ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Production of LiP by *Phanerochaete chrysosporium* MTCC 787 Through Solid State Fermentation of Wheat Straw and Assessing its Activity Against Reactive Black B

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Manuscript Info

Manuscript History:

Received: 15 November 2015
Final Accepted: 16 December 2015
Published Online: January 2016

Key words:
P. chrysosporium,
Ligninolytic,
Wheat straw,
LiP,
Reactive Black B

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Abstract

Lignin Peroxidase (LiP) is one of the key enzymes produced by several ligninolytic fungi including *P. chrysosporium* for degradation of lignin and several polyaromatic hydrocarbons. Textile azo dyes are also polyaromatic in nature and ligninolytic enzymes have been used for treatment of dye containing wastewaters. Solid state fermentation of wheat straw was attempted with *P. chrysosporium* MTCC 787 for production of LiP at 39ºC. Fermented residue was extracted with 0.2 M sodium tartarate buffer (pH 3) and was subjected to ammonium sulfate precipitation. Precipitates obtained were subjected to LiP assay, upon dialysis, using veratryl alcohol as substrate. Optimum pH, temperature and H_2O_2 were reported to be pH 3.5, 30ºC and 0.4 mM, respectively. Km and Vmax of LiP for veratryl alcohol were found to be 0.28 mM and 33.3 U/mg, respectively. Enzyme kinetics were also assessed against Reactive Black B (RBB), a widely used textile diazo dye, as substrate. Km and Vmax for this dye were observed to be 0.2 mM and 7 U/mg, respectively. These findings suggest higher affinity of LiP towards tested dye and hence the enzyme has a great potential in its application for treatment of RBB containing wastewater.

1. Introduction

Lignin is the second most abundant heteropolymer, next to cellulose (Grabber, 2005), present in woody plant tissues which confers rigidity to plants and resistance to attack by pathogens (Higuchi, 1990).White rot fungi (WRF) have ability to degrade lignin (Kirk and Farrell, 1987) and several other natural as well as synthetic polyaromatic hydrocarbons (Field et al., 1993; Hammel, 1995). These fungi degrade lignin by secreting enzymes collectively termed "ligninases", these can be classified as either phenol oxidases, such as laccase, or heme peroxidases, such as lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (Martinez et al., 2005). Lignin peroxidases and versatile peroxidases have been reported as key enzymes in the degradation of lignin (Tien and Kirk, 1983) whereas manganese peroxidases can oxidize small phenolic structures (Gold et al., 2000). These enzymes have been reported to oxidize several pollutants of polyaromatic nature (Vyas et al., 1994; Clemente et al., 2001; Silva et al., 2009). Due to this, several strains of white rot fungi have been used in bioremediation of PAH (Haritash and Kaushik, 2009).

Azo dyes are one of the several pollutants, extensively used in textile, paper, food, cosmetics, leather and pharmaceutical industries (Sartale et al., 2011), which are also polyaromatic in nature. They make up approximately 70% of all dyestuffs used worldwide by weight (Zollinger, 1987, Meng et al., 2012), making them the largest group of synthetic colorants and the most common synthetic dyes released into the environment (Chang et al., 2001; Zhao and Hardin, 2007; Saratale et al., 2009). For sulfonated azo dyes, both aromatic sulfonic and azo groups confer to

their xenobiotic nature as these are rare among natural products (Junnarkar et al., 2006). Approximately 10-15% of the dyes are released into the environment during manufacturing and usage. Since some of the dyes are harmful, dye-containing wastes pose an important environmental problem (Verma & Madamwar, 2003).

Ligninolytic enzyme systems such as LiP and MnP of *P. chrysosporium* have been reported to decolorize several synthetic dyes (Lankinen et al., 1991; Young and Yu, 1997; Fu and Viraraghavan, 2001). These enzymes from other fungi such as *Trametes versicolor*, *Bjerkandera adusta*, *Aspergillus ochraceus*, species of *Pleurotus* and *Phlebia*, and a variety of other isolates, also have been exploited for their ability to decolorize dyes (Saratale et al., 2011). Several approaches have been used by researchers for the production of ligninolytic enzymes ranging from submerged fermentation to solid state fermentation processes. Solid state fermentation offers several advantages over the submerged one such as superior productivity, simpler techniques, reduced energy requirements, low wastewater output and improved product recovery (Moldes et al., 2003). Several lignocellulosic substrates have been utilized in production of ligninolytic enzymes such as rice straw (Zeng et al., 2015), wheat straw (Fujian et al., 2001), corn cobs, coconut coir (Gupte et al., 2007) etc.

In this paper, we report our findings on production of LiP by *Phanerochaete chrysosporium* MTCC 787 through solid state fermentation of wheat straw, fortified with Kirk's medium. Characterization of partially purified LiP was attempted, its activity against Reactive Black B, a widely used textile diazo dye, was assessed and its affinity towards the dye was compared with its affinity towards veratryl alcohol, a routinely used substrate of LiP.

2. Materials and Methods:-

2.1 Chemicals:-

Textile diazo dye Reactive Black B (C. I. Reactive Black 5, λmax = 597 nm) was procured from Meghmani Chemicals Ltd., Vatva GIDC, Gujarat, India. Veratryl alcohol was purchased from Himedia Laboratories, India. All the other chemicals and reagents used were of analytical grade.

2.2 Organism:-

Phaenerochaete chrysosporium MTCC 787 was procured from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India. It was routinely subcultured on Malt Extract Agar (MEA, Himedia Lab., India) medium and preserved at 4ºC on MEA slants till further use.

2.3 Solid State Fermentation:-

P. chrysosporium was cultivated on MEA plates at 39ºC and six agar discs were punched and used as inoculum (per flask) for solid state fermentative production of LiP in 250 mL flasks containing 11.6 mL Kirk's medium and 5g wheat straw. Kirk's medium (1L) contained 100 mL Basal medium, 100 mL 10% glucose, 100 mL sodium acetate buffer (pH 4.2, 100 mM), 10 mL thiamine-HCl (100 mg/mL), 25 mL ammonium tartrate (8 g/L) , 1.35 mL veratryl alcohol (0.4M), trace element solution 60 mL, distilled water to make up the volume to 1L. Basal medium (1L) consisted of 20 g KH_2PO_4, 5 g $MgSO_4.7H_2O$, 1 g $CaCl_2$, trace element solution 100 mL and distilled water was added to make up the volume to 1L.Trace element solution (1L) was made by first dissolving 1.5 g of nitrilotriacetic acid in 800 mL water with adjustment of the pH to approximately 6.5 with KOH. The following were then added with dissolution: 0.5 g $MnSO_4 \cdot H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CoSO_4$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 10 mg $CuSO_4 \cdot 5H_2O$, 10 mg $AlK(SO_4)_2 \cdot 12H_2O$, 10 mg H_3BO_3, and 10 mg $Na_2MoO_4 \cdot 2H_2O$. Distilled water was added to make up the volume to 1L. Flasks were incubated at 39˚C till harvested for extraction of LiP. Control flasks were kept in which the culture was not inoculated and were processed as described above.

2.4 Preparation of extracellular enzyme extract:-

Flasks were harvested at an interval of 3 days upto 18 days of incubation (3, 6, 9, 12, 15 and 18 d) and the contents were suspended in 100 mL 0.2 M sodium tartarate buffer (pH 3) and kept under shaking condition (200 rpm) at 39ºC for 3 h. The entire content was filtered out by Whatman filter paper No.1 to obtain extracellular crude ligninolytic enzyme preparation. Proteins were precipitated from crude extract with ammonium sulfate to 80% saturation at 4ºC under continuous stirring and allowed to stand for 4h at 4ºC. Precipitates were collected by centrifugation at 15000 rpm for 30 min and dissolved in least quantity of 0.2M sodium tartarate buffer (pH 3) and were subjected to dialysis using Dialysis Membrane -70 (Himedia, India) against 0.02M sodium tartarate buffer (pH 3). The dialyzed samples were then subjected to LiP assay and the enzyme activities were compared with those observed in crude enzyme preparation. Protein estimation was done by the method described by Bradford (1976) using Bovine Serum Albumin as standard.

2.5 LiP assay:-

LiP activity was determined according to Kirk et al. (1986) using veratryl alcohol as the substrate. The reaction mixture (RM) contained 50 mM succinate lactate buffer (pH 3.5), 2 mM veratryl alcohol, and 0.1 mL enzyme and total volume was made to 2 mL with Milli-Q water. The reaction was initiated upon addition of 10 mM H_2O_2 to a final concentration of 0.4 mM and A_{310} was monitored after 5 min incubation at 30ºC with UV-Vis 100 Spectrophotometer (CyberLab, USA). One unit of enzyme was defined as the amount of enzyme required to release 1 µmole of oxidized product, veratrylaldehyde,($\varepsilon = 9300\ M^{-1}\ cm^{-1}$) per minute under standard assay conditions.

LiP activity against RBB were also reported by replacing the veratryl alcohol, as described above, with 0.1 mM RBB in the reaction mixtures and A_{597} was measured after 5 min at 30ºC. One unit of enzyme was defined as the amount of enzyme required to convert 1 µmol of RBB ($\varepsilon = 35550\ M^{-1}\ cm^{-1}$) into product per min under standard assay conditions.

All the assays were performed in triplicates and mean values of specific activities of LiP were reported as U mg^{-1} of protein. Maximum LiP activity was reported in 6 d enzyme sample and hence dialyzed sample was subjected to further characterization with respect to optimum pH, temperature and H_2O_2 concentration.

2.6 Effect of pH on LiP activity:-

LiP activity was assessed at varying pH (pH 2-6) using different buffers (50 mM) in the RM. Buffers used were 50 mM tartarate buffer (pH 2, 2.5 and 3); 50mM succinate lactate buffer (pH 3.5, 4 and 4.5); 50 mM citrate buffer (pH 5 and 5.5) and 50 mM phosphate buffer (pH 6).

2.7 Effect of temperature on LiP activity:-

LiP activity was monitored at different incubation temperature ranging from 15-45 ºC, with a 5ºC increment in the incubation temperature for RM using veratryl alcohol as the substrate.

2.8 Effect of H_2O_2 on LiP activity:-

To study the effect of H_2O_2 on LiP activity, assays were performed at varying concentration of H_2O_2 (0.02-0.6 mM) in RMs.

2.9 Effect of veratryl alcohol concentration on LiP activity:-

To study the effect of substrate concentration on LiP activity, assays were carried out at varying concentration of veratryl alcohol ranging from 0.2 to 5.0 mM in RMs as described above.

2.10 Effect of Reactive Black B concentration on LiP activity:-

Similarly, effect of RBB concentration on LiP was also assessed at varying concentrations of RBB ranging from 0.005-0.1 mM in the RMs. Km and Vmax of LiP for RBB was then calculated using LB Plot.

3. Results and Discussion:-

3.1 Effect of incubation period on LiP production

Flasks were harvested at an interval of 3 days and LiP activities were monitored in crude and dialyzed samples. Crude preparations from 3-9 d flasks had a dark brown color, intensity of which decreased in the similar preparations from 12-18 d flasks. Brown color may be attributed to the release of polyphenols from the lignocellulosic substrate being acted upon by the lignolytic enzyme system of *P. chrysosporium.* As illustrated in Fig. 1, the LiP activity increased steadily over time and maximum activity was reported after 6 d incubation. With further incubation, a gradual decline in the LiP activity was observed. In the dialyzed samples, nearly a 1.5 fold increase in the activity was observed, which may be attributed to the concentrated enzyme in the dialyzed samples.

Several white rot fungi have been studied for their ligninolytic enzyme production under different conditons. Orth et al. (1991) reported maximal extracellular LiP activity on day 5 under N limitation conditions (15 U/mg). For *P. chrysosporium*, maximum LiP activity was reported between 4-8 days of incubation by several workers (Kerem et al., 1992; Arora et al., 2002; Zeng et al., 2015).

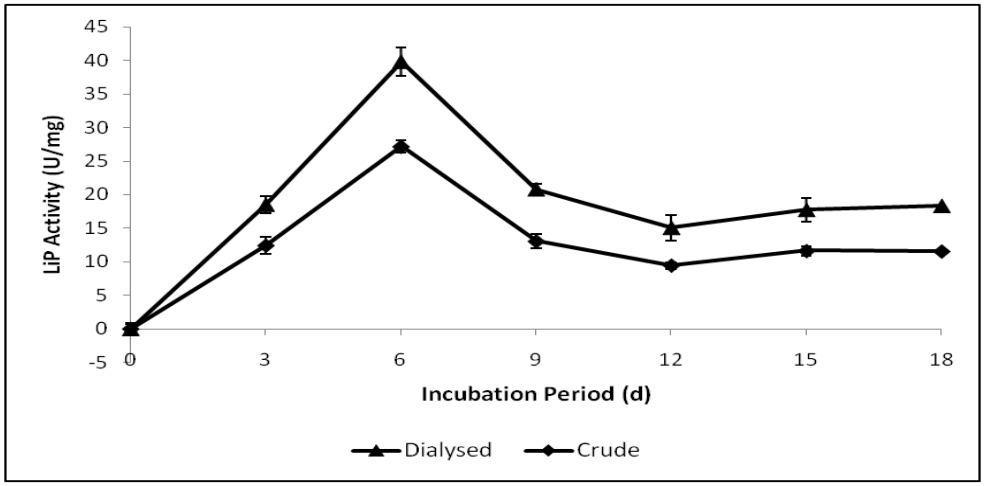

Fig 1: Effect of incubation period on LiP production by *P. chrysosporium* MTCC787 through solid state fermentation of wheat straw at 39ºC.

3.2 Effect of pH on LiP activity:-

pH is one of the most important parameter for enzyme activity. The LiP activity was checked at different pH by using various buffers (Fig. 2). The optimum activity was found at pH 3.5 (44 U/mg). Gradual increase in activity was observed as pH increased up to 3.5 there after the activity decreased upon increase in pH. This may be attributed to the fact that change in pH may alter the three dimensional structure of the enzymes. Optimum pH for LiP of *P. chrysosporium* has been reported in the range of pH 3.5 to 4.5 (Glenn and Gold, 1985; Deguchi et al., 1998; Zeng et al., 2013).

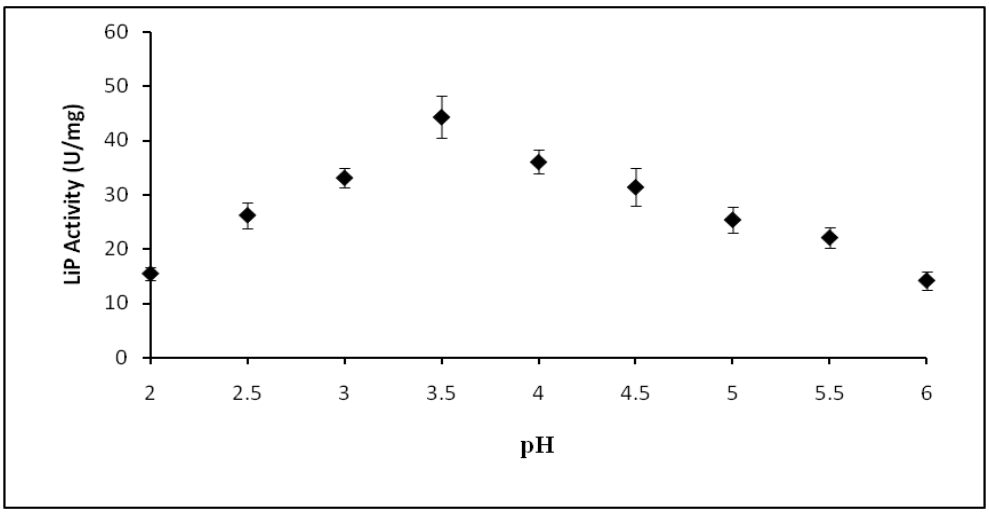

Fig 2: Effect of pH on activity of LiP from *P. chrysosporium* MTCC 787.

3.3 Effect of temperature on LiP activity:-

Temperature is an important parameter affecting the enzyme activity (Jacob et al., 2008). A typical temperature curve (Fig. 3) of LiP was observed against veratryl alcohol. Optimum temperature for LiP of *P. chrysosporium* MTCC 787 was observed to be 30ºC (42.4 ± 3.5 U/mg). Couto et al. (2006) reported the optimum temperature for LiP of *P. chrysosporium* BKM-F-1767 to be 34 ºC. Whereas, our findings were in accordance to those of Zeng et al. (2013), who reported 30 ºC as the optimum temperature for LiP of *P. chrysosporium* BKM-F-1767.

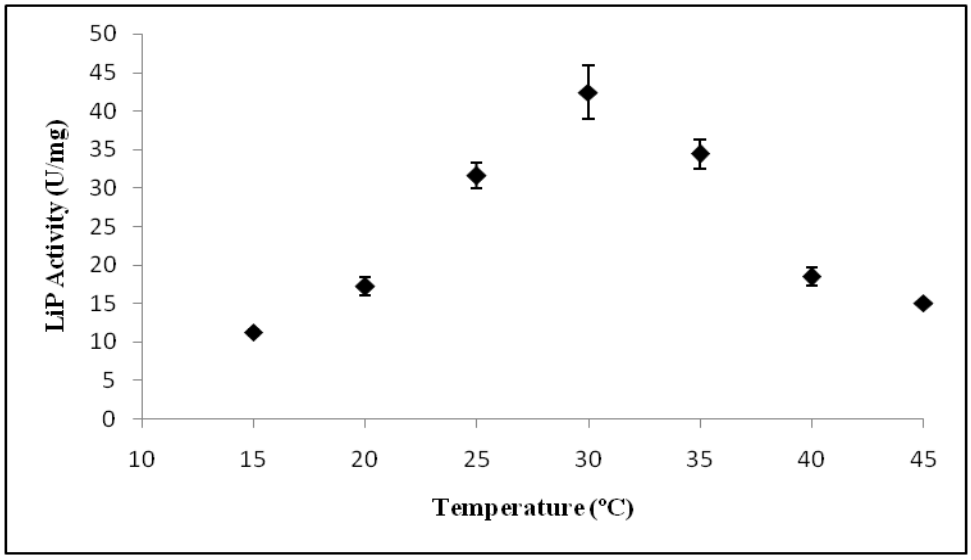

Fig 3: Effect of temperature on LiP activity of *P. chrysosporium* MTCC 787.

3.4 Effect of H_2O_2 on LiP activity:-

H_2O_2 is required for the activity of LiP. With increase in the H_2O_2 concentration, LiP activity increased gradually and was maximum at 0.4 mM concentration 20.41 ± 1.3 U/mg (Fig. 4). At high concentrations, H_2O_2 prevents LiP activity by bleaching the heme group (Have et al., 1997). Inhibitory activity of H_2O_2 was prevented in presence of tryptophan in the reaction mixtures where it functioned as a reductant fro Compound II in the same way as veratryl alcohol, for continuation of catalytic cycle (Collins et al., 1997).

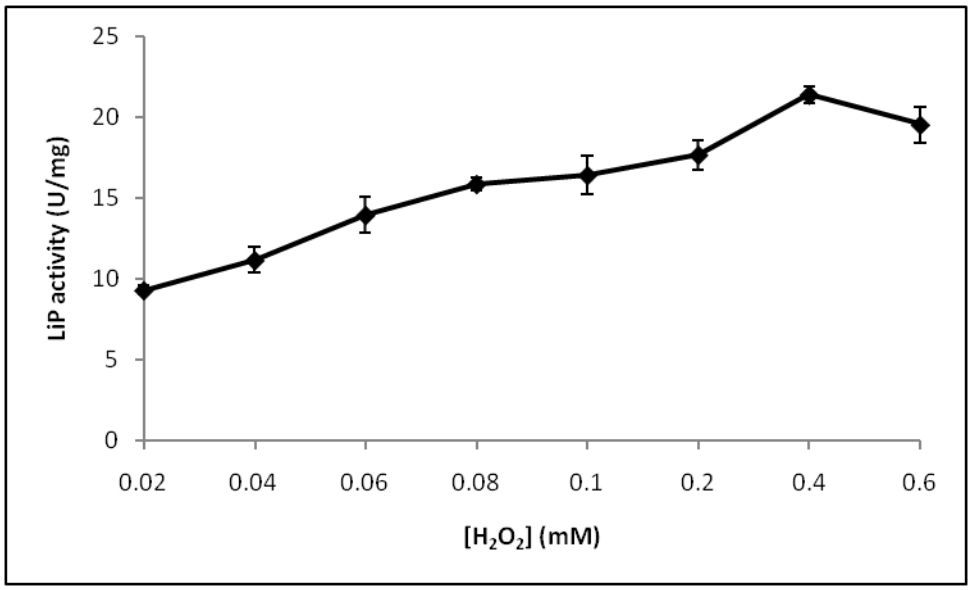

Fig 4: Effect of H_2O_2 concentration on activity of LiP from *P. chrysosporium* MTCC 787.

3.5 Kinetics of LiP against Veratryl alcohol and Reactive Black B:-

Kinetic studies of LiP from the fungal culture were performed against veratryl alcohol and RBB as substrate. For veratryl alcohol, Km and Vmax of LiP were reported to be 0.33 mM and 45.5 U/mg respectively (Fig. 5). Whereas, for RBB Km and Vmax of LiP were reported to be 0.02 mM and 7 U/mg, respectively (Fig. 6); indicating higher affinity of LiP towards RBB compared to veratryl alcohol.

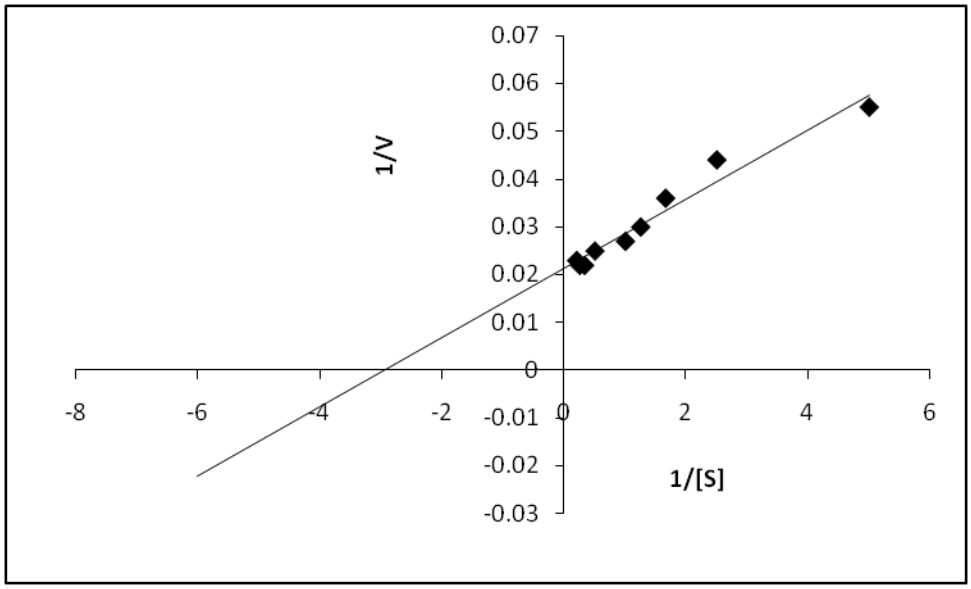

Fig 5: LB plot of LiP from *P. chrysosporium* MTCC 787 against veratryl alcohol as substrate.

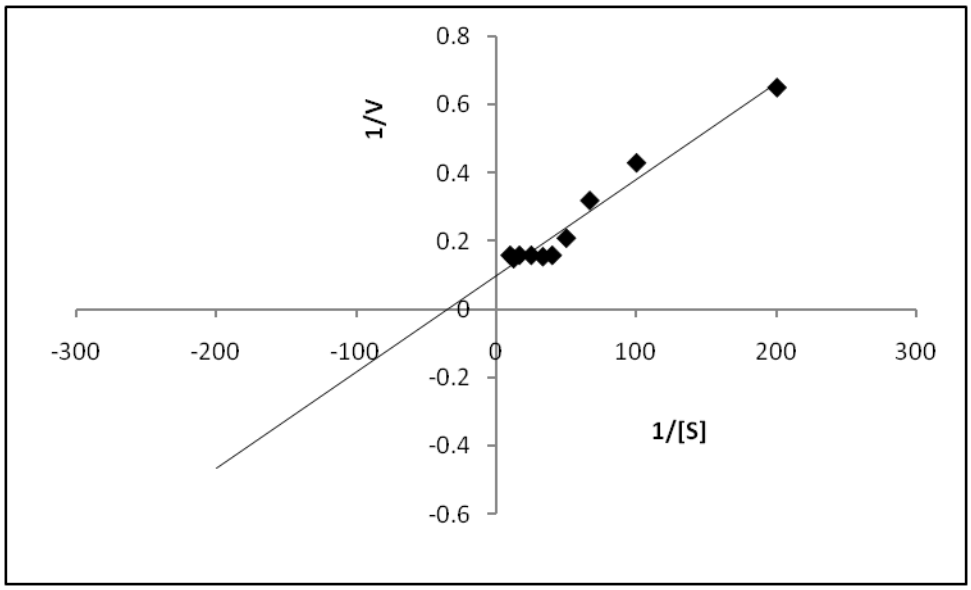

Fig 6: LB plot of LiP from *P. chrysosporium* MTCC 787 against RBB as substrate.

Dyes with different structures are decolorized at different intrinsic enzymatic rates and high dye concentration results in slower decolorization rate in general (Young and Yu, 1997).

4. Conclusion:-

In this study, LiP production by *P. chrysosporium* MTCC 787 through solid state fermentation of wheat straw resulted in maximum yield of LiP on 6th day. Characterization of partially purified LiP was attempted to reveal the optimum pH and H_2O_2 concentration, which suggested the stability of enzyme at low pH and high H_2O_2 concentration. The enzyme also exhibited higher affinity towards Reactive Black B. Thus, the present findings suggest the high potential of LiP for application in treatment of textile dye containing wastewater.

5. Acknowledgements:-

Authors are thankful to the management of Shree M. & N. Virani Science College for providing the research facilities to conduct this work. Authors are also grateful to Dr. Niraj T. Sheth-Associate Professor, Dept. of Microbiology, M. D. Gramseva Mahavidyalaya, Gujarat Vidyapith, Sadra, Gandhinagar; for his kind suggestions in this work.

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