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Immobilized Catalase on CoFoam Hydrophilic Polyurethane Composite

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Abstract

Catalase from bovine liver was covalently immobilized on hydrophilic polyurethane composite (CoFoam). The activity of the enzyme was assayed in the decomposition of H_2O_2 at pH 7.0 and 25°C. The effects of water-to-prepolymer ratio, the addition of a crosslinking agent, and the utilization of a spacer on enzyme activity were examined. The results of immobilization of the enzyme in a large-scale unit are reported. The advantage of the CoFoam composite lies in the low drop in pressure in a packed-bed reactor at fairly large flow rates. For example, at flow rates of 10–12 L/min, the drop in pressure is typically 3 kPa. Enzymes immobilized on CoFoam represent a novel use as catalysts in packed-bed reactors owing to the low drop in pressure.

Index Entries: CoFoam; catalase; hydrophilic polyurethane.

Introduction

Hydrophilic polyurethanes are well known for their ability to entrap or otherwise immobilize biologic materials *(1)*. Hypol 2000 and 3000 have been successfully used as immobilization matrices for living microbial cells in the biodegradation of toxic chemicals *(2)*. Fukushima et al. *(3)* immobilized invertase using hydrophilic urethane prepolymers such as Hypol 2000. Cellulase immobilized in foam made from Hypol 2002 was applied to the degradation of cellulose *(4)*. Foams made by these techniques have the advantage that the enzymes are covalently bound. They have the disadvantage that much of the enzyme is imbedded deeply into the foam macrostructure. Foams of this type have very poor mass transfer characteristics and are typically ground or cut into small pieces to increase the effective surface area *(5)*. Recent studies *(6–9)* have also demonstrated that the

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enzyme-foam interaction is covalent. Drevon and Russell *(10)* showed that the synthesis of polyurethane polymers in the presence of diisopropyl-fluorophosphatase enables the irreversible attachment of the enzyme to the polymeric matrix.

Hydrophilix has developed an innovative hydrophilic polyurethane composite, CoFoam, in which hydrophilic polyurethane foam is cast onto reticulated foam. It offers the water absorption advantage of hydrophilic polyurethane and the surface area, physical strength, and "low-resistance-to-fluid-flow" advantages of reticulated foam. CoFoam may be designed for a specific purpose. Design parameters such as the hydrophilicity, pore size, and density of the foam may be altered to meet application requirements. CoFoam can work between 4 and 107°C. CoFoam is not damaged by water, soap, most detergents, perspiration, or greases at ambient conditions.

In this article, we examine the effectiveness of various methods to covalently bind catalase to hydrophilic polyurethane CoFoam. These treatments included varying the ratio of water to prepolymer, mixing the enzyme into the prepolymer, adding a crosslinking agent, and utilizing spacers.

Materials and Methods

Catalase, Prepolymer, and Chemicals

Bovine liver catalase (C-9322) was obtained from Sigma (specific activity of 2700 IU/mg of solid; one international unit will decompose 1 µmol of H_2O_2/min at pH 7.0 and 25°C when the initial concentration of H_2O_2 is 0.01 *M*). High-purity bovine liver catalase (C-40; 26,640 IU/mg) was also obtained from Sigma.

The prepolymer Urepol 1002 was from Envirochem (Paso Robles, CA). We have used this prepolymer for a number of traditional hydrophilic polyurethane applications and have found it to be equivalent to the more traditional toluene diisocyanate–based prepolymers regarding hydrophilicity and texture. Some researchers have found that methyl diisocyanate–based prepolymers are more appropriate for immobilizations (specifically the entrapping of cells) *(5)*.

H_2O_2 solution (30%) stabilized with sodium stannate, glutaraldehyde (25% aqueous), acetone, and HCl was purchased from Fisher. Potassium phosphate monobasic anhydrous and 4-aminobutyraldehyde diethyl acetal were purchased from Sigma.

Preparation of CoFoam

A 10 × 10 cm sample of reticulated foam was cut from the bulk material. Exactly 0.05 g of catalase was dissolved in 10 mL of cold (refrigerated) phosphate buffer solution, pH 7.0. The cold temperature of the buffer served two purposes. First, it slowed the polymerization reaction, allowing the preparation of a more uniform CoFoam sample. Second, it reduced thermal

deactivation of the enzyme. Ten grams of prepolymer was weighed into a disposable plastic dish. Depending on the nature of the pretreatment or preparation technique, additional reagents were added (e.g., crosslinking agent or solvent). Next the enzyme solution was poured into the prepolymer. The pH of the mixture was close to 7.0, which is the optimum pH for both native and immobilized catalase in the decomposition of H_2O_2 *(11)*. The reaction mixture was mixed thoroughly with the reticulated foam to create a uniform CoFoam. The sample was left under a hood to dry for approx 1 h. At this point the sample was wrapped in Kimwipes and placed in a refrigerator to dry for 2 d.

Immobilized Catalase Assay

After the 2-d cure period, the mass of a 10×10 cm sample was recorded. A 2×2 cm sample was cut from the 10×10 cm sample and used in each run. This sample was then cut into four pieces. The mass of the four pieces was measured and considered the dry mass. Twenty milliliters of 0.01 *M* H_2O_2 (in phosphate buffer solution, pH 7.0) was measured into a beaker. The enzyme sample was added to the beaker and the mixture was agitated. The temperature was kept at 25°C. The concentration of H_2O_2 was monitored spectrophotometrically by measuring the absorbance at 230 nm. The concentration of H_2O_2 was recorded after 1 and 5 min, respectively. Enzyme leaching was checked by removing the enzyme sample and monitoring the concentration of H_2O_2 in a cuvet.

Enzyme Leaching

The enzyme sample was placed in a stirred vessel containing buffer solution. The buffer solution and sample were maintained at 10°C to reduce thermal inactivation. The vessel was stirred continuously overnight (approx 20 h). At this point the sample was removed from the vessel, equilibrated to 25°C, and the activity was determined a second time according to the procedure described in the previous paragraph. If all the enzyme were covalently bound to the CoFoam, the reaction would instantaneously terminate when the sample of H_2O_2 was removed from the reacting vessel. The concentration of H_2O_2 within the cuvet would not decrease with time. This was not always the case, owing to loosely bound enzyme, as well as the possibility of some very small pieces of enzyme/CoFoam on the edges of the foam flaking off in the reacting vessel owing to shear forces.

The method developed was to measure the decrease in H_2O_2 concentration of the sample in the cuvet over a 5-min period. From the data the change in concentration per unit time was calculated and multiplied by 10^6 (for ease of comparison).

Addition of Crosslinking Agent

Glutaraldehyde was added as a crosslinking agent. The purpose of the crosslinking agent was to determine whether it would reduce leaching of

Table 1
Treatments With Crosslinking Agent

Treatment 1	Glutaraldehyde was evenly distributed onto the reticulated foam. Then the enzyme solution was added and the CoFoam prepared.
Treatment 2	Glutaraldehyde was mixed with enzyme solution. This solution was mixed with the prepolymer and the CoFoam prepared.
Treatment 3	Glutaraldehyde and enzyme solution were added and mixed into the prepolymer at the same time. Then the CoFoam was prepared.
Treatment 4	Enzyme was mixed into prepolymer under an inert gas blanket for approx 4 h. Buffer solution was mixed with glutaraldehyde. The buffer/glutaraldehyde solution was added to the enzyme/prepolymer solution. Then the CoFoam was prepared.

the enzyme in the CoFoam. Four methods of glutaraldehyde addition were examined and are described in Table 1.

Addition of Spacers

A 10×10 cm sample of reticulated foam was cut from the bulk material. Ten grams of prepolymer was weighed into a disposable plastic dish. Twenty-eight microliters of butyraldehyde (90%) was stirred into 10 mL of deionized water. Next the butyraldehyde solution was poured into the prepolymer. The reacting mixture was mixed by hand using the reticulated foam to create the CoFoam. When the sample was no longer tacky, it was placed under a hood to dry for at least 3 h. To free the aldehyde groups, the sample was washed with deionized water followed by soaking overnight in 10% (v/v) glacial acetic acid at 40°C. Since the aldehyde groups are physically separated from the polymer surface, there are few stability issues. The sample was thoroughly washed with deionized water. In addition, wash-off from the sample was tested for pH to ensure complete removal of the acid. The sample was then soaked in enzyme solution (0.05 g of catalase/10 mL of buffer) in a refrigerator for approx 5 h. Finally, the sample was washed with buffer solution, dried, and stored in the refrigerator.

Immobilization of Catalase on CoFoam: Scale-Up

Immobilized enzyme on CoFoam was produced with catalase at the Hydrophilix facility. Each sample was made from 2.5 g of the enzyme stirred into 500 mL of cold buffer solution (pH 7.0). One of the samples also contained 50 mL of glutaraldehyde (25% aqueous). The glutaraldehyde was added and stirred into the enzyme solution just prior to CoFoam production. Next, 24 mL of the enzyme solution was emulsified with an equal volume of a methylene diisocyanate–based hydrophilic polyurethane prepolymer (Urepol 1002) solution (1 g/mL) using a Mixpac System 50 handheld meter mix-dispense device. Both liquids were mixed at room temperature. The emulsion was immediately applied to a 12 pores/cm reticulated polyurethane foam (Rogers Foam, Sommerville, MA) and

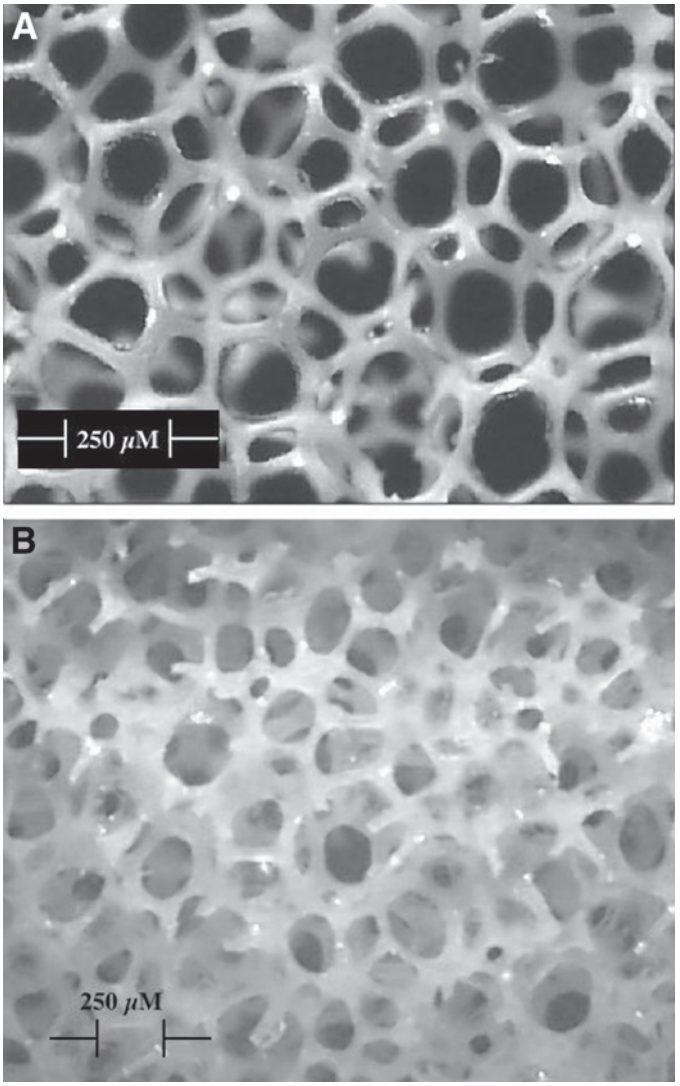

Fig. 1. **(A)** Reticulated foam of 12 pores/cm and **(B)** coated reticulated foam (CoFoam composite).

passed through a set of pinch rollers in such a way as to coat the entire inside structure of the foam. The foam was tack free in 2 min and fully cured in 1 h, after which it was air-dried at ambient temperature.

Results and Discussion

Foam Properties

Hydrophilic polyurethane CoFoam was used to immobilize several catalases. Figure 1A shows a picture of 45-ppi (pores per inch) plain reticulated foam, and Fig. 1B a hydrophilic polyurethane CoFoam. Unlike immo-

Table 2
Properties of CoFoam (As Tested)

Property	Value
Pores (linear cm)	18
Density (kg/m^3)	30.4
Deflection (kg/cm^2)	3.6×10^{-2}
Tensile strength (kg/cm^2)	1.1
Elongation (%)	180
Tear strength (kPa)	27.6
50% Deflection (compression, kg/cm^2)	1.07

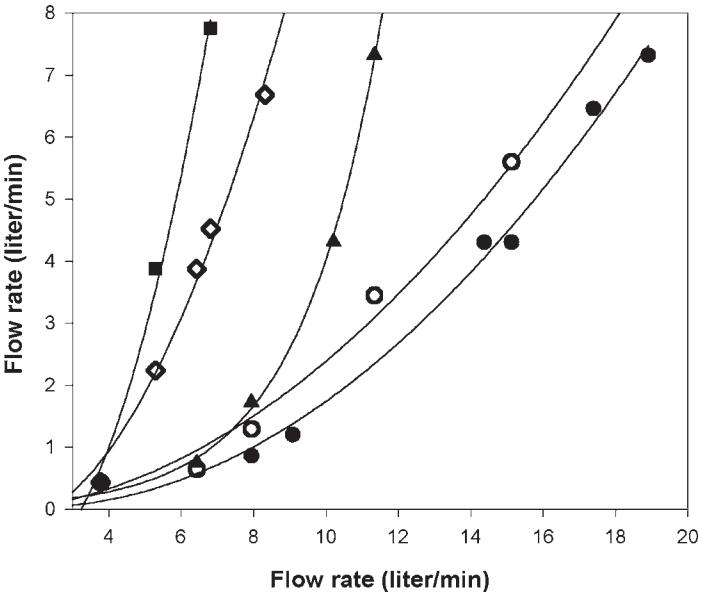

Fig. 2. Effect of flow rate on drop in pressure across CoFoam. H/R is the ratio of the mass of hydrophilic foam to the mass of the reticulated foam: (●) 0; (○) 1.1; (▲) 2.6; (◇) 3.7; (■) 4.2.

bilization on beads or gels, the product of immobilization yields a sheet of material of good durability. Table 2 provides the physical properties of CoFoam. The CoFoam sheet can be used as such or conformed to the inside of a pipe. It is important to note that the properties of CoFoam allow batch or continuous processes. One of the aspects of CoFoam is its low drop in pressure at high flow rates. Figure 2 shows the drop in pressure across a 0.15-m-long pipe (0.038-m id) using different mass ratios of hydrophilic polyurethane foam with respect to reticulated foam. As seen in Fig. 2, the drop in pressure across the foam increases with flow rate. When there is no hydrophilic foam in the CoFoam ($H/R = 0$), the drop in pressure through the reticulated foam is the least. However, as the ratio of the hydrophilic

foam to the reticulated foam increases, the drop in pressure across the foam increases as well.

It is a property of polyurethane prepolymer chemistry that both the nucleophilic groups in the enzyme and the water react with the isocyanate groups to cause polymerization (the water/isocyanate reaction) and chain termination (reaction with the enzyme). Thus, coupling of the enzyme to the support is through covalent linkage. By controlling the relative concentrations and the temperature, it is possible to modulate the physical properties of the composite and the activity of the foam *(12)*.

Effect of Varying Water-to-Prepolymer Ratio

The water content of immobilized enzyme on CoFoam immediately after preparation was measured using a Karl Fischer apparatus (Mettler, Model DL31) and found to be 35%. However, a sample that was air-dried and stored in a desiccator had a water content of only 1%. Since the area of the sample does not change, the activity of the enzyme is reported per unit area.

Catalase from bovine liver is peculiar in that it exhibits an initial burst of activity (α-phase activity) that is higher than the steady-state or β-phase activity. This initial α-phase activity lasts approx 1 min. The results sometimes refer to three different activity measurements. The first is the initial or α-phase activity. In addition, two β-phase results are reported. β-1 is the measurement taken after the initial burst of activity. Next the sample undergoes leaching studies (as described under Materials and Methods). Then the β-phase activity is determined a second time (β-2). The difference between the two measurements (loss of activity) is attributed to initial leaching. Reaction-in-cuvet (as described under Materials and Methods) is an additional measurement taken during the β-2 activity measurement.

Experiments were conducted in which the water-to-prepolymer ratio (WPR) was varied. The aim of these studies was to determine the optimum WPR. It is expected that a lower ratio would probably lead to more reaction between the enzyme and the available isocyanate groups. However, reduction in water probably leads to thermal inactivation of the enzyme owing to the heat liberated during reaction. The mass (activity) of enzyme was kept constant, and only the amount of buffer solution was changed. In this study, immobilized enzyme samples with mass ratios of 1:5, 1:2, and 1:1.25 were studied.

Table 3 presents the results of varying the WPR. The α-phase activity was higher than the β-phase activity in all three cases. In addition, there was not much leaching of the enzyme in all three samples (insignificant difference between β-1 and β-2 activities). The WPR has to be about 1:2 and there does not appear to be much difference between the ratios 1:2 and 1:1.25. However, as the amount of water was increased, the reaction-in-cuvet appeared to increase. As explained earlier, this may be attributed to either loosely bound enzyme or tiny pieces of the CoFoam flaking off during reaction owing to shear forces. As the amount of water is increased, it is surmised that there is less covalent bonding between the enzyme and the isocyanate groups in the prepolymer.

Table 3
Effect of Varying WPR

Ratio	Average α activity (U/cm^2)	β-1 activity (U/cm^2)	β-2 activity (U/cm^2)	Reaction-in-cuvet ($\Delta M \times 10^6$/min)
1:5	342	275	264	12
1:2	523	402	420	10
1:1.25	557	397	370	32

Effect of Adding Catalase to Prepolymer Instead of Water

The purpose of adding catalase to the prepolymer instead of water was to carry out a reaction between the enzyme and the prepolymer first before production of CoFoam. This permitted more contact time of the enzyme with the prepolymer. The prepolymer was placed in an Erlenmeyer flask under nitrogen blanketing. The viscous mixture required the addition of 2 mL of acetone for the magnetic stir bar to function. At this point 0.05 g of catalase was added. The mixture was stirred for approx 6 h at approx 15°C. The enzyme was uniformly distributed in the mixture even though some particles were still visible.

The initial α activity of this treatment was 352 U/cm^2. The β-1 and β-2 activities were determined to be 360 and 310 U/cm^2, respectively. Reaction-in-cuvet (after β-2 activity) for this treatment was 130 ($\Delta M \times 10^6$/min). This value is very high compared with that of other treatments. This may be attributed to the particles of the enzyme that were visible and had not gone into solution. Clearly, this method is not very useful.

Effect of Adding Crosslinking Agent

Glutaraldehyde was added to the samples as a crosslinking agent. The purpose of the crosslinking agent was to ascertain whether leaching was reduced. Four methods of glutaraldehyde addition as described in Table 1 were tested.

Figures 3 and 4 show the results for various treatments. As seen in Fig. 3, the β-2 activities for the first three treatments were similar. The reaction-in-cuvet was also very low as a result of glutaraldehyde addition (Fig. 4). The fourth treatment had a much lower activity and is not considered further.

Table 4 summaries all the results up to this point. Examination of β-2 activity and reaction-in-cuvet is useful, and it is clear from Table 4 that the addition of a crosslinking agent resulted in the lowest activity, but reaction-in-cuvet was also much lower for this sample. When the ratio of β-2 activity to reaction-in-cuvet is calculated for the various pretreatments described (*see* Table 1), the ratios are 2.5, 15, and 20, respectively. This indicates that the addition of a crosslinking agent results in minimal leaching and fairly reasonable enzyme loadings. It is not surprising that the addition of a crosslinking agent results in reduced initial activities of the immobilized enzyme.

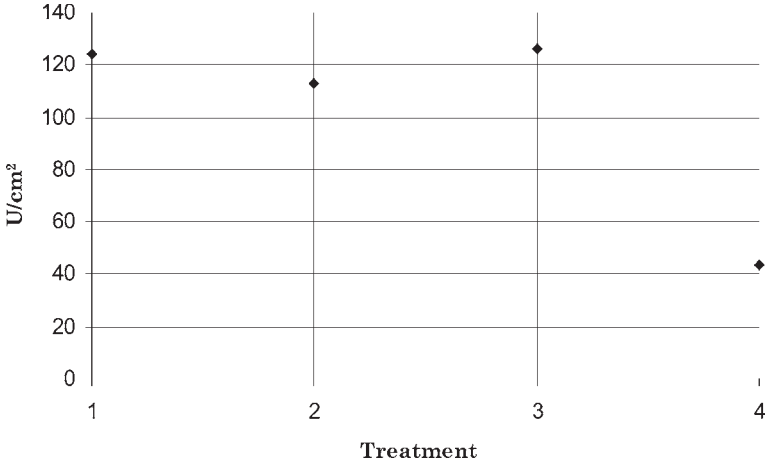

Fig. 3. Effect of crosslinking agent on β-2 activity: 4-cm^2 immobilized catalase on CoFoam, 0.01 *M* H_2O_2, 25°C, pH 7.0. Refer to Table 1 for treatments.

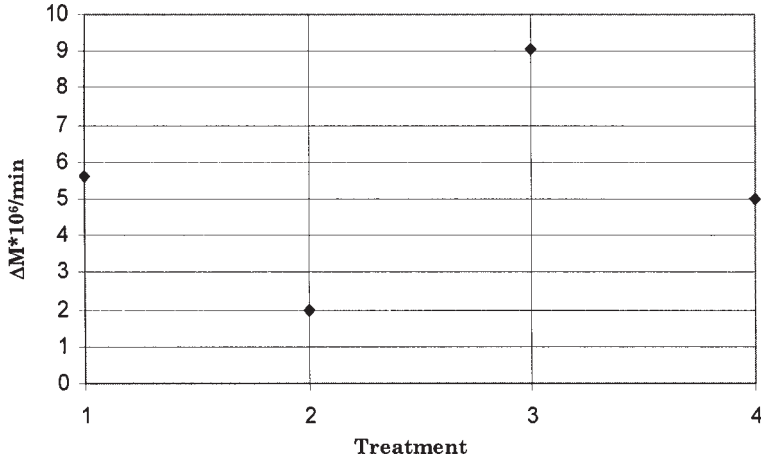

Fig. 4. Effect of crosslinking agent on reaction-in-cuvet: 4-cm^2 immobilized catalase CoFoam, 0.01 *M* H_2O_2, 25°C, pH 7.0. Refer to Table 1 for treatments.

Table 4
Comparison of Activity by Treatment

Treatment	α Activity (U/cm^2)	β-1 Activity (U/cm^2)	β-2 Activity (U/cm^2)	Reaction-in-cuvet (ΔM/min × 10^6)
Enzyme into prepolymer	352	360	310	130
WPR of 1:1.25	557	397	370	32
Crosslinking agent (average of treatments 1–3)	186	171	122	6

Table 5
Comparison of High-Purity Catalase Activity

Treatment	α Activity (U/cm^2)	β-1 Activity (U/cm^2)	β-2 Activity (U/cm^2)	Reaction-in-cuvet ($\Delta M/min \times 10^6$)
Glutaraldehyde	671	366	358	5
No glutaraldehyde	1461	404	344	50

Table 6
Summary of Hydrophilix Scale-Up Results

Treatment	α Activity (U/cm^2)	β-1 Activity (U/cm^2)	β-2 Activity (U/cm^2)	Reaction-in-cuvet ($\Delta M/min \times 10^6$)
Glutaraldehyde	294	200	172	4
No glutaraldehyde	384	236	200	12

High-Purity Catalase

Two samples were made with high-purity bovine liver catalase enzyme (C-40; 26,640 IU/mg of solid): the first sample with the addition of glutaraldehyde and the second without. Catalase activity prior to immobilization was approx 10 times higher than the enzyme used in all the other experiments. Table 5 summarizes the results.

The initial α-phase activity with and without the addition of glutaraldehyde was determined to be 671 and 1461 U/cm^2, respectively. The samples with the addition of glutaraldehyde were noticeably stiffer, probably owing to more crosslinking of the enzyme. Comparison of β-2 activities with and without glutaraldehyde showed that the activity per unit area was about the same, whereas reaction-in-cuvet was 10 times higher for the nonglutaraldehyde sample. This suggests that the addition of a crosslinking agent results in less leaching of the enzyme.

Effect of Spacer

The addition of a spacer was expected to reduce diffusional limitations. However, α-phase activity was determined to be 42 U/cm^2 and β-1 and β-2 activities were determined to be 24 and 16 U/cm^2, respectively. Reaction-in-cuvet was 22 $\Delta M/min \times 10^6$. Increasing the concentration of butyraldehyde did not enhance enzyme activity. Because the activity was low and leaching was high, this method was not pursued further.

Immobilization of Catalase on Scaled-Up Facility

Immobilized enzyme on CoFoam was produced at the Hydrophilix facility according to the procedure outlined under Materials and Methods; Table 6 summarizes the results. It was observed that the enzyme was uniformly distributed throughout the reticulated foam and, clearly, this was

the best way to prepare the sample. The results are in agreement with our laboratory-prepared samples. Higher loadings of the enzyme can be obtained by increasing the initial concentration of the enzyme and using a high-purity enzyme sample or reducing roller speeds.

Conclusion

The immobilization of catalase on CoFoam is effective and simple. The low resistance to fluid flow makes CoFoam a viable support to be used in large reactors. The enzyme can easily and quickly be loaded in any packed-bed reactor. In the case of CoFoam, a WPR of approx 1:1 gives the best results. The addition of a crosslinking agent during immobilization leads to the least amount of leaching while reducing the initial activity to some extent.

Acknowledgments

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