Appl Biochem Biotechnol (2009) 159:426–437
DOI 10.1007/s12010-008-8453-3

Lactose Hydrolysis by β-Galactosidase Covalently Immobilized to Thermally Stable Biopolymers

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Received: 3 September 2008 / Accepted: 25 November 2008 /
Published online: 10 December 2008

Abstract Lactose has been hydrolyzed using covalently immobilized β-galactosidase on thermally stable carrageenan coated with chitosan (hydrogel). The hydrogel's mode of interaction was proven by Fourier transform infrared spectroscopy, differential scanning calorimetry (DSC), and Schiff's base formation. The DSC thermogram proved the formation of a strong polyelectrolyte complex between carrageenan and chitosan followed by glutaraldehyde as they formed one single peak. The modification of carrageenan improved the gel's thermal stability in solutions from 35 °C to 95 °C. The hydrogel has been proven to be efficient for β-galactosidase immobilization where 11 U/g wet gel was immobilized with 50% enzyme loading capacity. Activity and stability of free and immobilized β-galactosidase towards pH and temperature showed marked shifts in their optimum pH from 4.5–5 to 5–5.5 and temperature from 50 °C to 45–55 °C after immobilization, which reveals higher catalytic activity and reasonable stability at wider pHs and temperatures. The apparent K_m of the immobilized enzyme increased from 13.2 to 125 mM, whereas the V_{max} increased from 3.2 to 6.6 μmol/min compared to the free enzyme, respectively. The free and immobilized enzymes showed lactose conversion of 87% and 70% at 7 h, respectively. The operational stability showed 97% retention of the enzyme activity after 15 uses, which demonstrates that the covalently immobilized enzyme is unlikely to leach. The new carrier could be suitable for immobilization of other industrial enzymes.

Keywords Biopolymers · Hydrogels · Covalent immobilization · β-Galactosidase · Biotechnology · Lactose intolerance

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Introduction

Lactose is the main carbohydrate contained in milk at a concentration between 5% and 10% (*w*/*v*) depending on the source of milk [1]. Lactose could be also found in whey permeate at higher concentrations. The consumption of foods with a high content of lactose is causing a medical problem for almost a 70% of the world population, especially in the developing countries, as the naturally present enzyme in the human intestine loses its activity during lifetime [2]. Unfortunately, there is no cure to lactose intolerance. This fact, together with the relatively low solubility and sweetness of lactose, has led to an increasing interest in the development of industrial processes to hydrolyze the lactose contained in dairy products (milk and whey) with both the free and immobilized conditions [3]. The studies have shown that glucose and galactose, two monosaccharides hydrolyzed from lactose, are four times sweeter than lactose, more soluble, more digestible [4], and can be consumed by 'lactose-intolerant' people [1, 5]. Hydrolysis of lactose present in whey permeate will produce lactose-free syrup, as solving an aquatic pollution problem as whey is usually thrown in water.

Immobilized enzyme is more favorable than free enzyme since it offers the possibility of continuous flow processing, so that easy regeneration of the immobilized enzyme and low-cost operation can be achieved in industrial processing.

Many techniques have been used previously for enzyme immobilization, including entrapment [6], cross-linking [7, 8], adsorption [9], or a combination of these methods [10]. Industrial surfaces such as glass beads [11], nylon-6 [12], and chitosan [13] have been used for this purpose.

β-Galactosidase has been immobilized onto a wide variety of solid supports such as Sephadex, alginate, κ-carrageenan, chitosan, porous glass, agarose, polyvinyl alcohol polymers, diethylaminoethyl cellulose, Eupergit C (epoxy-activated acrylic beads), nylon, polyurethane foams, or zeolite [14].

To our knowledge, carrageenan–chitosan systems were usually used to entrap enzymes and have not to any greater extent been used to covalently immobilize enzymes, with an exception of our recent work on carrageenan coated with chitosan [15]. It is known that the covalent technique is usually preferred in industry over entrapment, as the immobilized enzyme does not leak by reusability, whereas the entrapment does. The reusability of the immobilized enzyme has the advantage of reducing greatly the enzyme and the enzymatic products' cost by many folds.

In a previous work, we used a combination of advantages of two natural biopolymers, carrageenan and chitosan. Carrageenan is a water-soluble, sulfated galactan (polyanions) which is isolated from red seaweed and contains hydroxyl and sulfate groups. It can form gel easily by dissolving the carrageenan powder in hot water, and it sets at room temperature. Gel formation involves a conformational transition of the carrageenan molecule according to Wang and Qiang [16] mechanism, i.e., coil to helix to gel. This advantage enables the carrageenan gel to be shaped in any form [17]. However, a drawback of carrageenan is its low thermal stability [18] and the lack of active functional groups [19–21].

On the other hand, chitosan is a polysaccharide (polycation) obtained from the deacetylation of chitin, the second most abundant biopolymer present in nature after cellulose. The degree of deacetylation of typical commercial chitosan is usually between 70% and 95%, which means that chitosan possesses 70–95% primary amine groups (NH_2). Chitosan has the advantage of being a natural polycation with a high concentration of amino groups, which could be useful in forming a polyelectrolyte complex with the

carrageenan polyanion. The modification of carrageenan with chitosan imparts two extra benefits to carrageenan. The first is creation of a new functionality, amino groups (free NH_2); the second is improvement of the carrageenan gel's thermal stability by forming a polyelectrolyte complex (PEC) between the carrageenan $–OSO_3^-$ and the chitosan $–NH_3^+$ [15]. The free chitosan amino groups ($–NH_2$) were then activated with glutaraldehyde to covalently immobilize β-galactosidase.

The purpose of this research was to focus on providing valuable information on the immobilized β-galactosidase to cheap and naturally abundant biopolymers for the industrial use instead of the very expensive commercial carriers such as Eupergit C and agaroses. In view of the fact that the β-galactosidase is covalently attached, it will enable the immobilized enzyme to convert lactose to glucose and galactose without the enzyme being leached from the gel. The same immobilized enzyme could be reused to convert many batches of substrates, which will reduce the enzyme and the enzymatic products' cost. The enzyme is also unlikely to migrate to the food, and thus, it is unlikely to be consumed. For this rationale, factors which affect the free and immobilized enzyme activity and stability were investigated to obtain the optimum process conditions. This includes the effect of pH, temperature, and the Michaelis–Menten kinetics parameters in addition to the enzyme operational stability.

Materials

κ-Carrageenan (M_w, 154,000; sulfate ester ~25%) and chitosan (high viscosity 400 mPa s for 1% in 1% acetic acid at 20 °C) were supplied by Fluka. β-galactosidase (EC 3.2.1.23) from *Aspergillus oryzae*, 11.8 U/mg, was purchased from Sigma-Aldrich. Other chemicals were of Analar or equivalent quality. Parallel plate equipment was made in our laboratory for uniform gel sheets preparation. The gel disks dimensions were measured using a micrometer (Micro 2000, 0–25 mm).

Experimental Techniques

As a general rule, experiments were carried out in triplicate and data are means±SD (n=3) unless stated otherwise.

Preparation of κ-Carrageenan Coated with Chitosan Gel Disks

κ-Carrageenan gel was prepared as previously reported by Elnashar et al. [15, 17] by dissolving 2% (*w*/*v*) carrageenan in distilled water at 70 °C using an overhead mechanical stirrer until complete dissolution had occurred. Glass parallel plates equipment designed by Elnashar et al. [17] with 10-mm gaps were then immersed into the hot molten gel to produce uniform gel sheets. Typically, 4-mm diameter gel disks of average weight 145 mg were produced for immobilization.

The carrageenan gel disks were hardened using 0.3 M KCl for 3 h as a control [20] and with 0.75% (*w*/*v*) chitosan at pH 4 for 3 h as previously demonstrated by Elnashar et al. [15]. The gel disks were separated from the chitosan solution then thoroughly washed with distilled water and soaked in 2% (*v*/*v*) glutaraldehyde solution for 3 h.

Fourier Transform Infrared Spectroscopy

The infrared spectra of all formulations were recorded with Fourier transform infrared spectroscopy (FTIR-8300, Shimadzu, Japan). FTIR spectra were taken in the wavelength region 4,000 to 400 cm^{-1} at ambient temperature.

Differential Scanning Calorimetry and the Gel's Thermal Stability in Solutions

Differential scanning calorimetry (DSC) was performed to prove the formation of a strong polyelectrolyte complex between carrageenan and chitosan followed by glutaraldehyde. The thermal behavior of the different gel components (carrageenan powder, chitosan powder, carrageenan/chitosan powders physically mixed, and carrageenan/chitosan/glutaraldehyde gel) was characterized by DSC (SDT 600, TA Instruments, USA). Approximately 3 to 6 mg of the dried gels were weighed into an alumina pan. The samples were heated from 25 °C to 350 °C at a heating rate of 10 °C/min. For the gel's thermal stability in solutions, modified and unmodified gel disks were soaked in citrate phosphate buffer (100 mM, pH 4.5) solution at different temperatures (35 °C, 40 °C, and up to 95 °C). The stability of the gel disks were observed visually, where stable gels showed no debris and remained intact.

Immobilization Efficiency and Soluble Protein Determination

In a previous work by Elnashar et al. [15], the maximum enzyme immobilization efficiency percentage (I.E. %) was calculated using Eq. 2 and was found to be 28% using 10 ml enzyme of 3.4 U/ml, i.e., only ~1.0 U/ml enzyme was immobilized and the rest of enzymes remained in the supernatant.

$$\%\text{I.E.}\% = \frac{M_0 V_0 - M_f V_0}{w} \times 100 \tag{1}$$

Where M_0 is the initial enzyme concentration (U/ml), M_f the enzyme concentration of the filtrate (U/ml), V_0 the initial volume of the enzyme solution (ml), V_f the volume of filtrate (ml), and w the weight of carrier material used (g).

To increase the I.E. %, six disks of control and modified gel (10-mm height×4-mm diameter) were washed thoroughly with distilled water to get rid of any unbound glutaraldehyde groups and were incubated into 10 ml of enzyme solution of 2.0 U/ml prepared in 100 mM citrate phosphate buffer, pH 4.5, for 16 h. The immobilized enzyme was washed thoroughly and stored at 4 °C for further measurements. The supernatant and the wash were kept for soluble protein assay via Lowery assay using bovine serum albumin as a standard protein. The protein immobilized onto and into the carrier P_g (mg/g) was calculated using the following equation:

$$P_g = \frac{C_0 V_0 - C_f V_f}{w} \tag{2}$$

Where C_0 is the initial protein concentration (mg/ml), C_f the protein concentration of the filtrate (mg/ml), V_0 the initial volume of the enzyme solution (ml), V_f the volume of filtrate (ml), and w the weight of carrier material used (g).

Determination of β-Galactosidase Activity

β-Galactosidase activity was determined by the rate of glucose formation in the reaction medium. Known amount of immobilized or free enzyme were incubated into 10 ml of

200 mM lactose solution in citrate phosphate buffer (100 mM, pH 4.5) for 3 h at 37 °C and 100 rpm [22]. At the end of the time, 50 μl of reaction mixture was added to 950 μl buffer and boiled for 10 min to inactivate the enzyme and analyzed for glucose content using the glucose test. One enzyme unit (IU) was defined as the amount of enzyme that catalyzes the formation of 1 μmol of glucose per minute under the specified conditions.

Glucose concentration was measured spectrophotometrically with a glucose test based on the Trinder reagent [23]. Glucose is transformed to gluconic acid and hydrogen peroxide by glucose oxidase. The hydrogen peroxide formed reacts in the presence of peroxidase with 4-aminoantipyrine and *p*-hydroxybenzene sulfonate to form a quinoneimine dye as shown in Scheme 1.

The intensity of the color produced is directly proportional to the glucose concentration in the sample. The assay was performed by mixing 30 μl of a sample of unknown concentration and 3 ml of Trinder reagent; the reaction was allowed to proceed for 20 min at room temperature, and the absorbance of the unknown concentration was read at 510 nm.

K_m and V_{max} of Free and Immobilized β-Galactosidase

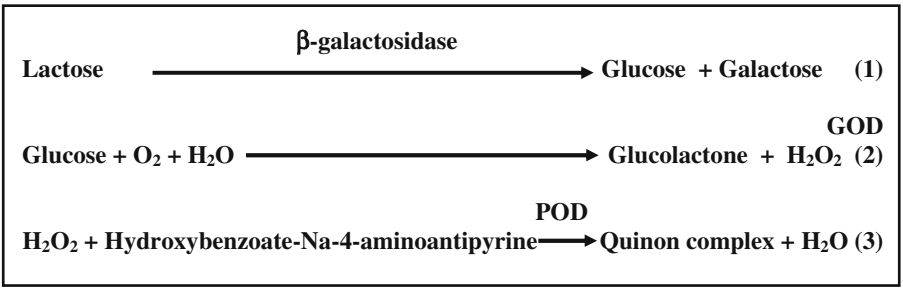
To obtain the Michaelis–Menten kinetic models adequate for the description of the hydrolysis of lactose by the free and the immobilized enzyme, apparent K_m and V_{max} of free and immobilized β-galactosidase were determined for lactose using the Hanes–Woolf plot method, Eq. 3. [24]:

$$\frac{[S]}{V_0} = \frac{1}{V_{max}} \times [S] + \frac{K_m}{V_{max}} \tag{3}$$

where $[S]$ is the substrate concentration (lactose), V_0 is the initial enzyme velocity, V_{max} is the maximum enzyme velocity, and K_m is the Michaelis constant and is defined only in experimental terms and equals the value of $[S]$ at which V_0 equals $1/2V_{max}$. Experimentally, the K_m from the plot is equal to $-[S]$, whereas the V_{max} is equal to 1/slope. The assay mixture comprised 5 U of free and immobilized enzyme and substrate concentration of 20–300 mM at 37 °C and pH 4.5 for 3 h.

Temperature Effect on β-Galactosidase Activity

The optimum temperature for the free and immobilized enzyme was examined. The free and immobilized β-galactosidase were incubated into 10 ml of 250 mM lactose at pH 4.5 and temperatures from 30 °C to 70 °C for 3 h. The data were normalized to 100% activity at



Scheme 1 Hydrolysis of lactose by β-galactosidase and glucose determination using a mixture of enzymes, glucose oxidase (*GOD*), and peroxidase (*POD*)

37 °C at which activities of the respective enzyme preparations were determined. The relative activity at each temperature is expressed as a percentage of the 100% activity.

pH Effect on β-Galactosidase Activity

The effect of pH on the activity of free and immobilized enzyme was examined. The free and immobilized β-galactosidase were incubated into 10 ml of 250 mM lactose at pH 3–6.5 at 50 °C for 3 h. The data were normalized to 100% relative activity at pH 4.5 at which activities of the respective enzyme preparations were determined. The relative activity at each pH is expressed as a percentage of the 100% activity.

Conversion of Lactose to Glucose

Forty units of free and immobilized enzyme was incubated into 10 ml of 250 mM lactose at pH 5.5 and 50 °C. Samples were withdrawn at interval times from 15 min to 7 h and analyzed for glucose content.

Operational Stability

The reusability of immobilized enzyme was studied using the modified gel disks. Forty units of immobilized enzyme was incubated into 10 ml of 250 mM lactose at pH 5.5 and 50 °C for 3 h, and the substrate solution was assayed for glucose content determination. The same gel disks were then washed with distilled water and re-incubated with another substrate solution; this procedure was repeated 15 times and the starting operational activity was considered as 100% relative activity.

Results and Discussion

Carrageenan Coated with Chitosan Elucidation Structure

Carrageenan gel sheets were prepared using the parallel plates apparatus [17]. The sheets were cut into disks and modified using partially protonated chitosan. The protonated chitosan

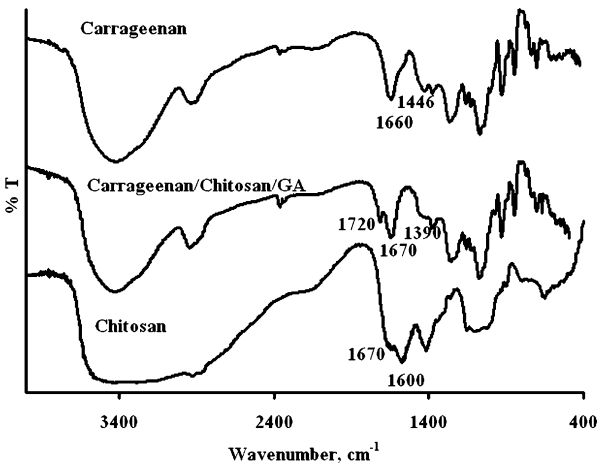


Fig. 1 FTIR of carrageenan, chitosan, and the carrageenan-coated with chitosan followed by glutaraldehyde (*GA*)

Table 1 Analysis of the FTIR data of carrageenan, chitosan, and carrageenan-coated chitosan followed by GA.

Polymers	Wavenumber (cm^{-1})	
	$-OSO_3^-$	–C=O
Carrageenan	1,446	–
Chitosan	–	1,670
Carrageenan/chitosan/GA	1,390	1,720, 1,670

amino groups ($-NH_3^+$) formed a PEC with the $-OSO_3^-$ of the carrageenan gel to increase the gel's thermal stability [25] and to incorporate a new functionality [26], free amino groups. The free chitosan amino groups ($-NH_2$) were used to form Schiff's base and covalently immobilize β-galactosidase via glutaraldehyde as a mediator.

The FTIR bands of the carrageenan, chitosan, and the modified gel (carrageenan/chitosan/glutaraldehyde) were shown in Fig. 1 and the data were tabulated in Table 1. The modified gel revealed the presence of carbonyl groups at 1,720 cm^{-1} which were expected to be for the glutaraldehyde-free aldehydic groups. The presence of glutaraldehyde –C=O was also confirmed visually, as it reacted with the chitosan $-NH_2$ group forming Schiff's base and changing color from pale yellow to brown-red according to the chitosan's pH [24]. The FTIR bands also revealed a decrease in intensity and a shift of the carrageenan $-OSO_3^-$ absorption band from 1,446 to 1,390 cm^{-1} after reaction with chitosan, which was derived from the ionic interaction between the carrageenan and the chitosan. This interaction evidenced the formation of strong polyelectrolyte complexes [27].

The DSC thermograms of the pure carrageenan and chitosan powders, the physically mixed carrageenan/chitosan powders, and the carrageenan/chitosan/glutaraldehyde gel were shown in Fig. 2. The pure carrageenan and chitosan powders revealed exothermic peaks at 239 °C and 303 °C, respectively, which represent their degradation. The physically mixed chitosan/carrageenan powders showed two exothermic peaks at 249 °C and 294 °C, which are in between those of the pure carrageenan and chitosan, respectively. However, the carrageenan/chitosan/glutaraldehyde gel showed a single peak at 252 °C [28]. This finding indicated formation of a strong polyelectrolyte complex due to ionic interaction between the polyanion

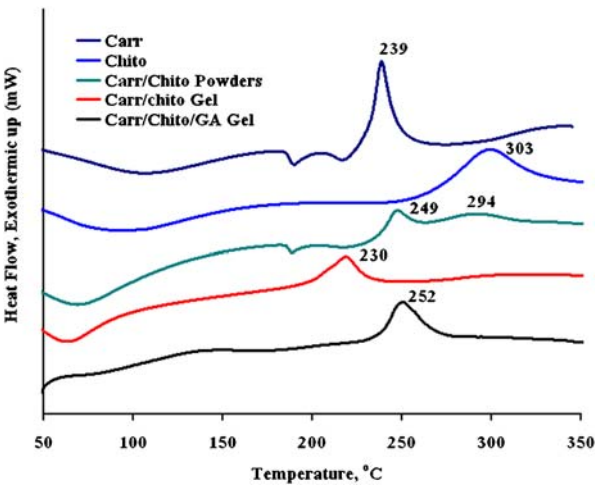


Fig. 2 DSC thermogram of the pure carrageenan and chitosan powders, the carrageenan/chitosan powders physically mixed, the carrageenan/chitosan, and the carrageenan/chitosan/glutaraldehyde gels

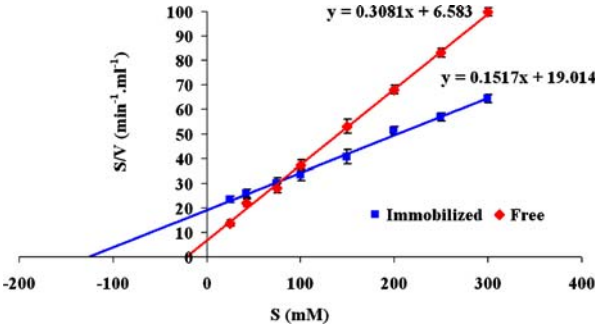


Fig. 3 Kinetic parameters of free and immobilized β-galactosidase using Hanes–Woolf plot

(carrageenan $-OSO_3^-$) and the polycation (chitosan $-NH_3^+$) [27, 29]. The modification of carrageenan using chitosan and glutaraldehyde improved greatly the carrageenan thermal stability from 239 °C to 252 °C. The gel's thermal stability in citrate phosphate buffer (100 mM, pH 4.5) solution was improved after the gel modification. Whereas the unmodified gel was completely dissolved in the buffer solution at 35 °C, the modified gel remained intact at 95 °C.

Immobilization Efficiency

In order to increase the immobilization efficiency percentage (I.E. %), 1 g gel was incubated in 20 U of enzyme (10 ml of 2 U/ml) instead of 34 U as previously used by the authors [15]. The results showed the immobilization of ~11 U/g wet gel, which reveals the increase of I.E. % from 28% to 50% using Eq. 2.

Kinetic Constants of Free and Immobilized β-Galactosidase

The kinetic constants of free and immobilized β-galactosidase were calculated using Hanes–Woolf plot method as shown in Fig. 3. The calculated values are shown in Table 2.

The apparent K_m after immobilization, 125 mM, is higher than that of the free enzyme, 13.2 mM, which indicates that a higher concentration of substrate is needed for the immobilized enzyme compared to the free enzyme. Higher K_m result (~187 mM) for the same immobilized enzyme was obtained by other authors [24]. It should be taken into consideration that the modification of enzyme's structure may result in the loss of its specificity towards lactose. However, no substrate or product inhibition by the increase of substrate concentration up to 300 mM could be observed during our experiment, as shown by the straight line of the Hanes–Wolf representation (Fig. 3). The maximum reaction velocity "V_{max}" values for the immobilized enzyme was astounding; it was found to double that of the free enzyme, i.e., it increased from 3.2 to 6.6 μmol/min.

Table 2 Kinetic constants of free and immobilized β-galactosidase.

β-Galactosidase form	Kinetic constants	
	K_m (mM)	V_{max} (μmol/min)
Free	13.2±0.03	3.2±0.005
Immobilized	125±0.025	6.6±0.007

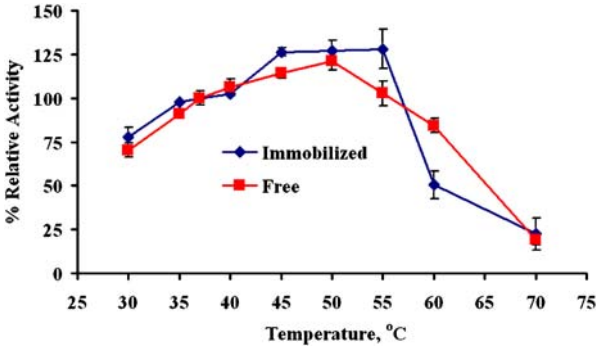


Fig. 4 Temperature–activity profile of free and immobilized β-galactosidase

Temperature–Activity Profile of Free and Immobilized β-Galactosidase

Figure 4 illustrates the temperature–activity profile of the free and immobilized β-galactosidase. The enzymatic activity is dependent on the temperature, as there is an optimum pH and temperature above which the enzymatic activity decreases due to the denaturation of the enzyme protein. As shown in Fig. 4, the immobilized enzyme was found to be stable at a wider range of temperature (45–55 °C) compared to the free enzyme (50 °C). The shift of the optimum temperature towards higher temperatures when the biocatalyst is immobilized indicates that the enzyme structure is strengthened by the immobilization process, and the formation of a molecular cage around the protein molecule (enzyme) was found to enhance the enzyme thermal stability [30]. The increase of the immobilized enzyme temperature tolerance may also be due to diffusional effects where the reaction velocity is more likely to be diffusion limited, so that improvements in thermal diffusion would correspondingly result in proportionally higher reaction rates.

pH–Activity Profile of Free and Immobilized β-Galactosidase

Figure 5 illustrates the pH–activity profile of the free and immobilized β-galactosidase. The optimum pH values for free and immobilized enzyme were 4.5–5 and 5–5.5, respectively, which showed that the immobilized enzyme was more stable at higher pH [31]. These properties could be very useful for lactolysis in sweet whey permeate, which has a pH range

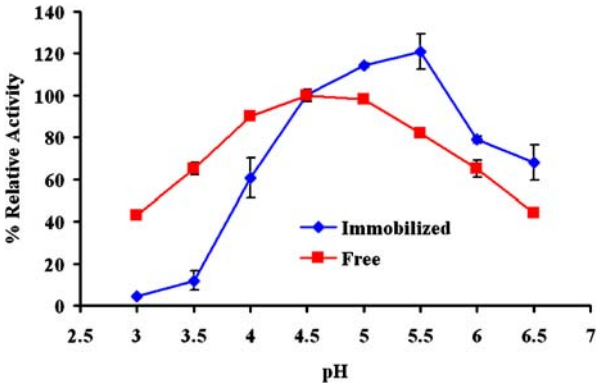


Fig. 5 pH–activity profile of free and immobilized β-galactosidase

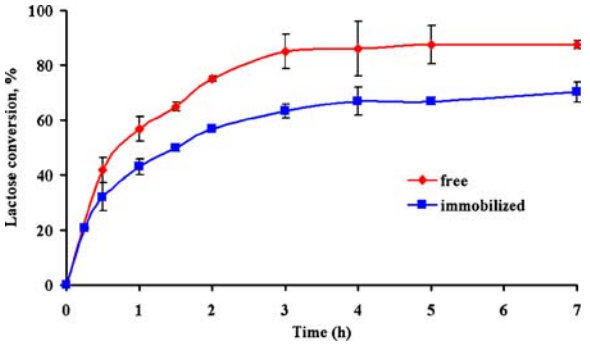


Fig. 6 Effect of time on lactose conversion to glucose using free and immobilized β-galactosidase

of 5.5–6 [32]. The shift in the pH–activity profile of the immobilized β-galactosidase may be attributed to the partition effects that were arising from different concentrations of charged species in the microenvironment of the immobilized enzyme and in the domain of the bulk solution [33].

Lactose Hydrolysis Using Free and Immobilized β-Galactosidase

This experiment has been carried out so that the immobilized enzyme could attain its maximum efficiency and acted with its highest velocity using double the K_m=125 mM concentration, 250 mM. The high concentration of substrate, 250 mM, was used in this study, as this enzyme was supposed to be suitable for hydrolysis of higher lactose concentrations found in mammal milk (88–234 mM lactose) [31] and whey permeate (85% lactose) [32]. The free enzyme reached a plateau and conversion of 87% of lactose after 3 h, whereas the immobilized enzyme reached 63% of conversion at that time and increased to 70% after 7 h as shown in Fig. 6. The decrease in substrate conversion after 3 h could be regarded to diffusion limitation [34]. In order to increase the enzyme substrate affinity and decrease the K_m value, a future trial will be carried out using smaller gel particles with high surface area and more gel pores to decrease the substrate/product diffusion limitation.

Operational Stability of Immobilized β-Galactosidase

The major advantage of immobilization of enzyme is easy separation and reusability. The data shown in Fig. 7 indicate almost no decrease in the enzyme activity after nine uses [35]. After the ninth use, the relative enzyme activity started to decrease gradually to attain 97%. The loss in activity was attributed to inactivation of enzyme due to continuous use [36].

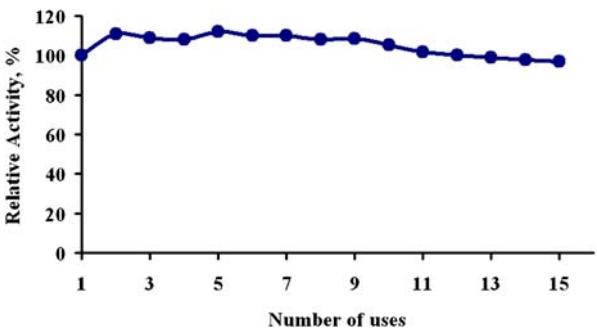


Fig. 7 Operational stability of immobilized β-galactosidase onto the modified gel (carrageenan/chitosan/glutaraldehyde)

Conclusion

β-Galactosidase from *A. oryzae* has been immobilized on natural biopolymers of carrageenan coated with chitosan. The two biopolymers having the advantage of being already permitted for use in the pharmaceutical or food industries was endorsed by an extra privilege, immobilizing β-galactosidase covalently. The new carrier increased the thermal stability of the carrageenan gel from 35 °C to 95 °C as well as the enzyme temperature and pH stabilities from 50 °C to 45–55 °C and from pH 4.5–5.0 to pH 5.0–5.5, respectively. The immobilized enzyme showed lactose conversion of 70% at 7 h compared to 87% for the free enzyme. However, the reusability of the immobilized enzyme for tens of times reduces the enzyme cost tremendously. For example, the operational stability retained 97% of the enzyme activity after 15 uses. In brief, the simplicity and effectiveness of the newly developed method for covalent immobilization of β-galactosidase, in addition to the promising results for lactose hydrolysis, are encouraging to use this method with lactose sources such as whey and milk.

Acknowledgment This work was supported by the National Research Centre, Centre of Scientific Excellence, Laboratory of Advanced Materials & Nanotechnology.

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