

A Novel Ceramic Membrane Reactor System For the Continuous Enzymatic Synthesis of Oligosaccharides

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Abstract

The continuous enzymatic production of galactosyl-oligosaccharides (GOS) from lactose as a substrate using a new type of ceramic membrane reactor system was investigated. GOS are non-digestible oligosaccharides and have recently attracted interest as prebiotics. However, the composition of oligosaccharides fraction and the variability in β -glycosidic linkages depends on the enzyme source. In the study presented below, native, physically immobilized, β -galactosidase from *Kluyveromyces lactis* (EC 3.2.1.23) was used as enzyme to catalyze transgalactosylation reaction to produce GOS, competed against the hydrolysis of lactose into its two component monosaccharides, glucose and galactose. To optimize GOS yielded, process conditions were varied: The average residence time of the enzyme was varied in the range of 13 to 24 min, the trans-membrane pressure (TMP) was in the range of 1 to 2 bar and the initial concentration of substrate was varied from 10 to 30 % (w/w). Regarding the conditions investigated here, the maximum oligosaccharide concentration exceeded 38% (w/w) when the average residence time was 24 min, the TMP was 2 bar and an initial lactose concentration of 30% (w/w) was adjusted.

1. Introduction

During enzymatic hydrolysis of lactose into its two component monosaccharides, glucose and galactose, using β -galactosidase (EC 3.2.1.23), 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). GOS consist of galactosyl-galactose chain terminated through glucose and are the desired by-products of the enzymatic catalysis [1, 2]. The composition of the GOS fraction varies in chain length and in the interconnection of the monomer units with varying β -glycosidic linkages depending on the enzyme source (Fig.1). Opposed to the hydrolysis of lactose, GOS are very slowly hydrolyzed both *in vivo* and *in vitro*. GOS produced here are low-molecular, not viscous, water-soluble liquid dietary fibers [3], which are stable at elevated temperatures and at low pH. GOS are considered as physiologically active functional food and enhance the growth of Bifido bacteria in the intestine [4].

Recently, various chemical and enzymatic methods for the production of GOS from disaccharide substrates have been developed. The chemical synthesis of GOS requires multiple protection and de-protection steps of specific molecular groups, possessing a complexity causing chemically driven synthesis little attractive for industrial applications. To

overcome this challenge, new strategies for the continuous production of GOS in innovative enzymatic membrane bioreactors have been previously developed [2-8]. Hence, enzymatic synthesis of oligosaccharides and their derivatives have been shown as promising alternatives to chemical synthesis. However, enzymatic synthesis of GOS and concurrent hydrolysis of lactose depend on several process parameters such as the initial concentration and nature of substrate (*i.e.*, lactose), enzyme source, membrane properties (*e.g.*, pore size and design) and the average residence time of the enzyme. Consequently, assessing continuous GOS production while varying the process parameters mentioned was addressed in the study presented below.

The application of a novel two-stage integrated ceramic membrane reactor system to physically immobilize β -galactosidase from *Kluyveromyces lactis* for the continuous production of galactosyl-oligosaccharides through enzymatic conversion of lactose as a substrate was investigated.

2. Materials and Methods

Chemicals

Deionised water, 5mmol/l potassium phosphate containing 5mmol/l MgSO_4 (pH 7.0) as a buffer in all experiments and food-grade lactose monohydrate (99.95% pure, Meggle GmbH, Wasserburg, Germany) were used. Other chemicals, entirely of p.a. quality, were purchased from VWR International GmbH (Darmstadt, Germany).

Membranes

Tubular ceramic membranes (Table 1) were used for ultrafiltration (atech innovations GmbH, Gladbeck, Germany). Membranes used have an asymmetric structure consisting of one support layer (Al_2O_3) with large pores and a low-pressure decay and one separation layer (TiO_2) to control the permeation flux. Membrane (1) has an outer diameter of 10 mm and a total active filtration area of $13.5 \cdot 10^{-3} \text{ m}^2$. Membrane (2) exhibits an inner diameter of 16 mm and a total active filtration area of $20.1 \cdot 10^{-3} \text{ m}^2$.

Enzyme

The commercially available enzyme used was β -galactosidases, Maxilact® L 2000 (Gist-Brocades NV, Delft, Holland), which was highly purified, liquid and prepared from *Kluyveromyces lactis* and hold an activity of 2000 NLU/g; whereas one NLU is defined as the quantity of enzyme that liberates $1 \mu\text{mol}$ of *o*-nitrophenol from *o*-nitrophenyl- β -D-galactopyranoside (ONPG) per minute under standard conditions. The enzyme had a molecular weight exceeding 135,000 Dalton [9]. Further properties specific to the enzyme are given (Table 2). Different concentrations of enzyme-buffer solution (2.5 and 5% (w/w)) were prepared adding different amounts of the enzyme to potassium phosphate buffer.

Continuous Ceramic Membrane Reactor System

Continuous production of oligosaccharides from lactose was performed using the laboratory equipment shown schematically (Fig. 2). A continuous stirred tank reactor (CSTR) was used together with a membrane module in a stainless steel housing. The ceramic membrane reactor is tubular and contains two coaxial, cylindrical monoliths with a mono flow channel for the substrate (Membrane (1)). The enzymes were confined in the annular space (volume about 43.5 ml) defined by two separating walls (Membranes (1, 2)). Oligosaccharides were synthesized at 40°C in 5mmol/l potassium phosphate buffer containing 5mmol/l MgSO_4 using various initial lactose concentrations ranging from 10 to 30% (w/w). The TMP was about 1 to 2 bar with shaking. During processing the lactose solution was pumped into the inner compartment of the membrane reactor (substrate flow channel) using a peristaltic pump (Fig. 2). The substrate permeated in radial direction across the inner membrane (1) to the annular

space where enzymatic hydrolysis occurred. Products (glucose, galactose, and oligosaccharides) contained in the permeate and the unconverted lactose passed through the outer membrane (2) due to the TMP. Assessing the driving force of the membrane separation, the TMP was determined measuring the inlet (P_1) and outlet (P_2) fluid pressures and averaging these values. The permeate was collected in the outer compartment. No permeate was recycled.

Membrane cleaning

Fouling of the membrane surface during processing decreases the productivity of the membrane and is responsible for limitation on performance of the enzyme membrane reactor system used. Membranes fouled through substrate, enzyme and products were cleaned with pure water and lye solutions (1 % (w/w) NaOH-Solution, Ultrasil P3-14, Ultrasil P3-10 for 30 to 60 min). Cleaning efficiency after membrane cleaning was evaluated determining the water flux recovery after cleaning relative to initial water flux.

Determination of carbohydrates

Carbohydrates containing glucose, galactose, oligosaccharides and the amounts of unconverted lactose were analyzed in the permeate utilizing high performance thin-layer chromatography (HPTLC) using a Camag system (Linomat 5, TLC Scanner 3, Camag GmbH, Berlin, Germany). A representative HPTLC chromatogram of products obtained from the production process described is shown (Fig. 3).

Determination of the enzyme activity

The initial and final enzyme activity was measured for each experiment incubating the enzyme and the substrate, o-Nitrophenyl- β -D-galactopyranosid (o-NPG), at 30°C for 5 min. The absorbance was detected at 420 nm.

Protein determination

Protein was determined utilizing a dye binding method (Bradford) and bovine serum albumin (BSA) as standard protein [10].

3. Results

Oligosaccharide concentrations at different average residence times

The amount and type of the oligosaccharides formed is determined by several parameters including enzyme source, concentration and nature of the substrate, degree of conversion of the substrate and reaction conditions [1]. Paradigmatically, data obtained through the continuous production of oligosaccharides as a function of the average residence time where β -galactosidase was physically immobilized using a two-stage integrated ceramic membrane reactor are shown (experimental conditions were: initial lactose concentration, 30%; volume of enzyme/buffer solution, 100 ml; enzyme concentration, 2.5% (w/w); pH 6.7; temperature, 40°C) (Fig. 4a and Fig. 4b). Using an average residence time of 16 min, the maximum oligosaccharide concentration exceeded 38% (w/w) (Fig. 4a). Besides, the permeate flux of membrane (2) decreased from initially 23 to 11 l/(h*m²) after 180 min run time at 2 bar TMP. Testing the enzyme activity in permeate, no enzyme leakage through the membrane was detected.

Oligosaccharide concentrations at different lactose concentrations

A main parameter affecting the yields of transgalactolysation products, substrate concentration increased shifts the equilibrium of the two reactions described towards the transgalactolysation [11, 12]. Thus, the initial lactose concentration was varied from 10% to 30% (w/w) and reactions were detected for 120 min (Fig. 5). Samples were taken at regular time intervals for further carbohydrate analytics. Data indicate increasing production of

oligosaccharides from 13% to 31% (w/w) after 1 h process time and using a higher initial lactose concentration as indicated (Fig. 5). This is evidence for lactose intensified competing with H₂O as an acceptor for galatosyl residues when the initial lactose concentration was increased.

4. Conclusions

Aiming at a new type of ceramic membrane reactor system for the continuous enzymatic synthesis of oligosaccharides from lactose using physically immobilized β -galactosidase from *Kluyveromyces lactis*, data obtained show that physically immobilized enzyme used in a two-stage integrated ceramic membrane reactor may perform well in the continuous production of GOS from lactose as substrate. Significant oligosaccharide concentrations were achieved in permeate, at different average residence times (Fig. 4a, 4b) and initial lactose concentrations (Fig. 5). A maximum oligosaccharide concentration yielded exceeded 38% (w/w) when an average residence time of 24 min, a TMP of 2 bar and an initial lactose concentration of 30% (w/w) were adjusted.

However, process optimization of the newly developed ceramic membrane reactor system in continuous mode including further investigation of process parameters used is needed. To maximize oligosaccharide yield in the permeate, the effect of process parameters (initial substrate concentration, various TMP, the use of different enzymes and different residence times) and statistical combinations of these parameters affecting the oligosaccharide yield have to be investigated.

Though, advantages of the novel type of ceramic membrane reactor presented include the possibility of continuous production of GOS, simple enzyme separation and recovery through physical immobilization, efficient optimization of average residence time of enzyme and reduced hydrolysis of newly formed GOS through rapid separation of the product from the enzyme. Thus, the process presented has proven its use aiming at oligosaccharide production and is promising when GOS production exceeding a laboratory scale is requested.

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Table1: Ceramic membrane material and properties.

| Membrane | MWCO | Material | pH range | Max. Temp. |
|---------------------|-----------------------|---------------------------------------------------|----------|------------|
| UF-TiO ₂ | 10,000 Da / 20,000 Da | AL ₂ O ₃ / TiO ₂ | 0 - 14 | 121°C |

Table 2: Source and properties of enzyme

| Trade name | Derived from | Opt. pH | Opt. Temp. | Activity [U/g] |
|-----------------|-----------------------------|-----------|------------|----------------|
| Maxilact L 2000 | <i>Kluyveromyces lactis</i> | 6.8 – 7.0 | 35 – 40 °C | 2000 |

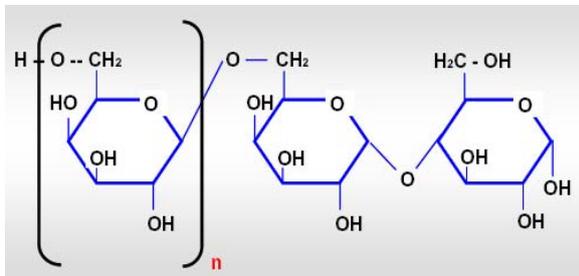
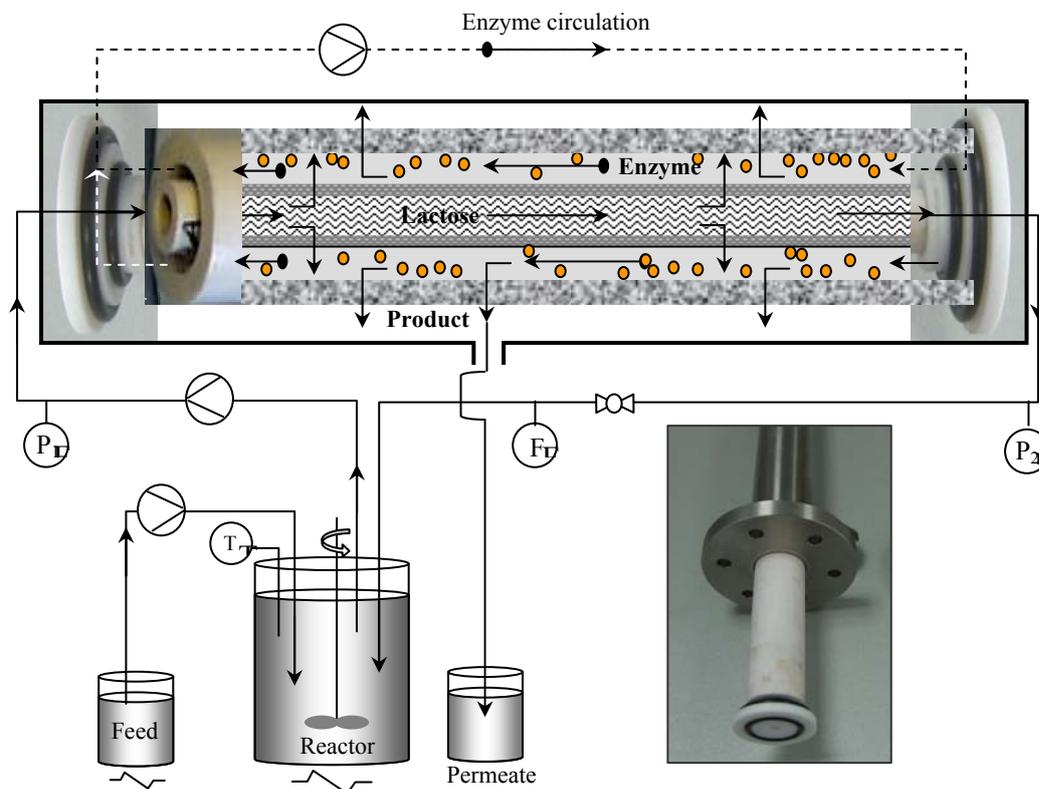


Fig. 1. General chemical structure of GOS



■ Membrane 1; ■ Membrane 2; ▨ Substrate flow; ● Enzyme flow

Fig. 2: Schematic diagram of continuous laboratory scale reactor/membrane system with physically immobilized β -galactosidase and enzyme circulation.

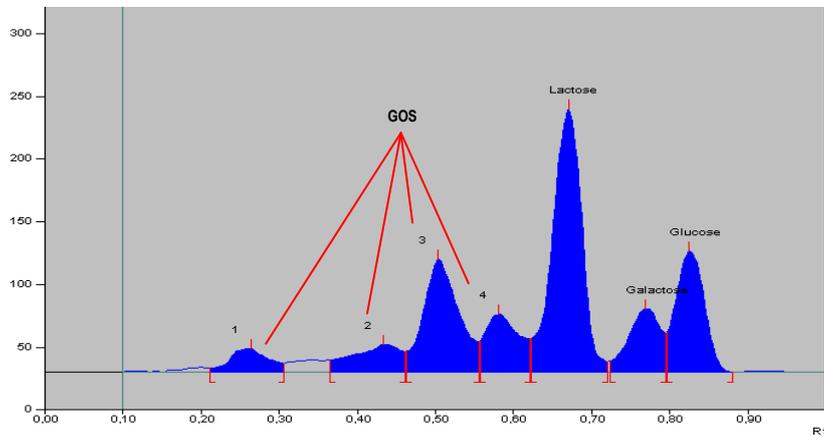


Fig. 3. HPTLC chromatogram of products obtained during continuous enzymatic synthesis of GOS (Sum of peaks 1-4) from lactose using enzyme Maxilact L 2000

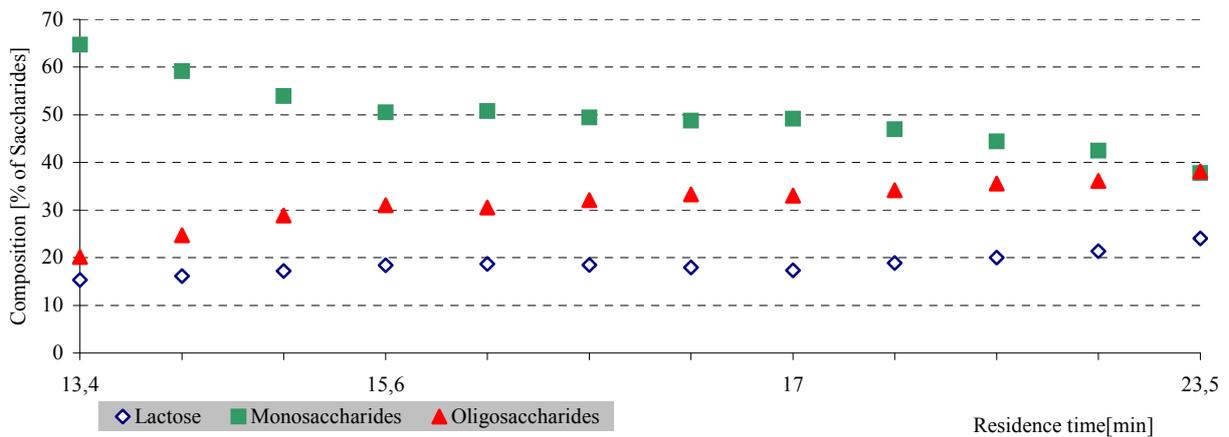


Fig. 4a) Continuous production of oligosaccharides from lactose in a two-stage integrated ceramic membrane reactor system as a function of the average residence time; process time, 180 min; initial lactose concentration, 30%; TMP, 2 bar; 40°C; pH, 6.7; 20,000 Dalton ceramic membranes were used.

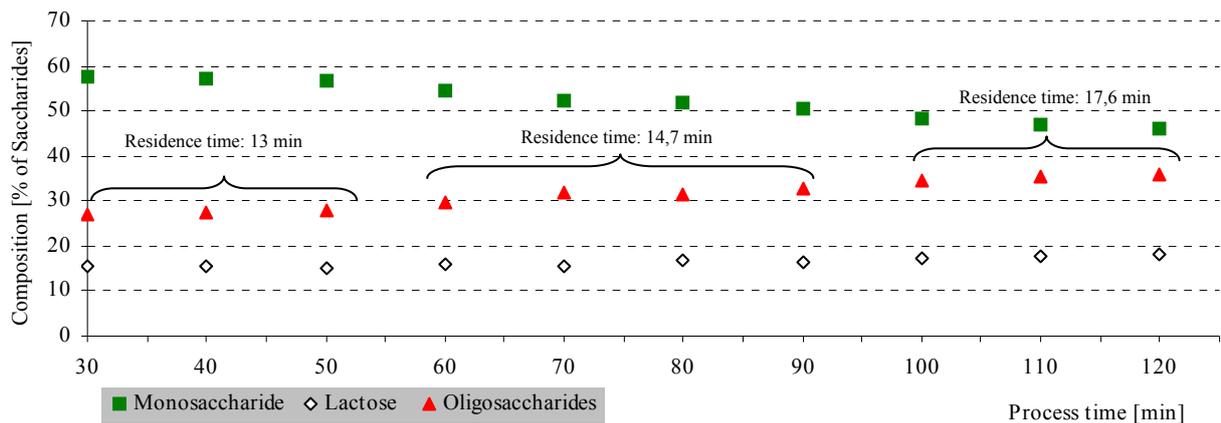


Fig. 4b) Continuous production of oligosaccharides from lactose in a two-stage integrated ceramic membrane reactor system as a function of the average residence time; process time, 120 min; initial lactose concentration, 30%; TMP, 1,7 - 1,8 bar; 40°C; pH, 6.7; 20,000 Dalton ceramic membranes were used.

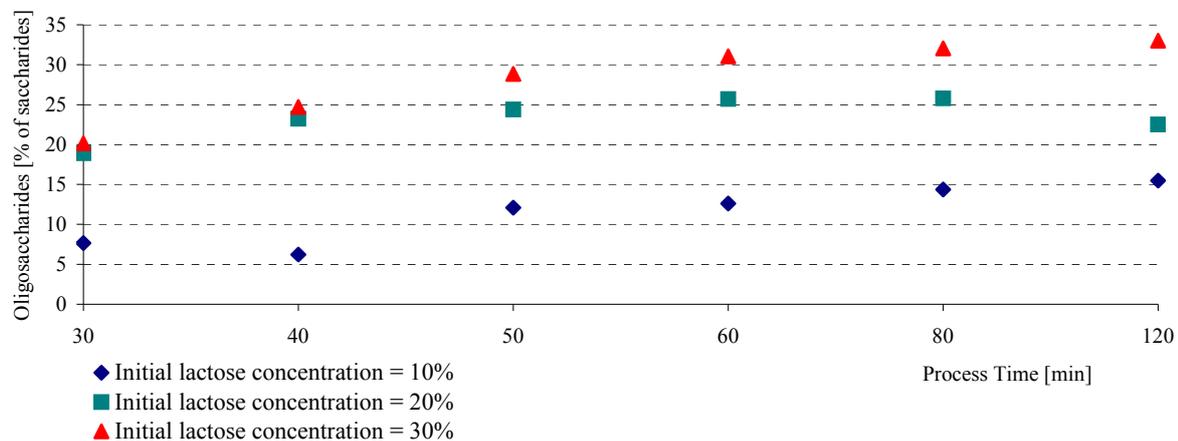


Fig. 5. Oligosaccharide concentration in permeate in a continuous mode membrane reactor system versus process time at different initial lactose concentrations 10, 20 and 30%; TMP, 1,8 bar; 40°C; pH 6.7; 20,000 Dalton ceramic membranes were used.