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Characterization and optimization of β-galactosidase immobilization process on a mixed-matrix membrane

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ABSTRACT

β-Galactosidase is an important enzyme catalyzing not only the hydrolysis of lactose to the monosaccharides glucose and galactose but also the transgalactosylation reaction to produce galacto-oligosaccharides (GOS). In this study, β-galactosidase was immobilized by adsorption on a mixed-matrix membrane containing zirconium dioxide. The maximum β-galactosidase adsorbed on these membranes was 1.6 g/m^2, however, maximal activity was achieved at an enzyme concentration of around 0.5 g/m^2. The tests conducted to investigate the optimal immobilization parameters suggested that higher immobilization can be achieved under extreme parameters (pH and temperature) but the activity was not retained at such extreme operational parameters. The investigations on immobilized enzymes indicated that no real shift occurred in its optimal temperature after immobilization though the activity in case of immobilized enzyme was better retained at lower temperature (5 °C). A shift of 0.5 unit was observed in optimal pH after immobilization (pH 6.5 to 7). Perhaps the most striking results are the kinetic parameters of the immobilized enzyme; while the Michaelis constant (K_m) value increased almost eight times compared to the free enzyme, the maximum enzyme velocity (V_{max}) remained almost constant.

1. Introduction

β-Galactosidases have been obtained from a variety of microorganisms and sources including fungi, bacteria, yeasts, plants, etc. β-Galactosidase is indeed an important enzyme in food industry and has found significant applications in enhancing sweetness, solubility, flavor and digestibility of dairy products [1]. A major application of β-galactosidase is lactose hydrolysis, a process that results in the formation of glucose and galactose. Lactose is the major sugar (4–5%) present in milk and its hydrolysis makes milk fit for consumption of lactose intolerant people [2]. Apart from lactose hydrolysis, β-galactosidase also finds application in galacto-oligosaccharide (GOS) formation via transgalactosylation reaction. During enzymatic hydrolysis of lactose into glucose and galactose, using β-galactosidase, the enzyme is also able to transfer galactose to the hydroxyl groups of galactose or glucose in a process called "transgalactosylation" and, thus, produces galacto-oligosaccharides (GOS) [3,4]. GOS are non-digestible oligosaccharides which are recognized as prebiotics thus stimulating the growth of Bifidobacteria in the lower part of the human intestine [5].

In case of β-galactosidase, enzyme parameters and price eventually determine the cost of lactose hydrolysis or GOS formation processes. The use of immobilized β-galactosidase for lactose hydrolysis in case of whey, has become economically feasible in spite of the cost of the enzyme and of the immobilization process. This has been mainly attributed to the facts that immobilized enzymes can be reused several times, and there is a possibility of developing a continuous hydrolysis process [6]. In general, the immobilization of enzymes on various supports is driven by the benefits such as continuous process, enzyme reuse, enhanced enzyme stability, i.e. resistance against extreme pH, temperature, high ionic strengths, etc. [1,2]. Moreover, it is believed that the possible disadvantages such as low retained activity as a consequence of the immobilization procedure are largely compensated by the possibility of the reuse of the enzyme [7].

β-Galactosidase has been immobilized on various supports including liquid aphrons (surfactant-stabilized solvent droplets), anion exchangers, magnetic beads, cotton cloth, polyethylene films, chitosan particles, etc. The resulting changes in activity and pH optimum, as a function of support material and immobilization methods, have been reported. These studies have been reviewed in detail by Husain [1]. Apart from conventional supports, in recent years membranes have been used for β-galactosidase immobilization [8,9]. Membranes have several advantages in enzyme immobilization such as the absence of mass transfer limitations and

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the convenient scale-up by adaptation of the available membrane surface area [10,11]. Furthermore, some level of product separation is also achieved along with biocatalytic conversion in enzyme membrane reactors (EMRs) [12]. Typically, membrane separations have the advantage of requiring only a limited amount of energy, because there is no phase change involved in such processes [12].

Immobilization of β-galactosidase on nylon and polysulphone membranes via grafting/crosslinking with epoxy groups have been investigated and changes in activity and stability were observed [7,13]. In terms of application, the immobilization of β-galactosidase on tubular ceramic membranes with Al_2O_3 support layer and TiO_2 separation layer were investigated for GOS formation thus aiming at a simultaneous conversion and separation process [3]. The benefits associated with application of immobilized enzymes in biotechnological processes have stimulated the interest of the researchers towards the research addressed to improve the performance of the biocatalytic membranes [13]. Therefore, finding an appropriate membrane for enzyme immobilization is a matter of great interest among researchers. Though the inherent properties of membranes such as hydrophobicity, hyrophilicity and molecular weight cut-off (MWCO) are of significant importance in enzyme immobilization, however, membrane's interaction with enzyme, permeability to substrates and products, chemical and biological stability, high mechanical strength and presence of certain chemical groups are often the prerequisites [8].

The aim of the present study is to immobilize β-galactosidase on a mixed-matrix membrane via adsorption. The focus is to estimate the enzyme immobilizing capacity of the membrane under optimal conditions of enzyme concentration, temperature, contact time and pH. The characteristics of immobilized enzymes were studied and compared to that of free enzyme to highlight the significant impacts of immobilization. This article presents the first results of β-galactosidase on an in-house prepared membrane. These results are being used to further tailor the membrane specifically for optimal β-galactosidase immobilization.

2. Materials and methods

2.1. Enzymes, reagents and membrane

Commercial enzyme β-galactosidase (EC 3.2.1.23) from *Kluyveromyces lactis* (with an activity of 75000 μmol *ortho*-nitrophenol released min^{-1} g^{-1} measured as described in Section 2.5.1) and the substrates 2-nitrophenyl β-D-galactopyranoside (oNPG) and lactose were purchased from Sigma–Aldrich NV/SA (Bornem, Belgium). The enzyme was stored at 4 °C and was used without further processing. Fifty mM Tris–HCl buffer containing 50 mM NaCl and 50 mM $MgCl_2$ (pH 5–9) was used in the experiments. All the other chemicals used in the study were of analytical grade. The membrane used for β-galactosidase immobilization in this study was a flat-sheet mixed-matrix membrane containing polysulphone and zirconium dioxide. This membrane was prepared in-house through phase inversion using a N-ethyl-2-pyrrolidone (NEP)/water system after casting the membrane dope (500 μm thick) on a plate. The water permeability and molecular weight cut-off of this membrane was 296 L/h m^2 bar and 13.8 kDa, respectively.

2.2. Enzyme immobilization procedure

Enzyme immobilization was conducted in 1.5 mL eppendorf tubes containing 0.6 mL of enzyme solution (diluted with Tris–HCl buffer). Wet membrane pieces of 1 cm^2 were used for immobilization. Before transferring the membrane into the eppendorf tubes, the 1 cm^2 membrane piece was cut into two halves to accommodate it well in the eppendorf tube as well as to allow its free movement inside the tube. These tubes containing enzyme solution were then placed on a rotary mixer set to rotate the tubes at approximately 50 rpm. After immobilization the membranes were transferred into new 1.5 mL eppendorf tubes containing 0.6 mL of buffer solution. This was done to rinse the membranes properly in buffer so that any traces of enzymes that are not attached to the membranes can be washed away. These rinsing steps were repeated 6 times. The first 3 times, the rinsing was conducted for 1 min on the rotary mixer and the last 3 times for 30 min on the rotary mixer or in the shaking water bath. All the tests were done in triplicate.

The amount of enzyme immobilized on membrane was measured as follows: the protein concentration of the enzyme-buffer solution was determined both before and after its contact with the membrane. This difference in the protein concentration (before and after immobilization) of the enzyme-buffer solution was considered as the amount of enzyme present on the membrane. Therefore, when referring to the amount of enzyme immobilized on the membrane the term "protein" has also been used throughout the manuscript. We also measured protein concentrations in all the membrane rinsing (washing) solutions but no detectable amount of protein was found.

2.3. Enzyme storage stability determination

After enzyme immobilization, a set of membranes and free enzyme were stored at 5 °C in a Tris–HCl buffer at pH 7. The stored membranes and free enzymes were used to measure the immobilized enzyme activity at regular time intervals until 7 days. Each day a fresh set of membrane was used to measure the enzyme activity.

2.4. Determination of kinetic parameters of free and immobilized enzyme

To investigate the differences between the kinetic parameters of the free and immobilized enzyme, the activity of the free and immobilized β-galactosidase was measured at different substrate concentrations. For the free enzyme oNPG ranging from 0 to 33 mM and lactose ranging from 0 to 29 mM were used. For the immobilized enzymes the concentrations ranged from 0 to 146 mM lactose. The kinetic parameters of the immobilized enzyme were only determined with lactose, due to the limited solubility of oNPG in the buffer solution (max 1% oNPG). The obtained activities were plotted in function of the substrate concentrations. The statistical program '*R*' was used to determine the kinetic parameters [14]. This was done by fitting a non-linear regression model to the data using the function 'drm' of the package 'drc' [15]. Such a fitting is preferred because it is much more accurate than the conventional linearizations [16].

2.5. Analytical methods

2.5.1. Free and immobilized enzyme activity

The activity of free enzyme (β-galactosidase) was measured after diluting it 250 times in 50 mM Tris–HCl buffer. For the activity determination, 50 μL of diluted enzyme solution was added to 2.5 mL of a 1% oNPG and 2.45 mL of 50 mM Tris–HCl buffer. The tube was incubated in a water bath for 5 min at 25 °C, while being shaken at 150 rpm. To stop the reaction, 1 mL of a 2 M sodium carbonate solution was added to the tube and subsequently the tube was vortexed. The samples were measured in a spectrophotometer (Shimadzu UV-1800) at 420 nm. For the activity determination on membranes, the buffer rinsed membranes were transferred into a 15 mL tube containing 2.5 mL of a 1% oNPG and 2.5 mL of 50 mM Tris–HCl buffer. The tube was incubated in the water bath during 5 min at 25 °C, while being shaken at 150 rpm. Again the reaction was stopped by adding 1 mL of sodium carbonate solution and subsequently vortexing the tube. Also these samples are measured in a spectrophotometer at 420 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol *o*-nitrophenol in 1 min at a substrate concentration of 0.5% (w/v) and at 25 °C.

2.5.2. Protein determination

Protein concentration was measured using the Coomassie (Bradford) protein assay kit obtained from Thermo Scientific (Rockford, USA). A calibration curve was constructed using bovine serum albumin (BSA) as the standard. Protein concentrations were determined by mixing 1.6 mL of Bradford solution with 0.4 mL of sample. Afterwards the sample absorbances were measured in a spectrophotometer at 595 nm.

3. Results

3.1. Enzyme immobilization

In case of enzyme immobilization on a support/membrane, achieving maximal enzyme loading seems the obvious aim, however, what matters most is actually the specific enzyme activity of the immobilized enzymes. Therefore, in order to achieve optimal enzyme immobilization, i.e. optimal enzyme loading as well as optimal specific enzyme activity on membranes, various parameters need to be optimized during the immobilization process. In this study, the immobilization of β-galactosidase was investigated in terms of variations in initial enzyme concentration, temperature, contact time and pH. The results are presented in the following sections.

3.1.1. Effect of initial enzyme concentration

To investigate the effect of initial enzyme concentration on enzyme immobilization on a mixed matrix membrane, various enzyme dilutions (ranging from 2 times to 500 times diluted