

## Effect of Nitrogen Sources and C:N Ratio on Production of Manganese Peroxidase using *Phanerochaete chrysosporium* NCIM 1197

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A lignin degrading enzyme, manganese peroxidase has detected in culture supernatants of *Phanerochaete chrysosporium* NCIM 1197 grown under various nitrogen sources. The present work was carried out to determine the effect of variable concentration of ammonium tartrate, ammonium chloride and urea on MnP production. Highest level of manganese peroxidase activity, (118.60 ± 2.26 U/ml) was observed in the production medium supplemented with urea (0.0108 mM) and glucose (55.55 mM). A very high C:N ratio in the medium favours the MnP production. The production of the MnP enzyme is a major demand in environmental biotechnology, so outcome of this study can help in scale-up of the enzyme.

**Key words:** Ligninolytic enzyme, Manganese peroxidase, Nitrogen source, *Phanerochaete chrysosporium*, White rot fungi.

Ligninolytic enzymes, such as manganese peroxidases (MnPs), lignin peroxidases (LiPs) and laccases (Lccs) are mainly secreted by white rot fungi and have ability to degrade a variety of recalcitrant organopollutants, like polynuclear aromatic hydrocarbons (PAHs), man-made polychlorinated biphenyls (PCBs) and various synthetic dyes due to their low substrate-specificity<sup>1,2</sup>. White rot fungi also have great potential to degrade the complex natural polymer, lignin, a major component of wood. Extracellular peroxidases are the most important part of

ligninolytic enzyme systems which are responsible for the initial depolymerization of lignin by a non-specific oxidation mechanism<sup>3,4</sup>. The principal role of MnP is related to the oxidation of Mn<sup>2+</sup> to Mn<sup>3+</sup> which then binds to an appropriate ligand, diffuses from the enzyme and finally oxidizes various phenolic substrates<sup>5</sup>.

In recent years, the ligninolytic enzymes are being used in many biotechnological applications, such as biopulping, biobleaching, bioremediation and biofuel production<sup>6-9</sup>. For these applications, large amounts of enzymes must be required at low cost. Although the production of ligninolytic enzymes have been investigated by optimizing culture medium and bioreactor design using *Phanerochaete chrysosporium* but their productivities have not reached industrial scale for the manganese peroxidase<sup>10</sup>.

Several studies on varying the constituents of media have been published but the effect of different nitrogen sources on MnP production are not attempted. Reports are available

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where only one type of nitrogen sources (ammonium tartrate, ammonium sulphate, yeast extract etc.) used for the manganese peroxidase production. Tien and Kirk studied the presence of ammonium tartrate is crucial to induce ligninolytic enzymes in *P. chrysosporium*<sup>11</sup>. Few reports suggested that nitrogen regulation is strain dependent and affected by the carbon source<sup>12,13</sup>. In the present work, the effect of variable concentrations of three nitrogen sources and glucose as main carbon source was studied for production of MnP using *P. chrysosporium* NCIM 1197 as producing strain.

## MATERIALS AND METHODS

### Microorganism

The white rot fungus *P. chrysosporium* NCIM 1197 was procured from National Chemical Laboratory, Pune, India and maintained at 4°C on potato dextrose agar (PDA) plates. The fungus was sub-cultured regularly till sporulation for 7 days at 37°C on PDA plates.

### Media

The standard basal medium used was composed of Basal III, 2,2-dimethyl succinate (pH 4.5), Thiamine HCl, Trace elements solution as described by Tien and Kirk<sup>14</sup>. The concentrations of all the nitrogen sources and glucose in the production medium were varied as per experiment (Table 1).

### Inoculum preparation and culture conditions

For inoculum preparation, the spores were scratched from freshly grown PDA plate, dispersed into sterilized water, filtered through glass wool, and the concentration was adjusted to approximately  $2 \times 10^5$  spores/ml, corresponding to an absorbance of 0.5 at 650 nm (Shimadzu UV 1601 UV-VIS spectrophotometer, Japan). The inoculum ratio used was 10% (v/v) based on total volume of production medium. The production of MnP by liquid culturing was carried out in 100 ml Erlenmeyer flasks containing 10 ml media as per experiment (Table 1). The inoculated flasks were incubated at 37°C in stationary condition for 11 days and MnP activity was monitored.

### Manganese peroxidase assay

Extracellular fluids were sampled aseptically and centrifuged at 10,000 rpm for 10 min at 4°C to remove the fungal biomass. After

separation of the cells, the supernatant containing manganese peroxidase enzyme (MnP) was used for determination of activity at 30°C by the method described by Paszczynski and co-workers<sup>15</sup>. The reaction mixture (500 µl) contained: 100 µl of sodium tartrate buffer (final concentration 0.1 M, pH 5.0 at 30°C), 10 µl of guaiacol (final concentration 0.2 mM), 10 µl of MnSO<sub>4</sub> (final concentration 0.2 mM) 100 µl of culture supernatant and final volume was made 490 µl with distilled water. The reaction was started by adding 10 µl H<sub>2</sub>O<sub>2</sub> (final concentration 0.2 mM) and increase in absorbance due to oxidation of guaiacol was measured at 465 nm with extinction coefficient,  $\epsilon_{465} = 12100 \text{ M}^{-1}\text{cm}^{-1}$ . One unit enzymatic activity was defined as the quantity of enzyme that produced 1 µmol of oxidized product per minute at standard conditions.

### Effect of carbon and nitrogen sources

Several experiments were conducted to explore the appropriate nitrogen source with glucose for the optimal production of MnP. Variable concentrations of carbon and nitrogen sources were used in production medium (Table 1).

### Statistical analysis

In all experiments the measurements were carried out in triplicate cultures. The MnP activities reported in table and figures are mean values with standard deviation.

## RESULTS AND DISCUSSION

It is well known that the MnP has several applications in industries, the present work was planned to enhance the production of MnP by using glucose as carbon and different nitrogen sources at variable concentrations and thus varying C:N ratio in the production medium. The surface culture (stationary condition) techniques has been employed in this study as the MnP production by this techniques was higher compared to submerged culture techniques (data is not shown).

### Effect of carbon source on MnP production

The effect of different concentrations of glucose (5.55 mM, 55.55 mM and 555.55 mM) and fixed concentration of ammonium tartrate as nitrogen source (10.80 mM) was used for optimization of production of MnP using *P. chrysosporium* NCIM 1197. It was observed that

the growth of fungus is favoured by high glucose concentration and reached to form a thick mycelia mat as the time of incubation progressed. However, the MnP production has not increased corresponding to the growth. Maximum production of MnP was observed only ( $1.34 \pm 0.01$  U/ml) on seventh day of incubation with high glucose concentration (555.55 mM) and reduced subsequently. When the glucose concentration in the medium was reduced to one tenth (55.55 mM) and one hundredth (5.55 mM), a significant increase in the MnP production was observed. The maximum MnP production was reached to ( $16.06 \pm 0.48$  U/ml) and ( $40.67 \pm 0.82$  U/ml) in the medium with glucose concentration of 55.55 mM and 5.55 mM, respectively (Fig. 1). Results clearly indicate that low glucose concentration in the medium with high nitrogen concentration (10.80 mM ammonium tartrate) gave better MnP production.

In order to check whether the MnP production is influenced solely by the glucose concentration in the medium or nitrogen has also the role in enhanced production of MnP, an experiment was planned with two low ammonium tartrate concentrations (1.08 mM and 0.0108 mM) keeping glucose concentration at 5.55 mM in the medium. The results are shown in Figure 2. The

maximum production of MnP attained was ( $46.47 \pm 0.77$  U/ml) and ( $40.44 \pm 0.79$  U/ml) in medium containing 1.08 mM and 0.0108 mM, respectively. Further reduction in concentration of both the carbon source (0.55 mM glucose) and nitrogen source (1.08 mM and 0.0108 mM ammonium tartrate) resulted in very low MnP production. The low MnP production in this case may be due to very low glucose concentration required for desired growth of the fungus as well as enzyme production (Fig. 3). Dosoretz and Grethlein also reported that synthesis of MnP partially repressed by the carbon starvation arising from a low C:N ratio<sup>16</sup>.

#### Effect of different nitrogen sources on MnP production

*P. chrysosporium* NCIM 1197 was grown with three nitrogen sources namely ammonium tartrate, ammonium chloride and urea at varying concentrations (0.0108 mM, 0.108 mM and 1.08 mM) in production medium containing 55.55 mM glucose as carbon source for MnP production. When the fungus was grown in production medium with 0.0108 mM ammonium tartrate as nitrogen source, the MnP production increased with time of incubation and reached its maximum ( $100.32 \pm 1.92$  U/ml) on 6<sup>th</sup> day of incubation and decreased subsequently. The higher concentration of

**Table 1.** Experiment plan and effect of C:N ratio on manganese peroxidase production

Exp. no.	Glucose concentration (mM)	Nitrogen concentration (mM)	C:N ratio	Maximum MnP (U/ml) production (DOI) <sup>a</sup>
1	555.55	10.80 (AT) <sup>b</sup>	137	$1.34 \pm 0.01$ (7)
2	55.55	10.80 (AT)	15	$16.06 \pm 0.48$ (9)
3	5.55	10.80 (AT)	3	$40.67 \pm 0.82$ (8)
4	5.55	1.08 (AT)	15	$46.47 \pm 0.77$ (7)
5	5.55	0.0108 (AT)	1355	$40.44 \pm 0.79$ (6)
6	0.55	1.08 (AT)	3	$2.40 \pm 0.02$ (8)
7	0.55	0.0108 (AT)	137	$2.34 \pm 0.02$ (7)
8	55.55	1.08 (AT)	135	$94.44 \pm 1.91$ (8)
9	55.55	0.108 (AT)	1343	$98.40 \pm 1.61$ (8)
10	55.55	0.0108 (AT)	13416	$100.32 \pm 1.92$ (6)
11	55.55	1.08 (AC) <sup>c</sup>	78	$95.42 \pm 1.70$ (9)
12	55.55	0.108 (AC)	780	$101.57 \pm 1.78$ (8)
13	55.55	0.0108 (AC)	7800	$105.24 \pm 1.89$ (5)
14	55.55	1.08 (U) <sup>d</sup>	44	$99.42 \pm 1.69$ (9)
15	55.55	0.108 (U)	437	$102.84 \pm 1.93$ (8)
16	55.55	0.0108 (U)	4366	$118.60 \pm 2.26$ (5)

<sup>a</sup>DOI = Day of incubation written in parentheses; <sup>b</sup>AT = Ammonium tartrate;

<sup>c</sup>AC = Ammonium chloride; <sup>d</sup>U = Urea

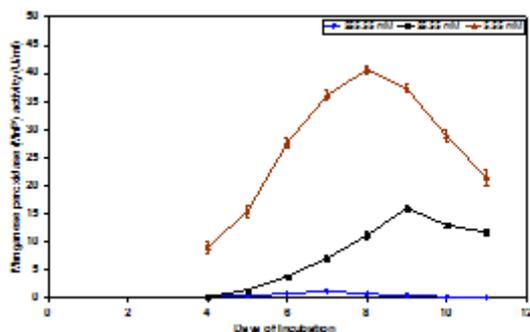
ammonium tartrate slightly lowers the yield and delayed the MnP production (Fig. 4).

When ammonium chloride was used as the main nitrogen source, the maximum MnP production was reached to  $(105.24 \pm 1.89 \text{ U/ml})$  on 5<sup>th</sup> day of incubation with lower concentration of ammonium chloride (0.0108 mM). Slightly lower yield and delayed MnP production was also observed in case of higher concentration of ammonium chloride in the production medium (Fig. 5). The highest MnP production  $(118.60 \pm 2.26 \text{ U/ml})$  was observed on 5<sup>th</sup> day of incubation in production medium containing 0.0108 mM urea. Slightly lower yield and delayed MnP production was also observed in case of higher concentration of urea in production medium (Fig. 6). However, the MnP production in 0.108 mM urea was higher compared to that of other two nitrogen sources. This increased MnP production in urea may be due to its organic nature.

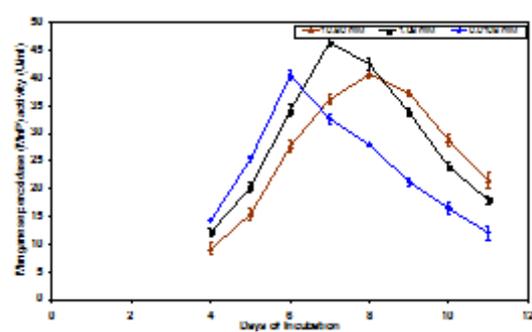
In all the experimental work the observed activity of MnP was highest in urea as nitrogen source  $(118.60 \pm 2.26 \text{ U/ml})$  whereas, the enzyme

activity of  $(100.32 \pm 1.92 \text{ U/ml})$  was obtained from the medium supplemented with the more commonly used ammonium tartrate as nitrogen source and 55.55 mM glucose. Based on the medium used by different researchers, glucose remains the first choice of carbon source, and ammonium tartrate as the nitrogen source<sup>17</sup>. Thakkar and co-workers had worked on biocatalytic decolourisation of molasses by *P. chrysosporium* NCIM 1197 and reported  $(0.14 \pm 0.04 \text{ U/ml})$  manganese peroxidase activities in stationary cultivation conditions in flat bottom glass bottles using ammonium tartrate as nitrogen source<sup>18</sup>. Recently, Singh and co-workers have reported  $(8.77 \pm 0.23 \text{ U/ml})$  MnP activity using *P. chrysosporium* ATCC 24725 where the medium contained mannose as carbon source, nitroacetate and S-adenosylmethionine as nitrogen sources<sup>19</sup>.

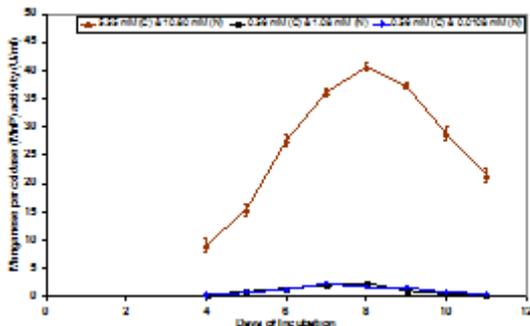
Wang and co-workers reported highest MnP activities in their experiments when media was supplemented with 55.55 mM glucose and 0.59 mM ammonium tartrate<sup>20</sup>. In contrast, they reported less MnP activity when nitrogen concentration was



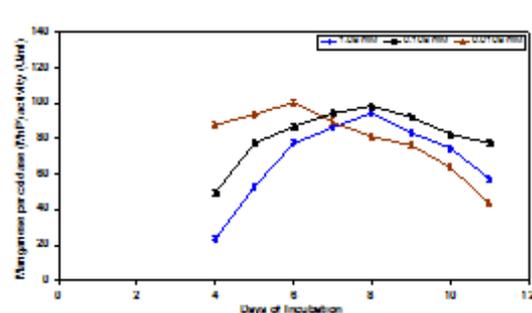
**Fig. 1.** Time course of MnP production (U/ml) by *P. chrysosporium* NCIM 1197 with constant ammonium tartrate (10.80 mM) and varying glucose concentrations



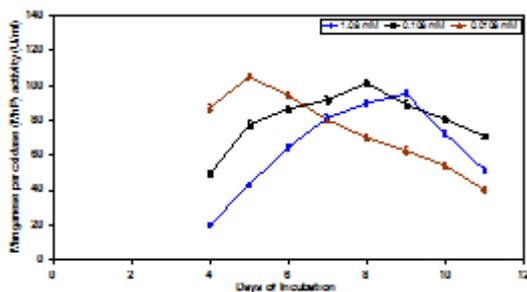
**Fig. 2.** Time course of MnP production (U/ml) by *P. chrysosporium* NCIM 1197 with constant glucose (5.55 mM) and varying ammonium tartrate concentrations



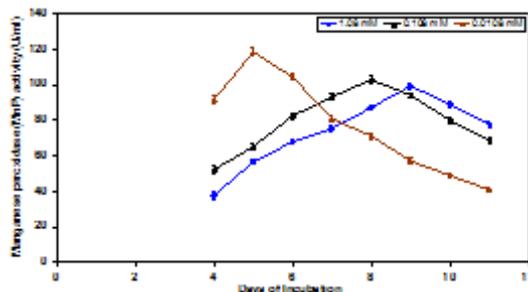
**Fig. 3.** Time course of MnP production (U/ml) by *P. chrysosporium* NCIM 1197 with varying glucose (0.56 mM and 5.55 mM) and varying ammonium tartrate (10.80 mM, 1.08 mM and 0.0108 mM) concentrations



**Fig. 4.** Time course of MnP production (U/ml) by *P. chrysosporium* NCIM 1197 with constant glucose (55.55 mM) and varying ammonium tartrate concentrations (1.08 mM, 0.108 mM and 0.0108 mM)



**Fig. 5.** Time course of MnP production (U/ml) by *P. chrysosporium* NCIM 1197 with constant glucose (55.55 mM) and varying ammonium chloride concentrations (1.08 mM, 0.108 mM and 0.0108 mM)



**Fig. 6.** Time course of MnP production (U/ml) by *P. chrysosporium* NCIM 1197 with constant glucose (55.55 mM) and varying urea concentrations (1.08 mM, 0.108 mM and 0.0108 mM)

0.30 mM but in the present work 0.0108 mM nitrogen concentration showed better enzyme production. This might be due to the strain specificity and incubation temperature. However, Buswell and co-workers found that high nitrogen concentration (26 mM) triggers the more ligninase activity in *P. chrysosporium* INA-12 than *P. chrysosporium* ME-446<sup>21</sup>. The appearance of MnP activity with high nitrogen conditions could be due to nutrient limitation into the mycelial mat<sup>22</sup>.

The level of nitrogen in the liquid medium is very important and crucial for the synthesis of ligninolytic enzymes in white rot fungi. These enzymes are produced during secondary metabolism, which is induced by limitations of carbon, nitrogen or sulphur in media<sup>23-25</sup>. Therefore, the levels of nitrogen in the liquid medium need to be low but still high enough for growth to be possible. Furthermore, the nitrogen concentrations need to favour the glucose oxidase production which is essential for the glucose metabolism<sup>26</sup>.

#### Effect of C:N ratio on MnP production

Table 1 shows the effect of the C:N ratio studied by altering carbon and nitrogen concentrations on MnP production. The results clearly indicated that the high C:N ratio in production medium enhances the MnP production as low nitrogen content triggered the early MnP production. The low C:N ratio (3.07) when sufficient glucose was present in the medium also help the fungus to produce the MnP in significant quantity ( $40.67 \pm 0.82$  U/ml) as observed in case of the medium containing 5.55 mM glucose and 10.80 mM ammonium tartrate. However when both glucose and ammonium tartrate were taken as one

tenth in the medium keeping the same C:N ratio the MnP production ( $2.40 \pm 0.02$  U/ml) was drastically reduced due to insufficient nutrients in the medium. The highest MnP production ( $118.60 \pm 2.26$  U/ml) was achieved in the medium containing 55.55 mM glucose and 0.0108 mM urea with a high C:N ratio of 4366 on 5<sup>th</sup> day of incubation.

Yu and co-workers also reported the effect of C:N ratio on the production of ligninolytic enzyme by immobilized *P. chrysosporium* using different glucose and ammonium tartrate concentrations in the medium<sup>27</sup>. The higher MnP production (90 U/l) were reported with a C:N ratio of 69 and 5 compared to 3.4. The significant production under nitrogen limitation (C:N ratio 69) or high carbon conditions (C:N ratio 5) and the repressed formation at a low carbon concentration (C:N ratio 3.4) suggested that the MnP synthesis preferred a high carbon content in the medium. It was also observed by Rothschild and co-workers that carbon starvation, resulting from a low C:N ratio strongly repressed MnP activity<sup>28</sup>. However, if the C:N ratio increased to very high level (more than 4000) as in case of present study, the MnP synthesis could be enhanced many folds.

#### CONCLUSION

In the present study we found that *P. chrysosporium* NCIM 1197 is an efficient organism to produce manganese peroxidase (MnP) enzyme with glucose (55.55 mM) and urea (0.0108 mM) as carbon and nitrogen source in media, respectively. The urea can be used as an alternative nitrogen source in case of different fungi involved in the

synthesis of MnP as it showed a good yield of MnP compared to ammonium tartrate and ammonium chloride.

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