

pH-Sensitive Chitosan Films for Baker's Yeast Immobilization

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Abstract

Dried baker's yeast cells were immobilized on a chitosan film, which is a natural polymer. Prepared chitosan films were treated with glutaraldehyde to facilitate the immobilization of the cells. The effects of the amount of glutaraldehyde, incubation time, pH, and temperature on immobilization were investigated. The amount of glutaraldehyde was chosen to be 0.01% (weight). The highest amount of yeast immobilization was obtained with 5 h incubation. It was determined that optimum temperature for immobilization is 25°C, and the optimum pH for immobilization is 6. Immobilized cells were allowed to stand for 3 d in distilled water and buffer solution (pH 6) to investigate the desorption, but no desorption was found. The maximum immobilization capacities were found to be 90 µg protein cm⁻² film in optimum conditions.

Index Entries: Yeast; cell; immobilization; chitosan.

Introduction

A large number of biological processes have been investigated in order to fully utilize immobilized biocatalysts. Among them, immobilized whole-cell systems have received considerable attention. Advantages of whole-cell systems include their catalyzing ability to synthesize various useful and complicated chemicals using multi-enzyme steps, and regeneration activity to prolong their catalytic life (1). There is a wide variety of materials

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that are currently used as supports for cell immobilization. Many gel-like materials are used as carriers, which may be based on unmodified and modified natural (alginate, carrageenan, agar, gelatine, chitin, chitosan, and so on) or synthetic (polyacrylamide, polyacrylate, polyurethane, and so on) precursors (2–6).

The use of an immobilized yeast cell system for alcoholic fermentation is an attractive and rapidly expanding research area because of its additional technical and economical advantages compared with the free cell system (7). Immobilization of yeast cells through application of various techniques (e.g., covalent bonding, encapsulation, entrapment in a gel matrix) or by γ -irradiation has been reported (8).

Chitosan is a poly(aminosaccharide), normally obtained by alkaline deacetylation of chitin, the principal component of living organisms such as fungi and crustacea. This naturally occurring polymer has a repeating unit of 2-acetamido-2-deoxy- β -D-glucose. Chitosan has been used in the applications such as immobilized enzymes (9) and drug carriers (10,11). It has also been used in immobilization of microbial cells or plant cells (12).

In our previous study, chitosan films were used for the immobilizing of catalase enzyme (13). The aim of the present study was designed to investigate immobilization of yeast cells on chitosan films that are noncrosslinked or crosslinked with glutaraldehyde.

Materials and Methods

Chitosan, bovine serum albumin, and glutaraldehyde were obtained from Sigma (St. Louis, MO). Dried baker's yeast was obtained from Pak Maya (Istanbul, Turkey). Other chemicals were obtained from Merck (Darmstadt, Germany).

Preparation of Chitosan Films

One gram of chitosan was dissolved in 100 mL acetic acid solution (0.8% w/v). The solution was uniformly deposited onto polymethylmethacrylate plates by a microsyringe dispenser. Films were then dried at 60°C for 30 h. They were washed with distilled water several times and finally redried. The chitosan films were conditioned in water, then treated with glutaraldehyde at room temperature and washed until washings were free of glutaraldehyde. They were cut in the dimensions of 1 cm \times 1 cm \times 0.09 cm, and used for the experiments.

IR Spectra of the Chitosan Films

The chitosan films were ground to the suitable size of powder and infrared spectra of the powder samples were obtained with a UNICAM Mattson 1000 FTIR spectrometer.

Thermomechanical Analysis of the Chitosan Films

To determine the penetration temperatures and maximum penetration, the thermomechanical analysis of the chitosan films were made with

a Shimadzu Thermomechanical Analyzer at the temperature range of 25–75°C under 1 g cm⁻² load.

Swelling Measurement

Noncrosslinked and crosslinked chitosan films were cut and were swollen in various pH values at 25°C. The percentage swelling degree (S%) was calculated from the following expression:

$$S\% = \frac{m_e - m_d}{m_d} \times 100$$

where m_e and m_d are the mass of sample for equilibrium swelling state and dry state, respectively.

The reversibility of swelling was also determined. The sample was first swollen in a solution of pH = 3, and the swelling degree was measured vs time. After certain time, the gel was transferred to a solution of pH = 8 and the swelling degree was measured vs time again.

Preparation of Cells

Two grams of active dried baker's yeast was dispersed in 200 mL water and heated 15 min with stirring on a boiling water bath, and then the cells were cooled.

Immobilization of Yeast Cells on Chitosan Films

One-half milliliter of the yeast solution was mixed with 20 cm² of glutaraldehyde-pretreated chitosan films in 10 mL phosphate buffer (pH 7) at 25°C. The suspension was incubated at 25°C under aerobic condition in a rotary shaker. After immobilization, the films were taken from yeast solution and washed until the washing was free of protein. Glutaraldehyde concentration, optimum incubation time, optimum temperature, and optimum pH were also investigated.

Determination of Immobilized Yeast Cell Protein

The protein content in the solutions was determined by the method of Bradford using bovine serum albumin as standard (14). The amount of immobilized cells as protein, Q , was calculated using the following relationship:

$$Q = \frac{C_0 - C_e}{A_c} V$$

where C_0 and C_e are initial and equilibrium concentration of protein ($\mu\text{g protein mL}^{-1}$) in the solution, A_c is area of chitosan films (cm²), and V is total volume of solution in mL.

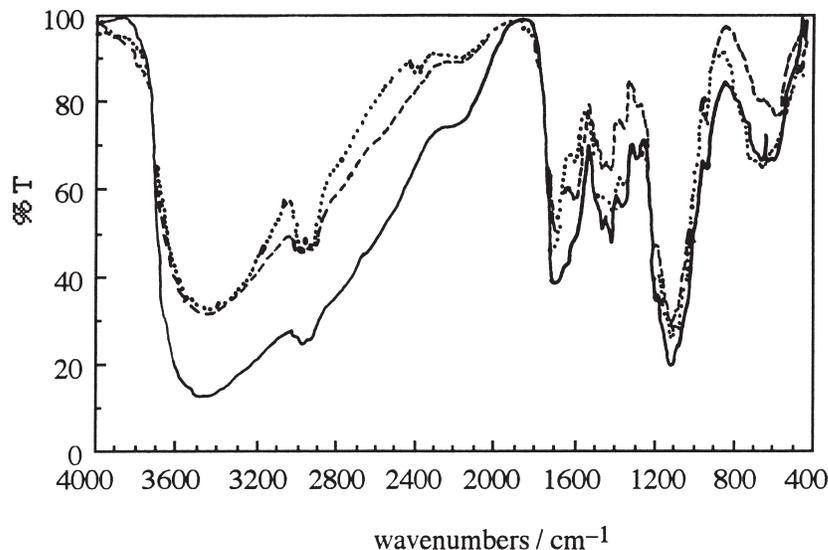


Fig. 1. The IR spectra of chitosan films: —, noncrosslinked (A); ···, crosslinked (B); - - -, yeast immobilized (C).

Determination of Desorption of Immobilized Yeast Cells

The chitosan films that were taken from yeast solution were allowed to stand for 3 d in distilled water and buffer solution (pH 6) at 25°C and then the protein concentration was determined.

Results and Discussion

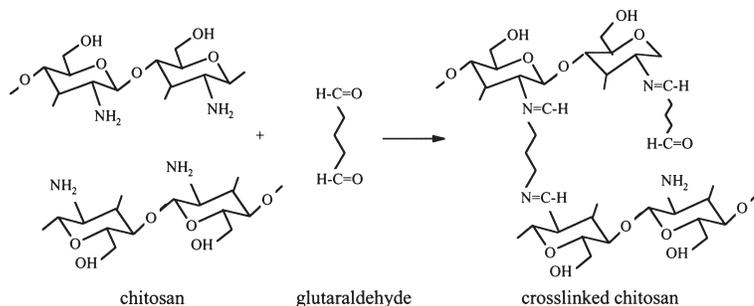
Preparation and Characterization of Chitosan Films

Chitosan was dissolved in acetic acid solution, and the solution was uniformly deposited onto polymethylmethacrylate plates, to form films. Prepared films were dried. Then, they were washed with distilled water several times and finally redried. The chitosan films were conditioned in water, then treated with glutaraldehyde at room temperature and washed until the washings were free of glutaraldehyde.

For the structural and thermomechanical characterizations of the chitosan films, IR spectra and thermograms were taken, and swelling tests were made.

IR Spectra of Chitosan Films

The IR spectra of chitosan (Fig. 1A) show 910 cm^{-1} and 1153 cm^{-1} peaks of the assigned saccharide structure and a weaker characteristic peak at around 1590 cm^{-1} (15). Figure 1B shows a significant peak at 1574 cm^{-1} , which can be attributed to the characteristic peak of C–N forming from the crosslinking reaction between chitosan and glutaraldehyde. The possible mechanism of this reaction can be presented schematically as shown in Scheme 1.



Scheme 1. Possible crosslinking reaction of chitosan with glutaraldehyde.

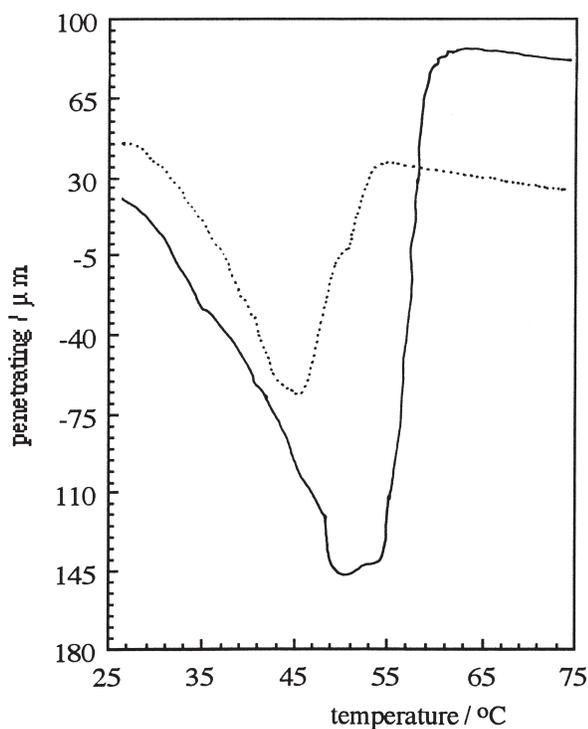


Fig. 2. The thermograms of chitosan films: ..., noncrosslinked; —, crosslinked.

Thermomechanical Analysis of Chitosan Films

Thermomechanical thermograms obtained for the chitosan films are shown in Fig. 2, and the values of maximum penetrating (l , μm) and the temperature at maximum penetrating (T , $^{\circ}\text{C}$) of chitosan films are presented in Table 1.

Figure 2 and Table 1 show that the values of maximum penetration and the temperature at maximum penetration are increased with the addition of crosslinker to the chitosan. Thus, it can be said that the thermomechanical stability of crosslinked chitosan films are higher than the noncrosslinked chitosan films.

Table 1
Some Thermomechanical Properties of Chitosan Films

Polymer film	l (μm)	T ($^{\circ}\text{C}$)
Noncrosslinked chitosan	65.84	45.29
Crosslinked chitosan	142.64	49.80

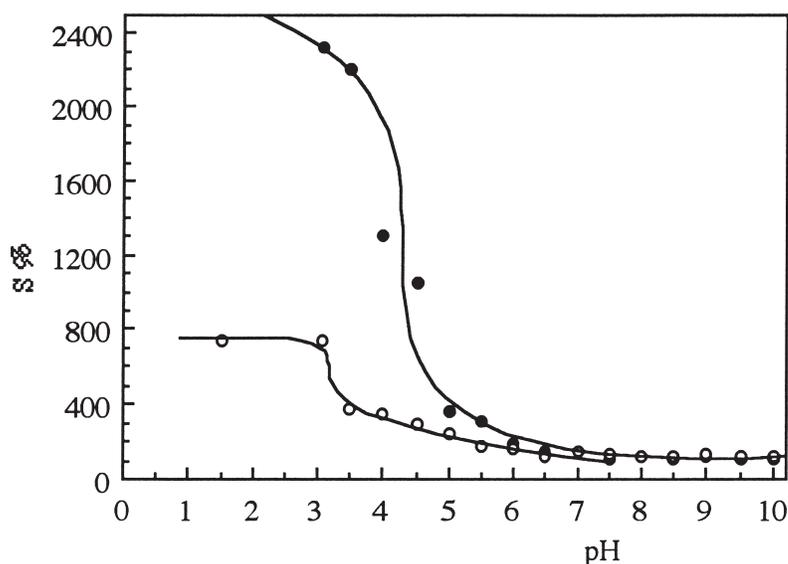


Fig. 3. The effect of pH on the swelling of chitosan films: ●, noncrosslinked; ○, crosslinked.

Swelling of Chitosan Films

The equilibrium degrees of swelling for noncrosslinked and crosslinked chitosan films at different pH are shown in Fig. 3. They all indicate that the degree of swelling drops sharply in high pH ($\text{pH} \geq 6$). This can be explained by the fact that the network is dissociated in an acidic medium, owing to amino groups on chitosan that ionized, but not as much as in an alkaline medium (15). At the same time, Fig. 3 shows the dependence of the equilibrium degree of swelling on the addition of the crosslinker glutaraldehyde to chitosan films. With the addition of glutaraldehyde, crosslink density of chitosan films becomes more restricted. However, in high pH ($\text{pH} \geq 6$), where amino groups are not ionized and hydrogen bonding associates, there is almost no affect of the addition of crosslinker on its swelling behavior and pH sensitivity.

The contrasting reversibilities of the swelling of chitosan films are shown in Fig. 4. It was observed that the degree of swelling of noncrosslinked chitosan films was higher than that of crosslinked films after the same time interval. On the other hand, Fig. 4 reveals that the swelling is reversible. At $\text{pH} = 3$, amino groups on chitosan are ionized, which leads

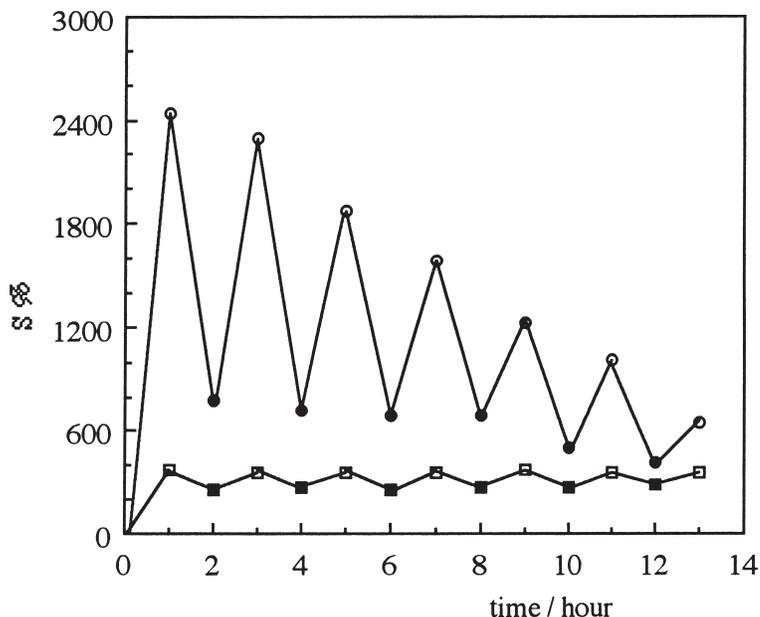


Fig. 4. Reversibility of the swelling of chitosan films: ○, noncrosslinked chitosan at pH = 3; ●, noncrosslinked chitosan at pH = 8; □, crosslinked chitosan at pH = 3; ■, crosslinked chitosan at pH = 8.

to the dissociation of hydrogen bonding in the network, whereas at pH = 8, ionized amino groups can revert to amino groups, which results in the association of hydrogen bonding in the network. This process is reversible.

Immobilization of Yeast Cells on Chitosan Films

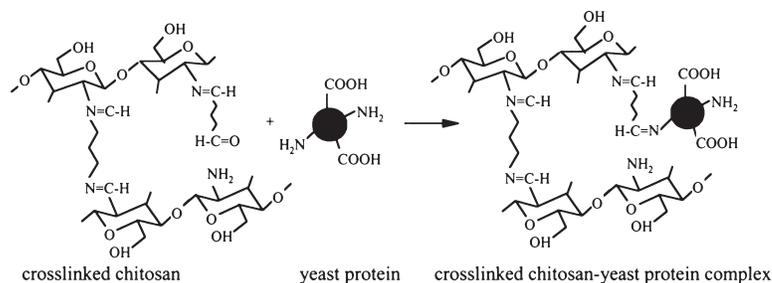
IR Spectrum of Yeast Cell Immobilized Chitosan Films

Infrared spectrum of yeast cell immobilized chitosan film was taken to investigate the immobilization of the cells onto crosslinked chitosan films with glutaraldehyde, and is presented in Fig. 1C.

In Fig. 1C there is a new peak at 1600 cm^{-1} , which can be attributed to the formation of C=N because of the imine reaction between amino groups from yeast cell protein and aldehyde groups in glutaraldehyde. Glutaraldehyde activation of supports is a method for irreversible immobilization of proteins through a single amino-support link. The possible mechanism of this reaction is presented schematically in Scheme 2.

The Effects of Immobilization Parameters

At this stage, the effects of glutaraldehyde concentration, incubation time, pH, and temperature on the immobilization of yeast cells on chitosan films were investigated. Desorption of yeast cells from chitosan films was also investigated.



Scheme 2. Possible immobilization of yeast cells on crosslinked chitosan.

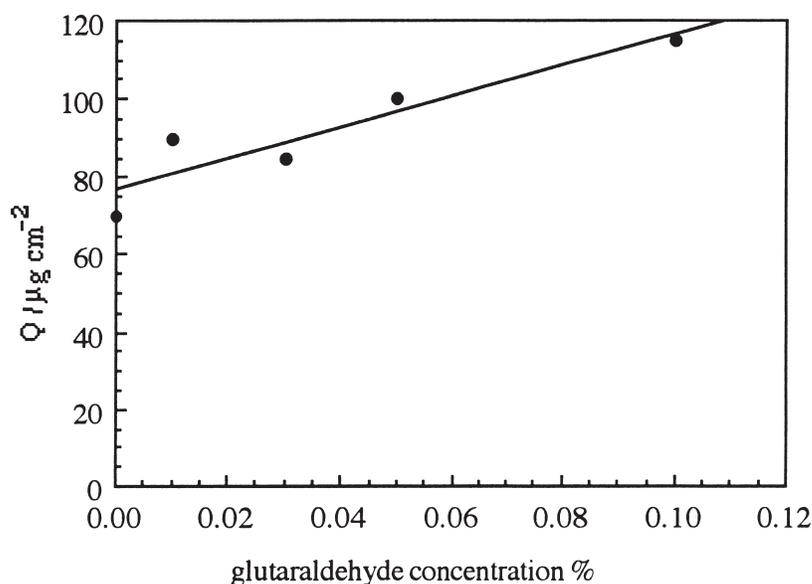


Fig. 5. The effect of glutaraldehyde concentration on immobilization.

Effect of Glutaraldehyde Concentration on Immobilization

To investigate the glutaraldehyde concentrations on the immobilization of yeast cells, chitosan films were treated with glutaraldehyde. The amounts of immobilized cells vs the concentrations of glutaraldehyde were plotted and are presented in Fig. 5. It can be seen in Fig. 5 that the immobilization of cells increased with the increase in the amount of glutaraldehyde. However, when the glutaraldehyde amount was increased, the films became brittle. Therefore, 0.01% was chosen as the optimum glutaraldehyde concentration.

Effect of Incubation Time on Immobilization

To investigate the optimum incubation time, the amounts of immobilized cells were plotted against the incubation times (Fig. 6). As shown in Fig. 6 maximum immobilization of cells was found at 5 h. Therefore, other assays were made in this time period.

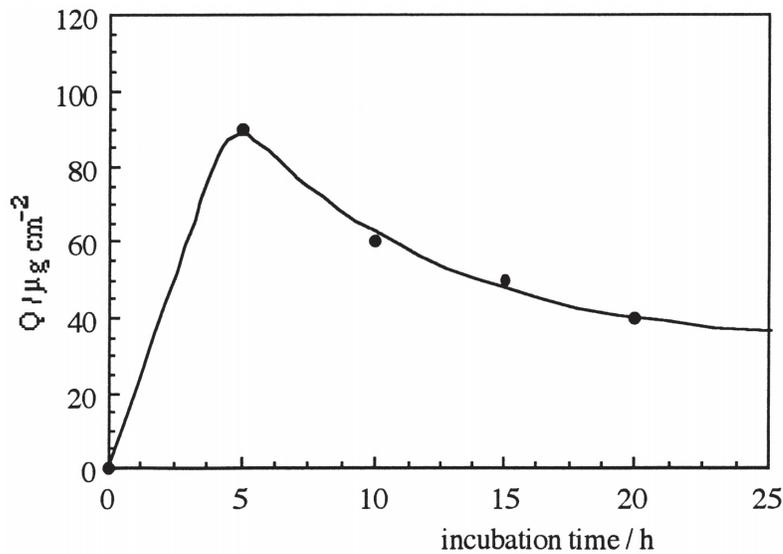


Fig. 6. The effect of incubation time on immobilization.

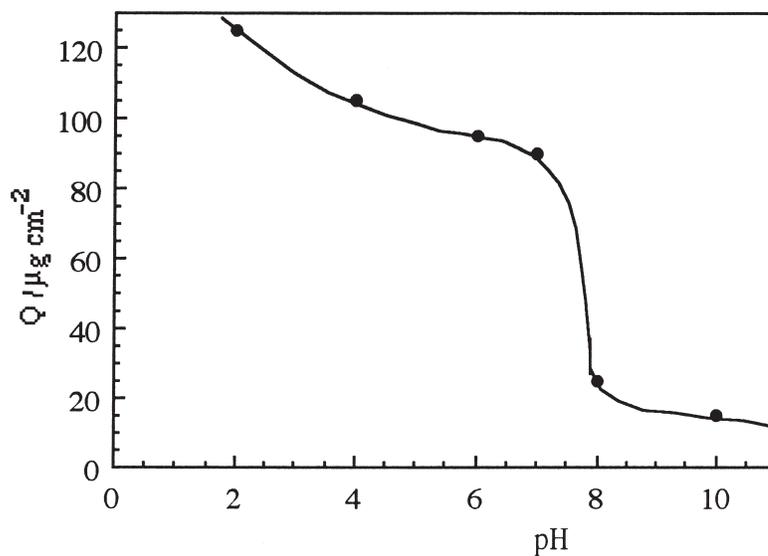


Fig. 7. The influence of pH on the immobilization of the yeast.

pH-Optimum Determination

To determine the optimum pH of the immobilization condition, the universal buffer (0.04 M phosphoric, acetic, and boric acids) was employed, and then the pH effect on immobilization was investigated. The pH-immobilized protein profile is presented in Fig. 7. Although, the immobilization was highest at pH 2, the cells were destroyed at this pH. Therefore, optimum pH was chosen to be pH 6.

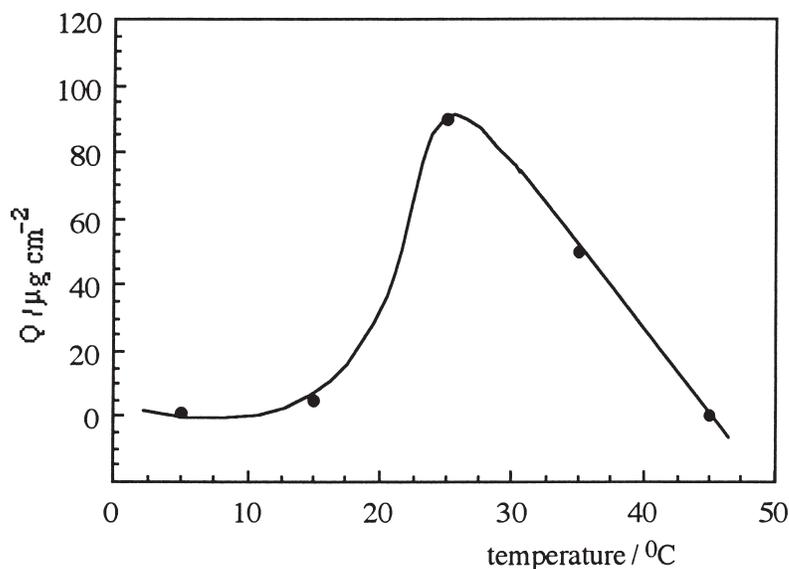


Fig. 8. The effect of temperature on immobilization.

Effect of Temperature on Immobilization

To determine the optimum temperature of the immobilization condition, the amounts of immobilized cells were plotted as a function of temperature (Fig. 8). Figure 8 shows that the maximum immobilization is found at about 25°C .

Desorption of Immobilized Cells

The chitosan films that were taken from yeast solution were allowed to stand for 3 d in distilled water and buffer solution at pH 6, but desorption of cells did not occur. These results suggest that the bond between chitosan and yeast was permanent and cannot be broken hydrolytically.

Conclusion

Chitosan [poly(1,4- β -D-glucopyranosamine)] is an amino polysaccharide polymer that possesses valuable properties as a biomaterial, a support for immobilization of cells or enzymes, and so on (16–18). It is a cationic polyelectrolytic gel in acidic media differing from the other hydrogels that are non-ionic or polyanionic (19–21).

For the optimum immobilization of yeast cells on chitosan films, first, pH-sensitive chitosan films were prepared by solvent evaporation, and spectroscopic properties, thermomechanical behavior, and swelling experiments were performed on the prepared films for characterization. Second, baker's yeast was immobilized on glutaraldehyde-pretreated chitosan films. Finally, the effects of glutaraldehyde concentration, incubation time,

pH, and temperature on the immobilization of yeast cells on chitosan films were evaluated and determined for optimum conditions.

The results of this study indicate that 0.01% of glutaraldehyde-pre-treated chitosan films were most suitable for immobilization of baker's yeast cells. The most appropriate experimental conditions were found to be about 25°C, at pH 6 for 5 h for immobilization of yeast cell. The maximum immobilization capacity of chitosan film was found to be 90 µg protein cm⁻² film in optimum conditions.

This study shows that yeast cells can successively be immobilized on pH-sensitive crosslinked chitosan films and it can be used for practical bioengineering applications such as a support for proteins, enzymes, or cells. Making use of immobilized yeast systems as a support especially on natural and/or biocompatible polymers such as chitosan for specific purposes, e.g., alcoholic fermentation, is an appealing and increasingly growing and used research area due to it is additional technical and economical advantages compared to the other free cell systems.

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