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# Immobilization of catalases from Bacillus SF on alumina for the treatment of textile bleaching effluents

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**Abstract**

A catalase preparation from a newly isolated Bacillus sp. was covalently immobilized on silanized alumina using glutaraldehyde as crosslinking agent. The effect of the coupling time of the enzyme-support reaction was determined in terms of protein recovery and immobilization yield and a certain balance point was found after which the activity recovery decreased. The activity profile of the immobilized catalase at high pH and temperature was investigated. The immobilized enzyme showed higher stabilities (214 h at pH 11, 30°C) at alkaline pH than the free enzyme (10 h at pH 11, 30°C). The immobilized catalase was inhibited by anionic stabilizers or surfactants added to the hydrogen peroxide substrate solution. 

*Keywords:* Catalase; Covalent immobilization; Thermo- and alkaline stability; Hydrogen peroxide

## 1. Introduction

Catalase (EC 1.11.1.6) is an abundant enzyme in nature decomposing hydrogen peroxide to water and molecular oxygen [1,2]. In the textile industry, this enzyme has a potential for the degradation of hydrogen peroxide after textile bleaching in order to prevent problems in subsequent dyeing [3,4]. The reaction rate of catalases is extremely fast, and under optimum conditions one mole of catalase is able to decompose 500 million of moles of hydrogen peroxide in one minute. Thus, the enzymatic treatment may enable considerable time, energy and water savings avoiding or shortening the extensive washing process after peroxide bleaching. Conventionally, effective peroxide bleaching is achieved at high temperature and alkalinity. Since most of the known catalases have their activity optimum at moderate temperatures (20–50°C) and neutral pH, application of existing commercial catalases required adjustments of the pH and the temperature of the bleaching liquor [5]. One approach to improve the enzyme stability is immobilization [6]. There are a number of papers reporting on catalase immobilization on different types of supports [7–12]. The method of covalent binding for catalase immobilization appears to be most appropriate when the enzyme will be used in drastic environments and high stabilities are required. In the present research the catalase was immobilized on alumina pellets, which were selected as carrier material due to their mechanical resistance at high pH and temperature. The effect of the immobilization conditions, such as carrier activation, and enzyme loading, was investigated with the aim to achieve maximum recovery in activity of the immobilized enzyme. The immobilized catalase was applied in a packed-bed reactor to study the stability and the half-life time of the enzyme for the degradation of hydrogen peroxide in the residual bleaching liquor.

## 2. Materials and methods

All determinations were in triplicate experiments and results shown are mean values with less than 2% of error.

### *2.1. Microorganism screening and fermentation*

A new *Bacillus sp.* was isolated from wastewater drain from a textile finishing company. The bacteria had its growth optimum at pH 9.5 and 65°C. The microorganisms

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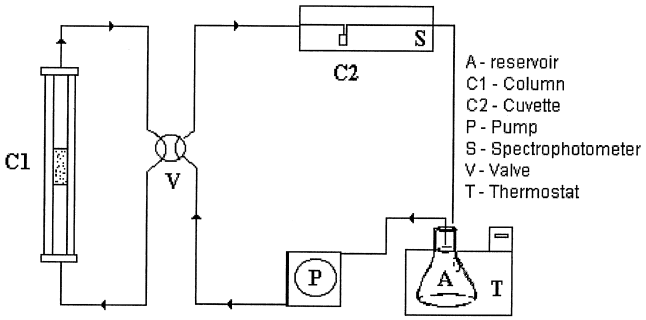


Fig. 1. Scheme of stability determination for immobilized catalase.

was deposited and identified by the German culture collection DSMZ (Braunschweig, Germany). The fermentation process carried out in an appropriate culture medium as described in our previous work [13].

### 2.2. Catalase immobilization

Alumina support (aluminum oxide pellets, 3 mm) was obtained from Aldrich. The alumina pellets were silanized by immersing them in a 4% (v/v) γ-aminopropyltriethoxy silane (Sigma) in acetone solution at 45°C for 24 h. The silanized pellets were thoroughly washed with distilled water and immersed in 2% (v/v) of aqueous glutaraldehyde (Aldrich) solution for 2 h at room temperature, after-washed with distilled water and dried at 60°C for 1h [14]. Thereafter, 5 *g* pellets were immersed in 20 ml of the crude enzyme preparation for 24, 48, and 72 h at room temperature.

### 2.3. Enzyme activity assay

Free catalase activity was determined spectrophotometrically at 240 nm (Unicam Heλios-α), following the degradation of 26 mM hydrogen peroxide in 50 mM phosphate buffer pH 7.0, 30°C, using a molar absorption coefficient of 39.4 $M^{-1}$ $cm^{-1}$ [15]. Activity units were expressed in terms of amount of substrate converted per minute. The immobilized enzyme activity was measured using a thermostated-jacketed column (length 15 cm, diameter 2.5 cm, total volume 74 ml) with an adjustable bed volume, purchased from Sigma. The reactor was loaded with 5 *g* of immobilized catalase (17 mg prot/g and 160 U/g support). Phosphate buffer (50 mM, pH 7.0) solution was pumped (1 $ml \cdot min^{-1}$) (Model Miniplus 3 Peristaltic Pump—Gilson) trough a flow cell and the absorbance at 240 nm was set as the zero baseline. In the next experimental step the absorbance maximum of the hydrogen peroxide at the same wavelength was measured. The enzymatic reaction started with the injection of the substrate solution (26 mmol/liter) into the reactor. The reaction was monitored for a period of 30 min for stability experiments, without recirculation of the substrate solution (Fig. 1).

### 2.4. Protein assay

The amount of protein in the supernatant solution after immobilization was determined according to the Bradford method [16], using bovine serum albumin as protein standard.

### 2.5. Protein recovery and immobilization yield

Protein recovery (RP%) and immobilization yield (YI%) were defined as: RP% = Uads/$U_0$ × 100; YI% = Uact/Uads × 100 where $U_0$ is the activity present in the immobilization solution, Uads are the absorbed units evaluated as the difference between $U_0$ and those remaining in the supernatant at the end of the adsorption procedure, Uact is the activity present on the support [17].

### 2.6. pH profile of the enzyme activity

Free and immobilized catalase preparations were incubated at 30°C, for 24 h at different pHs in appropriate 50 mM buffers—phosphate pH 7.0, tris pH 8.0–9.0 and carbonate pH 10.0–12.0, and the relative activity was measured under standard conditions.

### 2.7. Temperature profile of the enzyme activity

The temperature effect on enzyme activity was investigated in the range of 30°C–60°C for both free and immobilized catalase. The enzyme preparations were incubated in 50 mM phosphate buffer pH 7.0, for 24 h. After cooling, the remaining activity was assayed under the standard assay conditions.

### 2.8. Thermal stability measurements for free and immobilized enzyme

Thermal stability studies of free and immobilized catalase were performed measuring the residual activity of the enzyme exposed to three different temperatures—30°C, 50°C, and 60°C in 50 mM phosphate (pH 7.0), tris (pH 8.0–9.0) and respectively carbonate (pH 10.0–12.0) buffers. Samples were taken at different time intervals during incubation, and the remaining activity was measured as described in the enzyme assay.

### 2.9. Effect of the bleaching baths stabilizers and surfactants on the enzyme activity

Experiments were carried out to study the activity of free and immobilized catalase in the presence of surfactants: Invadine Lu—slightly anionic, Lutensit-A-LBN 50—anionic, and Lutensol A7N—non-ionic, and peroxide stabilizers: Calgon T, silicates, and Stabilizer CB—anionic. The substrate solution (26 mmol/liter) was prepared in phosphate buffer (50 mM, pH 7.0), and the stabilizers were

Table 1
Catalase immobilization on alumina support depending on the coupling time

| Time (h) | Efficiency of immobilization | |
|---|---|---|
| | RP (%)[a] | YI (%)[b] |
| 24 | 36.6 | 28.4 |
| 48 | 52.7 | 36.9 |
| 72 | 58.9 | 23.5 |

[a] RP = Protein recovery
[b] YI = Immobilization yield
Experimental conditions: [Catalase] = 2190 U/ml, [$H_2O_2$] = 26 mM at pH 7.0.

added in final concentration of 5 g/liter. Measurement of the enzyme activity was the same as described previously.

All the activity determinations were based on experiments, performed in triplicate.

## 3. Results and discussion

### *3.1. Influence of the coupling time on catalase immobilization*

The effect of the coupling time on the immobilization was investigated in terms of protein recovery and immobilization yield. The data in Table 1 clearly shows that coupling time of 48 h gives the highest protein recovery and immobilization yield (RP% = 52.7%, and YI = 36.9%). Comparing the results for reaction times of 48 h, and 72 h it can be noticed that the protein recovery's greater by ~6% for 72 h of coupling interaction. However, prolonging the reaction time up to 72 h decreases the immobilization yield by 10%. These results can be explained assuming that the increase of contact time between the enzyme and the support creates more bonds per enzyme molecule. Due to possible structural deformations during immobilization, the enzyme molecules can orientate themselves to remain with the active site blocked, thereby restraining the accessibility of the enzyme active site toward the substrate. Also, at longer incubation times the probability for covalent modifications close to the active site increases leading to enzyme inactivation.

### *3.2. Saturation of the support*

Data in Fig. 2 shows that the amount of catalase bound to the alumina increases significantly with the increase of the amount of enzyme initially brought in contact with the support. The increase of the amount of bound catalase describes a sigmoidal kinetic up to concentration of 10 mg/ml. Further increase of enzyme concentration gives poorer immobilization results, probably due to saturation of the given quantity (5 g) of support. The maximum protein recovery is achieved at an enzyme loading of 9,5 mg/ml (total volume 20 ml).

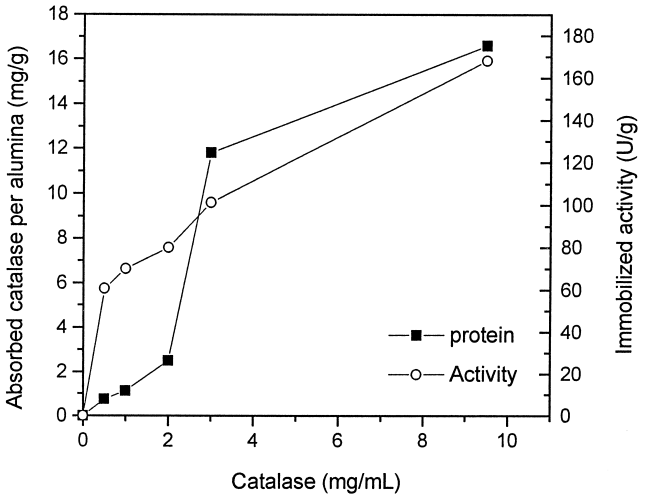


Fig. 2. Saturation of the alumina support (5 g) by catalase (20 ml), as a function of the enzyme concentration (0–10 mg/ml), where (-■-) is the amount of bound protein and (-○-) the activity of the catalase.

These results agree with the literature data, concerning catalase immobilization onto natural polymers [7].

### *3.3. Effect of pH on enzyme activity*

The effect of pH on the activity of free and immobilized catalase was studied in the pH range 7.0–12.0 (Fig. 3). No shift in the pH optimum of enzyme activity was detected for the immobilized catalase, and both free and immobilized enzymes showed their maximum activity at pH 8.0. However, at pHs higher than 10, the immobilized enzyme retained 60% of is original activity, while the free enzyme retained only 10% of its activity at pH 11 and was completely deactivated at pH 12. Treatment of the immobilized

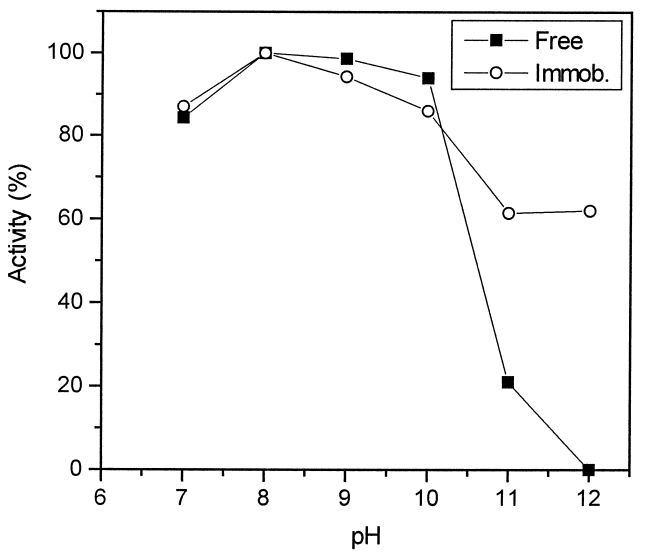


Fig. 3. Effect of pH on the activity of free (-■-) and immobilized (-○-) catalase. Activity was measured at standard conditions. Experimental conditions: [Catalase] = 2190 U · $ml^{-1}$, [$H_2O_2$] = 26 mM, 30°C.

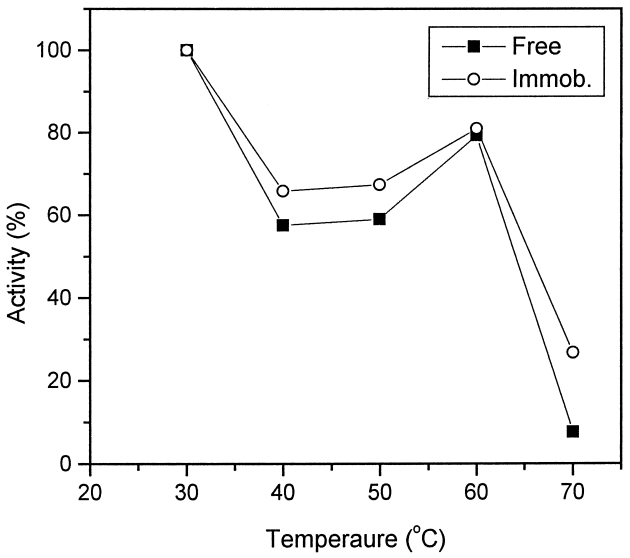


Fig. 4. Effect of the temperature on the activity of free (-■-) and immobilized (-○-) catalase. Activity was measured at standard conditions. Experimental conditions: [Catalase] = 2190 U · $ml^{-1}$, [$H_2O_2$] = 26 mM, pH 7.0, 24 h incubation.

preparations with glutaraldehyde further increased their ability to retain enzymatic activity at alkaline pHs. These observations suggest that the binding of the catalase onto the support results in significant alterations of enzyme microenvironment, improving the retention of activity at alkaline pHs [11]. The procedure of enzyme immobilization on insoluble carriers has a variety of effects on the protein conformation as well as on the state of ionization and dissociation of the enzyme and its environment, and it is not uncommon to result in changes in the relationship between pH and enzyme stability, and activity. It is known that in some cases, when the enzyme is coupled with a polyanionic carrier the pH optimum usually shifts in the alkaline direction whereas if the carrier is polycationic the shift is in the acid direction.

### *3.4. Effect of the temperature on the activity and stability of free and immobilized catalase*

The effect of the temperature on the activity of free and immobilized catalase was investigated in the temperature range from 30 to 70°C. Immobilized catalase exhibited a slightly improvement in the activity retention between 40°C and 50°C, compared to the free enzyme (Fig. 4).

The enzyme thermal stability is one of the important criteria for its long-term and commercial application. Generally, the immobilized enzymes are known to be more resistant against heat than the native enzymes. The thermal stability of the immobilized catalase was compared to that of the free enzyme in terms of half-life times after incubation at 30°C, 50°C, and 60°C, at different pHs (7.0–12.0) (Table 2). The immobilized enzyme showed considerably longer half-life times compared to those from the free enzyme. Thus the reported process of catalase immobilization stabilized the three-dimensional enzyme structure. Many inorganic matrices are inherently strong and are mechanically appropriate for use as enzyme supports. The reaction of γ-aminopropyltriethoxy silane with an oxide (aluminum oxide support) or hydroxide (glass support) results in a relatively stable organic ligand possessing reactive amino groups, integrated into inorganic matrix.

Table 3
Effect of stabilizers and surfactants on the free and immobilized catalase activity

| Stabilizers (5 g/l) | Activity (%) | |
|---|---|---|
| | Free catalase | Immobilized catalase |
| CB | 97,6 | 93 |
| Calgon | 79 | 64 |
| Silicate | 80 | 60 |
| Invadine-Lu | 87 | 50 |
| Lutensit A-LB 50 | 36 | 4 |
| Lutensol A 7N | 77 | 51 |

### *3.5. Influence of the bleaching bath stabilizers and surfactants on the activity of immobilized catalase*

The effect of different surfactants and peroxide stabilizing agents (CB, Calgon, Silicate, Invadine-Lu, Lutensit A-LB 50, and Lutensol A 7N), present in the bleaching liquor on the activity of immobilized catalase is shown in Table 3. Usually, the stabilizers are added to the bleaching bath to increase the stability of the alkaline-activated peroxide against the catalytic action of metal impurities in the radical degradation of the peroxide, which may cause chemical damage of the fibers. In the present experiment the

Table 2
Half-life time of free and immobilized catalase

| Temp. (°C) | Halftime-life (hours) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Free catalase | | | | | | Immobilized catalase | | | | | |
| | pH 7 | pH 8 | pH 9 | pH 10 | pH 11 | pH 12 | pH 7 | pH 8 | pH 9 | pH 10 | pH 11 | pH 12 |
| 30 | 216 | 240 | 240 | 148 | 10 | 1.5 | 662 | 663 | 660 | 642 | 214 | 120 |
| 50 | 15 | 20 | 48 | 15 | 12 min | 5 min | 158 | 44 | 43 | 42 | 10 | 1,3 |
| 60 | 22 | 42 | 24 | 4 | 10 min | 5 min | 94 | 43 | 64 | 20 | 2 | 1 |

stabilizers and the surfactants were applied in concentration 5 g/liter. As indicated in Table 3, the surfactant Lutensit A-LBN 50 reduced the activity of the immobilized enzyme by 96%. Lutensit A-LBN 50 is an anionic surfactant, sodium salt of $C_{10}C_{13}$ alkylbenzene-sulfonic acid. Studies on the influence of detergents and surfactants on the protein conformation reported that some surfactants are strong denaturants, and significantly affect the conformation of the protein [18]. These surfactants form associates with the active protein binding sites through electrostatic and/or hydrophobic interactions. The type and the size of the carbon chains might influence the protein stability as well. The quaternary structure of catalase enzyme is constituted by aggregation of 3 or 4 monomers and only in this form the enzyme is active. We have some experimental evidences [13] that our catalase represents an agglomeration of three molecules. The protein-surfactants interactions may result in disaggregation and deactivation of the enzyme trimer.

## 4. Conclusions

In this research, the utilization of aluminum oxide support and glutaraldehyde as a crosslinking agent for catalase immobilization enhanced the enzyme stability toward high pH and temperature. The half-life time of the immobilized enzyme was considerably longer compared to the free enzyme. In general, the evolution of the half-life time is regarded as an indicator for the efficiency of the immobilization process. The addition of anionic surfactants into the hydrogen peroxide solution decreased the activity of the immobilized enzyme. The inhibitory effect of the anionic surfactants could be attributed to electrostatic and/or hydrophobic interaction with the protein.

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