Novel Grafted Agar Disks for the Covalent Immobilization of β-D-Galactosidase

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ABSTRACT:

Novel grafted agar disks were prepared for the covalent immobilization of β-D-galactosidase (β-gal). The agar disks were activated through reacting with ethylenediamine or different molecular weights of Polyethyleneimine (PEI), followed by glutaraldehyde (GA). The modification of the agar gel and the binding of the enzyme were verified by Fourier Transform Infrared (FTIR) and elemental analysis. Moreover, the agar's activation process was optimized, and the amount of immobilized enzyme increased 3.44 folds, from 38.1 to 131.2 U/g gel, during the course of the optimization process. The immobilization of β-gal onto the activated agar disks caused its optimum temperature to increase from 45°C to 45–55°C. The optimum pH of the enzyme was also shifted towards the acidic side (3.6–4.6) after its immobilization. Additionally, the Michaelis-Menten constant (K_m) increased for the immobilized β-gal as compared to its free counterpart whereas the maximum reaction rate (V_{max}) decreased. The immobilized enzyme was also shown to retain 92.99% of its initial activity after being used for 15 consecutive times. © 2015 Wiley Periodicals, Inc. Biopolymers 103: 675–684, 2015.

Keywords: agar; covalent immobilization; β-D-galactosidase; polyethyleneimine; glutaraldehyde

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INTRODUCTION

The enzyme β-D-galactosidase (E.C. 3.2.1.23) catalyzes the hydrolysis of β-D-(1–3) and β-D-(1–4) glycosidic bonds in oligo- and disaccharides.[1,2] Lactose (β-D-galactopyranosyl-(1→4)-D-glucose) is the main substrate of β-D-galactosidase (β-gal). The enzymatic hydrolysis of lactose by β-gal has two main biotechnological applications; the production of low lactose milk and dairy products for consumption by lactose intolerant persons, and the utilization of whey, as its hydrolysates (glucose and galactose) have higher fermentation potential.[3]

Besides their hydrolytic activity on lactose, β-gal enzymes may also possess transgalactosylation activity. This transferase activity enables the synthesis of galacto-oligosaccharides (GOS), when lactose acts the acceptor of galactose.[4] GOS are considered as prebiotics because they selectively stimulate the growth of beneficial bacteria, such as *bifidobacteria* and *lactobacilli*, in the colon.[1] In addition to the prebiotic activity, GOS have also been reported to contribute to (i) reduction of serum cholesterol and lipid level; (ii) synthesis of B-complex vitamins; (iii) enhancing the absorption of dietary calcium; (iv) protection from infection and decreasing of pathogenic bacteria; (v) stimulating the absorption of some minerals.[5] Moreover, β-gal can also be used to attach galactose to other chemicals and consequently have imminent applications in the production of biological active compounds.[6]

Being an important industrial enzyme, β-gal was selected as a model enzyme for our immobilization study. Immobilization enhances the stability of enzymes. Furthermore, it facilitates

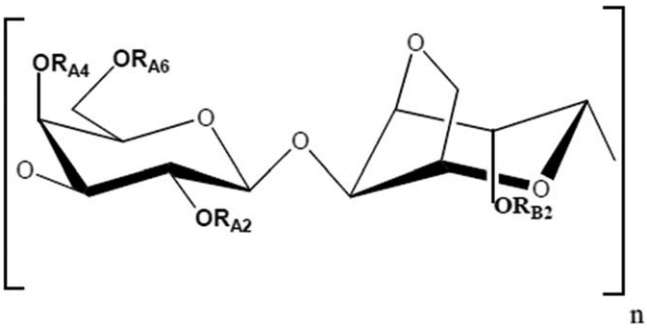

FIGURE 1 Schematic representation of agarobiose (R_{A2}; R_{A4}; R_{A6}; and R_{B2}: H) and agaropectin (R_{A2}: H, SO_3^-; R_{A4}: H, SO_3^-, Pyruvic acid (cyclic ketal with O_6); R_{A6}: H, CH_3, SO_3^-; Pyruvic acid (cyclic ketal with O_4), R_{B2}: H, CH_3, SO_3^-).

the separation of the enzyme from the product, thereby minimizing the chances of product contamination and allowing the easy recovery of the enzyme. This enzyme can then be reused in a continuous industrial operation.

However, efficient commercial carriers suitable for the immobilization of enzymes are fairly expensive.[7] Available carriers; such as, Eupergit C® or Agarose® are sold for € 6000 and € 3,250/kg, respectively.[8] Hence, there is a persistent need to prepare new enzyme carriers that are cheap. Agar (more correctly agar-agar) is a hydro gel that is available at a reasonable cost. Moreover, it is permitted for use in food industries.[9] Thus, it is an ideal candidate for enzyme immobilization.

Agar (Figure 1) is a polysaccharide consisting of a mixture of agarose and agaropectin. Agarose, the predominant component of agar, is responsible for the high strength gelling properties of agar. It is a linear neutral polymer, made up of the repeating monomeric units of agarobiose. Agarobiose is a disaccharide consisting of D-galactose and 3,6-anhydro-L-galactose. The other component of agar, agaropectin, is a charged polymer that has the same repeating units as agarose but with acid groups such as sulfate and pyruvate. Agaropectin is also methylated.[9]

There are many techniques to immobilize enzymes; such as, adsorption, covalent, encapsulation, entrapment, and cross-linking. Nevertheless, the covalent technique has the advantage of keeping the enzyme well bound to the carrier, avoiding enzyme leakage.[10] Thus, enabling the reuse of the immobilized enzymes tens of times, reducing their cost as well as their products' cost. This is why the covalent immobilization is widely preferred on the industrial scale. Agar was activated, for the covalent immobilization of enzymes, via its derivatization into glyoxyl agar (Agar-----O-CH_2-CHO).[11–13] In the present study, we attempted a new activation method which, to our knowledge, has never been applied to agar before. This activation method consisted of two steps (Figure 2) as follows:

- Step A which comprised the addition of different molecular weights of polyethylenimine (PEI) or ethylene diamine (EDA) to the agar disks. This polyamine compound contributed both protonated and free amino groups to the activation process. The protonated amino groups formed an ionic complex (network) with the sulfate and pyruvate anions of the agar gel.
- On the other hand, the free amino groups were employed during step B. This step involved the addition of the di-aldehyde spacer arm, glutaraldehyde (GA). The aldehyde groups present at one end of GA molecules reacted with the free primary amine moieties of PEI forming Schiff's base. Meanwhile, the unreacted GA side created free aldehyde groups for covalent immobilization of the enzyme.

In this study, β-gal, as a model enzyme, was covalently immobilized onto the novel activated agar disks. The activation process was optimized in order to attain the upmost amount of immobilized enzyme. Moreover, the alteration of the agar structure was elucidated by elemental analysis and Fourier Transform Infrared (FTIR). Finally the reusability of the immobilized β-gal was investigated.

EXPERIMENTAL

Materials

β-D-galactosidase (EC 3.2.1.23) from *Aspergillus oryzae*, PEI M_w 800, and PEI M_w 2000 were obtained from Sigma-Aldrich (Germany). PEI Mw 750,000 was bought from Fluka (Switzerland). GA solution was purchased Bio Basic (Canada). Agar was acquired from SD Fine chemicals (Mumbai). All other fine chemicals were of Analar or equivalent quality.

Methods

Preparation of Agar Gel Disks. An agar solution of 5% (w/v) was prepared in distilled water by heating. Then, a glass parallel plate equipment, with 10-mm gaps,[14] was immersed into the hot agar solution, and the agar was left to solidify in the fridge. The produced 10-mm thick homogeneous gel sheets were cut into disks using a cork borer. Typically, 4-mm diameter gel disks of average weight 180 mg were produced.

Activation of Agar Gel Disks. The agar disks were first soaked in an amine solution (PEI or EDA) for a specified period of time. The excess amine was then removed by thoroughly washing the disks with distilled water. Afterwards, the disks were soaked in a GA solution, washed with distilled water, and directly used to immobilize β-gal. It is worth mentioning that,

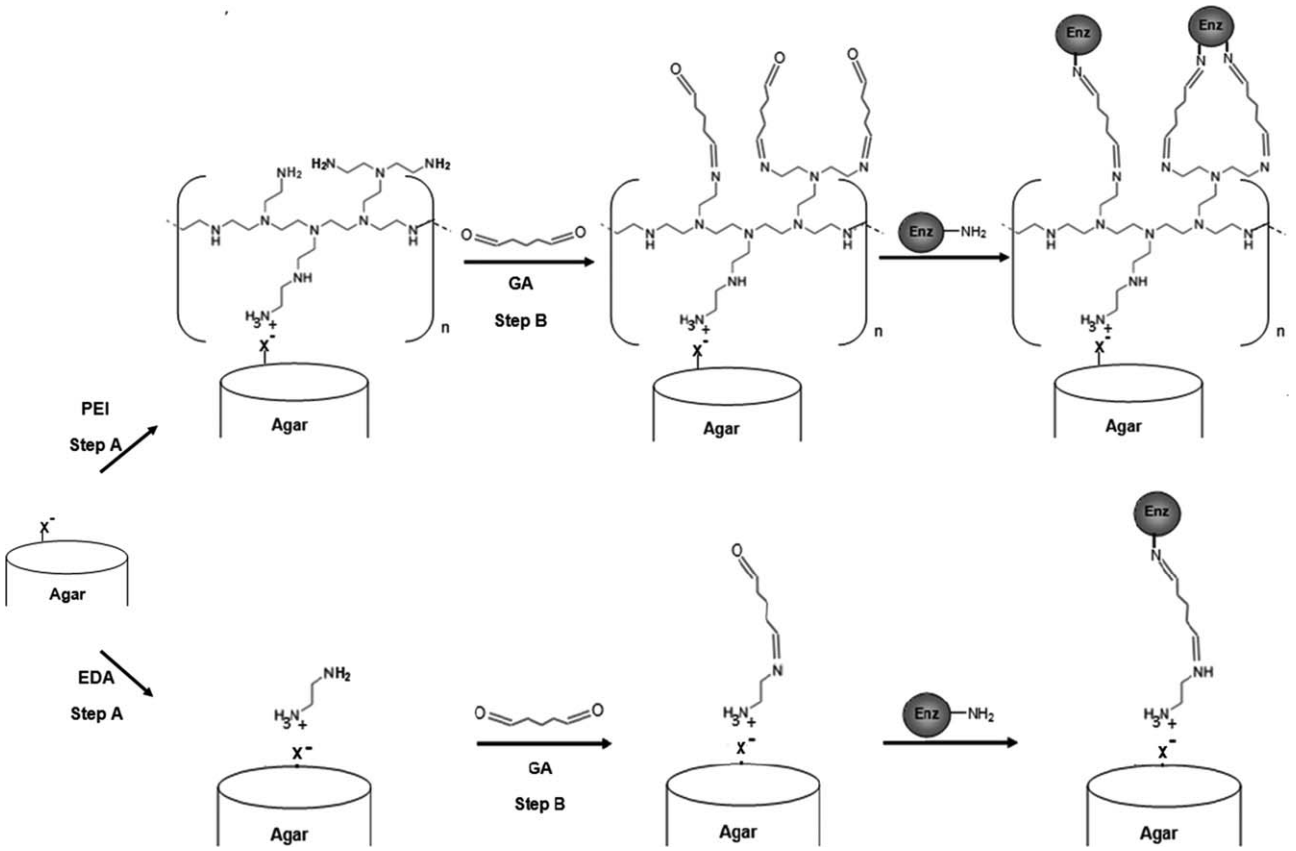

FIGURE 2 Schematic representation of agar gel activation and enzyme immobilization (X: sulfate or pyruvate).

the color of the agar disks changed from translucent to orange brown during the activation process. This orange brown color was due to the formation of Schiff's base (–N=CH–) between PEI amino groups and the GA's aldehyde groups. Noteworthy, the presence of the aldehyde group required for β-gal immobilization was proven through the Fourier Transform Infrared technique.

Covalent Immobilization of β-Gal onto the Activated Agar Disks. About 1 g of the activated agar disks was mixed with 5 ml of 0.3 *M* citrate-phosphate buffer (pH 4.5) containing ~200 U/ml β-gal. This mixture was agitated, using a roller stirrer, at room temperature for 18 h. Afterwards, the gel disks were filtered off, washed thoroughly with distilled water, and directly assayed for β-gal activity.

Determination of β-Gal Activity. The β-gal was assayed by using a modification of the procedure employed by Haider and Husain.[15] One β-gal loaded agar disk was soaked in 4 ml of 0.1 M acetate buffer (pH 4.6), followed by the addition of 1 ml of a 10 mM *o*-nitrophenyl β-D-galactopyranoside (ONPG) solution. The reaction was left to proceed for 15 min in a thermo-stated shaking water bath at 37°C. The agar disk was then removed, and the absorbance of the supernatant was measured at 405 nm.

Regarding the free β-gal, it was assayed by mixing 0.25 ml of the enzyme solution with 3.75 ml of 0.1 *M* acetate buffer (pH 4.6). Afterwards, 1 ml of a 10 mM ONPG solution was added to the reaction mixture. The reaction was left to proceed for 15 min in a thermos-stated water bath at 37°C. Then, the absorbance of the solution was measured at 405 nm. One unit of β-gal activity (U) was defined as the amount of enzyme that liberates 1.0 nmol of *o*-nitrophenol from the ONPG per min under standard assay conditions.

Structure Elucidation. **Elemental Analysis.** The elemental composition of four samples (agar, agar/PEI, agar/PEI/GA, and agar/PEI/GA/Enz) was determined by the elemental analyzer vario El (Elementar, Germany).

Fourier Transform Infrared. The infrared spectra of all formulations were recorded with Fourier Transform Infrared Spectroscopy (FTIR-4100, Jasco, USA). FTIR spectra were taken in the wavelength region from 4000 to 400 cm^{-1} at ambient temperature. Formula 1 (agar) formula 2 (agar/PEI), formula 3 (agar/

PEI/GA), and formula 4 (agar/PEI/GA/β-gal) were examined for the presence of the new functionalities using FTIR.

Assessment of the Covalent Bond Formed Between β-Gal and the Activated Agar Disks. The β-gal loaded agar disks were incubated with 1*M* NaCl at room temperature for 1 h. After that, enzyme activity was analyzed in the supernatant.[16]

Evaluation of the Catalytic Activity of β-Gal. **Effect of pH.** Both the free and the immobilized β-gals were assayed in buffers of various pH values (pH 2.6–8) as described in the section on determination of β-gal activity. The buffers used were the citrate-phosphate buffer (pH 2.6–5.6) and the sodium phosphate buffer (pH 6–8). These buffers were employed at a final concentration of 100 mM. The activity recorded at the optimum pH was taken as the 100% activity, and the activities offered at the other pH values were expressed as a percentage of this optimum 100% activity.

Effect of Temperature. The enzymatic reactions of both the free and the immobilized β-gals were carried out at different temperatures (37°C–65°C) as described in the section on determination of β-gal activity. The activity offered at the optimum temperature was taken as the 100% activity, and the activities recorded at the other temperatures were expressed as a percentage of this 100% activity.

Determination of the Kinetic Parameters. The Michaelis-Menten constant (K_m) and the maximum reaction rate (V_{max}) for the free β-gal and its immobilized counterpart were determined using the Hanes-Woolf plot. In order to do so, the enzymatic reaction was carried out in the presence of varying final concentrations of ONPG (0.5–5 mM) in 100 mM acetate buffer of pH 4.6.

Operational Stability (Reusability). The reusability of β-gal covalently immobilized onto activated agar disks was tested. The gel disks were assayed as in the section on determination of β-gal activity. Afterwards, the same gel disks were washed thoroughly with distilled water and re-assayed. This procedure was repeated 15 times, and the initial activity was considered as 100%. The relative activity was expressed as a percentage of the starting operational activity.

Statistical Analysis. Data were analyzed with the Microsoft Excel 2007. Results were expressed as mean ± standard error. The mean of the control was compared with the means of different treatments using the one way ANOVA. The P values less than 0.05 were considered statistically significant.

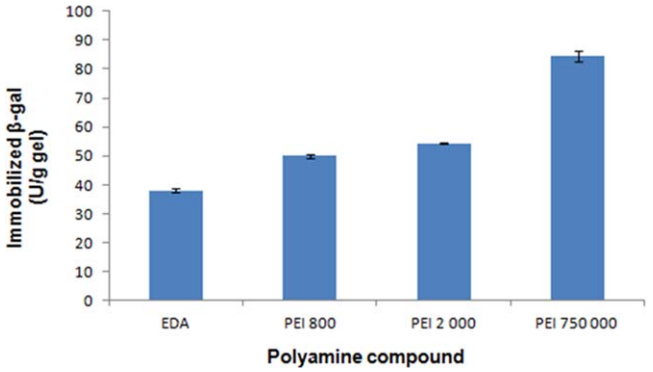

FIGURE 3 Effect of the type of the polyamine compound on the immobilization of β-gal (means ± S.E.).

RESULTS AND DISCUSSION

Optimization of the Activation Process

Optimization of Step A. Step A, which comprised the reaction of the agar disks with PEI (Figure 2), was optimized while keeping the reaction conditions for step B constant. In all the experiments, the agar disks were soaked in a 2% (v/v) GA solution for 1 h after their reaction with PEI.

Selection of the Polyamine Compound. EDA together with three different molecular weight PEI compounds (800, 2000, and 750,000 Da) were selected to test their efficiency in activating the agar gel. EDA was previously used during the activation of epoxy monolithic polyacrylamide cryogels[17] and poly(vinyl chloride) microspheres[18] for the covalent immobilization of enzymes. PEI was also used to activate carrageenan disks,[19] alginate beads,[20] and alginate-carrageenan beads.[21]

During the course of this experiment, the agar disks were soaked for 1 h in a 1% (w/v) solution (pH 8.5) of each of these four amine compounds. The pH 8.5 was selected as it is somewhat lower than the isoelectric point of EDA (PI 9.4).[22] Moreover, it was reported that about 18% of PEI would be protonated at pH 8.5.[23] Hence, all the tested amine compounds were partially protonated at pH 8.5. This partial protonation enabled the amine compounds to interact ionically with the negatively charged agar disks.

The results (Figure 3) showed that EDA offered the lowest immobilization yield (38.10 U/g gel). This could be due to the fact that each EDA molecule could only immobilize one enzyme molecule (Figure 2). EDA has only two amino groups; one to interact with the agar gel and the other to interact with one GA molecule and consequently one enzyme molecule.

It was noticed (Figure 3) that the amount of immobilized β-gal increased with the increase in the PEI's molecular weight till reaching a maximum of 84.44 U/g gel upon employing PEI 750,000. This could be attributed to the fact

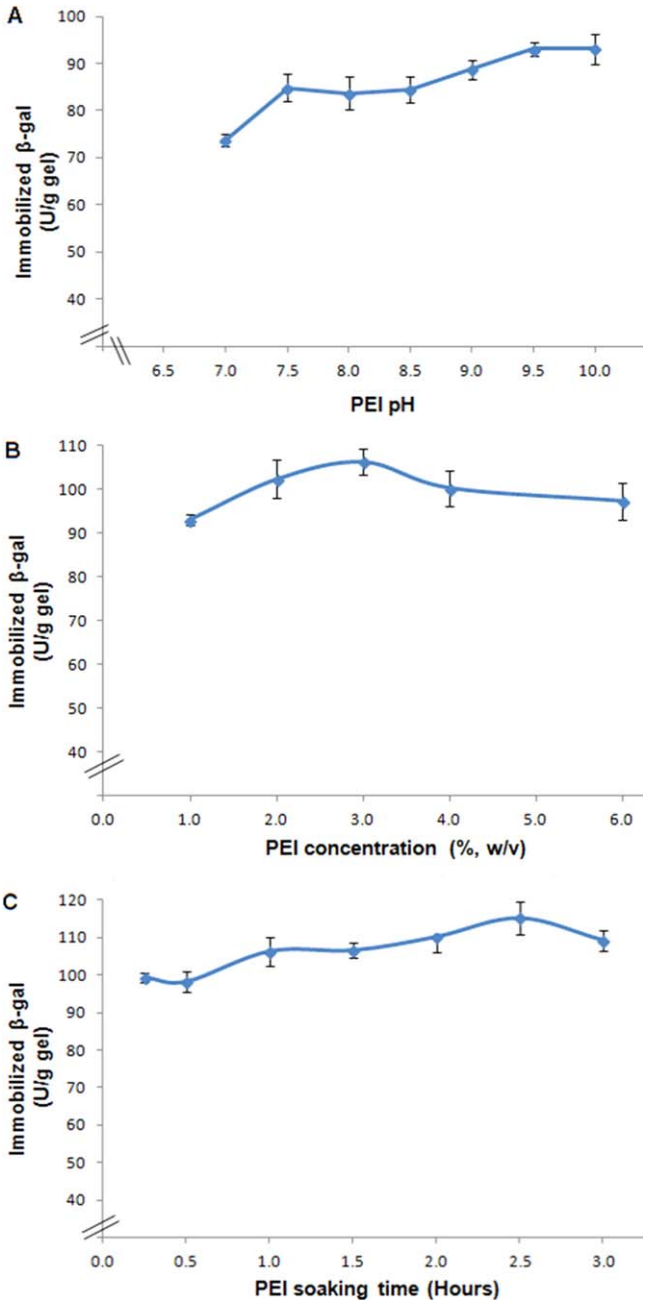

FIGURE 4 Effect of (A) PEI pH, (B) PEI concentration, and (C) PEI soaking time on the immobilization of β-gal onto the activated agar disks (means ± S.E.).

that the higher the molecular weight of PEI, the more branched it becomes (Figure 2), and the more primary amino groups it attains. Those primary amino groups are the ones responsible for the reaction with GA.[24] Thus, their abundance in the high molecular weight PEI would ensure the reaction of this PEI treated agar disks with plenty of GA and in turn plenty of enzyme molecules. Noteworthy, PEI 750,000 was also employed in the activation of poly(methyl methacrylate) microchannels for the covalent immobilization of glucose oxidase.[25]

Optimization of PEI 750,000 pH, Concentration, and Soaking Time. In order to mark out the optimum PEI pH, 7 different pH values (between 7 and 10) were examined. It could be seen (Figure 4A) that the maximum amount of immobilized β-gal was attainable at the highest inspected pH values. Where, a whole of 93.13 U/g gel were immobilized at either pH 9.5 or 10. These high pH values were favored for the enzyme immobilization, since they provided the most suitable ratio between the PEI's protonated and free amino groups. At such high pH values, the majority of the PEI's amino groups were in the free form and only few were protonated. Actually, it was previously reported that only ≈4% of PEI was protonated at pH 10.[26] This low level of protonation was enough for the PEI to interact ionically with the low amounts of negatively charged pyruvate and sulfate groups located on agar. Where, agar was previously reported to contain only 0.12–0.65% pyruvate[27] and about 2% sulfate.[9] The low sulfate content was also verified for the type of agar used in the study, as its elemental analysis showed it to exhibit only 0.596% sulfur. On the other hand, the high content of PEI's free amino groups, that was brought about by such high pH values, enabled the PEI treated agar disks to react with lots of GA molecules and accordingly to immobilize lots of enzyme molecules. It is worth mentioning that, a PEI 750,000 solution of pH 9.5 was also used during the activation of the waveguide sensor surface for the immobilization of antibodies.[28]

The PEI concentration and soaking time were optimized while utilizing a PEI 750,000 solution of pH 9.5. The results (Figure 4B) displayed a gradual increase in the amount of immobilized β-gal with the increase in the PEI's concentration till reaching a maximum of 106.29 U/g gel upon employing a 3% (w/v) PEI solution. It was also shown (Figure 4C) that the optimum soaking time of PEI with the agar disks was 2.5 h. These findings were close to those previously reported in literature where both alginate[29] and carrageenan[30] gel beads were soaked in a 4% PEI solution for 3 h during their activation for the covalent immobilization of penicillin G acylase. Moreover, Elnashar et al.[19] stated that it would be optimum to employ a concentration range of 3–5% PEI during the activation of carrageenan disks.

Optimization of Step B. Step B of the activation process involved the reaction of GA with the PEI treated agar disks. This reaction created free aldehyde groups capable of covalently immobilizing β-gal (Figure 2). The optimization of step B was fulfilled through the investigation of the optimum GA concentration and soaking time. Noteworthy, the optimum settings that were reached during the optimization of step A

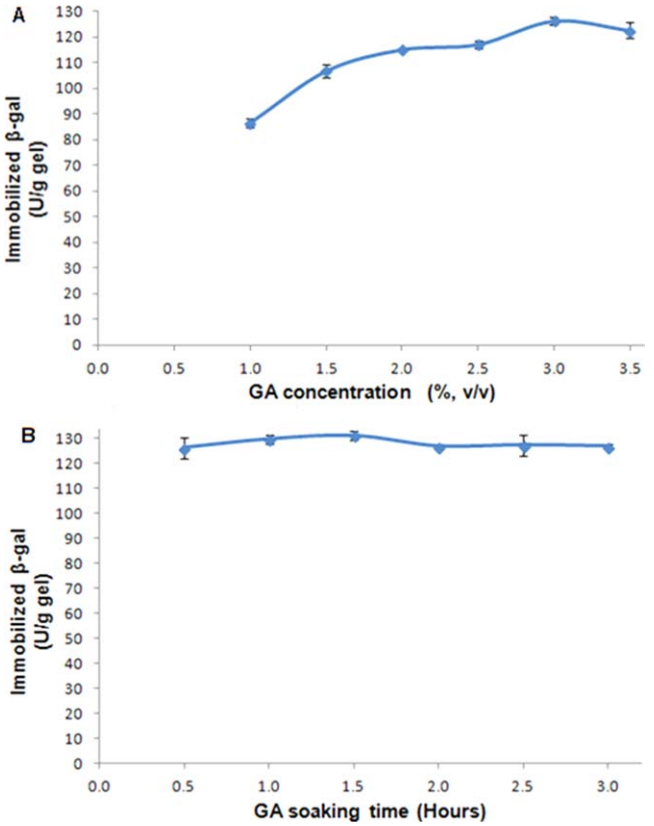

FIGURE 5 Effect of (A) GA concentration and (B) GA soaking time on the immobilization of β-gal onto the activated agar disks (means ± S.E.).

were employed here; i.e., the agar disks were soaked for 2.5 h in a 3% (w/v) PEI 750,000 solution of pH 9.5 prior to treating them with GA.

Optimization of GA Concentration. Figure 5A revealed that increasing the GA concentration from 1% to 3% (v/v) caused the amount of immobilized β-gal to increase from 86.56 to 126.23 U/g gel. This 1.46-fold increase in the immobilization yield reflected the increase in the amount of the free aldehyde groups capable of covalently immobilizing β-gal.

Further increasing the GA concentration to 3.5% (v/v) caused the amount of immobilized β-gal to drop slightly reaching 122.52 U/g gel. Thus, the optimum GA concentration was considered to be 3% (v/v). This value was close to the one reported by Kishore et al.,[31] who stated that employing a 3.34% (v/v) GA solution was optimal for the covalent immobilization of β-gal onto functionalized graphene nano-sheets.

Optimization of GA Soaking Time. In this experiment, six different GA soaking times, ranging from 0.5 to 3 h, were investigated. The amount of immobilized β-gal varied slightly, from 126.26 to 131.20 U/g gel, along the entire tested time range (Figure 5B). This slight variation (3.8%) could indicate that changing the GA soaking time from 0.5 to 3 h did not significantly affect the amount of immobilized β-gal. In order to verify such a postulate, we conducted a one way ANOVA test on the results of this experiment. The P value turned out to be 0.792, indicating that there was no significant difference between the amounts of immobilized β-gal obtained at any of the inspected GA soaking times. A similar situation was noticed by Elnashar et al.,[21] who reported that changing the GA soaking time from 0.5 to 3 h did not significantly affect the amount of penicillin G acylase covalently immobilized onto alginate-carrageenan gel beads. They debated that the reason behind this observation was the fact that GA crosslinks the PEI's free amino groups only. Most of these free amino groups would suffer from protonation upon incubation with a GA solution of low pH (4.52). Hence, after 30 min of incubation with the GA solution, the PEI treated agar disks would virtually have no more free PEI amino groups to offer for the reaction with GA. Thus, further increasing the soaking time would not significantly increase the amount of reacted GA and consequently would not significantly increase the amount of immobilized enzyme. For future work we recommend preparing the GA solution in a buffer of pH in the range of 7–9 to limit the protonation of the PEI's free amino groups.

Structure Elucidation

The incorporation of new functionalities into the agar gel structure, during the activation and the immobilization processes, was proven via the elemental analysis and the FTIR of agar, agar/PEI, agar/PEI/GA, and agar/PEI/GA/Enz formulations.

Elemental Analysis. The elemental analysis data for the agar disks was 36.29% (C), 7.126% (H), 0% (N), 0.596% (S), and 55.988% (O). Meanwhile, the elemental composition of the agar/PEI disks was 40.17% (C), 8.792% (H), 8.59% (N), 0.37% (S), and 44.078% (O). The absence of nitrogen in the agar disks and its presence in the PEI treated disks proved the incorporation of PEI in the agar gel. Elnashar et al.[19] also proved the incorporation of PEI into carrageenan gel by using the elemental analysis. Where, nitrogen was absent in case of carrageenan gel, but it was present at a concentration of 15.059% in the PEI treated carrageenan gel.

The elemental analysis of the agar/PEI/GA disks was 47.32% (C), 9.002% (H), 4.47% (N), 0.4% (S), and 38.808% (O). The rise in the carbon percent from 40.17%, in case of the agar/PEI disks, to 47.32%, in case of the Agar/PEI/GA disks, reflected the incorporation of GA ($C_5H_8O_2$) in the gel structure. Noteworthy, the incorporation of GA into alginate-carrageenan/PEI gel beads was also previously proven by the rise in the carbon percent from 30.64%, for the alginate-

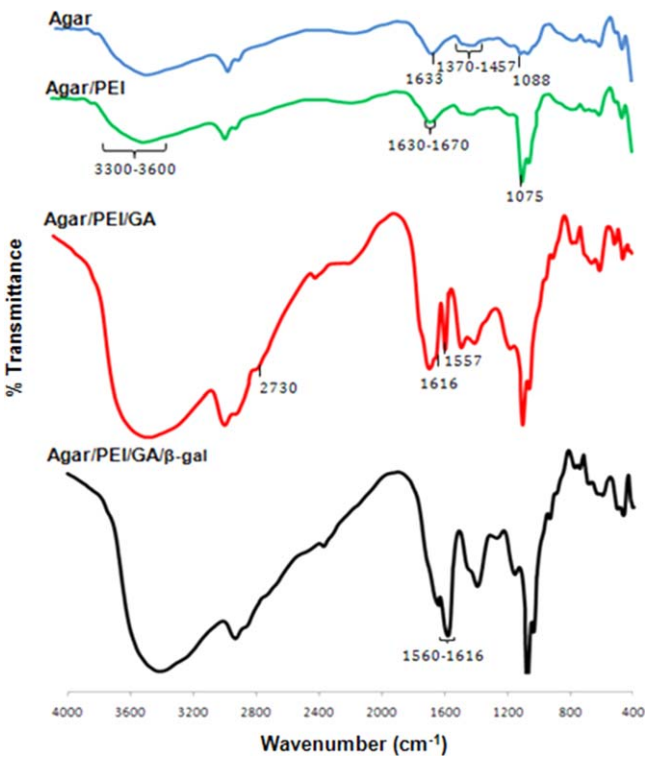

FIGURE 6 The FTIR bands of the (agar), (agar/PEI), (agar/PEI/GA), and (agar/PEI/GA/*β*-gal).

carrageenan/PEI beads, to 40.45%, for the alginate-carrageenan/PEI/GA beads.[21]

The agar/PEI/GA/Enz disks exhibited a somewhat different elemental composition from that of the agar/PEI/GA disks. The carbon, hydrogen, nitrogen, and sulfur contents of the enzyme loaded disks decreased reaching 41.21%, 8.19%, 4.24%, and 0.22%, respectively. Meanwhile, its oxygen content increased to the level of 46.14%. This alteration in the elemental composition of the agar/PEI/GA/Enz disks could be attributed to the covalent immobilization of *β*-gal onto the agar/PEI/GA disks.

Fourier Transform Infrared. The FTIR bands of the (agar), (agar/PEI), (agar/PEI/GA), and (agar/PEI/GA/*β*-gal) were shown in Figure 6. Regarding the agar formulation, it could be noticed that the agar's sulfate groups ($-OSO_3^-$) appeared as a band at 1088 cm^{-1}. Moreover, the pyruvate's carboxylate groups ($-COO^-$) were represented as a band at 1633 cm^{-1} and a broad band at 1370–1457 cm^{-1}. This broad band resulted from the overlapping of the carboxylate band with the bands of the agar's primary and secondary hydroxyl groups that were supposed to appear at 1400–1460 and 1345–1475 cm^{-1}, respectively. Considering the (agar/PEI) formulation, the PEI was expected to reveal two characteristic bands for the amine groups. These two bands were: a stretching vibration at 3300–3400 cm^{-1} and a bending vibration at 1590–1650 cm^{-1}. Both bands were overlapped with the broadband of the –OH and the –COOH groups at 3300–3600 cm^{-1} and 1630–1670 cm^{-1}, respectively. It is worth mentioning that, the sulfate band of the agar was perturbed from 1088 cm^{-1} to 1075 cm^{-1} after the formation of the gel complex with PEI. This observation could be due to the strong polyelectrolyte interaction between the positively charged $-NH_3^+$ of the PEI and the negatively charged $-OSO_3^-$ of agar. A similar shift was also observed by Elnashar et al.[21] where, the sulfate band of carrageenan was perturbed from 1050 cm^{-1} to 1030 cm^{-1} after the formation of a gel-complex with PEI.

As for the (agar/PEI/GA) formulation, it was postulated that the aldehyde groups present at one end of the GA molecules reacted with the free amine moieties of PEI forming Schiff's base. This Schiff's base (–C=N–) appeared as a shoulder at 1616 cm^{-1}. Moreover, it was also assumed that the unreacted GA side created free aldehyde groups for covalent immobilization of *β*-gal. These aldehyde groups appeared as a shoulder at 2730 cm^{-1}. This shoulder smoothed out in the (agar/PEI/GA/*β*-gal) FTIR indicating that the free GA aldehyde groups were employed to covalently immobilize *β*-gal through the formation of Schiff's base with the *β*-gal amino groups. The Schiff's base peak of the (agar/PEI/GA/*β*-gal) formulation overlapped with a band, that was previously located at 1557 cm^{-1} in the (agar/PEI/GA) FTIR, forming a single band from 1560–1616 cm^{-1}. Noteworthy, the Schiff's base formed between the aldehyde groups of GA and the amino groups of the immobilized enzymes was also noticed by Bahman et al.[32] and Ren et al.[33] at 1648 cm^{-1} and 1639 cm^{-1}, respectively.

Assessment of the Covalent Bond Formed Between *β*-Gal and the Activated Agar Disks

In order to ascertain that *β*-gal was covalently immobilized onto the activated agar disks and not just adsorbed onto the agar surface, the enzyme loaded disks were incubated with 1*M* NaCl. NaCl would cause the adsorbed enzyme to be eluted from the agar surface into the supernatant liquid, where it could be detected. The results of this experiment revealed that the supernatant was devoid of any *β*-gal activity. Hence, it could be concluded that all the immobilized *β*-gal was covalently attached to the agar disks and not adsorbed. Noteworthy, *β*-gal was shown to be covalently immobilized onto GA activated carrageenan-chitosan disk,[34] magnetic polysiloxane particles coated with polyaniline,[35] magnetic Fe_3O_4-chitosan nanoparticles,[36] and multiwalled carbon nanotubes.[37]

Evaluation of the Catalytic Activity of *β*-Gal

Effect of pH. The free *β*-gal was shown to exhibit a broad pH optimum (4.6–6.5) (Figure 7A). This optimum pH range was

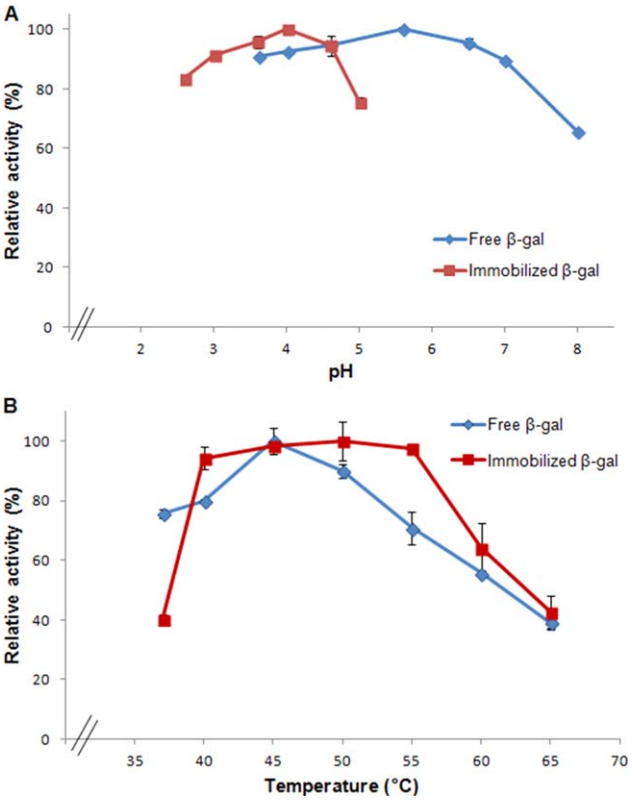

FIGURE 7 Effect of (A) pH and (B) temperature on the activities of the free and the immobilized β-gals (means ± S.E.).

somewhat broader than the one reported by Elnashar and Yassin[34] who stated that the *Aspergillus oryzae* β-gal exhibited an optimum pH range of 4.5–5. The immobilization of β-gal onto the activated agar disks caused its pH optimum to shift towards the acidic side (3.6–4.6). The shifting of pH optima to more acidic values after immobilization was previously reported for other enzymes, such as, the silica immobilized invertase[38] and the gelatin immobilized tannase.[39] The change in the pH optima of immobilized enzymes could be attributed to the ionic changes that occurred around the enzyme's active site as a result of the immobilization process.[40]

Effect of Temperature. Figure 7B revealed that the free *Aspergillus oryzae* β-gal offered its highest activity at 45°C. A comparable result was reported by Tanaka et al.[41] who stated that the *Aspergillus oryzae* β-gal exhibited a temperature optimum of 46°C. The temperature optimum for the agar immobilized β-gal was broadened from 45 to 55°C (Figure 7B). The immobilization of β-gal onto the cellulose acetate-polymethylmethacrylate membrane also caused its temperature optimum to broaden from 50 to 60°C.[42] The shift of an enzyme's optimum temperature towards higher values after its immobilization could be regarded to the formation of a molecular cage around the enzyme. This molecular cage protected the enzyme's molecule from the bulk temperature.[34]

Table I Kinetic parameters for β-gal catalyzed ONPG hydrolysis

	Kinetic parameters[a]	
Sample	K_m (mM)	V_{max} (μmol/min/mg enzyme)
Free β-gal	2.01	1.275
Immobilized β-gal	10.37	0.968

[a] Values of K_m and V_{max} were calculated from the Hanes-Woolf plot (Figure 8).

Determination of the Kinetic Parameters. The results (Table I) showed that the K_m for the free β-gal amounted to 2.01 mM. This value was close to the one reported by Vera et al.[43] who proved that the free *Aspergillus oryzae* β-gal exhibited a K_m of 2.27 mM. Immobilizing β-gal onto the activated agar disks caused the K_m to increase reaching 10.37 mM whereas the V_{max} decreased. Mass transfer resistance might be the reason behind the increase in K_m after immobilization. Mass transfer resistance was shown to be significant for macromolecular substrates such as ONPG because the substrate should contact the immobilized enzyme on its carrier. Additionally, the immobilization of β-gal might have restricted the enzyme's ability to undergo conformational changes intrinsic to the enzyme-substrate interactions. This restriction would cause the V_{max} of the immobilized enzyme to decrease.[42] The increase in the K_m and the decrease in the V_{max} for the immobilized β-gal were reported before for the *Kluyveromyces lactis* β-gal immobilized on modified carbon nanotubes[37] and the *Aspergillus oryzae* β-gal immobilized onto cellulose acetate-polymethylmethacrylate membrane.[42]

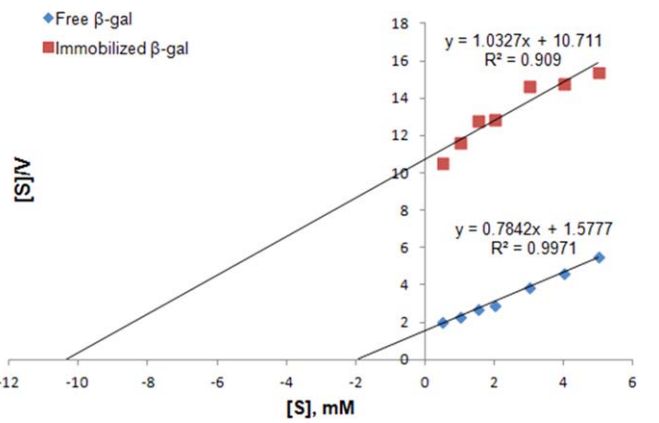

FIGURE 8 The Hanes–Woolf plot used to determine the kinetic parameters for the free and the immobilized β-gals.

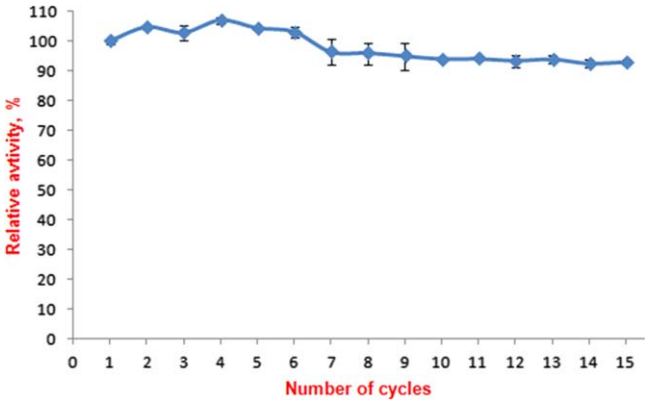

FIGURE 9 Reusability of immobilized β-gal (means ± S.E.).

Reusability

The reusability of β-gal covalently immobilized onto the activated agar disks was evaluated (Figure 9). The data revealed an initial increase in the enzyme's relative activity upon reusing it. This initial increase persisted up to the sixth reusability cycle (103.06%). The initial increase in the relative activity of immobilized β-gal was previously reported for both the covalently immobilized[34] and the entrapped β-gal.[44] It was hypothesized that the reason behind such an initial increase in the relative activity of covalently immobilized enzymes was the enzymes' need for a relaxation time in contact with their substrate in order to reach their maximum efficiency.[19]

The results also showed (Figure 7) that the immobilized β-gal retained a whole of 92.99% of its initial activity after 15 cycles. That is to say, the immobilized β-gal lost only 7.01% of its original activity after 3.75 working hours. This result was close to the one reported by Kishore et al.[31] who stated that β-gal covalently immobilized onto functionalized graphene nanosheets retained more than 92% of its enzymatic activity after 10 cycles of repeated use. On the other hand, the lactose pretreated β-gal covalently immobilized onto silica gel retained only 63.9% of its original activity being retained after 10 reuses.[45] Noteworthy, the decline in the enzyme activity by the fifteenth run could be attributed to the distortion in the active site of the enzyme due to its frequent encountering with the substrate.[46]

CONCLUSION

It could be concluded from the results of this study that the agar disks would be optimally activated through soaking them in a 3% (w/v) PEI solution (750,000 Da) of pH 9.5 for 2.5 h, followed by immersing them in a 3% (v/v) GA solution. As regards to the GA soaking time, a thirty minutes period would be enough, as further increasing the GA soaking time would not significantly increase the immobilization yield. These optimally activated agar disks managed to immobilize a whole of 126.26 U β-gal/g gel, and the immobilized β-gal retained 92.99% of its initial activity after being used for 15 consecutive times. Hence, this immobilized β-gal could be regarded as a good candidate for application in industry. These results also indicate the superiority of these novel grafted agar disks as enzyme carriers and would encourage using them to covalently immobilize other enzymes.

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