CHAPTER 2
EXPERIMENTAL TECHNIQUES

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2.1 Materials Used

<table>
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<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulphate</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>L-ascorbic acid</td>
<td>s. d. fine CHEM LTD. Mumbai</td>
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<tr>
<td>Diastase α-amylase</td>
<td>HiMedia Laboratories Pvt. Ltd. Mumbai</td>
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<tr>
<td>Dextrose anhydrous</td>
<td>s. d. fine CHEM LTD. Mumbai</td>
</tr>
<tr>
<td>3,5-Dinitrosalicylic acid</td>
<td>s. d. fine CHEM LTD. Mumbai</td>
</tr>
<tr>
<td>Folin &amp; Ciocalteu’s Phenol Reagent</td>
<td>Sisco Research Laboratories Pvt. Ltd. Mumbai</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>LOBA CHEMIE PVT. LTD. Mumbai</td>
</tr>
<tr>
<td>Aniline</td>
<td>s. d. fine CHEM LTD. Mumbai</td>
</tr>
<tr>
<td><em>O</em>-toluidine</td>
<td>SPECTROCHEM PVT. LTD. Mumbai</td>
</tr>
<tr>
<td>Starch soluble (potato)</td>
<td>s. d. fine CHEM LTD. Mumbai</td>
</tr>
</tbody>
</table>

2.2 Preparation of Polymers

2.2.1 Preparation of emeraldine salt (ES)

ES was prepared by chemical oxidation of 0.2 M of aniline hydrochloride with 0.25 M ammonium peroxydisulphate according to literature procedure [1, 2]. The efficient polymerization of aniline is achieved only in acidic medium; where aniline exist as anilinium cation. Here, hydrochloric acid was used in equimolecular proportion of aniline so that aniline hydrochloride formed *in situ* was used as the monomer. Peroxydisulphate is the most commonly used oxidant, and its ammonium salt was preferred to potassium counterpart because of its better solubility in water. The recommended stoichiometric ratio of peroxydisulphate to aniline was 1.25:1 [1]. The polymerization was completed within 1 hour at 0-2 °C, since the reaction was exothermic, low temperature was preferred.

Aniline (3.7 mL, 0.2 M) was dissolved in 0.2 M hydrochloric acid in a volumetric flask to 200 mL of solution. Ammonium peroxydisulfate (11.4 g, 0.25 M) was dissolved in distilled water to 200 mL as well. Both solutions were kept for 1 hour in an ice bath, mixed
in a beaker, stirred and left at rest to polymerize at room temperature for 1 hour. The green precipitate was collected on a filter, washed with 100 mL 1 M hydrochloric acid to remove the residual monomer, the oxidant and its decomposition products. The treatment with HCl provides more uniform protonation of ES. It was washed with distilled water and then 300 mL acetone to remove the low molecular weight organic intermediates and oligomers. It also prevents the aggregation of ES during drying, so that product was obtained as fine powder. The resulting protonated emeraldine was dried at room temperature for 3 hour and further in an oven at 70 °C for 3 hour.

2.2.2 Preparation of emeraldine base (EB)

The emeraldine base was prepared by deprotonation of emeraldine salt [3, 4]. For that ES was stirred with 0.1 M NaOH for 24 hour. It was then filtered, washed several times with distilled water and dried.

2.2.3 Preparation of poly(o-toluidine) salt (TS)

Poly(o-toluidine) was prepared in a manner analogous to polyaniline by oxidation of o-toluidine hydrochloride with ammonium persulphate in aqueous acidic media [5]. O-toluidine (4.4 mL, 0.2 M) was dissolved in 200 mL of 1 M HCl, and the solution was cooled to 0-5 °C in an ice bath. A few drops of FeSO_4 solution were added as catalyst. A pre-cooled solution of 4.6 g of (NH_4)_2S_2O_8 in 100 mL of distilled water was added drop wise with vigorous stirring during a period of 10 minutes. Ten to fifteen minutes after the reactants were mixed; the solution started to show a green tint and became intense green as a precipitate was formed. After ~2 hour, the precipitate was collected on a Buchner funnel. The blue-green powder was transferred into a beaker containing 200 mL of 1 M HCl to ensure complete protonation. After stirring at room temperature for 10 hour, the mixture was filtered, and the precipitate was washed with 500 mL of 1 M HCl until the filtrate became colorless. It was dried in air and stored.
2.2.4 Preparation of poly(o-toluidine) base (TB)

For the conversion of poly(o-toluidine) hydrochloride into poly(o-toluidine) base, the hydrochloride was suspended in aqueous NaOH (100 mL of 0.5 M) with stirring for 16 hour at room temperature [5]. The pH of the solution was periodically adjusted to ~10 (pH paper test) by the addition of drops of 1 M NaOH. The suspension was filtered, and the precipitate was washed with ~ 400 mL of 0.1 M NaOH followed by five 50 mL portions of a 1:1 mixture of methanol and 0.2 M NaOH and finally with distilled water and dried.

2.3 Activation of Polymers

2.3.1 Activation with glutaraldehyde

Activation of the support with glutaraldehyde followed by the immobilization of the enzyme is one of the most popular techniques to immobilize enzymes. The method is quite simple and even allows improving stability of immobilized enzyme [6]. All the polymers, emeraldine salt (ES), emeraldine base (EB), poly(o-toluidine) salt (TS) and poly(o-toluidine) base (TB) prepared were activated with glutaraldehyde for covalent immobilization of enzymes to obtain ES1, EB1, TS1 and TB1 respectively according to the methodology described by Olsson and Örgen [7]. 1 g each of the dried polymers was mixed with 2.5 % (v/v) glutaraldehyde solution prepared in 0.1 mol L⁻¹ potassium phosphate buffer on pH 6. The mixture was allowed to react under reflux for 2 hour. All supports were washed with the same buffer to free of excess glutaraldehyde and dried.

2.3.2 Activation of polymers with ascorbic acid

The bifunctional reaction of ascorbic acid (ASA) with amino compounds can be adapted in to enzyme immobilization on support materials which contain NH₂ groups [8]. 1 g each of ES, EB, TS and TB was stirred with 1 g (5.7 mmol) of ASA dissolved in 50 mL methanol. The reaction mixture was warmed slightly for 30 minutes. It was then filtered, washed with distilled water and dried to obtain ES2, EB2, TS2, and TB2 respectively.
2.4 Preparation of Immobilized Enzymes

In order to immobilize the enzyme, the polymer powder was mixed with equal volume of enzyme solution and buffer of the desired pH. It was shaken in a water bath shaker at room temperature for one hour and centrifuged [9, 10]. It was washed several times with the same buffer to remove the unbound enzyme. The biocatalyst was filtered using a sintered glass filter and dried in airflow. It was stored in refrigerator at 4 °C and used for further studies. The supernatant and washings were tested for any unbound enzyme.

2.4.1 Optimization of immobilization procedure

The influence of pH, time of incubation, and enzyme concentration on immobilization were determined by carrying out immobilization at various pH (3-8), contact time (15-90 minutes) and enzyme concentration of 0.5-2.5 mg glucoamylase and 1-6 mg α-amylase. The immobilized polymers were analyzed for enzyme activity under constant condition. Sodium acetate-acetic acid (0.2 M, pH 3-5.5) and sodium phosphate (0.2 M, pH 5.6-8) were used as buffers for optimization studies.

2.4.2 Estimation of protein

The amount of protein present in the solution before immobilization, both in the filtrate and washings were determined by the method proposed by Lowry et al. [11] using Folin & Ciocalteu Phenol reagent and measuring absorption at 730 nm in Spectro UV-Vis Double Beam UVD-3500 spectrophotometer. The amount of protein on the support was calculated from the difference between the amount of protein initially present in the reaction mixture and the amount of protein present in the filtrate and washing solutions after immobilization.

2.4.3 Immobilization efficiency

Immobilization yield (IY) was calculated by measuring concentration of protein in the supernatants before and after immobilization, according to equation 2.1 below.

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\[ IY (\%) = \frac{C1 - C2}{C1} \times 100 \]  

2.1

Where, \( C1 \) was the concentration of protein taken for immobilization and \( C2 \) was the concentration of protein present in supernatant after immobilization.

And the activity yield was determined by the equation 2.2.

\[ AY (\%) = \frac{\text{Activity of immobilized enzyme}}{\text{Activity of free enzyme}} \times 100 \]  

2.2

The immobilization efficiency,

\[ IE = \frac{AY}{IY} \]  

2.3

2.5 Determination of Activity of Enzymes

2.5.1 Activity of \( \alpha \)-amylase

Activities of free and immobilized \( \alpha \)-amylase were estimated by the hydrolysis of starch with the enzyme and subsequent determination of the residual starch with iodine-potassium iodide reagent [12]. 1 g soluble potato starch was dissolved in 100 mL boiling buffer of suitable pH. The solution was then cooled to 40 °C. A known amount of the free or immobilized enzyme in buffer was mixed with 2 mL of starch solution and incubated at optimum temperature in a water bath with constant shaking. After the particular time interval, the reaction was arrested by the addition of 0.1 mL 1 N HCl. 1 mL of above solution was withdrawn, added with 0.1 mL of iodine-potassium iodide reagent and was diluted with distilled water. The blue color developed was measured at 650 nm in a UV-Vis spectrophotometer. The results were compared with the absorbance of standard solution and the amount of starch converted was calculated. All tests were performed in triplicate and results are presented in average and standard deviation.
One enzyme unit (EU) of α-amylase activity was defined as the amount of enzyme, which converts 1 mg mL\(^{-1}\) of starch in 1 minute at optimum temperature and pH. Specific activity was calculated by the equation 2.4.

\[
\text{Specific activity} = \frac{\text{Enzymatic activity (mg mL}^{-1} \text{ min}^{-1})}{\text{Amount of protein (\(\mu g\))}} \tag{2.4}
\]

2.5.2 Activity of glucoamylase

The activity of glucoamylase (GMA) was determined by estimating the concentration of reducing sugar formed using DNS reagent [13]. 1 wt. % solution of soluble starch was prepared by dissolving starch in 0.2 M buffer of desired pH. In a test vial, a known amount of free enzyme or immobilized enzyme in buffer was placed. Subsequently, 1.0 mL starch solution in buffer was added and the system then incubated in a water bath with constant shaking at optimum temperature exactly for the particular time. The reaction was arrested by adding 0.5 mL of 3, 5-dinitrosalicylic acid reagent and with rapid cooling. It was placed in a boiling water bath for 10 minutes. The solution was cooled under running water and diluted with distilled water. The amount of reducing sugar produced was determined spectrophotometrically at 500 nm. A standard calibration curve was constructed with dextrose solution and the reducing sugar content was estimated. One unit (EU) of glucoamylase activity was defined as the amount of enzyme required to liberate 1\(\mu\)mol of reducing sugar per minute at optimum pH and temperature. Each result was an average of three separate set of experiments. Specific activity of GMA was calculated from the following equation,

\[
\text{Specific activity} = \frac{\text{Released reducing sugar (\(\mu\) mol mL}^{-1} \text{ min}^{-1})}{\text{Amount of protein (\(\mu g\))}} \tag{2.5}
\]
2.6 Biochemical Characterization

2.6.1 Effect of pH on activity

The influence of pH on activity of free and immobilized α-amylase and glucoamylase was tested at different pH using 0.2 M acetate buffer (pH 4-5.5) and 0.2 M phosphate buffer (pH 5.6-8).

2.6.2 Effect of temperature on activity and stability

The effect of temperature on the activity of free and immobilized α-amylase and glucoamylase was tested by subjecting them to various temperatures ranging from 30-70 °C at optimum pH with 1 % starch solution. For investigating temperature stability, free and immobilized enzymes were placed in a water bath at various temperatures from 30-60 °C for 1 hour. After being cooled to optimum temperature, the enzymatic reaction was carried out. Thermal inactivation curves at optimum temperature with respect to time was obtained by incubating enzyme at optimum temperature and after definite time intervals, known amount of enzyme was withdrawn and tested for activity by standard assay procedure. All results are presented with the highest value of each assigned the value of 100 % activity.

2.6.3 Determination of kinetic parameters

The kinetic constants $K_m$ and $V_{max}$ values of free and immobilized enzymes were determined by measuring the rates of the reaction at various substrate concentrations at optimum temperature and pH. $K_m$ and $V_{max}$ were calculated from the Lineweaver-Burk plots, and Hanes-Woolf plots.

2.6.4 Storage stability of free and immobilized enzymes

The storage stability of soluble and immobilized enzymes was measured by calculating their activities after being stored at 4 °C for a required period. The measurement was conducted at regular intervals of time. The activity was compared with initial activity and was represented as percentage initial activity retained.
2.6.5 Reusability study of immobilized enzymes

The reusability of the immobilized enzyme was tested by repeated batch experiments by measuring the residual activity of the immobilized enzyme preparation at optimum conditions at intervals of 30 minutes. After each run, the immobilized enzyme was removed, washed with the buffer solution and mixed with fresh substrate solution.

2.7 Physico-Chemical Characterization

2.7.1 FT-IR spectroscopy

Fourier Transform Infrared Spectroscopy (FT-IR) is a popular tool for identifying and characterizing polymer materials and their additives. It can be applied to the analysis of solids, liquids, and gasses. The term Fourier Transform Infrared Spectroscopy (FT-IR) refers to a fairly recent development in the manner in which the data is collected and converted from an interference pattern to a spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. FT-IR spectra of pure compounds are generally so unique that they are like a molecular "fingerprint".

The FT-IR spectra of synthesized, activated and α-amylase immobilized polymers were obtained using a FT-IR spectrometer using KBr pellet method. Changes in the absorption bands were investigated in the 500-4000 cm⁻¹ region and the resolution applied was 4 cm⁻¹. The spectra were corrected for the H₂O and CO₂ content in the optical path.

2.7.2 Surface area analysis

A Micromeritics Gemini 2360 Surface Area Analyzer was used to measure the nitrogen adsorption isotherms of the samples at liquid nitrogen temperature. From this specific surface area was determined. Prior to the measurement all samples were degassed at room temperature for 8-10 hours in nitrogen flow.
2.7.3 Thermogravimetric analysis

Thermogravimetric analysis (TGA) is an analytical technique used to determine a material's thermal stability and its fraction of volatile components by monitoring the weight change that occurs as a specimen is heated. The measurement is normally carried out in air or in an inert atmosphere, such as Helium or Argon, and the weight is recorded as a function of temperature. Sometimes, the measurement is performed in a lean oxygen atmosphere (1 to 5 % O\(_2\) in N\(_2\) or He) to slow down oxidation. TGA is used to determine polymer degradation temperatures, residual solvent levels, absorbed moisture content, and the amount of inorganic (non-combustible) filler in polymer or composite material. It can also assist in de-formulation of complex polymer products.

Thermal studies were performed on Perkin Elmer Pyris Diamond 6 Thermogravimetric Analyzer in nitrogen atmosphere in the temperature range of 40-600 °C and heating rate of 10 °C per minute. Powdered samples of about 3 mg were sealed in standard platinum pans. The instrument was calibrated using indium and tin as standards. Sample residual weight (TG curves) and its derivative (DTG curves) versus temperature were automatically generated by Pyris software.

2.7.4 Scanning electron microscopy

To determine the surface topography and morphology of samples SEM images were recorded using the LEICA 420 I scanning electron microscope in collaboration with Dr. C. P. Sebastian at Institute for Inorganic and Analytical Chemistry, Münster, Germany. SEM allows for studies of surface features at low nanometer scale. The study was performed on the synthesized polyaniline (ES and EB) and on poly(o-toluidine) (TS and TB). A sample for investigation was placed on a conducting polymer foil on an aluminum holder. The high magnification of the microscope allowed visualizing details of the crystal shape and taking photographs.
References


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