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Production of Catalase-Peroxidase and Continuous Degradation of Hydrogen Peroxide by an Immobilised Alkalothermophilic *Bacillus* sp.

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Summary

Catalase-peroxidase (CP) production by a *Bacillus* sp. (*Bacillus* KF) newly isolated from a textile finishing effluent and by this strain immobilised on light expanded clay was studied. In cultivations of *Bacillus* KF increased catalase activity (about 30-fold) was measured after the addition of H_2O_2 and Orange IV, while ascorbic acid, pyrogallol and Paraquat, seemed to be poor inducers. Catalases in the cell extract from *Bacillus* KF showed remarkable stability at high temperatures and pH values with half-lifes of 20 h at pH=9 and 60 °C while half-lifes based on the activity of catalases of only 2.2 h were measured in a column reactor during hydrogen peroxide degradation for the whole cells. However, after the addition of cultivation medium immobilised cells can be regenerated and thus used for textile bleaching effluent treatment.

Key words: catalase-peroxidase, *Bacillus* sp., bleaching

Introduction

Only little is known about induction of catalase-peroxidases (CP) in microorganisms compared to catalases. The *kat*G gene of *Escherichia coli* and *Caulobacter crescentus*, encoding CP, is known to be induced by hydrogen peroxide (*1*). *Kat*G CP of *C. crescentus* was induced 20-fold by treating cultures in the exponential phase of growth with 60 μM H_2O_2. In contrast, *Legionella pneumophila kat*B (as well encoding CP) was not inducible by H_2O_2 (*2*). Pyrogallol was used as an inducer because of its potential to generate O^{2-} and subsequently H_2O_2 (*3*). *Deinococcus radiophilus* was exposed to UV-irradiation and treated with H_2O_2 for inducing CP (*4*).

A gradual increase of catalase production in aging cultures reported in other organisms is not surprising since catalase and/or CP is one of the radical scavenging enzymes in cells in response to oxidative stress (*4*). This means that CP activity is increasing up to the end of the exponential phase. On the other hand, several organisms produce two or more CPs, whereby one enzyme was expressed at the end of exponential growth and during the stationary phase. This behaviour was observed in *E. coli*, *Pseudomonas putida*, *Streptomyces coelicolor* and *Arcobacter nitrofigilis* (*5–7*).

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Recently we have reported on the isolation of the alkalothermophilic *Bacillus* KF and about the potential of the CP in textile processing (*8,9*). In this study, various fermentation conditions of *Bacillus* KF with respect to production of CP were compared using different inducers. Additionally, the potential of immobilised *Bacillus* KF for continuous degradation of hydrogen peroxide was studied.

Material and Methods

Cultivation

Bacillus KF was grown in a medium consisting of the following mass concentrations: KH_2PO_4 3.5 g/L, $Na_2HPO_4 \cdot 7\,H_2O$ 7.5 g/L, yeast extract (Merck) 10 g/L, peptone from casein (Merck) 20 g/L, NH_4SO_4 2.5 g/L, $MgSO_4 \cdot 7\,H_2O$ 4.5 g/L, $MnSO_4 \cdot H_2O$ 0.2 g/L, iron citrate $\cdot H_2O$ 0.7 g/L and 2.5 % of a trace element solution containing $ZnSO_4 \cdot 7\,H_2O$ 100 mg/L, $MnCl_2 \cdot 4\,H_2O$ 30 mg/L, H_3BO_3 300 mg/L, $CuCl_2 \cdot 2\,H_2O$ 10 mg/L, $NiCl_2 \cdot 6\,H_2O$ 20 mg/L, $Na_2MoO_4 \cdot 2\,H_2O$ 900 mg/L, $CoCl_2 \cdot 6\,H_2O$ 200 mg/L.

Cultivation was carried out in 100 mL baffled Erlenmeyer flasks in a rotary shaker at 60 °C and 160 rpm or in a 10-L bioreactor capable of pH and aeration control. The bioreactor was equipped with 3 axial propellers (set to 300 rpm) and aeration by a membrane was usually controlled to give a dissolved oxygen concentration above 25 %. The pH was kept at pH=9.0 unless otherwise stated below. During the fermentations in the bioreactor samples of 50 mL were withdrawn and treated as described below.

Immobilisation of Bacillus KF

A 6-L standard bioreactor (DIN 38412, 1994) operating at 60 °C was used for continuous cultivation of *Bacillus* KF immobilised on light expanded clay (LECA) from Leca-Liapor Baustoffe (Vienna, Austria). The culture medium consisted of yeast extract (Merck) 5 g/L, peptone 5 g/L from casein (Merck) and KH_2PO_4 1 g/L containing w=1 % of a trace element solution as described above and was buffered with $NaHCO_3/Na_2CO_3$ (c= 50 mM) to pH=9.0.

Downstream-processing

Cells were harvested at the end of the exponential phase of growth (Erlenmeyer flasks) or from samples taken from the bioreactors (50 mL aliquots and carefully detached biomass from LECA, respectively), centrifuged for 15 min at 3000 g and the pellet was suspended in the equal volume of NaH_2PO_4 buffer (c=50 mM, pH=7.0). Cell disruption was carried out using a sonification unit (Bandelin Sonoplus HD 70, Berlin, Germany) and monitoring the progress under the microscope. Cell debris were removed by centrifugation for 20 min at 6500 g and the remaining supernatant was stored at 4 °C and will be referred to as cell extract.

Enzyme assay

Catalase activity was determined by monitoring the degradation of H_2O_2 at 20 °C spectrophotometrically at 240 nm as described previously by Aebi (*10*). The assay mixture contained cell extract 0.1 mL, H_2O_2 (Merck) stock solution 1 mL (c=26 mM) and NaH_2PO_4 buffer 0.9 mL (c=100 mM, pH=7). Catalase activity was expressed as Units (U) corresponding to the transformation of 1 micromole of substrate per minute (1 U = μmol/min). To determine catalase stability, 1 mL of cell extract was diluted with 9 mL buffer in test tubes ($NaHCO_3/Na_2CO_3$ (c=50 mM) for pH=9 and NaH_2PO_4 (c=10 or 50 mM) for pH=7 and pH=8), which were shaken at 50 rpm in a water-bath at different temperatures. Samples were withdrawn at various time intervals to measure catalase activity as described above.

Peroxidase activity was assayed in an incubation mixture containing 1 mM peroxoacetic acid and 1 mM of either guaiacol or *o*-dianisidine in phosphate buffer (c=50 mM, pH=7.0). The reaction was monitored spectrophotometrically as described previously (*11*). Protein concentrations were determined by the method of Bradford (*12*) (Bio-Rad, USA) using bovine serum albumin as a standard.

Influence of various substances on catalase production

Catalase production by both *Bacillus* KF and immobilised *Bacillus* KF was induced by the addition of various compounds (μM): hydrogen peroxide (30 % Selectipur, Merck) 150, Paraquat (Sigma) 3.9, L(+)-ascorbic acid (Merck), Orange IV and pyrogallol 1000 (Merck). Cultivation was carried out using 100 mL baffled Erlenmeyer flasks in a rotary shaker at 60 °C and 160 rpm for 12 h, while the inducers were added after 8 hours. Alternatively, cultivation was carried out in the reactors described above using the same medium as described for fermentation. The values in the results correspond to mean values of triplicate experiments with a standard deviation lower than 15 %.

H_2O_2 degradation with immobilised cells

A mass of 10 g of wet immobilised organisms from the 6-L reactor was transferred into a glass column (25 x 300 mm), which was kept at 50, 60 and 65 °C. A solution of H_2O_2 (300 mg/L) in 50 mM Tris HCl buffer (pH=9.0) was pumped through the column (2.5 mL/min) and the decrease of the H_2O_2 concentration in the effluent was monitored spectrophotometrically at 240 nm using a flow cell.

Results and Discussion

Catalases, which are stable at high temperatures and pH values, have a potential for the treatment of textile bleaching effluents, which we have previously shown for an immobilised CP from the *Bacillus* SF (equivalent to *Bacillus* KF) (*13*). Although the immobilised CP showed long half-life times of 64 h at pH=9 and 60 °C (*14*), at a certain stage down-stream processing and immobilisation of the enzyme obtained via production by this *Bacillus* KF had to be replaced. As an alternative, the potential of the whole organism for continuous degradation of hydrogen peroxide was assessed.

In the first step, production of CP by *Bacillus* KF was studied. *Bacillus* KF and immobilised *Bacillus* KF were

Table 1. Fraction of catalase activity (%) in *Bacillus* KF cell extract after the addition of various substances during cultivation

	blank	H_2O_2	ascorbic acid	Paraquat	pyrogallol	Orange IV
Bacillus KF	100	205	90	117	29	185
Immobilised *Bacillus* KF	100	180	123	74	59	120

treated with known catalase inducers as described above and compared with a blank. At the used inducer concentration there was no effect on growth. Paraquat and H_2O_2 were applied in lower concentrations than the other substances since growth inhibition was observed above the applied concentration. For both *Bacillus* KF and immobilised *Bacillus* KF, Paraquat seemed to be a poor inducer compared to H_2O_2 and Orange IV (Table 1). Although H_2O_2 is also generated after the addition of pyrogallol (3), this substance did not improve CP production.

In the second step, the influence of H_2O_2, Orange IV and pH on catalase production by *Bacillus* KF was studied in a 10-L bioreactor. The bioreactor also allowed controlled aeration or supply of oxygen. Compared to standard fermentation conditions, the addition of moderate concentrations of hydrogen peroxide increased catalase production over 20-fold yielding 590 U/mg cell dry weight (CDW) (Table 2). This is in good agreement with results reported in the literature where CP activity of *C. crescentus* was induced 20-fold by treating exponential cultures with the solution of H_2O_2 (60 μM) while CP of *L. pneumophila* was not inducible by H_2O_2 (2).

Table 2. Catalase activity in *Bacillus* KF cell extract cultivated in a 10-L bioreactor

Type	CDW	μ	CP	CP
	mg/mL	h	U/mg protein	U/mg CDW
pH=9 standard	4.3	0.29	37.6	27.2
pH=7	6.2	0.60	42	32
pH=8	2.2	0.50	3.5	17.7
pH=10	5.2	0.43	88.9	40.8
pH=9 oxygen	7.1	0.69	267	269
H_2O_2 (c=50 μM)	7.0	0.41	309	590
H_2O_2 (c=250 μM)	3.9	0.62	39	8
Orange IV	7.9	0.71	104	500
Orange IV anaerob	5.8	0.42	2.7	1.3

The ratio of peroxidase activity based on guaiacol or *o*-dianisidine as substrates to catalase activity remained constant during all experiments (data not shown) indicating that no additional catalase is produced by *Bacillus* KF after the addition of the tested substances. However, after the addition of Orange IV almost the same catalase activity per CDW (500 U/mg CDW) was measured as in the presence of H_2O_2 (590 U/mg CDW), while the activity per protein was much lower (104 to 309 U/mg protein). This could indicate the expression of other proteins (enzyme) after the addition of Orange IV. Alternatively, the added substances could also inactivate catalase and therefore lead to different specific activities.

Catalases from *Bacillus* KF showed remarkable stabilities at high temperatures and pH values with half-lifes of 20 h at pH=9 and 60 °C (Table 3). In contrast, in the same conditions only half-lifes of 2.2 h were measured in the column reactor for the whole cells (Table 4). However, when the fresh cultivation medium was supplied to the cells in between the hydrogen peroxide treatments, the efficiency of H_2O_2 decomposition did not basically decrease. In detail, after treatment of simulated bleaching effluent (300 mg/L H_2O_2) at pH=9 and 60 °C for 1 h and subsequent incubation with cultivation medium for 30 min the decomposition rate in another treatment of simulated bleaching effluent (300 mg/L H_2O_2) decreased only by 2 %.

Table 3. Half-life ($t_{1/2}$) of catalase activity of *Bacillus* KF cell extract (h)

pH	20 °C	40 °C	50 °C	60 °C	70 °C
7	weeks	15	9	4	0.25
8	weeks	24	12	22	0.33
9	weeks	36	30	20	0.17
10	weeks	23	22	5	0.08

Trial no. 2 showed the highest degradation capacity (Table 4), while the first trial gave the lowest catalase activity of the immobilised mixed population due to the high concentration of H_2O_2, which seemed to be toxic to the organisms. Trials no. 3, 4 and 5 form a homogenous group concerning the half-lifes. The increasing temperature obviously had a significant effect on the deactivation of the enzyme.

Table 4. H_2O_2 degradation with immobilised *Bacillus* KF in a column reactor

No.	t	m(clay)	flow	$\gamma(H_2O_2)$	$v_{deactivation}$	$t_{1/2}$
	°C	g	mL/min	mg/L	s	h
1	50	30	1.5	979	$3.5 \cdot 10^{-5}$	–
2	50	10	2.5	150	$3.6 \cdot 10^{-5}$	5.50
3	50	10	2.5	300	$7.0 \cdot 10^{-5}$	2.74
4	60	10	2.5	300	$8.5 \cdot 10^{-5}$	2.25
5	65	10	2.5	300	$8.7 \cdot 10^{-5}$	2.20

Bleaching effluents of textile finishing companies show hydrogen peroxide concentrations of up to 150 mg/L and pH values of 9 with peaks of up to pH=11. In trial no. 2 these conditions were simulated. On 10 g wet expanded clay 29.3 mg biomass (CDW) were attached. The initial H_2O_2 decomposition rate was 18.5 mg/L/min giving a specific decomposition rate of 631 mg/L/min/

g dry weight and 1.85 mg/L/min/g expanded clay, respectively. Considering the flow rate of 2.5 mL/min, 3.4 kg of wet Leca with immobilised microorganisms would be enough to degrade all peroxide in one cubic metre of bleaching effluent within 1 hour.

The stability of immobilised *Bacillus* KF was investigated in a 6-L reactor. At hydraulic retention time of 1 week (medium was refilled once a week) the same H_2O_2 decomposition rates were obtained with aliquots of immobilised *Bacillus* KF measured in the column reactor even after 6 months. After this period of time under insterile conditions at 60 °C and pH=9.0 no other organism could be detected in the reactor. This is not surprising since only few thermoalkalophilic bacilli potentially growing under these conditions have been described previously such as *Bacillus* sp. TAR-1 (*15*), *Bacillus thermocatenulatus* (*16*), *Bacillus thermoalcaliphilus* (*17*), or an anaerobic strain LBS3 (*18*). Interestingly, immobilised *Bacillus* KF stored without liquid in the reactor for 4 months could be employed for H_2O_2 decomposition after incubation in the cultivation medium for only 12 hours.

Conclusions

In this study we have shown that production of CP with high stability at high temperatures and pH values by *Bacillus* KF can be increased by addition of H_2O_2 and Orange IV. As an alternative to the application of these catalases for the treatment of hydrogen peroxide containing textile effluents, immobilised *Bacillus* KF also seems to have a potential for this application. While, for example, immobilised catalases have to be replaced after the addition of cultivation medium, immobilised *Bacillus* KF can be regenerated.

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Proizvodnja katalaze-peroksidaze i kontinuirana degradacija vodikova peroksida s imobiliziranim alkalotermofilnim stanicama *Bacillus* sp.

Sažetak

Ispitana je proizvodnja katalaze-peroksidaze (CP) u soju *Bacillus* sp. (*Bacillus* KF), nedavno izoliranom iz otpadnih voda tekstilne industrije, te njegova imobilizacija na svjetlosno ekspandiranoj glini. Uzgojem *Bacillus* KF povećava se aktivnost katalaze (oko 30 puta), uz dodatak H_2O_2 i Orange IV, dok su se askorbinska kiselina, pirogalol i Paraquat pokazali kao slabi induktori. Katalaze u staničnom ekstraktu *Bacillus* KF bile su stabilne pri visokim temperaturama i pH-vrijednostima. Poluživot staničnog ekstrakta iznosio je 20 sati pri pH=9 i 60 °C, a poluživot temeljen na aktivnosti katalaze samo 2,2 h. Ovi su rezultati dobiveni mjerenjima cijelih stanica u kolonskom reaktoru tijekom degradacije vodikova peroksida. Dodatkom podloge za uzgoj imobilizirane se stanice mogu regenerirati i tako primijeniti pri obradbi otpadnih tekstilnih izbjeljivača.