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RESEARCH PAPER

Immobilization of *Aspergillus oryzae* β-Galactosidase on Low-pressure Plasma-modified Cellulose Acetate Membrane Using Polyethyleneimine for Production of Galactooligosaccharide

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Abstract The aim of this study was to produce galacto-oligosaccharides (GOS) from lactose using β-galactosidase from *Aspergillus oryzae* immobilized on a low-pressure plasma-modified cellulose acetate (CA) membrane. Specifically, a novel method was developed for multilayer enzyme immobilization involving polyethyleneimine (PEI)-enzyme aggregate formation and growth on a CA membrane. A large amount of enzyme (997 μg/cm^2 membrane) was immobilized with 66% efficiency. The K_m value for the immobilized enzyme was estimated to be 48 mM, which indicates decreased affinity for the substrate, whereas the *Vmax* value was smaller. The immobilized enzyme showed good storage and operational stability. The half-life of the immobilized enzyme on the membrane was about 1 month at 30ºC and ~ 60 h at 60°C. Maximum GOS production of 27% (w/w) was achieved with 70% lactose conversion from 320 g/L of lactose at pH 4.5 and 60°C. Trisaccharides were the major types of GOS formed and accounted for about 75% of the total GOS produced. Based on these results, immobilized enzyme technology could be applied to GOS production from lactose.

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1. Introduction

Biocatalyst immobilization has gained increased attention for the synthesis of several industrial bioproducts. Galacto-oligosaccharides (GOS) are important bioproducts that play a range of crucial roles in biological systems as prebiotic food ingredients. Introduction of GOS into food products is desirable due to their many health benefits [1]. GOS are produced by enzymatic transglycosylation reaction of lactose on an industrial scale. β-galactosidase (EC 3.2.1.23) catalyzes lactose hydrolysis as a forward reaction and oligosaccharide synthesis as a reverse reaction [2]. The reaction conditions that favor trangalactosylation, or the reverse reaction, consist of high lactose concentration, elevated temperature, and low water activity in the reaction medium. β-galactosidase preparations that are thermally stable and exhibit high transgalactosylation activity need to be developed for efficient oligosaccharide synthesis [3].

Immobilization is a widely employed technique for the industrial application of enzymes with enhanced stability, reusability, cost-effectiveness, and viability [2]. Enzyme immobilization can be economically advantageous if there is a significant increase in enzyme stability and the support can be reused [4]. A wide variety of support materials and immobilization techniques can be applied for this purpose [5]. From a stability and reactivity point of view, immobilization via covalent bonding seems to be the best method

for enzyme attachment. Although many reactor designs and their potential advantages have been reported, enzymatic membrane reactors are generally an engineer's choice for commercial or industrial applications due to their high efficiency, ease of operation, and general simplicity. However, polymeric membranes usually do not possess the surface reactive groups that make covalent immobilization possible. Wet and dry chemical modification methods can be used to introduce functionalities onto a membrane surface [6]. Although low-pressure plasma treatment, a dry modification technique, can provide some desirable characteristics for enzyme immobilization on a membrane surface [7], the amount of immobilized enzyme is rather low and thus needs to be improved for industrial applications. In our previous study [8], plasma treatment was used to modify the pore size distribution of CA membranes instead of increasing surface reactivity, since many enzymatic processes in the food industry require selectively-permeable membranes in order to enhance product yield and quality on an industrial scale [9-11]. The goal of our present research was to develop a method featuring polyethyleneimine (PEI) for enzyme immobilization on plasma-modified membranes that can be used in a membrane biocatalyst reactor. PEI, a highly branched cationic chain polymer [12], has many applications in biochemistry due to its ability electrostatically interact with negatively charged species [13]. PEI has been an essential ingredient in many enzyme immobilization procedures, where it serves to coat an inert support such as porous glass microbeads [14] or charged insoluble carriers [15,16].

The present study explores an appropriate immobilization method for β-galactosidase that can be applied to oligosaccharide synthesis. The development of a novel technique involving PEI for the immobilization of β-galactosidase from *A. oryzae* onto a plasma-modified membrane support, which is then used in GOS production, was carried out in this study. Some properties of immobilized β-galactosidase, including optimum pH, temperature, and kinetic parameters, were investigated and compared to those established for the native enzyme. The stability and activity of the immobilized enzyme under various storage and operational conditions were evaluated. Finally, the immobilized lactase derivative was evaluated in GOS production from lactose in a membrane reactor.

2. Materials and Methods

2.1. Enzyme and reagents

β-galactosidase from *A. oryzae* (fungal lactase activity 100,000 U/g) was obtained from ENZECO® (Enzyme Development Corporation, USA). Lactose (99.9%) was purchased from Merck (Darmstadt, Germany). Polyethyleneimine [PEI; (C2H5N)*n*] (number average molecular weight 60,000; average molecular weight 750,000) and glutaraldehyde (GA) both as 50% (w/v) aqueous solutions were from Sigma (St. Louis, MO) and AppliChem (Darmstadt, Germany), respectively. Phosphate (Mallinckrodt Baker, Netherlands) and acetate (Sigma, USA) buffers were used to prepare the enzyme solution. Commercial asymmetric CA membranes having 0.2 μm pore size were purchased as sheets from Sartorius (Germany). All solutions for PEI, GA, and enzyme were prepared with double distilled water (Nanopure 185 Ultrapure System). The solution pH was adjusted when necessary using 0.1 N HCl or 0.1 N NaOH solution. Ethylenediamine (EDA), which was used as monomer for plasma deposition, was supplied from Sigma (USA).

2.2. Preparation of low-pressure plasma modified CA membranes

CA membranes were modified by plasma polymerization (PlzP) of EDA at a discharge power of 120 W and exposure time of 10 min. PICO type Low Frequency (LF) plasma equipment was supplied from Diener Electronics GmbH + Co (Germany). The plasma chamber was stainless steel (150 mm radius and 320 mm length). A 40 KHz LF generator (power range 0 ~ 200 W) was used to sustain the plasma in the reactor. PlzP processes were carried out in the fully closed and semi-automatic system. CA membranes were placed onto the ground electrode in the middle of the reactor. For plasma generation, low-pressure was created in the reactor using a vacuum pump (Trivac, Germany). Then, at a pressure of approximately 0.1 mbar, monomer EDA vapor was fed into the chamber. EDA vapor was allowed to flow at a special rate from 0.1 to 0.3 mbar. The plasma power was then adjusted, and the CA membranes were exposed to glow discharge. At the end of the process, the plasma generator was turned off automatically while the monomer inlet was closed manually. The plasma system was fed with argon gas for 10 min. Finally, the plasma system was placed in 0.1 mbar of vacuum pressure for 15 min. Argon feeding and vacuum applications were applied to deactivate free radicals in the plasma atmosphere.

2.3. PEI-enzyme aggregate formation

The procedures for formation of PEI-enzyme complex/aggregate by mixing PEI and enzyme in solution were detailed in our previous study [3]. Various amounts of PEI (0.01 ~ 0.2 mg in 0.1 mL of solution) were mixed with 1 mL of 1 ~ 50 mg/mL enzyme solution in microcentrifuge tubes in order to study the effect of PEI concentration (or the ratio of PEI to enzyme, PEI/enzyme) on the formation of PEI-enzyme aggregates. After ~ 5 min, 0.1 mL of 2.5%

(v/v) GA solution was added to the mixture. The resulting mixture containing PEI-enzyme aggregates was centrifuged at 15,000 g for 1 min. Initial enzyme activities associated with the PEI-enzyme slurry (containing GA) and supernatant were determined and compared to free enzyme (containing neither PEI nor GA). Morphology of the PEI-enzyme aggregates in cloudy turbid slurry was also shown in our previous study [3].

2.4. Multilayer enzyme immobilization on low-pressure plasma-modified CA membrane

Enzyme immobilization on plasma-modified CA membrane comprised three main steps: Adsorption of PEI solution to the membrane, introduction of enzyme to the PEI-containing membrane, and GA cross-linking of PEI-enzyme aggregates coated on the membrane.

Unless otherwise noted, 1 mL of PEI solution (pH 8.0) containing 0.018 ~ 0.18 mg/cm^2 of PEI was added to each 7 cm^2 portion of the membrane. The solution volume was sufficient enough to completely wet the membrane, thereby allowing a homogeneous distribution of PEI to contact the matrix. After adsorption of PEI, 8 mg of enzyme (5 mL of 1.6 mg/mL enzyme solution) was added. When a clear enzyme solution in distilled water was mixed with PEI solution, a cloudy, turbid, or “milky” slurry containing PEI-enzyme aggregates was instantaneously formed. The flasks were put into a shaker-incubator for 5 ~ 10 min. The white turbidity disappeared within 5 min, and the coupling solution was completely clarified. The clarified coupling solution was then slowly decanted, and PEI-enzyme-coated membranes were immersed in cold GA solution (2.5% (w/v), pH 7.0) for cross-linking for 5 min. The cross-linked membranes were washed extensively with distilled water and then with phosphate buffer (0.1 M, pH 7.0). All procedures were carried out in 125 mL Erlenmeyer flask.

2.5. Determination of optimum temperature, pH, and kinetic parameters

K_m, V_m, optimum temperature, and pH were determined by individually changing the conditions of the activity assay (pH from 3 to 9.0; temperature from 30 to 85°C; and lactose concentrations from 25 to 1,000 mM). Acetate (50 mM, pH 3.0 ~ 5.0) and phosphate (50 mM, pH 6 ~ 9) buffer solutions containing 4 mM $MgCl_2$ were used. Initial velocities for kinetic parameters were determined by carrying out the reactions for 1 min. K_m and V_m were calculated from Hanes-Woolf plots [17].

2.6. Thermal and pH stability of immobilized enzyme

The thermal stabilities of free and PEI-immobilized enzyme in 50 mM acetate buffer (pH 4.5) at various temperatures (30, 40, 50, and 60°C) were studied in a shake flask with 100 rpm. Reaction samples (100 μL) were taken in 2 min intervals from the reaction medium throughout the reaction period and analyzed by High Performance Liquid Chromatography (HPLC) (Section 2.9.2).

To determine the pH stabilites of the native and immobilized enzymes, soluble and immobilized enzymes were incubated in various buffers (pH 4.0 ~ 10.0) at room temperature (25 ± 2°C) for 18 h, and the remaining activities of these enzymes were determined under standard assay conditions (Section 2.9.1).

2.7. Storage stability and reusability of the immobilized enzyme

The soluble and immobilized enzymes were stored by cross-linking in phosphate buffer (pH 7.0) solution at 4°C at room temperature (25 ± 2°C). The immobilized enzyme in dried form was stored at room temperature for 28 days to evaluate the stabilities of the soluble and immobilized enzymes. The CA membranes stored in dried form were preconditioned in 5 mL of phosphate buffer (pH 7.0) for 15 min. The remaining activities of the soluble and immobilized enzymes were measured at their optimal temperature in 100 mM lactose solution.

2.8. GOS production in a membrane reactor

Batch production of GOS from lactose was studied in a membrane reactor [8]. GOS production with immobilized enzyme was carried out using a diffusion chamber with an effective membrane area of 7 cm^2. Experiments were carried out at 59 ± 2°C using a Perspex diffusion chamber consisting of two half cells held together with screw connections (A and B). Each half cell contained a round chamber of 150 mL volume. Enzyme-immobilized CA membrane samples were clamped between stainless steel discs. The chambers were secured with O-rings to obtain a water-tight connection. Both chambers were stirred using a headstirrer (Heidolph R2R 2102 Control, Germany) combined with a diffusion cell. Both chambers were filled with pH 4.5 acetate buffer and allowed to equilibrate for 15 min. The chambers were then emptied, after which chamber A and chamber B were simultaneously filled with lactose solution and acetate buffer (both pH 4.5), respectively, followed by rapid stirring. Samples (100 μL) from chambers A and B were then taken at proper time intervals (20 min intervals during initial 2 h of the reaction and 60 min intervals thereafter) and analyzed by HPLC (Section 2.9.2) and recorded. Experiments lasted around 360 min at a stirring rate of 100 rpm.

2.9. Analytical methods

2.9.1. Enzyme activity assay

Activity of the immobilized enzyme was measured with 100 mM lactose as the substrate in 50 mM acetic acid buffer (pH 4.5) at 60°C in a shaker-incubator at 150 rpm for about 1 min. After incubation, the membrane was removed from the reaction mixture and a volume of sample taken and mixed at a 1:1 ratio with 0.01 N NaOH to inactivate possible free enzyme activity leached during the activity determination. The glucose concentration in the sample was determined with HPLC (Section 2.9.2). The activity of the immobilized enzyme was determined by direct comparison of the reading with the standard curve of glucose concentration versus enzyme activity times reaction time (g/L vs (mg/mL/min)), which was obtained from free enzyme reactions and used to estimate the amount of active enzyme (mg/cm^2 membrane) and immobilization yield (%).

2.9.2. HPLC analysis

The concentrations of sugars in the sample solutions (glucose, galactose, lactose, and galactooligosaccharides) were determined by HPLC. ThermoFinnigan HPLC system integrated with an auto sampler, including temperature control for the column (SpectraSystem AS3000), a degasser system (SpectraSystem SCM1000), a quaternary gradient pump (SpectraSystem P4000), a refractive index detector (SpectraSystem RI-150), and a software package for system control and data acquisition (ChromQuest 4.0), were used for analyses. Sugars were analyzed with a Rezex RCM column (300 × 7.8 mm, Phenomenex) at 80°C using an RI detector. Deionized water was used as the mobile phase at a flow rate of 0.3 mL/min. Injection volume of the mobile phase was 20 μL. The concentrations (w/v) of these sugars (*e.g*., lactose, glucose, galactose, and oligosaccharides including tri-, tetra-, and, pentasaccharides) should be proportional to their peak areas with the same proportionality constant [3]. Thus, the normalized sugar concentrations, presented as weight percentages of total sugars, were determined from the peak heights and are reported in this paper.

3. Results and Discussion

3.1. PEI-enzyme aggregate formation

PEI-enzyme association and precipitation seemed to be the driving force of enzyme immobilization on a plasma-modified membrane. Therefore, the formation of PEI-enzyme aggregates in solution was studied first. The effects of PEI to enzyme ratio and pH on the activity of PEI-enzyme aggregates and the remaining activity in the supernatant after centrifugation of the cloudy solution were investigated. As shown in Fig. 1, the concentration of PEI slightly affected the enzyme activity at all ratios studied when the initial pH of the PEI solution was adjusted to 8.0. PEI-

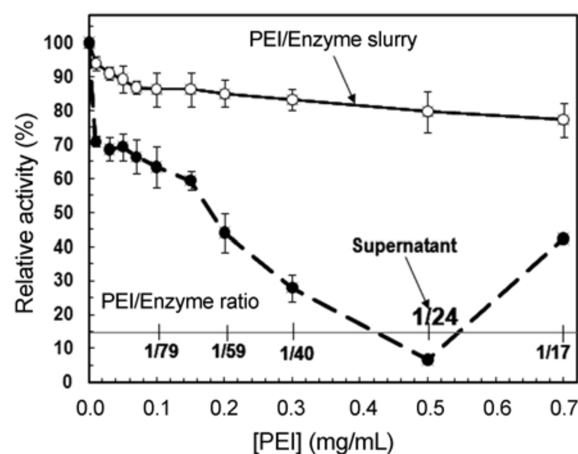

Fig. 1. Effect of PEI to enzyme ratio on enzyme activities of PEI-enzyme aggregates in solution and supernatant after centrifugation at 15.000 g for 1 min.

enzyme aggregate formation (cloudy solution) did not necessarily yield precipitation. The highest amount of enzyme precipitate was obtained at a PEI to enzyme weight (mg/mg) ratio of 1/20-1/40, whereas higher or lower ratios yielded ineffective particle formation that stayed in solution.

In addition to the PEI to enzyme ratio, pH was also found to be an important factor in affecting PEI-enzyme aggregate formation and the final activity of the complex. It was observed that the optimum pH range was between 6.0 and 8.0 (data not shown), and similar precipitation and activity levels were obtained. As the pH of the PEI-enzyme slurry was lowered to below 5.0, especially below 4.0, the turbid solution became clear and no precipitation occurred. At pH values above 8.0, aggregation and precipitation were not affected but enzyme lost its activity. Albayrak and Yang [3] reported that PEI-enzyme aggregate formation was totally reversible. The aggregates can be dissociated by lowering the pH, and the enzyme in the PEI-enzyme complex can be replaced by small, negatively charged species.

3.2. Enzyme immobilization on low-pressure plasma-modified cellulose acetate membrane

A wide range of PEI to enzyme ratios from 1/5 to 1/90 was first investigated to find the optimal PEI to enzyme ratio, which is a critical factor affecting the level of immobilized enzyme on a membrane surface. It was found that the maximum enzyme immobilization could be achieved when the PEI to enzyme ratio was near 1/20-1/40 (Fig. 2). This ratio was consistent with the optimal ratio for the formation of PEI-enzyme aggregates. Under this condition, a relatively constant immobilization yield of 65 ~ 70% was obtained.

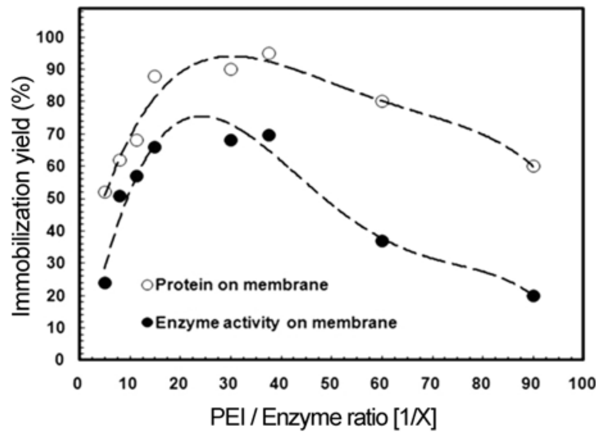

Fig. 2. Effects of PEI to enzyme ratio on immobilization yield.

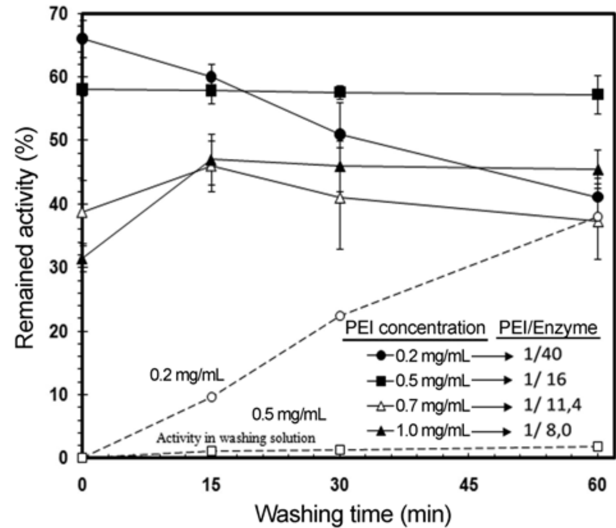

Fig. 3. Effects of different washing times on activity of enzyme on the membrane surface (PEI/Enzyme ratio: 1/8-1/40).

The effect of immobilization time at various PEI-enzyme ratios on remaining enzyme activity in adsorption solution was also investigated. There was almost no enzyme activity in adsorption solution after 15 min of adsorption at 0.2 mg/mL of PEI. Adsorption time of 5 min was not efficient for high yield immobilization at any PEI concentration. We did not find any significant differences between adsorption times of 15 and 30 min based on immobilization yield (data not shown).

Albayrak and Yang [3] also reported that enzyme immobilization on cotton cloth was affected by pH and temperature. The pH of the PEI-enzyme aggregates was driven by the initial pH and the concentrations of reactants, since no buffer was used in the preparation of either PEI or the enzyme solutions. When the pH of PEI solution was adjusted, the final pH of the PEI-enzyme cloudy solution was about 8.2 ~ 8.4. When the pH of PEI solution was adjusted to 6.0 ~ 8.0 and the enzyme was dissolved in distilled water (pH 6.6), insignificant differences were observed in the immobilization yield. When the pH of PEI-enzyme solution was reduced to 3.5, the solution lost its cloudy appearance and very little enzyme was immobilized. In this study, PEI solution was normally prepared with distilled water. When PEI solution was prepared in 0.05 M phosphate buffer, very low immobilization yield was achieved [3]. Clearly, phosphate-bearing negative charges competed with enzyme for interactions with PEI and essentially blocked the formation of PEI-enzyme aggregates. Positively charged ions, on the other hand, would cover the enzyme, and consequently, PEI would not be able to reach or would be repelled by the enzyme. Since ionized buffer species are small compared to enzyme, the immobilization capacity of PEI would be greatly reduced. Therefore, no buffer should be used, and the solution pH should be maintained between 6 and 8 during the PEI-enzyme coupling reaction.

The temperature for PEI enzyme immobilization also affected the activity of immobilized enzyme. The enzyme solution was kept on ice until added, but the temperature was not controlled during enzyme coupling. Cold enzyme solution (between 0 and 4°C) resulted in higher immobilized enzyme activity yield as well as more rapid enzyme immobilization (data not shown).

The final and crucial step of PEI-enzyme immobilization was GA cross-linking. In the absence of cross-linking, most of the enzyme is leached out from the membrane surface during enzymatic oligosaccharide synthesis. The immobilized membrane surface was washed in distilled water at 150 rpm for 15, 30, and 60 min in order to test immobilized membrane performance. As seen in Fig. 3, there were significant decreases in enzyme activity on the membrane surface, with the exception of 0.5 mg/mL of PEI. Although 0.2 mg/mL of PEI was determined to be the most effective immobilization parameter, from a stability point of view, there was significant enzyme loss at this PEI-enzyme ratio (1/40).

3.3. Kinetic parameters

For comparison purposes, we used standard experiments to measure the kinetic parameters of the free and immobilized enzymes using lactose as the substrate. The rate of enzymatic hydrolysis of lactose, υ, can be expressed by the Michaelis-Menten equation [17];

$$\upsilon = \left[\frac{V_{\max}[S]}{k_m + [S]}\right] \tag{1}$$

where K_m and V_{max} are kinetic constants and [S] is concentration of the substrate. This equation can also be expressed using Hanes-Woolf plots (Fig. 4);

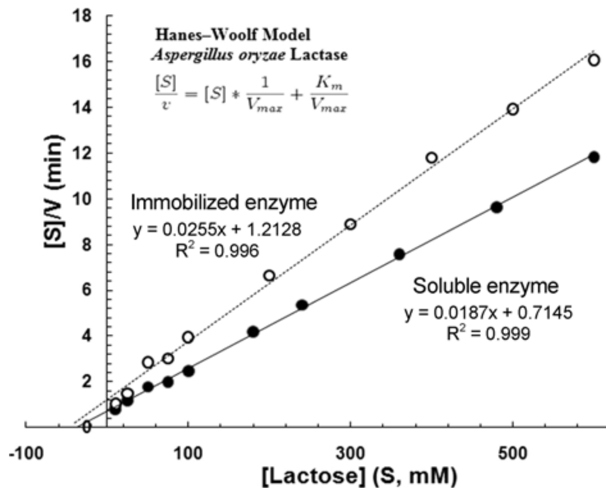

Fig. 4. Hanes-Woolf plots for free (●) and immobilized (○) enzymes.

$$\frac{[S]}{\upsilon} = [S] \times \frac{1}{V_{\max}} + \frac{k_m}{V_{\max}} \tag{2}$$

In this study, kinetic parameters for the free and immobilized forms of β-galactosidase were determined using lactose as the substrate in the range of 25 ~ 1,000 mM, as shown in Fig. 4. As shown in Table 1, the Michaelis Menten constants K_m and V_{max} for the free enzyme were found to be about 38 mM and 54 μmol of glucose/min/mg of enzyme, respectively. After the enzyme was immobilized onto the surface of CA membrane, K_m was increased 1.25-fold to 48 mM while V_{max} was slightly decreased to 39 μmol of glucose/min/cm^2 of support. Higher K_m values after β-galactosidase immobilization compared to those calculated for soluble enzymes have been reported by other authors with increases from 1.2-fold [20] up to 5.4-fold [21]. These results are most likely due to the fact that the immobilized enzyme surfaces are not accessible to all reacting species. The reduced catalytic activity can be attributed to several factors, such as protein conformational changes induced by the support, steric hindrances, and diffusional effects. These factors may operate simultaneously or separately, alternating the microenvironment around the bound enzyme [22].

3.4. Characterization of immobilized enzyme

To investigate the industrial feasibility of the immobilized enzyme process, loss of enzymatic activity during storage and application must be considered. The optimum temperature and pH values for soluble enzyme activity were found to be 55°C (Fig. 5A) and 4.5 (Fig. 5B), respectively. The optimal pH shifted to 5.5 upon enzyme immobilization, whereas the optimal temperature increased to 60°C. The immobilized enzyme derivative was slightly more stable at higher pH; the soluble and immobilized enzymes retained 5 and 45% of initial activity, respectively, at pH 8.0. These optimum pH values are within the range reported in the literature (4.5 ~ 5.5). Additionally, the optimum temperature value for the immobilized derivative was equal to those reported in the literature for immobilized β-galactosidase obtained from *A. oryzae* [20]. It is worthwhile to note that at 85°C, soluble enzyme lost almost all of its activity while the immobilized enzyme retained 65%

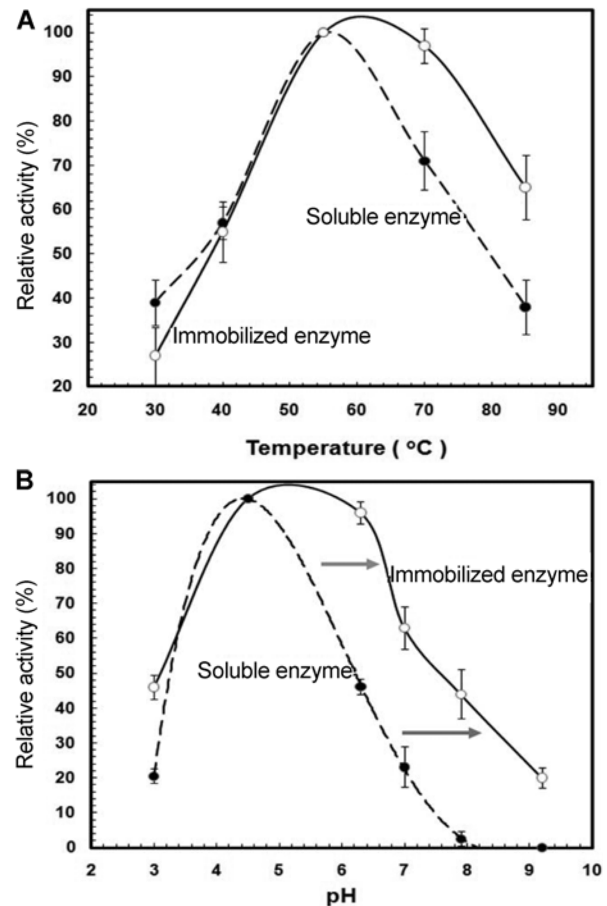

Fig. 5. Effects of temperature (A) and pH (B) on the activities of free (●) and immobilized (○) enzymes.

Table 1. Kinetic parameters of free and PEI-immobilized β-galactosidase from *A. oryzae*

Soluble enzyme		Immobilized enzyme	
V_{max} (μmol glucose/min/mg enzyme)	K_m (mM)	V_{max} (μmol glucose/min/cm^2 support)	K_m (mM)
54	38	39	48

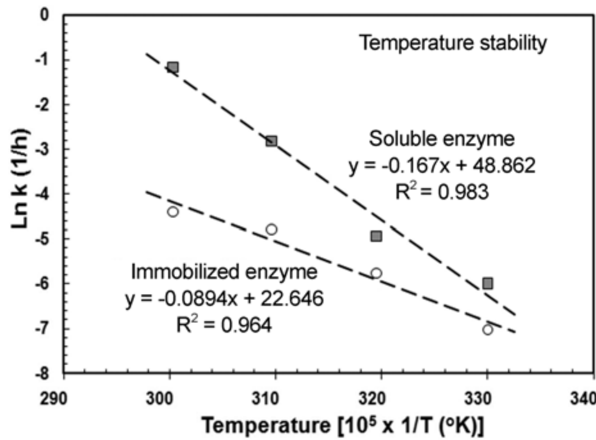

Fig. 6. Free (■) and immobilized (○) enzyme stabilities at different temperatures.

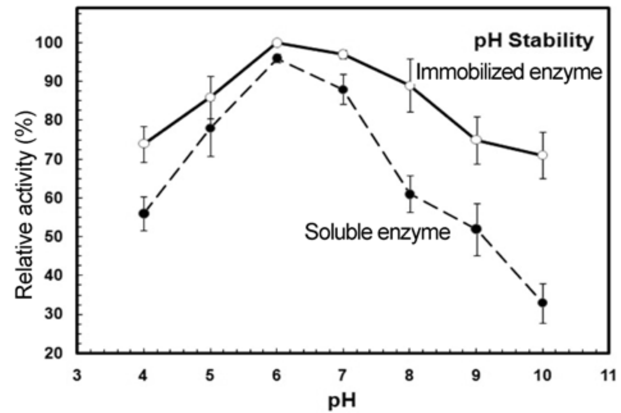

Fig. 7. pH stability of free (●) and immobilized (○) enzymes.

of its initial activity.

The higher thermal stability of β-galactosidase immobilized on low-pressure plasma-modified CA membrane compared to soluble enzyme is depicted in Fig. 6. Fig. 6 shows the decay in enzyme activity over time at various temperatures. It is clear that the immobilized enzyme was stable at 40°C under continous operational conditions and was much more stable than the free enzyme. Fig. 6 also shows that thermal deactivation of this enzyme followed first-order kinetics. The deactivation rate constants (k_d) under various temperatures can be estimated from the slopes of the semi-logarithmic plots. Based on the k_d data, the half-lives of the enzyme at various temperatures were estimated and are also listed in Table 2. The immobilized enzyme had an estimated half-life of more than 1 month at 30°C and 84 h at 50°C. At 60°C, the thermal stability of the enzyme was increased 27.5-fold by immobilization on the membrane surface, as compared to the free enzyme. The increased stability of the immobilized enzyme was attributed to a reduction in protein structure mobility caused by anchorage of the enzyme to the support, which was promoted by covalent bonding and subsequent translation of the rigidity at each anchorage point to the whole enzyme structure, thus protecting against the environment [23]. As seen in Table 2, the activation energy for the immobilized enzyme was considerably decreased from 139 kJ/mol for the soluble enzyme to 74 kJ/mol. El-Masry *et al.* [24] observed a decrease in the activation enegery of *A. oryzae* β-galactosidase (36.8 kJ/mol) after immobilization on nylon membrane (25.1 kJ/mol). As also shown in Fig. 7, immobilized enzyme was more stable at lower pH (4 ~ 5) and higher pH (8 ~ 10) levels.

Table 2. Comparison of thermal stabilities of free and PEI-immobilized enzymes[a]

Enzyme source	Temperature (°C)	Soluble enzyme[b]		Immobilized enzyme[c]	
		k_d ($10^{-4} \times h^{-1}$)	$t_{1/2}$ (h)	k_d ($10^{-5} \times h^{-1}$)	$t_{1/2}$ (h)
AOG	30	25	277	89	775
	40	72	96	314	220
	50	596	12	827	84
	60	3,129	2	1,222	57
	Ea (kJ/mol)	139		74	

[a]The deactivation rate constant k_d was determined from experimental data, which followed a first-order reaction kinetic model. The enzyme half-life was calculated from the k_d value.
[b]Incubated in pH 4.5 acetate buffer; activity was determined at various intervals.
[c]Immobilized enzyme in membrane reactor.

Immobilized β-galactosidase on plasma-modified membrane was successively reutilized for 10 cycles at 60°C, and the enzymatic derivative retained approximately all of its initial activity (Fig. 8). A decrease in activity was observed after 10 cycles, possibly due to the loss of mass of the enzymatic derivative during the reaction. The performance of the immobilized enzyme was also investigated

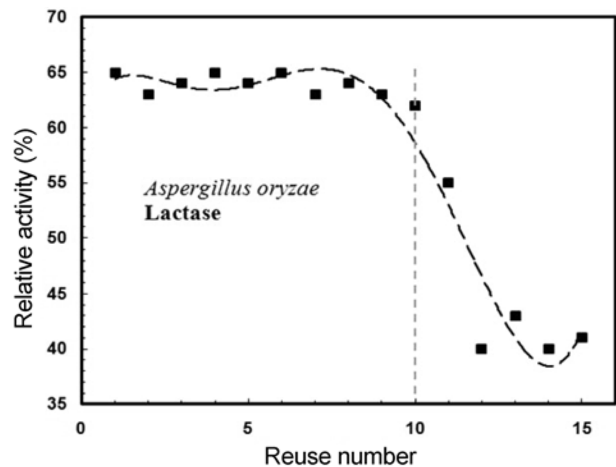

Fig. 8. Effect of repeated use capability on the activity of immobilized β-galactosidase.

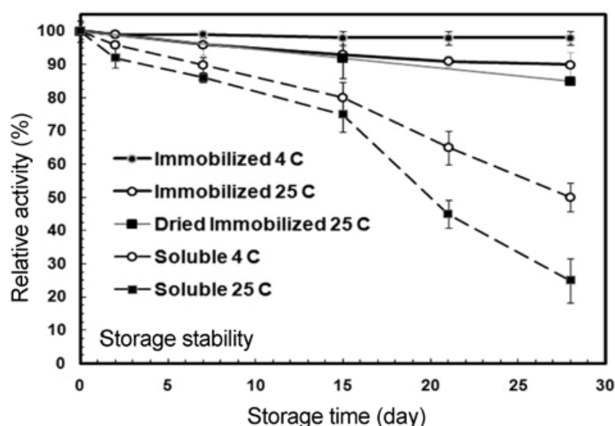

Fig. 9. Free and immobilized enzyme stabilities subjected to different storage conditions for 28 days.

during storage under three different conditions (Fig. 9). As seen in Fig. 9, the immobilized enzyme retained its activity during 28 days of storage in a refrigerator (+ 4°C), during which the activity was measured everyday by standard activity assay. Under the same conditions, free enzyme lost 50% of its initial activity during 28 days of storage in a refrigerator. The soluble and immobilized enzymes were stored for 28 days at room temperature (25 ± 2°C), and only immobilized enzyme was stored at room temperature in dried form. The changes in activities are shown in Fig. 9. Immobilized enzyme lost only 10% of its initial activity during 28 days of storage at room temperature in dried form. This result shows that the developed immobilization procedure provided notably easy transportation without significant activity loss.

3.5. GOS formation kinetics

GOS formation kinetics from lactose with the multilayered PEI-immobilized enzyme were studied in a plasma-modified membrane reactor. Fig. 10 shows the typical reaction kinetics for lactose hydrolysis and GOS formation. In general, a high rate of initial GOS formation was accompanied by a rapid decrease in lactose concentration. As the reactions continued, GOS formation leveled off and then decreased, whereas glucose and galactose continued to increase. The amount of galactose produced by lactose hydrolysis was less than that of glucose since galactose was also used to form GOS.

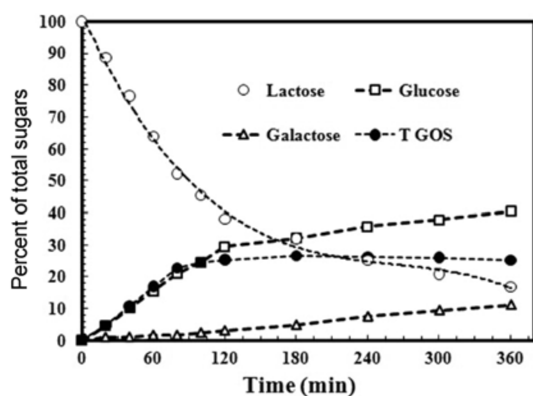

Fig. 10. Reaction kinetics of lactose hydrolysis and GOS formation catalyzed by PEI-immobilized enzyme in the membrane reactor at 60°C at an initial lactose concentration of 320 g/L.

Fig. 11 shows that the GOS production kinetics were affected by lactose conversion, which is defined as the amount of lactose converted to the other sugars. Maximum GOS production was obtained at ~ 70% lactose conversion. It was observed that the GOS produced by the reaction were primarily composed of trisaccharides (3-OS). Larger GOS such as tetra- and pentasaccharides were produced at lower levels.

Due to the viscosity of the lactose solution (320 g/L), formation of large GOS, and simultaneous release of small, inhibitory monosaccharides, mass transfer limitations could cause significant reduction in GOS formation. It was also found that the reaction kinetics and GOS formation were affected by the enzyme load (Fig. 12). Plasma-modified

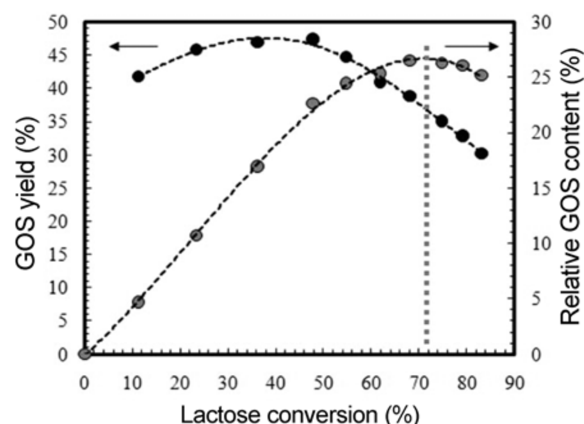

Fig. 11. Kinetics of GOS formation as affected by lactose conversion catalyzed by PEI-immobilized enzyme in the membrane reactor at 60°C at an initial lactose concentration of 320 g/L.

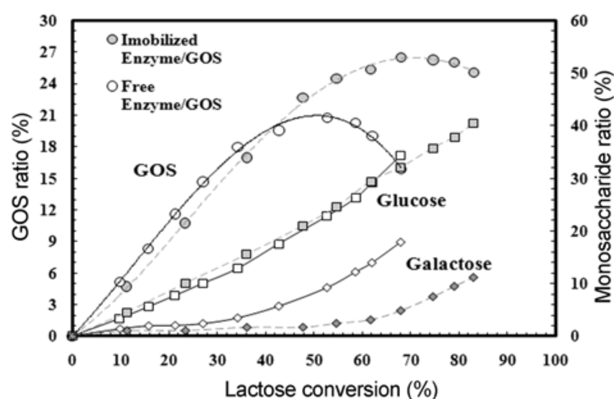

Fig. 12. Comparison of kinetics of GOS formation by lactose conversion catalyzed by free and PEI-immobilized enzymes.

Table 3. GOS production by various β-galactosidases in batch and continuous operations

Source of enzyme	Mode of process[a]	Reaction conditions			Max GOS[b] (w %)	Reference
		Lactose concentration (g/L)	T (°C)	pH		
T. Aquaticus	Batch (IE)	160.0	70	4.6	34.8	[25]
K. Lactis	Batch (FE, UF)	230.0	45	7.0	22.2	[9]
	Continuous (FE, UF)	200.0	45	7.0	31.0	[9]
B. Circulans	Batch (FE)	45.6	40	6.0	24.0	[26]
	Continuous (IE, CSTR)	45.6	40	6.0	40.0	[27]
A. Oryzae	Batch (FE)	380.0	40	4.5	31.0	[28]
	Continuous (IE, FBR)	400.0	40	4.5	26.6	[29]
A. Oryzae	Batch (FE)	320.0	55	4.5	20.8	This study
	Batch (IE, DU-PMMR)	320.0	60	5.5	27.0	This study

[a]FE: free enzyme, IE: immobilized enzyme, UF: ultrafiltration membrane reactor, CSTR: continuous stirred tank reactor, FBR: fibrous bed (cotton cloth) reactor, DU-PMMR: Diffusion unit-plasma modified membrane reactor.
[b]Max GOS is a weight percent of GOS based on the total sugars in the reaction mixture.

membrane reactor with immobilized enzyme provided a more reproductive enzymatic reaction via continuous seperation of inhibitory monosaccharides. The immobilized enzyme required a longer reaction time (360 min) as compared to the soluble enzyme (180 min) due to mass transfer limitations. However, higher lactose conversion (90%) and GOS content (27%) were achieved at the end of the reaction.

The maximum GOS contents of final products as reported by previous studies are compared in Table 3. β-galactosidase enzymes originating from *T. aquaticus* and *B. circulans* are more productive based on maximum GOS content. The maximum GOS content achieved by the free enzyme system in this study was relatively lower than those reported in the literature. However, it must be noted that the reaction conditions were different for each production unit. Further, it was reported that GOS content in the previuos studies also included disaccharides. The main significance of this research was partial seperation of monosaccharides having inhibitory effects on β-galactosidase using semi-permeable plasma-modified membranes. Higher GOS content (27%) was produced by the immobilized enzyme system compared to that of the free enzyme system (20.8%). Thus, much higher productivities were obtained, and the novelty of this research was demonstrated. Further, the method of multilayered enzyme immobilization should also be applicable to other enzymes reported in the literature.

4. Conclusion

Using PEI, multilayered enzyme immobilization on plasma-modified CA membrane, a novel biocatalytic membrane, was developed. In this study, CA membrane surfaces were modified by plasma polymerization in order to achieve desired pore size distribution for contunious sugar seperation. The plasma-modified CA membrane surface was coated with layers of immobilized enzyme; thus a large amount of enzyme was introduced in an active way. Membrane reactor systems offer great advantages, such as elimination of pressure drops over packed bed reactors during industrial-scale bioproduct formation. The method of multilayered enzyme immobilization should also be applicable to other enzymes. In comparing the numerous enzyme immobilization systems available, multilayer enzyme immobilization on a plasma-modified membrane is one of the cheapest, safest, fastest, and most successful. The method is simple and straightforward and requires no sophisticated expertise, which is unlike many other enzyme immobilization methods. The FDA has approved the use of PEI in the production of food ingredients. High enzyme loads (997 μg/cm^2 membrane) and immobilization yield (66%) were achived with this immobilization procedure. More importantly, a combined system based on simultaneous production and seperation at high enzyme loads resulted in high reactor productivity. Therefore, this immobilized enzyme technology should have important ramifications on GOS production from lactose.

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