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Immobilization of Invertase in Copolymer of 2,5-Di(thiophen-2-yl)-1-p-Tolyl-1*H*-Pyrrole with Pyrrole

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Immobilization of Invertase in Copolymer of 2,5-Di(thiophen-2-yl)-1-p-Tolyl-1*H*-Pyrrole with Pyrrole

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Immobilization of invertase in conducting copolymer matrix of 2,5-di(thiophen-2-yl)-1-p-tolyl-1*H*-pyrrole with pyrrole (poly(DDTP-co-Py)) was achieved via electrochemical polymerization. Kinetic parameters, Michaelis-Menten constant, K_m and the maximum reaction rate, V_{max} were investigated. Operational stability and temperature optimization of the enzyme electrodes were also examined.

Immobilized invertase reveals maximum activity at 50°C and; pH 8 and pH 4 for two copolymer matrices. Although the same two monomers are utilized for the copolymer synthesis, the way the copolymer is produced results in quite different responses in terms of enzyme activity, optimum pH and kinetic parameters. Excellent operational stability of the enzyme electrodes enables their repetitive use in the determination of invert sugar.

Keywords: Invertase, enzyme immobilization, electrochemical polymerization, conducting polymers, copolymers, copolymer matrices.

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1 Introduction

There are several different enzymes which are extraordinarily efficient and selective biological catalysts, catalyzing the reactions essential for life. Enzymes increase the rate of chemical reactions and weaken the chemical bonds by lowering the activation energy. Enzymes are delicate species as they can be affected by temperature and pH.

Immobilization is a technique which comprises attachment of enzyme to a solid matrix without loss of catalytic activity. Thus enzyme immobilization on solid support materials becomes a very effective way to stabilize them (1,2). Immobilization of enzymes has several technical and economical advantages; makes cost reduction of biocatalysts enabling repeated use, provides continuous operational mode, enhances rapid termination of reaction, easy separation of biocatalyst from product, increases the stability and activity of enzyme and accelerates the enzyme recovery (3–5).

Immobilization provides efficient use of enzymes in food technology, biotechnology, biomedicine and also analytical chemistry. Immobilization methods vary as adsorption, entrapment, covalent binding, encapsulation, crosslinking etc. (6). Enzymes which are immobilized usually show better thermal and pH stability. Moreover, immobilization is effective on several parameters like optimum temperature, Michaelis Menten constant (K_m) and the maximum reaction rate (V_{max}) which depend on the immobilization method and the nature of the carrier (7).

Invertase (β-D-fructofuranosidase EC 3.2.1.26), which has been found in bacteria, fungi, and plants, is the enzyme that mainly used for the production of invert sugar in food industry by hydrolyzing sucrose into glucose and fructose. Invert sugar is colorless and has lower crystallinity than sucrose. Its use in confectionery ensures the products remains fresh and soft even when kept for a long time.

Invertase has an industrial application in food industry for fructose syrups production which is widely used in the elaboration of sweetened carbonated drinks, candies, and canned food. In addition, soluble invertase is used in the production of artificial honey, jam and to a small extent liquid sugar in the sweetener industry (8–10). Immobilization of invertase on corn grifts (11), polyethylene (12), polyaniline (13), gelatin (14), carbohydrate moieties (15), polyelectrolytes (16), porous cellulose beads (17), diazonium salt of 4-amino-benzoylcellulose (18), and poly (ethylene-vinylalcohol) (19) has been studied.

Electrochemical polymerization is a technique for the synthesis of inherently conducting polymers which are very useful for physical entrapment of an enzyme in their matrices. This technique is attracting great interest, since it speeds up the formation of conducting film on the working

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electrode. The method also controls the thickness of the polymer via utilizing accurate and precise data. Furthermore, in this technique only small amounts of monomer are required (20).

In the present study, immobilization of invertase via electrochemical method in poly(2,5-di(thiophen–2-yl)-1-p-tolyl-1H-pyrrole-co-pyrrole) were investigated. Optimum pH, temperature and kinetic parameters (V_{max} and K_m) were examined for the immobilized enzymes. Also, operational stability and shelf life of the enzyme electrodes were determined. Surface morphologies of these modified electrodes were inspected.

2.1 Materials

Invertase (EC 3.2.1.26) Type V was purchased from Sigma and used as received without further purification. Pyrrole (Merck) was distilled before use and stored at 4°C. Sodium dodecylsulfate (SDS) was supplied from Merck. In order to prepare Nelson reagent, sodium carbonate, sodium potassium tartarate, sodium bicarbonate, sodium sulfate, copper sulfate pentahydrate, ammonium heptamolybdatetetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$) and sodium arsenate ($Na_2HAsO_4.7H_2O$) were provided from Aldrich.

2.2 Instrumentation

Potentioscan Wenking POS-73 and ST-88 potentiostats, Shimadzu UV-160-A model spectrophotometer and JEOL JSM-6400 model scanning electron microscope (SEM) were used for characterization.

2.3 Synthesis of Monomer

To a suspension of $AlCl_3$ in CH_2Cl_2, a solution of thiophene and succinyl chloride in CH_2Cl_2 was added dropwise. The red mixture was stirred at room temperature for 4 h. This was then poured into ice and concentrated HCl mixture. The dark green organic phase was washed with concentrated $NaHCO_3$ and brine, and then dried over $MgSO_4$. After evaporation of the solvent a blue green solid remained. Filtration and washing with ethanol yielded the 1,4-bis(2-thienyl)butane-1,4-dione.

The monomer (DTTP) was synthesized from 1,4-di(2-thienyl)-1,4-butanedione and p-toluidine in the presence of a catalytical amount of p-toluenesulphonic acid (PTSA). A round-bottomed flask equipped with an argon inlet and magnetic stirrer was charged with the 1,4-di(2-thienyl)-1,4-butanedione, p-toluidine, PTSA and toluene. The resultant mixture was stirred and refluxed for 24 h under argon. Evaporation of the toluene, followed by flash column chromatography (SiO_2 column, elution with dichloromethane: hexane (1:1)) afforded the desired compound as pale brown powder. The synthetic route of the monomer is shown in Figure 1 (21).

2.4 Synthesis of Copolymer of DTTP with Pyrrole via Two Different Routes

2.4.1 Route 1

Working electrode was coated with dichloromethane solution of DTTP (1%w/v). After evaporation of the solvent, the electrode was immersed into a typical three electrode

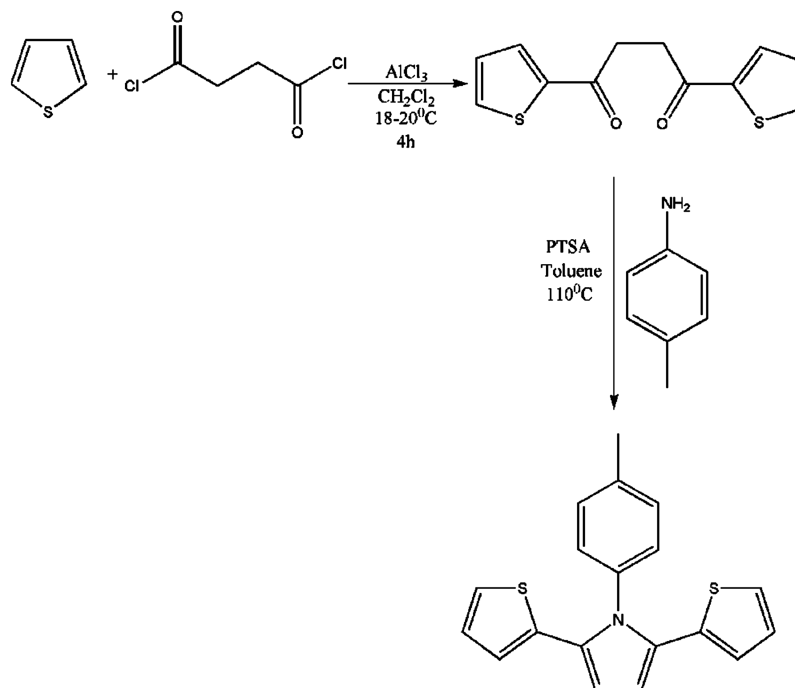

Fig. 1. Synthetic route of monomer (DTTP).

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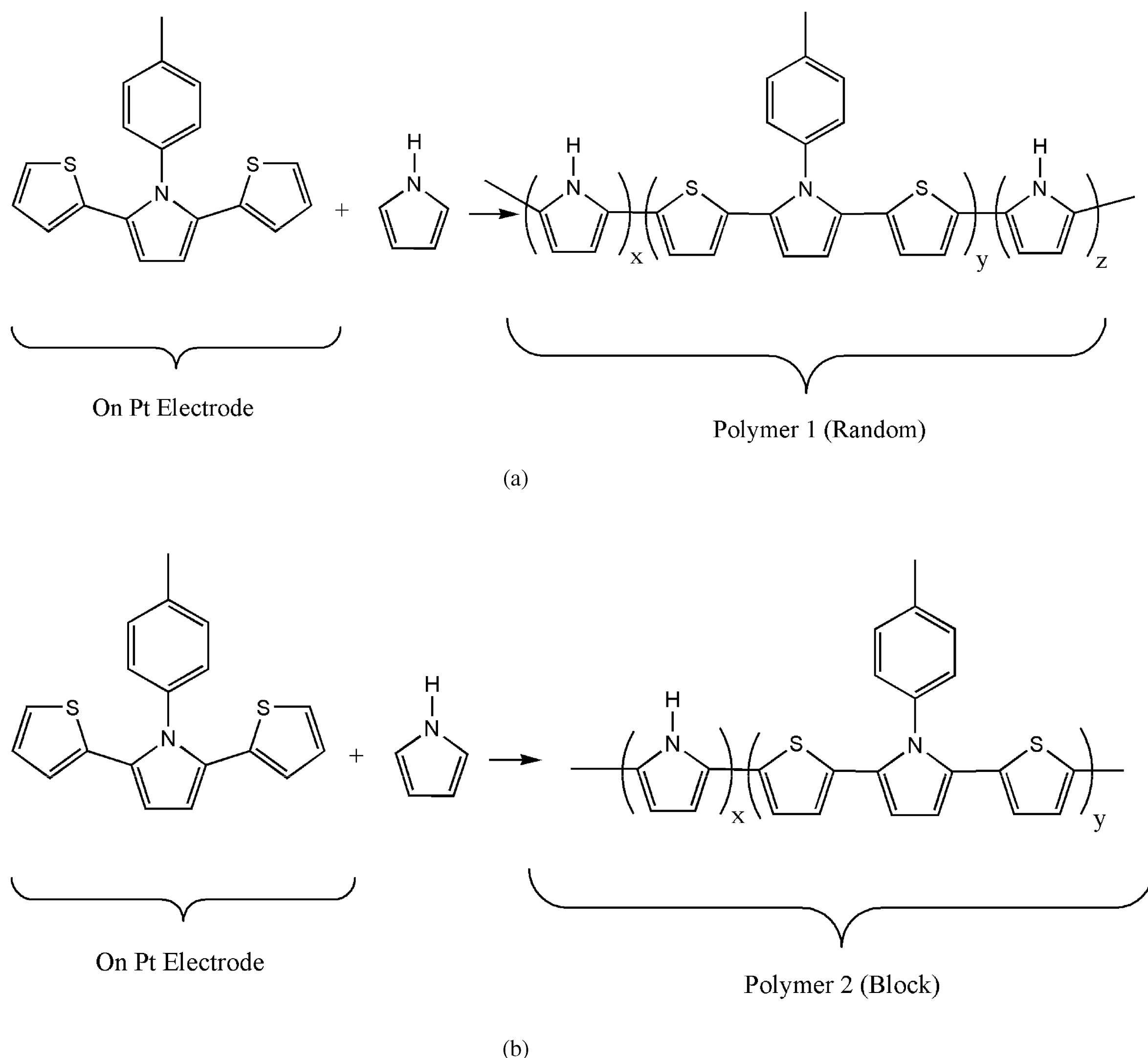

Fig. 2. (a) Method 1 for copolymerization of DDTP. (b) Method 2 for copolymerization of DDTP.

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cell containing acetate buffer, 0.01M pyrrole and SDS (1%w/v). Electro polymerization (30 min.) was performed by applying +1.0 V vs. Ag wire pseudo reference electrode. The resulting copolymer was coded as Polymer 1 (Fig. 2.a)

2.4.2 Route 2

Polymerization of DDTP was performed in a three electrode cell containing 10 mL acetonitrile, 0.1 M $NaClO_4$, 0.1 M $LiClO_4$ and 5%w/v DTTP. Polymerization reactions were carried out potentiodynamically with potentials between 0.0 V and 1.0 V for 60 min. Then coated electrode immersed in a solution of 4.2%w/v SDS, 0.01M pyrrole in acetate buffer. Homopolymerization was carried out by applying 1.0 V for 30 min. The resulting homopolymer was coded as Polymer 2 (Fig. 2.b)

2.5 Immobilization of Invertase in Polymer 1 and in Polymer 2 Matrices

Immobilization of invertase in Polymer 1 and Polymer 2 matrices was performed in a typical three electrode cell containing platinum foil (1 cm × 1 cm) working and counter electrodes and an Ag wire reference electrode by constant potential electrolysis at room temperature. Solution for the immobilization in Polymer 1 matrix is same as it is in synthesis of copolymer, only difference is that immobilization solution includes invertase (1%w/v). Solution for Polymer 2 matrix includes invertase (1.8%w/v) in addition to the solution of homopolymerization. Immobilizations were carried out by applying 1.0 V for 30 min. Immobilization of enzymes was carried out on bare and Polymer 1 and Polymer 2 coated electrodes. After electrolyses, enzyme electrodes were washed with distilled water in order to remove both the excess supporting electrolyte and the unbound enzyme.

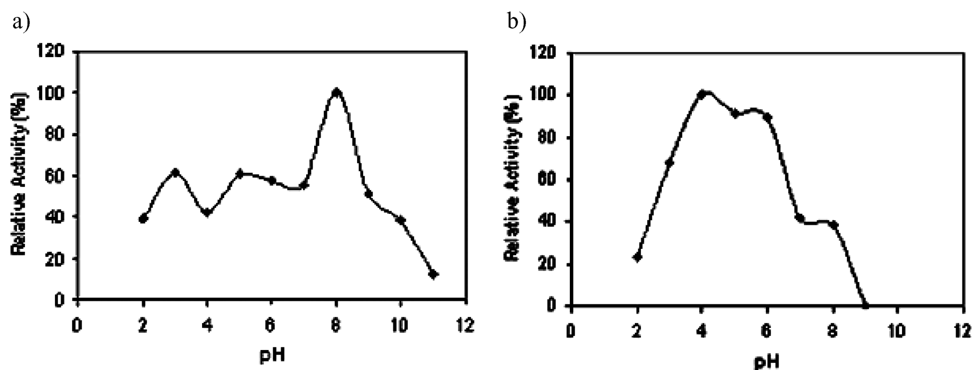

Fig. 3. Effect of pH on invertase enzyme activity of immobilized enzyme in (a) Polymer 1, (b) Polymer 2 enzyme electrodes.

Electrodes were kept in acetate buffer at 4°C when not in use.

2.6 Determination of Invertase Activity

Determination of immobilized and free invertase activities was performed using Nelson's method (22) for both matrices. Different concentrations of sucrose solutions were preincubated for 10 min at 25°C. Then, enzyme electrode was placed in sucrose solutions for specific reaction times (2, 4 and 6 min). After removing the electrode, 1 mL aliquots were drawn and added to 1 mL Nelson's reagent to terminate the reaction. The tubes were then placed in boiling water bath for 20 min, then they were cooled to room temperature and 1 mL arsenomolybdate reagent was added. Finally, 7 mL of distilled water was added to each test tube and mixed by vortexing. After mixing, absorbances for the blank and the substrate solutions were determined at 540 nm with a double beam spectrophotometer. One unit of invertase activity was defined as the amount of enzyme required to release 1 μmol glucose from sucrose per minute at pH 5 and 25°C.

2.7 Determination of Kinetic Parameters

In order to determine maximum velocity of the reaction (V_{max}) and the Michaelis–Menten constant (K_m) for immobilized enzyme, activity assay was applied for different concentrations of sucrose.

2.8 Determination of Optimum pH and Optimum Temperature

Optimum pH determination was carried out by changing pH between pH 2.0 and 11.0. Optimum temperature was determined by changing the incubation temperature between 10°C and 80°C. The rest of the procedure was the same as the invertase activity measurements.

2.9 Operational Stability

Operational stability of immobilized enzymes in polymer films was tested (at optimum activity assay conditions) by performing 40 activity assays in one day.

3. Results and Discussion

3.1 Kinetic Studies

Kinetic studies of immobilized invertase were carried by varying sucrose concentration while keeping temperature and pH constant. Maximum velocity, V_{max} and

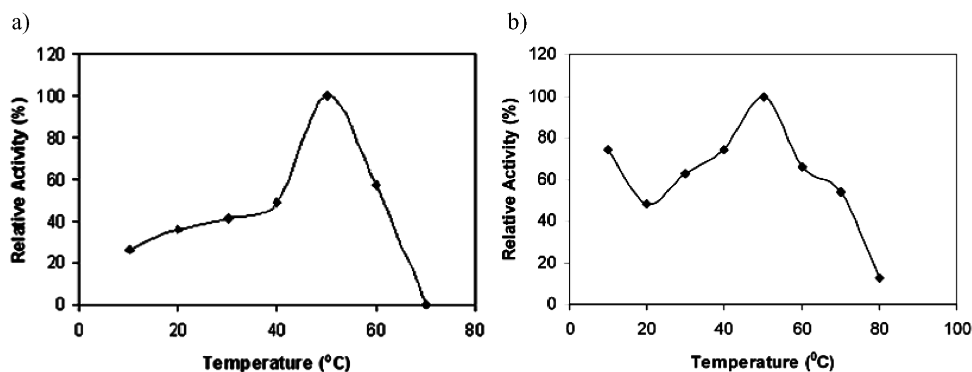

Fig. 4. Effect of temperature on invertase activity of immobilized enzyme in (a) Polymer 1, (b) Polymer 2 enzyme electrodes.

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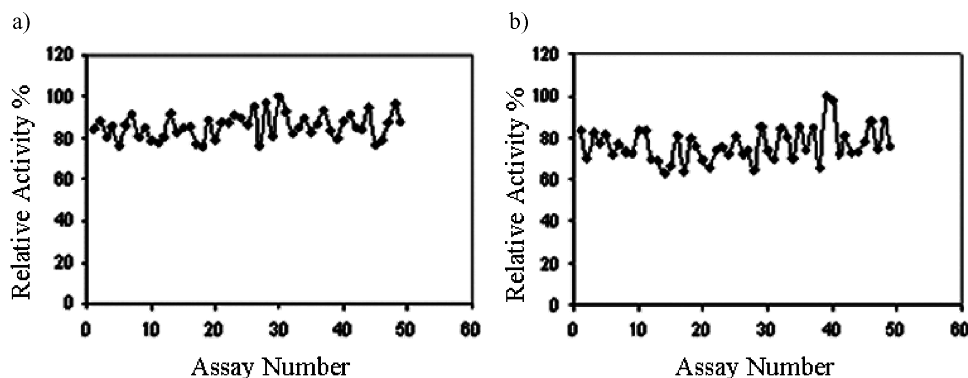

Fig. 5. Operational stability of invertase immobilized in (a) Polymer 1, (b) Polymer 2 enzyme electrodes.

Michaelis-Menten constant, K_m, were found using Lineweaver-Burk plot (23). For both matrices, immobilized invertase parameters are given in Table 1. The reason for different K_m values may be attributed to the differences in the porosity of each polymer film. Furthermore, it can be seen that there is a noticeable increase in V_{max} and K_m values compared to those for PPy matrix. K_m value of invertase in PPy matrix is lower than that of both in Polymer 1 and Polymer 2 matrices. Thus, the interactions between enzyme and substrate in PPy matrix are stronger that of both matrices since the lower K_m value means that the affinity between enzyme and substrate is higher. V_{max} values of invertase in both matrices are higher than that of invertase in PPy matrix. Lower V_{max} of invertase in PPy matrix can be due to the lower amount of enzyme entrapped in the matrix.

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3.2 Influence of pH on the Enzyme Electrode Response

Changes in pH can affect the enzyme structure and also cause denaturation. In order to prevent this, a buffer solution must be used. Invertase has an isoelectric point of 4.5 and therefore, it is negatively charged at pH 5.0 and 5.2. For this reason, the enzyme can be incorporated in the copolymer matrix as anions balancing a positively charged polymer and firmly entrapped by electrostatic interactions. Thus, pH measurements were performed at pH 5 for all enzyme electrodes. The maximum activity was observed at pH 8.0 for Polymer 1, and pH 4.0 for Polymer 2. They are illustrated in Figure 3. The pH of maximum activity of enzyme in Polymer 1 shifted to alkaline side and pH of maximum activity of enzyme in Polymer 2 shifted to acidic side compared to the free enzyme which might be explained by partitioning of protons. Negatively charged groups in matrix will tend to concentrate protons (thus lowering the pH) around the enzyme. Consequently, the pH around the enzyme will be lower than that of the bulk phase from which the measurement of pH is carried out. Polymer 1 has wider working range compare to Polymer 2. However Polymer 2 shows higher relative enzyme activities at three different pH.

Table 1. Kinetic parameters of invertase

	V_{max}	K_m (mM)
PPy/Invertase	0.3	2.8
Polymer/Invertase	2.6	40
Polymer 2/Invertase	2.1	85

3.3 Influence of Temperature on the Enzyme Electrode Response

The effect of temperature on enzyme activity was studied and illustrated in Figure 4. The maximum enzyme activity for both Polymer 1 and Polymer 2 was found to be at 50°C. Compared to Polymer 2, Polymer 1 has wider temperature range although it has lower enzyme activity. After 70°C, the response of invertase in Polymer 1 matrix decreases suddenly to zero. It is possible that the enzyme in the copolymer matrix denatures completely at 80°C.

3.4 Operational Stability of the Enzyme Electrodes

Forty measurements were performed in one day in order to determine the operational stability of immobilized invertase. Polymer 1 matrix reveals 85% activity even after 40 measurements (Figure 5(a)). On the other hand, activity of invertase in Polymer 2 is 80% for the same number of measurements (Figure 5(b)).

4. Conclusions

Immobilization of invertase was achieved by electrochemical polymerization. Kinetic parameters, operational and shelf life stability, optimum temperature and pH of enzyme electrodes were investigated for two matrices. Although V_{max} values for immobilized invertase are almost the same for both matrices, enzyme affinity was found to be higher to its substrate in Polymer 1 matrix. Immobilized invertase

reveals maximum activity at 50°C and; pH 8 and pH 4 for Polymer 1 and Polymer 2 matrices respectively. Polymer 1 supplies better operational stability and higher relative activity. This study shows that conducting copolymers of 2,5-di(thiophen-2-yl)-1-p-tolyl-1H-pyrrole with pyrrole can be used successfully as the immobilization matrices for invertase.

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