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An immobilised catalase peroxidase from the alkalothermophilic *Bacillus* SF for the treatment of textile-bleaching effluents

Abstract A catalase peroxidase (CP) from the newly isolated *Bacillus* SF was used to treat textile-bleaching effluents. The enzyme was stable at high pH values and temperatures, but was more sensitive to deactivation by hydrogen peroxide than monofunctional catalases. Based on the Michaelis–Menten kinetics of the CP, a model was developed to describe its deactivation characteristics. The enzyme was immobilised on various alumina-based carrier materials with different shapes and the specific activity increased with the porosity of the carrier. The shape of the carrier had an important influence on the release of oxygen formed during the catalase reaction from the packed-bed reactor and Novalox saddles were found to be the most suitable shape. Bleaching effluent was treated in a horizontal packed-bed reactor containing 10 kg of the immobilised CP at a textile-finishing company. The treated liquid (500 l) was reused within the company for dyeing fabrics with various dyes, resulting in acceptable colour differences of below $\Delta E^*=1.0$ for all dyes.

Introduction

More than 100 l of fresh water are currently consumed in the textile-finishing industry for the processing of 1 kg of textiles (Hillenbrand 1999). In particular, textile bleaching and the subsequent washing steps are water-intensive processes. Several methods have been suggested to

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degrade the bleaching agent, hydrogen peroxide, which would allow recycling of the bleaching effluent in the dyeing process. However, the addition of chemicals, such as sodium bisulphite or hydrosulphite, to reduce hydrogen peroxide would lead to unfavourably high salt concentrations in the process. Alternatively, the application of catalases has been suggested (Tzanov et al. 2001a, b).

Most commercial catalases would not withstand the conditions used during textile bleaching and new thermo-alkali-stable enzymes acting at temperatures above 60 °C and pH values above pH 9 are required. Recently, we isolated several alkalothermophilic bacteria from textile-processing effluents and a catalase-peroxidase from a new *Bacillus* sp. (*Bacillus* SF) showed promising stability at high pH and temperature (Gudelj et al. 2001; Paar et al. 2001).

Catalases (EC 1.11.1.6), which are present in all aerobic cells and decompose hydrogen peroxide to molecular oxygen and water, have been studied for more than 100 years (Loew 1901). The mechanism of mono-functional catalases has been elucidated and detailed models for the explanation of reversible and irreversible inactivation have been developed (Ghadermarzi and Moosavi-Movahedi 1999; Lardinois et al. 1996; Vasude-van and Weiland 1990). About 30 years ago, a new class of enzymes, the catalase peroxidase (CP), was identified (Childs and Bardsley 1975). The most interesting feature of bifunctional CP is its overwhelming catalase activity, with values for turnover number (k_{cat})/Michaelis–Menten constant comparable with monofunctional catalases (Regelsberger et al. 2000; Zamocky et al. 1995).

Although catalase is one of the most effective enzymes in terms of k_{cat} (Aebi 1983; Zamocky et al. 1995), the cost of enzyme for the degradation of hydrogen peroxide in bleaching effluents could be reduced by immobilisation and reuse of the enzyme. Furthermore, immobilisation would allow the reuse of the treated bleaching effluents for dyeing, which would otherwise not be possible, since protein (addition of free catalase) has been shown to interact with the dyeing process (Abadulla et al. 2000).

Many industrial processes use immobilised enzymes and catalases have been immobilised on numerous carrier materials, such as natural and synthetic polymers (Cetinus and Öztop 2000; Chatterjee et al. 1990; Gekas 1986; Horozova et al. 1997; Vasudevan and Weiland 1993). In this study, several alumina carrier materials were chosen for comparison as supports for catalases, due to their stability at high pH and temperatures. Furthermore, a model for the deactivation of the CP from the alkalothermophilic *Bacillus* SF by hydrogen peroxide was developed and a reactor system for treatment of bleaching effluents was constructed and tested in a textile-finishing company.

Fig. 1 Scheme for activity determination of immobilised catalase peroxidase (CP) from *Bacillus* SF

Materials and methods

Organism and enzyme

The newly isolated *Bacillus* SF (DSMZ 13198) was cultivated in a yeast and meat extract-based medium, as described by Paar et al. (2001). The *Bacillus* SF CP was purified by ammonium sulphate precipitation, hydrophobic interaction and size exclusion chromatography, according to Gudelj et al. (2001).
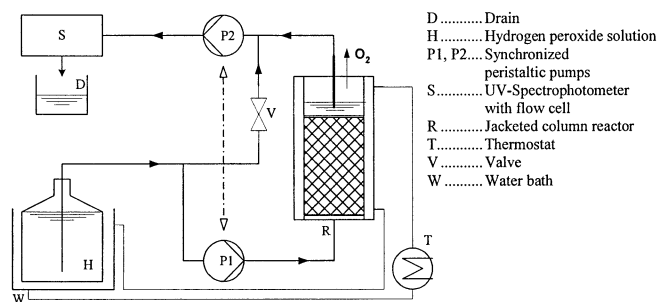
Immobilisation

The CP was immobilised by glutaraldehyde-coupling to the silanised support. The carrier materials used were: 3.5 mm α-Al_2O_3 balls (Sigma), 2 mm and 10 mm γ-Al_2O_3 balls (5-Continents, Newark, USA), 12.7 mm Novalox saddles (70% SiO_2, 25% α-Al_2O_3, 3% K_2O, 1,4% TiO_2, 1% Fe_2O_3, 0.4% MgO, 0.2% CaO, 0.15% Na_2O; VFF, Ransbach-Baumbach, Germany) and 6.35 mm Raschig rings (60% α-Al_2O_3, 34.2% SiO_2, 3.4% K_2O, 1.15% Fe_2O_3, 0.88% MgO, 0.14% CaO, 0.12% Na_2O, 0.054% TiO_2; Beggs, Cousland and Co., Glasgow, UK). The Novalox saddles and the Raschig rings were mixtures of Al_2O_3 and SiO_2 and were non-porous, due to sintering steps during production. Before silanisation, these supports were treated with 48% hydrofluoric acid at 30 °C. After 1 h, the hydrofluoric acid was decanted and the support was washed with distilled water. Sodium hydroxide solution (12 M) was added and the mixture was incubated in a water bath at 80 °C for 1 h. The support was washed with distilled water and dried at 65 °C.

All supports were silanised at 50 °C for 24 h in a solution of 3.0% (v/v) γ–aminopropyltriethoxysilane (Sigma) in acetone. The supports were re-silanised under the same conditions and, after washing with distilled water, were stored in a refrigerator. For immobilisation, the silanised supports were immersed in 2.5% (v/v) aqueous glutardialdehyde (Sigma) for 2 h at room temperature. Thereafter, the supports were washed with distilled water and then incubated with the crude enzyme preparation (20 mg ml^{-1}) for 48 h at 20 °C. The immobilised enzyme was washed with sodium phosphate buffer (50 mM, pH 7.0) and kept refrigerated until use. The progress of immobilisation was followed by enzyme activity measurements and by protein analysis, according to the method of Bradford (1976), using bovine serum albumin for calibration.

Enzyme activity assay

Free catalase activity was monitored spectrophotometrically. The assay mixture contained 100 µl of enzyme preparation and 900 µl of phosphate buffer (50 mM, pH 7.0). The reaction was initiated by the addition of 1,000 µl of a 26 mM H_2O_2 (Merck) stock solution and was monitored by the decrease in absorbance at 240 nm (A_{240}) at 20 °C (ε_{240} = 44.2 M^{-1} cm^{-1}). The linear range of the reaction

(30 s) was used to calculate the reaction rate. One unit of enzyme activity was defined as 1 µmol H_2O_2 decomposed min^{-1}. Deactivation of the purified CP by peroxide was measured by varying the substrate concentration from that used in the standard assay. The decrease in A_{240} was monitored at several peroxide concentrations until a stable value was reached.

## Activity assay of the immobilised CP

The activity of the immobilised enzyme was measured using a thermostatted glass column with an adjustable bed volume and a spectrophotometer (Hitachi U 2001) equipped with a flow cell (Fig. 1). The activity was calculated from the difference in A_{240} of the substrate solution and the solution at the outlet of the reactor. The column (3.6 cm diameter, length adjusted to the bulk volume of the biocatalyst) was loaded with CP immobilised on the carrier (total mass of 25 g) and sodium phosphate buffer (50 mM, pH 7.0) was pumped (4 ml min^{-1}) through the reactor system until a stable A_{240} was measured. This value was set as the zero baseline. In the next experimental step, the maximum A_{240} of the substrate solution containing hydrogen peroxide was measured at the same wavelength. The activity measurement was started by injection of the substrate solution (11 mM) into the column. For investigating the behaviour of oxygen release from the packed bed, the runs were prolonged.

Hydrogen peroxide decomposition in a packed-bed enzyme reactor

A horizontal column reactor (3.6 cm diameter, 5.11 cm long) was loaded with 60 g of the biocatalyst (immobilised enzyme + carrier). After rinsing the reactor system with sodium phosphate buffer (50 mM, pH 7.0) until a constant A_{240} was reached, this value was set to zero. The maximum A_{240} was determined via a bypass and then the measurement was started by pumping the hydrogen peroxide solution through the reactor system. Conversion was calculated from the difference in A_{240} at the beginning and at the end of the reactor system.

Industrial application of the reactor system

At a textile-finishing company, bleaching was carried out in a 1,000 l jigger machine (Norfil, Porto, Portugal) in batch configuration and washing was done in the same jigger machine but in continuous mode of operation. A 100-l sample of the bleaching liquor containing (as a percentage of the fabric weight; %fw) 4% hydrogen peroxide (of a 35% stock solution), 3.5% sodium silicate, 1% NaOH and 1% Na_2CO_3 (liquor:fabric ratio was 20:1) and the first 400 l of the washing water, containing only hydrogen peroxide, were pooled and stored in a tank. This liquor (pH 10, 45 °C, containing 12 mM hydrogen peroxide) was pumped (6 l h^{-1}) through a horizontal tank reactor system (for dimensions, see Fig. 2)
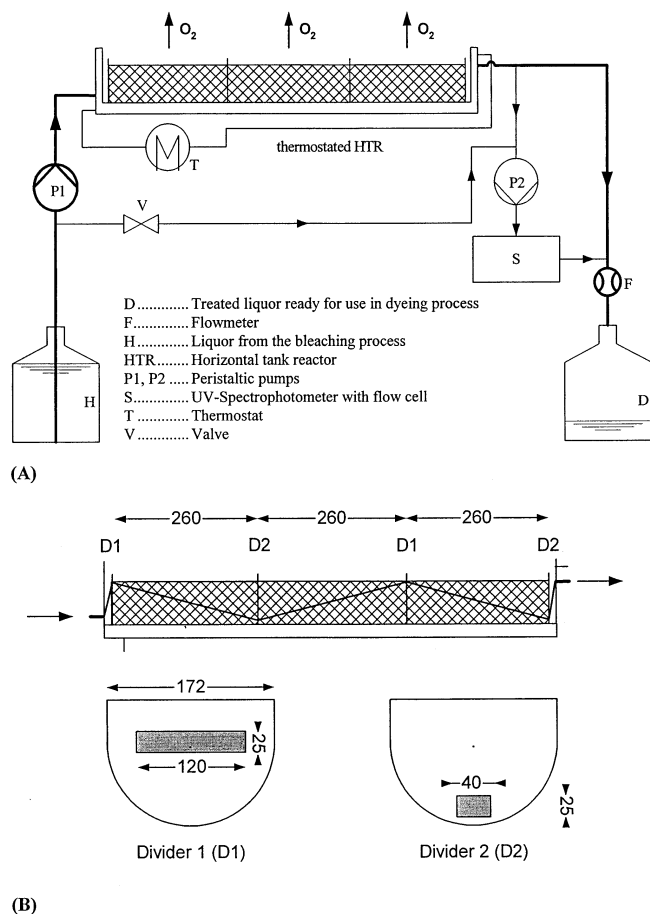
Fig. 2 A reactor system for the removal of peroxide from textile industry waste-water. A Scheme, wherein the *bold line* marks the flow of the main stream and the *thin lines* indicate the spectrophotometric monitoring of the process. B Dimensional sketch of the reactor system (in millimetres). The volume of one chamber is 2.66 l

loaded with 10 kg of a biocatalyst (immobilised enzyme + carrier) produced from the crude enzyme extract on a 3.5-mm alumina support. Merck test strips (0–25 mg l^{-1},0–400 mg l^{-1} H$_2$O$_2$) were used to monitor the decomposition of hydrogen peroxide. The treated liquor was then used for dyeing instead of fresh water.

Dyeing experiments

Cotton fabrics were bleached in a jigger machine at a textile-finishing company, with a liquor containing (%fw) 3.5% silicate, 1% soda ash, 1% sodium hydroxide, 4% hydrogen peroxide (35% stock solution) at 80 °C for 180 min and were then washed with water to remove any remaining hydrogen peroxide. The bleaching liquor and the washing water containing hydrogen peroxide were pooled. The hydrogen peroxide was decomposed by treating with immobilised catalase. Thereafter, the catalase-treated liquor was reused for dyeing. Three trichromatic mixtures of reactive dyes (Dyestar) were used: (1) green as a combination of Blue Royal G, Blue Sumifix BRF and Yellow Royal RS3R, (2) red as a combination of Red Remazol 3BS, Blue Sumifix BRF and Blue Royal RS3R and (3) blue as a combination of Red Remazol 3BS, Black Remazol B and Yellow Royal RS3R. The mixtures were prepared according to the Dyestar technical catalogue. Dyeing was performed in the presence of Glauber's salt (60 g l^{-1}) and soda ash (20 g l^{-1}) at 80 °C for 45 min. Dyeing was carried out in an Ahiba

Spectradye dyeing apparatus (Datacolor International, Luzern, Switzerland) at a liquor:fabric ratio of 20:1 (40 rpm; in step 1 the temperature was raised from 20 °C to 80 °C over 20 min, in step 2 80 °C was held for 45 min). Each dyeing experiment was repeated three times. Dyed fabrics were washed-off at the same liquor ratio with non-ionic detergent, Lutensol ON-30, for 30 min at 90 °C, to remove the unfixed dye. The diode array spectra of the dyes (both in standard dye baths and in dye baths containing enzymatically treated bleaching effluents) were recorded (TIDAS, J&M, Aalen, Germany). Colour differences of the dyed fabrics were determined using a reflectance-measuring apparatus (Spectraflash 600, Data-color) according to the Cielab colour difference concept at standard illuminance with a colour tolerance interval of 1 Cielab unit.

Other procedures

Fitting the model functions to the data was carried out by numeric solution of the coupled differential equations, using the Euler method (Epperson 2001).

Spectrophotometric measurements were made with a Hitachi U 2001 spectrophotometer, using quartz (UV) or polyacrylamide cuvettes (visible light).

Modelling CP kinetics

In contrast to monofunctional catalases, CPs show a kinetic behaviour which can be described by the Michaelis–Menten function (Eq. 1; Gudelj et al. 2001; Jakopitsch et al. 1999; Marcinkeviciene et al. 1995).

$$v = v_{Max} \cdot \frac{c_H}{K_M + c_H} = \frac{\partial c_H}{\partial t} \qquad (1)$$

v is the reaction rate, c_H is the substrate concentration, K_M is the Michaelis–Menten constant and t is time.

There is little information available about the interactions of enzyme and substrate in this relatively new class of bacterial enzymes and no information on deactivation, which so far has been modelled only for monofunctional catalases (Lardinois et al. 1996). However, for industrial applications, the behaviour of CP in terms of deactivation is extremely important. With a macroscopic view, it is possible to describe the time-dependent substrate decrease as a combination of enzyme deactivation (Eq. 2) and Michaelis–Menten kinetics. The two differential equations are coupled via the k_{cat} of the CP (Eq. 3; Gudelj et al. 2001).

$$\frac{\partial c_E}{\partial t} = k_d \cdot c_E^n \cdot c_H^m \qquad (2)$$

c_E is the enzyme concentration, k_d is the deactivation constant, n is the order of deactivation reaction with respect to enzyme concentration and m is the order of deactivation reaction with respect to H$_2$O$_2$ concentration.

$$v_{Max} = k_{cat} \cdot c_E \qquad (3)$$

Results

Deactivation of CP from *Bacillus* SF by hydrogen peroxide

The initial velocity of hydrogen peroxide degradation by the CP of *Bacillus* SF is substrate-dependent, according to the Michaelis–Menten model, as we have shown previously (Gudelj et al. 2001). However, after prolonged incubation, the enzyme seems to be inactivated. This is evident from the significant discrepancy between the
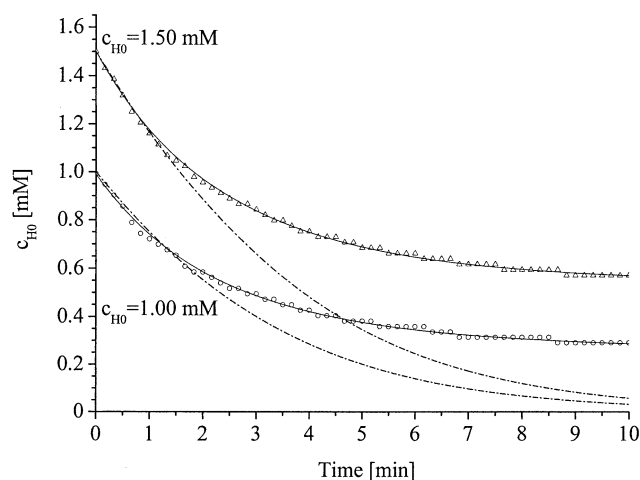
Fig. 3 Degradation of H_2O_2 by the CP from *Bacillus* SF, compared with the Michaelis–Menten model and the model developed in this study (see Results). *Circles, triangles* Measured H_2O_2 decay, *chain-dotted line* expected H_2O_2 decay with Michaelis–Menten model, *continuous line* expected H_2O_2 decay with new model, c_{H0} initial substrate concentration

measured decrease of the hydrogen peroxide concentration and the predicted decrease calculated using the Michaelis–Menten model (Fig. 3). Irreversible deactivation of CP from *Bacillus* SF was also shown by pre-incubation of the enzyme with hydrogen peroxide, which led to significantly lower degradation rates (data not shown). By combination of the Michaelis–Menten kinetics with enzyme deactivation (Eq. 2) via k_{cat} (Eq. 3), a more appropriate model describing the time-dependent decrease of the hydrogen peroxide concentration was obtained (Fig. 3). In the initial fitting of the equation to the measured curves, the variable parameters m, n and k_d were estimated: m was always very close to zero and n always had values near one. This means that the concentration of hydrogen peroxide has no influence on

the decomposition under physiological conditions up to a hydrogen peroxide concentration of 17.6 mM. In consequence, m was set to zero and n to one. The progression of hydrogen peroxide decomposition is regarded as a process which is dependent on enzyme concentration in the first order. A second modelling step was carried out to determine the remaining parameter, k_d. The values obtained for k_d were around a mean of 0.33 min^{-1} (Table 1).

Immobilisation on different supports

Bacillus SF was cultivated in a 10-l bioreactor and the crude enzyme preparation obtained had a catalase activity of 400 units·ml^{-1}. The crude enzyme preparation was used in excess in immobilisation experiments and the immobilisation yield is a function of the support material. The amount of enzyme immobilised and the specific activity both increased with the porosity of the support (Table 2). The highest specific activity obtained was with γ-Al$_2$O$_3$ balls, although Novalox saddles and Raschig rings performed better in packed-bed reactors, as oxygen forming during the decomposition of hydrogen peroxide was released easily. In contrast, using small spherical carrier materials, such as 2.0-mm alumina balls, oxygen bubbles were trapped in the reactor, reducing the active reactor volume. As a compromise between specific activity and the out-gassing behaviour, 3.5 mm alumina balls were chosen for further experiments.

Hydrogen peroxide decomposition in a laboratory-scale enzyme reactor

The horizontal reactor was loaded with *Bacillus* SF CP immobilised on 3.5-mm alumina balls. At pH 9.5 and 30 °C, there was a high conversion rate at the space time

Table 1 Model data for time-dependent H_2O_2 decomposition with the catalase peroxidase from *Bacillus* SF. c_{H0} Initial substrate concentration, v reaction rate, c_{E0} initial enzyme concentration, m order of deactivation reaction in respect to H_2O_2 concentration, n order of deactivation reaction in respect to enzyme concentration, k_d deactivation constant

| Parameters			First fitting			Second fitting
v (units ml^{-1})		c_{E0} (μM)	m	n	k_d	k_d (min^{-1})
1.0	317	1.71	<10^{-5}	0.90	0.27	0.27
1.5	406	1.66	<10^{-5}	0.92	0.31	0.31
3.76	599	1.52	<10^{-5}	0.93	0.36	0.31
7.5	571	1.15	<10^{-5}	0.93	0.37	0.35
13.0	769	1.38	<10^{-5}	0.95	0.47	0.39
17.6	860	1.48	<10^{-5}	0.94	0.38	0.33

Table 2 Specific catalase activity and amount of immobilised protein of the biocatalysts obtained by immobilisation of the crude enzyme preparation from *Bacillus* SF on different supports

Support	Specific catalase activity (U g^{-1})	Immobilised protein (μg g^{-1})
3.5-mm α-Al$_2$O$_3$ balls	1.2	60
2.0-mm γ-Al$_2$O$_3$ balls	1.9	95
10.0-mm γ-Al$_2$O$_3$ balls	0.7	35
12.7-mm Novalox saddles	0.9	45
6.35-mm Raschig rings	0.3	15

Table 3 Colour differences (ΔE^* values) for fabrics dyed in dyeing baths prepared either with water or with enzymatically treated bleaching effluent. Colours/mixtures were prepared according to the Dyestar technical catalogue

Colour	Components of the reactive dye	ΔE^* Value
Red	Red Remazol 3BS, Blue Sumifix BRF 150%, Blue Royal RS3R	0.97
Blue	Red Remazol 3BS, Black Remazol B 133%, Yellow Royal RS3R	0.42
Green	Blue Royal G 133%, Blue Sumifix BRF 150%, Yellow Royal RS3R	0.79
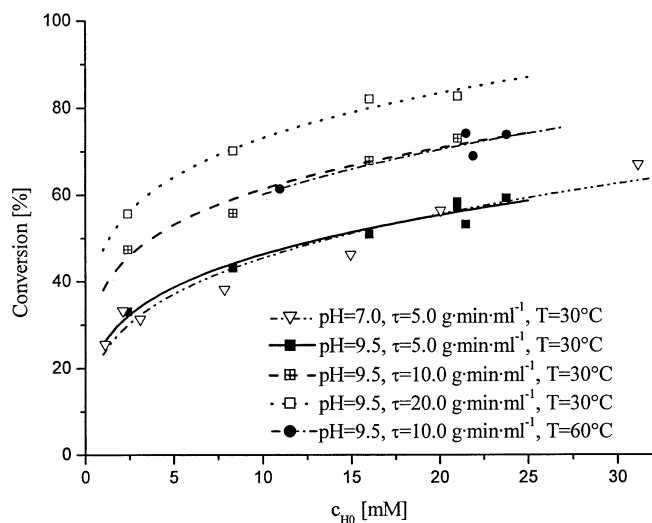
Fig. 4 H_2O_2 decomposition with CP from *Bacillus* SF immobilised on 3.5-mm α-Al_2O_3 balls in a packed-bed reactor at different pH values, space times and temperatures, as a function of the initial H_2O_2 concentration. τ Space time, T temperature

(specific retention time; Malikkides and Weiland 1982; Rovito and Kittrell 1973), of more than 10 g min ml^{-1}. As expected, the conversion rate increased with increasing space time (Fig. 4). Even at increased temperature (60 C), the conversion rate did not decrease. The conversion rate at pH 9.5 was only slightly lower than at pH 7.0, which was recently found to be the optimum pH for this enzyme (Gudelj et al. 2001). The conversion rate of hydrogen peroxide was lower at low initial hydrogen peroxide concentrations, due to transport phenomena within the porous biocatalyst.

Industrial application and dyeing experiments

A total of 500 l of bleaching effluents were treated using the enzyme reactor. For a final hydrogen peroxide concentration of 0.18 mM, which was previously found to be the upper limit tolerated in dyeing (Tzanov et al. 2001a), a specific retention time of 1.66 kg h^{-1} l^{-1} was determined for this reactor. At pH 10 and 45 °C, the half-life of the immobilised catalase was 22 days. The frequency of replacement of the biocatalyst will, however, depend on actual use of the reactor since lower storage temperatures when no treatment is performed or variations in pH or temperature during the treatment will influence the life of the catalyst. Periodic measurements

of the peroxide degradation with simple test-strips were accurate enough to determine when replacement of the catalyst was required. The treated effluent was used for the preparation of dyeing baths. Colour differences (ΔE^*) between fabrics dyed using the treated effluents and using water were below ΔE^*=1.0 for all three tested reactive dyes (Table 3). This means that no significant colour difference was found between fabrics dyed using fresh water or enzymatically treated bleaching effluents.

Discussion

Recently, we characterised a CP from the alkalothermophilic *Bacillus* SF and determined its kinetic parameters (Gudelj 2001). In this paper, we investigated the industrial application of this enzyme for the treatment of bleaching effluent, which required the determination of further deactivation of the enzyme by the substrate hydrogen peroxide.

CP from *Bacillus* SF seemed to be more sensitive to hydrogen peroxide than some monofunctional catalases (Lardinois 1996). However, most of the latter do not withstand the drastic conditions (pH 10–11, 50 °C) in bleaching liquor. Even CPs from other alkalothermophilic *Bacillus* spp were not as stable under these conditions as the CP from *Bacillus* SF (Gudelj et al. 2001; Yumoto et al. 1990). The deactivation constant (k_d) of the *Bacillus* SF enzyme was calculated as $k_d = 0.33$ min^{-1}. Deactivation constants (depending on the concentration of hydrogen peroxide) for catalases from *Aspergillus niger* have been reported to be in the range $k_d = 0.24$–0.30 M^{-1} min^{-1} (Tarhan 1995; Tarhan and Uslan 1990; Vasudevan and Weiland 1990, 1993). However, these values are not comparable with our data in terms of the biochemical deactivation mechanism, which seems to be different for monofunctional catalases and CPs (Lardinois et al. 1996).

The CP was immobilised covalently via glutaraldehyde-coupling (Malikkides and Weiland 1982) on alumina, which was previously described as a carrier for both CPs (Costa et al. 2001) and monofunctional catalases (Tarhan and Uslan 1990; Chatterjee et al. 1990). Glass-based carriers previously used for the immobilisation of catalases (Vasudevan and Weiland 1993) were not suitable in this case, due to their low stability at high pH. We previously showed that immobilisation increases the half-life of CP by up to ten-fold (Costa et al. 2001) and estimated the cost for immobilisation to be only around 15% of the cost for the enzyme needed to produce the alumina-based biocatalyst. Another advantage of

immobilisation is that no protein (the catalase) is added to the bleaching effluent, which has been shown to interact in the dyeing process (Paar et al. 2001; Tzanov et al. 2001a).

Natural polymers, like gelatine and chitosan, and other materials, like synthetic membranes from polyacrylamide, have also been used for the immobilisation of catalases, e.g. for sensors (Cetinus and Öztop 2000; Chatterjee et al. 1990; Gekas 1986; Horozova et al. 1997). Although immobilisation often enhanced the stability at higher pH and/or temperature, these carrier materials were not appropriate for bleaching effluent treatment at pH 10 and 50 °C. Furthermore, these materials are not available in the required shapes to allow release of the formed oxygen from the packed-bed reactor. From our studies, we conclude that the best available carrier for catalases used in bleaching effluent treatment are Novalox saddles of porous alumina.

At a given space time, with the laboratory-scale packed-bed reactor, similar peroxide conversion rates were measured for pH 7.0 and pH 9.5 at various peroxide starting concentrations. A larger horizontal packed-bed reactor containing the immobilised CP from *Bacillus* SF was constructed for the decomposition of the hydrogen peroxide from alkaline bleaching effluents. The reactor was operated at a space time giving a final peroxide concentration of 0.18 mM, which is not harmful in textile dyeing (Tzanov et al. 2001a). Colour differences between fabrics dyed using the treated effluents or fresh water were measured. These were expressed as ΔE^* values, which summarise the shift of the colour co-ordinates black–white, red–green and yellow–blue in the cylindrical colour space (Harold 1987). The correlation between A_{240} measurements in solution and reflectance measurements on dyed fabrics was evident (Ericson and Posner 1996). All ΔE^* values measured were below 1.0, which is acceptable to the industry (Baumann et al. 1987; Harold 1987; Steen 1998).

Since the concentration of hydrogen peroxide in the bleaching effluent was reduced to a level not harmful in textile dyeing (Tzanov et al. 2001a), these remaining colour changes could be caused by undefined substances extracted from the cotton, e.g. oils, waxes, pectins, proteins, organic acids, mineral matter and natural colouring-matter. The colour difference could be further reduced by adjustments of the dyeing bath composition, such as the concentration of dye, salts and additives (Tzanov et al. 2001b). Reusing the enzymatically treated textile bleaching- and washing-water in the dyeing process would lead to a saving of about 45 l of water per kilogram of textiles. Calculating an overall water consumption of more than 100 l for the processing of 1 kg of textiles (Hillenbrand 1999), the savings feasible with the catalase treatment will significantly contribute to cost reduction in textile processing.

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