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RESEARCH ARTICLE

SOLID STATE FERMENTATION OF WHEAT STRAW FOR PRODUCTION OF MNP BY *P. CHRYSOPORIUM* MTCC 787.

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Abstract

Solid state fermentation of wheat straw was attempted with *P. chrysosporium* MTCC 787 for production of MnP 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 MnP assay, upon dialysis, using MBTH and DMAB as substrate. Optimum pH and temperature were reported to be pH 4.5 and 30ºC respectively. Km and Vmax of MnP for MBTH were found to be 0.05 mM and 25 U/mg, respectively. Enzyme kinetics were also assessed against Reactive Black B (RBB), a widely used textile diazo dye, as substrate. Km and Vmax of MnP for RBB were reported as 0.2 mM and 7 U/mg, respectively. These findings suggest enormous potential of MnP of *P. chrysosporium* MTCC 787 for its application in treatment of RBB containing wastewater.

Introduction:-

Several white rot fungi have been utilized to produce lignin peroxidase (LiP), manganese peroxidase (MnP), laccase, versatile peroxidase (VP) and other ligninolytic enzymes through solid state fermentation of variety of lignocellulosic substrates (Asgher et al., 2014). Ligninolytic enzymes have found their applications in treatment of pollutant bearing wastewaters due to their ability to act on variety of substrates including textile dyes. Among the synthetic dyes, azo dyes are extensively used in textile, leather, paper, cosmetics, food and pharmaceutical industries (Saratale et al., 2011, Dellamtrice et al., 2017). Azo dyes are the most common synthetic dyes released into the environment during their usage (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).

In this paper, we report our studies on white rot fungus *P. chrysosporium* MTCC787 for the production of MnP through solid state fermentation of wheat straw, its characterization assessing its potential to transform textile di-azo dye Reactive Black B.

Materials and Methods:-

Chemicals and organism:-

Textile diazo dye Reactive Black B (C. I. Reactive Black 5, λmax = 597 nm) was procured from Meghmani Chemicals Ltd., Vatva GIDC, Gujarat, India. 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH), and

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3-dimethylaminobenzoic acid (DMAB) were purchased from HiMedia Laboratories, India. All the other chemicals and reagents used were of analytical grade.

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

Solid State Fermentation and preparation of enzyme extract:-
Solid State Fermentation (SSF) of wheat straw was carried out with *P. chrysosporium* MTCC 787 as described by Junnarkar et al (2016). *P. chrysosporium* was cultivated on MEA plates at 39ºC and agar discs were punched and used as inoculum (6 discs per flask) for solid state fermentative production of MnP in 250 mL flasks containing 11.6 mL Kirk's medium (Junnarkar et al., 2016) and 5g wheat straw. Flasks were incubated at 39˚C till harvested for extraction of MnP. Control flasks were kept in which the culture was not inoculated and were processed as described above.

Flasks were harvested at every 3d interval upto 18 d 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 for 3 h. Further processing was done as described by Junnarkar et al (2016). The dialyzed samples were then subjected to MnP 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.

MnP assay:-
MnP activity was determined according to Kirk et al. (1986) using MBTH and DMAB as the substrate. The reaction mixture (RM) contained 100 mM succinate lactate buffer (pH 2.0), 25 mM DMAB, 1 mM MBTH, 25 mM $MnSO_4$ and 0.1 mL enzyme and total volume was made 2 mL with Milli-Q water. The reaction was initiated upon addition of 10 mM H_2O_2 and A_{590} was measured after 10 min incubation at 25ºC. One unit of enzyme was defined as the amount of enzyme required to release 1 µmol of oxidized product (ε = 32.9 M^{-1} cm^{-1}) per min under standard assay conditions.

MnP activities against RBB were also reported by replacing MBTH and DMAB with 0.01mM RBB in the reaction mixtures and A_{597} was measured after 10 min to determine the MnP activity, at 25ºC. One unit of enzyme was defined as the amount of enzyme required to convert 1 µmol of RBB (ε = 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 and MnP were reported as U mg^{-1} of protein. Maximum MnP activity was reported in 6 d enzyme sample and the dialyzed sample was subjected to further characterization with respect to optimum pH, temperature and substrate concentration.

Effect of pH on MnP activity:-
MnP 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).

Effect of temperature on MnP activity:-
MnP activity was monitored at different incubation temperature ranging from 15-45 ºC, with a 5ºC increment in the incubation temperature for RM using MBTH-DMAB as the substrate.

Effect of MBTH concentration on MnP activity:-
To study the effect of substrate concentration, reaction mixtures were prepared having varying concentration of MBTH i.e. 0.2 mM, 0.4mM, 0.6 mM, 0.8mM, 1.0mM, 1.4mM, 1.8mM, 2.2mM in 2 ml of enzyme reaction mixture. Assay was performed in triplicates and mean specific activity of MnP was reported.

Effect of RBB concentration on MnP activity:-
Effect of RBB concentration on MnP was also assessed at varying concentrations of RBB ranging from 0.005-0.1 mM in the RMs. Km and Vmax of MnP for RBB was then calculated using LB Plot.

Results & Discussion:-

Effect of incubation period on MnP production under SSF conditions:-

Flasks were harvested at an interval of 3 days and MnP activities were monitored in crude and dialyzed samples. Crude preparations from 3-9d flasks had deep brown coloration, which may be due to the liberation of polyphenols from the lignocellulosic substrate being transformed by the ligninolytic enzyme systems of *P. chrysosporium* MTCC 787. With the increase in incubation period, decrease in intensity of brown color was observed in crude preparations from 12-18d flasks. As shown in Fig 1, the MnP activity increased steadily over time and maximum specific activity was reported after 6 d incubation (29.89 ± 1.07 U/mg). With further incubation, a gradual decline in the MnP activity was observed. In the dialyzed samples, nearly a 1.5-fold increase in the activity was observed, which may be attributed to the presence of concentrated enzyme in the dialyzed samples.

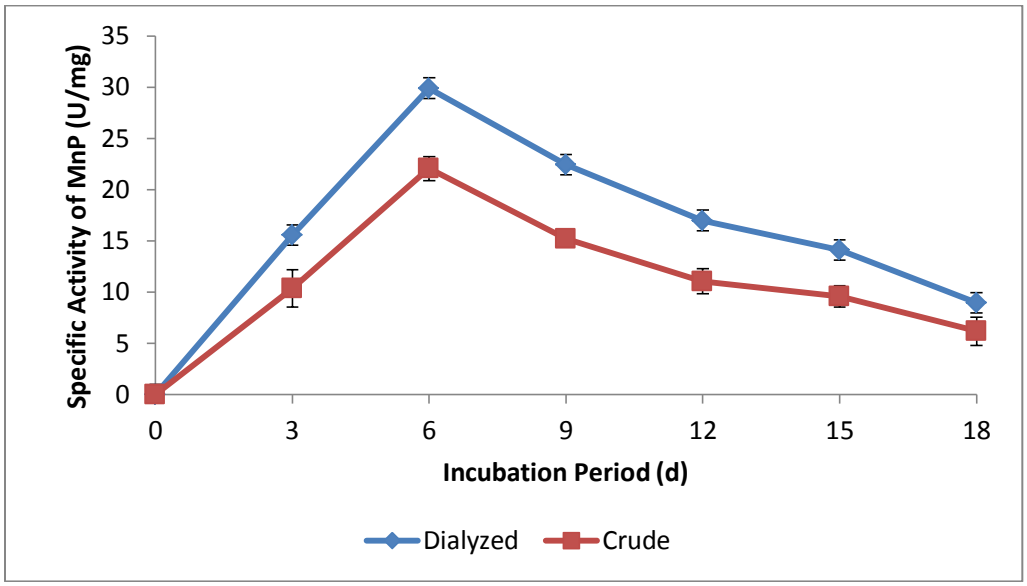

Fig 1:- Effect of incubation period on MnP production by *P. chrysosporium* MTCC787 at 39ºC under solid state fermentation conditions.

MnP production by *T. versicolor* was maximally reported on 12th day of incubation during SSF of wheat straw (Arora et al., 2002). For *P. chrysosporium*, maximum MnP activity was reported between 4-8 days of incubation by several workers (Kerem et al., 1992; Arora et al., 2002; Zeng et al., 2015). Maximum activity of MnP was reported on 7th day of incubation (1293 U/L) during ligninolytic enzyme production by *P. chrysosporium* in fixed bed reactor operating under semi solid-state conditions (Moldes et al., 2003).

Effect of pH on MnP activity:-

One of the most important parameter for enzyme activity is pH of the reaction mixture. The MnP activity in the enzyme samples was assessed at different pH by using various buffers in the reaction mixture; which is shown in Fig 2. Here, the optimum activity of MnP was reported at pH 4.5 (31.39 ± 2.27 U/mg). pH is critical for heme stability and activity, as it impacts on the ionic form of the enzyme active site residues and thus on the binding to the heme group (Zeng et al., 2015). Three-dimensional structure of enzyme is greatly affected by pH in reaction mixture. Such structural changes might have been the reason for decreased activity of MnP at pH other than its optimum pH. MnP was maximally reported at pH 5 by Sklener et al (2010). Optimum MnP activity of *P. chrysosporium* BKMF-1767 was reported at pH 4.5 by Zeng et al. (2013).

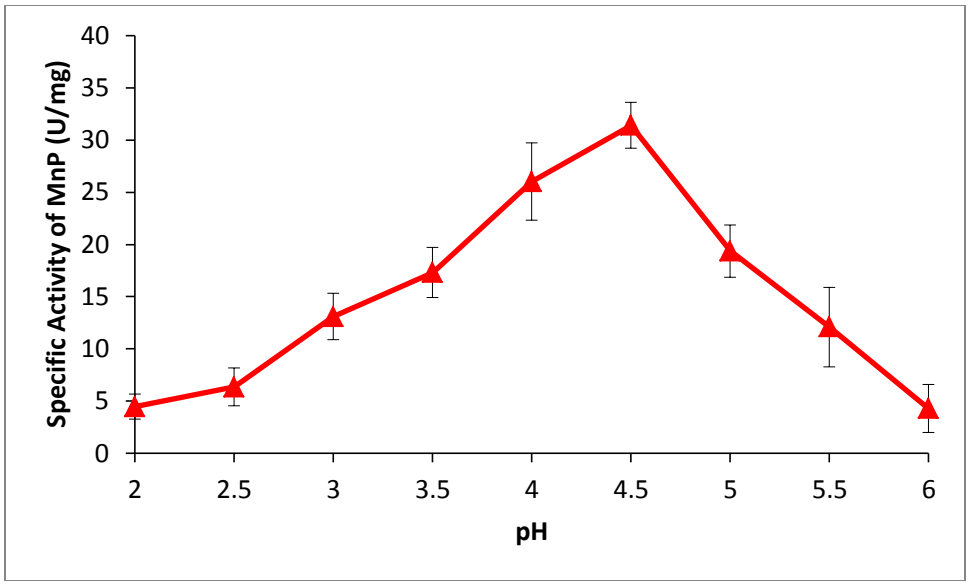

Fig 2:- Effect of pH on MnP activity of *P. chrysosporium* MTCC787.

Effect of temperature on MnP activity:-

A typical temperature curve for MnP was observed as shown in Fig 3. Optimum temperature for MnP of *P. chrysosporium* MTCC 787 was observed at 30ºC (32.8 ± 3.4 U/mg). Comparable results were reported for MnP of white rot fungal cultures by Wesenberg et al. (2003) and Ghodake et al. (2009).

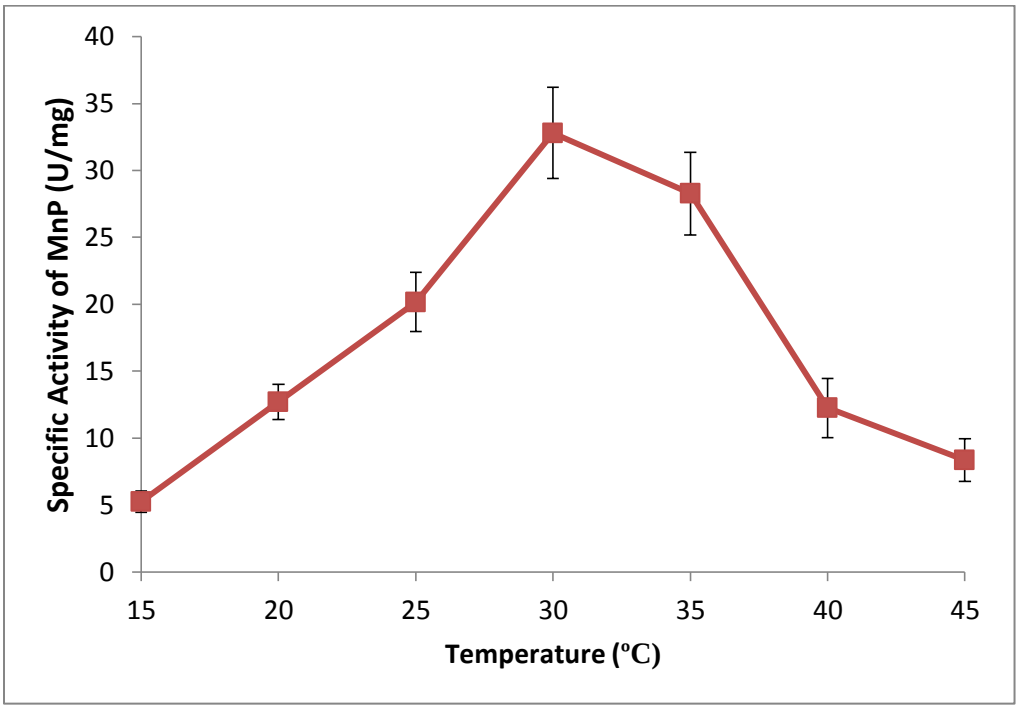

Fig 3:- Effect of temperature on MnP of the fungal cultures: *P. chrysosporium* MTCC787,

Effect of MBTH concentration on MnP activity of *P. chrysosporium*:-

Kinetic studies of MnP from the fungal culture were performed against MBTH and DMAB as substrate. Km and Vmax of MnP from *P. chrysosporium* towards MBTH were reported to be 0.16 mM and 20 U/mg, respectively.

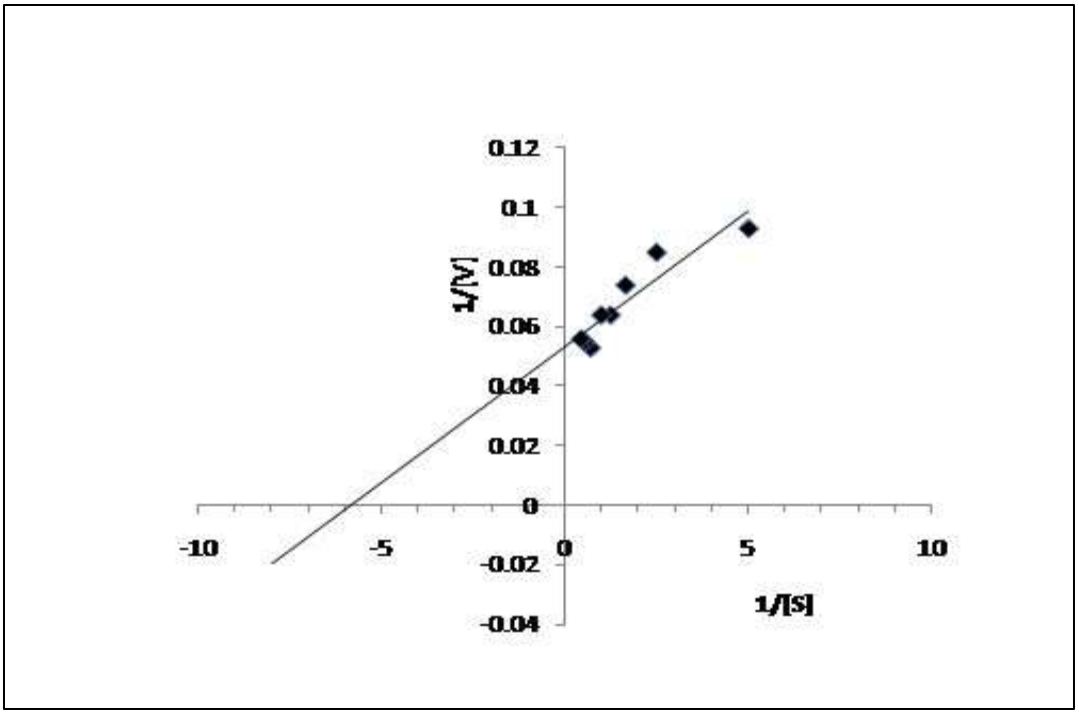

Fig 4:- LB plot of MnP from *P. chrysosporium* MTCC787 using MBTH-DMAB as substrate.

Effect of RBB concentration on MnP activity of *P. chrysosporium*:-

LB plot of MnP from *P. chrysosporium* using Reactive Black B as a substrate is shown in Fig 5 from which, the Km of MnP was calculated as 0.05 mM and Vmax was reported as 25 U/mg. Results indicate that RBB can be oxidatively transformed by MnP of the culture *P. chrysosporium* MTCC787.

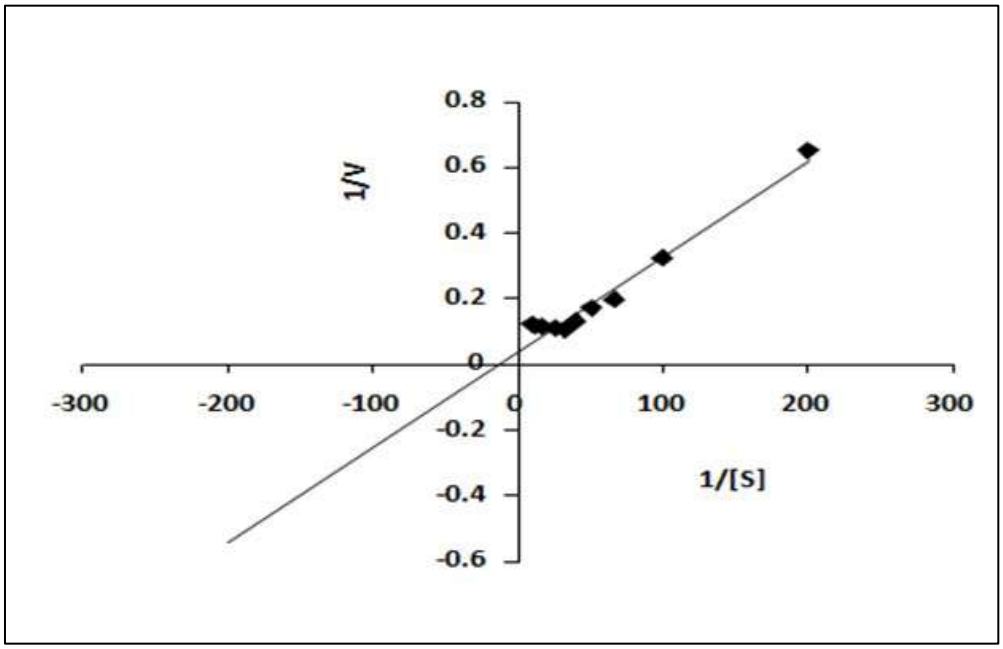

Fig 5:- LB plot of MnP from *P. chrysosporium* MTCC787 using Reactive Black B as substrate.

Conclusion:-

MnP of *P. chrysoporium* MTCC 787 exhibited comparable characteristics with respect to pH and temperature optima. Km and Vmax of enzyme for MBTH indicates its strong affinity for the substrate. MnP kinetics were also assessed against Reactive Black B (RBB), a widely used textile diazo dye, as substrate. Here, Km and Vmax of enzyme for RBB were reported as 0.2 mM and 7 U/mg, respectively. These findings suggest enormous potential of MnP of *P. chrysosporium* MTCC 787 for its application in treatment of RBB containing wastewater.

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