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IMMOBILIZATION OF YEAST CELLS IN SEVERAL CONDUCTING POLYMER MATRICES

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ABSTRACT

Immobilization of yeast cells *(Saccharomyces cerevisiae)* in different polymer matrices was performed by constant potential electrolysis. These matrices were polypyrrole (PPy); poly(methyl methacrylate)/polypyrrole (PMMA/PPy) and thiophene-capped poly(methyl methacrylate)/ polypyrrole (TPMMA/PPy). The characterization of PMMA/PPy copolymer was achieved by Fourier-transform Infrared Spectroscopy (FT-IR), Differential Scanning Calorimetry (DSC) and Scanning Electron Microscopy (SEM). The invertase activity of immobilized yeast cells was determined. Optimum temperature, Michaelis-Menten constants and maximum reaction rates of the enzyme electrodes were compared with those of free yeast cells. The operational and storage stabilities of three different immobilization systems were analyzed.

Key Words: Immobilization; Yeast; Invertase; Polypyrrole; Poly(methyl methacrylate)

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INTRODUCTION

Immobilization is a technique confining a catalytically active enzyme or cell within a reactor system and preventing its entry into the mobile phase which carries the substrate and product. Various advantages can be obtained upon immobilizing enzymes such as repetitive use and easy removal of enzyme from the substrate. Immobilization of cells containing specific enzymes has further advantages such as elimination of long and expensive procedures for enzyme separation and purification. The whole cell enzyme immobilization method gives high recovery of enzyme activity compared to the immobilization of purified enzyme [1]. Immobilized microbial cell derivatives are widely studied in biomedical, pharmaceutical and medical areas.

Immobilization of microbial cells can be carried out by encapsulation, by entrapment inside a polymer matrix, inside calcium alginate gels, calcium pectate gels, or by adhesion to wool. Yeast cells are widely used in industry due to exhibiting good invertase activity. Invertase is the enzyme, which catalyzes the hydrolysis reaction of sucrose into glucose and fructose. Since sucrose crystallizes more readily than invert sugar (fructose), the latter is used in the production of creams, jams and artificial honey. This enzyme has been also immobilized in pure form by using numerous techniques such as entrapment in fibers of cellulose triacetate [2], in poly (acrylamide) gel [3], binding to collagen by adsorption [4], covalently linking to several petroleum-based materials such as poly(ethylene-vinyl alcohol) membrane [5].

The immobilization of yeast cells exhibiting invertase activity has been performed by employing Ca-pectate gels [6], films of poly (2-hydroxyethyl methacrylate) [7], Ca-alginate gels [8], liquid-core alginate capsules [9], agar-agar [10] and wool [11] as carrier materials. Most studies concentrated on the production properties and bioreactor parameters. However, principal enzyme functions (kinetic parameters, optimum reaction temperatures, etc.) were not considered properly in immobilized systems. This work, therefore, aims to clarify the enzymatic parameters of the immobilized yeast cells in terms of the enzyme invertase.

In this study, the immobilization of yeast cells was achieved via entrapment within polypyrrole (PPy); poly(methyl methacrylate)/polypyrrole (PMMA/PPy) and thiophene-capped poly(methyl methacrylate)/polypyrrole (TPMMA/PPy) polymer matrices. The synthesis and characterization of TPMMA/PPy matrix was described earlier [12]. The idea of entrapping the whole cells instead of the invertase enzyme which was studied before [13, 14] lies in the fact that if the immobilization works, then one would not need to purify the material to obtain the pure invertase. Thus, if the immobilized whole cells can show a comparable activity to the pure invertase, it will be more feasible to entrap the whole cells.

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EXPERIMENTAL

Materials

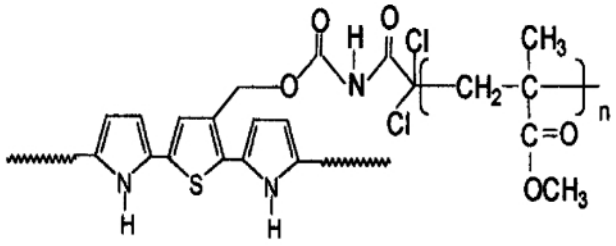
Baker's yeast *(Saccharomyces cerevisiae)* was purchased from Pak Maya (Turkey) and used without further purification. Pyrrole (Merck) was distilled and stored at 4°C. Sodium dodecyl sulfate (SDS) (Sigma) was used as received. For the preparation of Nelson reagent; sodium carbonate (Riedel de Haen), sodium potassium tartarate (Riedel de Haen), sodium bicarbonate (Merck), sodium sulfate (Merck), copper sulfate (Merck) and for the preparation of arsenomolybdate reagent; ammonium heptamolybdate (Merck), sodium hydrogen arsenate (Merck) were used as received.

Synthesis of PMMA/PPy Copolymer

1% solution (w/v) of PMMA ($M_w \sim 15{,}000$-GPC) was prepared in acetonitrile. Pyrrole was polymerized electrochemically on platinum (Pt) electrode that was previously coated with PMMA solution. SDS was used as the supporting electrolyte and electropolymerization yielded a black film on the electrode after 30 minutes of reaction by applying 1.0 V against Ag/Ag^+ reference electrode.

Immobilization of Yeast Cells in PPy, PMMA/PPy, TPMMA/PPy Matrices

Immobilization of yeast cells was performed by electropolymerization of pyrrole on bare, previously PMMA coated and previously TPMMA coated platinum electrodes. In the latter, pyrrole chains grow through the thiophene moiety of the TPMMA [12]. During the pyrrole polymerization yeast cells, which are present in the electrolysis medium, are expected to be entrapped in the copolymer matrix.



TPMMA/PPy

The electrolysis solution contained 0.4 mg/mL yeast; 0.4 mg/mL sodium dodecyl sulfate (SDS) as the supporting electrolyte, 0.02 M. pyrrole monomer and 50 mL 0.05 M. acetate buffer. After electrolysis, the electrode

was taken out; washed with distilled water several times to remove excess supporting electrolyte and kept in acetate buffer for further studies like enzyme activity; reaction rate; optimum conditions and operational stability. Triplicate experiments were performed for each study.

Invertase Activity Determination

The invertase activities of both free (EU/mL) and immobilized yeast cells (EU/electrode) were determined by measuring the initial reaction rates of sucrose hydrolysis at pH 4.8, at 50°C. Nelson method was used to determine reducing sugar concentration [13]. To convert the spectrophotometer readings to enzyme activities, a standard glucose calibration curve was prepared. One unit of yeast invertase activity (1 EU) was defined as the amount of yeast cells required to produce 1 μmol of glucose from sucrose per minute at pH 4.8, at 50°C.

Determination of Optimum Temperature

The reaction temperature was changed between 10°C and 70°C while sucrose concentration was kept constant at about 10 K_m for each system. The activities were determined as previously described.

RESULTS AND DISCUSSION

Synthesis and Characterization of PMMA/PPy Copolymer

1% solution (w/v) of PMMA was prepared in acetonitrile. Pyrrole was polymerized electrochemically on Pt electrode that was previously coated with PMMA solution. SDS was used as the supporting electrolyte and electropolymerization yielded a black film on the electrode after 1 hour of reaction by applying 1.0 V, against Ag/Ag^+ reference electrode. FT-IR, DSC, TGA analyses showed that copolymer of PMMA/PPy was formed.

The characteristic carbonyl peak of pure PMMA appearing at 1732 cm^{-1} also existed in the resulting copolymer with a small shift at 1738 cm^{-1} (Fig. 1). The DSC spectra of pure PMMA, PMMA/PPy films are studied (Fig. 2). The heating process was performed from 30°C to 530°C at a rate of 10°C/min. Pure PMMA showed glass transition temperature (T_g) around 92°C and a well-defined melting peak at 385°C. In the copolymers, two broad endothermic peaks were observed, the first one being at about 80°C due to the loss of water absorbed by the polymer during electropolymerization. The second peak was observed at around 258°C due to the

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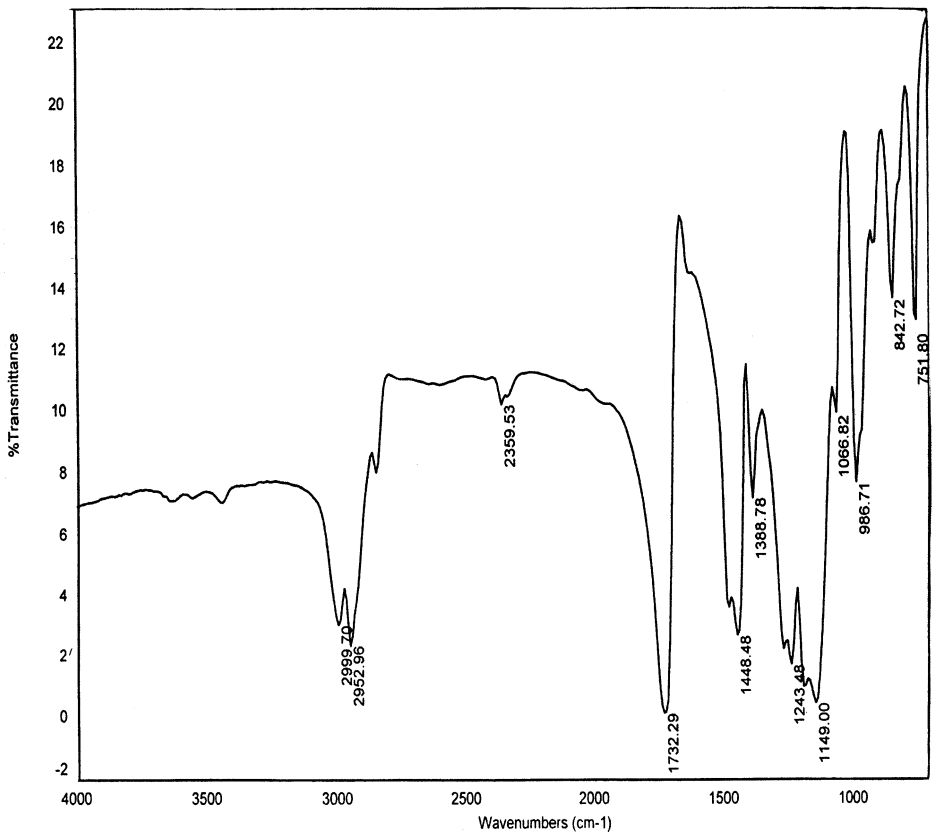

Figure 1a. FT-IR spectrum of pure PMMA polymer.

loss of dopant ion. No melting due to the PMMA segments was observed, indicating rather long PPy chains. Simple weighing measurements yielded that PPy was grafted on PMMA, with a higher percentage than did TPMMA, namely 77% for PMMA and 8% for TPMMA [12].

PMMA/PPy copolymer reveals a single weight loss pattern as does pristine PMMA (Fig. 3). The difference in their peak temperatures and the steepness of the peaks is yet another evidence for the copolymerization. Via copolymerization the resistance to heat is enhanced since ca. 45% remnant is found compare to that of 4% for PMMA.

Effect of Different Immobilization Matrices on the Invertase Activity of Baker's Yeast

The invertase activities of cells immobilized in three different matrices at 50°C, namely PPy; PMMA/PPy and TPMMA/PPy, are presented in Table 1. Approximately twice the activity was obtained for the copolymer matrices

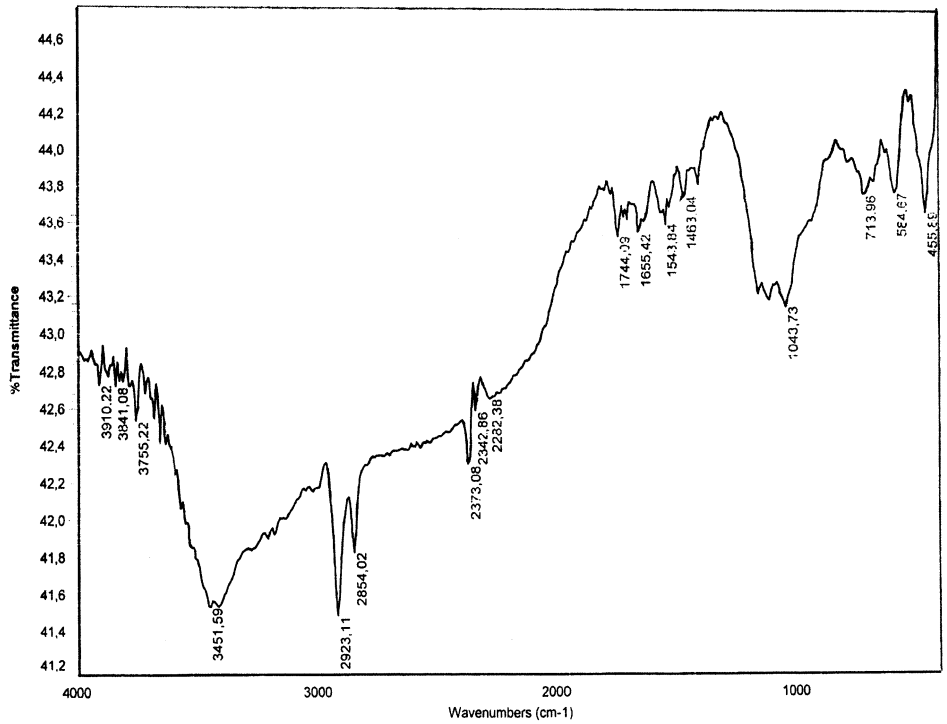

Figure 1b. FT-IR spectrum of PMMA/PPy copolymer.

compared to pure PPy and the highest invertase activity was seen in PMMA/PPy graft copolymer matrix. This may be related with either immobilization of more cells in these matrices, or more effective cell protection, or better substrate diffusion to cells, or better affinity to substrate in these matrices.

The lower grafting percent in case of TPMMA/PPy matrix means that it contained more pure PPy chains than did PMMA/PPy, i.e., TPMMA/PPy matrix behaves more like pure PPy towards yeast cells. Minimum invertase activity was observed in PPy matrix therefore, it is not surprising that PMMA/PPy matrix gave higher activity than the other copolymer matrix.

Kinetic Parameters

The Michaelis-Menten constant, K_m, and maximum reaction velocity, V_{max}, were obtained from Lineweaver-Burk plots and are given in Table 2. As observed, the K_m values for the immobilized cells within different matrices were almost the same, around 50 mM sucrose. This finding excludes the assumption of better diffusion properties in copolymer matrices since K_m values were the same as in PPy matrix. The K_m value for the free yeast cells

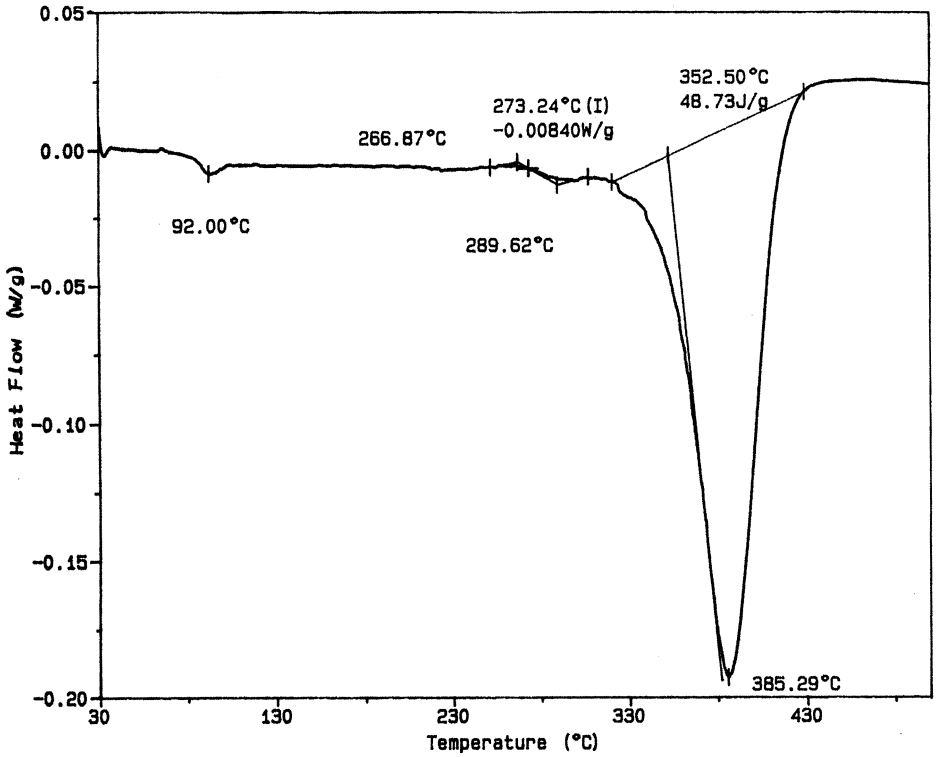

Figure 2a. DSC thermogram of pure PMMA polymer.

were obtained as 65 mM sucrose. Although a noticible decrease in substrate affinity is a general outcome of any immobilization experiment, the difference in K_m values of the free and immobilized cells is not large. In addition, these results showed lack of diffusion problems for all three matrices having almost the same K_m value. When V_{max} values were compared, again the highest reaction rate was obtained for PMMA/PPy matrix. (Table 2).

We have confirmed that yeast cells were not damaged or burst during electrolysis by checking protein and DNA concentrations within the solutions both before and after blank electrolysis (without monomer) by means of absorbance measurements at wavelengths 280 and 260 nm, respectively. Lack of significant differences in the protein and DNA concentrations indicated that yeast cells were not burst and the cell membranes were not broken. The spectrophotometer readings at 600 nm before and after electrolysis also did not reveal any difference in the cell density.

Kinetic Assay at 25°C

The kinetic parameters for TPMMA/PPy matrix were obtained at 25°C and compared with those of pure invertase [13] in order to examine the

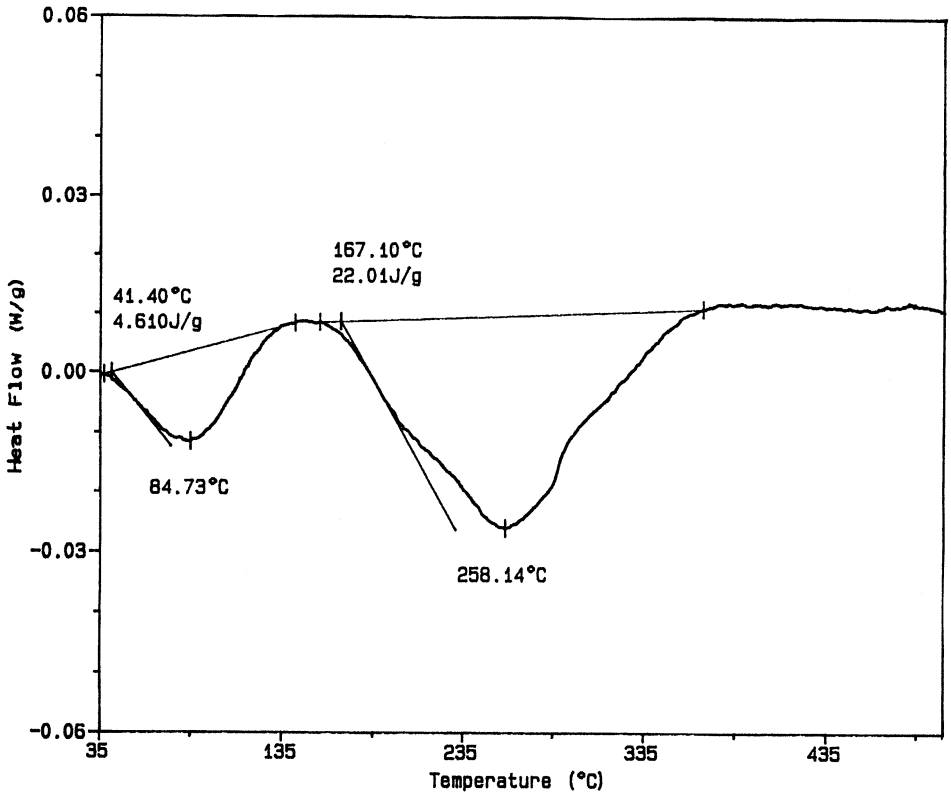

Figure 2b. DSC thermogram of PMMA/PPy copolymer.

efficiency of using immobilized invertase over immobilized yeast cells having invertase activity. K_m values were the same for both pure and yeast invertases, indicating that being confined in the cell did not cause any hinderance of the enzyme towards its substrate. For the reaction rates, the values were very similar that the challenged steps of enzyme purification did not show a benefit in terms of either substrate affinities or maximum reaction rates for the mentioned matrix. As a conclusion; it is practical to use whole yeast cells instead of pure invertase immobilized in TPMMA/PPy matrix.

Effect of Incubation Temperature on Invertase Activity

Optimum temperature for invertase activity of free yeast was found as 55°C. In a previous study for pure invertase, it was found as 50°C [13]. Figure 4 shows relative percent invertase activities versus temperature in three different immobilization systems, respectively. For all three matrices, the maximum invertase activity was obtained at the same temperature, which is 60°C. The temperature sensitivity of invertase activity of yeast cells can be deduced by

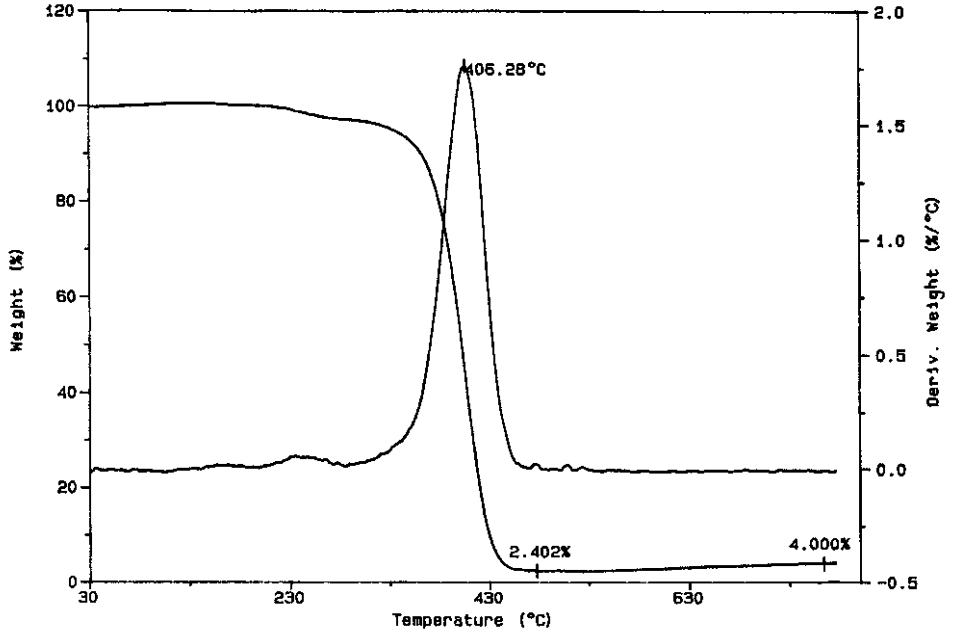

Figure 3a. TGA thermogram of pure PMMA polymer.

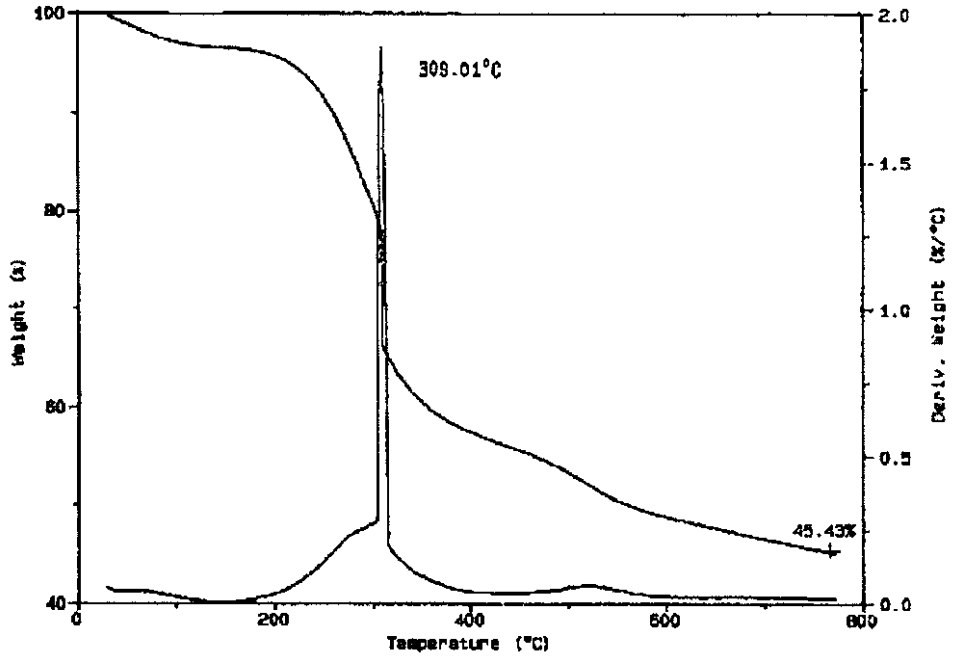

Figure 3b. TGA thermogram of PMMA/PPy copolymer.

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Table 1. Invertase Activities of Immobilized Yeast Cells in Different Matrices

System	Invertase Activity (EU/Electrode)
PPy	0.9
PMMA/PPy	1.8
TPMMA/PPy	1.4

Table 2. Kinetic Parameters for Invertase Activity of Yeast Cells

System	K_m (mM)	V_{max} (EU/Electrode)
Free	65	0.3*
PPy	54	1.2
PMMA/PPY	52	2.1
TPMMA/PPy*: (EU/mL)	50	1.5

the shape of the curves, which was a little bit different for pure PPy and the copolymers. Up to 60°C, sensitivity was higher in PPy than the two copolymer matrices. Yet, after the maximum point, yeast cells lost most of the invertase activity (20% left) in TPMMA/PPy matrix, while 95 and 80% of maximum activities were retained in PMMA/PPy and pure PPy, at 70°C. This may be attributed to the nature of TPMMA/PPy, which is a graft copolymer and the microstructure may as well be quite different than the network structures of the other two. This is somewhat revealed by the SEM pictures.

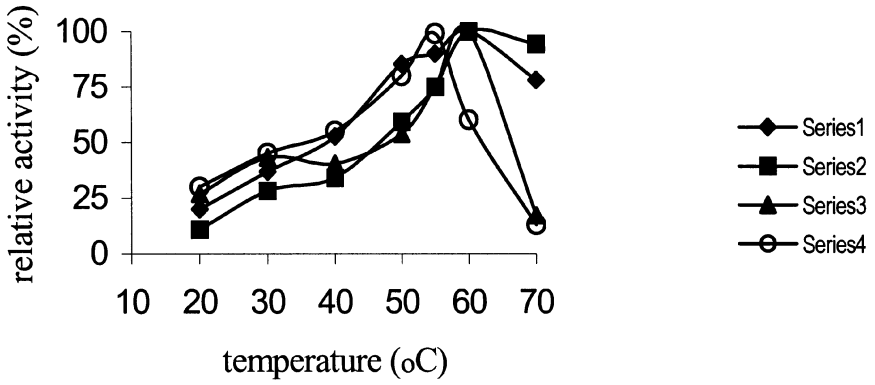

Figure 4. Effect of incubation temperature on invertase activity of yeast cells immobilized in PMMA/Ppy (Series 1, -◆-); Ppy (Series 2, -■-); TPMMA/Ppy (Series 3, -▲-); and free cells (Series 4, -⊖-).

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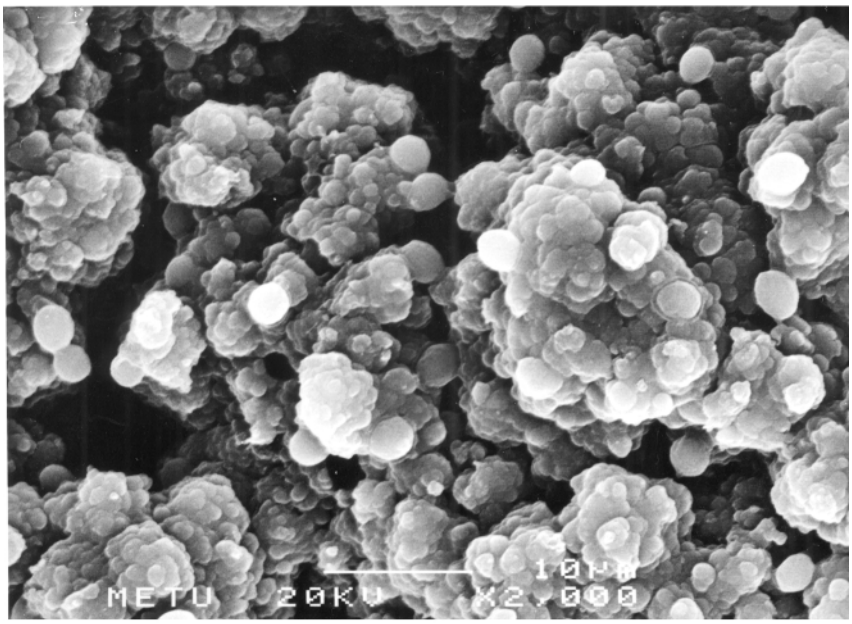
Morphologies of the Films

In order to determine the surface morphologies of polymer films, scanning electron microscopy (SEM) technique was used (JEOL JSM-6400). Scanning electron micrographs of Ppy/yeast cells, PMMA/Ppy/yeast cells and TPMMA/PPy/yeast cells are given in Fig. 5. The surface properties of PPy and TPMMA matrices without enzyme were given in previous studies [13, 14].

At the solution sides of PPy, PMMA/PPy and TPMMA/PPy films, the destruction of cauliflower-like structure was observed, being more drastic in the case of copolymers when compared to pure PPy. This may facilitate the substrate diffusion. However, there were no structural changes in the electrode sides for any matrix.

Operational and Storage Stability

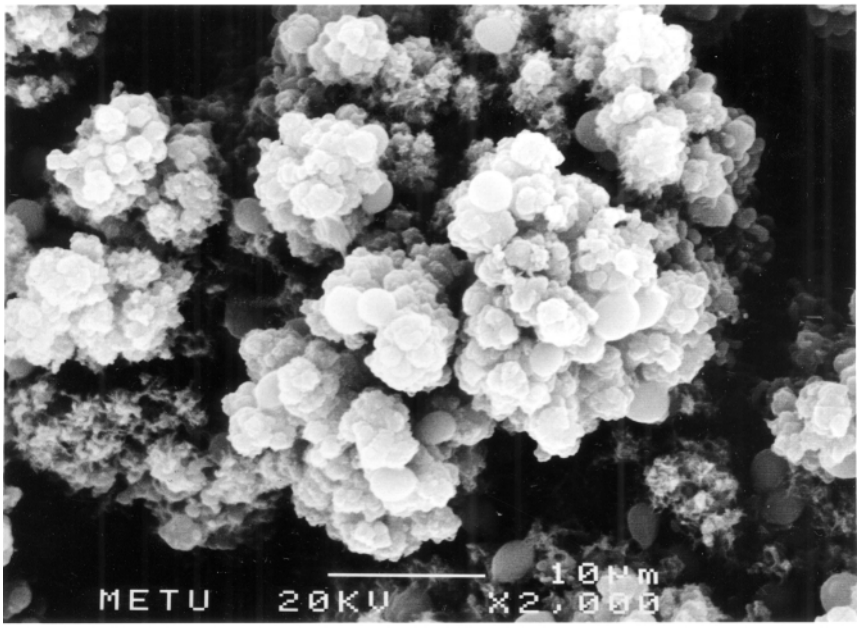
To determine the operational stability of immobilized yeast cells, electrodes were kept in 10 mL buffer solution at 4°C and the invertase activity was periodically measured at 50°C. For each, four measurements were performed. The results are shown in Fig. 6.



a

Figure 5. Scanning electron micrographs of: a) PPy film with yeast cells; b) PMMA/PPy film with yeast cells; c) TPMMA/PPy film with yeast cells.

(*continued*)



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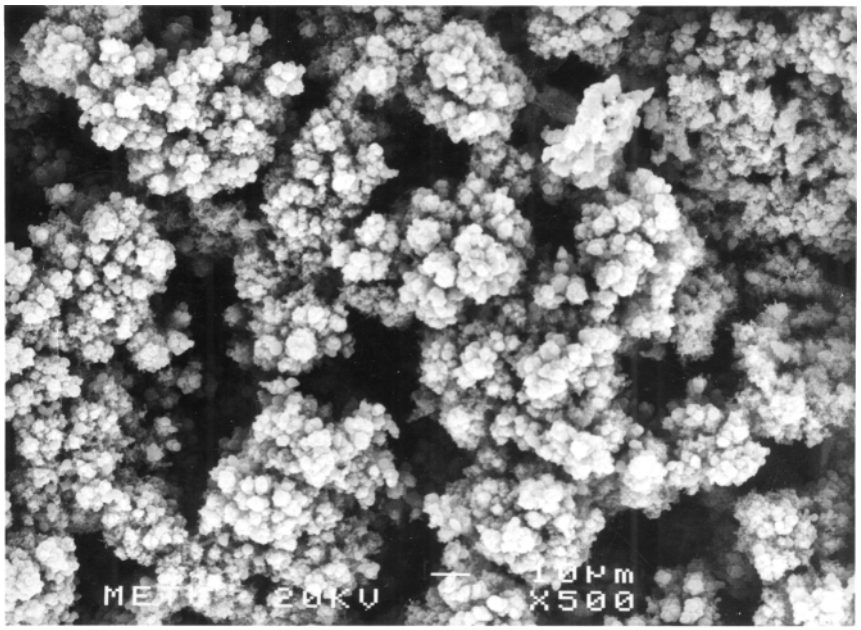

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Figure 5. Continued.

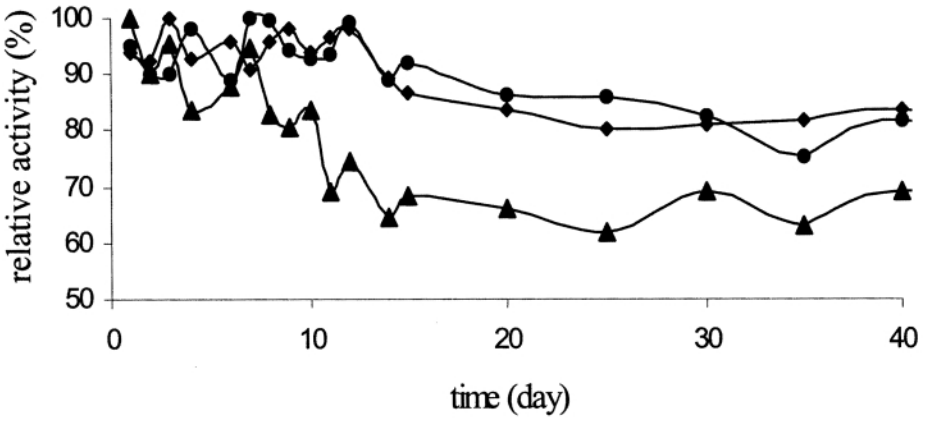

Figure 6. Operational and storage stabilities of PPy/yeast (◆); PMMA/PPy/yeast (●); and TPMMA/PPy/yeast (▲).

As revealed by the figure, PPy and PMMA/PPy enzyme electrodes showed better storage properties compared to TPMMA/PPy within the first 40 days. Moreover, PPy; PMMA/PPy and TPMMA/PPy electrodes retained 69; 56 and 63% of their initial activities.

Comparing the operational stabilities of invertase in yeast and pure invertase in PPy and TPMMA/PPy matrices [13]; after a period of 30 days, although yeast cells retained about 80% (PPy) and 70% (TPMMA/PPy) of their initial activity; pure invertase in the same systems retained about 60% and 80% of its initial activity, respectively.

CONCLUSION

In this study, yeast cells were used as a model for whole cell immobilization in several conducting polymer matrices and the invertase activity of the immobilized cells were investigated. The immobilized cell system showed comparable kinetic data with that of the immobilized enzyme. Morever, the immobilized cells showed good storage and operaional stabilities. The so-called 'enzyme electrodes', containing immobilized yeast cells, retained most of the initial invertase activity after 20 assays and 40-day storage at 4°C (PPy and PMMA/PPy: 80%, TPMMA/PPy: 70%).

Measurements performed at 25°C and comparison with pure invertase [13] revealed that the substrate affinity and maximum reaction rate of invertase immobilized in TPMMA/PPy matrix was not changed whether pure invertase or yeast cells were used. The substrate affinities, given by K_m values, were not different in case of all matrices and also for native yeast cells. This result may be attributed again to the protection of the enzyme by whole cell since it eliminated the effects of such a different environment (synthetic

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polymer matrix). As for the maximum reaction rate (V_{max}), the values obtained in copolymer matrices were higher than pure PPy, which might be caused by the better mechanical properties of insulating polymers incorporated into PPy in the copolymer. With their enhanced mechanical properties (flexibility), copolymer matrices may be more suitable for the immobilization.

As a result, it is worth using immobilized yeast cells instead of immobilized invertase. Since invertase is such a cheap enzyme, it is used rather in native form in the industry. However, this study can serve as a model for the immobilization of more expensive enzymes, in which case the usage of unpurified form would be economical.

ACKNOWLEDGMENTS

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