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Research Article

PRODUCTION AND PURIFICATION OF LIGNIN PEROXIDASE FROM *BACILLUS MEGATERIUM* AND ITS APPLICATION IN BIOREMIDATION

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ABSTRACT
Lignin is the second most abundant renewable biopolymer in nature after cellulose, yet it is degraded by only a small number of microorganisms. Ligninolytic enzymes are involved in the degradation of the complex and recalcitrant polymer lignin. This group of enzymes are highly versatile in nature and they find application in a wide variety of industries. In the present study lignin peroxidase producing bacteria *Bacillus megaterium* has been isolated and identified based on their morphological and biochemical tests. Further, Optimization of physical parameters like pH (7), temperature (37^{o}C), inoculum size (6%), agitation speed (180rpm) and nutritional parameters like carbon (lignin-1.5%) and nitrogen (peptone-2%) was carried out. Lignin Peroxidase was purified by using ultra filtration, ammonium sulphate precipitation and dialysis. The purity was checked by SDS-PAGE and the molecular weight of the enzyme was 65kDa. *Bacillus megaterium* decolorized the dyes cango red, methylene blue and malachite green to 94.90%, 63.23% and 6.40% within 96hrs of incubation. In waste water treatment *Bacillus megaterium* effectively acted on paper & pulp, textile red and textile blue samples with decolouration percentage of 93.70%, 48.57% and 31.64% respectively within 96hrs of incubation.

Keywords: *Lignin, Lignin Peroxidace, Bacillus Megaterium, Bioremidation*

INTRODUCTION
Lignin is the most abundant renewable carbon source on earth (Bo Zhang *et al.*, 2008). Lignin Peroxidase (EC 1.11.1.14) belongs to the family of oxidoreductases. Lignin Peroxidase (LiP) is an extracellular hemeprotein, dependent of H_2O_2, with an unusually high redox potential and low optimum pH. Due to their high redox potentials and their enlarged substrate range LiP have great potential for application in various industrial processes. LiP in contrast with laccases does not require mediators to degrade high redox potential compounds but it needs hydrogen peroxidase to initiate the catalysis.
Lignolytic enzymes are involved in the degradation of the complex and recalcitrant polymer lignin. This group of enzymes are highly versatile in nature and they find application in a wide variety of industries. Lignolytic enzymes are promising to replace the conventional chemical processes of several industries. Thus, there is a broad field of investigation that is almost entirely open to new findings and it is quite reasonable to propose that many new applications will be found in near future.
In biosphere, a wide variety of species are involved in lignin degradation including fungi and bacteria. To date, only few groups of organisms are capable of degrading complex lignin polymers. Till now, all the basics and applied research work has centred mostly on fungi. In case of industrial applications, the use of fungi is not feasible due to the structural hindrance caused by fungal filaments, requirement of particular culture conditions such as humidity, aeration, temperature and pH which are not compatible with industrial processing environments, requirement of long lag period and thus degrade lignin slowly. Bacteria are worthy of being studied for their lignolytic potential due to immense environmental adaptability and biochemical versatility. There is wide range of examples where bacteria like *Pseudomonas aeruginosa, Serretia marcescens, Nocardia, Arthrobacter, Flavobacteria, Micrococcus* and *Xanthomonas* have been identified as lignocellulosic degrading microorganisams. The bacterial ligninolytic potential is still largely unexplored and many novel ligninnolytic enzymes may await discovery. The lignin peroxidase activity is associated with primary growth of bacteria and thus the deligninification process is presumed to be the result of primary metabolic activity and not dependent

upon other factors such as stress to induce production. Thus, degradation of lignin by bacterial enzymes may be superior to their fungal counterparts with regard to specificity, thermo stability and mediator dependency. They may also have special advantages for the depolymerisation of the modified lignin residues typically encountered in waste streams from the pulping or second generation bio fuel based chemical industry.

In view of the potential application of LiP and the alarming rise in the accumulation of recalcitrant, the present work was undertaken. The proposed work focussed on the isolation and screening of bacterial strains with a high ligninolytic activity, enhancement of LiP production dye degradation studies by the isolated strain and its application in bio bleaching of effluents.

MATERIALS AND METHODS

Alkali Lignin Preparation

The sources used for the extraction of lignin were derived bark grinded to powder, dried straw grass. In 100gm of lignin source 50 ml of 1% sulphuric acid was added and heated for 20min for acid pre-treatment and allowed to cool to obtain the lignocelluloses mass. Further 100ml of 4% sodium hydroxide was added and heated for 30min. The dark brown coloured alkali lignin was filtered and autoclaved at 15 lbs. for 10 min (Bholay *et al*., 2012).

Screening of lignin peroxidase producing microorganisms

Collection of sample

The local soil sample were collected in screw cap bottles and used out for the isolation of microorganisms. 0.1 ml serially diluted sample was platted over nutrient agar for growing bacteria. The colonies were preserved on agar slants at temperature 4^{o}C.

Screening of bacteria in lignin supplemented broth

The bacterial colonies were inoculated into lignin broth having composition g/l lignin-15, peptone-20 with initial pH 7.0 at temperature 37^{o}C incubated for 48 hrs on a rotary shaker at 120rpm

Lignin peroxidase activity

Lignin peroxidase activity was determined spectrophotometrically at 310 nm through the oxidation of veratry alcohol to veratryl aldehyde (molar absorptivity ϵ_{310}=9300M^{-1} cm^{-1}).

The fermented culture solution was centrifuged at 10000 rpm for 10 minutes at 4^{0} C and supernatant was collected for estimation of lignin peroxidase activity. 0.5 ml supernatant was mixed with 1.0 ml of 0.3 M sodium citrate buffer (pH 0.7) and 0.5 ml of veratryl alcohol (8 Mm). The mixture was incubated for 5 minutes at 30^{0} C and the reaction was initiated by addition of 0.5 ml hydrogen peroxide. The obsorbance was immediately measured in 1 minute interval after addition of hydrogen peroxide. One unit (U) of LiP activity was defined as activity of an enzyme that catalyzes the conversion of µ mole of veratryl alcohol per minute (Atalla *et al.*, 2010).

Identification of Bacterial Strain

Colony morphology, microscopic examination and Physiological tests

The strain was examined for colony morphology, Gram's staining, motility test, sporulation etc., (Aneja, 2001). Various physiological tests like catalase test, Glucose fermentation test, Voges- Proskauer test, hydrolysis of casein, hydrolysis of gelatine etc., were performed as per standard methods (Aneja, 2001).

Optimization of physical parameters

Optimization of pH, temperature, inoculum size and agitation speed

In first set of experiment, the seed cultures were inoculated in 250ml Erlenmeyer flask containing 50ml of growth medium and was incubated at different pH ranging 6.0 to 9.0 with an interval of 1.0 at 37^{o}C at 120 rpm for 48hrs.

In another set of experiments, the cultures were inoculated at pH 7.0 of the medium and were incubated at different temperature ranging from 27^{o}C to 42^{o}C with an interval of 5^{o}C at 120 rpm for 48hrs.

In the next set of experiment, the inoculum size was varied from 2% to 8% with an interval of 2% with initial PH 7.0. The flasks were incubated at temperature 37^{o}C at 120rpm for 48hrs.

The fermentation was carried out at varying agitation speed. The medium with an initial pH 7.0 at temperature 37^{o}C inoculated with 6% culture incubated at different shaking speed ranging from 120-200 rpm with an interval of 20 rpm for 48hrs.

Optimization of Nutritional parameters

Optimization of carbon and nitrogen sources

The carbon source of the medium i.e Lignin was varied from 0.5% (w/v) to 2.5 % (w/v) with increasing concentration of 0.5 % (w/v). The initial pH 7.0 of the medium was inoculated with 6% (w/v) of cultures and was incubated at 37^{o}C at120rpm for 48hrs.

The nitrogen source of the medium, peptone was varied from 0.5% (w/v) to 2.5 % (w/v) with increasing concentration of 0.5 % (w/v). The initial pH 7.0 of the medium was inoculated with 6% (w/v) of cultures and was incubated at 37^{o}C at120rpm for 48hrs.

Purification of lignin peroxidase enzyme

The purification was carried out using crude enzyme extract. The enzyme was purified by the following steps at 0-4^{o}C, unless otherwise mentioned.

Ultrafiltration

Ultrafiltration was carried out by using membrane with molecular weight cut-off of 10kDa. The concentrated retentate was used for ammonium sulphate precipitation.

Ammonium sulphate precipitation and dialysis

Finely powdered ammonium sulphate was added to the crude extract. The lignin peroxidase activity was associated with the fraction precipitated at 40-60% saturation. The precipitate was collected by centrifugation at 10,000g for 10 min, dissolved in 0.3M Sodium Phosphate buffer pH 7.0 and dialyzed against the same buffer.

SDS- PAGE

SDS-PAGE was performed according to the method of Laemmli, with a separating acrylamide gel of 10% and stacking gel 5% containing 0.1% SDS. The gel was stained with coomassie brilliant blue R250 and destained with a solution of methanol, acetic acid and water in the ratio of 4:1:5. The following standard proteins were used for molecular weight determination 97.4, 66,43,29, 20.1 and 14.3 kDa.

Dye decolourization studies

Decolourization of lignin- mimicking dyes was assessed in liquid phase assays. The following dyes were selected: Malachite Green (MG), Cango Red (CR) and Methylene Blue (MB). For liquid assays, the individual strains were grown in Luria broth at pH 7.0. Dyes were added to 25mg/L and cultivation was continued for another 96h in 250ml at 37^{o}C with shaking at 180rpm. Cultures without inoculum were included as controls for spontaneous dye decolourization. Samples were drawn at various time intervals and centrifuged for 10min at 12,000 rpm. The decolourization of a specific dye was calculated as a percentage of the initial absorbance at λ_{max} (Luaine Bandounas *et al.*, 2011). Dye decolourization was studied at different ODs MG (615nm), CR (470nm), MB (665nm).

Decolourization rate was expressed as percentage decolourization and calculated using the formula

% Decolourization = A-B/A X 100

Where A is the initial absorbance

B is the observed absorbance

Application of Bacillus megaterium in waste water treatment

A total number of three different waste water samples were collected from textile (TR & TB), paper and pulp (PP) mill and used for the application studies. 24 hrs old culture broth grown on nutrient broth was inoculated at 6% inoculum size in a tube containing 25ml of each sample separately. Simultaneous controls were maintained for each sample. The inoculated tubes were incubated at 37oC for 96hrs and observed for decolourization at every 24hrs interval. Decolourization rate was expressed at percentage decolourization and calculated using the formula as mentioned above.

RESULTS AND DISCUSSION

Screening of Bacteria for lignin peroxidase production

A total of 10 bacterial strains were obtained by serial dilution methods. All strains were screened for lignin peroxidase activity in lignin supplemented broth. Among ten isolates, the isolate KRVCA-4 showed high lignin peroxidase activity of 4.0 IU

Table 1: Screening Of Bacterial Strains For Lignin Peroxidase Production

Sr. No	Bacteria	Lignin peroxidase activity (IU)
1.	KRVCA- 1	2.64
2.	KRVCA-2	2.98
3.	KRVCA-3	3.56
4.	KRVCA-4	4.00
5.	KRVCA-5	3.49
6.	KRVCA-6	3.23
7.	KRVCA-7	3.02
8.	KRVCA-8	2.87
9.	KRVCA-9	2.85
10.	KRVCA-10	2.73

Identification of bacterial strain (KRVCA-4)

Based on morphological, physiological and biochemical characterization, the strain was identified as *Bacillus magaterium*

Optimization of physical parameters

Optimization of pH, temperature, inoculum size, Agitation speed

Optimization of pH for lignin peroxidase production revealed that pH of 7.0 was found best for enzyme production by bacterial strain. The enzyme production was increased from pH 5.0 to 7.0 and thereafter production was decreased. Therefore, pH 7.0 was found to be best for enzyme production and the lignin peroxidase activity was 4.2 IU (Figure 1).

Optimization of temperature was carried out for lignin peroxidase production at different temperature ranging from 27^{o}C to 42^{o}C with an interval of 5^{o}C. The results showed that the lignin peroxidase production increased with increase in temperature from 27^{o}C to 37^{o}C thereafter the lignin peroxidase production ware decreased. Hence temperature of 37^{o}C was found best for enzyme production showing activity of 5.5IU. The results are depicted in Figure 2.

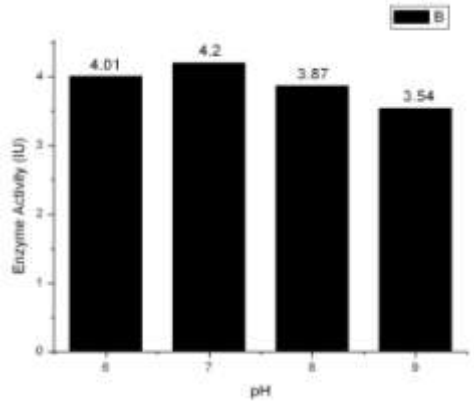

Figure 1: Optimization of pH

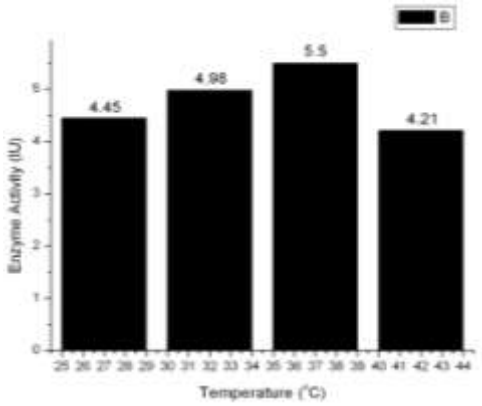

Figure 2: Optimization of Temperature

Optimization of inoculum size for the lignin peroxidase production was carried out at different inoculum size. The results showed that the lignin peroxidase production increased with increase in inoculum size from 2%(w/v) to 6%(w/v). The lignin peroxidase activity was 6.2IU at inoculum size 6% (Figure 3).

Optimization of agitation speed for the lignin peroxidase production was carried out at range of 120rpm to 200rpm. Lignin peroxidase activity was 7.0 IU at 180rpm. The results are depicted in Figure 4.

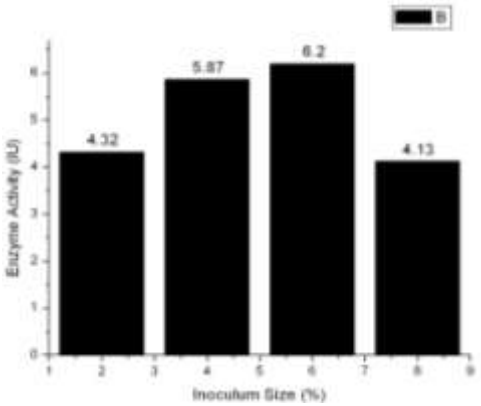

Figure 3: Optimization of Inoculum size

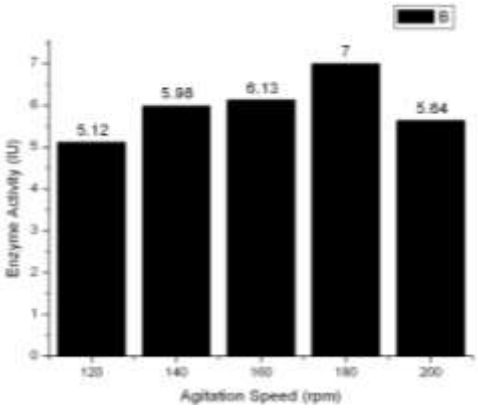

Figure 4: Optimization of Agitation speed

Optimization of nutritional parameters

Optimization of carbon and nitrogen source

Optimization of carbon source of the medium i.e. lignin is varied from 0.5% (w/v) to 2.5% (w/v). The result shows that the lignin peroxidase activity was increased with increase in lignin concentration from 0.5% (w/v) to 1.5(w/v), thereafter the enzyme activity was decreased. Therefore, 1.5% (w/v) of lignin concentration shows maximum enzyme activity of 8.6 IU (Figure 5).

Optimization of nitrogen source of the medium i.e peptone is varied at different concentration. The result showed that the enzyme production increased with increase in peptone concentration from 0.5% (w/v) to 2.0%(w/v), thereafter the enzyme production was decreased. Therefore, 2.0% (w/v) of peptone concentration shows maximum enzyme activity of 9.0 IU (Figure 6).

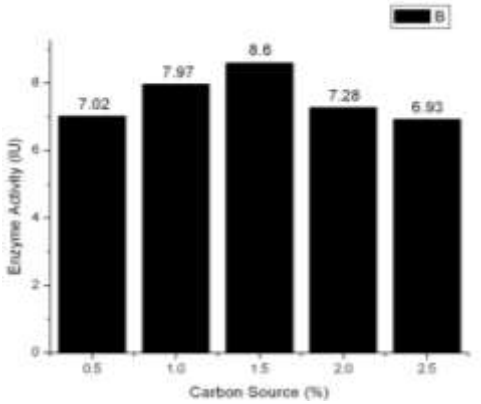

Figure 5: Optimization of Carbon Source

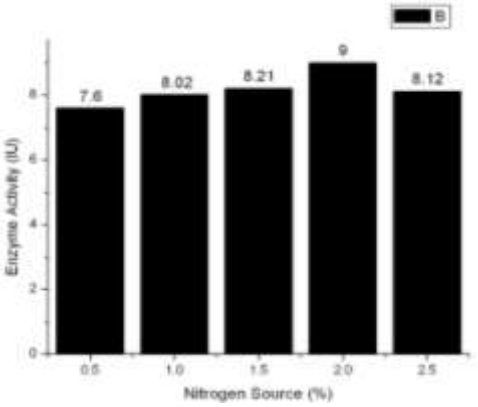

Figure 6: Optimization of Nitrogen Source

Purification of lignin peroxidase enzyme

The enzyme was purified by using ultra filtration, Ammonium sulphate precipitation and dialyses. The enzyme activity was associated with 60% fraction. The purity was checked by SDS-PAGE and has been found that the molecular weight of the enzyme 65kDa.

Dye decolourization studies

Bacillus Megaterium decolourize the dyes Congo red, Methylene blue and Malachite green to 18.13%, 10.87%, and 2.45% of their initial value within 24hrs in the liquid assays. The results are mentioned in table 2. Congo red proves effective dye compared to all (Figure 7).

Table 2: Dye Decolourization Studies

Sl.No	Sample	Percentage of decolourisation (%)			
		24hrs	48hrs	72 hrs	9rhrs
1	Congo Red	18.13	75.81	81.43	94.90
2	Methylene Blue	10.87	25.57	48.43	63.23
3	Malachite Green	2.45	5.83	5.97	6.40

Figure 7: Congo red decolourization

Application of Bacillus Megaterium in waste water treatment

Bacillus Megaterium acted on paper & pulp, textile red and textile blue water samples. The results are mentioned in table 3. *Bacillus Megaterium* could effectively act on paper & pulp mill sample compared to other samples (Figure 8).

Table 3: Application of Bacillus Megaterium in waste water treatment

Sl.No	Sample	Percentage of decolourisation (%)			
		24hrs	48hrs	72 hrs	9rhrs
1	Paper and Pulp	86.12	87.69	93.39	93.70
2	Textile Red	9.86	13.46	21.95	48.57
3	Textile Blue	21.45	23.52	26.75	31.64

(a) (b) (c)

Figure 8: Waste water treatment (a) Textile Red effluent (b) Paper and Pulp mill effluent (c) Textile blue effluent

DISCUSSION

The optimum pH for the production of lignin peroxidase is 7.0 with an activity of 4.2IU. The pH is important parameter which determines the growth of the organisms and lignin peroxidase production.

Bansal *et al.,* (2012) reported pH 7.5 was optimum for their work. The optimum temperature recorded was 37^{o}C. Our results match with findings of Renugadevi *et al.,* (2011) and Bansal *et al.,* (2012). The optimum inoculum size and agitation speed was 6% and 180rpm for maximum production of lignin peroxidase. According to Renugadevi *et al.,* (2011), the carbohydrates are sole energy source for most of the heterotrophic organisms. They show great influence on the production of enzyme. Lignin at 1.5% proved effective for maximum production of lignin peroxidase. Nitrogen source plays an important role in the biosynthesis o lignin by microorganisms. Peptone at 2% produces maximum lignin peroxidase.The molecular weight of enzyme has been found 48kDa. In the present studies, *Bacillus megaterium* decolourizes the dyes Cango red, Methylene blue and Malachite green. Congo red decolourization was observed to be highest. Bandounas *et al.,* (2011) also reported similar results with different dyes with *Bacillus sp*. LD003. *Bacillus megaterium* acted on paper & pulp, Textile red and blue effluents effectively proved efficient in decolourizing paper & pulp effluent. Bandounas *et al.,* (2011) reported lignin degrading activity by *Pseudomonas sp, Bacillus sp* etc.

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