BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, December 2013. Vol. **10**(2), 929-935

Hydrogen Peroxide Photoproduction by A Marine Cyanobacterium *Oscillatoria boryana* BDU 92181 with Potential Use in Bioremediation

D. Francisca Kalavathi* and G. Subramanian[1]

*Department of Microbiology, Bangalore City College, No. 160 Chellikere Main Road, Banaswadi Outer Ring Road, Kalyan Nagar Post, Bangalore - 560 043, India.
[1]National facility for Marine Cyanobacteria, Bharathidasan University, Tiruchirapalli - 620 024, India.

DOI: http://dx.doi.org/10.13005/bbra/1221

(Received: 18 August 2013; accepted: 29 September 2013)

The present study was aimed to find out the ability of the marine cyanobacterium *Oscillatoria boryana* BDU 92181 to produce H_2O_2 and to optimize its photoproduction with a view to harness its potential for decolorization of distillery effluent. The organism decolorized distillery effluent (5% V/V) by about 60% in 30 days. The mechanism of decolorization is postulated to be due to the production of hydrogen peroxide and molecular oxygen released by the cyanobacterium during photosynthesis. The effects of light intensity as well as different enhancers and inhibitors on the production of H_2O_2 in this organism were studied. Our results showed that, riboflavin and $MnSO_4$ positively influenced H_2O_2 production. Sodium metabisulphite and potassium cyanide inhibited H_2O_2 production.

Key words: Decolorization, Enhancers, Inhibitors, Hydrogen Peroxide, Photoproduction

In the recent past there is an upsurge of interest in employing technological applications of photo biological solar energy conversion (Roncel *et al*. 1989; Galvan and de la Rosa 1997; de la Rosa 1986; Navarro *et al*. 1987). The bioconversion of solar energy to a valuable renewable resource is not only attractive means, but also economical (de la Rosa 2001; Navaro *et al*. 1987) One of the products of biological conversion of solar energy is hydrogen peroxide, and was first shown to be photo produced in isolated chloroplasts (Mehler 1951). Patterson and Myers in 1973 were reported the photosynthetic production of H_2O_2 by the cyanobacterium *Anacystis nidulans*. A more extensive survey of blue green algal isolates from a variety of sources has revealed that H_2O_2 production is widespread among these organisms (Stevens *et al*. 1973). It has been demonstrated that the metabolic conditions of the algae in their different growth stages influence the capacity for hydrogen peroxide photoproduction. Hydrogen peroxide is a high-energy compound (100kJ/mol) and has been used as a fuel for motors and heaters. The significant advantage of using H_2O_2 in various fields is that it does not pollute the environment as it liberates energy by dismutation to H_2O and O_2. Biotechnological advances in the field of effluent treatment have resulted in a number of efficient technologies; however no biological treatment is available for colored textile effluents and pathogen elimination. Biodegradation of textile dyes has been demonstrated by H_2O_2 using *Pleurotus ostreatus.* (Vyas and Molitoris 1995), heterotrophs

* To whom all correspondence should be addressed.
Mob.: +91 9902697295; Fax: 080-25459958;
E-mail: dfkala@yahoo.com

deplete oxygen in the system and higher fungi are not easily adapted for aquatic habitats. Recently, it has been shown that prokaryotic, oxygen evolving photoautotrophic marine cyanobacteria are becoming important biotechnological tools especially in bioremediation and these unlike heterotrophs, oxygenate waterbodies (Subramanian and Uma 2001). Degradation/decolorization of recalcitrant distillery effluent pigment melanoidin (Kalavathi *et al.* 2001); triphenyl and azo dyes (Uma *et al*. 2000); and lignin (Malliga *et al.* 1996) by marine cyanobacteria has been documented. One of the reasons attributed for their degradation is the suspected production of hydrogen peroxide (Hayase *et al.* 1984; Malliga *et al*. 1996; Patil and Kapadnis 1995).

The present study was aimed to find out the ability of the marine cyanobacterium *Oscillatoria boryana* BDU 92181to produce H_2O_2 and to optimize its photoproduction with a view to harness its potential for bioremediating purpose, as it was already found to decolorize melanoidin.

MATERIALS AND METHODS

Organism Maintenance

Marine cyanobacterium used for the study was *Oscillatoria boryana* BDU 92181, a non-heterocystous filamentous cyanobacterium, obtained from the germplasm collection of National Facility for Marine Cyanobacteria, Bharathidasan University, Tiruchirappalli, India. The cultures were maintained in synthetic marine liquid medium with combined nitrogen source (ASN III) (Rippka *et al*., 1979) and incubated at 25±2°C under fluorescent light (20.15 mE m^{-2} sec^{-1}) with 14/10h L/D cycle

Effluent Source

Anaerobically digested molasses spent wash with pH8.3 was obtained from M/sTrichy distilleries Ltd, Tiruchirapalli, Tamilnadu, India.

Decolourization Experiment

To study the influence of cyanobacteria on decolourization of distillery effluent, various dilutions of effluents prepared were 0.5, 2.5, 5.0, 25, 50, 100% (v/v). The cultures treated with various dilutions of effluent were incubated at 25+/-2°C under fluorescent light at an intensity of 13.7W m^{-2}

Experimental design for hydrogen peroxide production

The experiments were grouped into 5 sets. All the experiments received uniform inocula prepared from mid-log cultures of *O.boryana* BDU 92181. The first set was incubated under varied light intensities of 20, 40, 80, 160 and 240µE m^{-2} sec^{-1}. As light intensity of 80µE m^{-2} sec^{-1}, was found to be the optimum for hydrogen peroxide production, further experiments were conducted at this light intensity. The second set was treated with various enhancers such as manganous sulphate, methyl viologen, riboflavin, and ascorbic acid at 50 and 100µM concentrations respectively. The third set received manganous sulphate enhancer of 50, 100, 150, 200, 250 and 500µM, in addition to the above tested concentrations. The fourth set was treated with inhibitors such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), sodium azide, potassium cyanide, phthalic acid, sodium metabisulphite, sodium thioglycolate at 50 and 100µM concentrations. Organism that did not receive inhibitor/enhancer served as controls.

Analytical methods

Biomass

After the incubation period, the cultures were washed with ASN III medium and Chlorophyll was determined following the method of Mac Kinney, (1941) in methanol extracts and the absorbance at 663nm (JASCO, HMC358, Japan).

Decolorization Assay

Decolourization of distillery effluent was measured by the absorbance (optical density) of supernatants of effluent containing cultures prior to and after the growth of the organism by a Beckman DU64 spectrophotometer (Beckman, Switzerland) at 475nm. Uninoculated samples served as controls.

H_2O_2 production

H_2O_2 was determined in aliquots by the chromogen formed using 4 aminoantipyrine and phenol (Green and Hill, 1984). The change in absorbance was measured at 505nm (JASCO, HMC358, Japan) and expressed as mmol of hydrogen peroxide $Chl.^{-1}$ mg^{-1} h^{-1} The values represented are the average of triplicates.

RESULTS AND DISCUSSION

Decolorization of distillery effluent

O.boryana BDU 92181 being non-heterocystous and non-nitrogen fixing, grew normally in marine synthetic liquid medium (ASN III) with a combined nitrogen source, but could not grow in ASN III nitrogen free medium. The maximum color reduction observed at 5% effluent concentration after 30days of treatment was about 60% (Fig 1, Plate 1). However higher concentration of distillery effluent above 5% did not support growth thereby less color reduction. This could be attributed to the lack of light penetration because of the very dark color of the effluent. This suggests that color reduction requires light and the consequent growth of the organism and also the result of several factors in effluent whose composition is different from the ASN III medium.

Hydrogen peroxide production

Hydrogen peroxide a form of active oxygen has been suggested to be responsible for decolorization of colored solutions, containing dyes such as ramazol brilliant blue and melanoidin (Hayase *et al*. 1984; Kelly & Reddy 1988; Vyas

Plate 1. Decolorization of distillery effluent by *O.boryana* BDU 92181

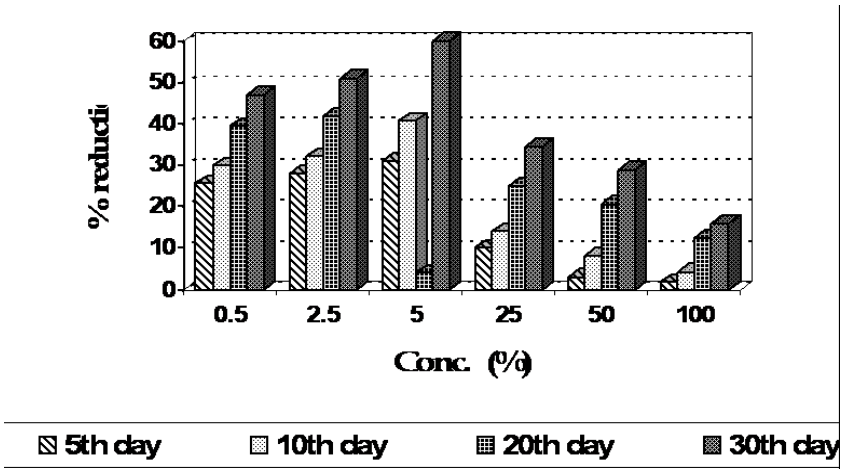

Fig. 1. Decolorization of distillery effluent by *O.boryana* BDU 92181

& Molitoris 1996). Algal systems as well as cyanobacterial systems were reported to produce hydrogen peroxide especially in the presence of light (Patterson and Myers 1973; Stevens *et al*. 1973; Roncel *et al*. 1989; Park *et al*. 1991).

Effect of light intensity

Cyanobacterial hydrogen peroxide is strictly a light dependent process, with a participation of PSI system (Park *et al*. 1991). Organism when exposed to different intensities of light showed different rates of H_2O_2 production (Fig.2). At lower intensities of light between 20 and 80 mE m^{-2} sec^{-1}, the rate of production was directly proportional to light intensity and were 27,48. and 81 mmol mg^{-1} $Chl.h^{-1}$ respectively, whereas at higher light intensities of 160 and 240mE m^{-2} sec^{-1}, there was only a moderate increase of 97 and 108 mmol mg $Chl.^{-1}$ h^{-1} (Fig.2). In dark, *O.boryana* BDU 92181 did not show any H_2O_2 production. The ability of the organism to photoproduce H_2O_2 at different light intensities exhibits the wide adaptability of the organism to suit different type of effluents where light penetration might vary.

Effect of enhancers

An attempt was made to identify possible enhancers of the process, so that colour reduction can be improved. Certain substances have been reported to enhance hydrogen peroxide production in different photosynthetic systems (Mehler 1951; Galvan and De la Rosa 1997). In the present study,

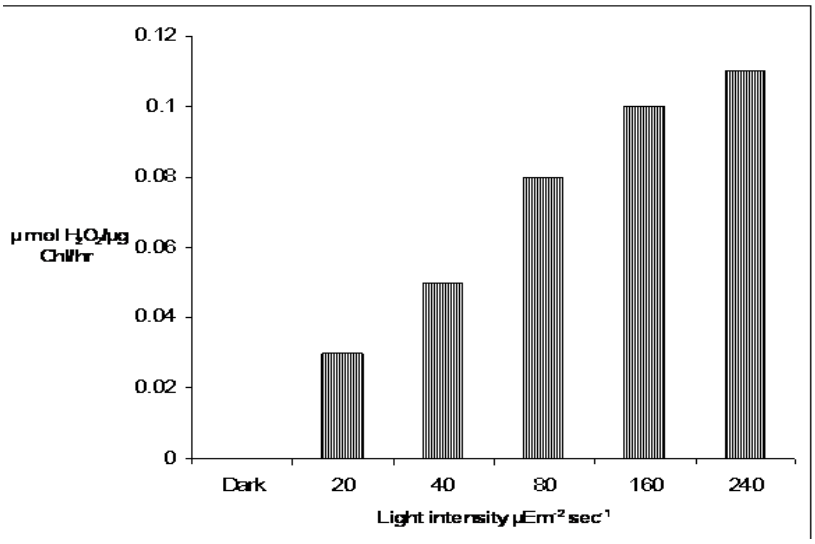

Fig. 2. Effect of Light on H_2O_2 Production of *O.boryana* BDU 92181

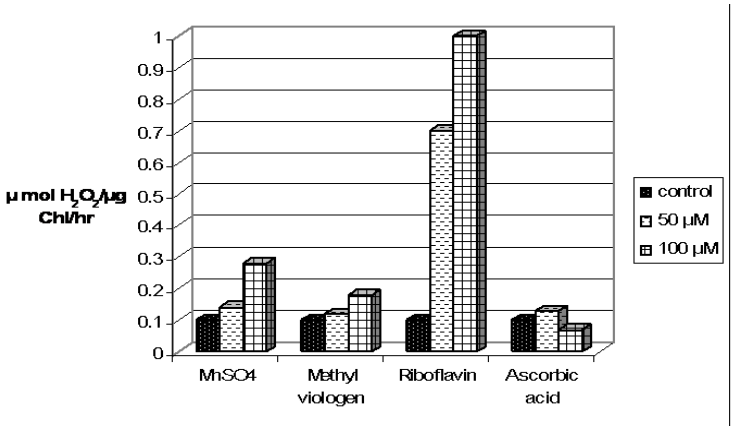

Fig. 3. Effect of enhancers on H_2O_2 Production of *O.boryana* BDU 92181

five substances have been tested for enhancing hydrogen peroxide production. The order of efficiency was riboflavin>manganous sulphate >methylviologen>ascorbic acid (Fig.3). Of the enhancers tested, riboflavin ranked first and at 100 mM, H_2O_2 production was almost 12 times more than that of control, whereas $MnSO_4$ which ranked second, at the same concentration produced three times higher H_2O_2 than control (Fig.3). This supports the finding of riboflavin catalysed H_2O_2 production serving as an electron acceptor at or near the PS I in isolated spinach thylakoids (De la Rosa *et al*. 1986). Use of riboflavin, for cyanobacterial hydrogen peroxide production is cost intensive and sustained production is always questionable or difficult. It is known that degradation/decolorization of coloured compounds is influenced by Mn dependent peroxidase (Paszczynski *et al*. 1985; Dehorter and Blondeau 1993; Kim and shoda 1999). Mn^{2+} acts as a substrate, redox coupler and mediator of manganese peroxidase, and thus the addition of manganese has influenced enzyme activity and in turn H_2O_2 production (Glenn *et al*.1983; Warishi *et al.* 1988). H_2O_2 produced in the present study showed a concentration dependent increase with exogenous addition of manganese revealing that H_2O_2 production is a Mn^{2+} dependent process in *O.boryana* BDU 92181 (Fig 4). The negative influence of ascorbic acid could be due to the fact

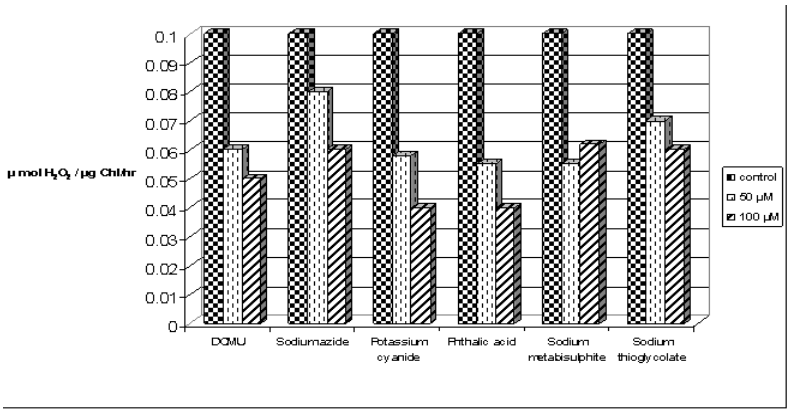

Fig. 5. Effect of inhibitors on H_2O_2 Production of *O.boryana* BDU 92181

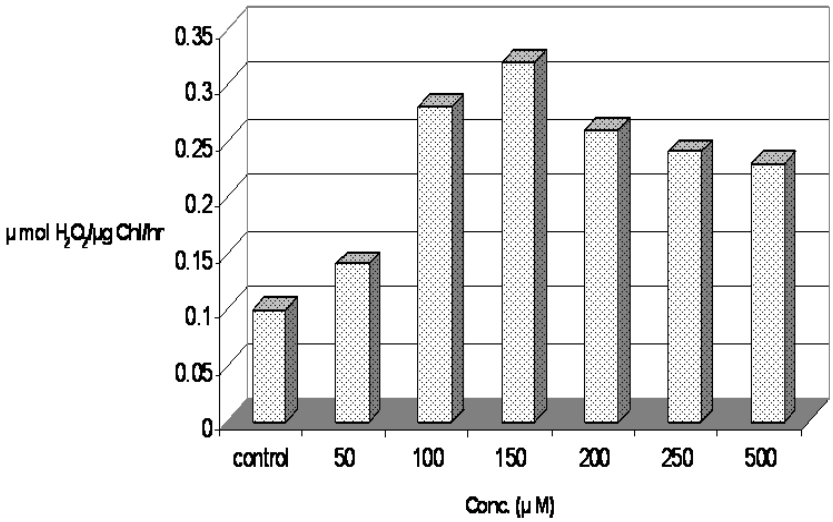

Fig. 4. Effect of $MnSO_4$ on H_2O_2 Production of *O.boryana* BDU 92181

that it could have acted as a chelator, scavenging the metal binding to dysfunction of peroxidase (De la Rosa *et al*. 1986). Methyl viologen is able to accept electrons at or near the terminal acceptor of PS I and reduced methyl viologen in turn reduces oxygen to superoxide radicals. If the electron acceptor is less, automatically H_2O_2 production will also be less. In the present study, methyl viologen supplied could have been low as the cyanobacterium has to face the competition with endogenous ferredoxin for electron from PSI also. Secondly living cells might impose permeability barrier by cell wall resulting in less penetration of methyl viologen.

Effect of inhibitors

Influence of known inhibitors of H_2O_2 were tested both to confirm the process of H_2O_2 production in the cyanobacterium and also to assess the extent of this influence since some of the effluents are likely to contain these or similar substances. In *O.boryana* BDU 92181, all the six different inhibitors tested for H_2O_2 production as expected had negative influence. There was no major difference among the inhibitors on their influence on H_2O_2 production, and none showed complete inhibition (Fig.5). This is the significant result of continuous replenishment of molecular oxygen by cyanobacteria during photosynthetic process. It is known that molecular oxygen is essential for the peroxidase enzyme reaction (Vyas and Molitoris 1996). Among the six inhibitors, potassium cyanide and phthalic acid, showed highest inhibition at 100 mM and the production was 50% less than control (Fig.5).

This could be because potassium cyanide is a strong inhibitor of peroxidase activity, while phthalic acid is a strong inhibitor of glucose oxidase activity as both the enzymes are involved in hydrogen peroxide production (Zhao and Janse 1996; Kelly and Reddy 1986). As sodium azide, sodium metabisulfite and sodium thioglycollate are inhibitors of oxidative enzyme reactions, and probably, this has negatively reflected on H_2O_2 production.

CONCLUSION

Oscillatoria boryana BDU 92181 a potent producer of hydrogen peroxide can be employed for large scale treatment of colored textile effluents containing azo and triphenyl dyes, distillery and paper mill effluents with manganese as electron source. *O.boryana* BDU 92181 though being a marine cyanobacterium has the ability to grow at zero salinity and an ideal organism, which needs only sunlight and carbon-dioxide for its optimal growth. This cyanobacterial biotechnological process involving hydrogen peroxide therefore would result in biodegradation of melanoidin present in distillery effluent.

ACKNOWLEDGMENTS

The authors thank the Department of Biotechnology, Govt. of India for fiscal support and M/s Trichy Distilleries for providing the effluent for the work. The first author acknowledged University Grants Commission, Govt. of India, for fellowship.

REFERENCES

1. Roncel, Mercedes, Jose A., Navarro Miguel, De La Rosa. Coupling of Solar energy to hydrogen peroxide production in the cyanobacterium *Anacystis nidulans. Applied and Environmental Microbiology.*, 1989; **55**:483-487.
2. Galvan, F.k., de la Rosa, F.F. Immobilized photosynthetic systems: Utilization of solar energy for production of chemicals and fuels. In: Handbook of photosynthesis, persarakli, M., Ed, University of Arizona, Arizona and Moreel Dekker, Basel, Hongkong.p.7 1997; 39-741.
3. De la Rosa, M.A., Rao, K.K., Hall, D.O. Hydrogen peroxide photoproduction by free and immobilized spinach thylakoids. *Photochem and Photophy* 1986; **11**: 173-187.
4. Navarro, J.A., Roncel, M., de la Rosa, F.F., de la Rosa M.A. Light-driven hydrogen peroxide production as a way to solar energy conservation. *Bioelectrochem Bioenergy* 1987; **18**: 71-78.
5. De la Rosa, F.F., Montes, O., Galvin, F. Solar energy conversion by green microalgae:A Photosystem for hydrogen peroxide production. *Biotechnology and bioengineering* 2001; **74**: 539-543.
6. Mehler, A.H. Studies on reactions of illuminated chloroplasts. I. Mechanism of the reduction of oxygen and other Hill reagents. *Arch Biochem Biophys* 1951; **33**: 65-67.
7. Stevens, S.E., Jr Patterson, C.O., Jack Myers. The production of hydrogen peroxide by blue green algae: A survey. *Journal Phycology* 1973;

9: 427-430.

8. Vyas, B.R.M., Molitoris, H.P. Involvement of an extracellular H_2O_2 dependent lignolytic activity of the white rot fungus Pleurotus ostreatus in the decolorization of Ramazol Brilliant Blue R. *Appl. and Environ.Microbiol.p* 1996; 3919-3927.
9. Subramanian, G., Uma, L., Potential applications of cyanobacteria in Environmental Biotechnology. In: Kojima, Hiroyuki, Lee, Yuan Kun edn. Photosynthetic microorganisms in Environmental Biotechnology pp 2001; 41-49.
10. Kalavathi, D.F., Uma, L., Subramanian, G. Degradation and metabolization of the pigment-melanoidin in distillery effluent by the marine cyanobacterium *Oscillatoria boryana* BDU 92181. *Enzyme microbial Technology* 2001; **29**: 246-25112.
11. Uma, L., Ramesh, M., Subramanian, G. Dye decolourization by the marine cyanobacterium *Oscillatoria boryana* BDU 92181. International Conference on Microbial Biotechnology Trade and Public Policy. 15-17thJuly. Osmania University, Hyderabad, India. 2000 p.122.
12. Malliga, P., Uma, L., Subramanian, G. Lignolytic activity of the cyanobacterium *Anabaena azollae* ML2 and the value of coir waste as a carrier for BGA biofertilizer. *Microbios*., 1996; **86**:175-183.
13. Hayase, F., Kim, S.B., Kato, H. Decolorization and degradation products of the melanoidins by hydrogen peroxide Agric. *Biol. Chem.,* 1984; **48**: 2711-2717
14. Patil, H.B., Kapadnis, P. Decolorization of melanoidin pigment from distillery spentwash. *Ind. J. Environ.Hlth.,* 1995; **37**: 84-87.
15. Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., Stanier, R.Y.. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol.,* 1979; **111**: 1-61.
16. Mac Kinney, G.. Absorption of light by chlorophyll solutions. *J Bio Chem.,* 1941; **140**: 314-322.
17. Green, M.J., Hill, H.A.O. Chemistry of dioxygen. In: Packer, L., (ed.) Oxygen radicals in biological systems. Methods in Enzymol., 1984; **105**: Academic Press. pp.3-22.
18. Kelly, R.L, Reddy, C.A. Glucose oxidase of *Phanerochaete chrysosporium*. In: *Methods in Enzymol*., 1988; **161**: 307-316
19. Patterson, C.O.P., Myers, J. Photosynthetic production of hydrogen peroxide by *Anacystis nidulans*. *Plant. Physiol*., 1973; **51**:104-109.
20. Park, I.H., Rao, K.K., Hall, D.O. Photoproduction of Hydrogen, hydrogn peroxide and Ammonia using immobilized cyanobacteria. *Int.J.Hydrogen Energy.,* 1991; **16:** 313-318
21. Paszczynski, A., Huynh, V.B., Crawford, R. Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochate chrysosporium*. *FEMS Microbiol. Lett* 1985; **29**: 37-41.
22. Kim, S.J, Shoda, M. Decolorization of molasses and a dye by a newly isolated strain of the fungus *Geotrichum candidium* Dec 1 *Biotechnology and Bioengineering*., 1999; **623**(2): 114-119.
23. Glenn, J.K., Gold, M.H., Decolorization of several polymeric dyes by the lignin degrading Basidiomycete *Phanerochaete chrysosporium*. *Appl and Environ. Microbiol* 1983; **45**(6):1741-1747.
24. Wariishi, H., Akileswaran, L., Gold, M.H. Manganese peroxidase from the basiodiomycete *Phanerochaetechrysosporium*: Spectral characterization of the oxidized states and catalytic cycle. Biochemistry., 1988; **27**:5365-5370.
25. Zhao, J., Janse, B.J.H. Comparison of H_2O_2-producing enzymes in selected white rot fungi. *FEMS Microbiol.Lett*., 1996; **139**: 215-221.