# INFLUENCE OF CULTURE CONDITIONS ON LIGNINOLYTIC ENZYMES PRODUCTION FROM *Ganoderma lucidum* USING AGRO-INDUSTRIAL RESIDUES

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## ABSTRACT

The present study was carried out to investigate the ligninolytic enzymes production potential of *G. lucidum* using different agro-industrial residues and their by-products. The enzyme profile of *G. lucidum* showed highest activities of manganese peroxidase (MnP) after 4$^{th}$ day of incubation on peanut shells followed by lignin peroxidase (LiP) and laccase after 6$^{th}$ and 7$^{th}$ day of incubation on orange peel and wheat straw, respectively. The enzyme with maximum activity (MnP) was optimized, therefore, through process optimization the production of MnP was substantially enhanced up to 2160 ± 212 U/mL, when fermentation medium of peanut shells inoculated with 5 mL spore suspension of *G. lucidum*. Other optimum conditions were: pH, 5; moisture level, 70%; temperature, 40$^{o}$C; carbon source, glucose (1%); nitrogen supplement, yeast extract (0.2%), and modulator, $MnSO_4$.

**KEYWORDS:** *G. lucidum*; ligninolytic enzymes; process optimization; still culture; white rot fungi

## 1. INTRODUCTION

White Rot Fungi (WRF) belong to class basidiomycetes and are among the most effective microorganisms [1], able to degrade lignin by the action of their oxidative enzymes system, mainly phenoloxidases (laccases) and peroxidases (LiP and MnP) [1-4]. Lignin Modifying Enzymes (LMEs) are industrially important, therefore, microorganisms able to produce them are interesting in view of the potential importance in industrial processes like bioremediation, bioleaching of pulp paper, degradation and detoxification of recalcitrant substances, textile and dying units and food industry [5-9]. A wide spectrum of lignocellulosic agro-industrial residues and their by products are available in abundance and considered as good substrates for the production of enzymes of industrial significance under solid state fermentation (SSF) by WRF [1].

MnP is considered unable to oxidize non-phenolic substrates, although it has potential capacity to depolymerize synthetic or natural lignins in vitro [6]. Laccase belongs to a family of multi copper oxidases, which has a wide range of reducing substrates like polyphenols and methoxy substituted phenols [2-4]. SSF is a process occurs under complete absence of free flowing water contents in the growth media. Enzyme production in solid-state fermentation is higher than in submerged fermentation and this is mainly because, there are several advantages of SSF, for example high productivities, extended stability of products and low production costs. With increasing progress and application of rational methods in engineering, SSF has achieved higher levels in standardization and reproducibility [2, 10].

Keeping in view the extensive industrial applications of ligninolytic enzymes, this paper reports the influence of culture conditions on ligninolytic enzyme production potential of *G. lucidum* under SSF technique. In the present study, a practical approach was adopted that dealt entirely with the use of different wastes (wheat straw, apple pomace, peanut shells, orange peel and municipal paper waste) to obtain useful products (ligninolytic enzymes). The present study was also focused on providing a potential solution for the management of a large magnitude of solid wastes.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and lignocellulosic substrates

All the chemicals used were of analytical grade. The agro-industrial wastes, *i.e.*, wheat straw, apple pomace, peanut shells, orange peel, were obtained from a local fruit market in Gujrat, Pakistan, while a large quantity of municipal paper waste was collected from the routinely distributed local newspapers. All of the substrates were

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crushed into pieces, dried, ground to fine particle size prior to the use for experimental work.

#### 2.2 Micro-organism and inoculum development

The pure culture strain of white rot fungi *G. lucidum* was grown on potato dextrose agar (PDA) slants. Aqueous spore suspension of *G. lucidum* was developed in 250 mL Erlenmeyer flask containing 100mL of Kirk's basal nutrient medium composed of (g/L); glucose, 10.0; ammonium tartarate, 0.22; $KH_2PO_4.7H_2O$, 0.21; $MgSO_4$, 0.05; $CaCl_2$. 2 $H_2O$, 0.01; thiamine, 0.001; 10% tween-80, 10mL; 100 mM veratryl alcohol, 10 mL and 10mL/L trace elements solution. The inoculated flasks were incubated at 30°C in a continuous shaking position at 120 rpm to get homogenous inoculum suspension of *G. lucidum*.

#### 2.3 Solid state fermentation procedure

All the samples for ligninolytic enzymes production were run triplically in 250mL laboratory scale Erlenmeyer flasks. Each flask contained 5g substrate initially moistened (60% w/w moisture) with Kirk's basal salt medium (pH 4.0). The prepared sample flasks were autoclaved (120°C) for 15 minutes and inoculated with fungal spore suspension (4-5mL) under sterilized environment. The flasks were incubated at 30°C in a temperature controlled incubator for 1-10 days.

#### 2.4 Harvesting and enzyme extraction procedure

At the end of each 24 h a set of incubated flasks were harvested for extraction of extra-cellular enzymes. To each flask 100mL distilled water was added and kept in shaker at 120 rpm for 30 min [6]. The contents were filtered through Whatman No.1 filter paper and washed twice with distilled water. The filtrates were centrifuged at 4,000×g for 10 min and supernatants were assayed to determine the activities for laccase, MnP and LiP.

#### 2.5 Enzyme activity assays

Lignin peroxidase (LiP) activity of supernatants was determined by following the $H_2O_2$ dependent oxidation of veratryl alcohol to verataldehyde at 25°C using UV/Visible spectrophotometer as described earlier [1]. Manganese peroxidase (MnP) activity of supernatants was determined using a UV/Visible spectrophotometer as described earlier [6]. While, the laccase activity was determined by the 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate) ABTS oxidation method as described earlier [4]. All of the recorded activities were expressed as U/mL.

#### 2.6 Determination of dry biomass

Fungal biomass was determined by direct method at the end of each experiment, the fermented biomass was transferred to a pre-weighed filter paper and dried in a hot air oven for 72 h at 80°C, and the weights (g) were recorded.

#### 2.7 Optimization of SSF culture conditions

Maximum production of enzyme require the optimization of different growth conditions so attempt was made to investigate the effect of various parameters including moisture levels, pH, incubation temperatures, additional carbon sources (glucose, fructose, sucrose, maltose, molasses), additional nitrogen sources (urea, yeast extract, beef extract, bacteriological peptone, ammonium sulphate), inoculum size and mediators on ligninolytic enzymes production in SSF from *G. lucidum*.

## 3. RESULTS AND DISCUSSION

#### 3.1 Screening of lignocellolosic substrates

*G. lucidum* was cultured in solid state fermentation of lignocellulosic substrates for the production of ligninolytic enzymes (laccase, MnP and LnP). After every 24 h triplicate flasks were harvested and cultured supernatants were analyzed for ligninolytic enzymes. The results of screening trial showed that maximum production of MnP (332 ± 6.66 U/mL) after 4$^{th}$ day of incubation on peanut shells followed by LiP (112 ± 1.10 U/mL) and laccase (55 ± 2.12 U/mL) after 6$^{th}$ and 7$^{th}$ day of incubation on orange peel and wheat straw, respectively. While using other substrates the enzyme production potential was substantially decreased. The results obtained are summarized in Table 1. Results of screening trial showed that microorganism takes some time in settling on the substrate and then showed its maximum activity after stipulated time period. On different substrates pattern of fungus growth may vary because of the difference in composition nature of each substrate. Based on the results presented in Table 1, the peanut shells as the best yielded growth substrate, was selected for further product optimization. The time taken for ligninolytic enzymes synthesis depends on the primary metabolism that varies with the chemical composition of lignocellulosic substrates [11]. Low cost substrates like wheat flour, wheat bran, straws, and molasses are suitable for fungal growth and enzyme production [4, 12].

#### 3.2 Optimization of SSF culture conditions

##### 3.2.1 Effect of moisture level

In order to optimize moisture content, varying levels of moisture (% w/w) were employed ranging from (50-75%) to the lignocellulosic substrate peanut shells. Peanut shells fermented at 70 % (w/w) moisture gave maximum MnP (545 ± 3.55 U/mL) production after 5 days of inoculation with *G. lucidum* however, an increase in moisture level more than 70 % showed decreasing trend in enzyme production (Fig.1). Higher moisture level had inhibitory effect on ligninolytics formation from microbes in secondary growth [1, 13]. The wet condition of the mold depends on an optimum moisture level and the water holding capacity of the substrate in SSF. Low moisture contents cause slower enzyme secretion from fungus due to reduced solubility of nutrients and low substrate swelling whereas, high moisture level results in decreased substrate porosity that leads to fungal growth inhibition due to poor accessibility of nutrients and limited aeration [1].

**TABLE 1 - Activities of ligninolytic enzymes produced by *G. lucidum* on different agro-industrial based lignocellulosic residues**

| Substrates (5g) | Enzymes | Fermentation time (Days) 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Wheat straw | MnP | 28±0.09 | 56±0.36 | 112±0.12 | 118±1.15 | 132 ±0.52 | 116 ±2.96 | 114 ±1.75 | 119 ±1.87 | 116 ±2.22 | 40 ±0.90 |
| | LiP | 32±0.06 | 45±0.35 | 56±0.14 | 52±1.22 | 70 ±1.02 | 88 ±2.36 | 99 ±1.29 | 66 ±3.85 | 45 ±1.85 | 42 ±1.10 |
| | Laccase | 9±0.00 | 12±0.15 | 25±0.09 | 32±1.10 | 38 ±0.50 | 48 ±3.45 | 55± 2.12 | 36±0.45 | 24±0.12 | 10±0.08 |
| Apple pomace | MnP | 52±1.25 | 66±0.65 | 78±1.25 | 111±1.86 | 152 ±2.12 | 111 ±1.36 | 99 ±1.85 | 42 ±1.32 | 52 ±2.33 | 22 ±0.06 |
| | LiP | 22±2.12 | 18±1.15 | 36±1.96 | 42±1.60 | 66 ±1.45 | 59 ±1.25 | 41 ±1.42 | 29 ±1.32 | 31 ±1.52 | 14 ±0.95 |
| | Laccase | 4±0.00 | 8±0.26 | 11±0.06 | 13±1.05 | 19 ±0.20 | 9 ±3.60 | 11 ±1.52 | 7 ±3.97 | 4 ±3.02 | 2 ±0.06 |
| Peanut shells | MnP | 145±2.22 | 199±1.33 | 288±2.36 | 332± 3.66 | 274±0.00 | 248±0.26 | 266±0.06 | 22±1.05 | 198 ±0.20 | 166 ±3.60 |
| | LiP | 32±0.00 | 58±0.26 | 66±0.06 | 86±1.05 | 45 ±0.20 | 88 ±3.60 | 95 ±1.52 | 49 ±3.97 | 29 ±3.02 | 15 ±0.06 |
| | Laccase | 16±2.20 | 29±1.22 | 32±1.15 | 22±1.15 | 44 ±1.32 | 41 ±2.23 | 19 ±1.52 | 23 ±2.12 | 11 ±1.21 | 8 ±2.32 |
| Orange peel | MnP | 99±3.23 | 145±3.56 | 188±2.66 | 255±2.45 | 266 ±3.32 | 132 ±3.22 | 111 ±3.45 | 99 ±2.56 | 79 ±1.66 | 66 ±3.65 |
| | LiP | 42±0.00 | 62±0.26 | 79±0.06 | 91±1.05 | 102 ±0.20 | 112± 1.10 | 98±0.00 | 64±0.26 | 39±0.06 | 44±1.05 |
| | Laccase | 5±0.05 | 14±0.36 | 22±0.09 | 31±1.40 | 27 ±0.66 | 18 ±1.11 | 14 ±1.15 | 11 ±0.65 | 9 ±0.02 | 12 ±0.06 |
| Municipal paper waste | MnP | 55±1.12 | 65±0.26 | 71±0.06 | 98±1.05 | 133 ±0.20 | 142 ±3.60 | 121 ±1.52 | 139 ±3.97 | 99 ±3.02 | 59 ±0.06 |
| | LiP | 22±0.09 | 28±0.33 | 48±0.12 | 61±1.74 | 52 ±0.65 | 75 ±2.21 | 51 ±0.09 | 38 ±1.25 | 32 ±1.24 | 36 ±0.66 |
| | Laccase | 14±0.04 | 15±0.45 | 39±0.12 | 37±1.11 | 66 ±0.85 | 52 ±1.24 | 22 ±1.52 | 26 ±0.08 | 12 ±0.05 | 10 ±0.06 |

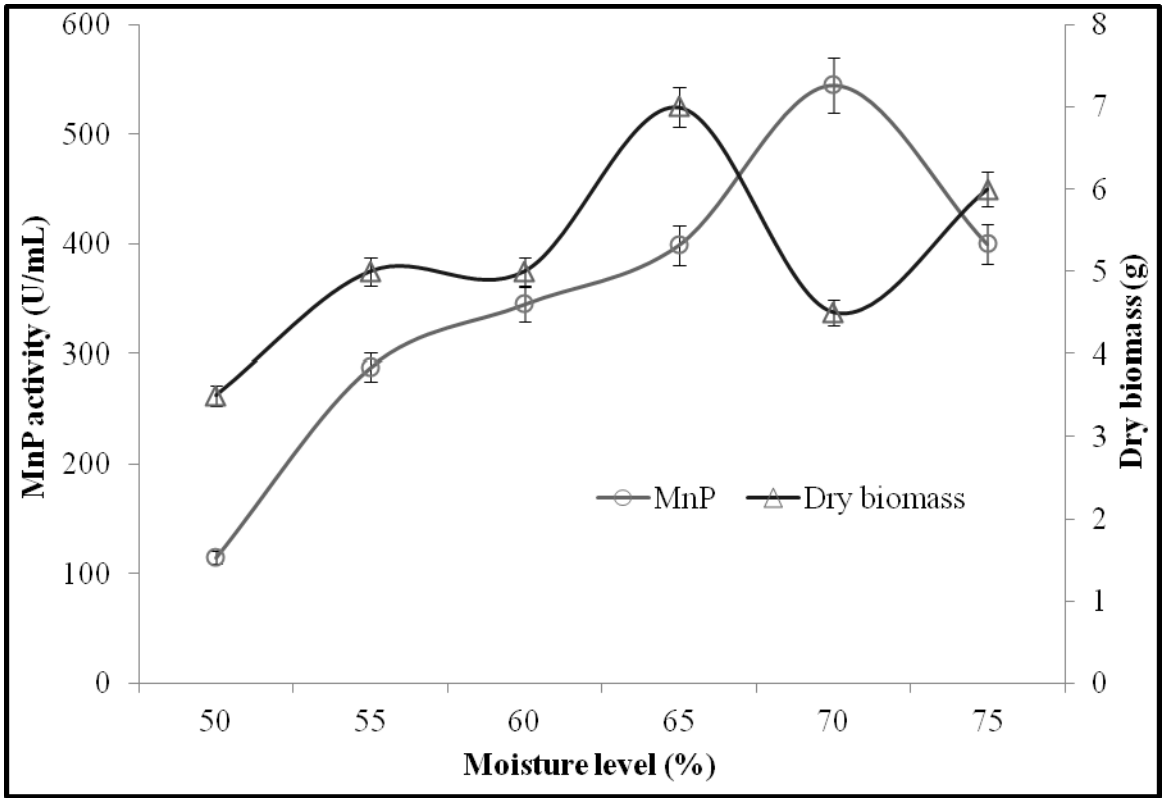


**FIGURE 1 - Effect of varying moisture levels on MnP production by *G. lucidum***

#### 3.2.2 Effect of pH

The maximum activity of MnP (688 ± 5.88 U/mL) was recorded in the SSF media containing peanut shells processed at pH 5. From the growth pattern of fungus and culture extracts it was observed that ligninolytic enzyme formation progressively increased with an increase in the pH of fermentation media and maximum activity was recorded at pH 5 (Fig. 2). Fungi are very susceptible to variation in the pH and dependent on chemical composition of the substrates and fermentation media as it is a crucial factor of SSF medium that had a significant influence on ligninolytics synthesis Radha et al. [14]. Metamora et al. [15] reported maximum ligninolytics activities in the pH range 4.0 to 5.0 at 25°C produced from *G. lucidum*. The study reveals that the enzyme secretion is greatly influenced by the change in initial pH of the medium.

#### 3.2.3 Effect of incubation temperature

A set of triplicate flasks were incubated under temperature controlled incubator and maximum enzyme activity was noted in the medium fermented and incubated at 35°C (Fig 3). Activities of ligninolytic enzymes of WRF were substantially decreased while cultured at temperatures elevated than 35°C. The incubation temperature showed

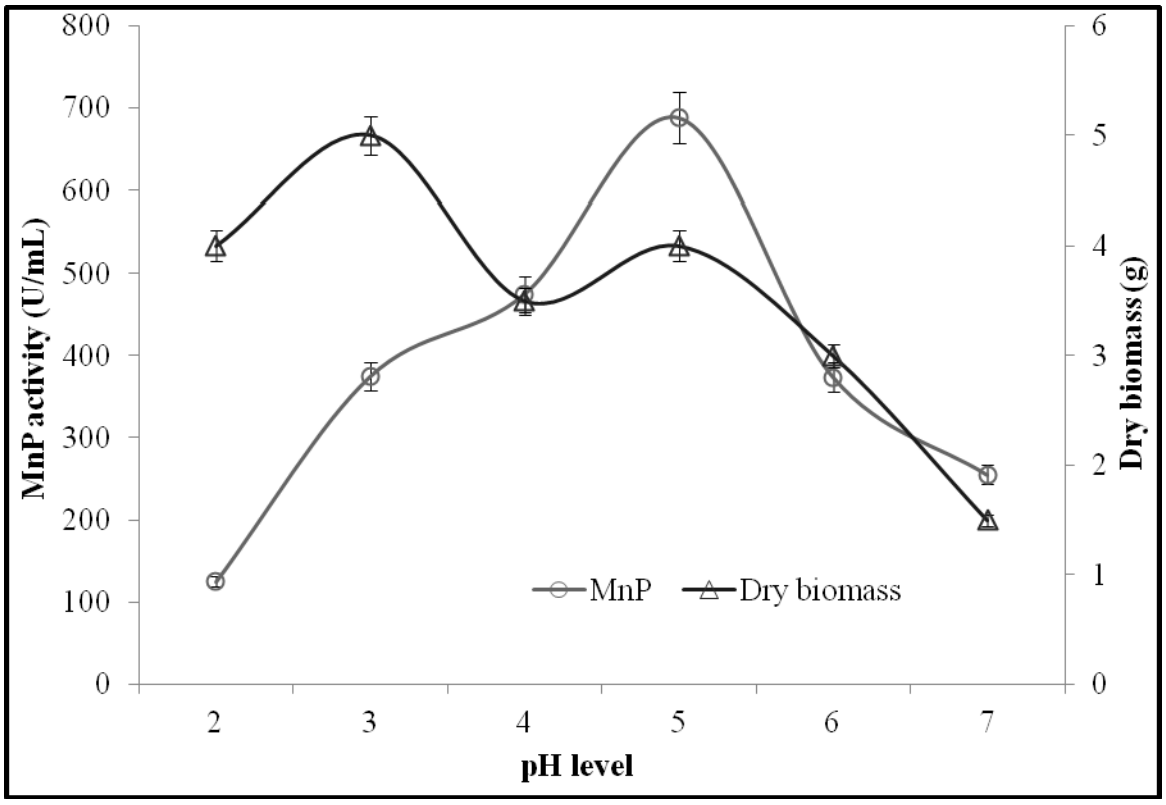


**FIGURE 2 - Effect of varying pH levels on MnP production by *G. lucidum***

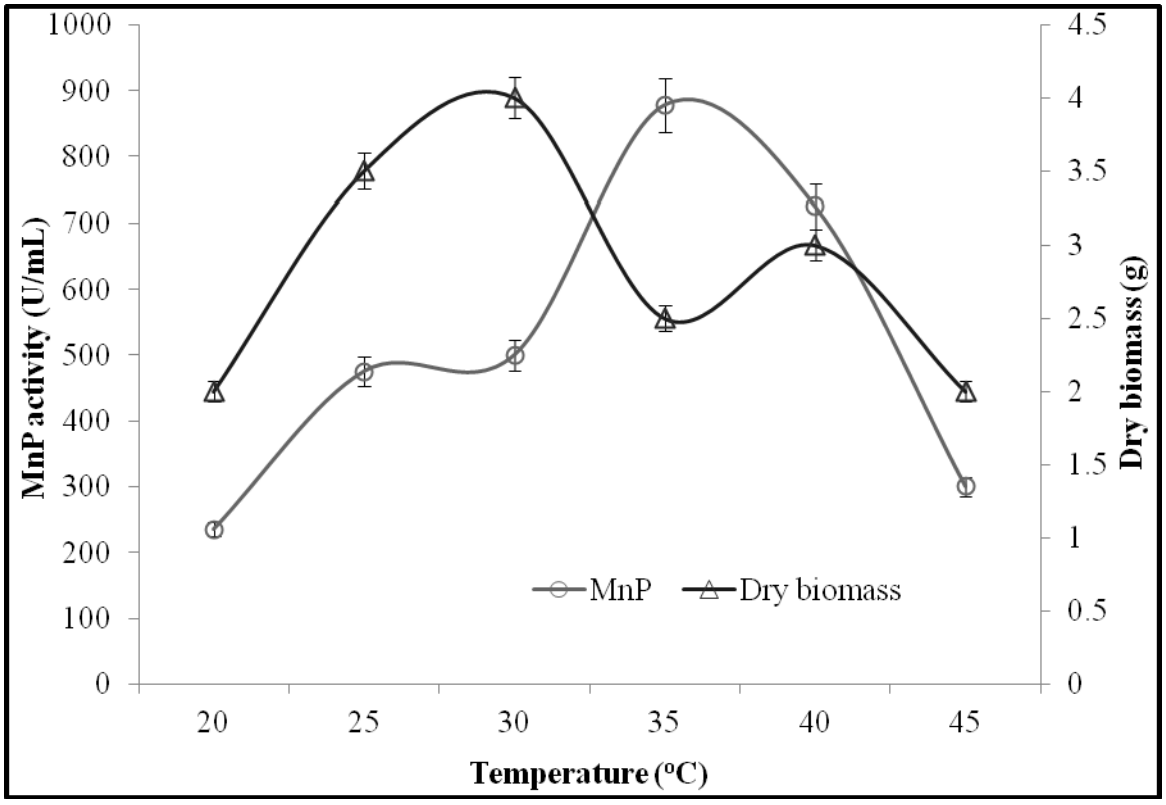


**FIGURE 3 - Effect of different temperatures on MnP production by *G. lucidum***

**TABLE 2 - Activities of ligninolytic MnP produced by *G. lucidum* with different carbon and nitrogen sources**

| Nitrogen sources (0.2% W/W) | Carbon sources (1% W/W) | | | | |
|---|---|---|---|---|---|
| | Glucose | Succrose | Fructose | Maltose | Molasses |
| **Urea** | 888±8.52 | 754±7.85 | 555±5.88 | 784±6.99 | 457±3.66 |
| **Yeast extract** | 1195±5.55 | 898±8.12 | 754±5.60 | 857±7.56 | 999±4.85 |
| **Beef extract** | 985±6.25 | 655±4.65 | 456±4.46 | 658±6.25 | 875±6.54 |
| **Bacteriological peptone** | 1002±3.52 | 457±9.85 | 654±4.36 | 555±4.85 | 422±5.25 |
| **Ammonium sulphate** | 758±6.52 | 658±6.54 | 754±6.56 | 657±6.54 | 245±3.56 |

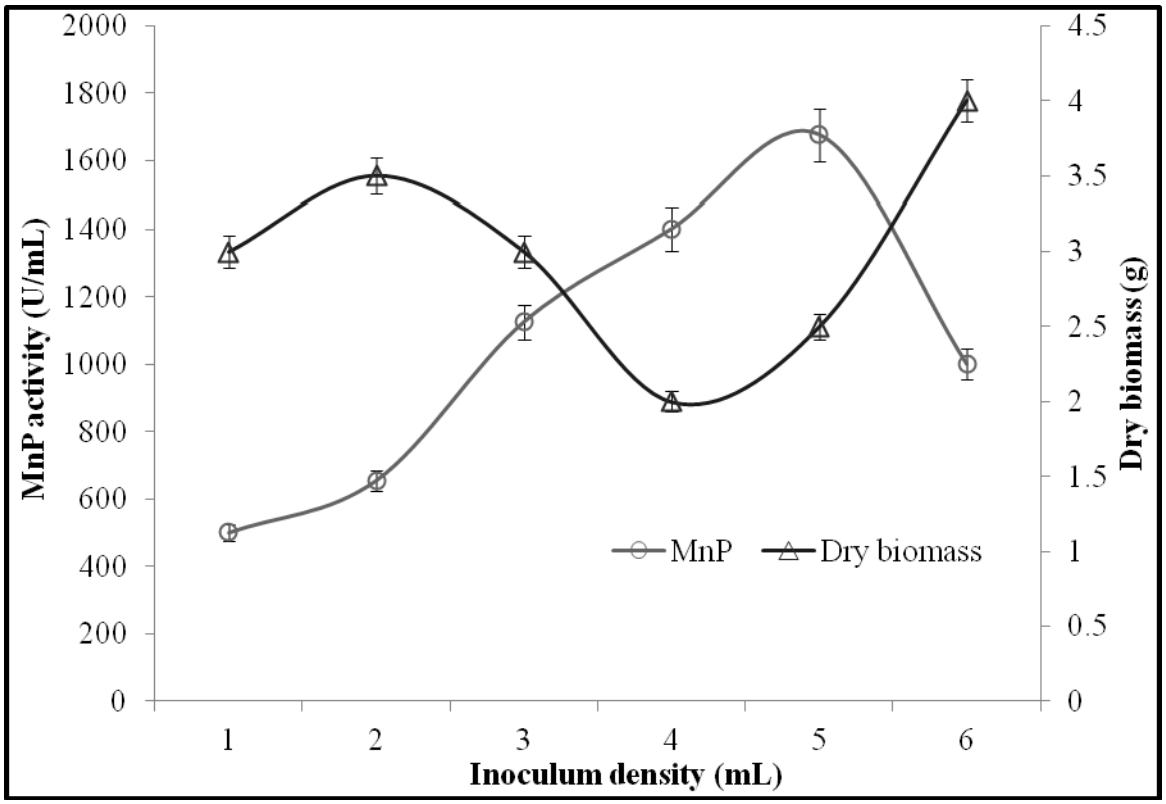


**FIGURE 4 - Effect of varying inoculums density on production of MnP by *G. lucidum***

significant impact on ligninolytic production. Tekere *et al*. [16] found that optimum temperature for cultures of *Coriolus* (*Trametes, Polyporus*) *versicolor* and *Phanerochaete chrysosporium* for ligninolytic synthesis in solid-state fermentation varied between 25-37 °C. A significant influence of incubation temperature on ligninolytic enzymes of *G.* sp. and *Dichomitus squalens* and other WRF has been reported and temperatures ranging from 25 to 37°C were found optimum [11, 17].

#### 3.2.4 Effect of carbon and nitrogen sources

To investigate the effect of carbon and nitrogen sources, Completely Randomized Design (CRD) was used. Glucose, fructose, sucrose, maltose and molasses (1%) as carbon sources along with (0.2%) nitrogen supplements like urea, yeast extract, beef extract, peptone and ammonium sulphate were used in different interaction for highest production of ligninolytic enzyme (MnP) to study their stimulatory/inhibitory effects under pre-optimized conditions. It was important to note that by the addition of carbon and nitrogen sources, the production pattern of enzymes changed; the combination of glucose and yeast extract proved best and gave maximal production of MnP (1195 ± 110 U/mL) (Table 2). The enzyme profiles varied with different carbon and nitrogen source combinations. The source and concentration of carbon and nitrogen are the powerful factors regulating the synthesis of ligninolytic enzymes by WRF [1, 18]. Different WRF show different growth and enzyme activity profiles with different additional carbon supplements because some WRF grow better under carbon and nitrogen limitations but others grow better in culture media contained sufficient carbon and nitrogen supplements. Selvam *et al*. [19] reported an easily available and utilizable additional carbon sources increase the growth and enzyme activity of WRF.

#### 3.2.5 Effect of inoculum size

Growth media containing 5g peanut shells with optimum (66.6 %) moisture content were inoculated with varying volumes of inoculum and incubated under optimum conditions. The maximum enzyme was produced in the flasks receiving 5 mL of inoculum (Fig. 4). The production of enzymes increased with an increase in inoculum from 1 to 5 mL. However, a further increase in an inoculums size caused decrease in enzyme production. Lower inoculum's level may not be sufficient to promote the fungal growth resulting in longer lag phase whereas, higher inoculum size causes faster depletion of available nutrients required for growth [1, 6, 20].

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#### 3.2.4 Effect of mediators

To study the effect of mediators on ligninolytic enzymes formation by *G. lucidum*, the flasks were supplemented with 1 mL of 1 mM solution of different mediators like veratryl alcohol, $MnSO_4$, 2,2-azinobis ethylthiazolinone 6-sulfate (ABTS), $H_2O_2$, Oxalate and 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (HBTO) to the SSF medium of peanut shells. The enzymatic analysis of extracted crude enzyme showed that different mediators have different effects on production of MnP. Veratryl alcohol and $MnSO_4$ showed notably enhancing outcome on the MnP production potential of *G. lucidum*. The maximum activity MnP (2160 ± 212 U/mL) was noted after four days in the culture supernatants of flasks inoculated with $MnSO_4$ followed by veratryl alcohol respectively (Fig. 5) whereas, ABTS had little or no affect on MnP activities. However, according to literature, reported by Lu *et al*. [21] ABTS acts as mediator for ligninolytic laccase and laccase mediated oxidation of lignin and a variety of pollutants.

## 4. CONCLUSIONS

*G. lucidum* showed remarkable potential for ligninolytic enzymes formation in SSF of peanut shells (an inexpensive lignocellulosic substrate) under optimized conditions. However, the suitability of the present enzyme for biotechnological applications can be investigated through further characterization as thermo-stability is a desired characteristic of an enzyme for its possible use in industry. In conclusion attempt was made towards finding the best growth conditions for successful cultivation of *G. lucidum*, and production of the ligninolytic enzymes.

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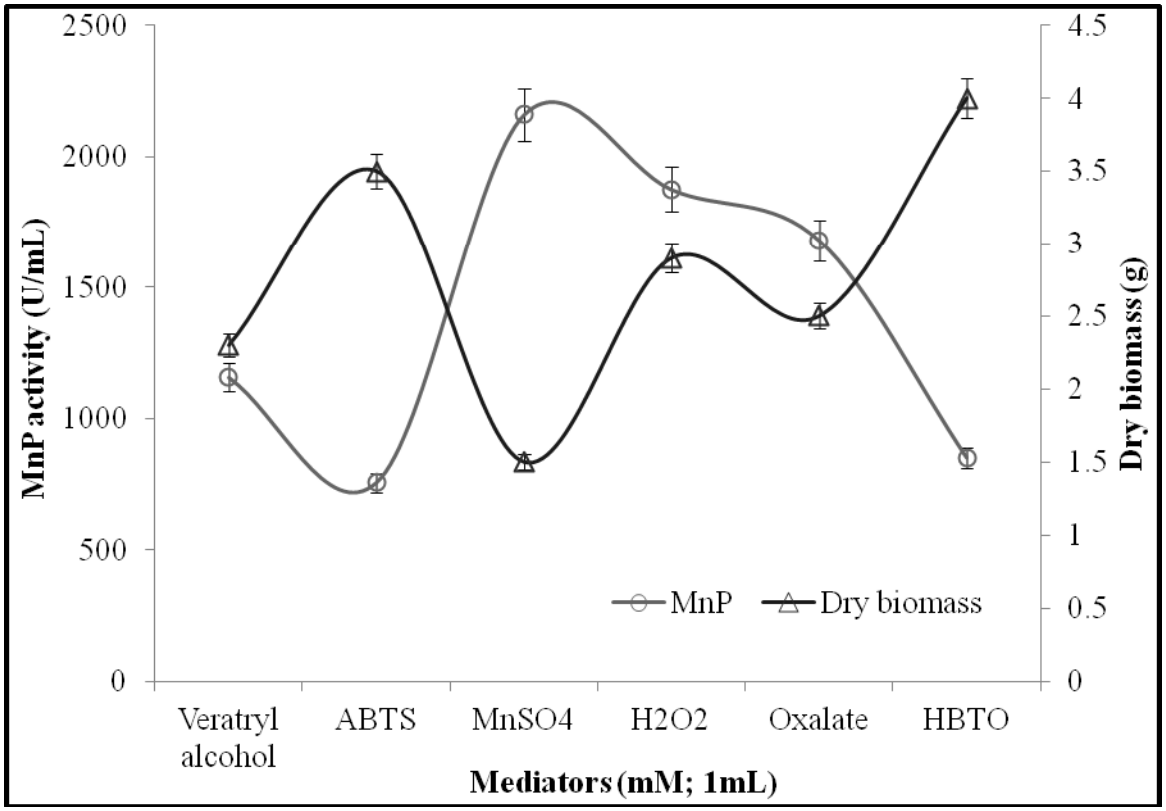


**FIGURE 5 - Effect of different mediators (1mM) on MnP production by *G. lucidum***

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