enzyme were reduced by sodium borohydride. Compared to the process without reduction, an almost twofold increase of the activity has been noticed.

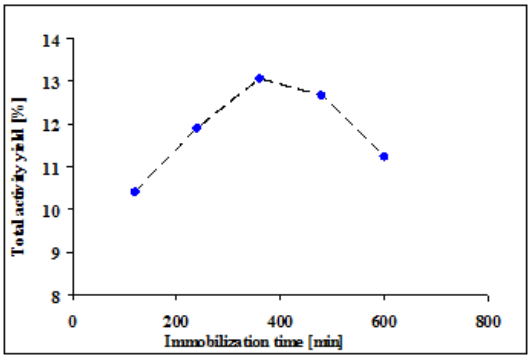

Figure 2. Influence of immobilization time on the catalytic efficiency of immobilized β-galactosidase

The values of activity and total activity yield reached 3.39 U/g dry support and 23.5%, respectively, while in the same immobilization conditions, but without reduction, the values were 1.89 U/g (dry support) and 13.06%, respectively (data taken from Table 1). The explanation is that reduction with sodium borohydride allows transformation of the weak Schiff bases to stable secondary amino bonds, in the same time as the remaining free aldehyde groups on the solid support are converted into inert hydroxyl groups, inducing a favourable conformational change of the immobilized enzyme.

3.2. Characterization and stability of the immobilized enzyme

3.2.1. pH profile of the native and immobilized β-galactosidase: Determination of the pH profile is important for characterization of the enzyme stability, because the enzymes are usually stable only inside a given pH domain and extreme pH values can lead to irreversible inactivation. One of the beneficial effects of immobilization can be an improvement of pH stability of enzymes [1]. To evaluate how covalent binding immobilization influence the activity of lactase at different pH values, the activity of native and immobilized enzymes were determined based on spectrophotometrical method (section 2.2.), within the pH range 5.5-11.0.

The results, expressed as relative activity related to the highest value, were depicted in Fig. 3, indicating that the covalently attached β-gal was lightly more stable at higher pH values compared to the native enzyme. This preparate retained around 50% of the activity at pH 9.

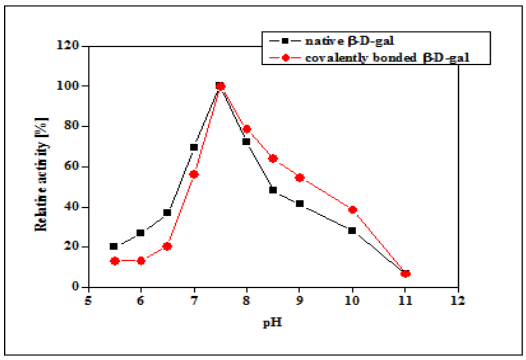

Figure 3. pH profile of native (■) and covalently attached (●) β-galactosidase

The results are in concordance with results reported by Neri *et al*., where β-gal from *Kluyveromyces lactis* was covalently immobilized onto a polysiloxane-polyvinyl alcohol magnetic composite by using glutaraldehyde [24]. An optimum pH value of 7.7 for immobilized β-gal from *Kluyveromyces lactis* on graphite was also cited [25].

3.2.2. Thermal stability of native and covalently immobilized β-gal: Thermal inactivation is one important barrier against scaling-up of industrial biocatalytic processes. Immobilization can be an important tool to improve the temperature stability of enzymes [1]. This thermal stability study was carried out by incubation at increasing temperatures, in the 30-60°C range, of native and covalently attached β-gal. Incubation time was 4h for the native enzyme and 8h for immobilized β-gal. Samples were taken after 30 min or 2 h, respectively, and the activities were assayed based on the described spectrophotometrical method. The results, expressed as percentage of the highest activity value, demonstrated that the bio-functionality of β-gal has been improved by immobilization (Fig. 4 and 5). The covalently bonded enzyme gradually lost its activity at each studied temperature, excepting at 30ºC where it was relative stable. At 40ºC and 50ºC after 2 h this preparate retained 60% of the initial activity.

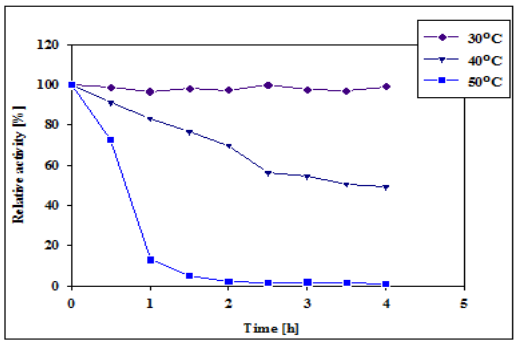

Figure 4. Effect of temperature on the activity of native β-galactosidase at 30ºC, 40ºC, 50ºC at pH 7.5, using ONPG as substrate, incubation time up to 4h.

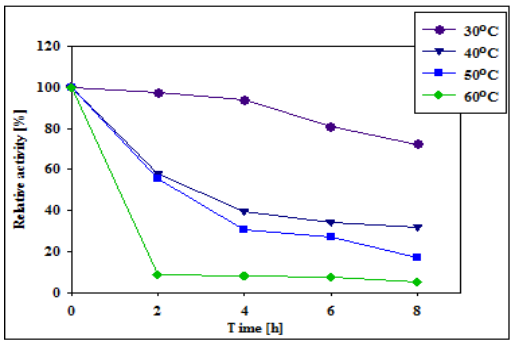

Figure 5. Effect of temperature on the activity of covalently attached β-galactosidase at 30ºC, 40ºC, 50ºC, 60ºC,at pH 7.5, using ONPG as substrate, incubation time up to 8h.

3.2.3. Fluorescence imaging of the immobilized β-gal labeled with FITC

At 60ºC, almost all activity was lost after 2h incubation (Fig. 5). Improvement of the thermal stability following the immobilization was observed only at 50ºC.

Fluorescein isothiocyanate (FITC) is one of the most used fluorochrome agent for protein labeling [26]. The protein distribution on chitosan microparticles was evaluated by fluorescence confocal microscopy. The native protein was tagged with FITC (described in section 2.8.), and the unreacted FITC was removed by several washes with 0.02 mol/L potassium phosphate buffer pH 7.5. After each washing step the UV-VIS spectra was collected in the range of 350-600 nm, and a decrease of the absorbance maximum at 493 nm, corresponding to FITC, was observed.

The labelled protein was immobilized by covalent binding as described in section 2.4. The fluorescent images of the immobilized β-gal-FITC complex indicate a uniform distribution on the chitosan microspheres surface (Fig. 6b). Control images were registered for unlabeled immobilized β-gal, where no fluorescence was detected (Fig. 6a).

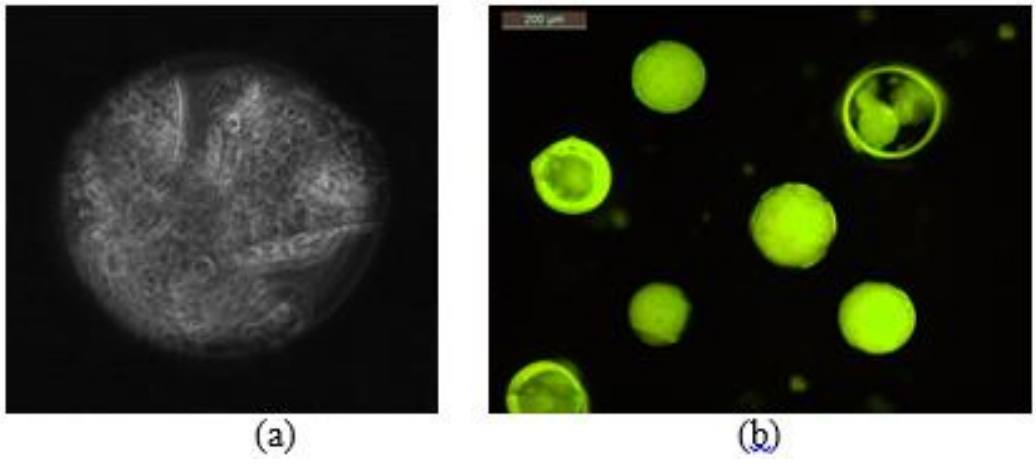

Figure 6. Fluorescence images of the covalently bonded unlabeled (a) and covalently bonded labeled (b) FITC-β-galactosidase

4. Conclusions

Immobilization of β-gal was accomplished by covalent binding to chitosan particles. The immobilization conditions were optimized by investigation of the main factors that influence the immobilization. Glutaraldehyde concentrations of 2% and 3% resulted in similar immobilization efficiency, but the latter concentration (3%) was considered as optimal one ensuring more coupling positions on the particles, because of the smaller size and higher specific surface of particles.

The binding capacity of chitosan microparticles cross-linked with 3% glutaraldehyde was 27 mg protein/g dry support. It was found that the proper reaction time for coupling of enzyme to the chitosan microparticle was 6 h, ensuring a maximal value of total activity yield. In the most favorable conditions the immobilization yield of protein reached 99% at 3.7 mg loaded protein per 1 g wet chitosan beads and the recovery yield of total enzymatic activity following immobilization was 13.06%. The reduction of Schiff bases with sodium borohydride after the covalent attachment, providing more flexible and stable secondary amino bonds, has increased the catalytic efficiency almost twofold resulting in a total activity yield of 23.5% which is a reasonable result for covalent immobilization.

The immobilized β-gal biocatalyst have been characterized, and its pH and temperature stabilities were determined. It was found that the covalently attached β-gal was slightly more stable at higher pH values compared to the native enzyme (showing about 50% residual activity at pH 9.0). The covalently immobilized enzyme showed improved thermal stability compared to the native enzyme at 50°C.

Acknowledgments: This work was performed through the Partnerships in priority areas - PN II program, developed with the support of MEN-UEFISCDI, project no. PN-II-PT-PCCA-2013-4-0734, and was partially supported by the strategic grant POSDRU/159/1.5/S/137070 (2014) of the Ministry of National Education, Romania, co-financed by the European Social Fund-Investing in People, within the Sectorial Operational Programme Human Resources Development 2007-2013.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

References

1. Guisan, J.M. (Ed.), *Immobilization of enzymes and cells*, Volume 22 of the series *Methods in Biotechnology*, 2nd Edition, Humana Press, Totowa, New Jersey, **2006**, doi:10.1007/978-1-59745-053-9.
2. Gänzle, M.G., Haase, G., Jelen P., Lactose: Crystallization, hydrolysis and value-added derivatives, *International Dairy Journal*, **2008**, *18*(7), 685-694, doi:10.1016/j.idairyj.2008.03.003.
3. Harju, M., Kallioinen, H., Tossavainen, O., Lactose hydrolysis and other conversions in dairy products: Technological aspects, *International Dairy Journal*, **2012**, *22*(2), 104-109, doi:10.1016/j.idairyj.2011.09.011.
4. Panesar, P.S., Panesar, R., Singh, R.S., Kennedy, J.F., Kumar, H., Microbial production, immobilization and applications of β-D-galactosidase, *Journal of Chemical Technology and Biotechnology*, **2006**, *81*(4), 530-543, doi: 10.1002/jctb.1453.
5. Ansari, S.A., Husain, Q., Lactose hydrolysis from milk/whey in batch and continuous processes by concanavalin A-Celite 545 immobilized *Aspergillus oryzae* β-galactosidase, *Food and Bioproducts Processing*, **2012**, *90*(2), 351-359, doi:10.1016/j.fbp.2011.07.003.
6. Taqieddin, E., Amiji, M., Enzyme immobilization in novel alginate–chitosan core-shell microcapsules, *Biomaterials*, **2004**, *25*(10), 1937-1945, doi:10.1016/j.biomaterials.2003.08.034.
7. Schoevaart, R., Wolbers, M.W., Golubovic, M., Ottens, M., Kieboom, A.P.G., van Rantwijk, F., van der Wielen, L.A.M., Sheldon, R.A., Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs), *Biotechnology and Bioengineering*, **2004**, *87*(6), 754-762, doi: 10.1002/bit.20184.
8. Lalonde, J., Margolin, A., *Immobilization of Enzymes*, in *Enzyme Catalysis in Organic Synthesis: A Comprehensive Handbook*, 2nd Edition (Eds. Drauz K. and Waldmann H.), Wiley-VCH Verlag GmbH, Weinheim, Germany, **2002**, doi:10.1002/9783527618262.ch6.
9. Brady, D., Jordaan, J., Advances in enzyme immobilization, *Biotechnoogy Letters*, **2009**, *31*(11), 1639-1650, doi:10.1007/s10529-009-0076-4.

10. Zhao, X.S., Bao, X.Y., Guo, W., Lee, F.Y., Immobilizing catalysts on porous materials, *Mateials Today*, **2006**, *9*(3), 32-39, doi:10.1016/S1369-7021(06)71388-8.
11. Saito, T., Yoshida, Y., Kawashima, K., Lin, K.H., Maeda, S., Kobayashi, T., Immobilization and characterization of a thermostable β-galactosidase from a thermophilic anaerobe on a porous ceramic support, *Appied Microbiology and Biotechnology*, **1994**, *40*(5), 618-621, doi:10.1007/BF00173317.
12. Krajewska, B., Application of chitin- and chitosan-based materials for enzyme immobilizations: a review, *Enzyme and Microbial Technology*, **2004**, *35*(2-3), 126-139, doi:10.1016/j.enzmictec.2003.12.013.
13. Cavaille, D., Combes, D., Characterization of β-galactosidase from *Kluyveromyces lactis*, *Biotechnology and Appied Biochemistry*, **1995**, *22*(1), 55-64.
14. Bradford, M.M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Analytical Biochemistry*, **1976**, *72*(1-2), 248-254, doi:10.1016/0003-2697(76)90527-3.
15. Denkbas, E.B., Odabasi, M., Chitosan microspheres and sponges: Preparation and characterization, *Journal of Applied Polymer Science*, **2000**, *76*(11), 1637-1643, doi:10.1002/(SICI)1097-4628(20000613)76:11<1637::AID-APP4>3.0.CO;2-Q.
16. Biró, E., Sz. Németh, Á., Sisak, C., Feczkó, T., Gyenis, J., Preparation of chitosan particles suitable for enzyme immobilization, *Journal of Biochemical and Biophysical Methods*, **2008**, *70* (6), 1240-1246, doi:10.1016/j.jprot.2007.11.005.
17. Secundo, F., Conformational changes of enzymes upon immobilization, *Chemical Society Reviews*, **2013**, *42*(15), 6250-6261, doi: 10.1039/c3cs35495d.
18. Pereira-Rodriguez, A., Fernandez-Leiro, R., Gonzalez-Siso, M.I., Cerdan, M.E., Becerra, M., Sanz-Aparicio, J., Structural basis of specificity in tetrameric *Kluyveromyces lactis* β-galactosidase, *Journal of Structural Biology*, **2012**, *177*(2), 392-401, doi:10.1016/j.jsb.2011.11.031.
19. Iyer P.V., Ananthanarayan L., Enzyme stability and stabilization - Aqueous and non-aqueous environment, *Process Biochemistry*, **2008**, *43*(10), 1019-1032, doi:10.1016/j.procbio.2008.06.004.
20. Becerra, M., Cerdan, E., Gonzalez Siso, M.I., Micro-scale purification of β-galactosidase from *Kluyveromyces lactis* reveals that dimeric and tetrameric forms are active, *Biotechnology Techniques*, **1998**, *12*(3), 253-256, doi:10.1023/A:1008885827560.
21. Pilipenko, O.S., Atyaksheva, L.F., Poltorak, O.M., Chukhrai, E.S., Dissociation and Catalytic Activity of Oligomer Forms of β-Galactosidases, *Russian Journal of Physical Chemistry A*, **2007**, *81*(6), 990-994, doi:10.1134/S003602440706026X.
22. Fernandez-Lafuente, R., Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation, *Enzyme and Microbial Technology*, **2009**, *45*(6-7), 405-418, doi:10.1016/j.enzmictec.2009.08.009.
23. Zhang, S., Gao, S., Gao, G., Immobilization of β-galactosidase onto magnetic beads, *Applied. Biochemistry and Biotechnology*, **2010**, *160*(5), 1386-1393, doi:10.1007/s12010-009-8600-5.
24. Neri, D.F.M., Balcao, V.M., Carneiro-da-Cunha, M.G., Carvalho, L.B.Jr., Teixeira, J.A., Immobilization of β-galactosidase from *Kluyveromyces lactis* onto a polysiloxane-polyvinyl alcohol magnetic (mPOS-PVA) composite for lactose hydrolysis, *Catalysis Communications*, **2008**, *9*(14), 2334-2339, doi:10.1016/j.catcom.2008.05.022.
25. Verma, M.L., Barrow, C.J., Kennedy, J.F., Puri, M., Immobilization of β-D-galactosidase from *Kluyveromyces lactis* on functionalized silicon dioxide nanoparticles: characterization and lactose hydrolysis, *International Journal of Biological Macromolecules*, **2012**, *50*(2), 432-437, doi:10.1016/j.ijbiomac.2011.12.029.
26. Pinto, M.C., Macias P., Determination of intraparticle immobilized enzyme distribution in porous support by confocal scanning microscopy, *Biotechnology Techniques* **1995**, *9*(7), 481-486, doi:10.1007/BF00159562.