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Immobilization of β-galactosidase on Carrageenan Gel Via Bio - inspired Polydopamine Coating

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IN THE CONTEXT of diligent efforts directed to develop new polymeric matrices for enzyme immobilization, we developed carrageenan gel disks activated with bio-inspired polydopamine layer. The carrageenan gel disks were firstly treated with polycationic polymer (hyperbranched polyamidoamine) followed by a polydopamine layer. Such layer can act as an active platform for the covalent immobilization of enzymes via Michael addition and Schiff base. The mechanical strength of the coated gel disks was obviously enhanced. *β*-galactosidase was successfully immobilized with loading efficiency of about 52 %. Furthermore, the immobilized *β*-galactosidase exhibited enhanced activity at broader range of pH and temperature. Additionally, the immobilized *β*-galactosidase preserved around 80 % of the initial activity during the eleven reusability cycles. Generally, activation of the gel disks with the bio-inspired polydopamine layer considered as a save alternative to the glutaraldehyde due to its cytotoxicity. Accordingly, the easily formed carrageenan gel disks coated with polydopamine could have potential as enzyme carrier in drug and food industries.

Keywords: Carrageenan, Dopamine, Immobilization, *β*-galactosidase, Lactose free.

Introduction

Inspired from nature, enzymes have been widely introduced as biocatalyst to activate many of chemical industrial reactions. The potential of enzymes lies in its high selectivity and activity. Additionally, enzymes consider as an eco-friendly biocatalyst that can replace the use of conventional hazardous catalyst and solvents which in turn make a process more economically feasible and biologically save. Hence, many enzymes exhibited great potential in many industrial sectors. Among these enzymes, penicillin G acylase, cholesterol oxidase, lipase and cellulase that are widely used in pharmaceuticals, clinical diagnosis, food and biofuel industries, respectively.[1-4] Particularly, *β*-galactosidase (*β*-gal) is an enzyme used for the hydrolysis of lactose into glucose and galactose. Thus, *β*-gal displayed a great potential in the production of lactose free dairy products which are highly demanded for those people who suffer from lactose intolerance.[5] In addition to lactose hydrolysis, *β*-gal can also catalyze transgalactosylation process at high lactose concentration providing a series of condensation products called galactooligosaccharides (GOS) which are used as a prebiotic.[6]

It is well known that immobilization of enzyme on solid matrices imparts many advantages on enzyme. One of the most advantages is the easily separation of the immobilized enzyme from the reactor and reuse of the immobilized enzyme for several cycles. This in turn leads to reduction in the production cost.[7, 8] Besides, enzyme immobilization enhances the enzyme stability under both storage and operational conditions such as temperature, pH, solvents and contaminants.[9] In this context, many matrices have been developed to immobilize *β*-gal such as magnetic particles,[10] glass,[11] polyethylene film,[12] carbon nanotubes,[13] and hydrogels.[14-16] Indeed, carrageenan gel is one of the promising matrices for cell and enzyme immobilization due to its

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features. Carrageenan is naturally abundant polysaccharides, cheap and approved from food and drug administration (FDA).[17] In addition it can easily form gel in different forms such as film, beads and disks.[18, 19] However, carrageen gel possesses sulfate and hydroxyl groups which cannot be used for the covalent immobilization of enzymes.[5] Accordingly, treating of carrageen gel with reactive functional coating is highly desired. This was usually achieved by formation of polyelectrolyte complex (PEC) between the anionic carrageenan gel and different polycations such as polyethyleneimine,[2] chitosan[20] and polyamidoamine.[21] Enzyme is then covalently bounded to the modified gel through small spacer like glutaraldehyde which is intensely used. However, the standing controversy around the toxicity of glutaraldehyde makes it unfavorable to be used in food and drug applications.[22]

As an alternative to glutaraldehyde, the current work aims to use the bio-inspired polydopamine as a reactive platform for enzyme immobilization. Particularly, carrageen gel disks were coated with hyperbranched polyamidoamine through PEC formation which were further coated with a polydopamine layer. Such layer was acting as reactive platform that bind to *β*-gal through Michael addition and Schiff base bonds. The developed gel disks were chemically and mechanically characterized. Furthermore, the effects of pH and temperature on the activity of the immobilized *β*-gal along with its reusability were investigated.

Experimental

Materials and Characterizations

Materials

Dopamine hydrochloride was purchased from Acros. Carrageenan, *β*-Galactosidase enzyme (*β*-gal, 11 U/mg) and diethylenetriamine were obtained from Sigma. Methyl acrylate was purchased from BDH Chemicals. Other chemicals were of analytical grade.

Characterizations

Universal mechanical testing equipment (Instron 5567, USA) has been used to carry out the compression test. Different gel disks with dimension of 10 mm diameter and 14 mm height been used. The compression load (N) was recorded at displacement of 10 mm. ATR-FTIR is recorded on JASCO instrument. Samples were scanned at resolution of 4 cm^{-1} over a wavenumber range of 400-4000 cm^{-1}. Enzyme activity measurements were conducted using UV-Vis spectrophotometer (JASCO V630).

Methods

Preparation of carrageenan gel disks (Cgel)

Carrageenan gel disks were prepared according to our previous report.[20] Particularly, Carrageenan (2% w/v) was dissolved in hot distilled water at 70 ºC. A homogeneous carrageenan gel sheet was obtained by pouring the hot carrageenan solution into pre-heated glass petri dish followed by cooling down to room temperature. Using cork borers, gel sheet was cut into uniform gel disks with dimension of 4 mm height and 3 mm diameter and average weight of 50 mg. For compression test, larger disks were cut with dimension of 10 mm diameter and 14 mm height.

Modification of gel disks

Hyperbranched polyamidoamine was synthesised as previously reported and used as polycationic polymer to form a polyelectrolyte complex with carrageenan.[21, 23] Mainly, the carrageenan gel disks were incubated in aqueous solution of polyamidoamine (4 % w/v) at pH 9 for 3 h. Then, gel disks (Cgel/PAMAM) were washed thoroughly with distilled water. The gel disks were further coated with a layer of polydopamine by incubating the gel disks into a freshly prepared dopamine solution (2mg/ml) in Tris buffer at pH 8.6 for 24 h. Finally the disks (Cgel/PAMAM/PDA) were washed with water and stored in Tris buffer for the further use.

Covalent immobilization of β-galactosidase (β-gal)

The polydopamine layer on the gel disks acted as an active platform for the covalent immobilization of *β*-gal. Particularly, the free amine groups of enzyme were covalently bound to the polydopamine via Michal addition or Schiff base formation. In details, one gram of wet gel disks were incubated into 10 mL of enzyme solution (2 U/mL) prepared in 100 mM citrate-phosphate buffer at pH 4.5 for 16 h. The immobilized *β*-gal was washed thoroughly with buffer solution to remove any unbound *β*-gal. Then immobilized *β*-gal was stored at 4 ºC for further measurements.

Determination of β-galactosidase activity

β-galactosidase activity was determined by measuring the rate of glucose formation in the reaction mixture. Known amount of immobilized or free *β*-gal were incubated into 2 ml of 200 mM lactose solution in citrate phosphate buffer (100 mM, pH 4.5) at 37 ºC and 100 rpm. After 30 min, the solution was boiled for 10 min to inactivate the free enzyme then the glucose content was determined using glucose kits. One enzyme unit (IU) was defined as the amount of enzyme that catalyses the formation of 1 µmol of glucose per minute under the specified conditions.

Effect of temperature and pH on β-galactosidase activity

To evaluate the optimum temperature at which the free and immobilized enzyme displayed a maximum catalytic activity, known unites of free and immobilized *β*-gal were incubated into 2 ml of 200 mM lactose solution in citrate phosphate buffer (100 mM, pH 4.5) for 30 min. The temperature was varied from 30 ºC to 70 ºC. However, the effect of pH was investigated under wide range of pHs (from pH 3 to pH 6.5) at 45 ºC for 30 min. The data were normalized to 100%.

Reusability investigation:

Reusability of the immobilized β-gal was studied using the polydopamine coated gel disks. Five disks of immobilized *β*-gal was incubated into 2 ml of 200 mM lactose at pH 4.5 and 45 ºC for 3 h then, the enzyme activity was determined by measuring the glucose content. The same gel disks were then washed with distilled water and re-incubated in a fresh lactose solution. This process was repeated 15 times and the enzymatic activity was normalized to 100% relative activity.

Results and discussions

Matrix preparation and characterization

A transparent carrageenan gel is directly formed by cooling down the hot carrageenan solution. However, the low mechanical properties of the obtained gel disks restrict their industrial applications. In addition, the lack of reactive functional groups on the carrageenan gel disks limits the covalent immobilization of enzymes. Therefore, the anionic carrageenan gel disks were coated with hyperbranched polyamidoamine (PAMAM) which used as a polycationic polymer to form a PEC.[21] As shown in Figure 1, the electrostatic interactions took place between the $–OSO_3^-$ groups of carrageenan gel and the protonated amine groups ($–NH^{3+}$) of PAMAM providing carrageenan gel disks coated with PAMAM which is rich with amine groups (Cgel/PAMAM). To immobilize enzyme on such gel disks, a coupling agent was needed to bind the amine groups derived from gel together with the amine groups of enzyme. Although, glutaraldehyde was extensively used as a coupling agent, its use in food and drug industries is not preferred due to its toxicity. In this context, the Cgel/PAMAM gel disks were further coated with mussel inspired polydopamine which act as active platform for enzyme immobilization. Firstly, by dipping the Cgel/PAMAM gel disks in dopamine solution at pH 8.6, dopamine was polymerized forming a deep brown layer of polydopamine which is strongly adhered to the gel surface through hydrogen bonding, Schiff base and Michael addition.[24] Finally, enzymes possess amine groups derived from their protein which can covalently bind to the polydopamine platform via Michael addition and Schiff base bonds (Figure 1).

Coating of carrageenan gel disks with PAMAM followed by a polydopamine layer was proved via ATR-FTIR spectroscopy analysis as shown in Figure 2. The spectrum of carrageenan gel (Cgel) displayed the typical characteristic bands of carrageenan. However, the spectrum of Cgel/PAMAM displayed presence of new bands at 1450 cm^{-1} and 2800-2900 cm^{-1} which are attributed to the bending and stretching vibrations of C-H group existing in PAMAM, respectively. Furthermore, typical bands were assigned at 1550 cm^{-1} and 1630 cm^{-1} resulting from the N-H and C=O of amide groups present in PAMAM. Such evidences confirmed the successful coating of carrageenan with PAMAM.[21] The presence of additional polydopamine layer was confirmed by appearance of a broad absorption band at 3300 cm^{-1} which corresponds to the stretching vibrations of -OH (phenolic hydroxyl) and N-H groups of polydopamine.[25]

Treatment the carrageenan gel with PAMAM followed by polydopamine provided not only reactive functional groups for enzyme immobilization but also improved the mechanical properties of the coated gel disks which is necessary to keep the gel disk intact during the repeated usage. As shown from the compression test illustrated in Figure 3, the pristine carrageenan gel disk (Cgel) resisted the applied load up to a value of 1 N leading to a displacement of 4 mm. Above that, the Cgel disk was collapsed indicating its low mechanical strength. However, coating the gel disk with PAMAM through PEC formation enhanced its mechanical stability where the Cgel/PAMAM disk displayed about 6.8 N at 10 mm displacement. Adding of polydopamine layer leaded to a further improvement in the mechanical strength where the Cgel/PAMAM/PDA gel disk recorded about 11 N at 10 mm displacement. This attributed to the dual function of the polydopamine layer which acts as a crosslinker for PAMAM via Michael addition and Schiff base bonds beside its role as an active platform for the enzyme immobilization.

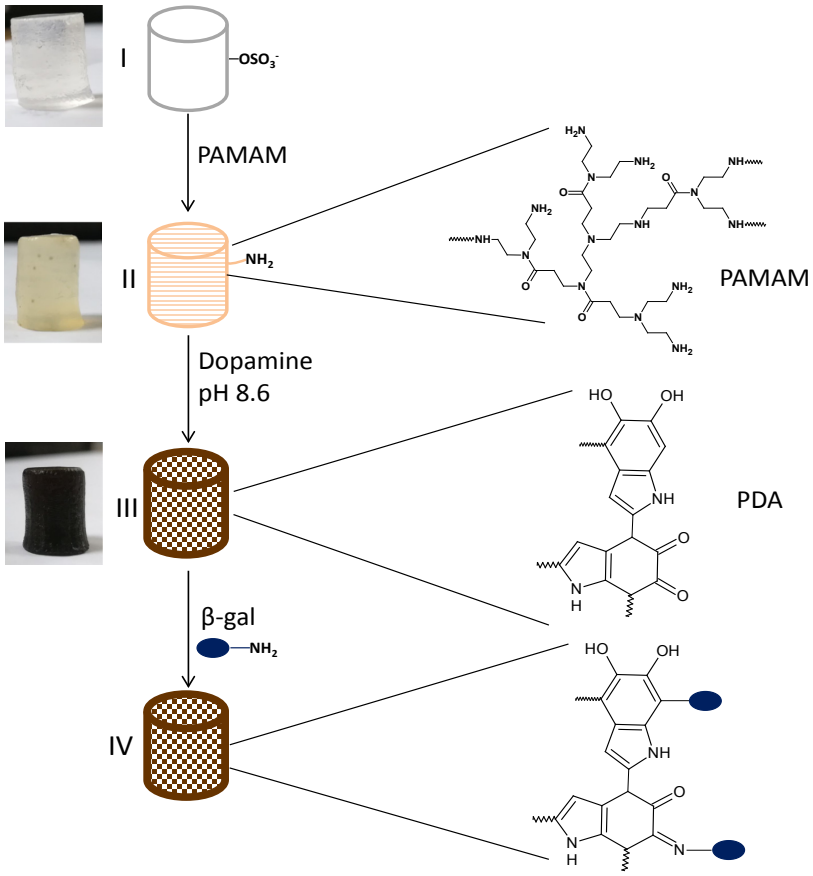


Fig. 1. Scheme representing the treatment steps of carrageenan gel with a polydopamine layer. Each step is presented with a photographic image for the gel disk along with the chemical structure of the formed layer. I) pristine carrageenan (Cgel), II) Cgel/PAMAM, III) Cgel/PAMAM/PDA and IV) Cgel/PAMAM/PDA/β-gal

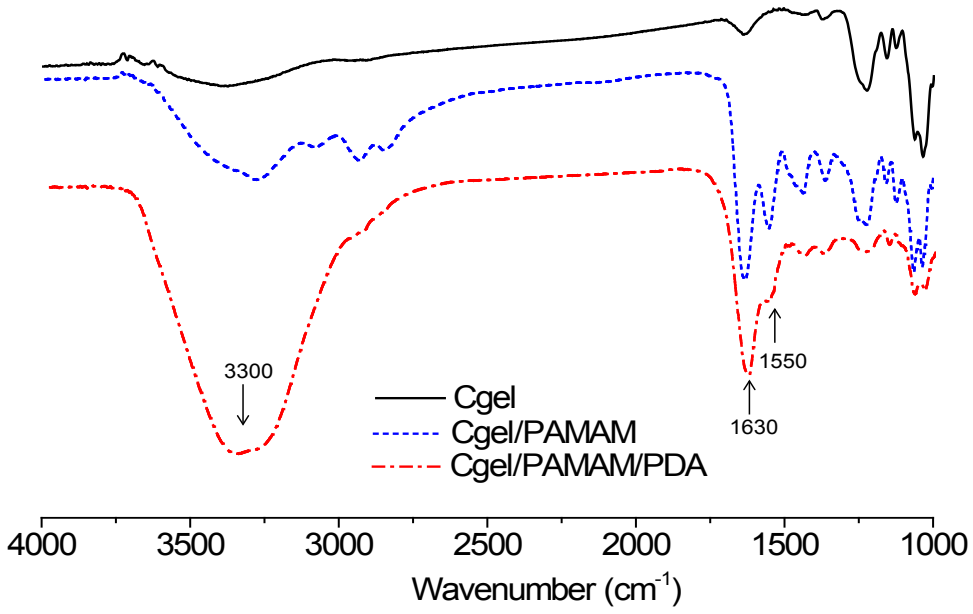


Fig. 2. ATR-FTIR of pristine carrageenan gel (Cgel), carrageenan gel coated with PAMAM (Cgel/PAMAM) and further coated with polydopamine (Cgel/PAMAM/PDA)

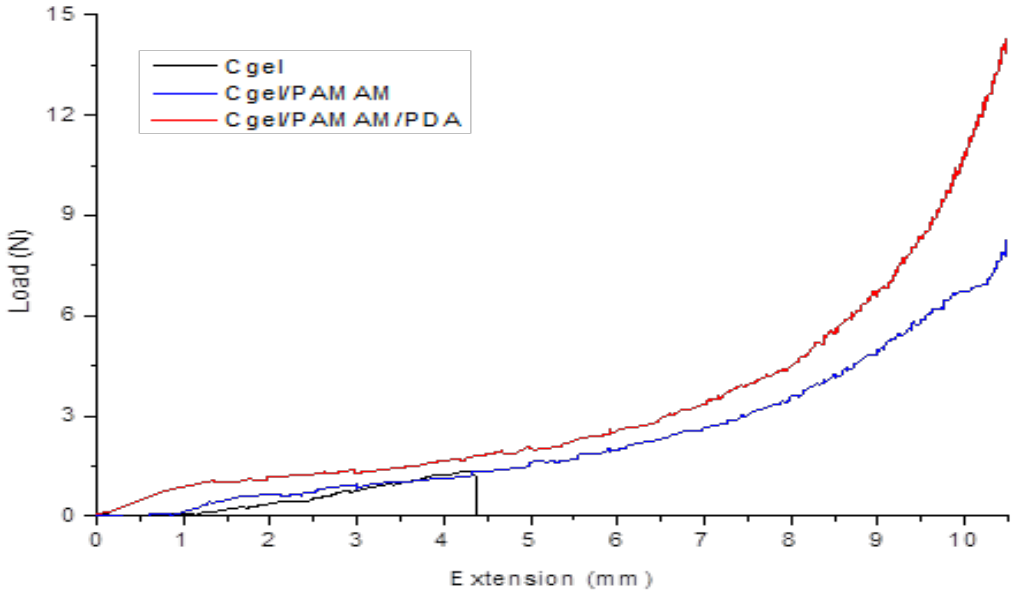


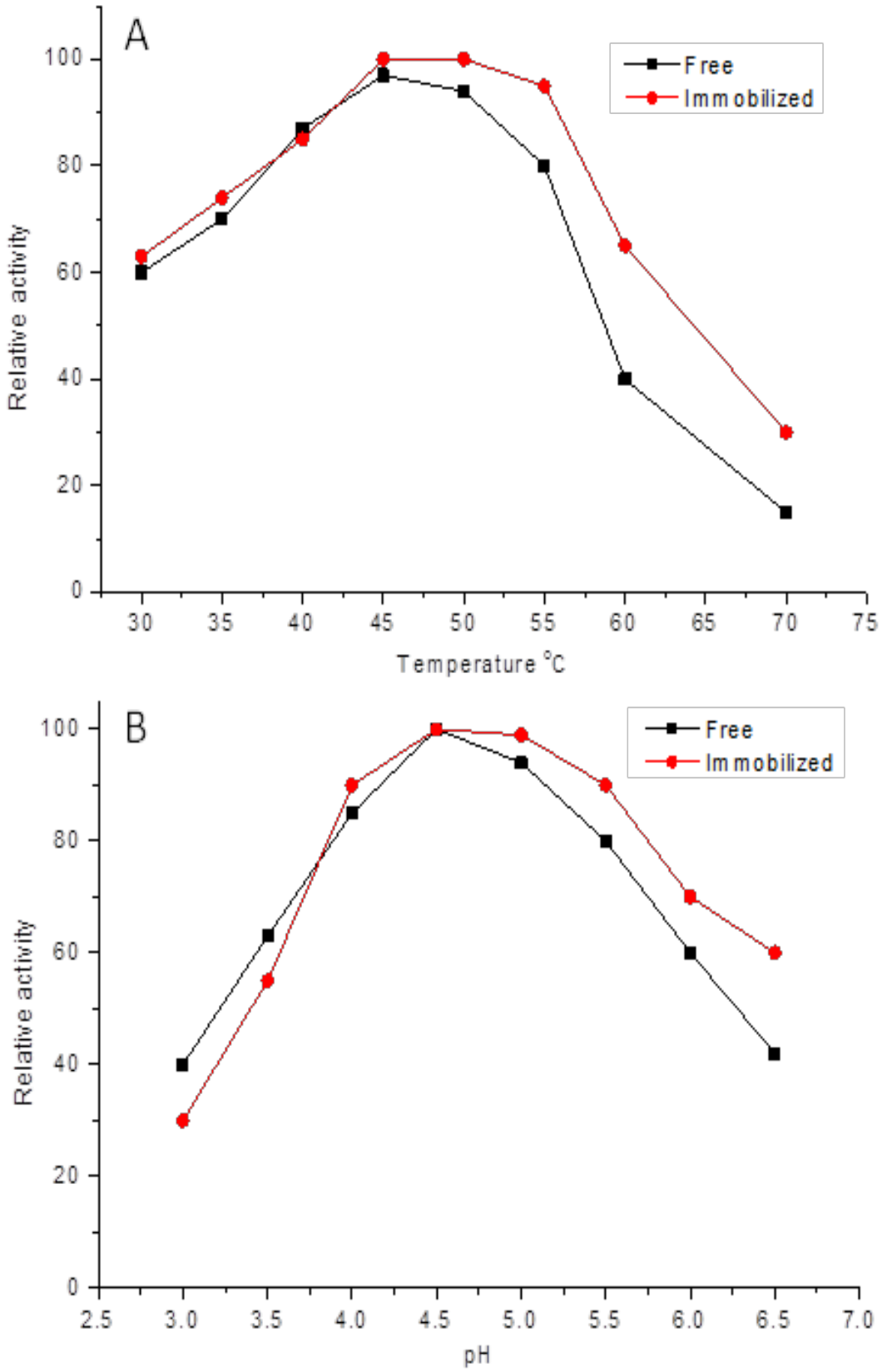
Fig. 3. Mechanical compression of the pristine carrageenan gel (Cgel), carrageenan gel coated with PAMAM (Cgel/PAMAM) and further coated with polydopamine (Cgel/PAMAM/PDA)

The established polydopamine layer on the surface of Cgel/PAMAM/PDA gel disk was used as reactive platform for the immobilization of *β*-galactosidase (*β*-gal). Particularly, polydopamine possess both catechol and quinone groups which can bind to the amine groups of *β*-gal via Michael addition and Schiff base bonds, respectively. *β*-galactosidase displayed relative high immobilization efficiency of about 52 % which can be attributed to the presence of large number of catechol and quinone groups on the polydopamine which gives high probability for the covalent binding of *β*-gal.

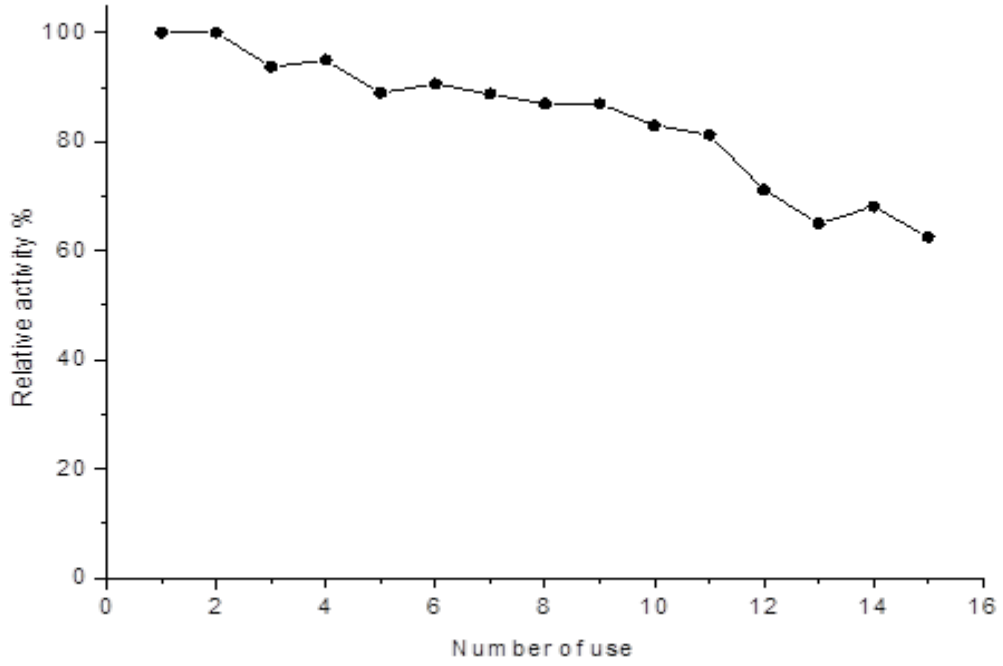
The catalytic activities of the free and immobilized *β*-gal were investigated at constant pH value (pH 4.5) while the temperature was varied between 30 ºC to 70 ºC. As shown in Figure 4A, the free *β*-gal revealed an optimum catalytic activity at 45 ºC however, the immobilized *β*-gal showed the same catalytic activity at broader temperature range from 45 ºC to 55 ºC. The temperature had no significant influence on the activity of the free and immobilized *β*-gal up to 45 ºC. However, at elevated temperature (above 45 ºC) activity of the free enzyme was significantly decreased however, the immobilized *β*-gal displayed tolerance towards higher temperature. For instance at 60 ºC, the free enzyme recorded catalytic activity of about 40 % compared to 65 % of the immobilized enzyme at the same temperature. This indicated that the polymeric gel disks protected enzyme from denaturation at elevated temperature.

To determine the optimum pH values at which maximum activity of the free and immobilized enzymes are recorded, the catalytic activities were evaluated within pH range from 3 to 6.5 at 45 ºC. As shown in Figure 4B, the optimum pH value of the immobilized *β*-gal lies in the range of pH 4.5 to pH 5 while the free enzyme displayed maximum activity at pH 4.5.

One of the most important features of enzyme immobilization is the easily separation of the enzyme from the reaction mixture and the ability to reuse the enzyme for several cycles. As shown in Figure 5, *β*-gal immobilized on Cgel/PAMAM/PDA disks conserved around 80 % of its activity during the eleven reusability cycles. This confirmed that *β*-gal was covalently bounded to the gel disk which in turn reduced the enzyme leakage from the gel disk. By the twelfth cycle the catalytic activity was gradually decreased to 65 % at the fifteenth cycle which can be ascribed to the end-product inhibition phenomena.[26]



Fig, 4. Catalytic activities of free and immobilized β-galactosidase at different temperature (A) and at variable pH values (B)



Fig, 5. Reusability of the immobilized β-galactosidase, Measurements were conducted at 45 ^{oC} and pH 4.5

Conclusion

β-galactosidase was successfully immobilized on new carrageenan gel disks activated with bio-inspired polydopamine as a biologically save catalyst in food industries. Modification of the carrageenan gel disks was carried out using hyperbranched polyamidoamine as polycationic polymer followed by a layer of polydopamine that ensure the covalent immobilization of *β*-gal through Schiff base and Michael addition bonds. It was shown that the coated gel disks have enhanced mechanical stability compared to uncoated ones as revealed by compression measurements. Compared to the free enzyme, the immobilized *β*-gal displayed higher activities at broader pH and temperature ranges. Moreover, the activity of the immobilized *β*-gal was nearly conserved over the first eleven cycles. Thus, such carrageenan gel disks coated with polydopamine can be considered as a bio-alternative route for enzyme immobilization instead of using the cytotoxic glutaraldehyde spacer.

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تحميل انزيم البيتا غالاكتوزيداز على جل الكاراجينان باستخدام اغلفة البوبى دوبامين

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في سياق الجهود الحثيثة الموجهة إلى تطوير مصفوفات بوليمرية جديدة لاستخدامها كحامل للإنزيمات ، قمنا بتطوير أقراص من جل الكاراجينان المغلف بطبقه من البوبى دوبامين المستوحاة من النظام الحيوية. حيث يمكن أن تعمل هذه الطبقة كمنصة نشطة لتثبيت الإنزيمات عبر روابط تساهمية يالاصافه الى دورها الفغال فى تحسين الخواص الميكانيكية للاقراص البوليمرية. تم تثبيت البيتا غالاكتوزيداز بنجاح على الاقراص البوليمرية بكفاءة تحميل تصل الى ٥٢ ٪. علاوة على ذلك ، أظهر الانزيم المحمل فاعليه افضل فى نطاق أوسع من درجات الحرارة و الاس الهيدروجينى. كما حافظ الانزيم المحمل على حوالي ٨٠ ٪ من نشاطه بعد استخدامه المتكرر لإحدى عشر دورة. بشكل عام ، تغليف أقراص الكاراجينان باستخدام البوبى دوبامين يعد بمثابة بديل آمن لتنشيط الاقراص البوليمرية يدلا من استحدام الغلوتارالدهيد ذات السمية.