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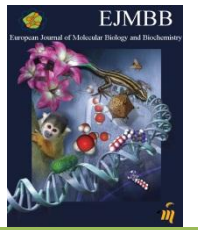

BIODEGRADATION OF *Prosopis juliflora* (SW.) D.C. WOOD BY THE MARINE CYANOBACTERIUM *Oscillatoria laetevirens*

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ABSTRACT

Biodegradation is recognized as a less expensive alternative to the physical and chemical degradation of organic pollutants. Cyanobacteria are firm candidates for such purposes and are one of the diverse groups of prokaryotes found in a wide variety of habitats. They are considered a potential resource in various biotechnological applications such as food, feed, fuel, fertilizer, medicine, industry and in combating pollution. In the present study, the lignin degrading ability of the marine cyanobacterium *Oscillatoria laetevirens* was investigated on wood of *Prosopis juliflora*. Results showed a significant increase in chlorophyll *a* when *O. laetevirens* was exposed to lignocellulosic waste. During this degradation process, the release of reducing sugar (63.6 µg/mL) and phenol (8.9 µg/mL) was found to be higher in *P. juliflora* degraded samples when compared to the control. Increased release of protein was followed by increased levels of nitrate and ammonia in *P. juliflora* treated cyanobacteria samples. Besides, this cyanobacterium was found to degrade 54.1% of lignin and eventually 46.7% of holocellulose from *P. juliflora* wood. Furthermore, the activity of degradative enzymes such as laccase, polyphenol oxidase and manganese independent peroxidase suggested a role of those enzymes in the degradation of *P. juliflora* by *O. laetevirens*.

INTRODUCTION

Microalgal technology has shown substantial promise due to the impact of these microbes on processes related to bioremediation [1]. Cyanobacteria provide an example of a unique class of Gram negative photosynthetic prokaryotes found in wetlands and aquatic ecosystems that provide suitable means for the remediation of various kinds of materials, as well as for the production of a variety of consumer products. Being photoautotrophs, they have advantages as potential biodegradation organisms. Cyanobacteria are capable of degrading various aromatic and phenolic compounds. Furthermore, they are capable of removing toxic metals from polluted waters.Since these microbes have simple growth requirements, they may provide interesting candidates for the production of valuable organic products [2].

Lignocellulose is a dominant renewable resource and constitutes a large proportion of agricultural waste. The tree *Prosopis juliflora*, native of the West Indies, Central America and Northern South America, is an invading species in India that competes with indigenous species. Although *Prosopis* has been recognized for its economical value, it is becoming an aggressive weed in several states of India. The invasion of *Prosopis* to grasslands, protected forests and nature reserves is alarming.

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This plant also affects the agricultural community by introducing it to irrigation channels and arable lands [3]. Lignin, although quite resistant to microbial attack, is ultimately degraded to humus, carbon dioxide and water. The lignin degrading ability of microorganisms is commonly studied by measuring the $^{14}CO_2$ production from ^{14}C labeled lignin preparations.

Applications of utilizing lignin-degrading organisms and their enzymes have attracted attention, since they may provide environment friendly technologies for the pulp and paper industry and for the treatment of many xenobiotic compounds, stains and dyes [4]. The complexity of the lignin structure provided most of the challenges for its degradation. Hence, the present investigation was carried out to study the degradation of lingo cellulosic waste *Prosopis juliflora* by the marine cyanobacterium *Oscillatoria laetevirens* and to estimate the renewable products released during the degradation process.

MATERIALS AND METHODS

Lignocellulosic Waste

Woody stems of *Prosopis juliflora* were collected from the Bharathidasan University campus, Tiruchirappalli, India. The finely chopped wood was dried under sunlight and ground into powder in a ball mill and passed through a 1-2 mm mesh sieve.

Organism and Culture Conditions

The marine cyanobacterium *Oscillatoria laetevirens* was obtained from the National Facility for Marine Cyanobacteria, Bharathidasan University, Thiruchirappalli, India. The culture was grown in ASN III medium under white fluorescent light of 13.8 E m^{-1} s^{-1} at 25 ±2 ºC at a 14/10 Dark/Light cycle.

Experimental Conditions

Exponentially grown cultures of *Oscillatoria laetevirens* were inoculated into ASN III medium along with the lignocellulosic waste *Prosopis juliflora* in a ratio of 1:3. The cultures were incubated at the above-mentioned conditions for 40 days. Medium containing *O. laetevirens* without the lignocellulosic waste *P. juliflora* and medium with *P. juliflora* devoid of *O. laetevirens* served as controls. Analytical experiments were performed at ten days interval for 40 days.

Biochemical Estimations

Chlorophyll *a* was estimated from centrifuged pellets to determine the cyanobacterial biomass by using the method of Mackinney [5]. In supernatants, sugars , phenols , proteins , nitrate and ammonia were analyzed. The supernatant was also subjected to spectral analysis at a wavelength ranging from 200-350nm (Jasco UV-550 spectrophotometer, Japan).

The dried pellet was assayed for its lignin content, following the modified klason lignin assay and the holocellulose estimation.

Colorimetric Enzyme Assay

The optimum temperature and pH for enzyme activities were elaborated for laccase, polyphenol oxidase and manganese independent peroxidase by following the methods of Caramelo et al. [6]. The rate of hydrogen peroxide produced during the lignolytic activity was quantified.

RESULTS AND DISCUSSION

The amount of chlorophyll *a* significantly increased during the degradation of *P. juliflora* by *O. laetevirens.* The chlorophyll content in the 30 day sample of *O. laetevirens* treated lignocellulosic waste was found to be higher (47.7 mg/g) when compared to control (*O. laetevirens* alone) (Fig. 1). Similar chlorophyll estimates were provided previously in some biodegradation processes. *Anabaena azollae* ML2 grown in the presence of coir pith showed an increase in growth rate in terms of chlorophyll *a* as reported by [7].

Evaluation of degradative efficiency of petroleum hydrocarbons by *Oscillatoria agardhi* and *Anabaena sphaerica* revealed an increase in biomass when compared to the control Parikh and Madamwar measured the growth of cyanobacteria during textile dye decolorization by measuring chlorophyll *a.* Increased chlorophyll *a* content in test samples showed that *P. juliflora* did not inhibit the growth of *O. laetevirens.* It is also possible that *O. laetevirens* may utilize the degraded lignocellulosic materials as a source of nutrients for its metabolic activities and growth.

The presence of reducing sugar in the supernatant was influenced by lignolytic activity of *O. laetevirens* and this may be due to the cleavage of complex polymers in the lignocellulosic waste to simple sugars. From the results obtained, it was observed that the concentration of sugar was considerably higher (63.6 µg/ml) in degraded *P. juliflora* culture filtrate. The control *O. laetevirens* and *P. juliflora* filtrate showed 36.6 µg/ml and 32.6 µg/ml of reducing sugar in 30 day samples (Fig. 2).

Our results are in accordance with earlier reports on biological delignification of *P. juliflora* by a fresh water cyanobacterium *Phormidium* sp. [8]. confirmed the influence of lignin and its degradation products on enzymatic hydrolysis of xylan by estimating the amount of reducing sugars in the media. Further, Ojumu et al. applied the presence of reducing sugar in the supernatant to confirm the presence of cellulose activity in *Aspergillus flavus* Linn isolate NSPR 101. Roy et al. used the Somogyi Nelson method (Nelson 1944) for measuring the reducing sugars released into the medium to confirm the xylanase activity in *Aeromonas caviae* W61 [9].

Similarly, Sigoillot et al., reported that the presence of reducing sugars in the spent media is due to xylanase in lignolytic and the hemicellulolytic system of *Pycnoporus cinnabarinus* . Kuhar and Yu et al. reported that biological delignification enhances the sugar production during enzymatic hydrolysis.The degradation of *P. juliflora* results in the release of some organic compounds such as phenol which was observed by a change of media from colorless to brown. The phenol content was considerably higher in *O. laetevirens* treated *P. juliflora* culture filtrate (8.9 µg/ml) in the 30 day sample when compared to control *O. laetevirens* (0.27 µg/ml) and *P. juliflora* (3.9 µg/ml) alone (Fig. 3).

This result was in accordance with the biodegradation of *P. juliflora* where phenol was quantified more in *P. juliflora* exposed to *Phormidium* sp. culture filtrate [10]. Similarly, biodegradation of lignocellulosic wastes coir pith, *P. juliflora* and *L. camara* by *Oscillatoria annae* showed a higher amount of phenol released into the media.

Table 1. Effect of pH on lignolytic enzyme activity of *O. laetevirens* treated *P. juliflora* on 30th day (*O. l - Oscillatoria laetevirens; O. l+ P. j- Oscillatoria laetevirens + Prosopis juliflora*).

Enzymes	Samples	Unit activity/ mg protein					
		pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
Laccase	*O. l*	0.028 ±0.004	0.0297 ±0.003	0.028 ±0.005	**0.032** ±0.002	0.028 ±0.002	0.025 ±0.003
	O. l + P. j	0.066 ±0.004	0.064 ±0.002	0.071 ±0.005	**0.096** ±0.007	0.049 ±0.003	0.037 ±0.004
Polyphenol oxidase	*O. l*	0.029 ±0.006	**0.034** ±0.005	0.028 ±0.003	0.022 ±0.002	0.019 ±0.004	0.014 ±0.003
	O. l + P. j	0.065 ±0.004	**0.14** ±0.016	0.048 ±0.007	0.039 ±0.006	0.025 ±0.003	0.015 ±0.005
Manganese Independent peroxidase	*O. l*	**0.134** ±0.016	0.078 ±0.004	0.066 ±0.002	0.039 ±0.005	0.033 ±0.003	0.028 ±0.006
	O. l + P. j	**0.186** ±0.023	0.175 ±0.018	0.076 ±0.006	0.053 ±0.004	0.047 ±0.003	0.043 ±0.004

Table 2. Effect of temperature on lignolytic enzyme activity of *O. laetevirens* treated *P. juliflora* on 30th day (*O. l - Oscillatoria laetevirens; O. l+ P. j- Oscillatoria laetevirens + Prosopis juliflora*).

Enzymes	samples	Unit Activity / mg protein	
		25º C	35º C
Laccase	*O. l*	0.028 ± 0.003	**0.032** ± 0.002
	O. l + P. j	0.057 ± 0.002	**0.063** ± 0.003
Polyphenol oxidase	*O. l*	0.038 ± 0.001	**0.045** ± 0.002
	O. l + P. j	0.062 ± 0.003	**0.093** ± 0.002
Manganese Independent Peroxidase	*O. l*	**0.156** ± 0.023	0.132 ± 0.012
	O. l + P. j	**0.293** ± 0.024	0.241 ± 0.018

Figure 1. Estimation of chlorophyll *a* in *O. laetevirens* treated *P. juliflora* in different days

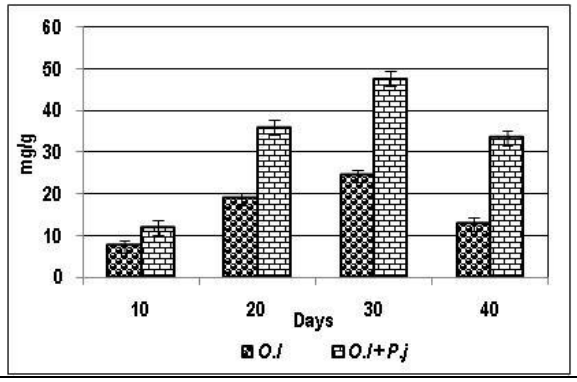


Figure 2. Estimation of sugar released during degradation of *P. juliflora* by *O. laetevirens* in different days

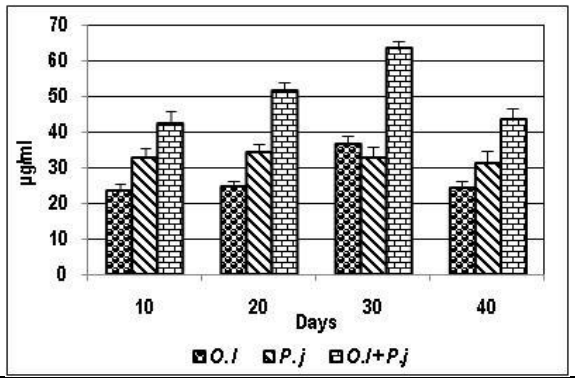


Figure 3. Estimation of phenol released during degradation of *P. juliflora* by *O. laetevirens* in different days

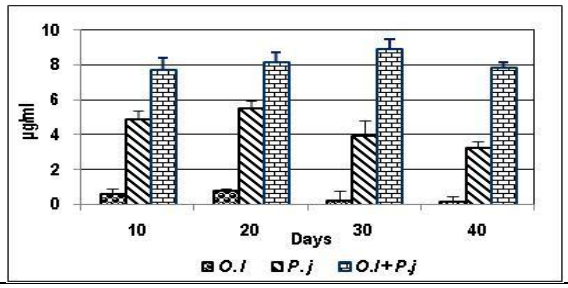


Figure 4. Spectral analysis during degradation of *P. juliflora* by *O. laetevirens* sp. after 30 days

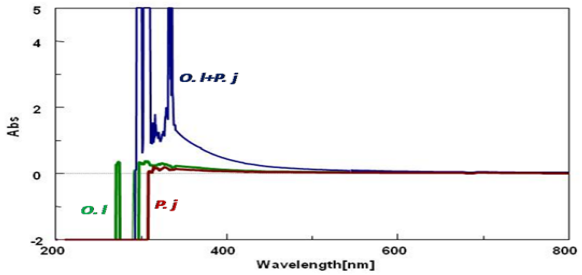


Figure 5. Estimation of protein released during degradation of *P. juliflora* by *O. laetevirens* in different days

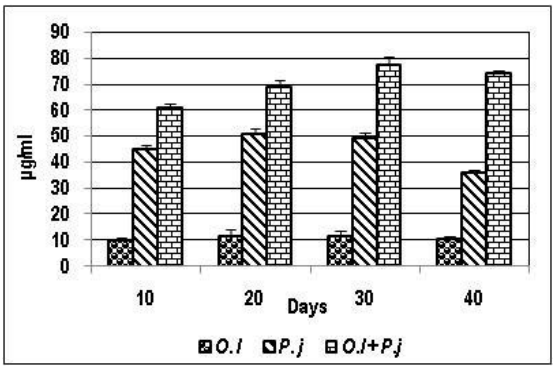
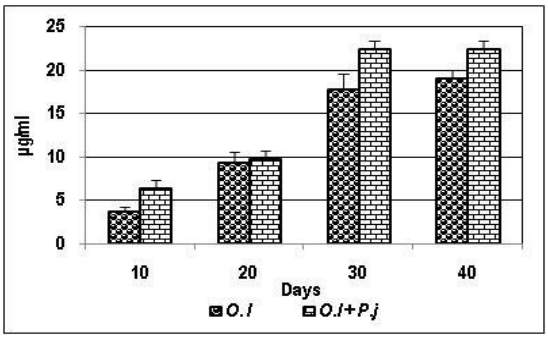


Figure 6. Estimation of nitrate released during degradation of *P. juliflora* by *O. laetevirens* in different days



(O. l - Oscillatoria laetevirens; P. j- Prosopis juliflora; O. l+ P. j- O.laetevirens + P. juliflora)

Figure 7. Estimation of ammonia released during degradation of *P. juliflora* by *O. laetevirens* in different days of *P. juliflora*

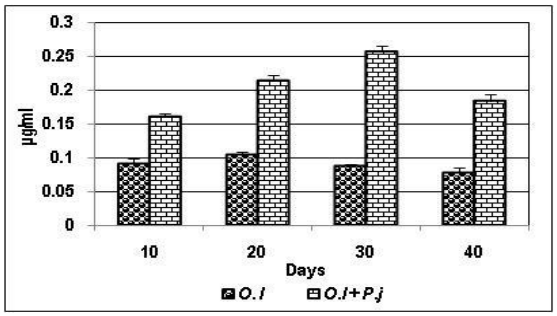


Figure 8. Estimation of lignin in degraded *P. juliflora* by *O. laetevirens* in *O. laetevirens* in different days

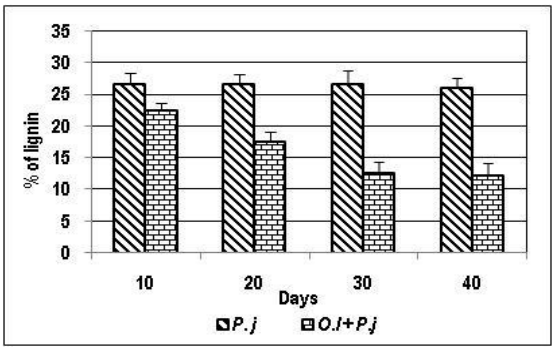


Figure 9. Estimation of holocellulose in degraded *P. juliflora* by *O. laetevirens* in different days

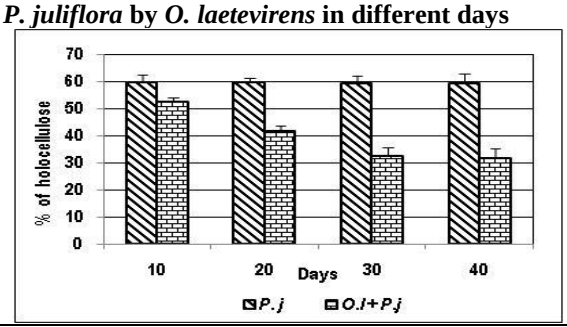
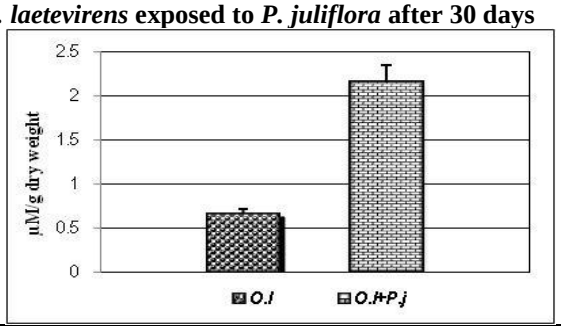


Figure 10. Estimation of hydrogen peroxide production by *O. laetevirens* exposed to *P. juliflora* after 30 days



(O. l - Oscillatoria laetevirens; P. j- Prosopis juliflora; O. l+ P. j- O.laetevirens + P. juliflora)

The presence of phenolic compounds in the culture filtrate was further confirmed by UV spectrum analysis at 270 nm showing high absorbance (Fig. 4) which was significantly higher than that of the controls. Camaerero et al. reported the phenolic and etherified units in natural and industrial lignin by mass spectrometry, where premethylation of isolated lignin was used as a tool to characterize phenolic and etherified moieties [11] reported the extracellular degradation of phenol and identification of muconic acid as the major product of phenol transformation by *Synechococcus* .

The concentration of protein was quantified as being higher (77.3 µg/ml) in *P. juliflora* treated *O. laetevirens* samples when compared to control *O. laetevirens* (11.6 µg/ml) and *P. juliflora* (49 µg/ml) alone (Fig. 5). The increase in protein content of test samples may be due to the release of lignolytic enzymes into the medium which is required for the degradation of lignin present in *P. juliflora*. The increase in protein can also be attributed to the *de novo* synthesis of phenol degrading enzymes and stress-related proteins.

Shashirekha et al. reported the growth of *Phormidium valderianum* 30501 during phenol degradation and observed that the test samples showed a higher protein content after exposure to phenol. Anandharaj showed degradation of coir pith, by *Oscillatoria annae* and estimated that coir pith treated cyanobacteria filtrate contain 63µg/ml of protein on day 30 [12]. Quantifying nitrate and ammonia is valuable for assessing the potential of the cyanobacteria to fix nitrogen. The nitrate present in the supernatant was found to be higher in *P. juliflora* treated *O. laetevirens* samples (22.3 µg/ml) when compared to control *O. laetevirens* in the 40 day sample (19.6 µg/ml)(Fig. 6).

Similarly, ammonia content was found to be higher in *P. juliflora* treated culture filtrate (0.25 µg/ml) when compared to the control, i.e. *O. laetevirens* alone (0.08 µg/ml) in the 30 day samples (Fig. 7). Stal and Krumbein reported the fixation of nitrogen under aerobic conditions by a non-heterocystous cyanobacterium *Oscillatoria* sp. [13]. According to their study, *Oscillatoria* sp. fixes nitrogen preferably in the dark when grown under alternating light-dark cycles. Similarly, providing light-dark condition in the lab and natural light-dark conditions in the field during the degradation of coir pith by *O. annae* might have resulted in nitrogen fixation and accumulation of nitrate and ammonia in the media .

The percentage of lignin and holocellulose content in *O. laetevirens* treated *P. juliflora* decreased during the process of degradation. The reduction of lignin content in control *P. juliflora* was negligible (2.2%), whereas 54.1% of lignin was degraded in *P. juliflora* exposed to *O. laetevirens* samples on day 30 and thereafter no significant improvement in lignin degradation was observed (Fig. 8). In another biodegradation study, the selected lignocellulose showed its highest lignin content in coir pith (37%), followed by *P. juliflora* (23%) and *Lantana camara* (22%). However, *Oscillatoria annae* treated lignocellulose showed a maximum reduction of its lignin content in *L. camara* (18.2%), followed by *P. juliflora* (17.4%) and coir pith (16.9%) after 30 days of incubation . A freshwater cyanobacterium *Phormidium* sp. was found to degrade 47% of lignin in *P. juliflora* and 22% lignin in coir pith within 30 days [14]. Gupta et al. reported that *Pycnoporus cinnabarinus* degrades about 7.69-10.08% of lignin in *P. juliflora* and 6.89-7.31% in *L. camara* and eventually enhance the holocellulose content by 2.90-3.97% and 4.25-4.61%, respectively. Holocellulose estimation revealed that *O. laetevirens* treated *P. juliflora* showed 46.7% of reduction while control *P. juliflora* showed feasible (0.8%) holocellulose reduction (Fig. 10). The obtained results correlate well with a previous study made by Viswajith who reported a maximum reduction of holocellulose in *Oscillatoria annae* treated *Lantana camara* (8.7%), followed by *P. juliflora* (8.3%) and coir pith (2.1%) which revealed that *O. annae* mediated lignin degradation is very similar to the first group of wood rot fungi [15].

The degradative ability of the cyanobacterium *O. laetevirens* was due to strong oxidative activity and a low substrate specificity of their ligninolytic enzymes which was confirmed by a colorimetric enzyme assay of laccase, polyphenol oxidase and manganese independent peroxidase. Optimization of temperature and pH of each lignolytic enzyme was performed where laccase showed maximum activity at 35°C (0.06 U mg^{-1} protein) and pH 7.0 (0.09 U mg^{-1} protein), whereas polyphenol oxidase showed optimal activity at 35°C (0.09 U mg^{-1} protein) and pH 5.0 (0.14 U mg^{-1} protein). However, manganese independent peroxidase showed optimum activity at 25°C (0.24 U mg^{-1} protein) and pH 4.0 (0.18 U mg^{-1} protein) in O. *laetevirens* treated *P. juliflora* (Table 1 and 2).

Pointing reported that Lignin peroxidase (LiP), manganese peroxidase (MnP), laccase and versatile peroxidase (VPs) are the major lignin modifying enzymes involved in lignin and xenobiotic degradation by wood rot fungi.Similar results have been reported in *Oscillatoria annae* treated coir pith, *P. juliflora* and *L. camara*. Anandharaj showed that polyphenol oxidase activity is highly induced in *Oscillatoria annae* exposed to coir pith when compared to control cyanobacteria alone, which is in accordance with our results [16]. An independent study revealed the presence of other manganese independent peroxidases, laccase, polyphenol oxidase, cellulolytic enzymes like endogluconase and xylanase in *O. annae*. In addition, Shashirekha et al. reported that a marine cyanobacterium *Phormidium valderianum* BDU 30501 was capable of degrading phenol through the activities of extracellular polyphenol oxidase and intracellular laccase . The absence of detectable activity of these enzymes in

control cultures shows the inducible nature of these enzymes in this cyanobacterium.

Hydrogen peroxide was also found at higher levels in *O. laetevirens* grown with *P. juliflora* (2.14 µM g^{-1}) when compared to control *O. laetevirens* (0.65 µM g^{-1}) (Fig. 9). Faison and Kirk studied the relationship between the production of reduced oxygen species, hydrogen peroxide (H_2O_2), superoxide (O_2) and hydroxyl radicals (OH^-) and the oxidation of synthetic lignin to CO_2 in the cultures of white-rot fungus *Phanerochaete chrysosporium* [17]. They also reported that the kinetics of the synthesis of H_2O_2 coincided with the appearance of the ligninolytic system.

It is reported that the H_2O_2 production was markedly enhanced by the growth of cultures at 100% O_2, mimicking the increase in ligninolytic activity under elevated oxygen tension. Hatakka reported that the extracellular enzymatic systems ligninolytic peroxidases, laccases and oxidases were responsible for the production of extracellular hydrogen peroxide (H_2O_2) [18].

These reports clearly indicate that the increased release of H_2O_2 from test samples was due to the lignolytic activity of *O. laetevirens*.

CONCLUSION

An abundance of organic waste materials worldwide demands the development of ecofriendly ways for its remediation or for its transfer into useful products. There is a wide range of microorganisms including bacteria, fungi and cyanobacteria, available for waste degradation, but reports on cyanobacterial degradation are scarce. Our results suggest that the marine cyanobacterium *Oscillatoria laetevirens* has the ability to degrade lignocellulosic waste of *Prosopis juliflora* fast and effectively without harmful effects on the environment. Further detailed studies on the degradation processes will be helpful in the exploration of value added products from degraded wood.

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CONFLICT OF INTEREST:
The authors declare that they have no conflict of interest.

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