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Thermo-alkali-stable catalases from newly isolated *Bacillus* sp. for the treatment and recycling of textile bleaching effluents

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Abstract

Three thermoalkaliphilic bacteria, which were grown at pH 9.3–10 and 60–65 °C were isolated out of a textile wastewater drain. The unknown micro-organisms were identified as thermoalkaliphilic *Bacillus* sp. Growth conditions were studied and catalase activities and stabilities compared. Catalases from *Bacillus* SF showed high stabilities at 60 °C and pH 9 ($t_{1/2} = 38$ h) and thus this strain was chosen for further investigations, such as electron microscopy, immobilization of catalase and hydrogen peroxide degradation studies. Degradation of hydrogen peroxide with an immobilized catalase from *Bacillus* SF enabled the reuse of the water for the dyeing process. In contrast, application of the free enzyme for treatment of bleaching effluents, caused interaction between the denaturated protein and the dye, resulting in reduced dye uptake, and a higher color difference of 1.3 ΔE^* of dyed fabrics compared to 0.9 ΔE^* when using the immobilized enzyme.

Keywords: Hydrogen peroxide; Catalases; Thermoalkaliphilic

1. Introduction

More than 100 l of water are currently consumed in the textile finishing industry for the processing of 1 kg of textiles (Hillenbrand, 1999). Particularly, textile bleaching is a water intensive process and thus, several methods have been suggested to 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 for the reduction of H_2O_2 would lead to unfavorable high salt concentrations in the process.

Alternatively, catalases could be used for the

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conversion of H_2O_2 into oxygen and water. However, most commercial catalases would hardly withstand the conditions used during textile bleaching. Thus, new thermo-alkali-stable enzymes acting at temperatures above 60 °C and pH values above 9 are required.

Catalases have been studied for a longer time than any other type of enzymes, with the first biochemical characterization reported almost 100 years ago (Loew, 1901). Catalase was one of the first enzymes isolated in a high state of purity, and its crystallization from beef liver extracts ranked among the early triumphs of biochemistry (Sumner and Dounce, 1937). However, research on catalases is still going on and this class of enzymes continues to surprise us (Loewen et al., 2000). There is very little known about catalases from alkalithermophilic micro-organisms, although enormous progress has been made over the last few years in the research area of extremophiles (Krulwich and Guffanti, 1989; Berkeley and Goodacre, 1992; Nakamura et al., 1995; Michaudsoret et al., 1998). Some thermoalkalophilic *Bacilli* are known, (Sarkar and Upadhyay, 1993; Prowe et al., 1996; Rua et al., 1997; Takahashi et al., 2000), however, alkalithermophiles seem to be more abundant among anaerobic organisms (Wiegel, 1998).

Although catalase is one of the most effective biocatalysts in terms of turn-over number (Aebi, 1983), cost of enzyme for the degradation of H_2O_2 of bleaching effluents could be reduced by immobilization of the enzyme. Many industrial processes use immobilized enzymes and catalases have been immobilized on numerous carrier materials such as alumina, gelatine, polyacrylamide and hen egg shell (Chatterjee et al., 1990), artificial membranes (Gekas, 1986), and carbon materials (Horozova et al., 1997). Alumina pellets were chosen as a carrier material for catalases due to their stability at high pH and temperatures.

In this study, catalases from three newly isolated thermoalkophilic *Bacillus* sp. were immobilized on alumina pellets for the treatment of bleaching effluents. The reuse of the treated bleaching effluent for subsequent dyeing would lead to savings in overall water consumption of up to 50%.

2. Materials and methods

2.1. Screening

Samples (1 ml) of a wastewater drain from a textile finishing company were added to 50 ml Standard I nutrient broth (Merck), buffered at pH 9.0 or 10.0, respectively, with 50 mM $NaHCO_3/Na_2CO_3$ and incubated in 100 ml Erlenmeyer flasks with chicanes in a rotary shaker at 60 °C and 160 rpm. Growth was monitored under the microscope and micro-organisms (1 ml) were transferred to fresh culture medium after 2 days. To isolate various strains, a dilution row was greased on Standard I nutrient agar (Merck) buffered with a 50 mM $NaHCO_3/Na_2CO_3$ buffer, pH 10. Agar plates were incubated at 60 °C and constant humidity; isolated strains were deposited and identified by the German culture collection DSMZ (Braunschweig, Germany), including partial sequencing of 16S rDNA.

2.2. Fermentation

Bacillus pallidus was grown in a medium consisting of (g l^{-1}) glucose 3, yeast extract (Merck) 5, peptone from casein (Merck) 5 and KH_2PO_4 1 at 60 °C and pH 9.0, buffered with 50 mM $NaHCO_3/Na_2CO_3$. *Bacillus* sp. LF was grown on (g l^{-1}) glucose 5, yeast extract 6, peptone from casein (Merck) 6, and KH_2PO_4 1 at 60 °C and pH 9.3, buffered with 50 mM $NaHCO_3/Na_2CO_3$. The culture medium for *Bacillus* sp. SF consisted of (g l^{-1}) yeast extract 8, extract from meat (Merck) 8, and KH_2PO_4 1 at 65 °C and pH 10.0, buffered with 50 mM $NaHCO_3/Na_2CO_3$. 1% (v/v) of a trace element solution containing (mg·l^{-1}): Na_2EDTA 2500, $ZnSO_4 \cdot 7H_2O$ 100, $MnCl_2 \cdot 4H_2O$ 30, H_3BO_3 300, $CaCl_2 \cdot 6H_2O$ 200, $CuCl_2 \cdot 2H_2O$ 10, $NiCl_2 \cdot 6H_2O$ 20, $Na_2MoO_4 \cdot 2H_2O$ 900, $Na_2SO_3 \cdot 5H_2O$ 30, $FeSO_4 \cdot 7H_2O$ 1000, was added to all incubation mixtures.

Cells were harvested at the end of the exponential phase of growth, centrifuged 15 min at $3000 \times g$ and the pellet was suspended in the equal volume with 50 mM NaH_2PO_4 buffer (pH 7.0). Cell disruption was carried out using a sonification unit (Bandelin Sonoplus HD 70, Berlin,

Germany) monitoring the progress under the microscope. Cell debris were removed by centrifugation 20 min at $6500 \times g$ and the remaining supernatant was stored at 4 °C and will be referred to as enzyme preparation.

2.3. Electron microscopy

Growing cells (exponential phase) were fixed by the addition of an equal volume of the fresh medium containing 8% (v/v) glutaraldehyde to stabilize proteins. The mixture was kept at room temperature for 1 h and then at 4 °C overnight. Samples were sedimented by centrifugation ($1000 \times$ g, 20 min, 20 °C), re-suspended in 0.1 M sodium cacodylate buffer (pH 7.2) containing 4% (v/v) glutaraldehyde and kept for several days. The fixed samples were washed with 0.1 phosphate buffer (pH 7.2), post-fixed with OsO_4 at 4 °C, dehydrated and embedded in epoxy resin. Sections stained with uranyl acetate and co-stained with lead citrate were analyzed in a transmission electron microscope (Philips CM 20 Analytical Electron Microscope).

2.4. Catalase assay and enzyme stabilities

Catalase activity was determined by monitoring the degradation of H_2O_2 spectrophotometrically at 240 nm as described previously (Aebi, 1983). The assay mixture contained 0.1 ml of enzyme preparation, 1 ml of a 26 mM H_2O_2 (Merck) stock solution and 0.9 ml of buffer.

To determine catalase stability, 1 ml of enzyme preparation was diluted with 9 ml buffer in test tubes (50 mM $NaHCO_3/Na_2CO_3$ for pH 9 and 10, 50 mM NaH_2PO_4 for pH 7 and 8) which were shaken at 50 rpm in a water-bath at 50 and 60 °C. Samples were withdrawn at various time intervals to measure enzyme activity.

2.5. Enzyme immobilization

Alumina pellets (Sigma) were silanised at 45 °C for 24 h in a 2.5% (v/v) solution of γ-aminopropyltriethoxy silane (Sigma) in acetone. The silanised pellets were washed with distilled water and immersed in 2% (v/v) aqueous glutardialdehyde (Sigma) for 2 h at 20 °C. Thereafter, the pellets were incubated with 60 mg protein l^{-1} of the crude enzyme preparation (obtained after ultrasonic treatment of the cells and removal of the cell fragments by centrifugation) for 5 h at 20 °C. The immobilized enzyme pellets were washed with potassium phosphate buffer (100 mM, pH 7.0) and kept refrigerated until further use. Immobilized protein was determined by protein analysis according to the method of Bradford using BSA for the calibration (Bradford, 1976).

2.6. Enzymatic treatment of bleaching effluents

Typically simulated bleaching effluents or 80 mg l^{-1} H_2O_2 solutions were continuously pumped (0.1 ml $^{-1}$ min, dual-piston-pump) through a column (15×300 mm^2) filled with 50 ml immobilized catalase corresponding to about 93.000 nkat catalase activity. Both the column reactor and the flow cell were kept at 30 °C and H_2O_2 degradation was monitored on a spectrophotometer equipped with a flow cell.

2.7. Dyeing experiments

Cotton fabrics were bleached in a bath containing (% of weight fabric) silicate 3.5, soda ash 1, sodium hydroxide 1, 35% hydrogen peroxide 4 at 90 °C for 180 min.

The bleaching bath, containing the fabric was treated with either free (93.000 nkat ml^{-1}) or immobilized catalase till all remaining hydrogen peroxide was converted to oxygen and water. Thereafter, the catalase treated liquor was reused for dyeing with Reactive Blue 198—a monochlorotriazine dye—(3% o.w.f depth of shade) in the presence of 60 g l^{-1} Glauber's salt and 20 g l^{-1} soda ash. Dyeing was performed at 80 °C for 60 min. Both dyeing and bleaching were carried out in an Ahiba Spectradye dyeing apparatus (Datacolor International, Luzern, Switzerland) at a liquor to good ratio of 20:1 (40 rpm, step 1: temperature was raised from 20 to 80 °C in 20 min; step 2: 80 °C, 60 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

Table 1
Characteristics of three isolated thermoalkaliphilic *Bacillus* sp.

	B. pallidus	*Bacillus* sp. LF	*Bacillus* sp. SF
Shape	Rods	Rods	Rods
Diameter	0.6–0.9 μm	0.6–0.9 μm	0.6–0.7 μm
Length	2–4 μm	2–4 μm	2–5 μm
Spore formation	+	+	−
Catalase activity (nkat mg^{-1})	8.300	63.000	107.900

min at 90 °C to remove the unfixed dye. Diode array spectra (TIDAS instrument from J&M, Aalen, Germany) of dyes both in standard dye baths and in dye baths containing enzymatically treated bleaching effluents were recorded. Color differences of the dyed fabrics were determined using a reflectance measuring apparatus (Spectraflash 600 from Datacolor) according to the CIELAB color difference concept at standard illuminant D_{65} (LAV/Spec. Excl., d/8, D_{65}/10°) with a color tolerance interval of 1 CIELAB unit.

3. Results and discussion

3.1. Isolated micro-organisms

Out of a textile finishing effluent, three thermoalkali-stable bacterial strains were isolated. One organism was identified as *B. pallidus*, while the other two organisms were new representatives of the *Bacillus* genus. Standard analysis of the cellular fatty acids indicated that all three isolated bacteria belong to thermophilic *Bacillus* sp. according to the DSMZ database. Similarly, the physiological conditions determined for the organisms clearly pointed to alkalophilic *Bacillus* sp. in all three cases. Partial sequencing of both the 16S rDNA of *Bacillus* SF and LF revealed below 92.5% similarity to other *Bacillus* sp. while the 16S rDNA of another strain showed 99.1% similarity to *B. pallidus* (data not shown). There are only few reports on thermoalkaliphilic species such as *Bacillus* sp. TAR-1 (Takahashi et al., 2000), *B. thermocatenulatus* (Rua et al., 1997), *Bacillus thermoalcaliphilus* (Sarkar and Upadhyay, 1993), or an anaerobic strain LBS3 (Prowe et al., 1996).

3.2. Catalases from the Bacillus sp.

B. pallidus, *Bacillus* LF and SF were cultivated in a 101 Bioreactor and crude enzyme preparations were obtained showing catalase activities of 8.300, 63.000 and 107.900 nkat·mg^{-1}, respectively (Table 1). The catalases of all three strains were quite stable at pH 10 and 25 °C, showing half-lives of at least 1 day (Table 2). Interestingly, at this temperature, all catalases showed substantially higher stabilities at pH 8 than at pH 7. At higher temperatures, the catalases of *Bacillus* SF were more stable than the enzymes from *B. pallidus* and *Bacillus* LF. At 60 °C, the *Bacillus* SF catalases had 13 and 10 times longer half-lives than the catalases from *B. pallidus* and *Bacillus* LF, respectively. Therefore, this strain and its enzymes were chosen for all further investigations.

Although catalases, which are produced by most aerobic micro-organisms are very well stud-

Table 2
Half-life $t_{1/2}$ (h) of catalases from three thermoalkaliphilic *Bacillus* sp.

Strain	pH	25 °C	50 °C	60 °C
B. pallidus	7.0	48	3	3
	8.0	96	3	4
	9.0	96	4	3
	10.0	48	1	1
Bacillus sp. LF	7.0	48	5	1
	8.0	72	6	3
	9.0	48	7	2
	10.0	24	3	0.5
Bacillus sp. SF	7.0	216	15	22
	8.0	240	20	42
	9.0	240	48	38
	10.0	148	15	4

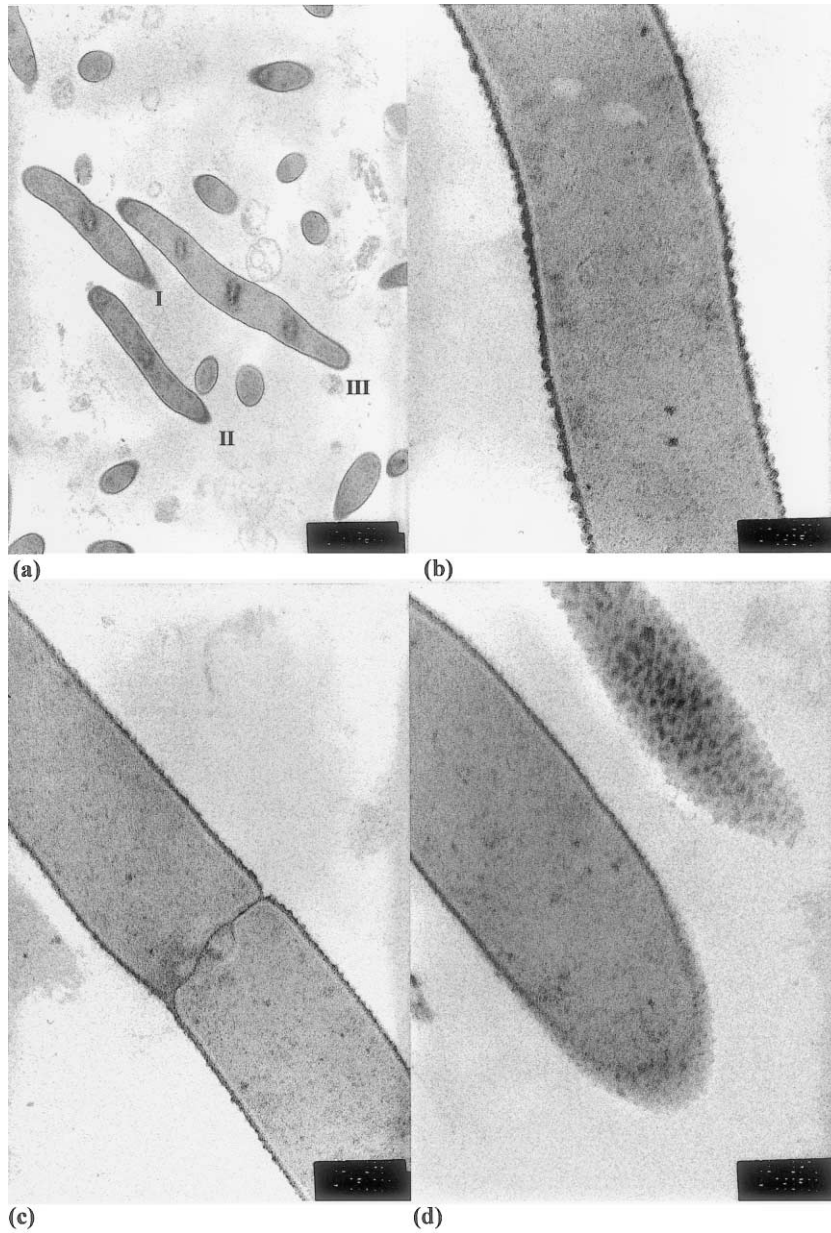

Fig. 1. Electron micrographs of cell walls of *Bacillus* sp. SF. Magnification: (a) 10.500; (b) 62.500; (c) 51.000; and (d) 62.500.

ied enzymes, there are only very few reports on thermo and/or alkali-stable catalases. Purified *Thermoleophilum album* catalase has its activity maximum at 35 °C with 56% of this activity at 60 °C, but retains 90% of its initial activity after incubation for 1 h at 80 °C and 93% activity after incubation for 24 h at 60 °C. This catalase retains 100% activity after 3 h of incubation at 25 °C in buffers over a range of 6.5–11.0 (Allgood and Perry, 1986).

3.3. Bacillus SF

Bacillus SF was studied under the electron microscope. Fig. 1(b) shows a cell wall with an ordered structure, which can also be seen from the surface (Fig. 1(d)). A number of micro-organisms show ordered structures such as S-layers which have been studied extensively (Taylor et al., 1982; Olabarria et al., 1996). However, the nature of the ordered layer of *Bacillus* SF remains to be investigated. The cell wall of *Bacillus* SF seems to be very thick (1b). Indeed, with a thickness of $18.6 + / - 2.5$ nm it was comparable to that of the alkalophilic *Bacillus lentus* ($21.5 + / - 2.2$ nm) (Aono et al., 1995), but it was thicker than the cell wall of a thermophilic *Cladorobacterium hydrogenophilum* with a thickness of 9–10 nm (Ludvik et al., 1994). Since the cell walls of alkaliphilic *Bacilli* is thought to be essential in providing a passive barrier to ion flux and elevation of cytoplasmic buffering capacity at highly alkaline growth pH (Krulwich et al., 1997), a thick cell wall with unusual shape of the new *Bacillus* SF is not surprising. Examination of the dark hemispherical structures in the cells (Fig. 1(a) – I, II and III) are most likely artefacts resulting from separation of the cytoplasm during the fixation of the cells.

3.4. Catalase immobilization, treatment and recycling of bleaching effluents

To allow repeated treatment of bleaching effluents, catalase preparations of *Bacillus* SF was immobilized. Preliminary experiments using glass-foam pellets as a support material were not successful because of the instability of glass-foam at high pH. On alumina, 93% of the enzyme protein in the catalase preparation was bound to the pellets and 87% of the activity was retained. This carrier material has recently been used for the immobilization of a laccase for the decolorization of textile dyeing effluents (Abadulla et al., 2000). The activity of the immobilized catalase was measured in an enzyme reactor monitoring continuously the difference between the H_2O_2 concentration in the influent and in the effluent. With this system, H_2O_2 was continuously degraded in a bleaching bath, which was further used for dyeing.

To compare the effect of free and immobilized catalase in the re-dyeing process, the shift of the co-ordinates of the color in the cylindrical color space L*, a* and b*, based on the theory that color is perceived by black–white (L), red–green (a) and yellow–blue (b) sensations (Harold, 1987), was summarized by the $\Delta E*$ value. The value of $\Delta E*$ represents the overall color difference between the sample and the standard (Table 3). Correlation between absorbance measurement in solution and reflectance measurements on dyed fabrics was evident (Ericson and Posner, 1996). Compared to the free catalase, the use of immobilized catalase for the treatment of bleaching baths gave a substantially lower $\Delta E*$ value in the dyeing process (Table 3). This indicated that the protein from the free enzyme might somehow interact with the dyeing process giving a larger color difference to a blank using water instead of enzymatically treated bleaching effluent for the preparation of the dyeing bath. Similar findings have been reported previously for laccase catalyzed decolorization of dyeing effluents, where enzyme protein had interacted with the re-dyeing process (Abadulla et al., 2000).

The $\Delta E*$ values for re-dyeing with bleaching baths treated with the immobilized catalase were around 1.0, which is acceptable to the industry (Baumann et al., 1987; Harold, 1987; Steen, 1998). However, these values might certainly be improved by proper adjustments of the standard dyeing protocols.

Table 3
Color difference between fabrics dyed in dyebaths prepared with water and fabrics dyed in dyebaths prepared with catalase treated bleaching liquor

	$\Delta E*$	Standard deviation
Free catalase	1.264	0.034
Immobilized catalase	0.886	0.022

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