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Kinetics of lactose conversion to galacto-oligosaccharides by β-galactosidase immobilized on PVDF membrane

Tapas Palai and Prashant K. Bhattacharya*

Department of Chemical Engineering, Indian Institute of Technology Kanpur, Kanpur 208016, India

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Experimental studies were made for immobilization of enzymes on microporous polyvinylidene fluoride (PVDF) membrane in order to carry out enzymatic reaction of lactose into galacto-oligosaccharides using β-galactosidase. The present work, however, is the first part in the direction of enzymatic membrane reactor studies for carrying out reaction followed by membrane based separation to purify galacto-oligosaccharides out of reaction mixture. The middle of the three compartment cell, separated by two immobilized (enzyme) membranes, was utilized to feed lactose solution; whereas, adjacent compartments were filled with distilled water. The reacted mixture solution was analyzed for tri-, tetra- and penta-forms of GOS. The formation of product GOS strongly depended on varying amounts of initial lactose concentration (ILC). Total GOS formation increased from 7% to 28% for ILC from 50 to 200 g/L. However, tri-saccharide was the major (67%) in comparison to tetra (27%) and penta (6%) forms of GOS. Further, based on Michaelis–Menten kinetics, a six-step-eleven-parameter model was developed. The model incorporated enzyme inhibition and formation of glucose and galactose separately. Simulated results from developed model matched exceeding well with experimental results.

[**Key words:** Enzyme immobilization; Galacto-oligosaccharides; Microporous membrane; Kinetic model; Lactose hydrolysis]

Recently, the concept of functional food, prebiotics and probiotics food ingredients got much attention in food technology (1). Galacto-oligosaccharides (GOS), important nutraceuticals, are one such kind of compounds of this class. The general structure of GOS may be represented as $(galactose)_n$-glucose, where n is 2–9, i.e., a galactosyl-galactose chain with a terminal glucose residue (2).

Whey is a co-product of cheese production. Whey or Milk Serum is the liquid remaining after milk has been curdled and strained when an edible acidic substance is added. One of the major and principle components of whey is lactose, which is present around 5–6% (3). Lactose, when recovered (3–5), may serve as food supplement; infant mammals nurse on their mothers to drink milk, which is rich in lactose. Considering a 3% annual increase in cheese production, lactose is a major byproduct of the dairy industry (4). Extensive research has been reported for better utilization of whey lactose, but dairy industries still need efficient technologies to produce marketable products out of lactose (6). In this regard, conversion of lactose into GOS (7) would be of greater market value than lactose. The GOS can be used in a variety of products, including fermented milk products, breads, jams and beverages (8).

Several studies on GOS synthesis from lactose, using enzymes from different sources, have been performed under free enzyme condition. The β-galactosidase enzymes are well established in production of oligosaccharides (7). The enzymatic hydrolysis of lactose, catalyzed by β-galactosidase, mainly yields glucose and galactose, but in the same biochemical reaction, GOS are also formed by a trans-galactosylation reaction (9). In aqueous systems, trans-galactosylation competes with hydrolysis. Therefore, considerable amount of unreacted lactose and byproduct monosaccharides always present in GOS mixtures (10). The separation and purification of GOS mixture is essential for its application as a prebiotic food ingredient (11,12). Goulas et al. (13) made an investigation for the purification of oligosaccharides mixture employing continuous diafiltration using nanofiltration membrane and found 81–98% yield of oligosaccharides.

However, one of the major concerns is the simultaneous formation of monosaccharides, a cause for enzyme inhibition, under free enzyme condition (14). Biocatalyst immobilization on solid supports like membrane got significant attention in food and dairy industries (15). Recently, a concept is being proposed to immobilize enzyme (16) and carry out the reaction with simultaneous removal of monosaccharides. This brings the application of enzymatic membrane reactor (EMR). Immobilization offers several other advantages like providing high surface area of reaction, enzyme reusability, continuous product formation (17), increase in reactor stability and productivity, improvement in product purity and quality (7), reduction in processing cost by decreasing number of processing steps (15) and above all ease of separation of enzyme after reaction. However, immobilization also poses certain limitations like membrane fouling (17) and diffusional problems. Several supports, such as ion-exchange resin, merckogel (18), chitosan

* Corresponding author. Tel.: +91 512 2597093; fax: +91 512 2590104.
E-mail address: pkbhatta@iitk.ac.in (P.K. Bhattacharya).

Abbreviations: BCA, Bicinchoninic acid; GOS, Galacto-oligosaccharides; ILC, Initial lactose concentration; ONP, *o*-Nitrophenol; ONPG, *o*-Nitrophenyl-β-D-galactopyranoside; PVDF, Polyvinylidene fluoride.

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beads (19,20), agarose beads (21), graphite (22) and cotton cloth (7) have been used for the immobilization of enzyme. Few studies on immobilization of enzyme on several polymeric membranes have also been reported in the literature (3,23,24). Further, a study (25) on economic evaluation of lactose hydrolysis showed that enzyme immobilization on some support is economically feasible irrespective of reactor types and configurations.

Choice of membrane, as support, in the present studies for immobilization was attempted with a purpose. It was thought if successful, eventually hollow fibers containing membrane contactor may be utilized in achieving much higher loading of immobilized enzyme as the said contactor provides significantly higher membrane surface area per unit volume. The enzyme can be immobilized on membrane surface by entrapment, gelification, physical adsorption, ionic binding, covalent binding or cross-linking (15). Owing to its loose binding with support, immobilization by adsorption offers better opportunity to reuse supports after simple washing but chances of enzyme leakage (26) is more prominent than other techniques. On the other hand, cross-linking, an irreversible immobilization method, provides better enzyme stability (27) and hence less chances of enzyme leakage. Gaur et al. (19) made a comparative study between different immobilization techniques and noticed that the covalent coupling is most suitable method for enzyme immobilization.

In the present study, main objective was to immobilize enzyme on microporous hydrophobic polyvinylidene fluoride (PVDF) membrane for conversion of lactose in to GOS using a commercial grade β-galactosidase from *Bacillus circulans*. Essentially, investigations were made to observe the influence of varying lactose concentration on GOS formation under immobilized enzyme condition. Further, it was thought to study kinetics in order to depict how different experimental conditions can influence the speed of a chemical reaction and yield information about the reaction's mechanism, as well as the construction of mathematical models that can describe the characteristics of a chemical reaction. Therefore, a model based on Michaelis–Menten kinetics was developed. In our earlier attempt (28), a simple four step reaction kinetics was developed under free enzyme condition. However, the model considered together the formation of glucose and galactose as monosaccharides while considering tri- and tetra-saccharides as GOS. Model though considered enzyme inhibition to be negligible. The present model, therefore, was attempted to include not only the inhibition of enzyme, it also includes glucose and galactose separately. However, tri-, tetra- and penta-saccharides are represented as GOS. It is expected that a membrane may have limited life span if it is repeatedly utilized for immobilization purpose. Thus, the objective was also to test number of times a single membrane would be immobilized after each specific experimental run.

MATERIALS AND METHODS

Chemicals Commercial grade β-galactosidase (EC 3.2.1.23, commercial name: Biolacta FN5) of specific activity of 1.2 U/mg-powder, extracted from *B. circulans*, was provided by Daiwa Kasei K.K., Japan. Lactose GR (monohydrate), D-galactose (extra pure, minimum assay 99%) and glutaraldehyde (25% aqueous solution) were purchased from Loba Chemie Pvt. Ltd., India. D-Glucose (anhydrous) was procured from Qualigens Fine Chemicals, India. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) (assay ≥ 98%) was supplied by Sigma Aldrich. Bicinchoninic acid (BCA) protein reagent kit was obtained from Novagen, USA. All chemicals were used without any further purification. Milli-Q (Millipore Corporation, India) water was used for the preparation of entire solution. Hydrophobic PVDF membrane (pore size: 2.2×10^{-7} m, thickness: 1.25×10^{-4} m, diameter: 0.047 m, mass: 0.134 g, porosity: 75%, water flow rate: 9000 L/m^{2}h and protein binding: 1.5 g/m^2) was supplied by Millipore Corporation, India.

Enzyme assay The activity of β-galactosidase was estimated using ONPG as substrate. The reaction was carried out as per earlier described procedure (28). The amount of *o*-nitrophenol (ONP) released during the reaction was measured in UV-visible spectrophotometer (Hitachi, U2900) by its absorbance at 410 nm. The protein content of the commercial preparation was estimated to be 0.171 g/g-powder enzyme by BCA method (29) using bovine serum albumin (BSA) as standard. One unit (U) of enzyme is defined as the amount of enzyme required to convert 1.0 μmol of ONPG to ONP and D-galactose per minute, at pH 6.0 and 40°C.

HPLC analysis As per procedure described earlier (28), the concentrations of lactose, glucose, galactose, and GOS (tri-, tetra- and penta-saccharides, etc.) were determined on an HPLC system (Waters), complete with all accessories; Sugar Pak-I column (300 mm × 6.5 mm; packed with calcium-loaded resin), a refractive index detector (2414, RID-10A), two pumps (515), a column heater, an auto-sampler (2707), an in-line degasser (DG2), and Empower-2 data processing software. Aqueous solution of ethylene-diamine-tetra-acetic acid calcium disodium (50 mg/L) was used as mobile phase at 0.5 mL/min flow rate. The temperatures of the column and detector were maintained at 75°C and 35°C, respectively.

Enzyme immobilization The β-galactosidase enzyme was immobilized on compacted PVDF membrane by cross-linking with glutaraldehyde (22). Since the three compartment cell had the provision of housing two membranes, thus the weights of both the membranes' were counted as each of them faced the feed solution. Accordingly, two PVDF membranes were equilibrated in phosphate buffer solution (pH 6.0) for 1 h. The membranes were activated for 4 h at 30°C with 4% v/v aqueous glutaraldehyde solution to improve covalent bonding ability; followed by buffer washing to remove excess glutaraldehyde. For completion of the cross-linking, membranes were immersed in enzyme solution (20 mL) of desired concentration (2.4–18 kU/L) for 18 h keeping in an incubator at 20°C followed by washing with brine (removal of adsorbed enzyme). Finally, membranes were stored in fresh buffer solution at 20°C. The amount of immobilized enzyme was calculated by difference of activity of enzyme solution before and after immobilization. Specific enzyme loading is defined as the units of enzyme (U) immobilized per unit mass of dry membrane (Eq. 1). On the other hand, enzyme loading is defined as the ratio of the units of enzyme (U) immobilized on membrane surface to units of enzyme (U) taken in solution (Eq. 2).

$$\text{Specific enzyme loading} = \frac{\text{Units of enzyme immobilized}}{\text{Mass of dry membrane}} \tag{1}$$

$$\text{Enzyme loading}(\%) = \frac{\text{Units of enzyme immobilized}}{\text{Units of enzyme (in solution) taken}} \times 100 \tag{2}$$

A schematic presentation of immobilized enzyme on membrane support using glutaraldehyde is shown in Fig. 1A.

Synthesis of GOS in three compartment cell A three compartment test cell, made out of perspex sheet, was designed and fabricated. Middle frames (25 mL) housed two immobilized membranes and filled with lactose solution dissolved in 50 mM phosphate buffer (pH 6.0) at 40°C. Other two sides were filled with water to support the membranes. The initial lactose (50–200 g/L) and enzyme solution concentrations (6–18 kU/L) were varied. At regular intervals, samples were withdrawn for analysis through HPLC till completion of the reaction (~30 h). They were then clarified by centrifugation at 10,000 rpm for 10 min, filtered through a 0.45 μm filter and the supernatants were diluted as required before HPLC analysis.

Membrane compaction and hydraulic membrane resistant determination The membrane was placed on the porous support and then the cell was assembled (30). In order to avoid any compaction of the membrane during operation, the membrane was a-priori allowed to be compacted in an ultrafiltration cell at a pressure (207 kPa) higher than the maximum operating pressure (179 kPa) till (~8 h) a constant water flux was observed. The constancy of water flux beyond this time interval suggests no further compaction of the membrane. This constant water flux was used to calculate (30) membrane hydraulic resistance (R_m).

Subsequent to membrane compaction, pure water flux (J_w) was measured at different trans-membrane pressure (TMP, range: 69–179 kPa). Thus membrane hydraulic resistance (R_m) was obtained from linear plot of J_w versus ΔP (Eq. 3).

$$R_m = \frac{\Delta P}{\mu_w J_w} \tag{3}$$

where ΔP is TMP and μ_w is the viscosity of water at experimental temperature.

To predict the change in membrane characteristics, J_w and R_m values of membranes were evaluated; for both states, before and after immobilization (Table 1). It is evident that the membrane resistance increases gradually with repeated immobilization; whereas J_w (at 179 kPa TMP) decreases. Flux reductions values (see Table 1) are reported as per Eq. 4. A reduction in water flux of ~74% was observed after third run. This is a clear indication of pore blocking due to the use of glutaraldehyde during enzyme immobilization and also may be due to accumulation of products on the surface or within the pores of the membranes (16).

$$\text{Flux reduction } (\%) = \frac{J_w^{BI} - J_w^{AI}}{J_w^{BI}} \times 100 \tag{4}$$

where J_w^{BI} and J_w^{AI} are the pure water fluxes before and after immobilization, respectively.