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Studies of Penicillin G Acylase Immobilization Using Highly Porous Cellulose-Based Polymeric Membrane

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Abstract The different ionic molecules/compounds were used as a ligand for the immobilization of penicillin G acylase on the highly porous cellulose-based polymeric membrane having buffer flux 1,746 LMH (L m^{-2} h^{-1}) at 0.5 bar pressure. The immobilized enzyme activity around 250 U_{App} was obtained with the ligand such as proline, tryptophan, casein acid hydrolysate, and brilliant green. Comparatively, proline showed less IMY% (percentage immobilization yield—58) but higher RTA% (percentage of activity retention—71) and specific activity (145 U_{App} g^{-1}). However, the crosslinked preparation of brilliant green obtained using glutaraldehyde showed 82±2.7% immobilized enzyme activity after the completion of successive five cycles. In comparison with the free enzyme, the enzyme immobilized on the brilliant green coupled membrane showed around 2.4-fold increase in K_m value (47.4 mM) as well as similar optimum pH (7.2) and temperature (40 °C). The immobilized enzyme retained almost 50% activity after 107 days and 50 cycles of operation. Almost 50% decrease in buffer flux after enzyme immobilization was observed. At the end of the 30 cycles, flux pattern shows around 38% decrease in buffer flux however, after 16 cycles of operation flux moves closer towards the steady state.

Keywords Penicillin G acylase · Immobilization · Proline · Brilliant green · Polymeric membrane

Introduction

Penicillin G acylase (E.C. 3.5.1.11) is a hydrolytic enzyme which acts on the side chains of penicillin G and related antibiotics to produce the β-lactam antibiotic intermediates such as 6-amino penicillanic acid. The total world market for β-lactam antibiotics is estimated to be around $15 billion, which corresponds to the 65% of the total world market for antibiotics [1]. The enzyme is also involved in synthesis of the anti-platelet agent and in enzymatic activation of pro-drugs in cancer therapy [2]. Penicillin G acylase is a heterodimeric protein

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formed by the processing of a single polypeptide precursor, which comprising of a small α-subunit and a large β-subunit [3]. This enzyme belongs to N-terminal nucleophile hydrolases that share a common fold around the active site bearing a catalytic serine, cysteine, or threonine at the N-terminal position [3, 4]. Because of industrial importance, the search of suitable carrier for the enzyme immobilization always remains a major thrust area of research. However, the mass transfer limitations in the carrier remain a major barrier to further improvement of the enzyme performance at industrial level [5]. The accumulation of product and generation of pH gradients are the major drawback in the conventional form of the carrier such as bead [5]. However, most of the recent studies indicate the dominance of a carrier with bead configuration for immobilization [6-10]. Comparatively, the use of unconventional source of a carrier for enzyme immobilization is not widely reported. There are some reports where polymeric membranes such as nylon [11] and porous polymer with oxirane groups [12] were used for enzyme immobilization. In comparison with the conventional sources, polymeric membranes appear to be suitable carrier for the enzyme immobilization. Because, substrate molecules can be convected through the membrane support rather than having to diffuse to a bead or particle to reach the active site of an enzyme. This helps in the enhancement of mass transfer efficiency significantly. However, irrespective of advantages such as easy scale up, high throughput etc., the fouling of polymeric membrane remains a major problem, which reduces productivity and increases maintenance and operating costs [13]. Particularly when the carries has to undergo different chemical reaction to immobilize the enzyme, this may result in the reduction of pore size and enhance the fouling. Therefore, it is desirable that the carrier should have the high pore size, chemical and mechanical stability, and biodegradability to overcome the after use disposal problem. In this work, we have used a highly porous cellulose-based polymeric membrane for enzyme immobilization to overcome the problem of mass transfer, fouling, and biodegrability. The different ionic molecules/compounds generally used for the protein adsorption were screened to select most appropriate molecules/compounds as a ligand for enzyme immobilization and its effect on the stability and functional properties of the enzyme were studied. The crude enzyme preparation and adsorption-crosslinking method [14] was used for the enzyme immobilization. This may enhance the selective binding of an enzyme to the carrier and also helps in the reduction of overall process cost.

Materials and Methods

Materials

Epichlorohydrin, glutaraldehyde , chloroacetic acid, sodium borohydride, and hydrazine hydrate (Loba, India); proline, tyrosine, tryptophan, gelatin, casein, peptone, casein acid hydrolysate, beef extract, meat extract, soya peptone, and tryptone (Himedia, India); polyethylemine, diethylaminoethyl chloride, and procion red (Aldrich Chemicals); and brilliant green (Fluka Chemicals) were used as received. All other chemicals used were of ACS grade.

Enzyme Production

Escherichia coli (NCIM 2400) was obtained from the National Collection of Industrial Microorganisms, Pune, and maintained routinely on nutrient agar slants. Cells were grown

in the defined medium g L^{-1} (3 g peptone, 3 g yeast extract, 3.5 g NaCl, 3 g K_2HPO_4, 0.3 g KH_2PO_4, 60.0 g black liquor, 1 g phenylacetic acid, and 0.1 g polypropylene glycol). The pH of the medium was adjusted to 7.2. The inoculum was developed in 250-mL conical flask containing 50-mL medium incubated at 23 °C for 24 h on a rotary shaker. The inoculum was transferred aseptically into a 1 L Gallenkamp fermenter with magnetic stirring containing 500 mL medium. The fermentation was carried out at 23 °C under agitation at 300 rpm and 0.5 vvm aeration for 24 h. After fermentation the cells were collected by centrifugation (10,000×*g*) using Remi C24. The harvested cells were suspended in 50 mM sodium phosphate buffer pH 7 and then disrupted in a Branson sonifier. The disrupted material was centrifuged at 10,000×*g* for 15 min and the supernatant was used as an enzyme penicillin G acyalse without any further treatment.

Enzyme Assay

Enzyme assay was carried out using 3.72% of penicillin G K^+ salt (Hindustan Antibiotics Ltd. Pimpri, India) as a substrate in 0.1 M phosphate buffer pH 7.0. To the mixture of 0.2 mL sample containing enzyme and 0.8 mL 0.1 M phosphate buffer pH 7.0, 1.0 mL substrate was added and then incubated at 40 °C for 1 h. The reaction was terminated by pipetting 0.2 mL of the reaction mixture into 3.8 mL of citrate-phosphate buffer, pH 2.5. The enzymatic activity was estimated by determining the amount of 6-aminopenicillanic acid (6-APA) formed using p-dimethylaminobenzaldehyde reagent [15]. The specific activity of the free enzyme was expressed as μmoles of 6-APA formed per minute per g of protein at pH 7.0 and 40 °C (U g^{-1}). Protein was determined using folin-ciocalteau's reagent [16].

Enzyme Immobilization

Membrane

The membrane was prepared as per the procedure described elsewhere [17]. The base polymer of the membrane was ethyl cellulose and the buffer flux (50 mM sodium phosphate buffer pH 7) of the membrane was 1,746 LMH (L m^{-2} h^{-1}) at 0.5 bar pressure. Figure 1 shows the attenuated total reflectance Fourier transform infrared (ATR/FT-IR) spectra which reveal the chemical structure of the membrane. It shows several characteristic peaks, the peak at 3,475 cm^{-1} corresponds to the –OH groups of closed ring structure of the polymer unit. The peak at 2,869 cm^{-1} corresponds to C–H bond whereas the peak at 1,374 cm^{-1} corresponds to C−N bond. The sharp peak at 1,074 cm^{-1} corresponds to –C−O (stretch).

Membrane Modification

The chemical modification of membrane was carried out to coupled ligand comprising different ionic molecules/compounds such as DEAE, chloroaceticacid, dyes, amino acids, and proteins as ligand to check their effect on enzyme immobilization (Table 1). After complete washing of the modified membrane to remove unbound chemicals, it was equilibrated with 50 mM sodium phosphate buffer pH 7 prior to enzyme immobilization. Enzyme immobilization was carried out by loading 5 mL of an enzyme on a modified membrane (13.4 cm^2) fixed in an amicon flow cell under mild stirring for 2 h. To remove unbound enzyme, sequentially four washing with 5 ml of 50 mM sodium phosphate buffer pH 7 and finally with 5 ml 1 M NaCl prepared in the same buffer was carried out. Sample of each washing was collected to estimate the enzyme activity and protein (Enzyme Assay).

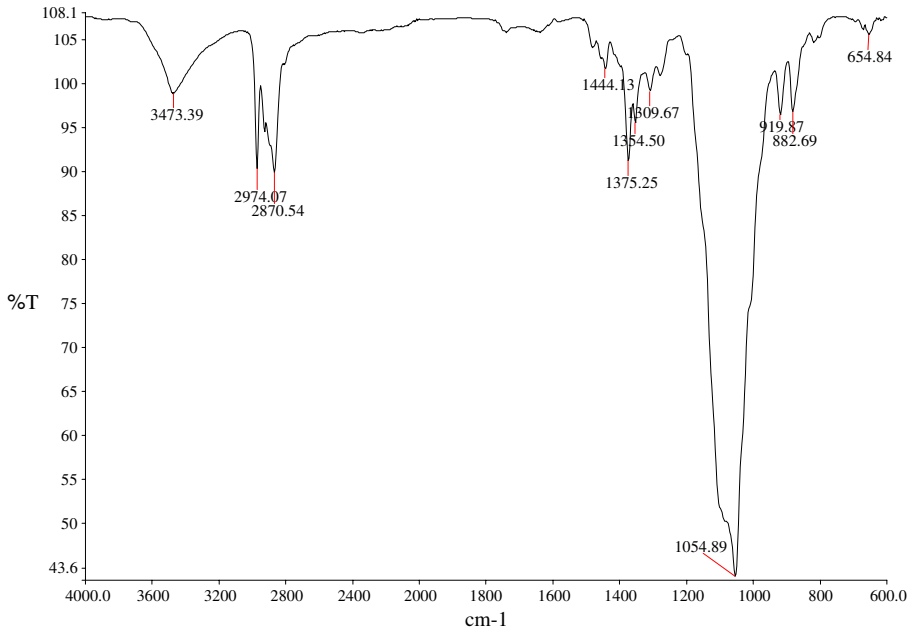

Fig. 1 Attenuated total reflectance Fourier Transform Infrared (ATR/FT-IR) spectrometry of the membrane. Spectra was obtained on Perkin Elmer FT-IR system, Spectrum GX and is given as it is

Immobilized Enzyme Activity

As mentioned above (Membrane Modification), the enzyme immobilized membrane was equilibrated with buffer and then 10 mL substrate (enzyme assay) was loaded and allowed to react at 40 °C for 1 h. After 1 h, sample was removed to measure the 6-APA (enzyme assay) and termed as test. The blank was treated similarly and the difference between test and blank was taken for the calculation of immobilized enzyme activity (Test−Membrane+Ligand+Enzyme; Blank−Membrane+Ligand). The apparent catalytic activity (U_{App}) was expressed as the μmoles of 6-APA produced per minute per square meter of membrane at pH 7.0 and 40 °C (μmoles min^{-1} m^{-2}). The specific activity (U_{App} g^{-1}) was expressed as the μmoles of 6-APA produced per minute per gram of protein adsorbed on the membrane at pH 7.0 and 40 °C (μmoles min^{-1} g^{-1}). These experimental conditions were always applied, except when indicated otherwise.

Treatment of Experimental Data

Every experimental point reported in the table and figures represents the average value of three experiments performed under the same conditions. The experimental errors did not exceed 5%.

Results and Discussion

In the present study we have used a crude enzyme preparation for immobilization. This may help to obtain more stable immobilized enzyme as well as reduction in the overall process

cost. It was reported that the use of highly purified penicillin G acylase for immobilization resulted in less stable preparation [18]. The enzyme immobilization to the membrane was carried out using adsorption-crosslinking method [14]. Because, this may facilitate the selective binding of an enzyme to the carrier as well as compensate the use of crude enzyme for immobilization to some extent. An enzyme immobilization using the combination of adsorption and crosslinking is a well-known method. The different carriers such as ion exchangers, hydrophobic adsorbents, and biospecific adsorbents can be used for this purpose [14]. Earlier, this concept has been used for the immobilization of penicillin G acylase on different carriers such as gelatin [19], chitosan-based support [20], derivitised crosslinked agarose, pheoxyacetylcellulose, oxirane-polyacrlamide resin, polyacrylamide foam, crosslinked polymethyl methacrylate/glycol dimethacrylate copolymer, crosslinked polystyrene, and porous glass, DEAE-cellulose, cellulose, CM-cellulose, ion exchangers, acrylamide gel, polyamides, silica gel, and Al_2O_3 [21]. However, the studies involving polymeric membrane for penicillin G acylase immobilization using the adsorption-crosslinking method are negligible. In the present paper, we have used different ionic molecules/compounds as ligand to modify the cellulose-based highly porous membrane to study penicillin G acylase immobilization. Most of these ionic molecules/compounds have been used earlier for an enzyme immobilization such as DEAE, chloroaceticacid (CM: carboxymethyl) [21], hydrazine hydrate [22], different dyes [14], polyethylimine [23], amion acid as ligand [24], proteins such as albumin [25], and gelatin [19]. The purpose of using these ionic molecules/compounds as a ligand was also to avoid harsh chemical reactions for membrane modification and enzyme immobilization. This may also help in developing a relatively inexpensive and nontoxic chemistry for enzyme immobilization.

Effect of Different Ionic Molecules/Compounds on Immobilization

The binding of different ionic molecules/compounds as ligand to the membrane was carried out to generate functional groups on the membrane for enzyme adsorption. Each step of the treatment was optimized using enzyme activity as the screening criteria. Although the different treatment was given to the membrane for the binding of different ligands but the enzyme immobilization procedure was uniform. The pH and ionic strength of the buffer was also not changed for the enzyme immobilization. Therefore, any change in enzyme activity after immobilization may be solely due to the interactions between carrier and enzyme. In Table 1 the enzyme activity and protein adsorption obtained for different ligands are presented. Table 1 show that there is no relation between the amount of protein adsorbed on the membrane and enzyme activity. The membrane without any modification showed significant protein adsorption but negligible enzyme activity. This suggests the need of modification of membrane to make it suitable for enzyme immobilization. On the other hand, DEAE showed comparatively higher protein adsorption but significantly less enzyme activity. The reason particularly in the case of DEAE may be the high amino-group concentration. It was reported that the carriers having high amino-group concentration results in multi-point bonding with penicillin G acylase, which deactivate the retained activity [26]. Another reason may be the presence of non-favorable interactions between the enzyme and the supporting matrix and microenvironment in which the immobilized enzyme is operating. Interestingly, the enzyme activity around 250 U_{App} was obtained with the ligand such as proline, tryptophan, casein acid hydrolysate, and brilliant green, this is not reported earlier. Table 2 shows the effect of these four ligand on IMY%, RTA% [26], and specific activity of immobilized enzyme. Comparatively, proline showed less IMY% but higher RTA% and specific activity. This indicates that the proline is most suitable for

Table 1 Effect of different ionic molecules/compounds (ligands) coupled to membrane on enzyme immobilization.

Ligands	Treatment	Treatment conditions	Preparation prior enzyme loading	Protein adsorbed (g m^{-2})	Immobilized enzyme activity (U_{App})
Nil	Membrane without any modification	Nil	Washing with DW and buffer	1.9	24.87
DEAE	Membrane + epichlorohydrin (0.5% *v/v*) prepared in 0.1 N NaOH + 0.2% sodium borohydride	40°C for 1 h at mild stirring	Washing with DW	5.62	44.77
	Membrane after treatment A + 2-diethylaminoethyl chloride hydrochloride (5.0% *w/v*) prepared in 0.1 N NaOH + 0.2% sodium borohydride	40°C for 1 h at mild stirring	Washing with 0.1 N NaOH followed by 0.1 N HCl, DW and buffer		
Chloroaceticacid	Membrane + chloroacetic acid 10% (*w/v*) prepared in 0.1 N NaOH	60°C for 1 h at mild stirring	Washing with DW and buffer	2.68	176.61
Polyethylimine	Membrane +2 N HCl prepared in DW	60°C for 1 h at mild stirring	Washing with DW	2.85	194.27
	Membrane after treatment A + polyethylimine (1.0% *w/v*) prepared in DW	RT for 1 h at mild stirring	Washing with DW and buffer		
Hydrazine hydrate	Membrane +10 mL hydrazine prepared in DW	RT for 24 h at mild stirring	Washing with DW	4.32	42.28
	Membrane after treatment A + glutaraldehyde (5% *v/v*)	37°C for 24 h	Washing with DW and buffer		
Procion red	Membrane + Procion Red MX-5B (2.5% *w/v*) prepared in 0.1 N NaOH	60°C for 1 h at mild stirring	Washing with DW followed by 2 M NaCl, 10% methanol, DW and buffer	2.46	144.40
Brilliant green	Same as procion red	Same as procion red	Same as procion red	3.2	316.29
Tyrosine	Membrane + 2 N HCl prepared in DW	60°C for 1 h at mild stirring	Washing with DW	3.16	131.09
	Membrane after treatment A + glutaraldehyde (5% *v/v*) prepared in DW	RT for 24 h at mild stirring	Washing with DW		
	Membrane after treatment B + tyrosine (5 mg/mL) prepared in 0.1 N NaoH	RT for 24 h at mild stirring	Washing with DW and buffer		

Proline	Same as tyrosine	Same as tyrosine	Same as tyrosine	2.34	341.29
Tryptophan	Same as tyrosine	Same as tyrosine	Same as tyrosine	4.15	254.10
Gelatin	Same as tyrosine	Same as tyrosine	Same as tyrosine	1.93	57.21
Casein	Same as tyrosine	Same as tyrosine	Same as tyrosine	3.46	149.37
Peptone	Same as tyrosine	Same as tyrosine	Same as tyrosine	2.6	141.91
Casein acid hydrolysate	Same as tyrosine	Same as tyrosine	Same as tyrosine	3.5	249.11
Beef extract	Same as tyrosine	Same as tyrosine	Same as tyrosine	3.0	199.29
Meat extract	Same as tyrosine	Same as tyrosine	Same as tyrosine	1.7	99.50
Soya peptone	Same as tyrosine	Same as tyrosine	Same as tyrosine	4.0	167.53
Tryptone	Same as tyrosine	Same as tyrosine	Same as tyrosine	2.4	161.69

U_{App} μmoles min^{-1} m^{-2} of membrane, *DW* distilled water, *RT* room temperature (28±2°C), *Mild stirring* ~100 rpm, *buffer* 50 mM sodium phosphate buffer pH 7

Table 2 Effect of ionic molecules/compounds (ligands) coupled to membrane on IMY%, RTA%, and specific activity of immobilized enzyme.

Ligands	IMY%	RTA%	Specific activity (U_{App} g^{-1})
Proline	58.22	71.74	145.85
Tryptophan	82.43	21.30	61.22
Casein AH	79.62	25.58	71.17
Brilliant green	71.72	39.41	98.84

Activity of free enzyme, 348.64 U g^{-1} ; IMY% (percentage immobilization yield)=(activity of free enzyme−activity of immobilized enzyme)/activity of free enzyme×100; RTA% (percentage of activity retention)=(activity of free enzyme−activity of immobilized enzyme)×100

AH acid hydrolysate, U_{App} g^{-1} μmoles min^{-1} g^{-1} of protein adsorbed on the membrane

selective binding of penicillin G acylase to the carrier. The selective binding of penicillin G acylase to the proline may be due to the presence of secondary α-amino group which is unique among the 20 protein-forming amino acids.

Effect of Glutraldehyde on the Enzyme Stability

The stability of immobilized enzyme is a critical factor from its practical application point of view. To verify this, further study was carried out using the ligands which showed relatively higher activity of the immobilized enzyme such as proline, tryptophan, casein acid hydrolysate, and brilliant green (Table 2). During repeated use, the possibility of leakage of adsorbed enzyme from the carries is evident. To prevent the leakage, crosslinking of adsorbed enzyme on proline, tryptophan, casein acid hydrolysate, and brilliant green with glutaraldehyde was carried out. Glutaraldehyde is a bifunctional crosslinking reagent and it can be used for post-immobilization crosslinking to avoid enzyme leakage [27]. It was reported that the higher retention of an enzyme activity and operational stability could be achieved using affinity adsorption of the enzyme on the carrier surface and then crosslinking with glutaraldehyde [28, 29]. Figure 2 shows a comparative study carried out using crosslinked and non-crosslinked enzyme preparation. The enzyme activity pattern of crosslinked and non-crosslinked preparations were studied up to five cycles carried out successively using batch mode of operation. This was done to select most appropriate ligand for further study. Comparatively, the non-crosslinked preparations of proline and brilliant green showed almost 75% immobilized enzyme activity up to two cycles and then it slide down below 10% around five cycles (Fig. 2a). On the contrary, the crosslinked preparations of brilliant green showed 82±2.7% immobilized enzyme activity after the completion of successive five cycles (Fig. 2b). This indicates that the brilliant green coupled membrane has the potential to be a suitable carrier for penicillin G acylase immobilization. Although the RTA% (Table 2) was significantly less (~39) for brilliant green than the proline (~71); however, it was higher than the tryptophan, and casein acid hydrolysate. The penicillin G acylase immobilized on chemically grafted nylon particles showed 30% retention of activity [26]. In conclusion, crosslinking with glutaraldehyde fail to protect the immobilized enzyme activity in case of proline, tryptophan, and casein acid hydrolysate. This may be due to the lack of reactive groups which is required to facilitate the crosslinking and forming the network to prevent enzyme leakage during repeated use.

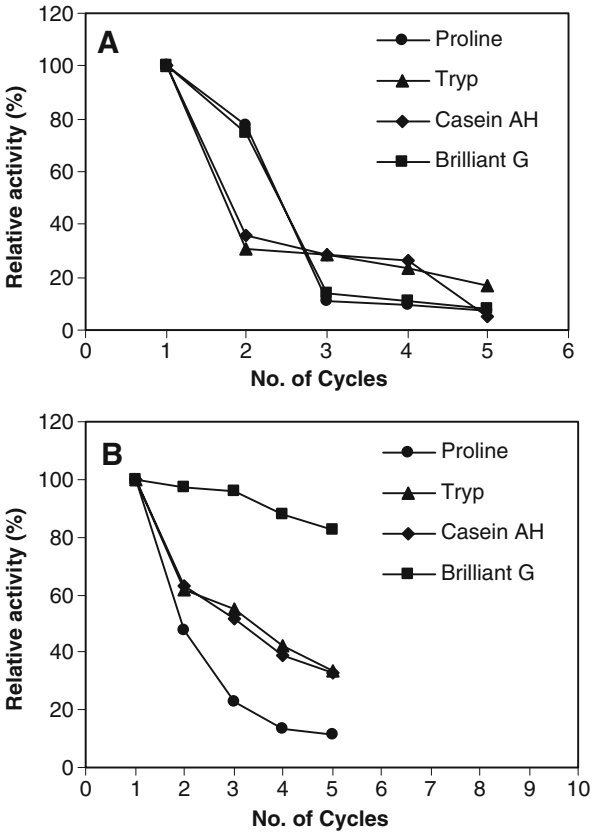

Fig. 2 Effect of glutraldehyde on the enzyme stability. **a** non-crosslinked; **b** crosslinked; glutaraldehyde treatment−membrane+ligand+5 mL 2% (*v*/*v*) glutaraldehyde prepared in buffer (50 mM sodium phosphate buffer pH 7) was allowed to react for 1 h under mild stirring. Relative activity (%) was calculated using higher enzyme activity obtained as 100%

Optimization of Immobilization and Characterization

As brilliant green appears to be the most suitable for penicillin G acylase immobilization, which is also not reported earlier. It is essential to evaluate its suitability for practical application. Therefore, the optimization of immobilization and characterization was studied to evaluate the most relevant factors such as brilliant green concentration, immobilization time, enzyme loading, effect of pH and temperature, effect on kinetic parameters, effect on storage, and operational stability and flux pattern of the enzyme immobilized membrane.

Effect of Brilliant Green Concentration

This study was carried out to select the optimum brilliant green concentration for the immobilization of an enzyme. The different concentration of brilliant green (0.05 to 0.25 g) was loaded on the membrane and the immobilization of an enzyme was checked. The higher enzyme activity and protein adsorption was obtained with 0.2 g brilliant green concentration (Fig. 3). The further study was carried out using 0.2 g brilliant green concentration.

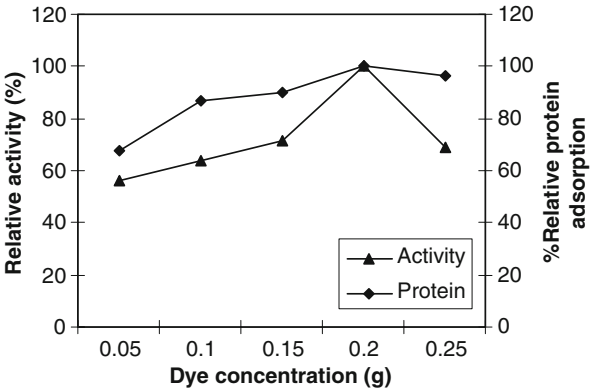

Fig. 3 Effect of brilliant green concentration. Membrane was treated with different concentration of brilliant green and the immobilized enzyme activity was estimated

Optimization of Enzyme Loading

To evaluate the amount of enzyme required to obtain higher activity, different volume of enzyme which corresponds to different amount of protein (2–33 g m^{-2}) was loaded on the membrane coupled with brilliant green. The enzyme activity increased with increase in protein loading from 2 to 22 g m^{-2} and thereafter it decreased (Fig. 4), the higher activity was obtained at 22 g m^{-2}. It appears that the higher loading of protein lead to embedding of active sites of enzyme during the immobilization process which results in the decrease of enzyme activity at higher loading. The further study was carried out using 22 g m^{-2} amount of protein for loading on the brilliant green coupled membrane.

Optimization of an Enzyme Loading Time

The time required for loading of an enzyme was optimized by measuring the amount of protein from the feed at various time intervals (Fig. 5). The 20% adsorption of protein was obtained within 10 min and it reaches to equilibrium in 170 min, which showed 27%

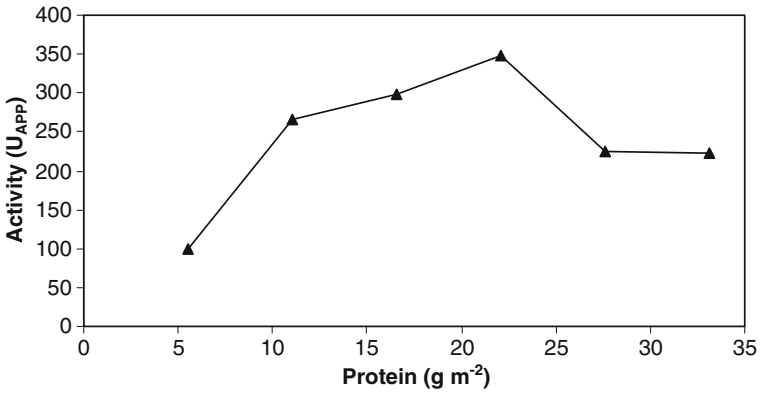

Fig. 4 Optimization of enzyme loading. Different volume of enzyme comprising 7.4 to 44.4 mg protein which corresponds to 5.5 to 33 g m^{-2} was loaded on the membrane coupled with brilliant green (0.2 g) and allowed to react for 1 h under mild stirring prior to the estimation of immobilized enzyme activity

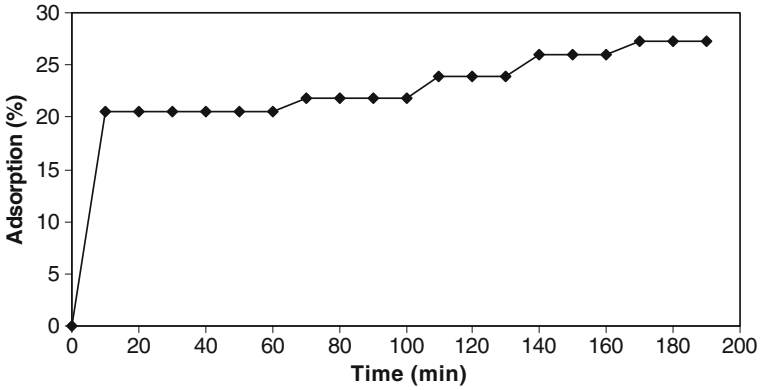

Fig. 5 Optimization of enzyme loading time. The enzyme comprising 29.6 mg protein which corresponds to 22 g m^{-2} was loaded on the membrane coupled with brilliant green (0.2 g) under mild stirring and 0.2 mL sample was removed from the top of the amicon flow cell after every 10 min to estimate the protein. Adsorption (%)=100−(protein concentration in sample×100/protein concentration in feed)

adsorption. The short time (10 min) required for the significant enzyme adsorption (20%) may be due to the presence of ionic interactions between brilliant green and enzyme. This might have results in the enhancement of specific adsorption of the enzyme. As well as in the rejection of nonspecific protein, as the crude enzyme preparation was used for the immobilization. The short time required for adsorption will be more beneficial at industrial applications where increase in operation time results in the increase of product cost. As there was no increase in adsorption up to 60 min, it was decided to carry out further study using 1 h as an enzyme loading time on the membrane.

Effect of pH

Generally, the environmental condition such as pH has significant effect on enzyme activity. In comparison to the free enzyme, the changes in optimum pH and pH activity curve of immobilized enzyme depends on the enzyme and or the support charges. These changes are responsible for the generation of partition effects in the microenvironment of the immobilized enzyme and in the domain of the bulk solution [11]. The activities of free and immobilized enzyme were examined at different pH values and the results are presented in Fig. 6. The optimum pH for free and immobilized occurred at the same pH 7.2. The pH 7.2 was also used for the production and assay of the enzyme. These results suggested that the microenvironments around the catalytic site of the immobilized enzyme and in the bulk remain unaltered. No change in pH pattern of free and immobilized PGA was also reported earlier [30, 31]. The curve pattern of free and immobilized enzyme was almost similar, however, immobilized enzyme curve was slightly broader than the free enzyme. This indicates that the immobilized enzyme is less sensitive to pH than the free enzyme.

Effect of the Temperature

The activities of free and immobilized enzyme were examined at different temperature and the results are presented in Fig. 7. The free and immobilized enzyme shows the same optimal temperature at about 40°C. The same temperature was also used for enzyme assay.

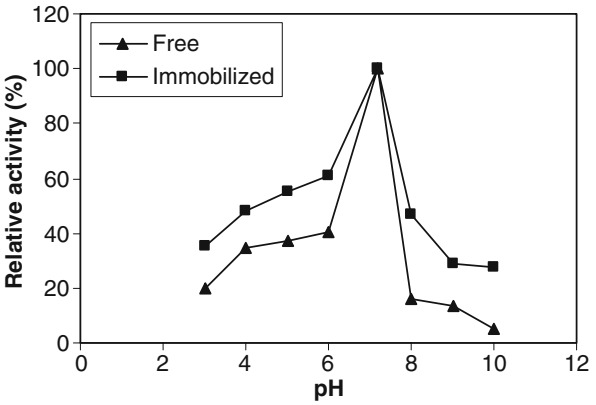

Fig. 6 Effect of pH. The immobilized enzyme activity was estimated using substrate prepared in different pH. The following buffers were used in the indicated pH ranges; at pH 3.0–6.0, 0.1 M citrate; pH 7.2, 0.05 M phosphate; and at pH 8.0–10.0, 0.1 M borate

The similar results were reported earlier for free and immobilized enzyme using different support [32]. The curve pattern of free and immobilized enzyme was almost similar, however, immobilized enzyme curve was slightly broader than the free enzyme curve below the optimum temperature. The immobilized enzyme also showed around 20% activity at 50 °C. This indicates that the immobilized enzyme is less sensitive up to 50 °C than the free enzyme.

Effect on Kinetic Parameters

Generally, the kinetic parameters (K_m and V_{max}) of an enzyme undergo variations after immobilization, which indicates change in affinity for the substrate. These variations may occur due to several factors such as protein conformational changes induced by the attachment to the support, steric hindrances, and diffusional effects. Effect of these factors results in decrease or increase of the value of apparent K_m. The decrease in the K_m indicates

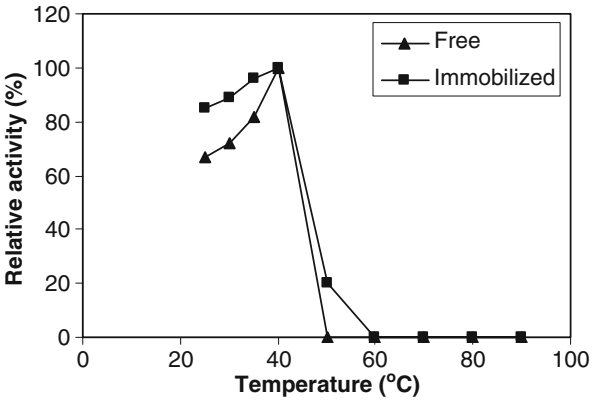

Fig. 7 Effect of the temperature. The immobilized enzyme activity was estimated at different temperature

faster reaction rate, whereas an increase of the K_m suggest the requirement of higher substrate concentration to achieve the same reaction rate observed for the free enzyme [33]. To evaluate the effect of immobilization on the kinetic parameters, the activity of enzyme was studied as a function of substrate concentration and the K_m and V_{max} values were determined from the Lineweaver–Burk plots (Fig. 8). After immobilization, an increase in K_m value of 19.52 mM to 47.4 mM was observed which corresponds to around 2.4-fold higher than the free enzyme. However, the V_{max} value showed the decrease from 1.4 (μmol min^{-1}) to 0.55 (μmol min^{-1}) which corresponds to around 2.5-fold lower than the free enzyme. This indicates that the immobilization results in lowering the affinity for the substrate with respect to free enzyme. Around 2.5-fold increase in K_m value after immobilization was also reported earlier [34].

Effect of Storage on Operational Stability

The stability of an enzyme activity over longer period of time is essential from its practical application point of view. To evaluate this, the enzyme activity was measured in a batch mode of operation and the gap between two batches was minimum 24 h. The effect of storage on operational stability of immobilized enzyme is shown in Fig. 9. Total 50 cycles of operations were carried out over 107 days and 84.05%±11.25% average enzyme activity for first 30 days and 22 cycles was obtained, which corresponds to around 16% loss of enzyme activity. Whereas, 48.88%±1.33% average enzyme activity for next 77 days and 28 cycles was observed, this corresponds to around 50% loss of enzyme activity. This indicates that the immobilized enzyme retained almost 50% activity after 107 days and 50 cycles of operation which appears to be significant. In general, the retention of operational stability varies from carrier to carrier. It was reported that the penicillin G acylase immobilized onto Amberlite XAD-7 showed a gradual activity loss upon repeated reuse and after modification its operational life time extended to >400 h at 37°C. Whereas, penicillin G acylase immobilized on Eupergit C showed operational stability around 60% over >800 cycles [5]. On the other hand, penicillin V acylase immobilized on the Amberlite CG-50 showed 52% loss of activity over 68 cycles [35].

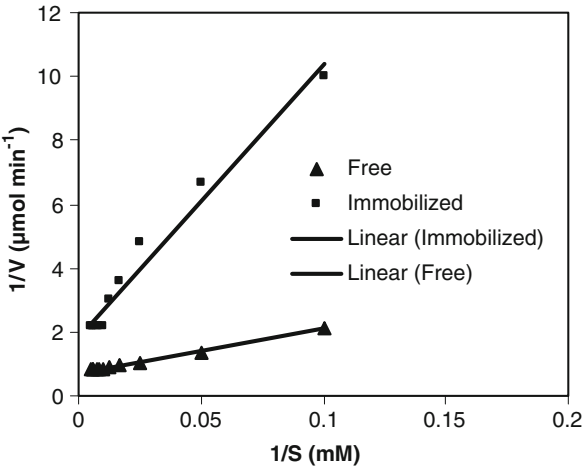

Fig. 8 Lineweaver–Burk plot. Kinetic parameters were determined using different concentration of substrate

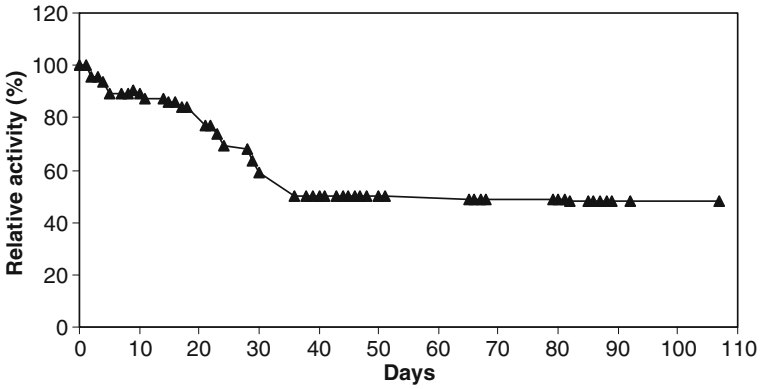

Fig. 9 Effect of storage on operational stability. The immobilized enzyme activity was estimated every day under identical experimental conditions. After completion of the experiment, membrane was washed with buffer (50 mM sodium phosphate buffer pH 7) and stored at 4°C

Effect on Flux Pattern

As the enzyme was immobilized on the membrane therefore, it was essential to understand the flux pattern. This will help in assessing operating life of the membrane. Figure 10 shows the results obtained for 30 cycles of operations. Almost 50% decrease in buffer flux after enzyme immobilization was observed. This indicates that the immobilization procedure has significant effect on the pore size of the membrane. The average buffer flux obtained for the first one to 15 cycles was 655±83 LMH whereas for 16 to 30 cycles it was 545±15 LMH. The average sample flux observed for the first one to 15 cycles was 436±94 LMH whereas for 16 to 30 cycles it was 310±11 LMH. These results suggest that as the number of cycle increases the deviation in flux value decreases and it moves closer towards the steady state. At the end of the 30 cycles, flux pattern shows almost 38% decrease in buffer flux which means around 60% active flux of the membrane remains intact. This suggests the good operating life of the membrane.

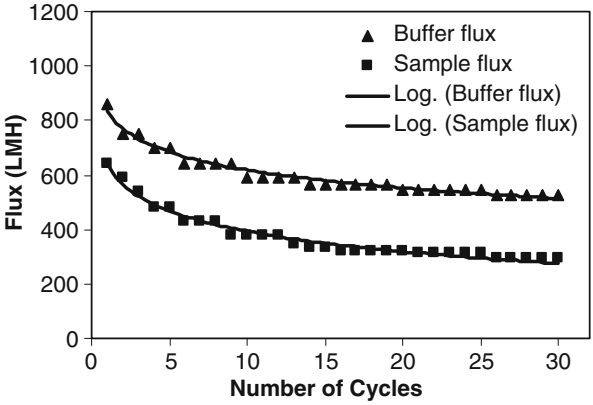

Fig. 10 Effect on flux pattern. *LMH*- L m^{-2} h^{-1}; *Buffer flux* flux measured prior to start of the experiment using buffer (50 mM sodium phosphate buffer pH 7); *Sample flux* flux measured during the removal of reacted substrate

Conclusion

Penicillin G acylase immobilization was studied using highly porous cellulose-based membrane modified with different ionic molecules/compounds. The novelty of the results was the findings of proline and brilliant green for penicillin G acylase immobilization. Proline showed selective adsorption of penicillin G acylase but with less stability, improvement in binding chemistry may enhance the stability. Comparatively, penicillin G acylase immobilized on the brilliant green coupled membrane showed significant activity retention as well as storage and operational stability and flux pattern. The use of an enzyme obtained from the modified microbial stain for immobilization will make this technology suitable for industrial applications.

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