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Resistance to Hydrogen Peroxide in Textile Waste Water *Bacillus sp.*

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ABSTRACT

A possible mechanism of resistance to hydrogen peroxide (H_2O_2) was studied for *Bacillus sp.* previously isolated from textile waste water. The results suggest that catalase is involved in the resistance and survival of this bacteria in the presence of low and high concentrations of H_2O_2. Inducible and constitutive catalase for exponential cells accumulated in extracellular, cytosolic and periplasmic fractions, with the majority compartmentalized in the periplasm. Protein electrophoresis showed a single band for catalase at 40 KDa. When periplasmic fraction was analyzed by Inductive Coupled Plasma (ICP), Fe and Mn were present suggesting that heme-dependent and manganese-dependent catalase might be present as a primary defense. UV-Visible spectrum showed two peaks relevant to catalase and peroxidase standards. Peroxidase activity suggests that HP I catalase-peroxidase is involved in the adaptation process, as a second defense, in response to active oxygen species. Catalase activity was constitutively present in *Bacillus* cells grown in the absence of H_2O_2 and its occcurrence was dependent on the age of cells, a trait known for HP II-type catalase. The survival of this bacteria in such high concentrations of hydrogen peroxide is a promising feature regarding treatment of waste water containing hydrogen peroxide.

Key Words: *Bacillus sp.*; Catalase; Peroxidase; Oxidative stress; Textile waste water

INTRODUCTION

The response of microorganisms to oxidative stress has been extensively examined for a number of physical and chemical agents (Riesenman & Nicholson, 2000) However, these studies were limited to short term oxidative stress few studies, were conducted on long term oxidative stress (Yumoto *et al*., 1999; Paar *et al*., 2003). Long term exposure to oxidizing agents is very important to understand the microorganisms living in highly oxidative environments and why such microorganisms can successfully adapt more than others. Under normal conditions a delicate balance exist between reactive oxygen species (ROS) such as hydroxyl radicals $^{\cdot}OH$, superoxide radicals O_2^- and hydrogen peroxide (H_2O_2) and the cellular antioxidant system. The presence of abnormal concentrations of ROS results in oxidative stress defined as the imbalance between proxidant and antioxidant (Dasgupta *et al*., 1997). Once this imbalance takes place, cellular molecules may be damaged by the predominant free radicals, this leads to oxidative modifications of the cellular molecules (Saada & Azab, 2001; Azab & El-Dawy, 2005).

Most microorganisms produce certain enzymes to counteract the damaging effects of hydrogen peroxide. Catalase (E.C 1.11.1.6) occur naturally in aerobically respiring organisms it is considered one of the mechanisms used to protect the cells against the damage caused by reactive oxygen species to cellular components, including nucleic acids, lipids and proteins (Miller *et al.*, 1997). It catalyzes the decomposition of exogenous or endogenous hydrogen peroxide (Harris *et al*., 2002). The overall reaction catalyzed by catalases is the degradation of two molecules of hydrogen peroxide to water and oxygen. This simple overall reaction can be broken down into two stages, but what is involved in each stage depends on the type of catalase. There are major classes of catalases, which are: monofunctional catalases, bifunctional catalase-peroxidases and manganese-containing catalases (Chelikani *et al.*, 2004).

A bacterium isolated from textile waste water exhibiting high tolerance to hydrogen peroxide was identified as *Bacillus maroccanus* type strain (Gomaa & Momtaz, 2006). The study of mode of protection and adaptation of bacteria against high levels of oxidative stress, represented by high concentrations of hydrogen peroxide, gives us an insight to the enzyme regulation system involved in response to oxidative stress for this strain, it is also a great potential in industrial applications related to high concentrations of hydrogen peroxide. Hydrogen peroxide is used in bleaching and as a microbicidal agent in paper, food, textile and semiconductor industries (Ichise *et al.*, 1999; Paar *et al*., 2003). It is crucial to remove hydrogen peroxide prior to any biological waste water treatment, because of its ability to damage microorganisms required for this treatment. This procedure is effective and is considered a low-cost treatment process rather than conventional methods for hydrogen peroxide removal (Mohey Eldein *et al.*, 2006).

In the present study, the mode of resistance of this bacterium to high concentrations of hydrogen peroxide was examined in order to study and understand its tentative potential in industrial applications and waste water

treatments.

MATERIALS AND METHODS

Bacterial strain and cultivation. *Bacillus sp.* (previously isolated from textile waste water), were cultivated aerobically on LB broth medium, 24 h cultures were used to inoculate LB broth with hydrogen peroxide added after sterilization. The cultures were incubated in the dark statically, cells were harvested in early exponential phase, time of incubation was chosen according to previous work (Gomaa & Momtaz, 2006).

Preperation of cytosolic and periplasmic extract. Cultures were centrifuged at 4°C for 30 min at 10000 rpm, then the pelleted cells were washed and centrifuged in phosphate buffer pH 7 for 15 min at 39000 rpm in a cooling ultracentrifuge SORVALL ULTRA 80 at 4°C, this process was done twice to obtain cytosolic and periplasmic cell fractions.

UV-visible spectrum. The periplasmic cell fraction, which was obtained in phosphate buffer pH 7, was taken and run on UV-visible spectrophotometer CECIL-3041 to obtain all possible peaks relevant to enzyme presence in comparison to catalase and peroxidase standard enzymes (purchased from Sigma, UK).

Incubation with low and high concentrations of hydrogen peroxide on *Bacillus* cells. Catalase, peroxidase and cell protein were assayed after incubation with low levels of hydrogen peroxide (1, 5 & 10 mm) and high levels of hydrogen peroxide (50, 150 & 250 mm).

Effect of time on *Bacillus* incubated with hydrogen peroxide. Catalase, peroxidase and cell protein were estimated every 30 min in cultures incubated with 250 mm hydrogen peroxide.

Metal analysis. Digestion of the samples was carried out by adding 5 mL of nitric acid to 0.5 mL of periplasmic extract the volume was completed to 100 mL in a volumetric flask. A standard curve for Fe and Mn was carried out using a SPEX plasma standard 1000 µg/mL. The concentration of the elements was determined from the standard curve. The metal analysis was carried out using Induced Coupled Plasma (ICP) JOBIN YVON HORIBA ULTIMA.

Enzyme and protein assay. Catalase was determined by monitoring the degradation of H_2O_2 in 1 min at 25°C using a Schimadzu UV 2100 spectrophotometer at 240 nm. The assay mixture contained enzyme extract and 1% H_2O_2 (Bergmeyer *et al.*, 1987). Peroxidase was assayed by monitoring the rate of formation of purpurogallin from pyrogallol in 20 seconds at 25°C spectrophotometrically at 420 nm (Sumner & Gjessing, 1943). Protein concentrations were determined by the method of Lowry (Lowry *et al.*, 1957) using bovine serum albumin as a standard. All samples were examined for their colony forming ability after incubation with hydrogen peroxide to ensure that the bacteria, was viable at the chosen concentration and time of the experiments.

SDS-PAGE. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed for periplasmic and cytosolic cell extracts at room temperature with 10% gels and Tris-glycine buffer (pH 8) at 125 V for 90 min to obtain the molecular weight of the enzymes, protein bands were stained by 0.05% coomasie brilliant blue R.

RESULTS AND DISCUSSION

A number of bacteria are capable of living under extreme environmental conditions, each having their own enzymatic defense mechanism to overcome abiotic stress. In a previous work (Gomaa & Momtaz, 2006) a bacteria, was isolated from textile waste water, it was characterized as extreme halotolerant, facultative psychrophilic, facultative alkalophilic and it exhibited tolerance to very high concentrations of hydrogen peroxide, which exceeded those reported normally for other bacteria. In this study, catalase was studied as the common enzyme that might contribute to scavenging hydroxyl radicals for this bacterium. Catalase was expressed when hydrogen peroxide was added to the media, being maximally expressed in late exponential phase cells when compared to early exponential cells (50% increase in activity), while changes in cellular proteins were not clearly distinct, probably, because of the low exposure time to hydrogen peroxide (Fig. 1). The fact that catalase was higher in the late exponential phase for *Bacillus* cells, even without the addition of H_2O_2 is a common feature for HP II type catalase in *Vibrio sp.* (Ichise *et al.*, 1999) and *E. coli* (Seaver & Imlay, 2001). This is a monofunctional catalase reported in many bacteria and is controlled as part of the σ^s regulon in stationary phase (Vattanaviboon & Mongkolsuk, 2000). In early exponential phase cultures, catalase was expressed constitutively in the absence of hydrogen peroxide and inducibly after the addition of hydrogen peroxide, a trait known for most bacteria in general.

Catalases were reported to be present in different cellular compartments its compartementaliztion depended on its age (Schnell & Steinman, 1995). It is located in extracellular, cytosolic and periplasmic fraction for early exponential cells in the presence and absence of H_2O_2, with the majority present in the periplasm (Fig. 2). In *E. coli*, catalase/peroxidase, were found in periplasmic and cytosolic fractions, being partially periplasmic and associated with the outer surface of the inner membrane (Heimberger & Eisenstark, 1988) (Heimberger & Eisenstark, 1988). The intracellular localization of the catalase/peroxidase shows 49% and 51% in periplasm and cytosol, respectively for wild type *C. crescentus* (Schnell & Steinman, 1995). A question raised in Schnell and Steinman's work: would the distribution of these two enzymes between cytosol and periplasm be growth stage dependent? The answer maybe positive as their distribution was almost equal in stationary phase cultures in their study, while the ones in this study

Fig. 1. Differences in catalase activity in early *Bacillus sp.* grown under H_2O_2- conditions (EC), *Bacillus sp.* grown under H_2O_2 + conditions (EH_2O_2), late *Bacillus sp*. cultures grown under H_2O_2- conditions(LC) and late *Bacillus sp.* cultures grown under H_2O_2+ conditions (LH_2O_2)

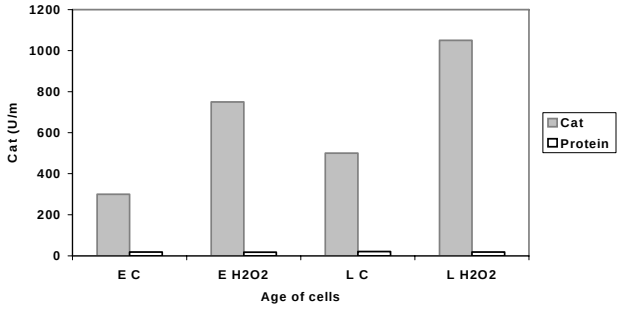
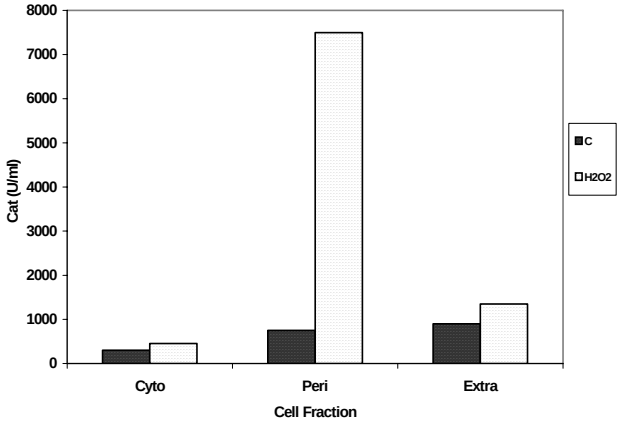


Fig. 2. Effect of H_2O_2 treatment on cytosolic, periplasmic and extracellular catalase activity for *Bacillus sp*. grown under H_2O_2 – conditions (C) and H_2O_2 + conditions (H_2O_2)



were 10% and 90% in cytosol and periplasm, respectively in early exponential phase cells and this relates compartmentalization to growth stages. The periplasmic locaization of catalase in young cultures suggest cell membrane involvement, hydrogen peroxide was reported to be a central signaling compound and that it is able to cross membranes to the place of detoxification (Bienert *et al.*, 2006). While for cultures reaching the stationary phase, spore coat enzymes, controlled by σ^F are expected to be more involved in the survival process (Bagyan *et al.*, 1998).

Protein electrophoresis of the cytosolic and perilasmic fractions in the presence and absence of H_2O_2 show a single band at 40 KDa (Plate 1), which is classified under small subunit (< 60 KDa) and is a characteristic for monofunctional, heme-containing catalase (Koltz *et al.*, 1997). This is a confirmation that catalase is present constitutivley and under hydrogen peroxide induction.

UV-visible spectrum of the inducible and constitutive periplasmic fraction was examined to determine if there are

Fig. 3. UV-visible spectrum of periplasmic fraction for *Bacillus sp.* grown under H_2O_2- conditions (A) and H_2O_2+ conditions, (B) A small peak was detected at 356 nm which is the same for standard catalase (purchased from Sigma, UK). It is shown that in (A) the peak is at 0.25 while in (B) after H_2O_2 addition it was 0.44. The peak for peroxidase is at 274 nm, but its also in the same range for protein fraction, therefore, it wouldn't be taken into account from this experiment

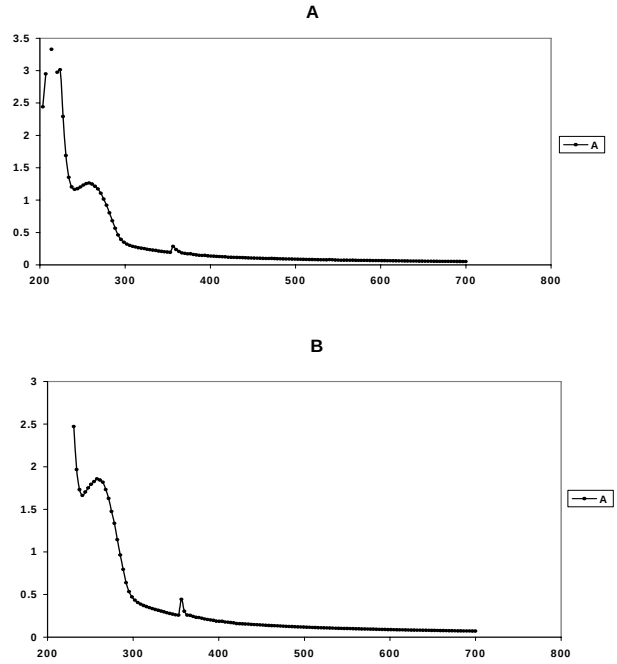
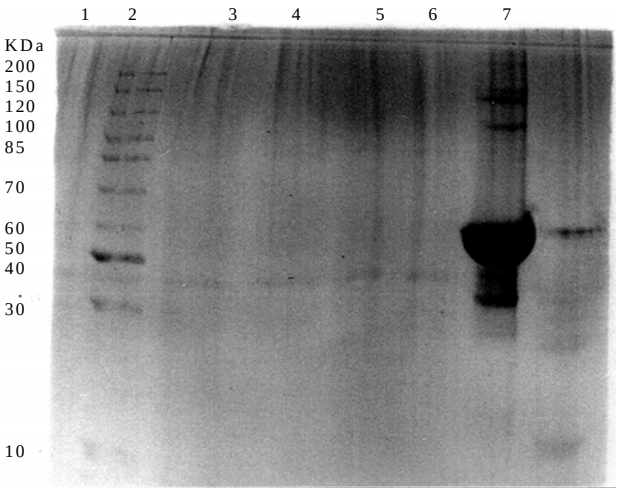


Plate 1. SDS-PAGE for Protein ladder 1, control sample2 and hydrogen peroxide induced cytosolic fraction 3, control 4 and hydrogen peroxide induced periplasmic fraction 5, standard catalse 6 and standard peroxidase 7, the gel was stained with coomassie brilliant blue



other enzymes responsible for hydrogen peroxide tolerance by *Bacillus sp*. vegetative cells, (Fig. 3A & B),the spectra show a peak at the wavelength for catalase, also there was a shoulder evident at the specific wavelength for peroxidase, both of which were confirmed by obtaining full spectrum for standard catalase and peroxidase, the peak for catalase is at 356 nm and that for peroxidase was 274 nm (data not shown). The spectrum represented in (Fig. 3B) is higher than that for (Fig. 3A), this is considered an additional confirmation that the inducible enzymes are highly pronounced than those for constitutive enzyme. The presence of peroxidase is an indication for the presence of bifunctional catalase-peroxidase, a feature for HP I catalase-peroxidase (Paar *et al.*, 2003), its controlled as part of the OxyR regulon, which senses active oxygen species (Chelikani *et al.*, 2004).

Low doses of hydrogen peroxide were reported as catalase inducer in fungi as an alternative source for oxygenation (Fiedurek & Gromada, 2000). Both catalase and peroxidase in periplasmic cell fraction of *Bacillus sp*. responded to low concentrations of hydrogen peroxide (Fig. 4). It is clear that the increase in hydrogen peroxide concentration is concomitant to the increase in both the catalase and peroxidase. The cellular protein content exhibited no alterations, probably, because of the low concenrations of hydrogen peroxide, which didn't exert any protein damage. The damage of protein by hydrogen peroxide is a well-known phenomenon that precedes damage to internal compartments (Riesenman & Nicholson, 2000), but the concentration used in this experiment was very low compared to that reported for this bacteria in earlier work, therefore no detectable change was evident.

On the other hand, when high concentration of hydrogen peroxide, were used catalases, peroxidases and cellular protein exhibited different behavioral changes (Fig. 5A & B). The figures show an increase in catalase directly proportional to the increase in hydrogen peroxide concentration, while the peroxidase was expressed maximally at 50 and 150 mm and decreased again at 250 mm. The protein content of cells decreased as the hydrogen peroxide concentrations increased, this is expected, because of the high concentrations of hydrogen peroxide, which attacked the protein of the cell membrane causing it to disintegrate.

The metals present in the periplasmic fraction of constitutive and inducible cultures were analyzed using the Induced Coupled Plasma (ICP) to search for specific metals representing the prosthetic groups for the enzymes present. The results shows the presence of iron (0.813 µg/mL) and manganese (0.441 µg/mL), both suggesting the presence of heme dependent and non-heme dependent catalase, together with catalase-peroxidase. UV-visible spectrum showed that catalase contained heme group as indicated by Shima *et al*. (2001). According to Chelikani *et al*. (2004), the presence of Fe^{2+} and Mn^{2+} suggests the presence of heme-dependent and manganese-dependent catalase and the presence of

Fig. 4. The effect of low concentrations of hydrogen peroxide on catalase Δ, peroxidase □and cellular protein • for *Bacillus sp.*

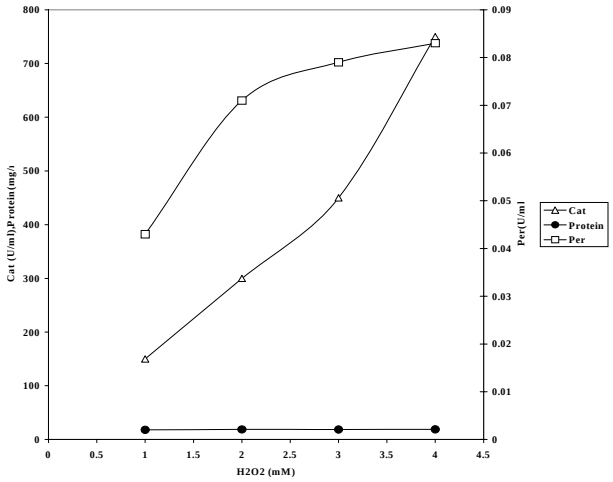
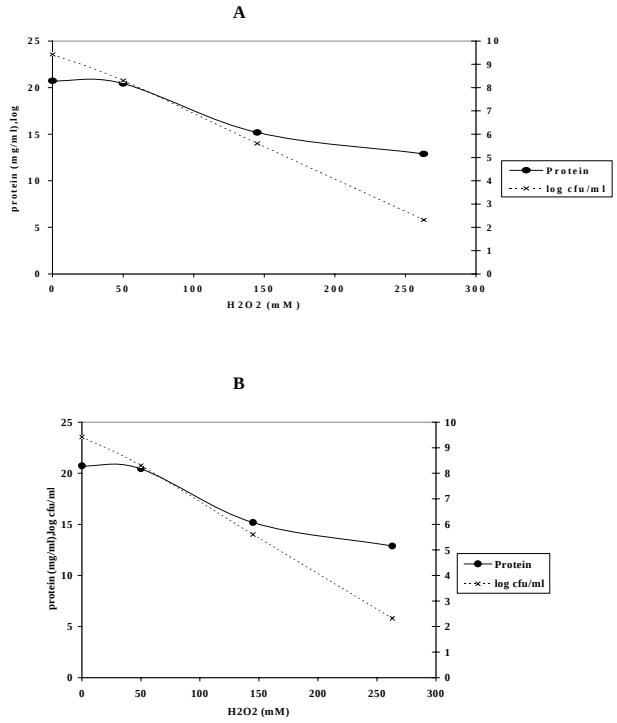


Fig. 5. Effect of high concentrations of hydrogen peroxide on catalase and peroxidase (A), log cfu and cellular protein content, (B) for Bacillus sp.



peroxidase suggests the presence of a bifunctional catalase/peroxidase, its therefore postulated that *Bacillus maroccanus* possesses 3 types of catalases as a mode of defense against hydrogen peroxide. Iron was reported in numerous reports as a metal related to oxidative stress response (OSR) through ferric uptake regulator (Fur) and Fur-like protein (Ppopham *et al.*, 1995; Harris *et al.*, 2002; Ricci *et al.*, 2002).

Specific catalase activity for *B. subtilis* was reported to be 16.4 U/mg of protein, while that for *V. rumoiensis* isolated from hydrogen peroxide fish pool was reported to reach 4.092.4 U/mg of protein, with a number of different strains giving intermediate values (Yumoto *et al.*, 1999). This puts the strain under study in a rank right below *V. rumoiensis*, as the catalase specific activity was calculated to be 3250 U/mg of protein.

In conclusion, this study represents the mode followed by *Bacillus maroccanus* for its survival through the formation of catalases. Three types of catalases were believed to take place in this defense against the oxidizing stress in young cells, each present according to time of incubation, the majority present in the periplasmic fraction in young cells. The ability of *Bacillus maroccanus* to defend itself against high concentrations of hydrogen peroxide was suggested in an earlier study as a suitable candidate for Advanced Oxidative Processes (AOP), it is also of great benefit in bioremediation of hydrogen peroxide containing waste water.

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