3.1 Significance of polypyrrole as support for enzyme immobilization

The incorporation of enzymes into conducting polymer matrices provides very convenient and stable biocatalyst interfaces that have important practical applications. Among the conducting polymers, polypyrrole has attractive applications as one of fundamental building materials in the design of various analytical tools [1].

Polypyrrole (PPY) is one of the most promising polymers suited for technological and biomedical applications because of its stability under environmental conditions [2], thermal stability [3], bio-compatibility [4,5] bio-degradability [6], and
Chapter 3

resistance to microbial attack [7]. The possibility of PPY synthesis in aqueous media and modification of its surface with various functionalizing agents makes this polymer a promising support for anchoring many enzymes and proteins [8,9].

The oxidation potential of polypyrrole is lower than that of pyrrole monomer; the polymer will be oxidized concurrently with pyrrole monomer during the polymerization reaction. Consequently, polypyrrole, will exist frequently in its oxidation state, carrying charges in the polymer, since a small amount of nitrogen atoms present in polypyrrole are positively charged. Although some of the counter anions present in the polymerization solution are integrated into the emergent polymer during the polymerization reaction to sustain neutrality of charge, yet the presence of the positive charges in the form of nitrogen atoms in the structure of the polypyrrole is beneficial in its applications in adsorption or filtration separation [223].

PPY is found to be promising, especially as an immobilization matrix in the design of various catalytic biosensors based on the catalytic action of enzymes, also, it is often used in the design of immunosensors and DNA sensors. Successful application of conducting polymers modified by enzymes in catalytic biosensors started by entrapment of glucose oxidase within polypyrrole [11], and employed for glucose sensing. S. Cete et al. have successfully immobilized uricase upon polypyrrole - ferrocenium film [12]. Moreover, PPY nanoparticles can be used for biosensor design. Self-encapsulation of redox enzyme- glucose oxidase E.C. 1.1.3.4 from Penicillium vitale (GOX) within conducting polymer polypyrrole was reported by Arunas Ramanavicius [10].

Recently, T. Sandu et al. reported immobilization of polyphenol oxidase on to polypyrrole functionalized with glutaraldehyde. The authors observed that peaks corresponding to C=O bonds seen in IR spectrum were characteristic of carbonyl and carboxyl groups which are formed through oxidative polymerization. These peaks were missing from IR spectrum of enzyme immobilized PPY which they reported as due to consumption during enzyme immobilization by the reaction with COOH or NH₂
groups from the enzyme. As per their conclusion enzyme immobilization could be done directly on pure PPY, without previous functionalization.

In order to perform as bioactive platform for the immobilization of biomolecules, it is necessary to carry out chemical modification of PPY. This can be achieved either by forming copolymers, conductive polymeric composites or blends with commercially available polymers or inorganic materials which offer better mechanical and optical properties, stability and processability [31].

3.2 Significance of Diastase α-amylase as the model enzyme for immobilization.

The Diastase α-amylase (EC 3.2.1.1; 1,4 α-D-glucan glucanohydrolase) enzyme which hydrolyzes starch to malto oligosaccharide is of great importance in present day biotechnology [13]. It was the first type of enzyme discovered in 1833, by Anselme Payen, who found it in malt solution. This enzyme catalyses the hydrolysis of α-1,4-glucosidic linkages in amylose, amylopectin and glycogen in an endofashion. It does not hydrolyse the α-1,6 linkages or any other branch points and so produces maltose and limit dextrins; the precise action pattern depends on the source of the amylase. Amylase is known to attack both insoluble starch and starch granules held in aqueous suspension.

Today diastase means α-, β-, γ-amylase (all of them hydrolases which differ in the way they attack the bonds of the starch molecules) that can break down carbohydrates. This starch degrading enzyme has received a great deal of attention because of its perceived technological significance and economic benefits.

Several methods have been developed for immobilization of α-amylase with each having its own advantages and disadvantages. α-amylase (Diastase) was immobilized on calcium alginate gel beads [14]. V. Singh et al. reported Silver nanoparticle (AgNPs) doped gum acacia-gelatin-silica nanohybrid as effective support for diastase amylase immobilization [15]. α-amylase was also found to be immobilized
onto poly (styrene-2-hydroxyethyl methacrylate) microspheres activated using epichlorohydrin [16].

Saville et al. have reported that amylase uptake on silaceous support was in the range 20-60% [17]. Aksoy et al. reported that α-amylase was covalently bound on poly (methyl methacrylate-acrylic acid) activated microspheres [18]. Kubrak et al. reported the immobilization of α-amylase via entrapment on Ca$^{2+}$-alginate beads [19]. Siso et al. immobilized α-amylase from Aspergillus oryzae onto corn grits and porous silica [20]. α-amylase was immobilized on celite by Ertan et al. via adsorption [21].

Bajpai et al. used semi-interpenetrating polymer network of poly (ethylene glycol), poly (vinyl alcohol) and polyacrylamide as support for immobilization of α-amylase [22]. P.C Ashly et al. immobilized α-amylase on poly (o-toluidine) [23] and polyaniline [24] which were prepared in both acidic and basic forms and functionalized with glutaraldehyde and ascorbic acid. Y. Ohtsuka et al. reported the immobilization of α-amylase on polymeric carriers having different structures and have found out that the amount of immobilized α-amylase mainly depended on the surface area of carriers, while the enzymatic activity depended on the texture of carrier surface [25].

In our study we carried out the immobilization of diastase α-amylase on to polypyrrole prepared using different strategies. The synthesis procedures adopted for the preparation of polymeric supports, biochemical and physico-chemical methods used for characterization of immobilized enzymes, optimization of immobilization parameters, estimation of protein and immobilization efficiency, are discussed in detail in this chapter. Finally the properties of immobilized enzymes are compared with that of free enzyme and kinetic parameters of both are evaluated.

### 3.3 Supports selected for our study

The supports prepared by different methods are designated as follows:

- Polypyrrole prepared by different oxidizing agents FeCl$_3$ and ammonium persulphate will be represented as PF and PA whereas PPY prepared via
interfacial polymerization will be represented as PI. Corresponding immobilized forms are represented as PFE, PAE and PIE.

- Polypyrrole prepared using surfactants SDS, CTAB and TWEEN 80 as the templates will be designated as PS, PC and PT respectively. Immobilized forms will be represented as PSE, PCE and PTE.

- Polypyrrole functionalized using glutaraldehyde as the spacer is designated as PG. Polypyrrole functionalized via coupling agent APTES is named as PN. Immobilized forms as PGE and PNE.

- Polypyrrole composites with silica and their functionalized form with APTES will be designated as PSi and PSiA respectively. The composite prepared by polymerization of pyrrole and 1-(3-aminopropyl pyrrole silica) nanocomposites will be named as PAM. Polypyrrole prepared using colloidal silica sol as templates will be designated as PM. The corresponding immobilized forms will be designated as PSiE, PSiAE, PAME and PME.

- Copolymers of polypyrrole and polyaniline will be named as PYPH. Copolymer of pyrrole and 1-(2-aminophenyl pyrrole) will be represented as PYPAE and PYPH.

### 3.4 Materials Used

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diastase α-amylase</td>
<td>Hi Media Laboratories Pvt. Ltd. Mumbai</td>
</tr>
<tr>
<td>Starch soluble (potato)</td>
<td>s.d fiNE chem. Ltd. Mumbai</td>
</tr>
<tr>
<td>Albumin Bovine</td>
<td>Sisco Research Laboratories Pvt. Ltd. Mumbai</td>
</tr>
<tr>
<td>Folin &amp;Ciocalteu’s Phenol</td>
<td>Sisco Research Laboratories Pvt. Ltd.</td>
</tr>
<tr>
<td>Reagent</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Chlorodimethyl vinyl silane</td>
<td>25ml (97%)</td>
</tr>
</tbody>
</table>
3.5. Immobilization of α-amylase on polypyrrole prepared in presence of different oxidizing agents and different methods

In this section we have discussed the preparation of polypyrroles and investigated the effect of different oxidizing agents and different methods on the morphology of polypyrroles and compared their efficiency as support for enzyme immobilization.
3.5.1 Preparation of Polypyrrole using APS as oxidizing agent

Preparation of polypyrrole has been carried out based on the IUPAC technical report prepared for publication by J. Stejskal in collaboration with R.G. Gilbert [26]. Pyrrole (extra pure) was first distilled under reduced pressure prior to the reaction. All other reagents and solvents were of reagent grade and were used as received.

The Polypyrrole was prepared using chemical polymerization method. An acidic environment is paramount to successful polymerization because it considerably inhibits the formation of simple dimmers and other oligomers which would otherwise occur [27]. Peroxydisulphate is the most commonly used oxidant, and its ammonium salt is preferred to potassium counterpart because of its better solubility in water. The mole ratio of monomer to oxidant affects the quality of the polymer formed. Hence the recommended stoichiometric ratio of peroxodisulphate to pyrrole, 1.25:1 was used for the polymerization reaction. The polymerization was completed within 1 hour at 0-2°C. Since the reaction was exothermic, low temperature was preferred.

The main disadvantage of using APS as the oxidant is that it is stoichiometrically consumed in the reaction, which means that the reaction requires a large amount of the chemical for mass production, resulting in a troublesome treatment of the acidic by-products of the oxidant [30].

Distilled pyrrole (40 mmol, 2.8 ml) was dissolved in 100 ml 1M HCl taken in a volumetric flask. Ammonium persulphate (50 mmol, 11.4095g) was also dissolved in 100 ml 1M HCl taken in another volumetric flask. Both solutions were kept for 1 hour in an ice bath, mixed in a beaker, stirred and was kept un-agitated for 24 hours at room temperature. The next day precipitated polypyrrole was filtered out under vacuum and washed with distilled water several times to remove any impurities present. The precipitate was then washed with three 100 ml portions of 0.2 M HCl to remove the residual monomer, oxidant and its decomposition
products. PPY thus formed was uniformly protonated with chloride counter ions. In addition to this sulphate or hydrogensulphate anions from the decomposition of peroxydisulphate also participated as counter anions.

The precipitate was finally washed with acetone in order to remove low molecular weight organic intermediates and oligomers. This prevented aggregation of PPY during drying and finely powdered samples were obtained, which was dried at room temperature for 2 days and further in an oven at 60°C for 3 hours. The equation is shown below [28]:

\[
\begin{align*}
\text{n} \begin{array}{c}
\text{H} \\
\text{N}
\end{array} + A^- & \rightarrow \text{n} \begin{array}{c}
\text{H} \\
\text{N}
\end{array} A^- + 2nH^+ + (2n+1) e^- \\
\end{align*}
\]

**Scheme: 3.1** Preparation of polypyrrole in the presence of APS

### 3.5.2 Preparation of polypyrrole using FeCl₃ as oxidizing agent

Synthesis of polypyrrole nanoparticles was achieved using micro emulsion polymerization system by oxidation of pyrrole monomer with iron (III) chloride hexahydrate. As the oxidant was added, the colour of the solution changed to deep greenish black which was an indication of oxidation of conducting polypyrrole. The reaction product polypyrrole was obtained in the form of a black powder. The black colouration is characteristic of polypyrrole and is due to the extended system of conjugated double bonds along the polymer backbone.

In most of the published papers on the chemical polymerization of pyrrole, usually, FeCl₃ was used as the oxidant because a much higher conversion was obtained than with APS. The oxygen may enter the PPY structure during the polymerization process itself as a consequence of the water presence in polymerization solution, as well as by reaction of the prepared polymer with atmospheric oxygen. These facts can explain the higher yield of polymerization reaction using FeCl₃ as oxidant. The reaction is represented as shown below [29].
Scheme 3.2: Preparation of polypyrrole in presence of FeCl₃

The optimum FeCl₃ / pyrrole molar ratio is 2:3 and it can vary in the range 0.5 to 4 [31] and we have chosen a mole fraction of 2 for our studies. Polypyrrole (PPY) was chemically synthesized in distilled water (100 ml) by mixing pyrrole (Py) 0.18 M (1.21 ml) with an oxidizing solution of FeCl₃ (0.36 M). The synthesis was allowed to proceed at 5-7°C. The pyrrole solution was kept in the ice bath before adding FeCl₃ (5.8 g). Since this is an exothermic reaction, the addition was done slowly and at low temperature. The synthesis was performed without agitation and kept for 24 hours. The precipitate was collected by filtration, rinsed first with distilled water, then with acetone and dried at 60°C in an oven [32].

3.5.3 Preparation of polypyrrole by interfacial polymerization.

Polypyrrole were synthesized based on already reported interfacial oxidative polymerization of pyrrole monomer. Pure pyrrole was distilled just prior to the reaction. Pyrrole (12.8 mmol, 0.9 ml) was dissolved in 40 ml of pure chloroform (spectroscopic grade). An oxidizing solution, comprising of 3.2 mmol (0.73 g) of ammonium persulphate dissolved in 40 ml of 2 M HCl was gently added to the monomer solution obtained above. The mixture was left undisturbed for 24 hours and a thin layer of the polymer was obtained at the interface. Sample was isolated by centrifuging the aqueous layer, washed with de-ionized water and was dried under vacuum [33].
3.5.4 Physico-chemical characterization

3.5.4.1 FT-IR Spectrum of Polypyrrole prepared by different oxidizing agents and different methods.

Polypyrrole consists of five-membered rings linked together to form a conjugated chain. FT-IR spectra of the PPY samples synthesized at different polymerization conditions indicate the typical characteristics of PPY which were consistent with literatures.

The FT-IR spectra are of good quality and the infrared bands are well defined. The FT-IR spectrum of PPY samples were plotted with the percentage transmittance as a function of wave number (cm\(^{-1}\)). The main absorption peaks of all samples were in the same region but with variations in their relative intensities depending on the method of preparation. The unique characteristics include nitrogen-hydrogen bonds which can participate in hydrogen bonding, and both C-N and C-H ring stretching.

![FT-IR Spectrum of polypyrrole prepared in presence of different oxidizing agents and their immobilized forms](image)

**Figure: 3.1** FT-IR Spectrum of polypyrrole prepared in presence of different oxidizing agents and their immobilized forms

The oxidized PPY are characterized by a very large absorption band located in the spectral domain between 4000 and 2500 cm\(^{-1}\), which is characteristic of the
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OH groups belonging to residual water molecules trapped in the polymer matrix [34]. The spectrum of the polypyrrole powder showed that the N-H stretching band of pyrrole ring also appeared at 3433 cm\(^{-1}\). The small peaks at 2925 and 2851 cm\(^{-1}\), are due to aromatic five-membered ring C-H ring stretching.

**Table 3.1** Assignment of main peaks in the spectra of PPY prepared in presence of different oxidizing agents (w-weak, vw- very weak, s-strong)

<table>
<thead>
<tr>
<th>Peak assignments (cm(^{-1}))</th>
<th>PA</th>
<th>PAE</th>
<th>PF</th>
<th>PFE</th>
<th>PI</th>
<th>PIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H stretch</td>
<td>3433</td>
<td>3428</td>
<td>3428</td>
<td>3424</td>
<td>3435</td>
<td>3446</td>
</tr>
<tr>
<td>C-H stretch</td>
<td>2929, 2926, 2856</td>
<td>2926, 2856</td>
<td>2926, 2852</td>
<td>2924, 2856</td>
<td>2926</td>
<td>2926, 2856</td>
</tr>
<tr>
<td>C=O</td>
<td>1710, 1745</td>
<td>1702, 1753</td>
<td>1708</td>
<td>1705, 1748</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C=N inter ring bending</td>
<td>1630</td>
<td>1656, 1596</td>
<td>1644</td>
<td>1640</td>
<td>1656, 1644</td>
<td></td>
</tr>
<tr>
<td>C=C stretch, C-H ring inplane bending</td>
<td>1549</td>
<td>1542</td>
<td>1544</td>
<td>1542</td>
<td>1547</td>
<td>1542</td>
</tr>
<tr>
<td>C-N stretch</td>
<td>1116</td>
<td>1110</td>
<td>1109</td>
<td>1108</td>
<td>1112m</td>
<td>1110</td>
</tr>
<tr>
<td>N-H ring out of plane bending (vs)</td>
<td>1045</td>
<td>1040</td>
<td>1044</td>
<td>1035</td>
<td>1027m</td>
<td>1009</td>
</tr>
<tr>
<td>Ring deformation</td>
<td>947</td>
<td>948</td>
<td>963</td>
<td>944</td>
<td>947</td>
<td>939</td>
</tr>
<tr>
<td>C=C-N in plane deformation</td>
<td>856</td>
<td>859</td>
<td>856</td>
<td>862</td>
<td>856</td>
<td>852</td>
</tr>
</tbody>
</table>

The intensity of the sharp bands located at 1710 cm\(^{-1}\) depends on the oxidation degree of the polymer. Indeed, this band is weak in the reduced form and is intensified in the oxidized form [34]. According to Lei and Martin polypyrrole is actually a copolymer of pyrrole and hydroxypyrrole [35].

The hydroxyl bands in the IR spectrum had been obscured by a broad absorption from 1600 cm\(^{-1}\) upwards due to the conductivity of polypyrrole. Reduction of the polymer had failed to completely eliminate this broad absorption because of partial oxidation by traces of oxygen. On the basis of these data, the band can be attributed to carbonyl groups fixed in the position of some pyrrole
rings which is the consequence of the nucleophilic attack of water molecules on pyrrole during preparation [36]. The hydroxyl groups introduced in the ring by this attack would finally produce carbonyl groups by keto-enol tautomerism [37].

The literature lists, a band at 1458 cm\(^{-1}\), due to the conjugated C-N bond stretching vibration in the ring. The bands located around 900 cm\(^{-1}\), 1200 cm\(^{-1}\) and 1550 cm\(^{-1}\) are characteristic of the oxidized PPY that have been associated by Zerbi with the effective conjugation coordination and show sensitivity to the oxidation level and to the conjugation length of the chain [38]. The band located at 1550 cm\(^{-1}\) in the spectrum is attributed to C=C / C-C stretching vibrations of the PPY chain.

The very weak peaks around 1384 cm\(^{-1}\) appear in most PPY spectra. These peaks are assigned to C=O bond from carboxyl groups which are formed through oxidative polymerization. These peaks are missing from PPY-E spectrum because they are consumed during enzyme immobilization, by reaction with COOH or NH\(_2\) groups from the enzyme. Similar results were also reported by T. Sandu et al. [39].

The peaks at 1344 cm\(^{-1}\) and 1315 cm\(^{-1}\) arise as a result of C-C in ring stretching. The peak near 1300 cm\(^{-1}\) is due to C-N stretching of the polymer and peak at 1380 cm\(^{-1}\) is because of C-N\(^{\text{+}}\) stretching and C-C vibration [40].

The band at 1296 cm\(^{-1}\) corresponds to C-H deformation. The IR spectra for pyrrole in water display intense narrow bands of plane vibrations of deformation at 1019 cm\(^{-1}\), 1045 cm\(^{-1}\), 1075 cm\(^{-1}\) respectively. The C=C stretching of aromatic compounds generally occur in the range of 1000-1100 cm\(^{-1}\).

The band observed near 950 cm\(^{-1}\) is due to the C-H out of plane bending. The peak at 750-780 cm\(^{-1}\) inferred the presence of polypyrrole indicating C-H, -N-H ring out of plane bending and peak near 800 cm\(^{-1}\) is due to the C-N stretch [28].

The spectra of all of the samples displayed the characteristic peaks of polypyrrole with noticeable differences due to shifts to either higher or lower frequencies, depending on the experimental conditions [41]. These shifts in the
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frequencies could be interpreted in terms of the nature of the dopant and its influence on the ring vibrations of polypyrrole [38].

Various IR bands and their assignments are given in table 3.1. The IR studies reflect difference in molecular orientation and crystallinity of each PPY sample. Similar spectral differences have been attributed to the changes in conformation, molecular ordering, and packing of the polymers as in previously reported IR studies [42].

When α-amylase was immobilized on to these samples characteristic additional peaks corresponding to enzyme structure was observed around 1650 cm\(^{-1}\). This can be ascribed to the amide I band that represents the stretching vibrations of C=O bonds in the backbone of the protein [43].

FT-IR spectra shows bands centered at 1648 and 1544 cm\(^{-1}\) that can be attributed to amide I and amide II, respectively. The amide II band arises from the combination of C-N stretching and N-H bending vibrations of the protein backbone [44], which confirms the incorporation of enzyme into the polymers [45].

The spectra also indicate an amide group at 3424 cm\(^{-1}\) (N-H stretching) for PFE, 3428 cm\(^{-1}\) for PAE and 3446 cm\(^{-1}\) for PIE. The bands at 1596 cm\(^{-1}\) (N-H bending), and 1656 cm\(^{-1}\) corresponds to C=O stretching. α-amylase contains a major percentage of aspartic and glutamic acids along with other amino acids [46]. The spectrum clearly indicates the presence of carboxylate ion groups at 1646 cm\(^{-1}\) a broad strong band [asymmetrical (C-O)\(_2\) stretch] and 1398 cm\(^{-1}\) [symmetrical (C-O)\(_2\) stretch], respectively. In addition to these bands, the other observed peaks are the asymmetrical (-NH\(_3^+\)) N-H band at 1317 cm\(^{-1}\), and the symmetrical (-NH\(_3^+\)) N-H band at 1510 cm\(^{-1}\) [22].
A band at 2932 cm$^{-1}$ in all immobilized samples is due to CH$_2$ groups of $\alpha$-amylase [47]. Almost all bands get broadened after immobilization of $\alpha$-amylase. Thus the peaks observed in the spectrum match well with the ones available in the literature confirming the formation of polypyrrole and its immobilized forms.

3.5.4.2 Thermogram of $\alpha$-amylase

The TG and DTG curves for free amylase were characterized by several weight loss peaks. The first weight loss in TG was at temperature range from 90°C to 140°C, which might be due to dehydration of the interstitial water that gets adsorbed in the free $\alpha$-amylase sample. From 200 to 360°C, continuous weight loss was observed indicating a complete decomposition of the organic structure of the enzyme as it could not withstand such elevated temperatures. The DTG curve shows two major peaks at 140 and 235°C and three minor peaks at 200, 292 and 362°C. The peak at 140°C corresponds to loss of water molecules that are trapped in the enzyme molecule. The other peaks might be the result of gradual decomposition of enzyme’s organic structure.

![Thermogram of $\alpha$-amylase](image_url)

**Figure: 3.2** Thermogram of $\alpha$-amylase
3.5.4.3 Thermogram of polypyrrole prepared in presence of different oxidizing agents and via different methods

The TG curves of polypyrrole prepared in the presence of different oxidizing agents and different methods are shown in the figure: 3.3. From the figure it is clear that the thermal stability of polypyrroles differ significantly as the oxidants and methods was varied. In all cases the initial weight loss was attributed to loss of water, the second weight loss was assigned to loss of any dopants and solvent molecules that get adsorbed on the surface of polymer backbone. Finally the third weight loss will be the decomposition of polymer structure.

The first significant weight loss occurs already at temperature between 30 and 100°C. It is known, that PPY is hygroscopic and during the heating to 100°C the residual water evaporates. For PA the main mass loss, which corresponds to polymer degradation, starts at about 187°C. The second minor weight losses corresponding to polymer decomposition starts at 336°C which continues up to 800°C.

But after immobilization PAE showed major weight loss at 157°C due to protein degradation after which weight loss occurred at 309°C, and is continuous till 800°C which corresponds to polymer decomposition.

For PF the main weight loss starts with a broad peak at 234°C and minor weight loss at 577°C. After immobilization of α-amylase the weight loss starts at 226°C which might be the result of protein degradation of α-amylase. The major weight loss at 277°C & 302°C corresponds to polypyrrole decomposition which is continuous till 800°C.

In the case of PI the major decomposition due to polypyrrole degradation starts at 255°C and minor weight loss starts at 415°C which continues upto 800°C. When α-amylase was immobilized the major decomposition peak starts at 194°C which is attributed to degradation of organic structure of enzyme. The weight loss corresponding to that of polymer backbone was observed at 296°C which continues up to 800°C.
Figure: 3.3: Thermograms of (i) PA, PF and PI (ii) PA and PAE (iii) PF and PFE (iv) PI and PIE

3.5.4.4 Surface Area analysis

BET surface area of polymers before and after immobilization of \(\alpha\)-amylase is given in the table 3.2. For all polymers prepared by conventional chemical polymerization method in the presence of oxidizing agents APS and FeCl\(_3\), the surface areas obtained were too low. The polymer prepared using APS as oxidizing agent was found to have higher surface area than that prepared with FeCl\(_3\) as the oxidant. For interfacial polymerization method the surface area was slightly more than that produced using FeCl\(_3\) via bulk polymerization method.
Table: 3.2 Surface areas of PPY polymers

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Surface area (m²/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>15.6</td>
</tr>
<tr>
<td>PF</td>
<td>4.6</td>
</tr>
<tr>
<td>PI</td>
<td>8.7</td>
</tr>
<tr>
<td>PAE</td>
<td>4.8</td>
</tr>
<tr>
<td>PFE</td>
<td>1.39</td>
</tr>
<tr>
<td>PIE</td>
<td>3.39</td>
</tr>
</tbody>
</table>

3.5.4.5 Scanning electron microscopy

The scanning electron microscopy was performed in order to investigate the dimensions and the surface morphology of polypyrrole samples.

Polypyrrole prepared in presence of ammonium persulphate as the oxidizing agent (PA) showed globular structure with particle size ranging from 116 nm-228 nm. Literature studies revealed that dopant anion has a profound influence on the morphology of PPY formed [48]. In the case of persulphate as the oxidant sulphate anions played the role of dopant anion in the synthesis medium. As persulphates are relatively strong oxidants (E₀=2.1V) it has much faster polymerization rate. The higher nucleation speed in the system increases the aggregation of nuclei and the stabilization is less effective which results to larger particles in the final dispersion.

The SEM micrographs of polypyrrole prepared in presence of FeCl₃ as oxidizing agent (PF) exhibited globular sized particles which have a distribution of dimensions between 120-440nm.

The SEM images of polypyrrole prepared by interfacial polymerization (PI) also showed globular structures with particle size ranging between 200-440 nm. It is because the reaction time and method of polymerization has significant impact on the resulting polymer morphology.
Figure 3.4: SEM images of (i) PA (ii) PF (iii) PI
3.5.5 Biochemical characterization

3.5.5.1 Coupling of α-amylase on polymer supports and their immobilization efficiency

The pH of the medium has a similar effect on both free and immobilized enzymes, but the pH stability relationship is of even greater concern with immobilized enzymes which have to undergo adverse conditions during coupling. Thus pH at which enzymes bind to a support is a critical parameter to be determined for any method of immobilization. It is enzyme activity which is of great concern and this is the product of total bound protein, which generally increases at a particular range of pH depending on the support and enzyme surface charges, and retains activity, which falls as the pH of coupling solution becomes more extreme.

Thus the optimum pH for binding may be a complex function and usually has to be determined empirically, taking into account of the enzyme’s stability and the known protein binding character of the support [49]. Amino, thiol, carboxyl, phenolic, guanidine, imidazole, disulphide, indole, thioether and hydroxyl groups of the aminoacid chain are responsible for linking to a support matrix [50]. The amount
of enzyme adsorbed depends on nature of support, type of enzyme, concentration of enzyme, medium used for immobilization, contact time, temperature, pH etc.

So the inevitable part of every immobilization study is the investigation of each of these parameters. The effect of pH of medium in which the enzyme is dissolved during immobilization on to polymeric supports is shown in the figure: 3.6.

\[\text{Figure: 3.6: Effect of pH of immobilization medium on the relative activity of immobilized } \alpha\text{-amylase}\]

Amylase adsorption was investigated in different pH’s of buffer solutions that are in the range 4-8, because amylase becomes unstable above pH 9.0 as its isoelectric point is around 4.6. The pH range for activity of diastase \(\alpha\)-amylase is in the range 5-8 with optimum pH between 5 and 5.5. PPY has an isoelectric point around 7 [51]. Both polypyrrole and amylase does not have a strong charge at pH close to its isoelectric point. Above isoelectric point both are positively charged and below isoelectric point both are negatively charged.

The maximum activity of the immobilized enzyme was observed in the range 5-6. The optimum pH is that which favours the interaction between the enzyme and support in such a way that maximum activity of biocatalyst can be retained with better immobilization efficiency. It was found that pH 5 was best for immobilization of \(\alpha\)-amylase on PA and PI and pH 6 for PF.
At pH 4 PPY have overall net positive charge and lysine and arginine amino acid residues on the protein surface of amylase have slight positive charge as it is close to its isoelectric point. Hence PPY should repel amylase. However, the results were different from what was expected. Hydrophobic interactions appear to dominate amylase adsorption at pH 4 for the polypyrrole adsorbent. This is because PPY is hydrophobic as it has large aromatic rings in the polymer backbone. Therefore, for these adsorbents, hydrophobic interactions dominate and the effect of pH is minimized. The small adsorption difference due to pH might originate from ionic effects, as a result of secondary amino groups of the pyrrole rings.

At pH 5, the secondary amino group possesses positive charge and it attracts α-amylase, which has a net negative charge. At pH 6 also the secondary amino group of PPY has net positive charge and these amino groups in polypyrrole are accessible to face to protein molecules without steric hindrance. Hence it attracts net negatively charged amylase molecules. At pH 7 PPY has no charge and hence hydrophobic interaction dominates over electrostatic attraction.

When the immobilization is carried out at higher pH, the same amount of enzyme is immobilized, however the activity of the immobilized enzyme is lower than when the immobilization process is carried out at pH 5 and 6. This is because at higher pH PPY adsorbents have overall net negative charge and amylase is also having net negative charge which results in electrostatic repulsion.

Thus the strength of the electrostatic interaction between the enzyme and the adsorbent is very important in maintaining the overall activity of the enzyme. The variation of activity with pH, within a range of 2-3 units each side of the pI, is normally a reversible process [52]. The curve profile became much broader between pH 5 and 7 although optimum pH of free enzyme was at pH 5.

The native enzyme could not survive with increase in pH towards alkalinity. This shows that the stability of the enzyme against pH was significantly improved upon immobilization. This is possible due to the stabilization of enzyme
molecules resulting from multipoint attachment on the surface of support and due to the charge effects of the support.

The difference in adsorption encountered among different adsorbents on immobilization pH can be explained based on their morphological difference as a result of their difference in preparation conditions. This was also evident from their characterization studies conducted via SEM and BET analysis. PA has smaller particle size compared to other two which correspondingly resulted in increase in surface area and hence increase in adsorbed amount of enzyme on it than the other two.

3.5.5.1.1 Effect of contact time on the activity of α-amylase

The contact time needed for enzyme to get adsorbed on PPY adsorbents is shown in the figure: 3.7.

![Figure: 3.7: Optimization of immobilization time needed for effective coupling of α-amylase.](image)

Adsorption occurs at the moment of contact when enzyme solution in a particular pH is added to PPY. Enzyme activity and adsorption first increases with increase in contact time and after that even if adsorption of enzyme increases, activity is found to decrease which might be due to lower accessibility of substrate as a result of multilayer adsorption of enzyme [53]. For all adsorbents optimum activity was obtained at 120 minutes of contact time.
Ball and Ramsden also mentioned that the amount of enzyme adsorbed increases with increase in enzyme concentration indicating that the electrostatic interactions between the molecules promote further adsorption [54]. This means that when the enzyme concentration is high, protein aggregation increases the surface adsorption capacity. But the active sites that are exposed to substrate get decreased as several of these sites get blocked due to overlaid protein layer one above other.

3.5.5.1.2 Effect of initial protein concentration on protein loading on to supports

The amount of protein bound to PPY adsorbents was analyzed based on the optimized conditions obtained.

![Figure 3.8: Change in protein loading with respect to initial amount of protein added to support](image)

The effect of initial protein concentration on protein loading is depicted in the figure: 3.8. Apparently, adsorbed amount of enzyme increases with increase in enzyme concentration. But after a particular concentration no further increase in adsorption occurs, instead a saturation point is reached and enzyme starts desorbed from the surface if loaded heavily. The amount of adsorbed protein depends on the strength of interaction between enzyme and the support and method of immobilization.

Even if maximum amount of enzyme get adsorbed at higher concentrations, optimum concentration was selected based on the optimum activity shown by the
immobilized enzyme at a particular concentration. As a result of this baseline kept throughout the study, many of the results were contradictory than expected. Usually we expect an increase in activity with increase in enzyme loading. Shift from this usual trend occurred as a result of the fact that during the immobilization process, the multipoint attachment to support unavoidably hampers the free conformation of enzymes and sometimes non-biospecific interactions of enzyme with the support results in the denaturation of enzyme protein, and thus resulting in its activity and stability losses [55]. It is thus important that the properties of support and immobilization processes should be well understood in order to improve activity retention of the immobilized enzyme [56].

3.5.5.1.3 Effect of initial protein concentration on the immobilization yield and activity of loaded enzyme.

Immobilization yield obtained for all adsorbents at various concentration taken are shown in the figure: 3.9.

![Figure 3.9: Effect of initial protein concentration on immobilization yield of enzyme](image)

From the figure: 3.9 it is clear that on addition of protein to the support, protein loading increases which then reaches a saturation point and then starts decreasing, which might be due to desorption at high loading due to weak interaction between enzyme and the support. Thus the optimum intake of protein
with maximum retention of its activity on PA, PF, and PI are 7.5 mg/g, 4.4 mg/g and 4.7 mg/g respectively. The corresponding immobilization yield was 71%, 44%, and 43% respectively.

Immobilization yield was not the prime parameter in optimizing protein loading in all cases; instead it is the activity of immobilized enzyme that played the key role in deciding the effectiveness of adsorption at a particular concentration. Because in the case of PA upon the addition of 7.5 mg of initial protein, optimum immobilization yield of about 71.4% was obtained. But maximum protein loading obtained at initial protein amount 10.5 mg with corresponding optimum activity 6.4 EU, and immobilization yield of only 71%.

But in the case of PF optimum activity and optimum immobilization yield (IY) was obtained at the same concentration. At initial protein amount of 10.1mg, IY of about 44% was obtained with maximum immobilized enzyme activity 7.7 EU.

For PI the optimum activity of 7.6 EU was observed when the protein load was 4.7 mg with initial protein amount of 15.6 mg.

![Figure: 3.10](image)

**Figure: 3.10** The trend of immobilized enzyme activity when initial protein amount was varied.

The trend of immobilized enzyme activity when initial protein amount was varied for all adsorbents is shown in the figure: 3.10. The increase in immobilization yield of PA compared to other two is supported by the increase in surface area obtained.
for it via BET analysis, than the other adsorbents. The SEM images also underline this fact as it is evident from the decrease in particle size of PA which results in increase in surface area.

The activity yield and immobilization efficiency are also evaluated. The results are tabulated in the table 3.3.

**Table: 3.3:** Immobilization efficiency of α-amylase on PPY prepared in presence of different oxidizing agents and different methods

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Initial protein (mg)</th>
<th>Immobilized protein mg/g support</th>
<th>Immobilization yield (%) IY</th>
<th>Initial activity (EU)</th>
<th>Immobilized enzyme activity (EU)</th>
<th>Activity yield (%) AY</th>
<th>Immobilization efficiency (%) IE=AY/IY</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>10.5</td>
<td>7.5</td>
<td>71</td>
<td>34.5</td>
<td>6.4</td>
<td>19</td>
<td>26</td>
</tr>
<tr>
<td>PF</td>
<td>10.1</td>
<td>4.4</td>
<td>44</td>
<td>22.9</td>
<td>7.7</td>
<td>34</td>
<td>77</td>
</tr>
<tr>
<td>PI</td>
<td>15.6</td>
<td>4.7</td>
<td>43</td>
<td>21.3</td>
<td>7.6</td>
<td>36</td>
<td>82</td>
</tr>
</tbody>
</table>

Various binding capacities and preserved activities are given in the literature for α-amylase immobilized systems. When adsorption was achieved chemically onto polystyrene and polyaniline supports, coupling capacities and preserved activities are reported as 3 and 2.2 mg/g support and 7 and 18%, respectively [75,24].

### 3.5.5.2 Effect of pH on enzyme activity

The procedure of immobilization usually has a variety of effects on the conformation as well as on the state of ionization and dissociation of an enzyme and its environment; it results in changes in the relationship between pH, stability and activity of immobilized enzyme. These changes in pH would alter or totally inhibit the enzyme from catalyzing a reaction.
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The pH effect on the activity of the free and immobilized forms of $\alpha$-amylase has been studied in buffer solution at different pH in the range 4-8 and the results are presented in the figure: 3.11 and table: 3.4.

**Table 3.4:** Optimum pH found out for PAE, PFE and PIE

<table>
<thead>
<tr>
<th>pH</th>
<th>Free enzyme</th>
<th>PAE</th>
<th>PFE</th>
<th>PIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 &amp; 5.5</td>
<td>5.5</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

**Figure: 3.11:** Effect of pH on percentage relative activity of free and immobilized $\alpha$-amylase

The optimum activity is represented as 100% and other activities are expressed relative to this optimum activity. Free enzyme exhibits maximum activity in the pH range (5-6) with optimum activity at 5 and 5.5. At pH 4 and 8 a decrease of the enzymatic activity is observed for both the immobilized and the free enzyme; however, at pH 8 the residual activity of the immobilized enzyme in most samples is significantly higher than that of free enzyme.

The native enzyme could not survive with increase in pH towards alkalinity. The immobilization process provides structural stability, preventing an irreversible unfolding of the enzymatic protein.

The immobilized enzyme has the same optimum pH in the range 5-6 but with a much broader profile, which was also beneficial for their applications. The inhibition of activity in the lower pH ranges may be due to two reasons: a lower
loading and a possible change of the enzyme conformation due to an unfavourable charge distribution on the amino acid residues [57]. A change in pH will affect the intra-molecular hydrogen bonding thus leading to a distorted conformation that will reduce the activity of the enzyme.

For amylase immobilization, shifts in the optimum pH towards both the acidic and alkaline directions have been observed [16, 58]. For PAE the optimum pH was at 5.5, for PFE it was at 5 and for PIE it was at 6. The slight differences in pH might be due to morphological differences in the PPY adsorbents that occurred as a result of difference in synthesis procedures adopted. Thus the charge on the carrier surface also changes the microenvironment of the enzyme bound to it thereby resulting in shift of the pH of the reaction medium after immobilization [224,225].

At very high pH, enzyme inactivation occurs via oxidation of sulphur containing aminoacids and/or deamidation of glutamine residues [59].

3.5.5.3 Effect of temperature on the activity

The effect of temperature on the activity of free and immobilized α-amylase was studied by carrying out the reaction in such a way that the incubation temperature during the reaction was varied in the range 30-60°C. The results are shown in the figure: 3.12.

![Figure: 3.12: Effect of temperature on the activity of free and immobilized α-amylase](image_url)
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The optimum temperature of native enzyme was 50°C which, as a result of immobilization, get shifted to 40°C for PAE and PFE but to 50°C for PIE. This change in temperature might arise from alterations that arise in the conformation of enzyme structure by immobilization which thus favoured amylase activity in a broad range.

The changes in optimum temperature among adsorbents occurred as a result of morphological differences among them which might affect the mode of adsorption that could cause significant alterations in the structural conformation of enzyme at the active site. Handa et al. have reported a 5°C decrease in optimum temperature when α-amylase was immobilized on to polyacrylonitrile [65]. Ashly et al. also reported a similar decrease in optimum temperature when α-amylase was immobilized on polyaniline and poly (o-toluidine) [24,23].

The decrease in the optimum temperature might be due to change in conformational integrity of the enzyme structure by immobilization which favoured amylase activity below 50°C [16, 66, 67].

On elevated temperature the denaturation of an enzyme corresponds to the unfolding of the enzyme by disruption of non-covalent intra-molecular interactions which affected the conformation of protein and resulted in an alteration of enzyme-substrate affinity [68].

3.5.5.4 Thermal stability of the free and immobilized enzymes

Besides affecting the biocatalyst, temperature, like pH, has an impact on substrate stability. Like all other proteins, enzymes are susceptible to thermal denaturation, whether they are immobilized or in the free state. In many cases, however, the rate of inactivation and denaturation of an immobilized enzyme is less than that of the free enzyme.

Since the mobility of enzyme is restricted upon immobilization, it retards the unfolding of protein thereby maintaining activity. But, as the time passes this reduction in mobility is eliminated due to weak nature of enzyme-support
interactions like electrostatic, Van der Waals, ionic and hydrophobic interactions and therefore results in protein unfolding and corresponding reduction in activity.

Thermal stability of both free and immobilized enzymes was investigated by incubating them in buffer solution of optimum reaction pH obtained, for 1 hour at various temperatures in the range 30-60°C in a water bath. The results are shown in the figure: 3.13.

**Figure: 3.13:** Effect of temperature on the stability of both free and immobilized α-amylase

Both free and immobilized enzyme showed maximum activity when incubated at 30°C for 1 hour. As the temperature increased, the stability dropped significantly for both free and immobilized amylase. At 40°C, both free and immobilized enzyme retained up to 80-90% of their activity. At 50°C for 1 hour the immobilized enzyme was inactivated at a much lower rate than the free enzyme. Similar increase in thermal stability was observed when Pessela et al. performed immobilization of β-galactosidase from *Thermus sp. T2* via ionic adsorption onto two different supports: a new anionic exchanger resin, based on the coating of Sepabeads internal surfaces with polyethylenimine (PEI) polymers (Mw-25,000), and traditional DEAE-agarose [69]. The free enzyme lost almost 90% of its activity at 60°C after 1 hour treatment whereas, immobilized amylase lost 80% of its activity. Figure: 3.14 shows effect of pre-incubation time on the activity of each immobilized enzyme.
About 50-60% of immobilized enzymes maintained their activity when subjected to 120 minutes of pre-incubation time at their respective optimum temperature whereas; free enzyme could retain only 10% of their initial activity when subjected to same period of time. These results suggest that the thermal stability of α-amylase increased considerably as a result of immobilization on to PPY adsorbents and is suitable for long term applications. Improved thermal stability of α-amylase was reported by Sanjay et al. while immobilizing α-amylase on clay supports; Reshmi et al. while immobilization of α-amylase on to zirconia, Ashly et al. during the immobilization of α-amylase on polyaniline and poly (o-toluidine) polymers and Bryjak when using acrylic carriers as the support for α-amylase immobilization [23,24,70,71].

![Figure: 3.14: Effect of pre-incubation time on the activity of free and immobilized α-amylase](image)

In the case of adsorption increase in thermal stability was ascribed to enhanced enzyme rigidity due to strong direct electrostatic interaction of support with the enzyme thus preserving its tertiary structure from conformational transition that might occur at elevated temperatures. Similar enhancement in the thermal stability after immobilization was observed by Zhao et al. [72]. The authors immobilized glucoamylase from *Aspergillus niger* onto functionalized magnetic SBA-15 (FeSBA-15) as a regenerated support through metal-ion affinity interactions. After particular temperature the denaturation of an enzyme encounters
which corresponds to the unfolding of the enzyme by disruption of noncovalent intra-molecular interactions. This can be induced by any change in the enzyme environment because of increase in temperature.

3.5.5.5 Determination of kinetic parameters

The velocity of enzyme reaction is decisively influenced by the concentration of its substrate. In cases this relationship obeys Michaelis-Menten kinetics, $K_m$ the Michaelis constant and $V_{max}$, the maximum rate at which the reaction proceeds at infinite substrate levels are the two most important parameters used to characterize the kinetic properties of the enzyme. These are determined by varying the concentration of starch in the reaction medium. The Michaelis constant provides a measure of affinity of enzyme active site for its substrate and apparent $K_m$ values obtained were higher than those for native enzyme. This difference in $K_m$ value occurred as a result of obstruction in the access of substrate to active site of the enzyme by diffusion barriers. This hindrance might be due to micro-environmental effects of the carrier as a result of immobilization method which thus alters the proper orientation and conformation of protein structure preventing it from being more accessible to substrate [73].

On the other hand, the $V_{max}$ which measures the maximum catalytic potential of the enzyme follows an opposite trend. It usually shows a decrease in trend during immobilization of enzymes as is widely observed and reported in most studies [23,70]. This loss in activity may be attributed to either a loss in native conformation on account of immobilization or steric hindrance in immediate vicinity of the enzyme molecule caused by diffusional limitation of carrier or shielding effect of carrier.

The kinetic parameters of free and immobilized enzyme are shown in table 3.5. Lineweaver-Burk plots and Hanes-Woolf plots of free and immobilized $\alpha$-amylase are shown in figure: 3.15
Immobilization of Diastase $\alpha$-amylase on to Synthetic Polymers

(i) FREE ENZYME

(ii) PAE

(iii) PFE

(vi) PAE

(vii) PFE
Figure: 3.15: Lineweaver-Burk plots for evaluation of $K_m$ and $V_{max}$ for free and immobilized $\alpha$-amylase (i) free enzyme (ii) PAE (iii) PFE (iv) PIE. Hanes-Woolf plots for (v) free enzyme (vi) PAE (vii) PFE (viii) PIE.

Table 3.5: Kinetic parameters for free and immobilized $\alpha$-amylase

<table>
<thead>
<tr>
<th>Lineweaver - burk plot</th>
<th>Free enzyme</th>
<th>PAE</th>
<th>PFE</th>
<th>PIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (mg/ml)</td>
<td>0.50 ± 0.04</td>
<td>0.67 ± 0.01</td>
<td>0.63 ± 0.03</td>
<td>0.73 ± 0.01</td>
</tr>
<tr>
<td>$V_{max}$ (mg/ml/min)</td>
<td>7.40 ± 0.05</td>
<td>2.45 ± 0.04</td>
<td>3.13 ± 0.04</td>
<td>3.20 ± 0.06</td>
</tr>
</tbody>
</table>

3.5.5.6 Storage stability of Immobilized $\alpha$-amylase

It was observed that storage stability of an immobilized enzyme in the wet state is often better than that of free enzyme in a similar solution due to high local concentration of protein in the medium. If the enzyme is used soon after preparation, maximum activity was found to be obtained when it was suspended in buffer and stored at 4°C. While storing enzyme for long periods sometimes removal of water may be necessary for which freeze drying was found to be more effective than simple drying in retaining maximum activity. Simple drying is inadvisable since this can cause loss of the three dimensional structure of both enzyme and matrix.

In most cases results obtained showed that storage stability of the immobilized enzyme was significantly better than free enzyme. In order to
compare the efficiency in storage, free enzyme and immobilized enzymes were stored in buffer solution under same conditions. The free enzyme lost all of its activity within 2 days whereas; under storage less reduction in activity was observed for immobilized enzymes. The decrease in stability of immobilized enzyme during 6 months of storage is shown in the figure: 3.16.

![Figure: 3.16](image)

**Figure: 3.16:** Storage stability of immobilized enzymes at 4°C

From the bar diagram it is evident that all immobilized enzymes retained more than 50% of initial activity after 3 months of storage with PFE and PIE retaining more activity compared to PAE. This might be due to morphological differences among them which were favourable for immobilization of enzyme on PI and PF than PA. The results also suggest that immobilized enzyme exhibits improved storage stability over the free enzyme.

In the context of protein stability, there is evidence that adsorption onto solid surfaces may alter the conformation, i.e., lead to interfacial denaturing of some protein [60,61], while others appear to resist significant conformational change [62-64].

Lim et al. reported a 10% loss of activity in every 12 days of storage for amylase on silanized silica particles [74]. Ashly et al. reported 50% of original activity for α-amylase after checking the activity over a period of four months [24]. The stability achieved in the case of all immobilized enzymes can be attributed to
improved life of biocatalyst due to efficiency gained against thermal inactivation and other denaturing agents by decreased flexibility which enhanced proper orientation of enzyme on the support.

3.5.5.7 Reusability

In our study activity for 12 cycles of use for the immobilized enzymes was monitored and the results are shown in the figure: 3.17.

![Figure: 3.17: Reusability studies of immobilized enzymes](image)

Each time after the reaction, washing of immobilized biocatalyst was carried out using buffer solution to free the immobilized enzyme from any traces of initial substrate added. PAE retained about 69% of its initial activity; PFE retained about 44.4% of its initial activity while PIE retained 14% of its initial activity. Similar improvement of reusability after immobilization of α-amylase was observed by Cakmakci et al. while immobilizing the enzyme on to epoxy containing thiol-ene photocurable materials [76].

3.6 Significance of polypyrrole prepared in presence of surfactants as templates and its role as a support for enzyme immobilization

Obtaining PPY with excellent chemical and physical characteristics becomes more and more attractive because of its excellent characteristics that have
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led to wide potential applications in various fields such as sensors, actuators etc. [77,78]. For this purpose, the polymerization of pyrrole in different surfactant systems has been developed. In tailoring the nanostructures of polypyrrole during polymerization, concentration of pyrrole monomer and surfactant have been proved to play a major role which thereby reflects in their morphology. This is because surfactants can induce pyrrole to grow in a certain manner and result in PPY with an ordered morphology, which will show properties superior to those from a conventional aqueous solution [79, 80].

The advantage of using these soft template materials is that they are easy to remove after the synthesis, and in the meantime, the micro-/nanostructures of the resulting polymers are formed. This template directed synthesized polypyrrole nanostructures have their potential applications in chemical and electrochemical sensors [81].

Micro-emulsion polymerization has been developed to prepare polymer nanostructures. This technique allows particles to transfer into spherical aggregates through the surfactant template. Surfactant creates a micro-reactor vessel via micelle formation, where monomer is restricted in a localized environment originated from encapsulation by the surfactant [82]. Compared with the aqueous solution and conventional emulsion polymerization, the micro-emulsion polymerization of PPY increased the extent of the pi-conjugation along the polymer backbone, and the ordered arrangement of the macromolecular chains.

Zhang et al. reported the controllable synthesis of PPY nanostructures with different kinds of surfactants, including Octyltrimethylammonium Bromide, Cetyl Trimethyl Ammonium Bromide (CTAB), Dodecyl Trimethyl Ammonium Bromide (DTAB), poly (ethylene glycol) mono-p-nonylphenyl ether (Opi-10), and sodium dodecyl sulfate (SDS) [81]. Grady et al. reported the formation of nanostructured PPY with controlled morphologies on atomically flat surfaces with adsorbed surfactant molecules as templates [83]. Omastova’ et al. conducted the synthesis of PPY in the
presence of anionic, cationic, and non-ionic types of surfactants [84]. Kwon et al. reported that PPY prepared without surfactant showed an arbitrary shape whereas, the PPY samples with surfactant showed a spherical shape [85]. From all these studies, it can be concluded that the surfactant provides a space to control morphology as template does.

### 3.6.1 Preparation of polypyrrole in the presence of surfactants

The 0.1 mol of anhydrous FeCl₃ (16.2g) was dissolved in 100 ml distilled water in a reactor vessel containing magnetic stirring bar. To this solution each surfactant with concentration above their cmc (SDS, CTAB, and Tween 80) dissolved in 100 ml distilled water was added and mixed. The whole mixture is then stirred for 30 minutes until surfactant is completely dissolved. The 0.15 mol freshly distilled pyrrole (10.4ml) was first dispersed in 50 ml of distilled water and then inserted drop wise into the stirred mixture of an oxidant and surfactant. Immediate formation of black PPY was clearly observed right after addition of the monomer. The polymerization was carried out for 24 hours at room temperature with moderate stirring. Finally, the PPY is washed with acetone and dried in oven at 60°C for 2 hours.

The PPY prepared in presence of anionic surfactant was treated with an excess of 1M NaOH for 12 hours, then filtered and washed with distilled water and dried at 60°C in oven. This will help to remove the anionic surfactant leaving behind pure polypyrrole with controlled morphology. The cationic and non-ionic surfactants were removed by washing with methanol [84].

![Scheme 3.3 Preparation of polypyrrole through surfactant template](image)
3.6.2 Physico-chemical characterization

3.6.2.1 FT-IR Spectra of polypyrrole prepared in presence of surfactants

The FTIR spectra of PPY prepared in presence of different types of surfactants are shown in figure: 3.18, and the main peak positions are listed in the table: 3.6. The characteristic peaks of PPY can be clearly observed. For instance, the peak at about 3436 cm$^{-1}$, 3434 cm$^{-1}$ and 3430 cm$^{-1}$ correspond to N-H stretching vibrations in the pyrrole ring, of PS, PC and PT respectively. The peak near 2928 cm$^{-1}$ and 2852 cm$^{-1}$ corresponds to the C-H stretching vibration of the methylene group. The intensity of these peaks are so weak which indicates that surfactants have been completely removed from these polymers. These demonstrated that the resulting polypyrrole nanostructures were pure [81].

The most pronounced change after the deprotonation is the reduction of absorption above 1800 cm$^{-1}$. An additional peak at about 1748 cm$^{-1}$ and 1710 cm$^{-1}$ can be observed for all samples, indicating that PPY is slightly over oxidized during the growth process. The oxygen may enter the PPY structure during the polymerization process itself as the consequence of the water presence in polymerization solution, as well as by reaction of the prepared polymer with atmospheric oxygen.

The peak around 1028 cm$^{-1}$ indicates N-H ring out of plane bending which is shifted to 1024cm$^{-1}$ for PC and 1020 cm$^{-1}$ for PT. The C-H ring out of plane bending around 592 cm$^{-1}$ was seen in all samples.

For all samples there was a shift to lower wave number after enzyme has been immobilized. For all three samples characteristics peaks with respect to enzymes were also obtained. As per the literature review the main peaks around 1656 cm$^{-1}$, 1646 cm$^{-1}$, 1617 cm$^{-1}$, 1596cm$^{-1}$, 1542cm$^{-1}$ and 1397 cm$^{-1}$ are the characteristic peaks confirming the presence of $\alpha$-amylase.
Figure: 3.18: FT -IR Spectrum of PPY prepared in presence of surfactants

Table: 3.6 Characteristic peaks corresponding to PPY prepared in presence of surfactants. (w - weak, vw-very weak, s-strong)

<table>
<thead>
<tr>
<th>Peak assignments(cm⁻¹)</th>
<th>PS</th>
<th>PSE</th>
<th>PC</th>
<th>PCE</th>
<th>PT</th>
<th>PTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H stretch</td>
<td>3436</td>
<td>3449</td>
<td>3434</td>
<td>3454</td>
<td>3430</td>
<td>3467</td>
</tr>
<tr>
<td>C-H stretch</td>
<td>2828, 2923</td>
<td>2852, 2859</td>
<td>2828, 2852</td>
<td>2826, 2852</td>
<td>2831, 2854</td>
<td></td>
</tr>
<tr>
<td>C=N</td>
<td>1748, 1712</td>
<td>1752, 1710w</td>
<td>1748, 1712w</td>
<td>1747, 1710w</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-N.C ring-in plane deformation</td>
<td>1636</td>
<td>1656, 1646, 1617w</td>
<td>1656, 1646</td>
<td>1628.8, 1617</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C.N,N-H ring in plane bending(s)</td>
<td>1518</td>
<td>1548, 1542, 1596</td>
<td>1550, 1518w, 1596</td>
<td>1596m, 1542w, 1524w, 1596</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-N stretch(w)</td>
<td>1460</td>
<td>1467.2</td>
<td>1454w</td>
<td>1467vw, 1408vw</td>
<td>1459w</td>
<td>1462w</td>
</tr>
<tr>
<td>C=C.N in plane deformation</td>
<td>851</td>
<td>881</td>
<td>857</td>
<td>851vw</td>
<td>857</td>
<td>892,867vw</td>
</tr>
</tbody>
</table>
3.6.2.2 Thermogram of polypyrrole prepared in presence of surfactants

Thermogravimetric analysis showed that the PPY prepared in presence of SDS was relatively thermally stable in N₂ up to approximately 272°C. The subsequent weight loss curve (180°C - 350°C) is significantly steeper than that observed for either the PC or PT. The major weight loss was at temperature 272°C and minor weight loss starts at 430°C which continues upto 800°C.

When α-amylase get adsorbed on to this polymer the major weight loss starts at 196°C which is assigned to protein degradation and the other major weight loss corresponding to PPY structure starts at 332°C.

In the case of PC the major weight loss was observed at 211°C which is due to the decomposition of polymer backbone along with minor amounts of dopants if present. The other minor weight losses occur at 303°C and 490°C.

After immobilization the decomposition starts at 198°C which corresponds to degradation of amylase molecular structure. At 309°C the polymer decomposition starts which then continuously decompose up to 800°C.

For PT the decomposition of PPY initiates at 226°C with subsequent weight loss at 353°C. α-amylase immobilized on PT started decomposing at temperature above polymer decomposition as enzymes have delicate backbone that could not withstand elevated temperature. Thus at 176°C the amylase decomposition might have occurred subsequently followed by decomposition of the polymer organic structure at temperature of 363°C. The TG curves obtained are shown in the figure: 3.19
3.6.2.3 Surface area analysis

The BET surface area of polymers prepared are given in table: 3.7.

**Table: 3.7** Surface area of PPY prepared in presence of surfactants and their enzyme immobilized forms

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Surface area (m²/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>9.4</td>
</tr>
<tr>
<td>PC</td>
<td>11.9</td>
</tr>
<tr>
<td>PT</td>
<td>26</td>
</tr>
<tr>
<td>PSE</td>
<td>5.96</td>
</tr>
<tr>
<td>PCE</td>
<td>9.07</td>
</tr>
<tr>
<td>PTE</td>
<td>23.3</td>
</tr>
</tbody>
</table>
3.6.2.4 Scanning electron microscopy

The SEM images show that good spherical nanoparticles were obtained using surfactants as template during PPY synthesis. The surfactants played a major role on the surface morphology of products because upon the removal of the surfactant template the total surface area increases. There occurs agglomeration of particles in all the samples prepared. This is due to the close interactions between the polymer chains.

Figure: 3.20 SEM images of (i) PS (ii) PC (iii) PT
3.6.3 Immobilization of α-amylase on polypyrrole prepared in presence of different surfactants.

3.6.3.1 Influence of pH during immobilization of α-amylase on polymer supports

To find the optimum binding condition necessary for the enzyme, we measured immobilized α-amylase activity under the conditions of different pH values. The effect of pH of the medium on the relative activity of the immobilized enzyme is shown in the figure: 3.21.

From the figure it is evident that the immobilized enzyme activity was highest in the pH range 5-6. The decrease in activity above and below this pH may be due to lower loadings which occurred as a result of change in conformation of tertiary structure due to unfavourable charge distribution of amino acid residues as a result of change in pH. Since the free enzyme used in this experiment was stable in the narrow pH range 4.5-5.5, the possible denaturation of enzyme in alkaline region was also expected.

**Figure: 3.21** Effect of pH of immobilization medium on the relative activity of immobilized α-amylase

Here we observed that pH 5 was best for the immobilization of α-amylase on PS and PT whereas, for PC the best retention of enzyme activity was observed at
pH 6. At pH 5 and 6, since polypyrrole is positively charged and amylase negatively charged, strong adsorption occurred in the case of all supports. This is due to significant electrostatic interaction between supports and the enzyme.

Furthermore, all of the polymers have conjugated rings for hydrophobic interactions. At pH 4, PPY adsorbents have overall net positive charge and lysine and arginine amino acid residues on the protein surface of amylase have slight positive charge as it is close to its isoelectric point. Hence PPY adsorbent should repel amylase enzyme. However, adsorption of proteins was still observed at pH 4 because hydrophobic interactions appeared to dominate over electrostatic interaction at pH 4. The small adsorption difference due to pH might originate from ionic effects, due to secondary amino groups of the pyrrole rings.

At pH 7 PPY has no charge whereas amylase is negatively charged. Hence the observed adsorption might have occurred as a result of hydrophobic interaction. When the immobilization is carried out at higher pH, the same amount of enzyme is immobilized, however the activity observed is lower than that attained at pH 5 and 6. This is because at higher pH PPY adsorbents have overall negative charge and amylase is also having net negative charge which results in electrostatic repulsion. Thus overall activity of the enzyme is very much dependent on the strength of the electrostatic interaction between enzyme and the support.

**3.6.3.1.1 Effect of contact time on the activity of α-amylase**

The contact time needed for enzyme to get adsorbed on PPY adsorbents is shown in the figure: 3.22.
Figure: 3.22 Effect of contact time on immobilized enzyme activity

For PS the adsorption capacity was maximum within 180-240 minutes contact time. Whereas in the case of PC and PT contact time was 240 minutes and 120 minutes respectively in order to have maximum protein adsorption. The decrease in activity after this optimum time might be due to the fact that, as the first adsorption occurred the surface of the support get saturated with the enzyme and remaining enzymes in solution had to bind with the support surface via second adsorption site which needed more energy. This could have weakened the adsorption efficiency.

3.6.3.1.2 Effect of initial protein concentration on protein loading on to polymeric supports

The amount of protein bound to PPY adsorbents were analyzed based on the optimized conditions obtained; it is shown in figure: 3.23.

The effect of initial protein amount on protein loading is depicted in the graph shown. Adsorbed amount of enzyme increases with increase in enzyme concentration taken but after a particular concentration no further increase in adsorption occurs instead a saturation point is reached and enzyme starts desorbed from the surface if loaded heavily [86].
3.6.3.1.3 Effect of initial protein concentration on immobilization yield and activity of loaded protein

Immobilization yield obtained for all adsorbents at various concentrations taken are shown in the figure: 3.24.

From the graph, it is clear that enzyme loading increases as concentration increases which then reaches a saturation point and then starts decreasing or remains constant which might be due to desorption at high loading with increase in enzyme amount as a result of multilayer adsorption which causes weak binding of enzymes on to supports.
For PS optimum immobilization yield was 34% when 12.8 mg of initial protein was added. Protein load was also the maximum at this concentration with immobilized enzyme activity 26.5 EU.

For PT even if maximum loading of 65.5% was obtained when initial protein was 14.6 mg and protein load of 9.6 mg, the optimum immobilized activity 12.2 EU was obtained at the initial protein concentration of 17.9 mg with protein load of 10.6 mg. Similar is the case with PC.

Thus there are several unpredictable interactions that can cause change in protein conformation and thus cause deviation from theoretical trends. The trend of immobilized enzyme activity when initial protein amount was varied for all adsorbents is shown in the figure: 3.25.

![Figure 3.25: Effect of initial protein concentration on immobilized enzyme activity](image_url)

The immobilization yield, activity yield and immobilization efficiency were also evaluated. The results are tabulated in the table 3.8.

Table 3.8: Immobilization efficiency of PPY prepared in presence of surfactants

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Initial protein (Mg)</th>
<th>Immobilized protein mg/g</th>
<th>Immobilization yield (IY)</th>
<th>Initial Activity EU</th>
<th>Immobilized enzyme activity EU</th>
<th>Activity yield (%)</th>
<th>Activity yield efficiency (%)</th>
<th>IE = AY/IY</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>12.8</td>
<td>4.4</td>
<td>34</td>
<td>26.5</td>
<td>8.5</td>
<td>32</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>17.9</td>
<td>6.1</td>
<td>44</td>
<td>27</td>
<td>9.3</td>
<td>34</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>PT</td>
<td>17.9</td>
<td>10.6</td>
<td>59</td>
<td>27</td>
<td>12.2</td>
<td>45</td>
<td>76</td>
<td></td>
</tr>
</tbody>
</table>
Because of comparatively small particle size and hence high surface area, protein loading was the maximum for PT compared to PS and PC. Correspondingly activity yield was also found to be higher for PT. But the immobilization efficiency was the highest for PS compared to PC and PT. This might be due to lower affinity of amylase towards PC and PT when compared to PS. Thus results indicate that loading efficiency was affected by increasing loading amount [87].

3.6.3.2 Effect of pH on enzyme activity

An enzyme’s apparent response to pH may change when it is in a heterogeneous environment associated with polymer matrices. Figure: 3.26 show the effect of different pH on enzyme activity.

Free and immobilized α-amylase exhibits similar activity in the range from pH 4.5 to 5.5. Above pH 6.0 immobilized α-amylase showed better performance than free α-amylase. In the case of PSE optimum activity was observed at pH 5 whereas, optimum activity in the case of PCE and PTE was at pH 6.

![Figure: 3.26: Effect of pH on the activity of free and immobilized α-amylase](image)

**Table: 3.9** Optimum pH for free and immobilized α-amylase

<table>
<thead>
<tr>
<th>pH</th>
<th>Free enzyme</th>
<th>PSE</th>
<th>PCE</th>
<th>PTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 &amp; 5.5</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

At pH 4 and 8 a decrease of the enzymatic activity is observed for both the immobilized and the free enzyme; however, at pH 8 the residual activity of the
immobilized enzyme in most samples is significantly higher than that of the unmodified form.

A greater bulk pH is required in providing an optimum pH in the microenvironment of the enzyme and hence a shift to higher value is encountered. Thus, the immobilization process provides structural stability, preventing an irreversible unfolding of the enzyme protein.

The enzyme is inactivated at lower pH values (pH < 5) [88]. The curve profile for all immobilized enzymes became much broader when compared to that of free enzyme.

3.6.3.3 Effect of temperature on the activity

The inactivation rate of an enzyme increases with temperature. Every enzyme therefore shows optimum activity at a particular temperature at which it assumes its more stable conformation. This can be directly related to the efficiency of immobilization process and hence will have a profound impact not only on enzyme activity but also on enzyme stability. Influence of temperature on activity of free and immobilized α-amylase is depicted in the figure: 3.27.

![Figure: 3.27: Effect of temperature on the activity of free and immobilized α-amylase](image)

The 10°C decrease in the optimum temperature combined with thermal stability exhibited by PCE and PTE was an interesting finding of this work. Similar
Immobilization of Diastase α-amylase on Synthetic Polymers

decrease in optimum temperature was reported by Su et al. when they immobilized β-glucosidase on alginate by combining cross-linking with entrapment and again, cross-linking. The energy reduction and lower time to cool the reaction mixture represents advantages acquired with the immobilization process.

In the case of PSE there was an increase in optimum temperature by 10°C compared to free enzyme. Since in the bound state enzymes are less mobile they resist denaturation of protein [89]. The decrease in optimum temperatures of PCE and PTE may be due to less activation energy required for starch hydrolysis because of the conformational change that occurred at the enzyme active site after immobilization. But increase in temperature for PSE might be due to increase in activation energy required for starch hydrolysis as a result of structure changes encountered at the active site after immobilization.

This can be either due to excess energy required to orient the enzyme into its native conformation or due to improper transport of substrate molecules from the bulk to the enzyme active site on account of diffusional resistances to mass transfer [90].

3.6.3.4 Thermal stability of the free and immobilized enzymes

Thermal stability obtained after immobilization conferred to PPY adsorbents a very good performance as can be seen in figure: 3.28. After pre-incubation at various temperatures in the range 30-60°C for 1 hour with the support in respective buffer solution, PCE and PTE showed 80% of their initial activity whereas PSE showed 60% of its initial activity. This improvement in denaturation resistance of the immobilized α-amylase was probably a consequence of the multipoint attachment acquired in the immobilization process.

All the results obtained were very much better, as free enzyme cannot withstand such a prolonged period of thermal treatment [91]. For industrial application the enzyme should be stable towards temperature fluctuations. The
immobilized enzyme shows moderate decrease in activity, which emphasizes that the rate of inactivation is lowered upon immobilization.

**Figure: 3.28** Thermal stability of free and immobilized α-amylase

As the temperature increases, the stability drops significantly for both free and immobilized amylase. At 40°C, both free and immobilized enzyme retain up to 70-80% of their activity. At 50°C the immobilized enzyme was inactivated at a much lower rate than the free enzyme. The free enzymes lost almost 90% of their activity at 60°C after 1 hour treatment whereas immobilized amylase lost only 20-40% of its initial activity. Figure: 3.29 show the effect of pre-incubation time on the activity of each immobilized enzyme.

**Figure: 3.29** Effect of pre-incubation time on the activity of free and immobilized α-amylase
About 70-80% of immobilized enzymes maintained their activity when subjected to 120 minutes of pre-incubation at their respective optimum temperature whereas, free enzyme could retain only 10% of their initial activity when subjected to same treatment.

These results suggest that the thermal stability of α-amylase increases considerably as a result of immobilization on to PPY adsorbents and is suitable for long term applications. Such an improvement in thermal stability was observed by Talekar and Chavare when they carried out immobilization of α-amylase on to calcium alginate via entrapment method [92]. It is reported that immobilization can help to distribute the thermal energy imposed to the protein at higher temperatures and hence it is less susceptible to temperature induced conformational changes.

3.6.3.5 Determination of kinetic parameters

$K_m$ and $V_{max}$ were calculated from the Lineweaver-Burk plots and Hanes-Woolf plots. Both plots are presented in the figure: 3.30.

Table 3.10: Kinetic parameters determined for free and immobilized α-amylase

<table>
<thead>
<tr>
<th></th>
<th>Free Enzyme</th>
<th>PSE</th>
<th>PCE</th>
<th>PTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (mg/ml$^{-1}$)</td>
<td>0.50 ± 0.04</td>
<td>0.916 ± 0.01</td>
<td>1.07 ± 0.04</td>
<td>1.49 ± 0.05</td>
</tr>
<tr>
<td>$V_{max}$ (mg/ml/min)</td>
<td>7.40 ± 0.05</td>
<td>2.34 ± 0.03</td>
<td>4.41 ± 0.02</td>
<td>3.44 ± 0.02</td>
</tr>
</tbody>
</table>

(i) (iv)
The $K_m$ values were found to be increasing and $V_{max}$ values decreasing in the case of PSE, PCE and PTE [93,94].

This demonstrates that there is a significant role played by mass transfer restrictions due to diffusional limitations. The change in affinity of the enzyme to its substrate is probably caused by structural changes in the enzyme as a result of immobilization on to a solid support and by lower accessibility of the substrate due to improper diffusion of the substrate to the active sites of enzyme [91]. Since
immobilization process does not control proper orientation of the enzyme on the supports, this may cause variation in the usual native conformation and thus changes the property of the active site thereby hindering the active site from binding the substrates [95].

3.6.3.6 Storage stability of Immobilized \( \alpha \)-amylase

In the free form, enzyme has very short life time and hence gets easily inactivated with minor fluctuations in its local environment. Hence its reuse is impossible which impose a heavy wastage of enzymes and thus economically not beneficial. Immobilization of enzymes enables long-term storage of the enzyme and thus becomes available for various applications. In the dry form, the immobilized enzymes cannot maintain their stability and activity for long term storage. But, when stored in buffer solution under low temperature of 4°C immobilized enzymes exhibit better activity and could retain its stability.

On the other hand free enzyme even when stored in buffer solution under low temperature conditions lose complete activity within 7 days. Whereas PCE could retain 53% of its initial activity, PTE retained 60% of its initial activity and PSE 40% of its initial activity, all of which are far better than free enzyme as can be seen in the figure: 3.31. This decrease in activity among the immobilized enzymes can be explained as the time-dependent natural loss in enzyme activity which occurs due to conformational change in the active site of enzyme on long term storage that is caused by changes in its micro environment. Similar enhancement in storage stability after immobilization has been reported by other authors [76,96].
3.6.3.7 Reusability

In our systems reusability was checked for 20 continuous cycles and results obtained were given in the figure: 3.32. After 15 cycles PSE and PCE retained 50% of their initial activity whereas, PTE retained almost 60% of its initial activity. It was observed that the immobilized enzyme activity decreased when recycling number was increased. Similar results were obtained by other authors. Jaiswal et al. reported that immobilized α-amylase on gelatin was reusable upto seven cycles. Almost 90% activity was retained upto three cycles, but with subsequent runs, there was a decline in the activity of the immobilized enzyme. The activity loss could be due to weakening in the strength of binding between the matrix and enzyme on repeated use and hence the enzyme might leach out from the matrix [97]. Besides, the frequent encountering of the substrate into the same active site might distort it which would dwindle the catalytic efficiency either partially or fully [98]. Om Prakash et al. reported that α-amylase immobilized on agarose and agar matrices could retain its activity upto 5 cycles after which there was a subsequent decrease in activity which may be due to enzyme denaturation and due to physical loss of enzyme from the carrier [226].
3.7 Significance of functionalized polypyrrole as support for enzyme immobilization

PPY is also associated with some limitations such as its roughness, rigidity, lack of processability and absence of any functional group for surface immobilization of bioactive molecules. In order to perform as bioactive platform for the immobilization of biomolecules, it is necessary to carry out chemical modification of PPY [31]. The polymers were functionalized by introducing reactive groups that are able to react with -COOH and -NH₂ groups of the enzyme molecule. All these modifications opened up an easiest route for covalent attachment of drugs and biomolecules.

Recently, T. Sandu et al. reported a work based on functionalization of polypyrrole after polymerization is completed. The functionalization agent was glutaraldehyde, and the functionalization purpose was to create binding sites at certain distances from the polymer surface. This is necessary in view of covalently immobilize enzymes on polypyrrole [99,100].

Polypyrrole is an inherent biocompatible polymer. Here in our study the functionalization was not realized during polymerization process, but after polymerization. The functionalizing agents were glutaraldehyde and...
aminopropyltriethoxysilane. The functionalization was done with the view that functional groups introduced will serve as binding sites that can react with functional groups of the enzyme. The α-amylase was immobilized on to glutaraldehyde activated PPY via covalent binding and to APTES activated PPY via adsorption method.

Amylases contain a number of reactive lysine groups on their surfaces which are capable of reacting with the aldehyde groups from glutaraldehyde for enzyme rigidification. Glutaraldehyde is a bi-functional agent which could also serve as a spacer arm in enzyme immobilization [101,102]. A Schiff base was formed through the reaction between CHO groups from glutaraldehyde and -NH groups from polypyrrole. The unreacted CHO groups that remain free on the other side of bonded aldehyde are available for subsequent bonding with the enzyme [99,100].

Amino propyl triethoxy silane has an organic functional group (NH$_2$-) and three alkoxy groups. The chemical reaction began with the hydrolysis of the alkoxide groups into silanols that may condense to form siloxane bonds. Silane molecules can absorb, condense, or interact with the substrate, influencing coupling effectiveness. After silanization active sites were introduced on the surface or at the end of silanized polymer, which can be bonded with bio-molecules to attain sensitive elements for biosensor application by direct coagulation or through the format of amino bridge bio-molecules [103].

It was reported by Bernard et al. that silanization of crushed magnetite, synthetic magnetite or bio-magnetite with APTES further derivatized with glutaraldehyde does not yield a highly stable silane coating [104]. Hence we didn’t activate APTES but direct linkage of APTES with amylase was adopted as the method.

### 3.7.1 Functionalization of polypyrrole with Glutaraldehyde

PPY was synthesized from pyrrole through the chemical oxidation-polymerization method, with Fe$^{3+}$ as oxidant and water as solvent. In brief, 1.75 ml of pyrrole (0.025mol) was added drop wise into 150 ml of aqueous FeCl$_3$·6H$_2$O
Immobilization of Diastase \( \alpha \)-amylase on to Synthetic Polymers

(13.5g, 0.05mol) solution with stirring. The mixture was stirred for 3 hours to allow the oxidation-polymerization reaction to be fully completed. Then, the resultant black precipitate was separated by filtration, thoroughly washed with deionized water and methanol to remove any possible iron residues, and then dried in a vacuum desiccator for 24 hours. The powder so obtained were PPYCl that is PPY doped with chloride. This PPYCl is then functionalized using the procedure reported by T. Sandu et al. [99].

A liquid phase was first prepared by mixing water (80ml), glutaraldehyde (20ml) and sulphuric acid (1.36ml) respectively and was poured into polypyrrole solid phase. Polypyrrole (1g) thus mixed with liquid phase was then heated at 70°C for 2 hours. The final mixture was then filtered and washed with distilled water to remove the impurities. The precipitated polypyrrole powder is then dried at 40°C.

In this reaction hydroxyl groups were said to be formed through functionalization reaction between NH groups from polypyrrole and glutaraldehyde according to the scheme: 3.4.

\[
\text{NH} + \overset{\text{O}}{\text{HC}} - (\text{CH}_2)_3 - \overset{\text{CH}}{\text{O}} \rightarrow \overset{\text{N}}{\text{CH}} - (\text{CH}_2)_3 - \overset{\text{CH}}{\text{O}}
\]

**Scheme: 3.4** Functionalization reactions between polypyrrole and glutaraldehyde

Goldstein and Menecke suggested that the glutaraldehyde reaction most probably involves conjugated addition of protein amino groups to ethylene double bonds of \( \alpha-\beta \) unsaturated glutaraldehyde oligomers, since the linkages formed between the protein and glutaraldehyde are irreversible and survive extremes of pH and temperature [105]. The possible reaction is as depicted in scheme: 3.5. Therefore, the possibility of Schiff base (aldehydine) formation has been kept to a minimum due to the reaction’s reversibility in aqueous media, particularly at low pH values.
**Scheme 3.5:** Enzyme immobilization reaction between free CHO groups from glutaraldehyde and H₂N groups from the enzyme \[106\]

### 3.7.2 Functionalization of polypyrrole with Aminopropyltriethoxy silane.

PPYCl powder was dispersed in an APTES solution (2 wt%) with pH being adjusted to 4 by the addition of acetic acid. After 2 hours, the resultant precipitate was filtered, washed by soaking in a 0.01M NaCl solution at pH 4 and then separated and dried in a vacuum desiccator for 24 hours \[107\]. The reaction is depicted in the scheme: 3.6 \[108\].

**Scheme 3.6:** Functionalization of polypyrrole with Aminopropyltriethoxy silane
3.7.3. Physico-chemical characterization

3.7.3.1. FT-IR Spectra of functionalized polypyrrole

FTIR spectra were obtained for the PN as shown in figure 3.34. There were three peaks at the wave numbers at about 1550 cm\(^{-1}\), 1470 cm\(^{-1}\) and 1309 cm\(^{-1}\) for PN, which stand for the C=C stretching, C-N stretching and C-C in ring stretching respectively. The peak at 3107 cm\(^{-1}\) stands for the C-H stretching on the ring of PN and the peak at 2924 cm\(^{-1}\) and 2854 cm\(^{-1}\) were assigned to a (C-H) stretching band of a (N-C-H) unit slightly shifted from the normal (C-H) position. A new peak at 2972 cm\(^{-1}\) (indicating the -C-NH\(_2\) stretch vibration) was also observed in the spectrum. The FT-IR results prove that mixing APTES into the polymer powder is an efficient way to functionalize the polymeric nanostructure [109].

There was a C-N stretching peak for secondary nitrogen on PPY at 1179 cm\(^{-1}\) and the peaks at 1041 cm\(^{-1}\) and 1427 cm\(^{-1}\) were assigned to the C-N stretching for primary amine and the N-CH\(_2\) stretching for tertiary amine on PN respectively.

The major change in the transmittances can be observed at the wave number 3448 cm\(^{-1}\) (N-H stretching in secondary amine) for the PPY, which was replaced by two split peak at 3394 cm\(^{-1}\) and 3460 cm\(^{-1}\) for N-H stretching in primary amine and the other peak at 788 cm\(^{-1}\) (N-H deformation). Moreover, an additional peak appeared at around 1624 cm\(^{-1}\), which was assigned to the NH\(_2\) in-plane deformation for PN. All these changes indicate that the surface amination process added some primary amine groups on the surface and changed some secondary aromatic amine groups in PPY into tertiary amine groups.

It is observed that spectra has featureless shape above 1600 cm\(^{-1}\), which may be caused by the conjugate molecular structure of PPY and the free charge carriers present in the polymers. At the wave numbers below 1600 cm\(^{-1}\), the spectra show a characteristic series of seven absorption peaks which are intensified and sharp peaks [110]. This may be attributed to the assumption that the aminated chains bonded to the nitrogen atoms in the PPY backbone may disturb the conjugate structure of PPY and
thus limited the extent of charge delocalization along the polymer chain, leading to the increase of spectral features.

In particular, the intensities of the two peaks at 1550 cm\(^{-1}\) and 1309 cm\(^{-1}\) increased but the peak at 1470 cm\(^{-1}\) decreased significantly for the PN adsorbent, in comparison with those of un-aminated PPY-based adsorbent [107].

After immobilization of α-amylase for PN the peaks in the region of 400-1600 cm\(^{-1}\) were enhanced when compared to the spectra of PN before enzyme adsorption. As reported by Tian and Zerbi the relative intensities of the infrared bands at 1550 cm\(^{-1}\) and 1470 cm\(^{-1}\) were particularly sensitive to the extent of delocalization along the PPY polymer chain [111].

An intense sharp peak at 1680 cm\(^{-1}\) corresponds to C-N stretching whereas very weak peak at 1638 cm\(^{-1}\) corresponds to C=C stretching. Liang et al. proposed that the conjugation length of the doped PPY were inversely proportional to the ratio of the intensities of bands at 1550 cm\(^{-1}\) and 1470 cm\(^{-1}\). Hence it was concluded by X. Zhang et al. that the ratio changes in the intensities of bands at 1550 cm\(^{-1}\) and 1470 cm\(^{-1}\) are an indication of the decrease of conjugation length and extent of delocalization along PPY chain after the enzyme adsorption [107].

From the differences in the two spectra it is clear that the chemical interactions of enzyme have occurred directly with the PPY backbone. Thus X. Zhang et al. concluded that as a result of electrostatic interaction between some of the carboxyl groups of the protein with the positively charged nitrogen atoms in the PPY, some complexes might have formed which thus limit the extent of delocalization and the conjugation length and cause changes of the FT-IR bands in the region of 400-1600 cm\(^{-1}\) [107].

For PG a peak at 3428 cm\(^{-1}\) corresponds to NH stretching of pyrrole. Two strong bands in the region 2932 cm\(^{-1}\) and 2865 cm\(^{-1}\) was assigned to CH\(_2\) groups which confirm the polypyrrole functionalization using glutaraldehyde [106]. A peak at 1718 cm\(^{-1}\) corresponds to carbonyl group. This peak which was common in PPY as a
result of over-oxidation is otherwise very weak. But after functionalization with glutaraldehyde this peak becomes so intense which confirms the presence of aldehyde groups from glutaraldehyde.

In the case of PG the additional peaks that are the characteristic of $\alpha$-amylase was observed in the spectrum. Thus a new peak at 1643 cm$^{-1}$ results which is the representative of peptide bonds from enzyme. This peak was absent in the spectra of PG before immobilization. Again another peak at 1283 cm$^{-1}$ was present in spectra of PG which was absent in spectra of PGE. This is due to the presence of C=O groups in the parent compound which was utilized during immobilization and hence is absent in the spectra of immobilized enzyme. This confirms the fact that immobilization has occurred via glutaraldehyde functional groups through covalent bonding. The intensity of other peaks got increased after immobilization. Similar results were reported by T. Sandu et al. while immobilizing polyphenol oxidase on functionalized polypyrrole [106].

Peak assignments characteristic to that of PN, PNE and PG, PGE were presented in table 3.11.

Figure: 3.34: FT-IR Spectra of PPY prepared in presence of surfactants and its immobilized forms.
Table 3.11 Peak assignments for immobilized enzymes and supports

<table>
<thead>
<tr>
<th>Peak assignments (cm⁻¹)</th>
<th>PG</th>
<th>PGE</th>
<th>PN</th>
<th>PNE</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H stretch</td>
<td>3436</td>
<td>3443m</td>
<td>3432,3107vw,</td>
<td>3394</td>
</tr>
<tr>
<td>C-H stretch</td>
<td>2936,2862vs</td>
<td>2937,2866</td>
<td>2924,2854vvw</td>
<td>2923,2849vvw</td>
</tr>
<tr>
<td>C-O</td>
<td>1718,1683vs</td>
<td>1722w,1686s</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C-N ring -in plane deformation</td>
<td>1638vvw</td>
<td>1656</td>
<td>1548,1544s</td>
<td>1542</td>
</tr>
<tr>
<td>C-N , N-H ring in plane bending(s)</td>
<td>1550</td>
<td>1542</td>
<td>1550</td>
<td>1544m</td>
</tr>
<tr>
<td>C-N stretch(w)</td>
<td>1454</td>
<td>1448m</td>
<td>1456</td>
<td>1464</td>
</tr>
<tr>
<td>C-C in ring stretch(s)</td>
<td>1349mb,</td>
<td>1334bm</td>
<td>1295</td>
<td>1309w</td>
</tr>
<tr>
<td></td>
<td>1315</td>
<td>1322</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C inter ring stretch(w)</td>
<td>1165</td>
<td>1164vvw</td>
<td>1175</td>
<td>1189</td>
</tr>
<tr>
<td></td>
<td>1279bm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-H,N-H ring out of plane bending (vs)</td>
<td>1043m,</td>
<td>1051</td>
<td>1042</td>
<td>1055</td>
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<tr>
<td></td>
<td>788</td>
<td>785 vvw</td>
<td>775</td>
<td>788 w</td>
</tr>
</tbody>
</table>

3.7.3.2 Thermogram of functionalized polypyrrole

In the case of PG, the major degradation starts around 207°C which accounts for removal of moisture and other small chain fragments of polymer chain along with solvent molecules. The maximum degradation occurred around 425°C. This might include the degradation of polymer chain along with the methylene ammonium salt formed as a result of reaction between glutaraldehyde and PPY, which was reported to be a stable compound.

Thus it can be concluded that the functionalized PPY is more stable than pure PPY. Also similar to the reported literature by T. Sandu et al. maximum degradation was found to be higher for the functionalized sample when compared to pure polypyrrole [100].
Figure: 3.35 TG curves of (i) PG and PN (ii) PG and PGE (iii) PN and PNE

In the case of PN the major decomposition peak was at 232°C. The minor decomposition peaks at 348°C, corresponds to removal of 1-(2-amino propyl pyrrole) - silica that remains unreacted along with small chain organic fragments of polymer chain. The minor peak that continues from 546°C corresponds to degradation of polymer chain backbone.

3.7.3.3 Scanning electron microscopy

The SEM images obtained for the functionalized supports are depicted in figure: 3.36. PN granules agglomeration was observed, thus giving the morphology being similar to PPY.
In the case of PG these particles are agglomerated exhibiting cluster morphology. After PPY functionalization with glutaraldehyde a cluster compactation also takes place which could be explained by aldehyde groups attachment on PPY chains or by PPY chains crosslinking [106].

![Figure: 3.36: SEM images of PN and PG](image)

3.7.3.4 Energy Dispersive X-ray spectroscopy

Elemental data obtained from Energy Dispersive X-ray analysis confirmed the functionalization of APTES on PPY. The higher C and N content and decreased Cl content in the modified sample compared to unmodified sample is a direct evidence for organo silane modification on the polypyrrole.

**Table 3.12: Elemental data obtained from Energy Dispersive X-ray analysis**

```plaintext
<table>
<thead>
<tr>
<th>Element</th>
<th>(keV)</th>
<th>Mass%</th>
<th>Atom%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C K</td>
<td>0.277</td>
<td>50.57</td>
<td>72.64</td>
</tr>
<tr>
<td>N K</td>
<td>0.392</td>
<td>2.24</td>
<td>2.75</td>
</tr>
<tr>
<td>O K</td>
<td>0.525</td>
<td>2.77</td>
<td>2.99</td>
</tr>
<tr>
<td>Cl K</td>
<td>2.621</td>
<td>44.42</td>
<td>21.61</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Element</th>
<th>(keV)</th>
<th>Mass%</th>
<th>Atom%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C K</td>
<td>0.277</td>
<td>77.56</td>
<td>87.22</td>
</tr>
<tr>
<td>N K</td>
<td>0.392</td>
<td>3.63</td>
<td>3.5</td>
</tr>
<tr>
<td>O K</td>
<td>0.525</td>
<td>4.56</td>
<td>3.85</td>
</tr>
<tr>
<td>Cl K</td>
<td>2.621</td>
<td>14.25</td>
<td>5.43</td>
</tr>
</tbody>
</table>
```
Immobilization of Diastase $\alpha$-amylase on to Synthetic Polymers

Figure: 3.37 (i) EDS Spectrum of PPY (ii) EDS Spectrum of PN.
3.7.4 Immobilization of \( \alpha \)-amylase on polypyrrole functionalized using glutaraldehyde and APTES as coupling agent

3.7.4.1 Optimization of immobilization parameters

The effect of pH of medium in which the enzyme is dissolved at the time of immobilization on to polymeric supports is shown in the figure: 3.38.

![Figure: 3.38: Effect of pH of immobilization medium on the relative activity of immobilized \( \alpha \)-amylase](image)

Glutaraldehyde was reported to be most reactive in neutral or basic conditions. Immobilization can bring about a shift in pH towards acidic or alkaline side. From the graph it is clear that PGE was found to have highest activity when immobilization was carried out in the pH range 6-7 with optimum activity at pH 7. Similar shift in pH was reported for RSDA immobilized on agarose using spontaneous cross-linking method with glutaraldehyde as a cross linker [112].

Strong interactions between amylase and support via free aldehyde groups that remain on glutaraldehyde bonded to the support, affected intra-molecular forces responsible for maintaining the enzyme conformation, leading to a resultant change in enzyme activity. The stability of immobilized enzyme at neutral pH is an added advantage as this will promote storage of amylase for longer periods [113].
At lower pH activity was found to be decreasing which might be due to unfavourable environment that could not hold the enzyme on support efficiently. As the pH increases beyond 7 also there was a decline in activity which might be due to change in active conformation that caused denaturation of enzyme protein to a considerable extent.

In the case of PN, however, the amino-terminated chains were flexible and could extend far into the solution. The amino groups on the surfaces of PN can thus easily interact with enzyme molecules in the solution for adsorption, regardless of the feature of the net surface electrostatic interaction.

Although amylase was adsorbed on PN in whole pH range examined, the highest activity was obtained in the range 5-6 with the optimum activity at pH 5. This is due to the fact that at this pH range amino groups on PN gets protonated and surface charge of PN adsorbent turns positive. Amylase has negative charge at this pH due to its isoelectric point of 4.6. This results in specific interaction between the positively charged amino groups of PN and negatively charged carboxyl groups at the enzyme surface to form R-NH\textsuperscript{3+}…OOC-R complexes, which enhanced the amylase adsorption on PN.

At pH lower than 5 both amylase and PN are positively charged and hence electrostatic repulsion will prevent adsorption. Also, at pH greater than 7 both amylase and PN are negatively charged which again results in electrostatic repulsion thereby causing hindrance to amylase molecules from getting strongly attached to PN.

But from the graph it is clear that significant amounts of adsorption occurs at these lower and higher pH’s which may be explained from the macromolecular feature of the proteins and the location of the amino groups on PN being at the exterior ends of the chain, which allowed some parts of the protein molecules to penetrate through the electrostatic barrier and form complexes with amino groups
on PN to undergo effective adsorption. The results obtained led to the conclusion that surface complexation may be another important adsorption mechanism for enzymes attached to polymeric support [107].

**3.7.4.1.1 Effect of contact time on the activity of immobilized enzyme**

The period of contact needed for enzyme and support so as to achieve maximum loading are shown in the figure 3.39.

![Figure: 3.39: Influence of contact time on immobilized enzyme activity](image)

PGE showed an optimum activity after a contact period of 1hour. Further increase in time will lead to multilayer adsorption of enzyme and active sites on the surfaces get blocked with the enzyme that gets added in excess. This will cause corresponding reduction in activity.

For PNE the contact time of 180 minutes was sufficient to attain optimum activity. Since the same type of enzymes carried the same type of electric charges, after 180 minutes repulsive electrostatic interaction between already adsorbed enzymes on PN and the enzymes to be adsorbed from the solution would always occur, which can hinder further adsorption of enzymes from the solution on to the adsorbents. So, activity did not further get enhanced even if time of contact between support and the enzyme was increased.
3.7.4.1.2 Effect of initial protein concentration on protein loading and activity of immobilized enzyme

The trend of protein load that follows when initial concentration of enzyme was varied is shown in the figure: 3.40.

In the case of PG when concentration of initial protein was increased protein load also increased and then after an optimum of 19.5 mg it reached a plateau and further increase did not show any increase in loading. The optimum concentration indicates the maximum amount of enzyme that support can hold with retention of its activity. But this may not be the same for all supports because as multilayer adsorption comes into play, masking of number of active sites available may occur at optimum concentration and that will result in decrease in activity for some supports.

PN also showed an increase in loading of enzyme as concentration of initial protein was increased. This is because an increase in concentration will result in an increase of transport rate from the bulk solution to the solid-liquid interface and lead to the formation of clusters by aggregation of protein molecules [114,115].

For PN at initial concentration of 19.5 mg maximum protein load of 8.38 mg was obtained. This can be also explained based on the increase in surface area for PN when compared to PG, which allowed maximum amount of protein to be adsorbed on PN than on PG. After this range loading get decreased and this might be due to desorption of enzymes as result of weakening of the bonds that hold enzyme to the support.

It has been concluded that maximum adsorption occurs at near the isoelectric point [116,117]. Thus PN had its highest adsorption of amylase at pH 5. So this pH was selected as pH of immobilization medium to find out further optimization parameters.
The trend of immobilized enzyme activity for PGE and PNE when initial protein concentration was varied is shown in the figure: 3.41.

The higher the concentration of anchor groups, the higher will be the amount of bound protein and the probability of the multipoint attachment of proteins to the carrier increases. As a consequence of such relation, three kinds of responses in the immobilized enzyme activity could be noticed. “Firstly, the overloading of carrier’s surface may cause steric hindrance of large starch molecule and enzyme active site, lowering the enzyme activity. Secondly, decrease of activity as a result of changes in the enzyme structure by multipoint covalent modification. Thirdly, greater number of linkages between protein and support should result in more stable immobilized preparation” [71,118].

In the case of PNE activity enhanced when compared to PGE. This is because of the increase in surface positive charge for PN which arises as a result of protonation of amino groups at their surfaces. The increase in positive charge will result in strong electrostatic interaction between PN and amylase particularly near their isoelectric point. Hence change in structural conformation of enzyme at its active site will be negligible and hence the resultant loss in activity is not as much as that occurs via covalent bonding.
For PG the optimum activity was 8.5 EU at initial protein concentration of 19.5 mg and for PN it was 12.24 EU at the same initial protein concentration.

As the amount of bound enzyme increased the total activity of immobilized enzyme increased also but the rate of increase slackened as more enzymes was bound [119]. The results are shown in the figure: 3.41.

**Figure: 3.41** Influence of initial protein concentration on immobilized enzyme activity

3.7.4.1.3 Effect of initial protein concentration on the immobilization yield and activity of loaded enzyme.

Immobilization yield obtained for all adsorbents at various concentration taken are shown in the figure: 3.42.

**Figure: 3.42** Variation of immobilization yield with initial protein concentration
Immobilization using glutaraldehyde activated support was done via rapid “intra-molecular” reaction between nucleophiles of the enzyme and very near glutaraldehyde groups in the support. Immobilization yield was not as much as expected which might be due to cross-linking that occurred between glutaraldehyde reactive end groups as a result of which few number of activated ends was available for enzyme binding.

For PG when 19.4 mg of initial protein was added the immobilized enzyme showed maximum activity even if immobilization yield was low. This might be due to availability of more active sites at this concentration for the enzyme to carry out starch hydrolysis with maximum retention of its activity. The change in conformation of enzyme structure is the final parameter upon which the optimum activity depends and not on the immobilization yield which increases with increase in protein concentration.

Thus immobilization yield for PN was found to be 43% which was higher than PG. This is because of presence of amino groups at the surface of PN which enhanced the enzyme adsorption capacity as a result of strong positive charge near its isoelectric point.

Immobilization efficiency and activity yield can thus be summarized as follows in the table: 3.13. As shown in the table PG has the immobilization efficiency of 95% whereas, PN has 86%. But activity yield was higher for PN than for PG which was 37% and 26% respectively.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Initial protein (mg)</th>
<th>Immobilized protein mg/g support</th>
<th>Immobilization yield (%IY)</th>
<th>Initial activity (EU)</th>
<th>Immobilized enzyme activity EU</th>
<th>Activity Yield (%AY)</th>
<th>Immobilization efficiency (%)</th>
<th>IE=AY/IY</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>19.5</td>
<td>5.24</td>
<td>27</td>
<td>33</td>
<td>8.5</td>
<td>26</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>PN</td>
<td>19.5</td>
<td>8.38</td>
<td>43</td>
<td>33</td>
<td>12.2</td>
<td>37</td>
<td>86</td>
<td></td>
</tr>
</tbody>
</table>
3.7.4.2 Effect of pH on enzyme activity.

The variation in enzyme activity with change in pH of the reaction medium is shown in the figure: 3.43. The reaction pH was varied in the range 4-8 for all supports and it was found that both covalently bound amylase and adsorbed amylase followed a different trend.

Table 3.14: Optimum pH obtained for free enzyme, immobilized PG and PN

<table>
<thead>
<tr>
<th>pH</th>
<th>Free enzyme</th>
<th>PGE</th>
<th>PNE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 &amp; 5.5</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

For PNE the pH range in which maximum activity observed was within the range 6-7 with optimum pH at 6. This shifts results due to change in structural configuration of the immobilized enzyme. According to Singh et al. the strength of the electrostatic interaction between the enzyme and the support is very important in deciding the overall activity of the enzyme. As pH was increased the activity of both free and immobilized enzyme declined. The activity of immobilized enzyme is higher as compared to free enzyme in the studied pH range [167].

Figure: 3.43: Effect of pH on the activity of free and immobilized α-amylase

For PGE, immobilized enzyme was active in the range pH 5-6 with optimum at pH 5. This enhanced activity over a wider pH range could result from the change in acidic and basic amino acid side chain ionization in the microenvironment around the active site, which was caused by the newly formed
interactions between basic residues of enzyme and glutaraldehyde activated support [120,121].

The decline in amylase activity in the presence of glutaraldehyde may be the result of steric hindrance caused by the presence of the cross linking agent, which may have affected the accessibility of the substrate to amylase [122]. This can be explained by the fact that covalent bonding between enzyme and support restrict chain mobility within protein molecules and therefore conformational changes that are essential during catalysis are disabled. However, despite this rather low relative activity, immobilized enzymes with an activity approximately the same as the free enzyme have the potential to be used in industry. The costs saved by recovery and reuse of the enzymes can compensate for the loss of activity [123].

3.7.4.3 Effect of temperature on the activity

The temperature of the free and immobilized enzymes was determined by incubation at temperatures ranging from 30 to 70°C.

From the figure: 3.44 it is clear that the maximum activity of PGE was observed at 55°C. The soluble enzyme exhibited 27% activity at 60°C, whereas, PGE had around 69% activity at 60°C. At 70°C PGE showed 54% of activity whereas, free enzyme could give only 14% of its initial activity at same temperature. The increase in optimum temperature was caused by the change in physical and chemical properties of the enzyme as a result of immobilization. The covalent bond formation via amino groups of the immobilized enzyme might have also reduced the conformational flexibility, thereby resulting in higher activation energy for the molecule to reorganize and attain the proper conformation for binding to substrate [124].

For PNE the optimum temperature shifted to 60°C with the retention of 95% of its activity. Similar shift by 10°C to higher temperature was observed by Singh et al. in their work where they have immobilized Pullulanase from Bacillus
Immobilization of Diastase $\alpha$-amylase on to Synthetic Polymers

$acidopullulyticus$ on to the hydrophobic synthetic macroporous resin Duolite XAD761 via covalent method, through the formation of a Schiff base [125]. Even at 70°C 68% of activity was retained by PNE.

Both the immobilization systems could provide better heat resistance compared to free enzyme allowing activity at elevated temperatures. In short, the free preparations showed a remarkable decrease of the activity surpassed their optimal temperature, whereas, the immobilized preparations exhibited an activity that was never less than 60% in the entire interval of temperatures considered. This might be due to the presence of coupling agents which prevents intense rigidification and unfavourable conformational changes in the enzyme structure [126].

Figure: 3.44 Effect of temperature on the percentage relative activity of free and immobilized enzymes

3.7.4.4 Thermal stability of the free and immobilized enzymes

The thermal stability of the enzyme preparations was determined by incubation in appropriate buffer at temperatures ranging from 30° to 60°C for 1 hour. The results of thermal stability experiments showed that both covalent and adsorbed enzymes have improved thermal stability than free enzyme.

The activity is less for covalently immobilized enzyme compared to adsorbed one. Stability at higher temperature when analyzed was more for covalently
immobilized enzyme. This is due to the fact that covalently immobilized enzyme PGE was protected from the conformational changes caused by the environment.

The increase of thermal stability after immobilization can be explained by the fact that immobilization of the enzyme in their respective supports were able to preserve the tertiary structure of the enzyme from conformational changes caused by the environment [127]. The results are summarized in the figure: 3.45 shown below.

![Figure: 3.45 Thermal stability of free and immobilized enzyme](image)

When the immobilized enzymes were subjected to pre-incubation at regular time intervals in their respective buffer solutions similar trend was again repeated and results obtained are shown in the figure: 3.46 below.

Both the immobilized forms retained 80-90% of their initial activity after 40 minutes of pre-incubation time. With increase of pre-incubation time PGE retained 76% of its initial activity whereas, PNE retained 72% after 60 minutes at 40°C. After 80 minutes of pre-incubation time PGE retained 65% of its initial activity and PNE retained 61%. Finally an activity of 55% was retained by PGE and 45% by PNE after 100 minutes of pre-incubation time. This indicates greater rigidity of the immobilized α-amylase leading to enzyme molecules that are more resistant to unfolding at higher temperatures than the free form.
Figure: 3.46: Effect of pre-incubation time on relative enzyme activity of free and immobilized enzymes.

The surface on which the enzyme gets immobilized is responsible for retaining the tertiary structure in the enzyme through hydrogen bonding or the formation of electron transition complexes. These links will prevent vibration of the enzyme and thus increase thermal stability [128,129]. The stability of immobilized enzymes at low pH and its increased activity at high temperatures implies that the enzyme can be applied in starch hydrolysis at low pH and moderately in high temperatures of 60°C and above to minimize contamination during processing [130,112].

3.7.4.5 Determination of kinetic parameters

When a biocatalyst is immobilized, kinetic parameters $K_m$ and $V_{max}$ undergo variations with respect to the corresponding parameters of the free form, revealing an affinity change for the substrate [131].

The effect of immobilization on kinetic parameters was studied by measuring the rates of starch hydrolysis by free and immobilized amylase at various concentrations of starch. The Lineweaver-Burk plot and Hanes-Woolf plots for the free and immobilized $\alpha$-amylase is given in figure: 3.47. The $K_m$ and $V_{max}$ values were calculated from the slope and intercept of the straight lines, respectively and results are tabulated in the table 3.15.
An increase of the $K_m$ suggests the requirement of higher substrate concentration to achieve the same reaction rate observed for the free enzyme [132].

**Table 3.15:** Kinetic parameters for immobilized $\alpha$-amylase on PG and PN

<table>
<thead>
<tr>
<th></th>
<th>Free enzyme</th>
<th>PGE</th>
<th>PNE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (mg/ml)</td>
<td>$0.50 \pm 0.04$</td>
<td>$1.88 \pm 0.03$</td>
<td>$3.57 \pm 0.04$</td>
</tr>
<tr>
<td>$V_{max}$ (mg/ml/min)</td>
<td>$7.40 \pm 0.05$</td>
<td>$1.80 \pm 0.02$</td>
<td>$3.56 \pm 0.03$</td>
</tr>
</tbody>
</table>

**Figure: 3.47:** Lineweaver-Burk plots for $\alpha$-amylase immobilized on (i) PG (ii) PN and Hanes-Woolf plots of (iii) PG and (iv) PN

Since the enzymatic reactions take place through a carrier medium the affinity of the enzyme towards the substrate is not the same in every carrier media.
Thus higher $K_m$ value can be attributed to the diffusional resistance of the carrier against substrate and or product and less porous structure. This occur either due to structural changes in the enzyme induced by the applied immobilization procedure or due to the lower accessibility of the substrate to the active site of the immobilized enzymes [133,134]. In other words, the increase in $K_m$ values after immobilization may be partially due to mass transfer resistance of the substrate into the immobilization medium.

The decrease in $V_{max}$ value as a result of immobilization should be related with the increase in $K_m$ value. Since immobilization brings about conformational changes of the enzyme, which usually decreases the affinity to the substrate. As a result of this partial inactivation of all or the complete inactivation of the part of the enzyme molecules may occur and hence decrease of $V_{max}$.

Hanefeld et al. reported that the difference in kinetics between lipases immobilized on different supports was ascribable to conformational changes induced upon enzyme-polymer interaction [135]. A similar observation had been reported for immobilized invertase on white and black lahar (volcanic mudflow) by the silane-glutaraldehyde method [136]. Immobilization brought about an increase in the $K_m$ but a decrease in the $V_{max}$ and these changes were correlated to immobilization induced conformational changes in the enzyme [137].

### 3.7.4.6 Storage stability of Immobilized α-amylase

When stored in buffer solution the starch hydrolyzing activity of the α-amylase immobilized via covalent coupling decreased at a slower rate than that of the adsorbed enzyme. Upon 4 months of storage, the adsorbed enzyme preserved 40% of its initial activity whereas; covalently bound enzyme retained 55% of its initial activity. This is because conformational changes due to immobilization help the enzyme to suitably orient its active site towards the substrate [138].
The immobilization of enzyme to a support often limits its freedom to undergo drastic conformational changes and hence results in increased stability. Storage stabilities of free and immobilized enzymes studied at 4°C is depicted in the figure: 3.48. The decrease in activity was explained as time-dependent natural loss in enzyme activity.

![Figure: 3.48: Storage stability studies of α-amylase immobilized on PG and PN](image)

### 3.7.4.7 Reusability

The operational stability of the α-amylase was evaluated in a repeated batch process. The results in figure: 3.49 indicated that the catalytic activity of the immobilized enzyme was durable under repeated use.

In the case of PNE up to 7 cycles 70% activity was retained which decreased drastically for further cycles. PGE the immobilized enzyme was able to maintain good activity up to 60% even after five runs. There was no drastic decrease in percent hydrolysis even after five uses, which could be due to glutaraldehyde treatment of polypyrrole which prevented the leakage of enzyme. Similar results were reported by S. Talekar et al. [139]. As per their study α-amylase CLEAs retained 65% activity after 4 reuses with 30 min of reaction time.

Ates and Mehmetoglu found that after treatment with glutaraldehyde the Cu-alginate immobilized enzyme could be used 8 times with high activity. This
improved stability of immobilized $\alpha$-amylase can be attributed to the improved resistance to denaturation and conformational changes of the enzyme in buffer solution, as a result of the covalent bonding procedure of the amylase molecules on PG [140].

![Figure: 3.49](image)

**Figure: 3.49**: Reusability studies and for $\alpha$-amylase immobilized on PG and PN

### 3.8 Significance of polypryrole copolymers as support for enzyme immobilization

In order to further enlarge the application of PPY in sensors, much effort has been carried out in recent years to fabricate functionalized PPY derivatives through either modification of the pyrrole monomer’s structure or the preparation of block and graft copolymers [141,142]. The former method, namely, the synthesis of pyrrole monomer substituents, is very appealing because of the ease of polymerization and the wide variety of functional groups that can be linked to pyrrole, which enables post functionalization of the resulting polymer. Surface modification of these materials with biological moieties is desired to enhance the biomaterial-tissue interface and to promote desired tissue responses.

Copolymerization of a pair of monomers will lead to an increase in the number of conductive polymers that can be made from the same set of monomers
Moreover, it is also likely that the copolymer will incorporate the unique properties of homo-polymers. A growing interest in copolymers as immobilization supports can be observed, since by changing the ratio between the monomers, the properties of the copolymer (hydrophilicity / hydrophobicity, amount of functional groups, mechanical properties, porosity, etc.) can be desirably changed. Therefore custom-made supports for enzyme immobilization of all kinds for different purposes can be produced [144].

Among the various conductive polymers studied, polyaniline (PANI) and polypyrrole (PPY) have been of particular interest due to their high electric conductivity, environmental stability and low cost of production and favourable physico-chemical properties associated with the chain heteroatoms. Another reason for the great deal of attention given to PANI-PPY copolymers is probably attributed to the great difficulty to synthesize new conducting polymers with electric properties and stability that are better than polyaniline and polypyrrole. Fusalba et al. have reported that the main motivation for preparing copolymer composites lies in the possibility that these materials overcome the limitation of the rareness of new conjugated pi bond containing monomers [145]. Both polyaniline and polypyrrole have secondary amino groups in their polymer backbone. In polyaniline, steric effects of benzene rings might block access by the amino groups in the polymer backbone. Amino groups in polypyrrole are accessible to face protein molecules without steric hindrance. So for polyaniline adsorbent very low loading was observed during enzyme immobilization [146]. Copolymerization will enhance the enzyme loading capacity as it has the added advantage of polypyrrole along with polyaniline in the polymer backbone.
Another way of increasing enzyme adsorption efficiency is by copolymerizing with a modified monomer having any substituted functional group. 3-substituted and N-substituted pyrroles are the most used derivatives of pyrrole. When compared to 3-substituted pyrrole which are asymmetric molecules, N substituted pyrrole are fundamentally symmetric. Thus polymerization of N-substituted pyrrole will result in an increased order and a planarity of polymer backbone. Consequently N-substituted pyrrole derivatives are more desirable forms than their 3-substituted counterparts. Thus amylase immobilization was carried out on two copolymers namely pyrrole-aniline copolymer and pyrrole - 1-(2- amino phenyl) pyrrole. Songul et al. have reported the use of N-substituted polypyrrole derivatives towards glucose sensing electrodes [147].

### 3.8.1 Preparation of polypyrrole co-polymers

#### 3.8.1.1 Preparation of poly (Pyrrole -co-1-(2-aminophenyl pyrrole)

1-(2- aminophenyl pyrrole) \((4.0 \times 10^{-3} \text{mol}, 0.632 \text{ g})\) was dissolved in 200ml CHCl₃. This solution was treated with an ultrasonic bath for 15 minutes to obtain the best dispersion of NoaPy. Pyrrole \((4.0 \times 10^{-3} \text{mol}, 0.278\text{ml})\) was added to this solution. The solution was maintained in an inert N₂ atmosphere and under
magnetic stirring, whereas FeCl₃ (0.01 mol, 0.812g) in 200 ml of CHCl₃ were slowly dropped to the monomer solution during 30 minutes. n_{ox}/n_{mon} ratio was taken as 2.5 for the synthesis. After polymerization time 24 hours, precipitated polymer was filtered and washed with firstly CHCl₃ then with ethanol until the filtrate was colorless. Finally, the polymer was dried at 50°C for 24 hours under vacuum environment [147].

The reaction is depicted as follows:

![Scheme 3.8 Copolymerization of pyrrole with 1-(2-aminophenyl pyrrole)](image)

### 3.8.1.2 Preparation of poly (aniline-co-pyrrole)

Copolymerization of pyrrole and aniline was carried out similar to the polymerization of pyrrole. Typical experimental procedure (aniline: pyrrole = 1:1) was as follows. Aniline (0.9313g, 0.01mol) was added to 100ml 0.1M HCl solution, which was soon followed by addition of pyrrole (0.67g, 0.01mol) dissolved in 100ml 0.1M HCl with stirring. Final mixture was stirred vigorously for 30 minutes in an ice bath. To this pre-cooled solution of 10 ml 2M APS was added drop wise with constant stirring and the temperature is maintained at 0-5°C. Further agitation was applied for 24 hours after the dropping process was completed. The product was then washed and filtered three times with de-ionized water to eliminate unreacted oxidants and the oligomers. Finally black powder obtained was washed with methanol [148,149].
3.8.2 Physico-chemical characterization

3.8.2.1 FT-IR Spectra of polypyrrole copolymers.

Pyrrole-aniline copolymer:

For the PYPA copolymer, the spectra recorded show most of the characteristic bands of PPY and PANI. The peaks at 1385 cm\(^{-1}\), 1288 cm\(^{-1}\) and 1196 cm\(^{-1}\) is due to various C-N stretching vibrations. It is observed that the peaks shifted to a lower wave number for the copolymer (poly (aniline-co-pyrrole)) demonstrating the presence of neighbouring aniline and pyrrole constitutional units [150].

Another interesting point of the spectra is that the quinonoid phenyl ring C-C stretch band of PANI at 1600 cm\(^{-1}\) and the C=C / C-C stretching mode of PPY at 1560 cm\(^{-1}\) have combined to form a broader band at around 1588 cm\(^{-1}\). A new band at 1112 cm\(^{-1}\) can be observed which can be attributed to the C-H in plane bending on the 1,2,4-substituted benzene of PANI. From this new band, it may be deduced that the pyrrole units in the copolymer are likely to be attached to the 1,2 or 4 positions on the benzene ring [151].
Secondly, the para-substituted aromatic C-H out of plane bending band of PANI at 840 cm\(^{-1}\) has greatly diminished in copolymer spectra and is shifted to a lower wavelength which is only a very weak band at 832 cm\(^{-1}\). This observation is likely to be caused by the replacement of aniline units by pyrrole units along the chains.

The C-H out of plane bending mode of copolymers show more complicated absorption bands than that of polyaniline or polypyrrole.

After immobilization of \(\alpha\)-amylase wavenumber gets shifted for both copolymers. Thus for poly (pyrrole- co- aniline) the wave number at 3454 cm\(^{-1}\) corresponds to N-H stretching vibration. The bands at 2928 cm\(^{-1}\) and 2854 cm\(^{-1}\) are attributed to C-H stretching vibration. The bands at 1648 cm\(^{-1}\), 1542 cm\(^{-1}\), 1504 cm\(^{-1}\) and 1397 cm\(^{-1}\) were of increase in intensity and is attributed to that of \(\alpha\)-amylase. The other bands were lowered in intensity and wave number compared to parent polymer.

**Pyrrole-1-(2 aminophenyl pyrrole) copolymer**

FTIR spectra of PYPH copolymer were shown in figure: 3.50. The sharp peak at 1512 cm\(^{-1}\) is one of the characteristic C=C aromatic stretching vibration. Whereas the peaks around 1000 cm\(^{-1}\) are out of plane C-H vibrations, absorption band at 1462 cm\(^{-1}\) is attributed to in plane C-H stretching vibrations of pyrrole and phenyl rings [152].

The peaks appeared in the range of 1265 cm\(^{-1}\) and 1045 cm\(^{-1}\) are the characteristic peaks for pyrrole rings [153]. The bands, which belongs to either pyrrole or phenyl ring were observed from the FTIR spectra. The peak at 1162 cm\(^{-1}\) and 1090 cm\(^{-1}\) confirm the presence of benzene ring in the structure.

The peaks at around 793 cm\(^{-1}\), 742 cm\(^{-1}\) and 621 cm\(^{-1}\) in the spectra were assigned to the out-of-plane vibration of three adjacent carbon-hydrogen bonds which reflected the substituted benzene ring [147, 154].
The peak at 609 cm$^{-1}$ is attributed to C-H aromatic in plane vibration. Several bands occurring in the regions, 1609-1581 cm$^{-1}$ and the 1574-1490 cm$^{-1}$ can be attributed to the phenyl $\nu$C=C stretching vibrations.

The bands located in the 1498-1424 cm$^{-1}$ region are due to the C=C pyrrolic ring stretching vibrations. The intensities of the absorption band characteristic for the collective vibration mode located around 1550 cm$^{-1}$ decrease due to the presence of substituted pyrrole monomer. This fact also indicates a reduction of the effective conjugation length of the polymeric chains due to the conformational modifications induced by the substituted pyrrole [155].

For immobilized poly (pyrrole-co-1-(2-aminophenylpyrrole)) decrease in intensity of characteristic bands occur. Along with that some additional bands assigned to $\alpha$-amylase was also observed at 1656 cm$^{-1}$, 1542 cm$^{-1}$, 1398 cm$^{-1}$, 1317 cm$^{-1}$ which was absent in the spectra of parent polymer.

Peak assignments for copolymers and their immobilized forms are given in the table 3.16.

**Figure: 3.50** FT-IR spectra of copolymers
Table 3.16 Peak assignments for copolymers before and after enzyme immobilization

<table>
<thead>
<tr>
<th>Peak assignments (cm⁻¹)</th>
<th>PYPH</th>
<th>PYPH E</th>
<th>PYPA</th>
<th>PYPAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H stretch</td>
<td>3433</td>
<td>3440</td>
<td>3396</td>
<td>3452.</td>
</tr>
<tr>
<td>C-H stretch</td>
<td>2965,2929,2857</td>
<td>2961,2824, 2854</td>
<td>2961, 2926, 2852</td>
<td>2924,2855</td>
</tr>
<tr>
<td>C – O</td>
<td>1688 vvw</td>
<td>1708,1747</td>
<td>1710</td>
<td>1748,1704</td>
</tr>
<tr>
<td>C-N-C ring -in plane deformation</td>
<td>1646, 1656b</td>
<td>1590</td>
<td>1650bm,</td>
<td></td>
</tr>
<tr>
<td>NH₂ in plane deformation</td>
<td>1615bm</td>
<td>1621</td>
<td>1624</td>
<td>1617</td>
</tr>
<tr>
<td>N-H ring in plane bending(s)</td>
<td>1550</td>
<td>1542</td>
<td>1582, 1492, 1457</td>
<td>1542</td>
</tr>
<tr>
<td>C-C in ring stretch(s)</td>
<td>1337</td>
<td>1336,1317</td>
<td>1336, 1316</td>
<td>1363,1317</td>
</tr>
<tr>
<td>Aromatic C-H in plane bend</td>
<td>1158,benz</td>
<td>1176</td>
<td>1171</td>
<td>1170</td>
</tr>
<tr>
<td>C-H ring in plane bending(w)</td>
<td>1112,</td>
<td>1115</td>
<td>1112,1123 sub,benz.</td>
<td>1100</td>
</tr>
<tr>
<td>C=C-N ring in plane deformation</td>
<td>1094</td>
<td>1070</td>
<td>1072</td>
<td>1061</td>
</tr>
<tr>
<td>Aromatic C-H out of plane bend</td>
<td>783,742,621</td>
<td>782</td>
<td>784</td>
<td>780</td>
</tr>
</tbody>
</table>

3.8.2.2 Thermogram of copolymers

Thermogram of copolymers PYPA and PYPH are shown in the figure: 3.51. In the case of PYPA a minor weight loss occur at 76°C due to water desorption. The major weight loss was observed in the range 264-564°C. This slow weight loss below 564°C may be due to degradation of low molecular weight and small chain segments. The next weight loss above 564°C is assigned to the structural decomposition of the polymer backbone.
In the case of immobilized PYPA, the minor peak at 198°C and 270°C is attributed to protein degradation. The major weight loss at 339°C and 568°C is assigned to degradation of copolymer structure. The minor weight loss which continues to occur after this temperature is the result of structural decomposition of polymer backbone.

Thermogram of PYPH showed more thermal stability than the PYPA which might be due to the presence of phenyl ring in the former. Two major decompositions and two minor decompositions were observed in the PYPH. The minor weight loss was observed in DTG (not shown) at 156°C which might be due to elimination of water molecules and unreacted monomers. The major weight loss at 305°C and 338°C might be due to the decomposition of copolymer of pyrrole-1-(2-amino phenyl pyrrole). The peak at 595°C corresponds to degradation of polymer backbone.

After immobilization the major weight loss was in the range 170°C -513°C, which correspondingly showed peaks in DTG at 305°C, 372°C and 428°C. These are assigned to protein organic structure decomposition, water desorption and polymer degradation.
3.8.2.3 Surface area analysis

For PYPH appreciable surface area was there and it comes in the range 37mg/g but for PYPH surface area was too low. This might be due to the presence of aminophenyl groups on the surface of copolymer that resulted in reduction of surface area when compared to bare polypyrrole.

The enzyme immobilized samples of all polymers showed corresponding decrease in surface area. Protein loading was found to be proportional to surface area of polymers. Thus as surface area increases loading capacity of the supports also increased.

3.8.2.4 Scanning electron microscopy

The copolymer formed between pyrrole and aniline PYPH showed spherical structures that are agglomerated. The size of the particles varied in the range 90-100 nm. It can be seen that pyrrole and aniline monomer affected the morphology of the obtained copolymer.

The copolymer of pyrrole and 1-(2-amino phenyl pyrrole) (PYPH) was found to have rough surface with flaky structures. The particles seems to aggregate into irregular shape and hence distributed non-uniformly.

Figure: 3.51: TG curves for (i) PYPH and PYPH (ii) PYPH and PYPHE
(iii) PYPH and PYPHE
3.8.3. Immobilization of α-Amylase on Polypyrrole Copolymers

3.8.3.1 Optimization of immobilization conditions

PYPH, co-polymer has (2-amino phenyl) functional group at N-position. So the conjugated backbone will be available for hydrophobic interaction and amino end will be favouring electrostatic interactions. Thus it is well documented that no particular property dominates the adsorption process; rather it is a combination of some, if not all, of the processes that determine the adsorption characteristics of proteins.

For PYPA copolymer both monomers are having conjugated rings which leads to hydrophobic interaction with proteins. But electrostatic interaction also has a secondary role here.

Even though isoelectric point of copolymers is not available it can be assumed from isoelectric point of homopolymers. As isoelectric point of polypyrrole is 7 and that of polyaniline is around 7.6, isoelectric point of copolymer must be close to this [146]. Also, in the case of PYPH isoelectric point will be close to that for polypyrrole. The variations in the adsorption capacity of copolymers are attributed to the influence of groups in the copolymers and also their surface area.
The pH dependence of the immobilized enzyme activity was compared with that of the free enzyme in the pH range of 3-8 at 30°C, and the results are presented in figure: 3.53.

The best pH for immobilization was found to be at pH 4 for PYPH and at pH 5 for PYPA. At pH 4 - 4.5 both amylase and amine groups on the support surfaces are positively charged and hence they repel each other. But results are contradictory showing adsorption in this region. This is because hydrophobic interaction might have dominated here which thus minimised the pH effect and electrostatic interaction is only having a secondary role.

Immobilization affects the three dimensional structure and the distribution of the functional groups of enzyme and as a result, a change in the microenvironment of the enzyme is expected. Therefore the stable substrate-enzyme transition complex may form at different pH values compared to that of free enzyme [156].

Below pH 4 and above pH 5 the relative activity of immobilized PYPH decreased sharply. Similarly below pH 5 and above pH 6 for PYPA the relative activity of the immobilized enzyme suffers a decline in activity.

These two observations might be due to the fact that difference in charge separation on surface of both supports have affected the free enzyme differently and that may owe to desorption of enzyme from the carrier or instability of pure enzyme in the regions below and above the optimum immobilization pH’s of both immobilized enzymes. This happens because the changes in pH bring about apparent changes in enzyme structure, which influence its activity.
Figure: 3.53 Effect of pH on the immobilization of α-amylase on to copolymers

3.8.3.1.1 Effect of contact time on the activity of immobilized enzymes

The contact time needed for enzyme to get adsorbed on PPY adsorbents is shown in the figure: 3.54.

Figure: 3.54: Effect of contact time on immobilized enzyme relative activity

It was optimized by allowing free enzyme in buffer solution to be in contact with the supports for different known time intervals. The support gets saturated with the enzyme after a particular interval of time. The maximum activity was represented as 100% and other activities are represented as relative to this optimum activity. After this particular contact time enzyme starts to get desorbed from the
support due to multilayer adsorption and hence will show corresponding loss in activity when compared with optimum.

Thus PYP shows maximum adsorption rate when kept in contact with enzyme for 240 minutes, and PYPH shows maximum adsorption rate within 180-240 minutes of contact time. Above this time interval there was decrease in enzyme loading on the support which thereby reflected in its activity.

3.8.3.1.2 Effect of initial protein concentration on the protein loading

![Graph](image)

**Figure: 3.55:** Effect of initial protein concentration on the loading capacity of copolymers (i) PYP (ii) PYPH.

The amount of protein loaded to PPY copolymers, is given in figure: 3.55.

On the addition of protein to support, immobilization occurs immediately and loaded amount increases and reaches a saturation point. Further addition of enzyme did not make any remarkable increase in loading. The saturation point depends upon the properties of the support and the methods of immobilization.

Effects of immobilization depends on several parameters like surface area of supports, accessibility of support surface for enzymes, number of activated functional groups on the support, distance between bound enzyme and the surface of the support and conformation at the active site. Mono- or multipoint binding of
Immobilization of Diastase α-amylase on Synthetic Polymers

an enzyme to the support, chemical affinity of the protein to the material of the support are further factors influencing immobilization [157,158].

Here, in the case of PYPH surface area obtained was higher than PYPH and that correspondingly reflected in loading of protein on to the supports. Consequently protein load of PYPH is found to be maximum compared to PYPH. For PYPH enzyme loading of 4.3 mg/g support was the maximum whereas, for PYPA 12 mg/g support was the optimum loading.

After the saturation point for both supports immobilization rate starts decreasing because multilayer adsorption will result in lowering of available active sites of enzyme thus favouring only weaker interaction of proteins with the support. This will result in easy desorption of protein molecules anchored strongly on to the support.

3.8.3.1.3 Effect of initial protein on immobilization yield and activity of loaded enzyme

Immobilization yield obtained for all adsorbents at various concentrations taken are shown in the figure: 3.56.

For PYPA as concentration of enzyme increases immobilization yield which gives the percentage of protein loaded, increases, up to 17 mg which then starts decreasing. The optimum percentage yield thus obtained for PYPA is 67%.

Even though optimum yield obtained for PYPH was 74% the higher protein load was at lower concentration of 9.3 mg where immobilization yield was only 46%. This lower loading is due to the multilayer adsorption which results in poor interaction between enzyme and support. As a consequence chances of enzyme desorption from the surface increases or it may cause lesser exposure of all active sites of the enzyme present on the support.
Figure: 3.56: Effect of initial protein concentration on the immobilization yield of protein bound on copolymers (i) PYPA (ii) PYPH

Corresponding variation in immobilized enzyme activity is shown in the figure: 3.57. In both cases optimum immobilized enzyme activity was shown at the concentration in which optimum loading occurred. This might be due to the presence of greater number of available active sites with proper orientation, which was favourable for the substrate to undergo chemical reaction easily and hence resulted in enhanced activity of immobilized enzyme.

For PYPA optimum immobilized enzyme activity of 14 EU was at protein load of 12 mg and for PYPH the optimum activity of 7 EU was at optimum protein load of 9 mg. To get overall results of enzyme support interaction evaluation of activity yield and immobilization efficiency is inevitable. Hence they were calculated using standard equations using results obtained above and is tabulated as shown below.

Table 3.17: Immobilization efficiency of α-amylase on copolymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Initial protein (mg)</th>
<th>Immobilized protein (mg/g support)</th>
<th>Immobilization yield (%)</th>
<th>Initial activity (EU)</th>
<th>Immobilized enzyme activity EU</th>
<th>Activity Yield (%)</th>
<th>Immobilization efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYPA</td>
<td>18</td>
<td>12.1</td>
<td>67</td>
<td>40</td>
<td>14.4</td>
<td>36</td>
<td>54</td>
</tr>
<tr>
<td>PYPH</td>
<td>9.3</td>
<td>4.3</td>
<td>46</td>
<td>26</td>
<td>4.7</td>
<td>18</td>
<td>39</td>
</tr>
</tbody>
</table>
Figure: 3.57: Effect of initial protein concentration on immobilized enzyme activity in the case of copolymeric supports (i) PYPH (ii) PYPA

The maximum activity yield obtained for PYPA was 36% which has the highest value for immobilization efficiency of 53.7%. Indicating that immobilized enzymes retained their original activity.

PYPH loading was lower due to its low surface area and hence activity yield was only 18% with immobilization efficiency of about 39%. This is the consequence of lower loading and improper conformational orientation of enzyme that get anchored to the support. The enzyme-substrate affinity correspondingly gets lowered which is reflected in the poor activity yield and subsequent decline in the immobilization efficiency. Hasirci et al. studied poly (dimer acid-co-alkyl polyamine) particles that were activated by CDI, EDA, and HMDA, respectively [159]. The amount of bound enzyme was found as 7.6, 6.5 and 39.3 mg/g of each particle respectively. Several studies reported shows that binding capacity of the support materials is labile due to characteristic properties of prepared materials [160].

3.8.3.2 Effect of pH on enzyme activity

The pH dependent activity profiles of both free and immobilized amylase are shown in figure: 3.58.
Figure: 3.58: Effect of pH on the relative activity of free and immobilized enzymes on copolymers

Table 3.18: Optimum pH for immobilized enzymes

<table>
<thead>
<tr>
<th></th>
<th>Free enzyme</th>
<th>PYPae</th>
<th>PYPHE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5 &amp; 5.5</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

In the pH range 5-7 all the copolymers are having positive charges on their surfaces which thus favours strong electrostatic interaction with amylase which is negatively charged at this pH [162]. For both copolymers optimum reaction pH was 6. This shift is due to the increase in positive charge on amine groups on both copolymers. As a result, the concentration of H⁺ ions in the microenvironment of the immobilized enzyme decreases which means that the pH of the immobilized region is more alkaline than that of external solution. Immobilization often times lead to a shift in pH either towards the acidic side or towards the alkaline side [163,164].

Factors which influence the pH of immobilized enzyme include surface and residual charges on solid matrix and the enzyme bound pH in the micro-environment as compared to the bulk environment [165]. According to Dhingra et al. optimum pH of an enzyme shifts upon immobilization, particularly when the support material is charged. Strong interactions between enzyme and support affect intra-molecular
forces responsible for maintaining the enzyme conformation leading to a resultant change in enzyme activity [161]. Increased stability of immobilized α-amylase over a wide range of pH (4 to 8) is an indication of greater insensitivity of the enzyme to changes in environmental pH, brought about by conformational changes following immobilization [166].

**3.8.3.3 Effect of temperature on the activity**

The effect of reaction temperature on the activity of both free and immobilized enzymes are shown in the figure: 3.59. In fact the free preparation showed a remarkable decrease of the activity, surpassed their optimal temperature, whereas, the immobilized preparations exhibited an activity that was never less than 60% in the entire interval of temperatures considered.

For PYPH the optimum temperature was at 50°C which is same as that of free enzyme. Similar result showing no change in optimum temperature was also observed by Tanriseven and Olcer [168].

For PYPA, optimum temperature shifted to 60°C. Similar shift to higher temperature after immobilization has been observed by other authors [169,170]. Thus the results again confirmed that the immobilized α-amylase holds greater heat resistance than that of free enzyme.

The decrease of relative activity of the free enzyme is probably due to its thermal denaturation [167], while the relative activity of the immobilized enzyme decreased only slowly because of some protections of the carrier for the immobilized enzyme. The increase of optimum temperature for the immobilized enzyme may arise from the change of the conformational integrity of the enzyme structure upon adsorption to the carrier materials [165,171].
Compared to the free enzyme, the higher activity of immobilized enzymes at elevated temperatures and ability to hydrolyze starch would help overcoming problems related to gelatinization of starch during hydrolysis [172].

3.8.3.4 Thermal stability of the free and immobilized enzymes

Thermal stability of both free and immobilized enzymes were investigated and the results are shown in the figure: 3.60.

Both free and immobilized enzyme showed maximum activity when incubated at 30°C for 1 hour. As the temperature increases, the stability dropped significantly for both free and immobilized amylase. At 40°C, both free and
immobilized enzyme retained 80-90% of their activity. At 50°C for 1 hour the immobilized enzyme was inactivated at a much lower rate than the free enzyme. The free enzyme lost almost 75% of their activity at 60°C after 1 hour treatment whereas, immobilized amylase lost only 20-30% of their activity [174]. Figure: 3.61 shows effect of pre-incubation time on the activity of each immobilized enzyme.

About 50-60% of immobilized enzymes maintained their activity when subjected to 120 minutes of pre-incubation time at their respective optimum temperature whereas free enzyme could retain only 10% of their initial activity when subjected to same period of time. These results suggest that the thermal stability of α-amylase increased considerably as a result of immobilization on to PPY adsorbents and is suitable for industrial applications. Thermal stability upon immobilization is the result of molecular rigidity and the creation of a protected microenvironment [173].

Similar increase in thermal stability was also reported by N. Tuzmen et al. [175]. The authors have carried out adsorption of α-amylase on to Magnetic poly (2-hydroxyethylmethacrylate)/Cibacron blue [mPHEMA]/CB beads. Immobilization brings about more stable conformation by restricting the mobility of the enzyme due to which thermal denaturation of enzyme native conformation via unfolding is hindered and hence enzyme can withstand distortion or damage by heat exchange.

Similar observation was also observed by P. Singh et al. when α-amylase was immobilized on cation exchange resin [176]. The stability of α-amylase at low pH and its increased activity at high temperatures implies that the enzyme can be applied in starch hydrolysis at low pH and moderately in high temperatures of 60°C to minimize contamination during processing [112,177].
3.8.3.5 Determination of kinetic parameters

Enzyme immobilization may produce both conformational and micro-environmental effects that will affect the kinetics of enzyme catalyzed reactions [178].

Conformational effects refer to the structural changes produced in the enzyme molecule as a consequence of the immobilization procedure. Alteration of the native three-dimensional structure of the enzyme protein and the steric effects due to its close proximity to the surface of the support are the conformational changes that may produce differences in kinetic behavior of immobilized enzyme with respect to the free enzyme.

Micro environmental effects refer to partition and mass transfer limitations. Partition of substrates and products to the biocatalyst and products, transport from it back into the bulk reaction medium affect the kinetics of the enzyme catalyzed reaction [179]. The kinetic parameters of free and immobilized enzyme are shown in table 3.19. Lineweaver-Burk Plots and Hanes-Woolf plots of free and immobilized α-amylase is shown in figure: 3.62
Figure: 3.62: Lineweaver-Burk plots for α-amylase immobilized on copolymers
(i) PYPA (ii) PYPH. Hanes-Woolf plots (i) PYPA (ii) PYPH

Table 3.19 Kinetic parameters for free and immobilized enzymes on copolymers

<table>
<thead>
<tr>
<th></th>
<th>Free enzyme</th>
<th>PYPA</th>
<th>PYPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (mg/ml)</td>
<td>0.50 ± 0.04</td>
<td>2.73 ± 0.06</td>
<td>2.47 ± 0.04</td>
</tr>
<tr>
<td>$V_{max}$ (mg/ml/min)</td>
<td>7.40 ± 0.05</td>
<td>4.21 ± 0.04</td>
<td>2.95 ± 0.03</td>
</tr>
</tbody>
</table>

3.8.3.6 Storage stability of Immobilized α-Amylase

The stability of immobilized α-amylase stored at 4°C in buffer solution was determined by periodically analyzing samples over 6 months for its activity retention. The trend in stability during 6 months of storage is shown in the figure: 3.63.
PYPA retained 50% of its initial activity and PYPH retained 25% of its initial activity after 6 months. Similar improvement in storage stability for a longer period was reported by S. Dhingra et al. [180]. This difference in activity between two immobilized enzymes is dictated by many factors such as number of bonds formed between enzyme and support, the nature of the bonds, the degree of confinement of enzyme molecules in the matrix, and immobilization conditions [172,181,182].

On the basis of results obtained it was concluded that these supports provide better stabilization effect, minimizing possible distortion effects, which might be imposed from aqueous medium on the active site of the immobilized enzyme. PYPA shows outstanding performance compared to PYPH. The generated multipoint ionic interactions between enzyme and matrixes also conveyed a higher conformational stability to the immobilized enzyme.

![Graph showing relative activity over months for PYPA and PYPH.]

**Figure: 3.63** Storage stability studies of α-amylase immobilized on copolymers

### 3.8.3.7 Reusability

In the case of immobilized enzymes reusability is a vital parameter as it is necessary to make enzyme cost effective. The number of successive cycles for which a biocatalyst can be efficiently reused depends on several parameters like biocatalyst stability upon immobilization, its biochemical parameters like pH in which it is undergoing reaction, temperature, nature of linkage to the support etc.
Here we have carried out reaction for 14 cycles in order to check the feasibility of reusing the catalyst. Also, after each reaction immobilized enzymes were thoroughly washed with buffer so as to remove the products completely from the medium.

PYPAE could be used without much loss in its activity up to 14 cycles after which there was reduction in activity due to the natural inactivation of enzyme as a result of its continuous usage.

PYPH but retained its activity significantly only for 6 cycles after which it showed decline in activity that might have occurred due to poor adsorption capacity of support to hold enzyme in a proper orientation for long time. Similar results are reported by other authors for example Tanyolac et al. have reported complete reusability for \( \alpha \)-amylase immobilized onto nitrocellulose membrane up to 10 successive cycles followed by a 35% reduction in activity [183]. The results are shown in the figure: 3.64.

![Figure: 3.64 Reusability studies of immobilized enzymes on copolymers](image)

**3.9 Significance of polypyrrole composites as support for enzyme immobilization**

Polymeric nanocomposites are special class of hybrid materials formed by the intimate combinations of one or more organic or inorganic nanoparticles with a
polymer so that unique properties of former can be taken together with the existing qualities of the latter. From the point of versatility of synthesis techniques, properties, and broadness of the scope of application, these materials have raised a great deal of scientific and technological interest and have led the research in materials science in a new direction [184].

It was found that unfunctionalized PPY-silica particles are much more effective than the corresponding PPY bulk powders in adsorbing human serum albumin (HSA) [185]. Saoudi et al. have reported DNA adsorption onto PPY powder, a colloidal silica sol, and three PPY-silica nanocomposite particles (untreated and amine- and carboxylic acid-functionalized) [186].

From the reports available it is clear that because of the attractive properties together with ease of synthesis in aqueous media, conductivity, intrinsic deep black colour suitable for visual diagnostic tests [187], redox chemistry and long term stability, PPY composites are suitable candidate in the fabrication of biosensors [188], and as adsorbents of enzymes and proteins [189,190].

Literature reviews also shows that enzyme adsorption rather than covalent grafting can be sufficient for the development of a new assay using polypyrrole based material as a carrier [187].

3.9.1 Preparation of polypyrrole composites

3.9.1.1 Preparation of polypyrrole by template directed synthesis method

Polypyrrole was prepared using commercial colloidal silica sol as template via chemical polymerization method. Surface of colloidal silica was first modified prior to reaction. In order to achieve this 2 ml of chlorodimethylvinylsilane (CDVS) was added into a solution containing 4 ml of Ludox SM-30 colloidal silica sol solution and 26 ml of distilled water, and the mixture solution was stirred overnight for the surface modification of colloidal silica particles by CDVS.
Distilled 1.34 ml of pyrrole was added to the prepared solution, and stirred for 3 hours. Then 7.46 g of ferric chloride was added and stirred for 3 hours at room temperature to polymerize the pyrrole containing the surface modified silica particles. The product was washed with distilled water to remove free silica particles. To fabricate PPY from the PPY/Silica nanocomposites, silica templates were etched by 1M NaOH solution in water/ethanol mixture [191].

3.9.1.2 Preparation of Polypyrrole-silica composites

The synthesis of PPY-silica composites were done using the method described by Goller et al. As per the method reported by them aminated polypyrrole silica particles were produced via two synthetic routes. The main advantage of Route 1 is that the precursor homopolypyrrole-silica particles can be readily obtained with uniform particle size in a narrow range. Since such particles are known to have silica-rich surfaces the protocol described by Goodwin et al. for the amination of silica was applied [192]. In the Route 2 the aminated particles are synthesized in a single step. However, the amine-functionalized pyrrole comonomer required for Route 2 is not commercially available and therefore was synthesized prior to the reaction.

3.9.1.2.1 Materials

Pyrrole, 1-(2-cyanoethyl)pyrrole, ammonium persulfate, iron(III) chloride, lithium aluminium hydride (1.0M solution in diethyl ether), anhydrous diethyl ether, sodium chloride, potassium hydroxide and acetic acid were obtained from Aldrich. Pyrrole was distilled and stored at 4°C prior to use. Water was de-ionized and doubly distilled.

3.9.1.2.2 Synthesis of nanocomposites

In order to prepare homopolypyrrole-silica nanocomposites pyrrole (1.0 ml) was added to a vigorously stirred dispersion of colloidal silica (3.47 w/v%) in
Chapter 3

deionized water containing (NH₄)₂S₂O₈ (3.84 w/v%) as oxidant. Subsequently, the mixture was stirred for a further 24 hours to allow the pyrrole polymerization to proceed to completion. The resulting colloidal nanocomposite particles were isolated from the soluble inorganic byproducts and excess silica sol via four centrifugation/re-dispersion cycles (6000 rpm for 30 min), with successive supernatants being replaced with water. [193,194].

3.9.1.2.3 Silylation of nanocomposites

To functionalize the nano-composite particles 1 wt% of APTES at pH 4 (adjusted by the addition of acetic acid) was added to 3.1 wt% of the particles weighed and stirred the mixture for 2 hours at 25°C. The resulting silylated sols were purified by four centrifugation/redispersion cycles at 6000 rpm for 40 minutes and were re-dispersed in 0.01M aqueous NaCl solution, adjusted to pH 4 with 0.1M HCl [195].

3.9.1.3 Nanocomposite preparation via copolymerization

The comonomer 1-(3-aminopropyl) pyrrole comonomer was synthesized by the reduction of 1-(2-cyanoethyl) pyrrole in anhydrous diethyl ether [196]. 1-(2-cyanoethyl) pyrrole (5g) was added drop wise to a two molar excess of LiAlH₄ in anhydrous diethyl ether. The reaction was stirred under nitrogen atmosphere for 2 hours and then quenched by successive additions of water and sodium hydroxide (20 wt %). The ethereal layer was decanted and the product was collected as yellow oil after evaporation of the ether phase. The comonomer was characterized by 1H NMR spectroscopy using a 250 MHz instrument (CDCl₃ solvent and TMS reference).
3.9.2 Physico-chemical characterization

3.9.2.1 FT-IR spectra of polypyrrole composites

FT-IR spectra recorded for polypyrrole-silica (PSi) nanocomposites and its APTES functionalized derivative (PSiA) prepared in presence of FeCl₃ as oxidant is shown in the figure: 3.65.

Bands attributed to both the doped polypyrrole and silica components are observed. The very weak bands at 2928 cm⁻¹ and 2856 cm⁻¹ are attributed to C-H
stretch of pyrrole ring. The intensities of these bands increased after immobilization due to CH₂ groups of amylase.

The major peak at about 1110 cm⁻¹ (varying with different samples in the range of 1000-1200 cm⁻¹) that is attributed to the asymmetric stretching vibrations of Si-O-Si bonds of silica can be found in the hybrids. The bands present around 802 cm⁻¹ and 788 cm⁻¹ is assigned to scissor deformation of commercial silica particles [197]. Also bands at 700 cm⁻¹ are attributed to Si-O-Si asymmetric stretching and that at 462 cm⁻¹ is assigned to Si-O-Si asymmetric bending vibrations.

For PSiA broad peak in the range 3591-3213 cm⁻¹ is assigned to NH₂ stretching vibration. Peaks around 3300 cm⁻¹ indicate the NH₂ stretch vibration and 2972 cm⁻¹ indicates the -C-NH₂ stretch vibration [198]. On the other hand, the peak at 2954 cm⁻¹ indicating the C-H stretch that was observed in the PSi disappeared in the PSiA.

Bands in the range of 2965-2853 cm⁻¹ are attributed to aliphatic CH₂ stretching vibration. Broad band at 1605 cm⁻¹ is assigned to NH bending vibration. A weak band at 1522 cm⁻¹ is due to NH₂ deformation. In addition to all these bands, the characteristic bands due to polypyrrole also appear in the spectra of PSiA, which imply that PPY chains have been formed. However, these peaks shift to higher wave number compared to pure PPY suggesting that PPY chain in nanocomposite is shorter than in pure PPY.

For PAM broad peak in the range 3425-3392 cm⁻¹ is assigned to NH₂ stretching vibration. Bands in the range 2928 and 2852 cm⁻¹ are attributed to aliphatic CH₂ stretching vibration. The weak band at 2969 cm⁻¹ is assigned to C-NH₂ stretching vibration. A broad band at 1606 cm⁻¹ with strong intensity is due to NH bending vibration. The other characteristic peaks which are assigned to pyrrole ring confirmed the formation of poly-1-(2-aminopropyl) pyrrole silica nanocomposites.
For PM characteristic pyrrole ring stretch absorption bands were observed in the region 1600-1100 cm\(^{-1}\). But majority of the bands were shifted to lower wave number than in the pure polypyrrole [199]. No peaks corresponding to silica was obtained as it was used as template during the synthesis and has been removed [191].

For immobilized samples PSiE, PSiAE, PAME and PME the characteristic peaks due to presence of enzymes were observed in the spectra of all samples.

For PSiE the characteristic peak of N-H stretching vibration was shifted to lower wave number 3432 cm\(^{-1}\). The peak of aliphatic CH\(_2\) stretching vibration was also shifted to 2926 cm\(^{-1}\) and 2854 cm\(^{-1}\). Peaks corresponding to silane groups have either completely disappeared or decreased in intensity confirming the fact that enzymes have been anchored on to support via those functional groups. The characteristic peaks of PPY were also present with shifts to lower wave number. The characteristic peaks due to enzyme at 1646 cm\(^{-1}\) be ascribed to the amide I band that represents the stretching vibrations of C=O bonds in the backbone of the protein . In turn, obtained results could indicate adsorption of proteins on the surface of the polymers. FT-IR spectra shows bands centered at 1548 cm\(^{-1}\) that can be attributed to amide II band, spectrum clearly indicates the presence of carboxylate ion groups at 1646 cm\(^{-1}\) and 1377cm\(^{-1}\) respectively [22]. All bands get broadened after immobilization of \(\alpha\)-amylase.

For PSiAE and PAME the characteristic bands at 3449 cm\(^{-1}\) and 3454 cm\(^{-1}\) was observed for NH\(_2\) stretching vibration of pyrrole ring. The bands at 2926 and 2854 cm\(^{-1}\) were assigned to CH\(_2\) stretching vibration for PSiAE whereas for PAME these bands were at 2924 and 2856 cm\(^{-1}\) respectively. The rest of characteristic bands were also present in both polymers with shift in wave number with respect to different functional groups present. Bands at 1656, 1547, 1515, 1317 and 1377cm\(^{-1}\) were attributed to that of \(\alpha\)-amylase.

For PME the characteristic bands due to enzyme were present at 1656 cm\(^{-1}\), 1646 cm\(^{-1}\), 1544 cm\(^{-1}\), 1510 cm\(^{-1}\)and 1315 cm\(^{-1}\) [200].
Figure: 3.65: FT-IR Spectra of PPYSi composites before and after α-amylase immobilization (i) Spectra of PM and PSi (ii) Spectra of PAM and PSiA

Table 3.20: (i) Peak assignments for PM and PSi before and after enzyme immobilization (ii) Peak assignments for PAM and PSiA before and after enzyme immobilization.

<table>
<thead>
<tr>
<th>Peak assignments (cm⁻¹)</th>
<th>PSi</th>
<th>PSiE</th>
<th>PM</th>
<th>PME</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H stretch</td>
<td>3438</td>
<td>3443</td>
<td>3438s</td>
<td>3436</td>
</tr>
<tr>
<td>C-H stretch</td>
<td>2923vw, 2854vww</td>
<td>2928, 2854vw</td>
<td>2928, 2851vw</td>
<td>2928, 2854vw</td>
</tr>
<tr>
<td>C=O</td>
<td>1710w, 1748vww</td>
<td>1745, 1710w</td>
<td>1718vw</td>
<td>1745, 1707bw</td>
</tr>
<tr>
<td>C-N-C ring in plane deformation</td>
<td>1657vww</td>
<td>1656bm</td>
<td>1654, 1631.6s</td>
<td>1656, 1638</td>
</tr>
<tr>
<td>N-H ring in plane bending</td>
<td>1523</td>
<td>1545</td>
<td>1526</td>
<td>1542, 1521m</td>
</tr>
<tr>
<td>C-N stretch</td>
<td>1476vw</td>
<td>1456w</td>
<td>1476</td>
<td>1488, 1459w</td>
</tr>
<tr>
<td>C-C in ring stretch</td>
<td>1339vw</td>
<td>1379, 1342vww1316</td>
<td>1394, 1317</td>
<td>1340, 1316, 1398w</td>
</tr>
<tr>
<td>C-C inter ring stretch</td>
<td>1243</td>
<td>1298vw</td>
<td>1256</td>
<td></td>
</tr>
<tr>
<td>C=C-N ring in plane deformation</td>
<td>1113bs</td>
<td>1160w, 1113w</td>
<td>1121vw</td>
<td></td>
</tr>
<tr>
<td>C-H,N-H ring out of plane bending</td>
<td>1051</td>
<td>1008</td>
<td>1024</td>
<td>1012</td>
</tr>
<tr>
<td>C=C-C, ring in plane deformation</td>
<td>881w</td>
<td>880</td>
<td>852</td>
<td></td>
</tr>
<tr>
<td>C-H,N-H ring out of plane bendings</td>
<td>792</td>
<td>770m</td>
<td>787</td>
<td>779w</td>
</tr>
</tbody>
</table>

(i)
Immobilization of Diastase α-amylase on to Synthetic Polymers

<table>
<thead>
<tr>
<th>Peak assignments</th>
<th>PSi A</th>
<th>PSi AE</th>
<th>PAM</th>
<th>PAM E</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-H stretch</td>
<td>3420</td>
<td>3447</td>
<td>3419</td>
<td>3438</td>
</tr>
<tr>
<td>C-H stretch</td>
<td>2924w</td>
<td>2857w</td>
<td>2924, 2853w</td>
<td>2924w, 2856w</td>
</tr>
<tr>
<td>C=O</td>
<td>1746, 1710v</td>
<td>1746, 1710w</td>
<td>1745</td>
<td>1745, 1708v</td>
</tr>
<tr>
<td>C-N-C ring in plane deformation</td>
<td>1622bm</td>
<td>1658bm, 1642s, 1622m</td>
<td>1606, 1632m, 1640m</td>
<td></td>
</tr>
<tr>
<td>N-H ring in plane bending(s)</td>
<td>1596w, 1546w</td>
<td>1544, 1526, 1515</td>
<td>1596w</td>
<td>1544w, 1521.8w</td>
</tr>
<tr>
<td>C-N stretch(w)</td>
<td>1454</td>
<td>1462, 1480</td>
<td>1467w</td>
<td>1462.2, 1425</td>
</tr>
<tr>
<td>C-C in ring stretch(s)</td>
<td>1334, 1310</td>
<td>1338, 1317w</td>
<td>1318w, 1367</td>
<td>1376w, 1340.8v, 1316.8</td>
</tr>
<tr>
<td>C-C inter ring stretch(w)</td>
<td>1259.6</td>
<td>1230m</td>
<td>1204</td>
<td>1233, 1244m</td>
</tr>
<tr>
<td>C-N stretch,N-H ring in plane bending(w)</td>
<td>1116.8m, 1107mb</td>
<td>1155w, 1110, 1100w</td>
<td>1113mb, 1110vw, 1116.8w</td>
<td>1115.4w</td>
</tr>
<tr>
<td>C-C,ring in plane deformation</td>
<td>873vw</td>
<td>886, 854w</td>
<td>859</td>
<td>894vw</td>
</tr>
<tr>
<td>C-H,N-H ring out of plane bending(s)</td>
<td>803w</td>
<td>789w</td>
<td>780</td>
<td>774vw</td>
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<tr>
<td>C-H ring out of plane bending(w)</td>
<td>731vw</td>
<td>734</td>
<td>723</td>
<td>744vw</td>
</tr>
</tbody>
</table>

(ii)

3.9.2.2 Thermogram of polypyrrole composites

Figure: 3.66 show the thermograms of PSi, PSiA and PAM.

The thermograms have roughly similar shapes at high temperature showing a plateau value of weight loss vs. temperature. The thermogram of PSi shows a higher mass loss compared to bare PPY. The weight loss of PSiA is also significantly higher than PSi which is mainly due to removal of the APTES moiety.

After immobilization weight loss enhances again which is the result of adsorption of enzyme on to these polymers. The increase in weight loss was found to be more in PSiAE and PAME than on PSiE which is due to presence of -NH₂ groups on the other two. Moreover APTES is an adhesion promoter which favoured the specific interactions of lewis acid-base type between the basic amino group and the acidic N-H bonds and the positively charged polypyrrole backbone.
Figure 3.66: TG curves for composites before and after immobilization. (i) TG curves of PSi, PSiA, PAM, PM (ii) TG curves of PM and PME (iii) PSi and PSiE (iv) PSiA and PSiAE (v) PAM and PAME.
In all cases the initial loss is mainly attributed to the elimination of residual water, dopant molecules and unreacted monomer.

In the case of PSi the major sharp peak at 266°C would be the beginning of degradation of polypyrrole chains. At 383°C minor weight loss starts which along with the decomposition of polypyrrole also includes silica particles. The continuous weight loss starts at 465°C and continues up to 800°C. After immobilization of α-amylase the decomposition due to enzyme protein structure occurs at 209°C. The next major loss occurs in the range 241-392°C which is a broad peak. The other minor loss at 635°C might be the polymer decomposition.

In the case of PSiA minor weight loss at 218°C might be the beginning of polypyrrole backbone. The major weight loss occurred in the range 283-332°C which accounts for polymer degradation, APTES removal, and silica decomposition. The continuous degradation of polymer structure then occurs at 541°C which gradually occurs till 800°C.

When thermogram of PAM was analyzed the initial decomposition due to residual water occurs before 100°C and the polymer decomposition occurs at 209°C. The other decomposition which includes the removal of unreacted (1-(3-aminopropyl pyrrole)) silica comonomer occurs at 339°C. When α-amylase was immobilized the corresponding weight loss was observed in the thermogram. It was at 164°C the protein structure degradation observed in PAME. The other major weight loss occurs at 257°C which corresponds to initial decomposition of polymer backbone. The subsequent decomposition occurs at 307°C which correspondingly reflects the decomposition of 1-(3-aminopropyl group)) silica and the polymer structure.

3.9.2.3 Surface area analysis

BET surface area of PPYSi composites before and after immobilization of α-amylase is given in the table 3.21.
Highest surface area is shown by PPY-silica composite (PSi) and polypyrrole prepared using silica as template (PM).

For PSiA the surface area is found to reduce. This might be due to the fact that pores of silica on polypyrrole might get prefilled or have blocked with APTES. In other words APTES might have grafted on the outermost layers of silica or sorbed in the pores but near the outermost surface of silica. Similar reduction in surface area was reported by several authors.

Luo et al. have clearly shown that the silica pretreatment entails a significant decrease of both porosity and pore radii in addition to specific surface area compared to the untreated silica gel particles [222]. C. Perruchot et al. have reported that specific surface area of PPY-silica particles matched that of untreated silica gel, whereas the APTES treatment of silica yields a sharp decrease in the surface area of the latter [201].

In the case of PAM also surface area was reduced than PSi. This might be due to the fact that addition of pyrrole and 1-(3-aminopropyl pyrrole) mixture on to silica gel followed by polymerization step have promoted the growth of copolymer on the surface of silica thereby reducing its surface area. Whereas, etching of silica after the polymerization process have resulted in the formation of pores on polypyrrole produced and thereby increase in surface area in the case of PM.

### 3.9.2.4 Scanning electron microscopy

The SEM images of PPYSi composites prepared were shown in the figure: 3.67 below
The samples prepared with colloidal silica as templates (PM) were found to have flaky structures. It is known that the interaction between the pyrrole main chains is very strong. Therefore it has a tendency to aggregate into irregular morphology, which is commonly observed in the polypyrrole obtained by the chemical oxidative polymerization. The SEM image showed a rough surface morphology with non-irregular particle size.

Polypyrrole silica composites (PSi, PSiA and PAM) exhibit uniform texture with a granular morphology, with non-regular shape particles. PSi particles obtained
were found to have rough surface morphology with particles of granular shape ranging from 67-120 nm. This might be due to high silica rich surface which on further amination with APTES (PAM) becomes weakly flocculated. The particle size is in the range 53-97 nm for PAM. PAM is much more spherical compared to other two.

3.9.3 Immobilization of α-Amylase on Polypyrrole Composites

3.9.3.1 Optimization of immobilization conditions

In this section we have reported a study of interaction of Diastase α-amylase with PPY powder (PM), and three nanocomposite systems-unfunctionalized PPY-silica (PSi), aminated PPY-silica (PSiA) and copolymerized poly (pyrrole-co-1-(3-aminopropyl)-pyrrole-silica (PAM) nanocomposites. The amylase adsorption capacities of various PPY adsorbents at various immobilization pH are shown in the figure: 3.68.

α-amylase was adsorbed on to all adsorbents via both electrostatic and hydrophobic interactions [186]. Thus depending on the relative surface hydrophobicity and presence or absence of surface functional groups, either hydrogen bonding and/or hydrophobic interaction may enhance the extent of amylase adsorption. All these phenomena can be clearly explained based on isoelectric points of PPY adsorbents.

PM has a high surface area when compared to bare PPY and expected isoelectric point approximately 7 as that of PPY. When solution pH is below 7, PM will be positively charged and above 7 negatively charged [201]. α-amylase having isoelectric point around 4.6 will be positively charged at pH 3 and 4. So at pH 3 and 4 less adsorption occurs on PM as both amylase and PM having similar charge faces electrostatic repulsion.
When pH > 5, amylase is negatively charged whereas, PM is positively charged up to pH 6. So, strong electrostatic attraction encounters at pH 5 and 6. At pH 7 no charge exists for PM and amylase is negatively charged. Hence considerable adsorption was not expected but results were contradictory showing appreciable adsorption at this pH which might be due to hydrophobic interaction between PM and negatively charged amylase which dominated over electrostatic attraction. This is because polypyrrole is hydrophobic as it has large aromatic rings in the polymer backbone [202]. Therefore, for these adsorbents, hydrophobic interactions dominate and the effect of pH is minimized.

At pH 8 and 9 again electrostatic repulsion occurs as both enzyme and support are negatively charged, but it might be due to dominant hydrophobic interaction that resulted in adsorption capacity at this pH too even if it is not significant as at other pH. That is activity obtained at this pH is less compared to other pH [146]. The optimum pH for PM was found to be at pH 6.

In the case of PSi optimum adsorption occurred at immobilization pH 4. This is because for PSi isoelectric point is at pH 2. As reported by C. Perrchot et al., the X-ray photoelectron spectroscopy studies of these nanocomposites exhibit silica rich surfaces an observation that was subsequently confirmed by zeta potential measurements [201].

But at pH above 4 since both amylase and PSi are negatively charged poor adsorption occurs due to prominent electrostatic repulsion. Still very low adsorption occurs which is driven by hydrophobic interaction between support and enzyme.

PSiA nanocomposites show a different trend. As reported by M.I Goller et al. from zeta potential measurements PSiN has isoelectric point around 7.5 an approximate charge balance between the anionic silanol groups and cationic -NH₃⁺ groups [203]. At pH 7.5 surface amine sites on the PSiA particles
are extensively protonated as -NH$_3^+$ groups. Thus a net positive surface charge is not a prerequisite; the attractive electrostatic interactions between amylase and isolated -NH$_3^+$ groups are apparently sufficient for amylase adsorption.

The optimum immobilization pH obtained for PSiA was at pH 5. Thus immobilized enzyme has the same pH optimum as the free enzyme (pH 5), and so immobilization did not change the optimal pH of α-amylase. Similar observation was reported by T. Kalburcu et al. when α-amylase was immobilized on metal ion affinity nanospheres [204]. This is because PSiA was positively charged below 7.5 and negatively charged above 7.5. Therefore most prominent and effective electrostatic interaction occurs in the pH range 5-7 as amylase will be negatively charged in this range and PSiA will be positively charged. Above and below this range adsorption occurs mainly due to hydrophobic interactions between enzyme and the support. This might be the reason for lower loadings at higher and lower pH.

PAM on the other hand has an isoelectric point of about 8.5 as reported by Goller et al. [203]. Hence most proper orientation of enzyme with the support occurs in the pH range 5-7 where more favourable electrostatic interaction comes to play. The optimum pH for amylase adsorption occurred at pH 6 for PAM. The lower loadings at pH above 7 and below 5 are the result of hydrophobic interaction which dominates over electrostatic repulsion. The variation of activity with respect to change in pH of the environment, within the range 2-3 units each side of the pI is normally a reversible process [205].

In addition to these, both PSiA and PAM have higher adsorption than PSi at higher pH values. This happens because of the presences of amino groups which accounts for greater zeta potential and thus have stronger electrostatic interaction with amylase than PSi. When pH is below 5, PSi has more adsorption capacity and activity compared to other two aminated PSi nano composites.
Similar results were obtained by Y. Nakashimada when lysozyme and BSA was adsorbed on polypyrrole synthesized on to model silica gel through APTES [146].

**Figure: 3.68** Effect of pH at the time of immobilization on the relative activity of α-amylase

3.9.3.1.1 Effect of contact time on immobilized enzyme activity

The contact time needed for enzyme to get adsorbed on PPY composites is shown in the figure: 3.69. As soon as the addition of enzyme to these adsorbents, immediate interaction occurs and this varies with the nature of the support.

In the present case for PM immediate saturation of the support with the enzyme occurs as contact time reaches 120 minutes. For PSi the optimum level was attained when the contact time of 240 minutes was allowed. This might be due to poor adsorption capacity of PSi compared to others which is the consequence of its surface charges. PSiA and PAM reached maximum adsorption limit when kept for 180 minutes of contact time. The easily accomplished adsorption rate might be due to the presence of surface functional groups on these polymers which favoured electrostatic interactions effectively and hence resulted in subsequent loading of enzymes on support while retaining maximum activity.
3.9.3.1.2 Effect of initial protein on protein loading of immobilized enzyme

The amount of protein bound to PPY adsorbents was analyzed based on the optimized conditions obtained; it is shown in figure: 3.70.

Figure: 3.70 Influence of initial protein concentration on protein loading on to supports (i) PSi, PSiA, PAM (ii) PM

As far as nanocomposites are concerned surface functionalization with amine groups produced much higher amylase adsorption in the order PAM > PSiA > PSi.

For PM, since the surface area is high, loading was also high and was about 6.8 mg. PAM has amino group at the surface and was found to hold more proteins.
strongly than PSiA particles. This might be due to difference in distribution of amino groups at the surface of both composites which ultimately favoured the copolymerized one. But both have better binding capacity compared to unfunctionalized PSi, which has a poor binding capacity due to its isoelectric point which is unfavourable for its strong electrostatic interaction with amylase. In case of other three, electrostatic interactions play a key role that facilitates proper orientation of enzyme with the support so that less conformational changes were encountered during the immobilization process that would have caused alterations in their activity.

In all three cases as the initial protein concentration increases protein load also get increased gradually which then reaches a saturation point after which loading gets decreased [204]. This might be either due to insufficient binding sites at higher loadings or due to desorption of enzyme from the surface of the supports as a result of multilayer adsorption of enzymes [206]. The corresponding activity when evaluated showed similar trend for PSi and PAM, but a different trend for PSiA.

For PSiA optimum immobilized enzyme activity was obtained not at maximum protein load but on the concentration below it. This might be due to masking of many active sites on higher loading which caused mass transfer diffusional limitation of substrate molecules towards the active site. From the graph it is clear that protein load of PSi, PSiA, PAM and PM was 6.1, 6.8, 9.2 and 7.1 mg respectively.

3.9.3.1.3 Effect of initial protein concentration on immobilization yield and activity of loaded enzyme.

Immobilization yield obtained for all adsorbents at various concentrations taken are shown in the figure: 3.71.
From the graph it is evident that for PM immobilization yield was about 43% at initial protein concentration of 40.3 mg. PSi showed maximum immobilization yield of 28% at initial concentration of 30.5 mg whereas, PSiA showed maximum yield of 44% at initial protein concentration of 30.5 mg and PAM showed maximum immobilization yield of 53% at initial protein concentration of 19.2 mg.

For PSi maximum activity of 6.5 EU was obtained at maximum loading but with less immobilization yield. PSiA showed maximum activity of 9.8 EU at enzyme loading of 6.2 mg with immobilization yield 44%, both were not the optimum. For PAM maximum activity of 9.2 EU was obtained at initial enzyme concentration of 32.7 mg with 8.7 mg protein load having immobilization yield of 53%. Finally for PM immobilized enzyme activity was a maximum of 7.1 EU at initial enzyme concentration of 15.6 mg which attained the protein loading of 6.8 mg with an immobilization yield of 43%. All results are depicted in the graphs below in figure: 3.72.
Immobilization of Diastase α-amylase on to Synthetic Polymers

Figure: 3.72: Effect of initial protein concentration on the immobilized enzyme activity

Immobilized enzyme activity yield and immobilization efficiency are also evaluated. The results are tabulated in the table 3.22.

Table 3.22: Immobilization efficiency of α-amylase on polypyrrole composites

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Initial protein (mg)</th>
<th>Immobilized protein (mg/g support)</th>
<th>Immobilization yield (%)</th>
<th>Initial activity (EU)</th>
<th>Immobilized enzyme activity (EU)</th>
<th>Activity Yield (%)</th>
<th>Immobilization efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSI</td>
<td>40.3</td>
<td>6.1</td>
<td>28</td>
<td>36.2</td>
<td>6.5</td>
<td>18</td>
<td>64</td>
</tr>
<tr>
<td>PSIA</td>
<td>28.3</td>
<td>6.2</td>
<td>44</td>
<td>30.8</td>
<td>8.8</td>
<td>32</td>
<td>72</td>
</tr>
<tr>
<td>PAM</td>
<td>32.7</td>
<td>8.7</td>
<td>53</td>
<td>26.3</td>
<td>9.2</td>
<td>35</td>
<td>66</td>
</tr>
<tr>
<td>PM</td>
<td>15.6</td>
<td>6.8</td>
<td>43</td>
<td>25.9</td>
<td>7.1</td>
<td>27</td>
<td>63</td>
</tr>
</tbody>
</table>

3.9.3.2 Effect of pH on enzyme activity

The optimum pH of an immobilized enzyme may be different from that of free one because of non-uniform distribution of charges between the microenvironment of enzyme and the bulk solution. This effect becomes more predominant when support contains ionizable groups at its surface [207].

The immobilized enzyme demonstrates greater stability than the free form and hence shows a much broader pH profile. This is because during immobilization enzymes movement is restricted as it is bonded to support in a favourable orientation retaining its activity at the optimum. Hence immobilized enzyme can withstand
sudden conformational changes thereby lowering the rate of inactivation [208,209,210]. Diffusional limitations or secondary interactions between the enzyme and the carrier may also influence this change [211,212]. Similar observations have been reported for immobilization of α-amylase and other enzymes [213].

The pH effect on the activity of the free and immobilized forms of α-amylase has been studied in buffer solution at different pH in the range 4-8 and the results are presented in the figure: 3.73.

Figure: 3.73: Effect of pH on the relative activity of immobilized α- amylase

The optimum activity is assigned as 100% and other activities are expressed as relative to this optimum activity. A change in pH will affect the intra-molecular hydrogen bonding leading to a distorted conformation that will reduce the activity of the enzyme [214].

For amylase immobilization, shifts in the optimum pH towards both the acidic and alkaline directions have been observed [58, 165, 226]. This is because, when an enzyme is linked to a support, some strain is enforced on the enzyme, which causes slight unfolding of its native conformation and at this juncture contact with buffer solution causes additional strain that may be beneficial or destructive. The nature of linkage between enzyme and support also decides the shift of pH which becomes more prominent when surface functional groups are present on the support.
Free enzyme exhibits maximum activity in the pH range (5-6) with optimum activity at 5 and 5.5. At pH 4 and 8 a decrease of the enzymatic activity is observed for both the immobilized and the free enzyme; however, at pH 8 the residual activity of the immobilized enzyme in most samples is significantly higher than that of free enzyme. Thus, the immobilization process provides a structural stability, preventing an irreversible unfolding of the enzymatic protein. The native enzyme could not survive such a shift towards basic side.

In the case of PSi optimum activity was obtained at pH 5. For PSiA optimum activity was obtained at reaction pH 6 and the shift towards the basic side is required to maintain the enzyme conformation after immobilization. In the case of PAM also optimum pH was obtained at 6.

**Table 3.23**: Optimum pH obtained for immobilized α-amylase on Polypyrrole composites

<table>
<thead>
<tr>
<th></th>
<th>Free enzyme</th>
<th>PSi</th>
<th>PSiA</th>
<th>PAM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5 &amp; 5.5</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>5.5</td>
</tr>
</tbody>
</table>

**3.9.3.3 Effect of temperature on the enzyme activity**

The effect of temperature on the activity of α-amylase on PPY composites and PPY powder was analyzed and the results are shown in the figure: 3.74.

**Figure: 3.74**: Effect of temperature on the relative activity of free and immobilized enzyme
From the graph it is evident that optimum temperature for all composites and PPY powder was 50°C. But the added advantage is that activity of about 89-96% was retained even at 60°C whereas, free enzyme could retain only 25% of its initial activity. For PSiAE maximum activity was shown in the range 40-50°C. Enzyme thermal inactivation is the consequence of the weakening of the intermolecular forces responsible of the preservation of its three-dimensional structure leading to a reduction in its catalytic activity.

3.9.3.4 Thermal stability of the free and immobilized enzymes

Thermal stability of both free and immobilized enzymes was investigated and the results are shown in the figure: 3.75.

![Figure: 3.75: Thermal stability studies of free and immobilized enzyme](image)

From the figure: 3.75 it is evident that the immobilized enzymes show moderate decrease in activity, which emphasizes that the rate of inactivation is lowered upon immobilization. This is because immobilization brings about a restriction in the free movement of the enzyme by fixing it on a solid support. As a result, thermal denaturation that might result due to unfolding of protein structure is hindered and hence the enzyme can withstand higher temperature compared to free state.
The added advantage of using composites is very much clear from the graph. Enzymes immobilized on composites are found to have more thermal stability than PPY powder. At 60°C, 80-90% of activity was retained by immobilized enzymes as it is less susceptible to conformational changes. Further increase of temperature will lead to loss of activity, as for adsorbed enzymes, enzyme-support interaction is weaker resulting in unfolding of protein structure.

After incubation at 30°C for 1 hour the activity showed by free and immobilized enzyme was taken as optimum and activity at other temperatures are expressed as relative to this activity. As the temperature increased, the stability dropped significantly for both free and immobilized amylase. At 40°C, enzyme immobilized on composites retained up to 95-97% of their activity whereas, only 63% activity was retained by PM. At 50°C for 1 hour the immobilized enzyme was inactivated at a much lower rate than the free enzyme. 80-90% activity was retained by enzymes on composites and 40% by enzymes on PM. The free enzyme lost almost 90% of its activity at 60°C after 1 hour treatment whereas, immobilized amylase lost 20-30% of its activity.

Figure: 3.76 show the effect of pre-incubation time on the activity of each immobilized enzyme.
PSiAE and PAME maintained 60-77% of their initial activity when subjected to 120 minutes of pre-incubation time at their respective optimum temperature whereas, 46% of initial activity was maintained by PSiE; PME could retain 20% and free enzyme could retain only 10% of their initial activity when subjected to thermal treatment for same period of time.

This thermal stability results suggest lower flexibility due to multi point attachment on to supports enhances the stability of enzyme which makes the potential utilization of such enzymes extensive [215].

### 3.9.3.5 Determination of kinetic parameters

The $K_m$ and $V_{max}$ was calculated using Lineweaver-Burk plot [216].

$K_m$ increase and $V_{max}$ decrease after immobilization. There are several reasons that can explain the difference in behaviour of free and immobilized amylase. First, the immobilized amylase resides in an environment that is quite different from that of free enzyme in bulk solution. In addition, attachment of amylase to composite particles will cause some change in conformation or steric hindrance. This conformational change might have resulted in decreased activity and decreased affinity of enzyme for its substrate. The ionic, hydrophobic or other interaction between the enzyme and the matrix which produce micro-environmental effects may also result in changed $K_m$ and $V_{max}$ values. These essentially reversible effects are caused by the variations in the dissociation equilibria of charged groups at the active centre. Again non uniform distribution of substrate and/or product between the enzyme matrix and the surrounding solution affects the measured (apparent) kinetic constants. Similar increase in $K_m$ and decrease in $V_{max}$ was observed by other authors [217,218].

**Table 3.24: Kinetic parameters for α-amylase immobilized on composites**

<table>
<thead>
<tr>
<th></th>
<th>Free enzyme</th>
<th>PSiE</th>
<th>PSiAE</th>
<th>PAME</th>
<th>PME</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (mg/ml)</td>
<td>0.50 ± 0.04</td>
<td>1.6 ± 0.03</td>
<td>0.84 ± 0.02</td>
<td>0.669 ± 0.05</td>
<td>0.59 ± 0.06</td>
</tr>
<tr>
<td>$V_{max}$ (mg/ml/min)</td>
<td>7.40 ± 0.05</td>
<td>1.94 ± 0.06</td>
<td>2.86 ± 0.09</td>
<td>2.21 ± 0.08</td>
<td>3.91 ± 0.07</td>
</tr>
</tbody>
</table>
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(i)  (v)

(ii)  (vi)

(iii)  (vii)
3.9.3.6 Storage stability of Immobilized α- amylase

The storage stability during 4 months is shown in the figure: 3.77.

The results showed that in dry form immobilized enzyme could not retain its stability and activity for long term usage. But in wet form about 90% of activity was found to be retained by all PPY adsorbents. After 1 month of storage the loss in activity occurs due to natural inactivation of enzyme as a result of time dependent denaturation of enzyme protein. The rate of denaturation varies with type of support as it is based on how strongly and perfectly the enzyme is anchored to the support.

PM is found to retain stability up to 50% after four months. PAM could retain 30%, PSiA retained 38% and finally PSi retained only 10% of its initial activity after 4 months of storage in buffer solution.
Reusability

In order to check reusability of immobilized enzymes a continuous assay of amylase immobilized on PPY adsorbents was performed. The results obtained are shown in the figure: 3.78. The activity loss of immobilized enzymes upon reuse could be due to the weakening of binding strength between the enzyme and the support. Moreover there also exists the possibility of distortion of the enzyme structure as a result of frequent encountering between the active site and substrate which consequently retards its catalytic efficiency [219].

PM retained about 80% of its initial activity; PAM retained about 25% of its initial activity while PSiA and PSi retained 45% and 30% of their initial activity respectively, which are all far better than using free enzyme without immobilization. The loss of activity may be caused by the restriction of the support which resulted in limited mobility and accessibility of the active sites [220].

Similar loss of activity after repeated use of immobilized $\alpha$-amylase was observed by Mobasher [217] while immobilizing $\alpha$-amylase from *Aspergillus niger* on to natural polymers consisting of chitosan and alginate and synthetic polymer consisting N- isopropyl acrylamide and alginate via entrapment method. Similar results were reported in other immobilization studies [215,221].
3.10 Conclusions

Immobilization of Diastase α-amylase on to polypyrrole and its derivatives were successfully carried out via adsorption method. Functionalization of polypyrrole with glutaraldehyde was also performed as per the reported procedure by T. Sandu et al. and α-amylase has been immobilized on to the functionalized PPY via covalent binding method. The optimal immobilization conditions for the α-amylase were found out by the process of trial and error so as to ensure the highest possible retention of activity of the enzyme, its operational stability and reusability. The major outcomes of the work can be summarized as follows:

- The optimum pH for free enzyme was found to be in the range 5 -5.5 and temperature 50°C. The enzyme lost almost 90% of its activity at 60°C after 1 hour treatment. The kinetic parameters for starch hydrolysis when evaluated gave 0.50 mg/ml as $K_m$ and 7.40 mg/ml/min as $V_{max}$. When stored under 4°C free enzyme lost all its activity within 2 days.

- All the PPY supports and its derivatives were prepared as per the reported procedures. The physico-chemical characterization of supports before and after enzyme immobilization were carried out using the FT-IR, TG, SEM, BET and EDS analytical techniques.
Diastase α-amylase when immobilized on to PPY prepared in the presence of different oxidizing agents and different methods showed enhanced activity and stability when compared to free enzyme. Evaluation of optimal binding parameters was carried out. It was found that all the immobilized enzymes showed a broader pH profile compared to free enzyme. The optimum pH for PA, PF and PI were 5.5, 5 and 6 respectively. PA having surface area higher than other two possess highest immobilization yield. But activity yield was higher for PI. The optimum temperature for PA and PF get shifted to 40°C, whereas for PI the optimum temperature was about 50°C. The kinetic parameters $K_m$ and $V_{max}$ were also determined via Lineweaver-Burk plot. In most cases results obtained showed that storage stability of the immobilized enzyme was significantly better than free enzyme. In our study activity for 12 cycles of use for the immobilized enzymes was monitored and PF exhibit better reusability than other two.

PPY prepared in presence of surfactant templates were found be suitable supports for enzyme immobilization as PPY particles formed are having controlled well tailored morphology when compared to those prepared in absence of surfactants. Biochemical characterization conducted revealed that optimum pH for PS is pH 5 which is same as that of free enzyme, whereas for PC and PT it gets shifted to pH6. The 10°C decrease in the optimum temperature combined with thermal stability exhibited by α-amylase immobilized on PC and PT was an interesting finding of this work. α-amylase immobilized on PS showed an increase in the optimum temperature. The $K_m$ values were found to be increasing for PS, PC and PT. The $V_{max}$ values of immobilized enzymes were decreasing for PS, PC and PT. Enzyme immobilized on PS retained 40% of its initial activity even after 6 months of storage in buffer solution at 4°C whereas, enzyme immobilized on PC and PT retained 53 and 60% respectively under similar
conditions. The reusability was checked for 20 continuous cycles. After 15 cycles PS and PC retained 50% of their initial activity whereas, PT retained almost 60% of its initial activity.

✔ α-amylase was successfully immobilized on PPY functionalized with glutaraldehyde (PG) via covalent binding. Whereas in the case of PPY modified with APTES (PN), immobilization of amylase occurs via adsorption. The optimum pH for PGE was found to be at pH 5 which is same as that of free enzyme but for PNE it was shifted to pH 6. The optimum temperature for PGE was obtained at 55°C whereas for PNE the optimum temperature gets shifted to 60°C. Immobilization efficiency for PGE was found to be 95% and for PNE it was about 86%. But the activity yield was higher for PNE than for PGE. The kinetic parameters evaluated showed an increase in $K_m$ and decrease of $V_{max}$ for both immobilized enzymes. Upon 4 months of storage, the adsorbed enzyme preserved 40% of its initial activity whereas; covalently bound enzyme retained 55% of its initial activity. The reusability studies showed that in the case of PNE up to 7 cycle 70% activity was retained which decreased drastically for further cycles. Whereas PGE was able to maintain good activity up to 60% even after five runs.

✔ The copolymers prepared were found to exhibit enhanced enzyme loading capacity than the bare PPY. For both PYPAE and PYPHE the optimum pH get shifted to pH 6. The optimum temperature for PYPH was same as that of free enzyme that is 50°C but that of PYPA get shifted to 60°C. About 50-60% of immobilized enzymes maintained their activity when subjected to 120 minutes of pre-incubation time at their respective optimum temperature whereas free enzyme could retain only 10% of their initial activity when subjected to same period of time. $K_m$ value for PYPA was found to be 2.73 mg/ml and that for PYPH was about 2.47 mg/ml which
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was comparable. But the $V_{\text{max}}$ values of both immobilized enzymes varied significantly as 4.21 mg/ml/min for PYPH and 2.95 mg/ml for PYPH. On examining the storage stability it was found that PYPH retained 70% of its initial activity and PYPH retained 25% of its initial activity after 6 months. The reusability studies indicated that PYPH adsorbed enzyme could be used without much loss in its activity up to 14 cycles. PYPH but retained its activity significantly only for 6 cycles after which it showed decline in activity.

Both functionalized and unfunctionalized PPY silica composites possess higher surface area compared to other PPY supports previously discussed and hence the enzyme loading capacity of these PPY composites also get enhanced. As far as nanocomposites are concerned surface functionalization with amine groups produced much higher contents of amylase adsorption in the order PAM > PSiA > PSi. Immobilization efficiency was maximum for aminated PSi composite. The optimum pH for PSi was same as that of free enzyme. For aminated PSi composites optimum pH shifted towards pH6. The optimum temperature for PPY composites was same as that of free enzyme. But the added advantage is that activity of about 89-96% was retained even at 60°C whereas, free enzyme could retain only 25% of its initial activity. For PSiA almost 100% activity was shown in the range 40-50°C. The free enzyme lost almost 90% of its activity at 60°C after 1 hour treatment whereas, immobilized amylase lost 20-30% of its activity. The kinetic parameters when evaluated showed an increase in $K_m$ value and decrease of $V_{\text{max}}$ value for all PPYSi composites. The maximum in activity was obtained in the case of aminated polypyrrole composites compared to other three. The stability during 4 months of storage at 4°C when analyzed it was found that PAM could retain 30%, PSiA -38% and finally PSi retained only 10% of its initial activity. The reusability when
checked it was observed that PM retained about 80% of its initial activity; PAM retained about 25% of its initial activity while PSiA and PPYSi retained 45 and 30 % of initial activity respectively, which are all far better than using free enzyme without immobilization.

3.11 References


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