Membrane-Chromatography-Reactor-System for the Continuous Synthesis of Galactosyl-Oligosaccharides

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1. Introduction

ß-Galactosidase (EC 3.2.1.23), commonly known as lactase, catalyses not only the hydrolysis of lactose to the monosaccharides glucose and galactose but also the transgalactosylation reaction to produce galactosyl-oligosaccharides (GOS) [1-4].
GOS are non-digestible oligosaccharides which are recognized as prebiotics. Prebiotics have been found to reach the human colon without being hydrolyzed or absorbed in the upper part of the gastrointestinal tract. GOS selectively stimulate the growth of bifidobacteria in the lower part of human intestine. Increase in the growth of bifidobacteria is usually accompanied by suppression of potentially harmful bacteria such as *Clostridia* and *Bacteroides* species in the intestine [1,4,5,6].
GOS consist of a number of oligosaccharides with varying ß-glycosidic linkages depending on enzyme source. There have been several investigations on the synthesis of GOS by ß-galactosidases from various sources. Especially, ß-galactosidase from *Kluyveromyces lactis* has been extensively studied. The enzyme was reported to have stronger hydrolytic activity than transferase activity and produced a high proportion of trisaccharide in the synthetic GOS mixtures [1, 2,7].
Some techniques have been developed for immobilization of ß-galactosidase including non-covalent adsorption, covalent binding, entrapment and encapsulation [1-3,8-11].
The newly developed synthetic micro porous membrane adsorbers as chromatographic media are an attractive alternative to traditionally used packed bed chromatography, which has several limitations [12]. The adsorption of enzymes on ionic exchange resins is still the most popular, simplest and oldest technique for reversible immobilization of enzymes.
The chromatography membranes are an ion exchange support containing functional quaternary amines or sulphopropyl groups e.g. supported cross-linked polyethersulphone (PES) or regenerated cellulose. The pores in the membranes

are large enough to allow the biomolecules access to all the binding sites by direct fluid convection. This results in a very high capacity for large biomolecules [13,14]. Pressure filtration forces the liquid through the micro pores of the membrane, bringing target substances into direct contact with the binding sites. This direct convection to the binding sites minimizes diffusion limitation of mass transfer without sacrificing capacity.
The main applications of membrane adsorbers are separation and analysis of proteins. In our study, the membrane adsorbers have been investigated as an alternative support for immobilization of enzyme to produce GOS. Some of the advantages of immobilized enzymes via physical adsorption of enzyme on activated support are: the chemically inertness of activated supports, good stability during storage, controlled residence time on the enzyme, the recovery of the enzyme after usage, the possibility of a continuous process in enzyme reactors and the elimination of the enzyme from the final product [3,10].
This present study focuses on developing of a method for immobilization of ß-galactosidase in chromatography membranes, investigating the properties of immobilized enzyme and the possibility of using the immobilized enzyme system for the synthesis of GOS from lactose. The ß-galactosidase from *Kluyveromyces lactis* was chosen as a model enzyme for this study. A strongly basic anion exchanger membrane was used here to achieve optimal conditions for the enzyme.

2. Material and Methods

2.1 Chemicals and Materials

Chemicals for the immobilization and synthesis were from VWR International GmbH (Darmstadt, Germany).
ß-lactose was a gift from Meggle GmbH (Wasserburg, Germany).
Enzyme Maxilact L 2000 from *Kluyveromyces lactis* with an activity of 2000 NLU/g was kindly provided by Gist Brocades (Delft, The Netherlands).
Buffer: 50 mM potassium phosphate containing 5 mM $MgSO_4$ (pH 7,0) was used in all experiments.

2.2 Membrane

Properties of membrane used in this work are given in Table 1.
Mustang Q membrane adsorbers (PES) obtained from Pall GmbH (Dreieich, Germany) were used in form of a flat sheet (dimension: 9 cm disc) in a filter holder.

Table 1: Membrane specification(as indicated by the manufacturer)

membrane material	supported cross-linked polyethersulphone (PES)
functional group	quaternary ammonium -R-CH_2-N^+-$(CH_3)_3$
	strongly basic anion exchanger
bed height [mm]	0,138
bed volume for 9 cm disc [cm^3]	0,878
pore size [µm]	0,8
binding capacity (for BSA)	60 mg/ml of membrane volume

Figure 1: Filter holder Type TPP-90 and membrane adsorber Mustang Q.

Filter holder Type TPP-90 (Fig.1) were from Amafilter Deutschland GmbH (Düsseldorf, Germany).

2.3 Continuous membrane chromatography reactor system

The Mustang Q membrane was packed in the filter holder (figure 1). The feed reservoir containing substrate solution was kept on a heater with stirrer to maintain the constant reaction temperature. The substrate solution in the mixed reservoir was continuously circulated through the membrane chromatography reactor at a flow rate of 34 ml/[min · cm^3]. All experiments were carried out at the same flow rate. The flow was directed in a spiral pattern using channels (height about 1 mm) in the top of the filter holder. The retentate was back flushed to the mixed reservoir. No permeate was recycled. The permeate was accumulated in the product reservoir.

2.4. Immobilization of ß-galactosidase

For enzyme immobilization the system was rinsed with buffer (equilibration) in cleaning mode (Fig. 2, c). After system equilibration the enzyme-buffer solution (pre-cooled, if needed), pH 7,0 was pumped for 30 min from mixed reservoir to the membrane reactor in recycle mode (Fig. 2, a). The immobilization was carried out at 40, 15 and 4 °C.
After immobilization the membrane were washed with buffer at 40 °C in cleaning mode (Fig. 2, c) in order to remove non-bound enzyme.
To determine enzyme activity after immobilization the membrane was removed from filter holder and incubated for 10 min in 40 ml 0,1 molar lactose solution at 30 °C (static experiment).

2.5 Synthesis of galactosyl-oligosaccharides

For all synthesis experiments the steps like equilibration, immobilization, washing and determination of enzyme activity were similar to the steps described above. Continuous enzyme reactions were carried out in a thermostat water bath from GFL (Burgwedel, Germany) at 40 °C in continuous mode without product return

(Fig. 2, b). The substrate solution was prepared by dissolving lactose (20 % wt/wt) in buffer. The reaction was followed for at least 1 h and samples were taken every 10 minutes.

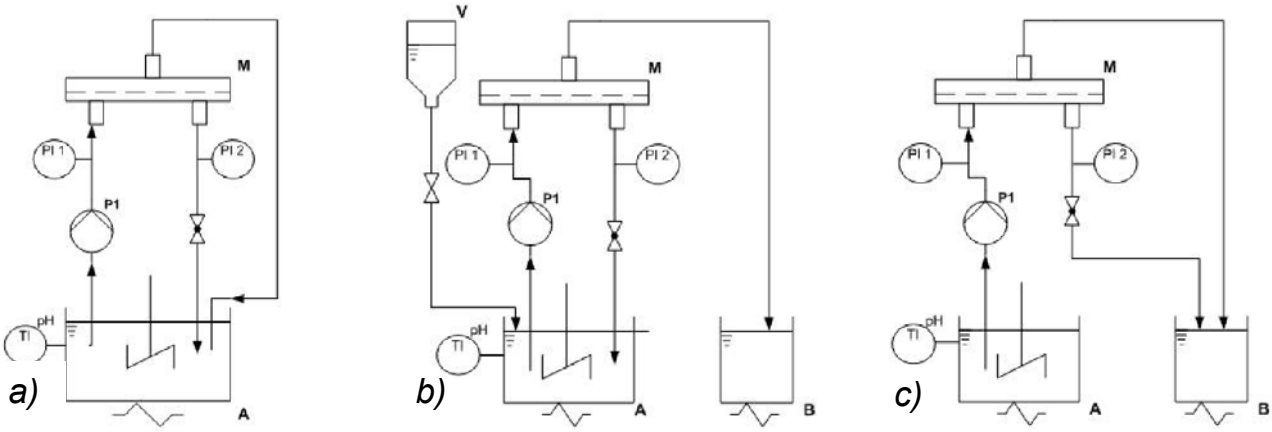

Figure 2: Laboratory scale chromatography membrane reactor system. Different modes used for enzyme immobilization and GOS-synthesis: recycle mode (a), continuous production mode (b) and cleaning mode (c). A - mixed reservoir, B - product reservoir, V - feed reservoir, M - reactor with immobilized enzyme, P1-P3 - pump.

2.6 Analytics

The Bradford method was used for protein determination. The enzyme activity was measured by using o-Nitrophenyl-β-D-galactopyranosid (o-NPG) as the substrate by 30 °C and an incubation time of 5 min. The absorbance was read at 420 nm. The millimolar extinction ε_{420} was 4,60 per mM. One neutral lactase unit (NLU) of enzyme activity is defined as that quantity of enzyme hydrolyzing 1µmol of o-NPG under the conditions stated above.
Thin film chromatography (HPTLC) was used to detect the oligosaccharides content.

3. Results

3.1 Immobilization of ß-galactosidase

ß-Galactosidase was successfully immobilized on an anion exchange membrane (Table 2). Average protein capture efficiency, as defined by the amount of protein adsorbed on the membrane divided by the amount of protein in the original solution, was between 30 and 36 % depending of immobilization temperature.
The highest activity of immobilized enzyme in static experiment was achieved at 15 °C immobilization temperature. The activity of immobilized enzyme in dynamic experiment (convective flow of substrate through the membrane – Fig. 2 b) was 3158 NLU/ cm^3 membrane volume at 15 °C immobilization temperature.

Table 2: Protein capture efficiency and resulting activity for immobilized ß-galactosidase on chromatography membrane

Immobilization temperature [°C]	Protein capture efficiency [%]	Activity of immobilized enzyme, static experiment, 30°C / 0,1 mol lactose / pH7,0 [NLU/cm³ membrane]	Activity of immobilized enzyme, dynamic experiment, 40°C / 0,6 mol lactose / pH 7,0 [NLU/cm³ membrane]
40	30	164	-
15	30	314	3158
4	36	215	-

3.2 Reactor performance

The results reported below were all achieved with steady-state continuous runs. The feed flow was held equal to the permeate flow (permeate flux was always 34 ml/[min·cm^3]).

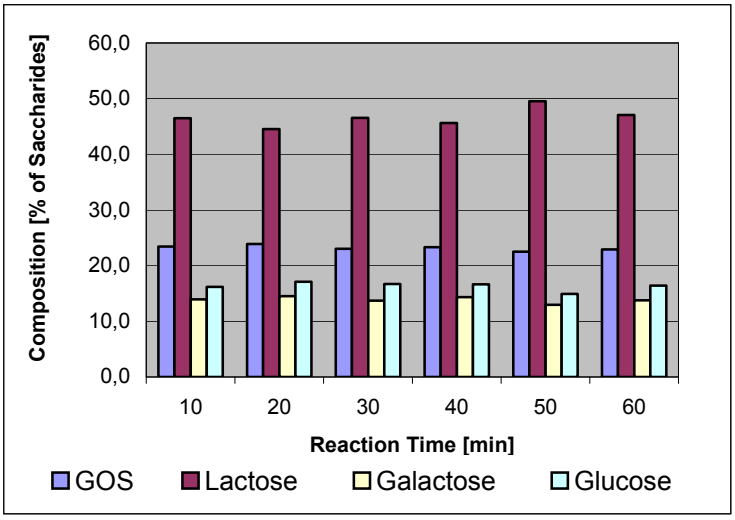

Figure 3: Production of oligosaccharides from lactose in a continuous membrane adsorber reactor (enzyme: Maxilact L 2000, T = 40°C, pH = 7,0, membrane: Mustang Q (PES), 20% [wt/wt] lactose solution)

Figure 3 shows representative results with the adsorber membrane at different reaction times. It is clear that the conversion to oligosaccharides is quite significant. The oligosaccharide yield was nearly constant over the whole reaction time which indicates the steady-state conditions. Our results show that a continuous membrane-chromatography-reactor CMCR can perform well in the production of oligosaccharides from lactose.

4. Conclusion

The corresponding reactor productivity for the production of GOS from lactose with continuous membrane-chromatography-reactor system was 98,7 g GOS per hour and cubic centimeter membrane volume, which is higher than those previously reported.

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