Effect of immobilization conditions on the properties of β-galactosidase immobilized in xanthan/chitosan multilayers

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Abstract. The effect of lactose concentration on the activity of the immobilised enzyme β-galactosidase from Aspergillus niger has been evaluated, considering future applications for the production of galactooligosaccharides with prebiotic potential. The following enzyme was immobilized in xanthan and chitosan polyelectrolyte multilayers (PEMs) deposited by dip coating method on polylactic acid positively corona charged pads. The pads were charged in a corona discharge system, consisting of a corona electrode (needle), a grounded plate, and a metal grid placed between them. Positive 5 kV voltage was applied to the corona electrode. 1 kV voltage of the same polarity as that of the corona electrode was applied to the grid. The chitosan layers were crosslinked with sodium tripolyphosphate (Na-TPP). The enzyme showed a temperature optimum at 50°C and a pH optimum at 5.0. The immobilization was carried out over the different adsorption time and optimum conditions were determined. These results give insights for further optimization of transgalactosydase reactions in order to produce galactooligosaccharides with specific structure and having pronounced better prebiotic properties. For the determination of the surface morphology of the investigated samples an atomic force microscope was used and root mean square roughness was obtained.

1. Introduction

β-Galactosidases (EC 3.2.1.23) also known as lactases are enzymes belonging to glycoside hydrolase families 1, 2, 35, 42 and 59 (GH1, GH2, GH35, GH42 and GH59) [1, 2]. These enzymes catalyze the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactoside substrates. In the case of lactose as a substrate the products of hydrolysis – namely glucose and galactose, are transferred to water. When the initial concentration of lactose is more than 15 %, an acceptor of galactose residue can be another carbohydrate present in the reaction mixture – for example lactose, fructose or lactulose, and a transgalactosylation reaction is observed, resulting in production of galactooligosaccharides (GalOSs) with a different degree of polymerization (DP) [3, 4]. A large part of world population has lactose intolerance. According to M. Heyman lactose intolerance is a clinical syndrome of 1 or more of the following: abdominal pain, diarrhea, nausea, flatulence, and/or bloating after the ingestion of lactose or lactose-containing food substances [5]. On the other hand the synthesized by β-galactosidases GalOSs contain a synthesized variety of bonds including β-(1→4), β-(1→6) and β-(1→3), which determines their application as food additives with significant prebiotic potential and other beneficial effects [4, 6].
The amount and structure of the synthesized GalOSs are significantly affected by enzyme source, the acceptor molecules and the reaction conditions (substrate concentration, pH and temperature) [7-9].

Immobilization of the enzyme lactase on polyethylene polymer films enables development of an active packaging film to produce lactose-free milk products. Using layer by layer immobilization chemistry technique increases the amount of enzyme that can be immobilized per unit area of packaging film. This technique makes it possible to increase the functional efficiency of enzyme, enhance the reproducibility of the processes, improve the process control and ensure stable supply of the products in the market [10]. Various applications of immobilized β-galactosidases can be found in the food industry, medicine and research. Other industrial applications of immobilized β-galactosidases include laboratory-scale organic synthesis and analytical and medical applications.

One of the suitable and mostly used natural polymers for enzyme immobilization is chitosan. It is characterized as biocompatible, nontoxic, physiologically inert, and hydrophilic, offer the unique characteristic of a remarkable affinity to proteins and has been widely applied in medicine and biological research. Furthermore, chitosan is positively charged in acidic aqueous solutions and its charge density is high [11].

The effect of the support size on the properties of enzyme immobilization was investigated by using chitosan macroparticles and nanoparticles in [12]. Alginate–chitosan core-shell microcapsules were prepared in order to develop a biocompatible matrix for enzyme immobilization, where the protein is retained either in a liquid or solid core and the shell allows permeability control over substrates and products. In [13] alginate–chitosan core-shell microcapsules were prepared as a novel biocompatible matrix system for β-galactosidase enzyme immobilization where the catalyst is confined to either a liquid or solid core and the transport properties of the substrate and product are dictated by the permeability of the shell. Thus, the biological agent is protected in the inner biocompatible alginate core and the outer chitosan shell dictates the transport properties.

The multilayer immobilized enzyme can be employed in a biocatalyst reactor for production of galacto-oligosaccharides from lactose and the hydrolysis of lactose to glucose and galactose. The method of surface modification by layer-by-layer (LbL) polyelectrolyte multilayers allows very precise control and changes in a wide range of the carrier’s physicochemical properties – thickness, charge, hydrophilic–hydrophobic balance [14]. Such multilayer coatings with included functional components (nanoparticles, enzymes, and dyes) may be used in microelectronics, optics, biotechnology, and pharmacy [15]. The LbL technique is based on sequential deposition of oppositely charged polyelectrolytes from their solutions via electrostatic interactions. The assembly is based on spontaneous adsorptions, no stoichiometric control is necessary to maintain surface functionality, and the assembled films have good thermal and mechanical stability [16].

The aim of the present work is to describe the immobilization of the β-galactosidase enzyme in xanthan/modified chitosan/multylayers deposited on polylactic acid corona charged pad. Emphasis was focused on the effect of the number of polyelectrolyte multilayers (PEMs) and the immobilization time on the immobilized enzyme functional properties.

2. Materials and methods

2.1. Enzyme activity
A commercial fungal β-galactosidase (from Aspergillus niger) was used in the current kinetic studies. One unit of β-galactosidase activity is defined as the amount of enzyme catalyzing the release of 1 μmol min⁻¹ glucose or fructose at 37 °C and pH 5.0. The influence of the substrate concentration on the initial velocity of the enzyme reaction was studied at a range 0.01 M – 1.30 M lactose. β-Galactosidase activity was studied in the presence of lactose at 5% and 10% and mixtures of chitosan (0.1%) and lactose (5% and 10%). The concentrations of the released glucose was determined enzymatically [17]. The β-galactosidase activity assays were carried out additionally using ONPG(ortho-Nitrophenyl-β-galactopyranoside) with substrate prepared in acetate buffer solution. One β-galactosidase unit (U) was defined as the amount of enzyme which liberated 1 μmol of ONP(ortho-Nitrophenyl) per min per mg of
protein at 37 °C and pH 5.0. Protein concentration was assayed by the method of Bradford [18]. All the analyses were performed at least in triplicate. Programmable scientific calculation “CITIZEN” SRP-45N and SigmaPlot 12.0 (Systat Software, Inc) were used for data analysis.

2.2. Samples preparation
Polyactic acid (PLA) with inherent viscosity (0.55 ÷ 0.75) dL/g, purchased from Lactel Absorbable Polymers (USA), was used for the preparation of the biodegradable pads. The PLA pads were casted from 2% w/v PLA chloroform solution and then dried at 35 °C for 48 hours. After that the pads obtained were stored in an exicator at room temperature (T = 25 °C) and relative humidity (RH = 54%). Immediately prior to the deposition process, the pads were treated in corona discharge in order to achieve positive surface charge excess. The corona discharge system consisted of corona electrode (needle), grounded plate electrode, and a grid placed between them. The pads were placed on the grounded plate electrode and a voltage of 5 kV was applied to the corona electrode. One kV voltage of the same polarity as that of the corona electrode was applied to the grid. The PLA pads were charged for one minute under room conditions (T = (21÷23) °C and RH = (40÷60)%). After charging the electrets, the surface potential was measured by the method of the vibrating electrode with compensation by which the estimated error was better than 5%.

2.3. Polyelectrolyte multilayers deposition
Chitosan (low molecular mass, degree of deacetylation > 75%) and xanthan gum were purchased from Sigma-Aldrich and were used as received. The layer-by-layer (LbL) deposition technique was applied for obtaining of multilayer structures. For obtaining of this structures were used 1% w/v chitosan and 1% w/v xanthan solutions of acetate buffer (pH 4.5 and ionic strength 0.1 M). The deposition was done by the dip-coating process. The first built-up layer was of xanthan because at these conditions the xanthan was negatively charged and the PLA pads were charged in a positive corona. For dip-coating assembling a programmable slide stainer (Poly Stainer IUL, Spain) was used with deposition program: (1) 15 min xanthan solution dipping - adsorption of polyelectrolyte molecules; (2) followed by 5 min washing in the acetate buffer (pH 4.5 and ionic strength 0.1 M); (3) 15 min chitosan solution dipping - adsorption of polyelectrolyte molecules of opposite charge; (4) 20 min dipping in 1.5 % sodium tripolyphosphate (Na-TPP) water solution – the layer was crosslinked; (5) 30 min dipping in 0.1% glutaraldehyde (GA) water solution - the layer was activated; (6) 5 min washing in the same acetate buffer. After that the samples were dipped in 1% solution of β-galactosidase for 1 hour or 2 hours. The procedure was repeated until obtaining the desired numbers of layers (4 or 8) xanthan/chitosan. After obtaining the polyelectrolyte structures, they were dried in hot air and then stored in exicator, at RH = 54%.

2.4. Immobilisation of the Enzyme.
As a support to enzyme immobilization, a chitosan layer was washed in 1 M acetic acid solution with 1.5% w/v Na-TPP crosslinking agent. Thereafter, the chitosan layer was washed with deionized water until neutrality. For activation, chitosan layer was incubated in 0.1% GA solution under constant stirring. The excess GA was washed off and suspended in 0.025 M of acetate buffer at pH 4.5. For immobilisation, the activated chitosan layer was incubated in 1% β-galactosidase solution under constant stirring. The excess enzyme was washed off and suspended in 0.025 M of acetate buffer at pH 5.0.

2.5. Atomic force microscope
Chitosan and xanthan polyelectrolyte multilayers (PEMs) surface morphology roughness was investigated by means of Atomic force microscope (AFM NANOSURF FlexAFM). The samples were scanned with a standard silicon cantilever (type Tap 190Al-G) and the measurements were performed in ambient atmosphere, and dynamic operating mode. The applied force was always minimized, not to deform the samples. The surface topographic images of the PEMs films were taken.
3. Results and discussion

3.1. Effect of type of PEMs on immobilization and enzyme activity.

The effect of the conditions of β-galactosidase immobilization on the enzyme activity was studied. Chitosan was selected as a material for polyelectrolyte multilayer preparation for several reasons – it is cationic biopolymer with intra- and intermolecular hydrogen bonding ability; it has unique approach to modify the surface of chitosan by anionic biopolymer xanthan to improve biocompatibility; and exists the possibility to prepare chitosan membranes with controlled pore size and density. Four different PEMs were obtained by chemical modification of the chitosan layer using NA-TPP and GA. The results are shown in table 1. The enzymatic activity of immobilized β-galactosidase was compared with that of free enzyme using ONPG as a substrate. The storage stability of all immobilized systems for 5 h exposition was studied (table 1). It was found that the most stable PEMs are PLA pad with 4 multilayers dipping in enzyme solution for 1 h. Immobilization efficiency indicated that the significant amount of enzyme was bond to PEMs with 4 layers of modified xanthan/chitosan. In contrast immobilization efficiency is 5 times less in PEMs with 4 multilayers dipping in enzyme solution for 2 h. Obviously, in order to achieve a good immobilization efficiency of β-galactosidase the optimal configuration is PLA pad with 4 xanthan/chitosan multilayers dipping in enzyme solution for 1 h.

Table 1. β-Galactosidase activity in different types of PEMs after incubation in acetate buffer.

| No | Type of PEMs                                      | Enzyme activity (U/mg protein) | Enzyme activity (U/mg protein) | Enzyme activity (U/mg protein) | Enzyme activity (U/mg protein) |
|----|--------------------------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| 1  | PLA pad with 4 multilayers dipping in enzyme solution for 1 h | 0.022                          | 0.019                          | 0.029                          | 0.021                          | 0.017                          |
| 2  | PLA pad with 8 multilayers dipping in enzyme solution for 1 h | 0.056                          | 0.025                          | 0.088                          | 0.067                          | 0.054                          |
| 3  | PLA pad with 4 multilayers dipping in enzyme solution for 2 h | 0.119                          | 0.109                          | 0.126                          | 0.118                          | 0.090                          |
| 4  | PLA pad with 8 multilayers dipping in enzyme solution for 2 h | 0.084                          | 0.123                          | 0.123                          | 0.083                          | 0.085                          |

3.2. Effect of lactose concentration in 0.1 % chitosan solution on enzyme activity

The operational stability of the studied enzyme was evaluated by the hydrolysis of buffered lactose solutions 5% and 10% w/v, pH 5.0 at 37 °C.

Lactose hydrolysis was performed at 5 and 10 percent concentrations of lactose and with PLA pad with 4 multilayers dipping in enzyme solution for 1 h. As illustrated in table 2, there is not any inhibition effect of different percent of lactose. From the tested concentrations of lactose, the highest value of enzyme hydrolysis was measured in the presence of 5% of this sugar (15.24% of total substrate). When the enzyme reactions were performed in the presence of 0.1%, a chitosan light decrease of hydrolyzing reaction was observed in reaction without any chitosan concentration (15.04% of total substrate). On the other hand lactose hydrolysis performed with PLA pad with 4 multilayers dipping in enzyme solution for 1 h in the presence of 10% lactose resulted in 10.78% of lactose conversion in 3 h reaction time. When the enzyme reactions were performed in the presence of 0.1% chitosan in acetate buffer, a light increase of hydrolyzing reaction was observed in reaction with substrate 10% lactose (11.52% of total substrate).
Table 2. Lactose hydrolysis performed with immobilized β-galactosidase in PLA pad with 4 multilayers dipping in enzyme solution for 1 h.

| Time (h) | Glucose (mg/ml) | Glucose (mg/ml) |
|----------|-----------------|-----------------|
|          | 5.0% Lactose    | 10.0% Lactose   | 5.0% Lactose in 0.1% Chitosan | 10.0% Lactose in 0.1% Chitosan |
| 0        | 0.00            | 0.00            | 0.00                           | 0.00                           |
| 0.5      | 0.38            | 0.79            | 0.28                           | 1.40                           |
| 1.0      | 1.63            | 1.77            | 1.85                           | 2.06                           |
| 1.5      | 1.84            | 2.68            | 1.93                           | 2.98                           |
| 2.0      | 2.31            | 3.12            | 2.29                           | 3.59                           |
| 2.5      | 2.72            | 4.02            | 2.66                           | 4.06                           |
| 3.0      | 3.81            | 5.39            | 3.67                           | 5.76                           |

3.3. Morphology investigation

The surface topographic images of all the investigated PEMs were taken. The root mean square roughness was calculated. Surface morphology of the PEMs carried out by AFM was judged by their root mean square roughness Sq presented in Table 3. In figures 1(a) and 1(b) surface topographic images of PLA pads with 4 multilayers dipping in enzyme solution for 1 h and 2 h are presented.

Table 3. Values of the root mean square roughness for type of PEMs listed in Table 1.

| No of type of PEMs | 1     | 2     | 3     | 4     |
|--------------------|-------|-------|-------|-------|
| Root mean square roughness - Sq, nm | 2.5   | 3.3   | 12.8  | 12.4  |

Figure 1. AFM images of PLA pads with 4 multilayers dipping in enzyme solution for:
a) 1 h and b) 2 h.

The results obtained (see figure 1 and table 3) clearly show that the roughness of PLA pads dipping in enzyme solution for 1 h is much smaller in comparison with that dipping in enzyme solution for 2 h independently of the layer numbers. The PEMs dipping in enzyme solution for 1 h are the samples with greater β-galactosidase activity (see table 1). The PLA pad with 4 multilayers dipping in enzyme solution for 2 h has the great roughness and the smallest activity. Consequently, smaller roughness is associated with greater activity, which is most likely due to uniformly stable engagement of the enzyme in all layers.

When comparing the activity of the immobilized enzyme with that of the free enzyme, a similar inhibition has been observed due to galactose in the activity of the immobilized enzyme and a decrease in the activation energy of Michaelis–Menten constant of the immobilized enzyme compared to that of the free enzyme at 37 °C. The activity of the enzyme after immobilization was in all cases equal or higher than 50% of the activity of the free enzyme (data not show).
In the present work, we have studied the influence of different concentrations of lactose on the initial velocity of β-galactosidase. Such kind of kinetic information for lactose hydrolysis by β-galactosidases is scarce in the literature, despite of the fact that this disaccharide is a substrate by transgalactosylation or used in many studies as a donor/acceptor of galactose/fructose units during the synthesis of GalOSs having specific structures and bioactive properties [19-21].

From studies performed by other authors it is known that the galactosyltransferase reaction for synthesis of GalOSs is favored when the lactose concentration in the reaction mixture is more than 15%. In these conditions the content of free water is low and the transfer of galactose moiety of lactose to suitable acceptor molecules is more likely to occur [7, 22]. It is necessary to perform additional studies concerning the distribution, composition and yield of the obtained transgalactosylation products during the reactions with increasing concentrations of lactose and fructose molecules.

4. Conclusion
The current study is the first time to compare the influence of chitosan on the initial velocities of reaction catalyzed by β-galactosidase. The studied immobilizing enzyme in multilayer biofilms showed substantial differences in its affinity to the lactose concentration. The obtained results will be further applied and extended in experiments dealing with the direction and optimization of the GalOSs production.

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