Mineralization of Melanoidin by H₂O₂ Producing Enzymes from Marine Cyanobacteria *Oscillatoria boryana* BDU 92181

D. Francisca Kalavathi

ABSTRACT

Intracellular enzymes of *Oscillatoria boryana* BDU 92181 exhibited mineralizing activity on melanoidin, a recalcitrant pigment present in the distillery wastewater. Melanoidin decolorization was postulated to be due to the production of hydrogen peroxide and molecular oxygen released by the cyanobacterium during photosynthesis. The present study was aimed to find out the efficacy of the marine cyanobacterium *O. boryana* BDU 92181 in producing H₂O₂ and enzymes involved in hydrogen peroxide production with a view to utilize its potential for decolorization of melanoidin pigment in the distillery effluent. The enzymes involved in the melanoidin degradation have not so far been attempted with cyanobacteria.

The results obtained in the present work suggested the activity of the glucose oxidase and Manganese peroxidase enzymes in a marine cyanobacterium *Oscillatoria boryana* BDU 92181 and whose activity was found to be enhanced in the presence of melanoidin.

Keywords: Mineralization, decolorization, melanoidin, glucose oxidase, manganese peroxidase.

Published Online: May 28, 2021

ISSN: 2684-5199

DOI: 10.24018/ejbio.2021.2.3.198

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I. INTRODUCTION

Molasses spent wash (MSW) is a strongly acidic, dark brown coloured effluent with strong objectionable odour has been recognized as environmental hazards for many decades. The coloured nature of MSW is due to the presence of natural polymers called melanoidins, formed by the Maillard amino-carbonyl reaction [1]. Recalcitrant nature of this pigment is due to the fact that it escapes from the various stages of wastewater treatment processes and finally enters into the environment. Since the physio-chemical methods are not efficient for decolorization, various alternative biotechnological treatment has been found to be effective and also ecologically friendly as well as economical. Microorganisms such as *Aspergillus fumigates*, *Coriolus* and *Phanerochaete chrysosporium* have been shown to degrade melanoidin [2]-[8]. The heterotrophic nature of these microorganisms renders them unsuitable for aquatic habitats.

Cyanobacteria, oxygen-evolving photoautotrophs would be ideal for the treatment of MSW as they apart from degrading the polymer, also oxygenate waterbodies [9]. Degradation/decolorization of recalcitrant MSW pigment melanoidin [10] by marine cyanobacteria has been documented. One of the reasons attributed for their degradation is the suspected production of hydrogen peroxide, hydroxyl anions and molecular oxygen, released by the cyanobacterium during photosynthesis. Some of the

enzymes reported to be involved in the production of H₂O₂ and the degradation of melanoidin are glucose oxidase, manganese dependent and independent peroxidases [2], [11].

The present paper was aimed to investigate mineralisation of melanoidin shown by H₂O₂ producing enzymes such as glucose oxidase and peroxidase from the marine cyanobacterium *Oscillatoria boryana* BDU 92181.

II. MATERIALS AND METHODS

A. Effluent Source

Anaerobically digested MSW with pH 8.3 was obtained from M/S Trichy Distilleries, Tiruchirappalli, Tamil Nadu, India.

B. Extraction of Melanoidin Pigment

Melanoidin pigment from the effluent was extracted with isopropanol. The resulting pigment was air dried and used for experiments.

C. Organism

Marine cyanobacteria *Oscillatoria boryana* BDU 92181 was obtained from the germplasm of the National Facility for Marine Cyanobacteria, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. The culture was maintained in marine synthetic liquid (ASN III) combined nitrogen medium (pH 7.5) and incubated at 25±2 °C under

fluorescent light at an intensity of 13.7 W m^{-2} .

D. Experimental Design for Hydrogen Peroxide Production and Enzyme Assays

For the estimation of hydrogen peroxide production and all the enzyme assays, organisms grown in combined nitrogen served as control and organism grown in 5% effluent and 0.1% melanoidin pigment were used. To study the enzyme assays, cultures were inoculated in the respective media 24 hours before the assay period. All enzyme reactions were carried out at 4°C . Cyanobacterial pellet was homogenized with respective assay buffer and centrifuged at $15000 \times g$ for 15 min at 5°C . The supernatant was used as the intracellular enzyme source.

Peroxide estimation. Hydrogen peroxide produced was estimated by the chromogen formed by 4-aminoantipyrine and phenol [12]. The change in absorbance was measured at 505 nm and expressed as mmol of hydrogen peroxide $\text{Chl}^{-1} \text{mg}^{-1} \text{h}^{-1}$. The values represented are the average of triplicates.

E. Enzyme Assays

1. Assay of Glucose Oxidase

The glucose oxidase enzyme activity was measured by increase in absorbance at 460 nm for 3 to 5 min. Specific activity (U/mg protein) was measured by using a molar extinction coefficient of 8.3. One unit of activity represents the oxidation of $1 \mu\text{mol}$ O-dianisidine per min at 37°C [13]. Protein concentrations were estimated by the method of [14].

2. Assay of Manganese Dependent peroxidase (MnP)

Manganese dependent peroxidase activity was monitored by increase in absorbance at 238 nm during the first 5-30 s of reaction. [15].

3. Assay of Manganese Independent peroxidase (MIP)

Manganese Independent peroxidase activity was measured by increase in absorbance was noted for 2 min at 436 nm. [16]

F. Protein and Enzyme Electrophoresis

The protein samples were estimated and used for SDS-PAGE. Electrophoresis was carried out at $20 \pm 2^\circ\text{C}$ with 1.5 mm thick polyacrylamide gel in Tris glycine buffer (pH 8.3). Samples were electrophoresed at 60 V through the stacking gel (6%) and 120V through the separating gel (10%). SDS gels were fixed and stained with Coomassie brilliant blue R-250 for 3 h and destained.

G. Native gel-Staining for Peroxidase Isozymes

Activity of Peroxidase Isoenzyme was detected by incubating the native gel in 100 ml of 0.1M sodium acetate buffer (pH 4.6) containing 30 mg 3,3'-diaminobenzidine tetra hydrochloride. The reaction was initiated by adding 250 μl of 30% hydrogen peroxide and allowed till intense brown band appears [17].

III. RESULTS AND DISCUSSION

The application of microorganism for bioremediation has been developing in the last few decades. Increasing attention of researchers has been directed towards microbial decolorization through mineralization of melanoidin in

distillery effluent. [18], [19]. It is now realized that microbial treatment provides a safer, more efficient, and less expensive alternative to physio-chemical methods for decolorization as well as mineralization of melanoidin. Cyanobacteria as pollution mitigator was found only in a few reports [9], [20] and there is none on the mineralization of melanoidin. In my previous paper, we reported the ability of a marine cyanobacterium *O. boryana* BDU 92181 utilized the recalcitrant biopolymer melanoidin as nitrogen and carbon source leading to decolorization. [10]. The organism decolorized pure melanoidin pigment (0.1% W/V) by about 75% and crude pigment in the distillery effluent (5% V/V) by about 60% in 30 days). Although the enzymatic system related with the degradation of melanoidins is yet to be completely understood and it seems greatly connected with fungal ligninolytic mechanisms. Several studies regarding the degradation of melanoidins, humic acids and related compounds by microorganisms have also suggested the participation of different categories of enzymes.

It has been established that Hydrogen peroxide was responsible for decolorization of solutions containing various dyes such as melanoidin and ramazol brilliant blue. [21]-[23]. It was reported that, cyanobacteria and few of the algal systems produce hydrogen peroxide especially in the presence of light [24]-[27].

Hence it was suspected that melanoidin in the distillery effluent could be degraded by hydrogen peroxide produced by the cyanobacterium used in the present study. The current study examined *Oscillatoria boryana* BDU 92181 and found that hydrogen peroxide being produced both under control conditions as well as in the presence of melanoidin or effluent (Fig. 1). Therefore, production of hydrogen peroxide by *O. boryana* BDU 92181 could be a major cause of melanoidin degradation. The hydrogen peroxide production was significantly more in pigment solution compared to control, while it was half the quantity produced by the control in the presence of effluent. Since the pigment solution was prepared in N-ASN III medium, there was no combined nitrogen available for the organism unlike the control. Therefore, it could be expected that the organism has enhanced its hydrogen peroxide production in order to degrade melanoidin and obtain the nitrogen source. There was a considerable reduction in hydrogen peroxide production with effluent indicates the presence of substances inhibitory to this process in the effluent. A number of substances are known to inhibit hydrogen peroxide production and even in the presence of catalase can rapidly remove the hydrogen peroxide produced [26]-[28].

The pH of the medium influenced the decolorization and degradation of synthetic melanoidin in the alkaline range due to hydroxyl ions [21]. Further, the cyanobacteria during photosynthesis generated hydrogen peroxide, which reacts with hydroxyl anion to form mainly perhydroxyl anion [HOO^-] which help decolorization of pigment [29]. In this study the pH of the cyanobacterium had grown in melanoidin containing medium was between 9-9.5. Hydroxyl radical generation in cyanobacteria has been shown by superoxide dismutase resulting in shift in pH to alkalinity. Colour reduction can also be brought about by active oxygen released during the photolysis of water by the cyanobacterium. The observation that decreases of

melanoidin pigment was evident only in light and not in dark supports the possible involvement of active oxygen. [10].

Fig. 1. Hydrogen peroxide production by *O. boryana* BDU 92181.

Glucose oxidase is an important enzyme involved in hydrogen peroxide production and it was found that hydrogen peroxide as a secondary product during the oxidation process. There is a correlation with decolorization of Molasses spent wash with glucose oxidase activity. Oxidases which convert glucose to gluconic acid in *Coriolus*. [21]. Hydrogen peroxide produced as a result of glucose oxidase activity acts as a bleaching agent on MSW. [30]. But the activity of this enzyme has not so far been shown in cyanobacteria. The activity of glucose oxidase was higher in both effluent and pigment grown cultures compared to control. (Fig. 2). The activity of this enzyme in marine cyanobacterium was found to be enhanced in the presence of melanoidin.

Fig. 2. Glucose Oxidase activity of *O. boryana* BDU 92181.

The role of a number of peroxidases with special reference to H₂O₂ production has been discussed in literature especially in fungal systems [15], [11]. It has been reported that melanoidin decolourisation by *Coriolus hirsutus* pellet was mainly due to the production of extra cellular hydrogen peroxide and peroxidases [8], [21], [6]. In *O. boryana* BDU 92181, H₂O₂ production appears to be due to the activity of both oxidases and peroxidases. The culture fluid contained two major extra cellular peroxidases, one manganese

independent peroxidase (MIP) and other manganese dependent peroxidase (MnP) since both MIP and MnP showed melanoidin decolourizing activity in presence of H₂O₂.

The enzyme activity has direct co-relation with the melanoidin decolorization. In addition to high levels of glucose oxidase activity, the activity of both MnP and MIP is detectable in *O. boryana* BDU 92181 even in the control. The activity of Mn dependent peroxidase was twofold higher in effluent grown cultures compared to control and nearly two-fold higher in pigment grown cultures compared to effluent grown ones. (Fig. 3). It is therefore highly suggestive of the involvement of this enzyme in H₂O₂ production like glucose oxidase. The activity of MIP was fairly higher in melanoidin pigment grown and five-fold higher in effluent grown cultures than control cultures (Fig. 4). When the protein profiles of the control and treated organisms were compared, effluent and pigment grown cultures showed 3 distinct bands with molecular weights of 80, 47, 40 KDa and these bands were quite feeble in the control (Plate 1). It has been reported in the literature that the enzyme glucose oxidase has a molecular weight of 80 KDa ([20]. In the present study the activity of the enzyme was significantly higher in the effluent and pigment grown cultures compared to control. Therefore, it is likely that this distinct band seen in the effluent and pigment grown cultures at 80 KDa could be glucose oxidase.

Fig.3. Manganese dependent peroxidase activity of *O. boryana* BDU 92181.

Fig. 4. Manganese independent peroxidase activity of *O. boryana* BDU 92181.

The Manganese dependent peroxidase from fungal sources was reported to vary from 43-47 KDa in molecular weight [29]. The very distinct band in the pigment grown cultures at 47 KDa, the fairly distinct band in effluent grown and feeble band in control cultures at the same molecular weight could be peroxidase of the Mn dependent type. Manganese independent peroxidases are of a wide variety [31]. The Mn independent peroxidase enzymes were localized in native gel which further confirmed the enzyme activity (Plate 2). Thus, the participation of these H₂O₂ producing enzymes for melanoidin degradation by marine cyanobacterium *O. boryana* as a potential biotechnological tool for the treatment of distillery effluent.

Plate 1. SDS-PAGE Protein profile of *O. boryana* BDU 92181 stained by Coomassie brilliant blue R-250.

M – Marker, C – Control (*O. boryana*), E – *O. boryana* grown in Effluent, M – *O. boryana* grown in Melanoidin pigment.
80 k.D – Glucose oxidase, 47 k.D – Mn dependent peroxidase 40 k.D – Mn independent peroxidase.

Plate 2. Native Agarose Gel Electrophoresis showing peroxidase enzyme stained by 3,3-diaminobenzidine hydrochloride.

C – Control (*O. boryana*), E – *O. boryana* grown in Effluent, M – *O. boryana* grown in Melanoidin pigment.

IV. CONCLUSION

The study indicated that *Oscillatoria boryana* proved to be a promising strain and a potent producer of hydrogen peroxide, glucose oxidase and Manganese dependent and independent peroxidases. Hence it was confirmed that hydrogen peroxide producing enzymes from *Oscillatoria boryana* were involved in the decolorization of melanoidin pigment from the distillery effluent. Cyanobacterial decolorization can be utilized for an eco-friendly and cost-effective biotechnology platform for the treatment of distillery effluent. *O. boryana* could be used in bioreactors for treatment of wastewaters or scaling up for enzyme productions. Moreover, large scale cultivation of this marine cyanobacterium is also simple and economical.

ACKNOWLEDGEMENTS

The author acknowledged University Grants Commission, Govt. of India for fellowship and M/s Trichy Distilleries for providing the effluent for the work.

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