

Hyperproduction and Characterization of a Cost-Effective Manganese Peroxidase from *Pleurotus ostreatus* Using Response Surface Methodology under Solid State Fermentation

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Manganese peroxidase (MnP) is a lignin-degrading enzyme required in the ligninolytic process catalyzing oxidation of Mn^{2+} to Mn^{3+} . It has the ability to degrade nitroaromatic compounds, chlorophenols, and polycyclic aromatic hydrocarbons, all of which contribute to organic pollution. In this work, an indigenous strain of white-rot fungus *Pleurotus ostreatus* (oyster mushroom) was employed for hyperproduction of MnP using response surface methodology (RSM) under solid state fermentation through screening of local biomass. Among the parameters, 14 days incubation period, pH 4, 30 °C temperature, 8.0 g substrate concentration, 4.5 mL inoculum size, 60% moisture content, 2.87 g nitrogen supplement, and 0.082 g magnesium sulphate were found as the optimized conditions for production of manganese peroxide. Peanut shell was found as the best substrate for maximum production of enzyme (74.70 U/mL). The degradation of guaiacol as a substrate by MnP was also confirmed through Fourier Transform Infrared spectroscopy, which showed the absence of a peak of $-C=O$ at 1636.6 cm^{-1} and $-OH$ at 3331.4 cm^{-1} as structural components of guaiacol, after degradation by MnP. Peanut shell is easily available as agriculture residue. Therefore, hyper-produced MnP from *P. ostreatus* could lead to cost effective exploitation of further enzymes for industrial applications.

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INTRODUCTION

Manganese peroxidase (MnP) is an oxidoreductase enzyme with a molecular weight of approximately 40 to 50 kDa (Hoshino *et al.* 2002; Jarvinen *et al.* 2012). This enzyme can be obtained from brown-rot, soft-rot, and white-rot fungi (Eichlerová and Baldrian 2020). Moreover, MnP requires hydrogen peroxide for its activity. Therefore, it can be called a heme-peroxidase. This enzyme is involved in the oxidation of Mn^{2+} to Mn^{3+} . Because of this unique characteristic, MnP has the ability to oxidize several phenolic compounds, leading to degradation of biological pollutants (Bermek *et al.* 2004; Muhammad and Asgher 2013).

Manganese peroxidase also has potential to oxidize and break down synthetic and organic synthetic lignin (lignocellulosic material). Fungi can generate some biological acids that help chelate and stabilize Mn^{3+} ions. This enzyme has countless applications from an industrial perspective when used in pure form after being produced from various microbes. It is involved in bioremediation, as it breaks down chlorinated solvents, pollutants, and toxic organic complexes. This property enables MnP to treat polluted water and soil, helping to mitigate the harmful impact of industrial byproducts (Martin 2002; Rashid *et al.* 2018; Dao *et al.* 2019). This enzyme can selectively break down lignin, aiding in efficient fiber separation, which helps to maintain the quality of paper. It plays a role in decolorization and bleaching of textile industry waste. Industrial bleaching processes mostly have relied on harsh and toxic chemicals, posing risks to the environment. However, today MnP is being utilized to treat pulp and paper mill waste effluents, providing a safer and more ecofriendly solution of environmental pollution (Saleem *et al.* 2018; Zhang *et al.* 2018; Kumar *et al.* 2020; Chang *et al.* 2021). Manganese peroxidase can break down various harmful substances, such as dyes, antibiotics, phenolic polymers, and aromatic hydrocarbons, making it a valuable biocatalyst for reducing toxic environmental contaminants in wastewater (Kariminiaae *et al.* 2007; Mahmood *et al.* 2023). This enzyme also plays a role in pre-treatment of biomass, degradation of lignin, improvement of cellulose fiber, and hemicellulose availability, which is necessary for subsequent conversion into bio-fuel. It can also be employed in the pharmaceutical industry to catalyze the oxidation of organic polymers for the process of chemical conversion. Due to its ecofriendly properties, MnP can replace traditional catalysts, offering a more sustainable method of chemical production (Radhika *et al.* 2022; Saikia *et al.* 2023; Riffat *et al.* 2024).

Saprophytic fungi have been known as potential sources for production of the MnP enzyme. This can be efficiently produced from white rot fungus (WRF). Particularly, many scientists have worked on a WRF known as *Phanerochaete chrysosporium* (Kukreti *et al.* 2024). Several species of WRF can release enzyme MnP, for instance *Trametes hirsute*, *T. ochracea*, and *T. Versicolor*. This can also be expressed in the form of an extracellular peroxidase in some species of fungi including *Rigidoporus lignosus*, *Bjerkandera adusta*, *Dichomitus squalens*, and *Lentinula edodes* (Singh *et al.* 2025).

Manganese peroxidase can be produced from various species of fungi and bacteria. However, *Pleurotus ostreatus* (oyster mushroom) being globally cultivated fungus, stands out as the most effective fungus for this purpose. This fungus is an edible mushroom (strain of WRF) from kingdom fungi, phylum Basidiomycota and genus *Pleurotus* (Toros *et al.* 2022; Hasan *et al.* 2023). The oyster mushroom features a wide cap with an oyster shape that ranges in color from white to grey to dark brown. Oyster mushroom's gills are white and cream colored, while its margins are wavy as well as smooth (Yehia and Rodriguez-Couto 2017). This mushroom grows on the trunks of numerous trees. Cultivation of *P. ostreatus* is of significant interest and offers greater benefits than other strains. This strain offers several advantages, such as rapid mycelium growth, ability to grow on various substrates, and no requirement for fertilizer. It also grows more quickly at high temperature and across a wide pH range, produces ligninolytic enzymes, and provides significant environmental benefits. Additionally, this fungus increases yield of biomass and enhances nutritional as well as medicinal properties (Lesa *et al.* 2022; Iqbal *et al.* 2024).

Solid state fermentation (SSF) and submerged fermentation techniques are being employed to produce MnP using different substrates. In submerged fermentation, nutrients are evenly distributed, making them readily available to microorganisms. In contrast, SSF involves culturing microorganisms on the surfaces of solid substrates. The substrates

commonly used for fermentation encompass lignocellulose and grains waste, rice straw, wheat husk, barley, peanut shells, orange peel, sugarcane bagasse, banana peels, corn cobs, corn stalks, and paper waste (Robinson and Nigam 2003; Kucharska *et al.* 2018; Chen *et al.* 2024).

The enzyme's production utilizing the process of SSF is considered more reliable, as this process has a number of advantages over submerged fermentation. These advantages include easy to perform cultivation of enzyme, improved stability, increased productivity, reduced process expenses, and greater yield. Through using simple machines and utilizing less energy, SSF has reduced the procedure cost and has potential of reforming the food deposits and agricultural litters from food and agriculture industry into raw materials (Couto *et al.* 2005). Better-quality yields and successful production of MnP can have a substantial impact on generation of industrially produced useful substances. Many types of advanced and traditional procedures are being used to enhance the degradation ability of enzymes. The most common processes are optimization of parameters, purification of enzyme, biochemical analysis, or characterization. Manganese peroxidase purification and characterization is mostly accomplished by using a particular microbial strain. Due to the exceptional properties of purified MnP, such as thermal and alkaline stability, along with salt tolerance, this enzyme has numerous industrial applications (Hoshino *et al.* 2002; Kanayama *et al.* 2002; Manan and Webb 2017).

Response Surface Methodology (RSM) is an advanced statistical tool which uses designed experiments to predict one or more response variables based on effects of different independent factors and relationships between those factors. This design is applied in various studies for enhancing enzyme yields. However, this approach is not suitable for establishing any correlation between dependent and independent variables. To overcome such challenges RSM can be employed (Bezerra *et al.* 2008). This method is used to optimize all variables and analyze the final products. A range of parameters is established to plan and design the experiments and identify the optimal conditions. This optimization methodology is being utilized in different research fields including organic chemistry, biochemistry, and nanochemistry. The strategy of RSM facilitates observation, analysis, and optimization of various factors of fermentation leading to enhanced enzyme yield and activity. It can be used either for observation of impact of only explanatory variable or the collective effect of all fermentation parameters (Gassara *et al.* 2011; Yasmeen *et al.* 2013; Abdullah *et al.* 2024).

The vast amount of agro-industrial wastes has been a big problem for the agriculture sector in the world. Strategies need to be designed for efficient waste-management system resulting in decreasing agriculture pollution. Subsequently, researchers around the globe have focused on finding the ways to recycle such wastes and byproducts and to convert these wastes into valued products using enzymes. Manganese peroxidase is capable of utilizing hydrogen peroxide to oxidize various organic polymers. The primary role of manganese ions generated by MnP is to degrade lignin. As a result, MnP products have potential to be explored for a wide range of applications (Adnan *et al.* 2024; Riffat *et al.* 2024). Because of the nontoxic nature of oyster mushrooms, they have gained significant global and local attention. Additionally, they are highly important for their nutritional benefits and ease of cultivation to produce enzymes. Therefore, the present research work was designed to use the local biomass for the cultivation of *P. ostreatus* and to enhance the production yield, purification, and activity of MnP in an economical way through RSM under solid state fermentation.

EXPERIMENTAL

Materials and Methods

Growth and sporulation of Pleurotus ostreatus

Pleurotus ostreatus, an edible strain of white-rot fungus, was isolated indigenously from District Gujrat, Punjab, Pakistan. The fungus was grown on Potato Dextrose Agar (PDA) media and an optimized synthetic nutrient agar (SNA) media to compare the growth pattern and spore formation (Olana and Negassa 2020). The SNA media were prepared containing calcium chloride (0.05 g/100 mL), ammonium sulphate (0.05 g/100 mL), potassium dihydrogen phosphate (0.02 g/100 mL), and magnesium sulphate (0.02 g/100 mL) in addition to carbon and nitrogen sources. The chemicals used were analytical grade (99.99 % pure) and purchased from Sigma-Aldrich, USA. Both media were transferred into sterilized petri plates under laminar air flow and a thin slice of *P. ostreatus* was gently placed in the center of petri plates and placed in an orbital shaker (150 rpm) at 30 °C for 15 days to observe maximum fungal sporulation.

Biomass Screening

Different lignocellulosic biomass, including rice straw, peanut shells, wheat straw, and discarded paper, were collected from local areas of Gujrat, Punjab, Pakistan and used as substrates for enzyme production using SSF. All substrates were dried, ground to small particles using substrate grinder, and sieved for eliminating unwanted particles or impurities. Each of the substrates was tested with fungal inoculum media to identify the most suitable for MnP production.

SSF

Solid state fermentation was carried out for enzyme production using substrates in solid form. For this purpose, a 250-mL Erlenmeyer flask was filled with 5 g of rice straw, peanut shells, wheat straw, and discarded paper. These substrates were moistened using 5 mL to 8 mL of distilled water and subjected to autoclave to maintain sterility. To screen out the suitable substrate for enzyme production, each substrate was fermented with inoculum media. To carry out SSF, each flask comprising of solid substrate was inoculated with 3 mL inoculum of *P. ostreatus* and incubated at 30 °C for 15 days.

Enzyme Extraction

Enzyme extraction was performed by adding 50 mL of distilled water in each inoculated flask. The flasks were put into orbital shaker at 150 rpm, 30 °C, for 60 min. The fermented biomass from each flask was filtered using cheesecloth. The filtrate was poured in 50 mL falcon tubes and centrifuged at 4400 rpm for 30 min at 4 °C. Following this, supernatant containing enzyme was stored at 4 °C till further use.

Enzyme Assay

The enzyme activity of MnP was determined using guaiacol as substrate. Control and sample reaction mixtures were put in separate test tubes. For the reaction mixture, manganese sulphate 600 µL, guaiacol 300 µL, sodium succinate buffer 300 µL (4.8 pH), crude-enzyme extract 300 µL, and distilled water 1200 µL were added to the final volume (3 mL). For preparing the control, 300 µL distilled water was substituted for crude enzyme extract. Both test tubes were incubated at 30 °C for 2 min, followed by addition of hydrogen peroxide (300 µL). Reddish brown color appeared in the test tube due to oxidation of

guaiacol, and absorbance was measured at 465 nm wavelength using a UV/Visible Spectrophotometer (T80+, PG Instruments, UK) (Atalla *et al.* 2010).

RSM

The synergistic and simultaneous effect of different parameters of SSF for MnP production from *Pleurotus ostreatus* was investigated through response surface methodology. For this purpose, the effect of eight different parameters with various experimental combinations including temperature (25, 30, 35, 40, and 45 °C), pH (2.50 to 8.50), substrate concentration (2, 4, 6, 8, and 10 g), inoculum size (1, 2, 4.5, 7, and 9.5 mL), incubation period (2 to 18 days), moisture content (20 to 120%), magnesium sulphate (0.015 to 0.2175 g), and nitrogen supplement (0.15 to 2.87 g of urea) on MnP production was investigated in the first and second phases of RSM. Minitab 19 software (Minitab LLC, State College, PA, USA) was employed for the analysis of obtained experimental data. The findings of RSM were assessed, and the regression fit model was put into practice for formulating an equation and the model fitness was also evaluated. For a response variable y and predictors $x_1, x_2, \dots, x_k$, the quadratic model is,

$$y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i < j}^k \beta_{ij} x_i x_j + \epsilon \quad (1)$$

where β_0 is the intercept (mean), β_i items are linear coefficients, β_{ii} items are quadratic (squared) coefficients, β_{ij} are interaction coefficients (2FI terms), and ϵ denotes the random error.

Purification of MnP

Ammonium sulphate precipitation

For initial purification, ammonium sulphate was used to precipitate crude MnP extract as extra-cellular enzyme. Ten falcon tubes were prepared with 10 to 100% ammonium sulphate concentrations. After saturation, extract of crude enzyme was placed at ambient temperature, stirred for 60 min, and centrifuged for 15 min at 10,000 rpm. A total of 500 μ L of guaiacol was added into 0.5 mL of pellet and 1 mL of sodium acetate buffer (pH 5) and mixed well to prepare enzyme assay solution. Following incubation for 2 min, absorbance was determined at 280 nm wavelength. The pellet leading to maximum absorbance was subjected to dialysis. For this purpose, enzyme was suspended in phosphate buffer having a pH of 5 (Gassara *et al.* 2010). The mixture was put into a dialysis bag, allowed to submerge into a beaker with buffer and incubated for 24 h in a shaking incubator. The buffer was replaced during the process for removal of ammonium sulphate from the enzyme.

Gel filtration chromatography

After dialysis, the enzyme was further purified using gel filtration chromatography (SP Sephadex C-50, Sigma-Aldrich, St. Louis, MO, USA). For this, 6 g silica gel was dissolved in 100 mL distilled water and subjected to autoclave. The column was set up right on a stand. Sand was initially added, and silica gel mixture was added over it. The mixture was retained at 20 °C for 2 h. Following this, the column was equilibrated and a phosphate buffer (pH 5) was initially run in the column. The dialyzed enzyme was poured into the gel filtration column. The enzyme was eluted from the column at a flow rate of 0.5 mL/min and protein fractions were collected in different tubes. The enzyme activity was subsequently estimated through guaiacol assay at 280 nm.

Determination of Protein Content

To determine protein content of the purified enzyme, the Bradford assay procedure was followed using Bovine Serum Albumin (BSA) as standard (Ali *et al.* 2016).

Characterization of Manganese Peroxidase

The enzyme was characterized, and the effects of different physical and chemical factors including temperature, pH, and metal ions on its activity were investigated. The enzyme activity was determined through oxidation of guaiacol as substrate. For determination of optimum temperature, incubation of purified enzyme was performed at 20, 25, 30, 35, 40, and 45 °C for 1 h. For optimum pH, enzyme activity was determined at different values of pH ranging from 3 to 9, using the appropriate buffer. For determination of metal ions effect, 0.5 mL of purified MnP was mixed with different concentrations (100 to 500 mM) of CaCl₂ and ZnSO₄ prepared from 1 M stock. The solution was incubated at 30 °C for 60 min, and absorbance was measured using a UV/Visible spectrophotometer (T80+, PG Instruments, UK).

Fourier Transform Infrared Spectroscopic Analysis of Substrate Degradation

The production of enzyme was also confirmed through studying structural changes in substrate (guaiacol) *via* its degradation. For this purpose, Fourier transform infrared spectroscopy (FTIR) analysis was performed before (control) and after the addition of MnP on substrate. For control, guaiacol was mixed in distilled water instead of enzyme. The IR spectra of both control and enzyme-based sample towards degradation of guaiacol were compared to confirm MnP oxidation activity. The analysis was conducted from 500 to 4000 wavenumber (cm⁻¹) using a diamond attenuated total reflectance (ATR) FTIR plate (Model Nicolet iS 5, Thermo Fisher Scientific, Waltham, MA, USA).

RESULTS

Growth of *Pleurotus ostreatus*

An indigenous strain of *Pleurotus ostreatus* (WRF) was isolated locally from Gujrat, Punjab, Pakistan and grown on PDA media at 30 °C.

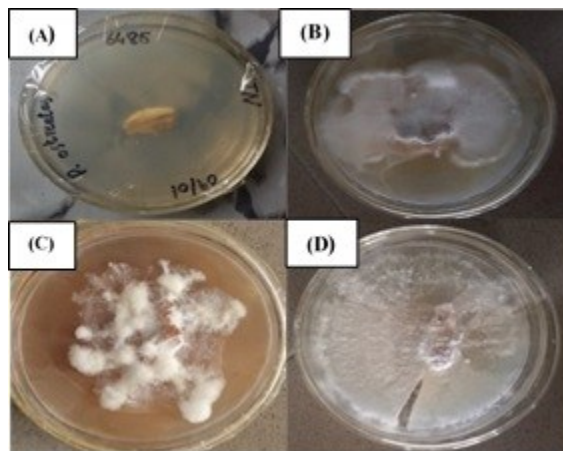


Fig. 1. Growth of *P. ostreatus* on PDA medium (A) Day 1 of incubation, (B) 5 days incubation, (C) 10 days incubation, and (D) maximum growth was observed at 14 days incubation at 30 °C

For appropriate mycelium growth, plates were incubated at 30 °C for 15 days. The fungal colonies with white color were visible after 5 days of incubation and maximum growth of *P. ostreatus* was achieved at 14 days of incubation (Fig. 1).

Sporulation of *P. ostreatus*

For sporulation, PDA and an optimized SNA media were incubated for 14 days at 30 °C. Fungal growth and spore formation were compared between both media. It was observed that PDA liquid culture exhibited less spore production (Fig. 2A), while maximum white colored fungal spores appeared on SNA medium, as shown in Fig. 2B.

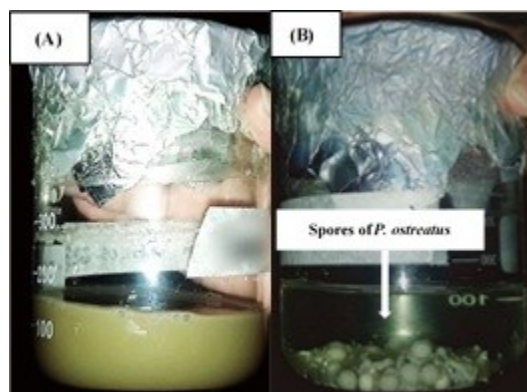


Fig. 2. Spore formation of *P. ostreatus* using different media: (A) PDA medium exhibited negligible sporulation, (B) SNA medium exhibited maximum sporulation of *P. ostreatus* after 14 days of incubation at 30 °C

Screening of Biomass for Manganese Peroxidase Production

Rice straw, peanut shells, wheat straw, and discarded paper were employed for enzyme production. Lignocellulosic biomass was chosen because of its easy availability, low cost, and eco-friendly features. The substrates were ground and dried prior to their use for enzyme production. Enzyme activity was determined using guaiacol, which showed substantial color deviations from reddish brown to the light brown. These color changes (Fig. 3) were observed due to oxidation of guaiacol by the produced MnP.

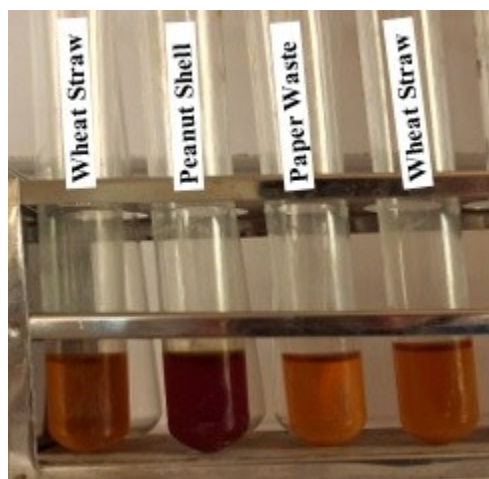


Fig. 3. MnP assay using different cellulosic substrates showing color variation due to oxidation of guaiacol

The absorbance was noted at 465 nm and enzyme activity was determined. It was found that peanut shell when employed as substrate, maximally produced manganese peroxidase enzyme with an activity of 55.7 U/mL after 14 days of incubation (Fig. 4). Therefore, peanut shell was chosen as best substrate for further experimentation.

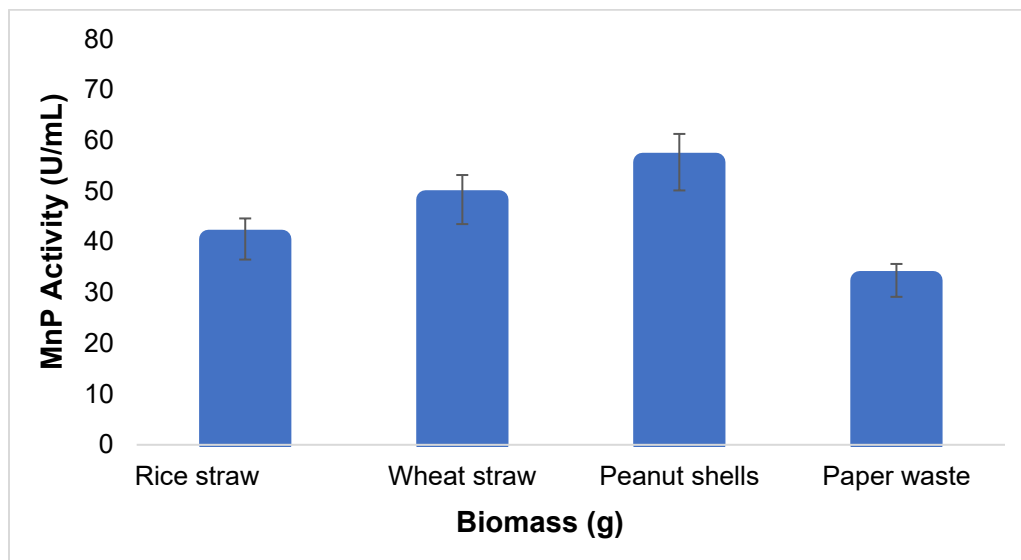


Fig. 4. Screening of different substrates for MnP production from *Pleurotus ostreatus* before applying RSM design; the maximum enzyme activity (55.73 U/mL) was shown by peanut shells as substrate.

Optimization of Fermentation Parameters Using RSM

During the 1st phase of RSM, a maximum enzyme activity of 74.70 U/mL was achieved following specific values of influencing parameters as shown in Table 1.

Combined Interaction of Fermentation Parameters During 1st Phase of RSM

The analysis of variance (ANOVA) among interaction of multiple factors to observe their significance for production of manganese peroxidase during 1st phase of RSM is shown in Table 2. Surface plot analysis was conducted to determine the combined effect of different values of pH and temperature on enzyme production. The blue area of the plot shows maximum MnP activity at 30 °C temperature with pH of 4 (Fig. 5A). The interaction between incubation period and pH was also investigated, and 14 days incubation period with pH of 4 were found as optimum values for enzyme production from *P. ostreatus* (Fig. 5B).

While studying the combined effect of substrate concentration and pH, the maximum production of MnP was found at 8 g substrate concentration and pH of 4 (Fig. 5C). The dark blue shaded area in surface plot of Fig. 5D shows maximum MnP activity at 30 °C temperature with 14 days of incubation. It was further found that when the mixture was incubated at 30 °C along with 8 g lignocellulosic substrate, maximum activity was observed (Fig. 5E) and beyond these values of temperature and substrate concentration, the activity was decreased. While comparing substrate amount vs incubation time, 8 g substrate and 14 days of incubation period were determined as optimum values for MnP production (Fig. 5F).

Table 1. Optimization of Fermentation Parameters during First Phase of RSM

Sr#	pH	Temperature (°C)	Incubation Time (Days)	Substrate Conc. (g)	MnP Activity (U/mL)
1	5.5	35	2	6	7.56
2	5.5	35	10	10	26.78
3	5.5	35	10	6	21.41
4	5.5	35	10	6	23.41
5	5.5	35	10	6	22.92
6	5.5	35	10	6	21.12
7	5.5	35	10	6	21.65
8	5.5	25	10	6	61.12
9	5.5	45	10	6	3.33
10	5.5	35	10	2	19.13
11	8.5	35	10	6	7.344
12	2.5	35	10	6	22.18
13	5.5	35	18	6	36.21
14	4.0	30	14	8	74.70
15	7.0	40	6	8	5.41
16	4.0	30	14	4	54.45
17	4.0	40	14	4	9.71
18	7.0	40	14	8	8.30
19	4.0	40	14	8	22.32
20	7.0	40	6	4	5.86
21	7.0	30	6	4	24.55
22	4.0	40	6	4	6.37
23	7.0	30	14	8	54.52
24	4.0	30	6	8	34.64
25	7.0	30	14	4	58.67
26	4.0	30	6	4	29.74
27	4.0	40	6	8	16.91
28	5.5	35	10	6	21.56
29	7.0	40	14	4	15.65
30	7.0	30	6	8	24.40

The bold values show optimum values of parameters for maximum production of MnP

First Phase RSM Equation

The presented equations (Eqs. 2 and 3) mathematically describe the relationship between manganese peroxidase (MnP) activity (response variable, Y) and four critical fermentation parameters: pH (A), temperature (B), incubation time (C), and substrate concentration (D). Eq. 2 represents a comprehensive quadratic model incorporating linear, interaction, and quadratic terms, while Eq. 3 presents a refined model retaining only the most statistically significant parameters.

The full model (Eq. 2) provides complete theoretical understanding of all potential interactions, valuable for fundamental studies of MnP activity. The reduced model (Eq. 3) may provide greater applicability for industrial applications, where simpler implementation is preferred. Both models consistently identify pH-substrate interactions and temperature-incubation time effects as critical control parameters. The models' predictions should guide future experimental designs aimed at maximizing MnP yields for industrial applications, particularly in bioremediation and biopulping processes where this enzyme shows significant promise,

$$\text{MnP Activity (U/mL)} = +140.7 + 6.491A - 9.578B + 12.43C + 5.50D \\ + 0.093A \times B + 0.035A \times C - 1.258A \times D - 0.336B \times C$$

$$-0.034B \times D + 0.051C \times D - 0.440A^2 + 0.135B^2 + 0.049C^2 + 0.264D^2 \quad (2)$$

$$y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_4D + \beta_{14}AD + \beta_{23}BC + \beta_{22}B^2 + \epsilon \quad (3)$$

where y is the response variable, A is pH, B is temperature, C is incubation time, D is substrate concentration, β_0 is the intercept (constant term), β_1 to β_4 are linear coefficients, β_{14} and β_{23} are interaction coefficients, β_{22} is the quadratic coefficient for temperature, and ϵ is the random error term.

Table 2. Analysis of Variance (ANOVA) to Observe Interaction of Multiple Factors for Production of Manganese Peroxidase during 1st Phase of RSM

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	9581.26	14	684.38	39.26	< 0.0001	significant
A-pH	274.44	1	274.44	15.74	0.0012	significant
B-Temperature	6039.23	1	6039.23	346.45	< 0.0001	significant
C-Incubation time	1798.02	1	1798.02	103.15	< 0.0001	significant
D-Substrate concentration	110.44	1	110.44	6.34	0.0237	significant
AB	7.95	1	7.95	0.4559	0.5098	non-significant
AC	0.7279	1	0.7279	0.0418	0.8408	non-significant
AD	227.90	1	227.90	13.07	0.0025	significant
BC	723.16	1	723.16	41.49	< 0.0001	significant
BD	1.88	1	1.88	0.1081	0.7469	non-significant
CD	2.66	1	2.66	0.1528	0.7014	non-significant
A ²	26.90	1	26.90	1.54	0.2332	non-significant
B ²	312.84	1	312.84	17.95	0.0007	significant
C ²	17.17	1	17.17	0.9848	0.3368	non-significant
D ²	30.76	1	30.76	1.76	0.2039	non-significant
Residual	261.47	15	17.43			
Lack of Fit	257.20	10	25.72	30.12	0.0008	significant
Pure Error	4.27	5	0.8540			
Cor Total	9842.73	29				

R-sq = 97.34%, R-sq(adj) = 94.86%

Factor Coding: Actual

3D Surface

MnP activity ((U/mL))

3.3382 74.7032

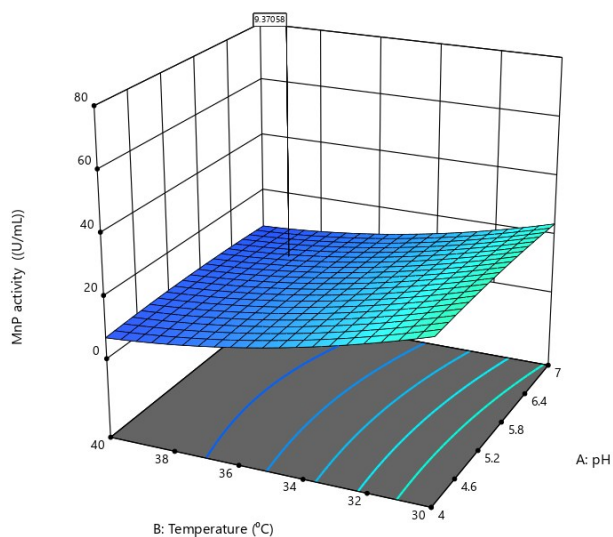
X1 = A

X2 = B

Actual Factors

C = 6.20539

D = 4.46671

**Fig. 5A.** Surface plot analysis showing interaction between pH vs. temperature for MnP activity

Factor Coding: Actual

3D Surface

MnP activity ((U/mL))

3.3382 74.7032

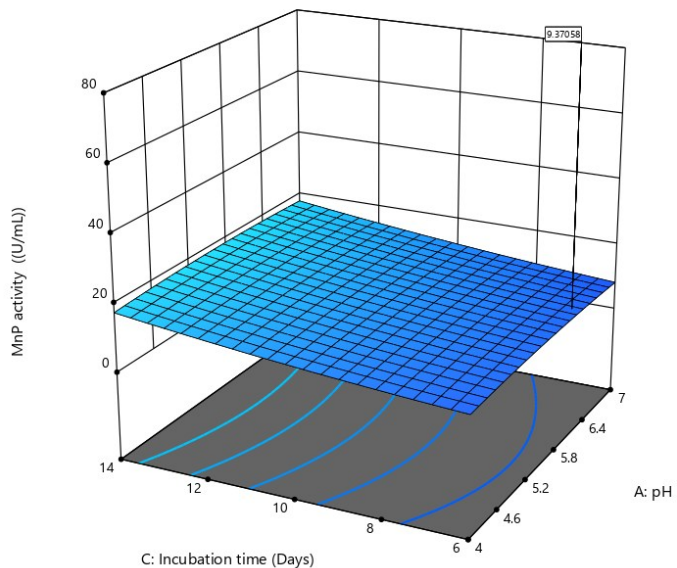
X1 = A

X2 = C

Actual Factors

B = 37.7622

D = 4.46671

**Fig. 5B.** Surface plot analysis showing interaction between pH vs. incubation time for MnP activity

Factor Coding: Actual

3D Surface

MnP activity ((U/mL))

3.3382 74.7032

X1 = A

X2 = D

Actual Factors

B = 37.7622

C = 6.20539

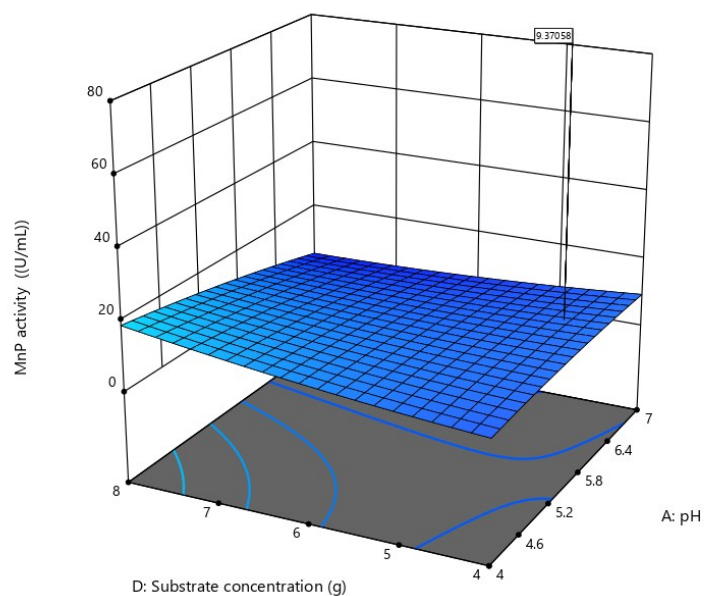


Fig. 5C. Surface plot analysis showing interaction between pH vs. substrate concentration for MnP activity

Factor Coding: Actual

3D Surface

MnP activity ((U/mL))

3.3382 74.7032

X1 = B

X2 = C

Actual Factors

A = 6.19594

D = 4.46671

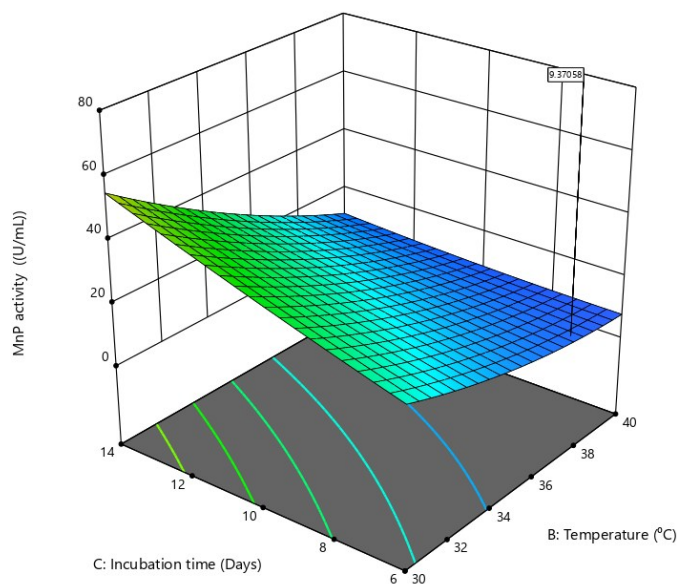


Fig. 5D. Surface plot analysis showing interaction between temperature vs. incubation time for MnP activity

Factor Coding: Actual

3D Surface

MnP activity ((U/mL))

3.3382 74.7032

X1 = B

X2 = D

Actual Factors

A = 6.19594

C = 6.20539

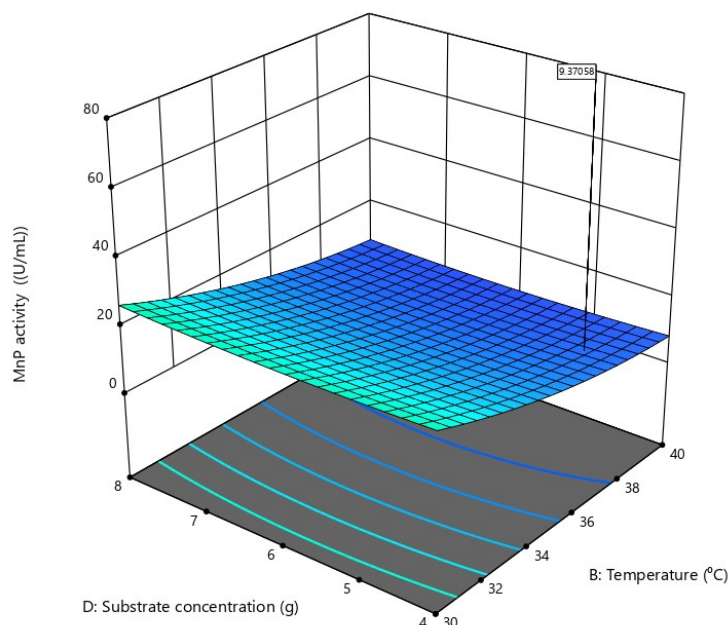


Fig. 5E. Surface plot analysis showing interaction between temperature vs. substrate concentration for MnP activity

Factor Coding: Actual

3D Surface

MnP activity ((U/mL))

3.3382 74.7032

X1 = C

X2 = D

Actual Factors

A = 6.19594

B = 37.7622

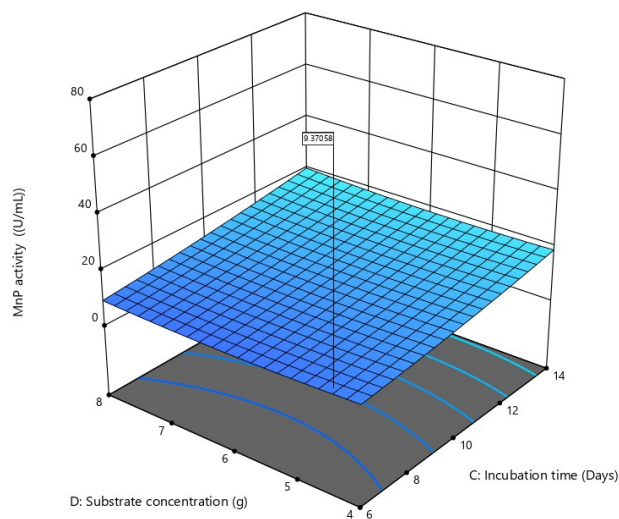


Fig. 5F. Surface plot analysis showing interaction between incubation time vs. substrate concentration for MnP activity

Table 3. Optimization of Fermentation Parameters During Second Phase of RSM

Sr#	Inoculum Size (mL)	Moisture Content (%)	Nitrogen Supplement (g)	Magnesium Sulphate (g)	MnP Activity (U/mL)
1	7.0	30	2.000	0.0150	7.49
2	2.0	90	2.000	0.1500	20.32
3	4.5	60	2.875	0.0825	62.24
4	7.0	90	0.250	0.1500	17.37
5	2.0	30	0.250	0.0150	3.63
6	2.0	30	2.000	0.1500	45.97
7	2.0	90	0.250	0.0150	4.19
8	7.0	90	2.000	0.0150	11.55
9	7.0	30	0.250	0.0150	22.18
10	2.0	90	2.000	0.0150	13.49
11	7.0	90	0.250	0.0150	18.76
12	2.0	90	0.250	0.1500	8.34
13	2.0	30	2.000	0.0150	17.50
14	7.0	30	0.250	0.1500	18.99
15	7.0	30	2.000	0.1500	29.11
16	2.0	30	0.250	0.1500	20.50
17	7.0	90	2.000	0.1500	14.00
18	4.5	60	1.125	0.2175	51.94
19	4.5	20	1.125	0.0825	16.39
20	4.5	60	1.125	0.0100	25.40
21	4.5	60	1.125	0.0825	31.47
22	4.5	60	1.125	0.0825	28.32
23	4.5	60	1.125	0.0825	29.15
24	4.5	60	1.125	0.0825	30.65
25	1.0	60	1.125	0.0825	2.29
26	4.5	60	0.150	0.0825	31.64
27	4.5	120	1.125	0.0825	2.580
28	9.5	60	1.125	0.0825	3.812
29	4.5	60	1.125	0.0825	31.62
30	4.5	60	1.125	0.0825	30.49

The bold values show the optimum values of parameters for maximum production of MnP.

Combined Interaction of Fermentation Parameters During 2nd Phase of RSM

The interaction of the remaining parameters of SSF was revealed during the second phase of RSM design for production of MnP. The findings of RSM during this phase were assessed, and the regression fit model was put into practice for formulating an equation as mentioned below and the model fitness was also evaluated. The statistical analysis was performed using Design Expert software (version 13). The comprehensive pattern of design using different values of selected parameters is shown in Table 3, which indicates maximum MnP activity of 62.2 U/mL. Analysis of variance (ANOVA) among interaction of multiple factors to observe their significance for production of manganese peroxidase during 2nd phase of RSM is shown in Table 4.

Table 4. Analysis of Variance (ANOVA) to Observe Interaction of Multiple Factors for Production of Manganese Peroxidase during 2nd Phase of RSM

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	5663.85	14	404.56	12.37	< 0.0001	significant
A-Inoculum Size	78.97	1	78.97	2.42	0.1410	non- significant
B-Moisture Content	115.26	1	115.26	3.53	0.0800	non- significant
C-Nitrogen Supplement (g)	248.48	1	248.48	7.60	0.0147	significant

D-Magnesium Sulphate (g)	570.59	1	570.59	17.45	0.0008	significant
AB	39.65	1	39.65	1.21	0.2881	non- significant
AC	358.82	1	358.82	10.98	0.0047	significant
AD	84.76	1	84.76	2.59	0.1282	non- significant
BC	36.22	1	36.22	1.11	0.3092	non- significant
BD	167.22	1	167.22	5.11	0.0390	significant
CD	115.23	1	115.23	3.52	0.0801	non- significant
A ²	1729.33	1	1729.33	52.90	< 0.0001	significant
B ²	996.62	1	996.62	30.48	< 0.0001	significant
C ²	388.40	1	388.40	11.88	0.0036	significant
D ²	28.88	1	28.88	0.8835	0.3621	non- significant
Residual	490.39	15	32.69			
Lack of Fit	481.87	10	48.19	28.30	0.0009	significant
Pure Error	8.51	5	1.70			
Cor Total	6154.24	29				

R-sq = 92.03%, R-sq(adj) = 84.59%

While comparing the interaction of inoculum size vs. moisture content, it was found that maximum activity was achieved using moisture content of 60% with fungal inoculum size of 4.5 mL, as shown in Fig 6A. The surface plot analysis between inoculum size and nitrogen supplement exhibited 4.5 mL inoculum size of *P. ostreatus* and 2.87 g urea as optimized conditions (Fig. 6B).

Factor Coding: Actual

3D Surface

MnP activity (U/mL)

2.2994 62.2459

X1 = A

X2 = B

Actual Factors

C = 1.42174

D = 0.10168

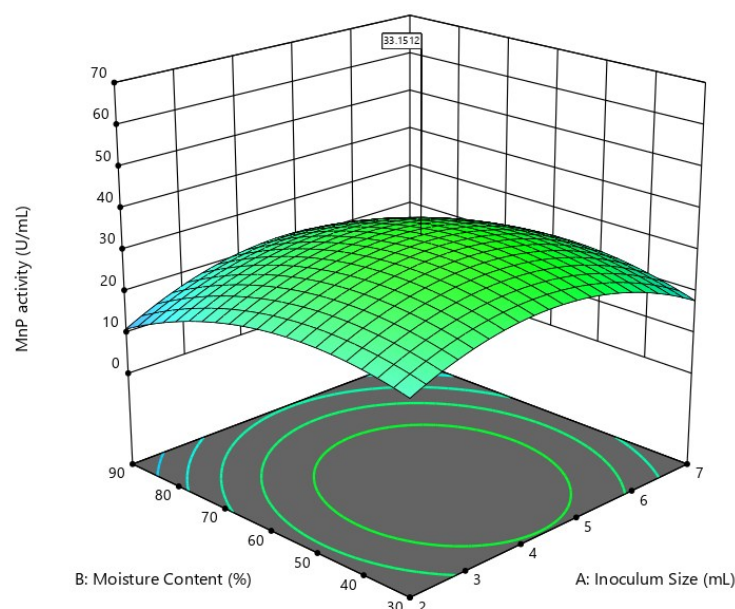


Fig. 6A. Surface plot analysis between inoculum size and moisture content for maximum production of MnP from *P. ostreatus*

Factor Coding: Actual

3D Surface

MnP activity (U/mL)

2.2994 62.2459

X1 = A

X2 = C

Actual Factors

B = 60.1322

D = 0.10168

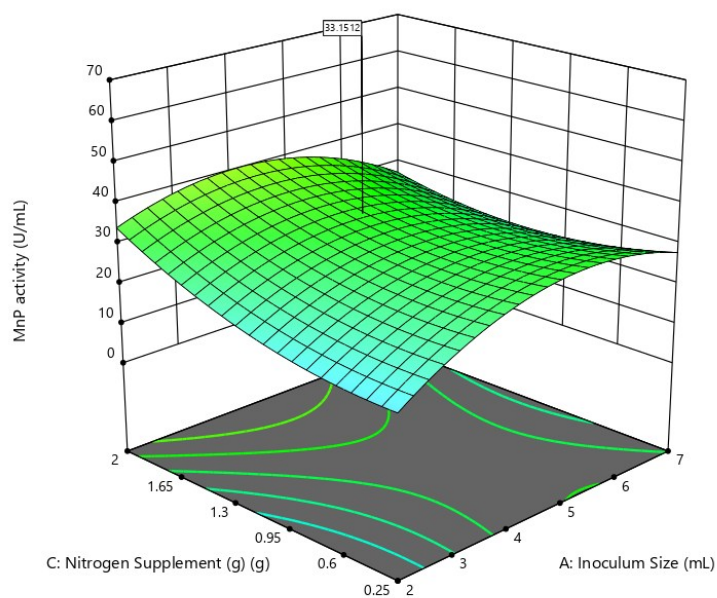


Fig. 6B. Surface plot analysis between inoculum size and nitrogen supplement

Factor Coding: Actual

3D Surface

MnP activity (U/mL)

2.2994 62.2459

X1 = A

X2 = D

Actual Factors

B = 60.1322

C = 1.42174

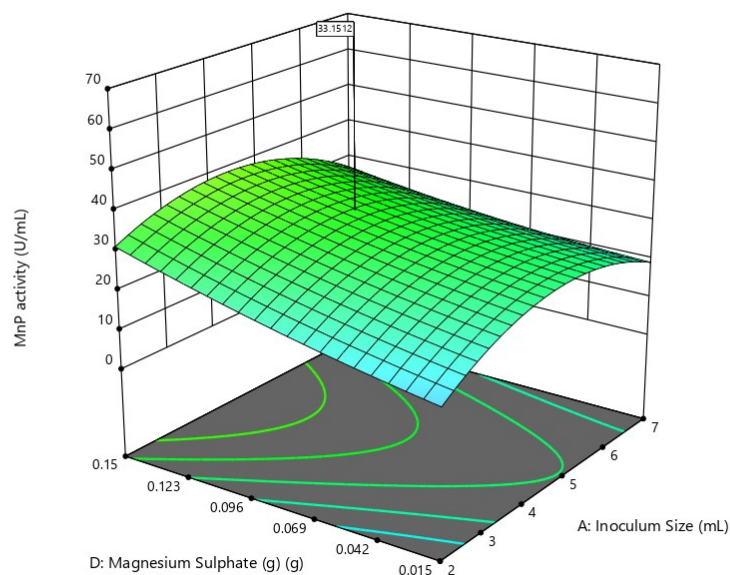


Fig. 6C. Surface plot analysis between inoculum size and magnesium sulphate

Factor Coding: Actual

3D Surface

MnP activity (U/mL)

2.2994 62.2459

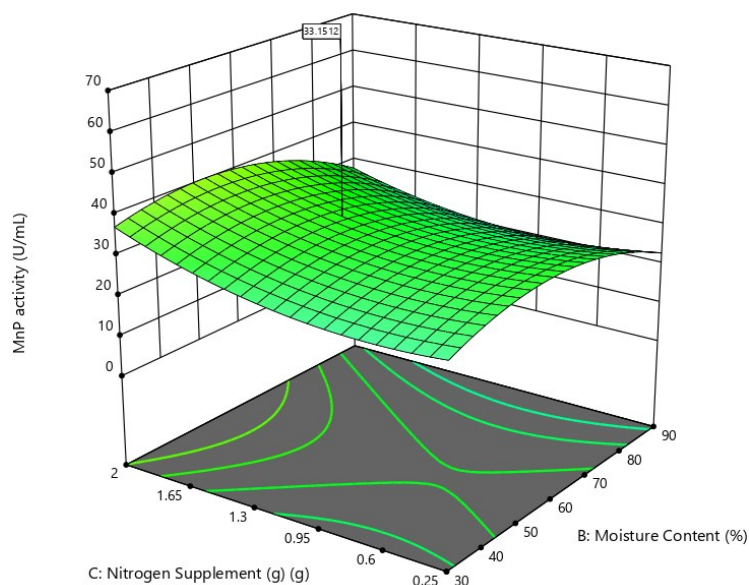
X1 = B

X2 = C

Actual Factors

A = 4.70628

D = 0.10168

**Fig. 6D.** Surface plot analysis between moisture content and nitrogen supplement

Factor Coding: Actual

3D Surface

MnP activity (U/mL)

2.2994 62.2459

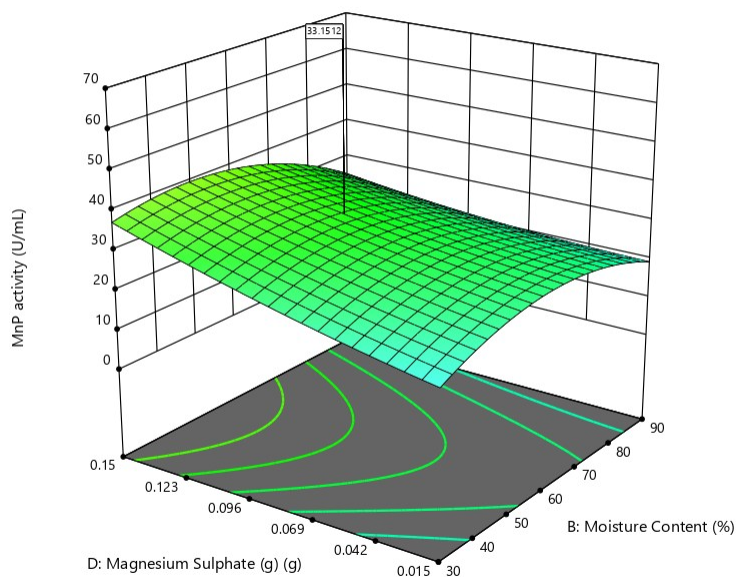
X1 = B

X2 = D

Actual Factors

A = 4.70628

C = 1.42174

**Fig. 6E.** Surface plot analysis between moisture content and magnesium sulphate

Factor Coding: Actual

3D Surface

MnP activity (U/mL)

2.2994 62.2459

X1 = C

X2 = D

Actual Factors

A = 4.70628

B = 60.1322

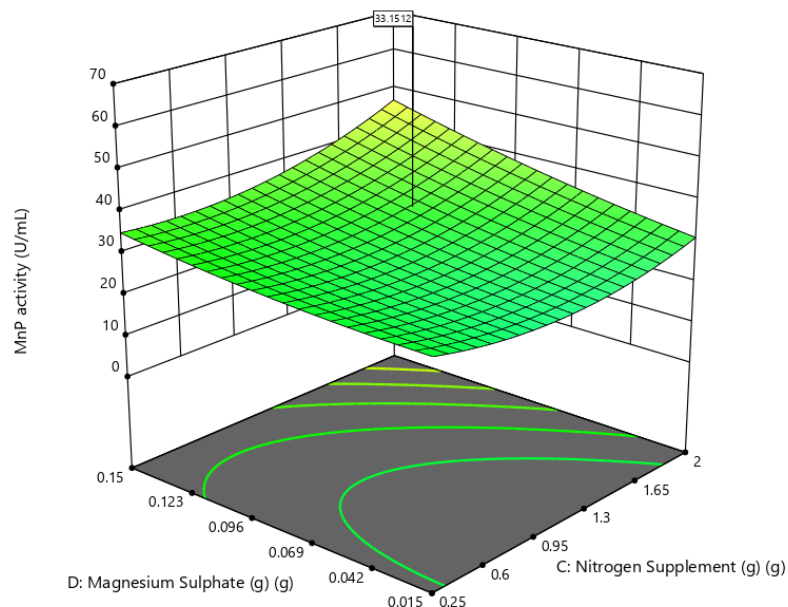


Fig. 6F. Surface plot analysis between nitrogen supplement and magnesium sulphate

The interaction between inoculum size vs. magnesium sulphate showed that more MnP activity can be obtained at 4.5 mL inoculum size and 0.217 g MgSO_4 , as shown in Fig. 6C. Meanwhile, 60% moisture content and 2.87 g nitrogen supplement (urea) revealed maximum production of enzyme, as shown in blue shaded region of Fig. 6D. The interaction between moisture content and magnesium sulphate was also analyzed through surface plot and 60% moisture content with 0.082 g of MgSO_4 were found as optimum values. In the last, the interaction of nitrogen supplement vs. amount of magnesium sulphate revealed that 0.0825 g of MgSO_4 when employed along with 2.87 g urea yielded maximum MnP from *P. ostreatus*.

Combined Interaction of Fermentation Parameters During 2nd Phase of RSM

The interaction of the remaining parameters of SSF was revealed during the second phase of RSM design for production of MnP. The comprehensive pattern of design using different values of selected parameters is shown in Table 3, which indicates maximum MnP activity of 62.2 U/mL. Analysis of variance (ANOVA) among interaction of multiple factors to observe their significance for production of manganese peroxidase during 2nd phase of RSM is shown in Table 4. Equations 4 and 5 are presented as regression models that describe the relationship between manganese peroxidase (MnP) activity (response variable, Y) and four key fermentation parameters: inoculum size (A), moisture content (B), nitrogen supplement (C), and magnesium sulphate concentration (D). Equation (4) represents a full quadratic model incorporating linear, interaction, and quadratic effects, while Equation (5) is a reduced model retaining only the statistically significant terms. Both models highlight magnesium sulphate (D) and inoculum size (A) as dominant factors in MnP production, with nitrogen (C) and moisture (B) playing secondary but interactive

roles. The full model (Eq. 4) showed complex interactions, while the reduced model (Eq. 5) provides a more streamlined approach for optimization.

Second Phase RSM Equation (MnP Activity, U/mL)

The fitting equations were as follows:

$$\begin{aligned} \text{MnP Activity (U/mL)} = & 49.47 + 16.96A + 1.014B - 0.662C + 139.3D \\ & + 0.020A \times B - 2.164A \times C - 13.63A \times D - 0.057B \times C - 1.596B \times D \\ & + 45.43C \times D - 1.542A^2 - 0.008B^2 + 6.316C^2 + 290.9D^2 \end{aligned} \quad (4)$$

$$y = \beta_0 + \beta_3C + \beta_4D + \beta_{13}AC + \beta_{24}BD + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \varepsilon \quad (5)$$

where Y is the response variable, A is the inoculum size, B is the moisture content, C is the nitrogen supplement (g), D is the magnesium sulphate (g), β_0 is the intercept, β_3 and β_4 are linear coefficients for C and D , β_{13} and β_{24} are interaction coefficients, β_{11} , β_{22} , and β_{33} are quadratic coefficients, and ε is random error

Purification of Manganese Peroxidase

After RSM, the produced crude enzyme was purified using different techniques. For this purpose, ammonium sulphate precipitation was performed to recover the extracellular MnP. It was found that enzyme was maximally precipitated at 70% ammonium sulphate concentration. The protein concentration of enzyme was estimated through absorbance at 280 nm. The enzyme was further purified by means of dialysis and gel filtration chromatography. Cell free extract of culture from fermented batch of *P. ostreatus* revealed the MnP activity of 3735 U/mL with specific activity of 34.2 U/mg. After ammonium sulphate precipitation, specific activity 38.3 U/mg was achieved with 70% saturation and 1.11 purification fold. Through dialysis followed by gel filtration chromatography, the enzyme was further purified up to 2.36 fold with specific activity of 80.8 U/mg. The purification summary of MnP from *P. ostreatus* is shown in Table 5.

Table 5. Summary of MnP Purification from *P. ostreatus* Using Peanut Shells as Substrate

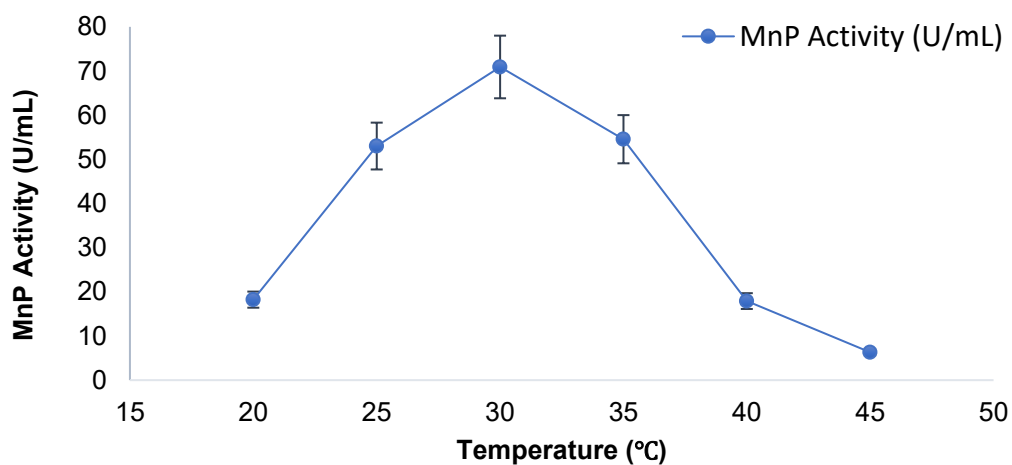
Purification Stage	Total Volume (mL)	Total Enzyme Activity (U/mL)	Total Protein Content (mg)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Cell-free supernatant	50 mL	3735	109	34.2	1	100
Ammonium sulphate precipitation	10 mL	2569	67	38.3	1.11	68.8
Dialysis	5.0 mL	1145	25	45.8	1.34	30.7
Gel filtration chromatography	1.5 mL	469	5.8	80.8	2.36	12.6

Characterization of Manganese Peroxidase

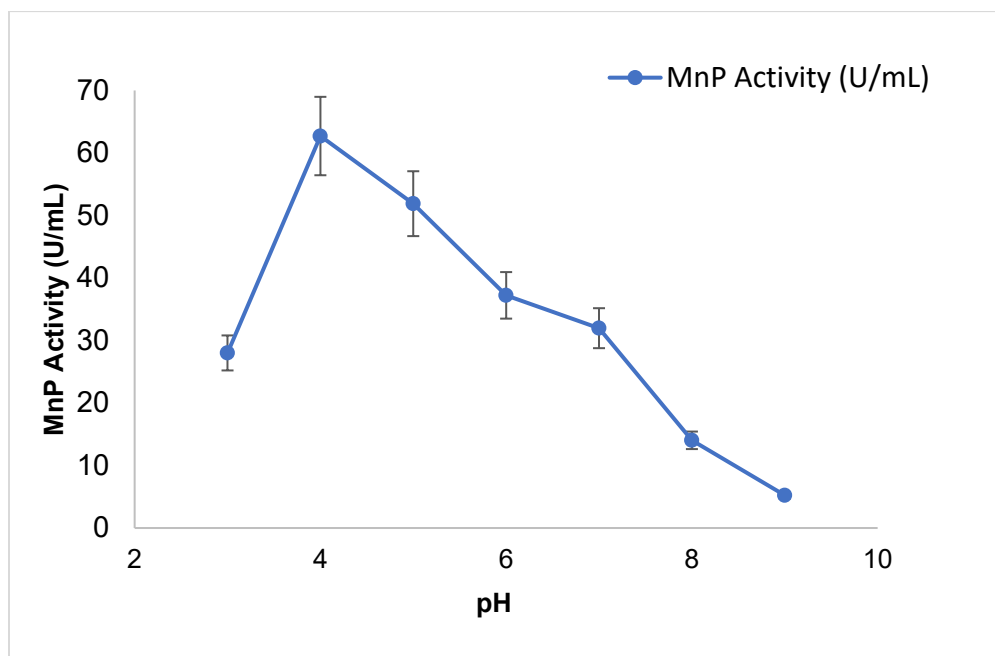
For determination of optimum temperature, the activity of purified MnP was assessed at different temperatures in the range 20 to 45 °C using peanut shell as substrate. The maximum activity of MnP (70.9 U/mL) was observed at 30 °C. However, it was decreased after 30 °C, and the enzyme was denatured at 45 °C as shown in Fig. 7A. Among different values of pH, the maximum activity was found at pH of 5 (Fig. 7B). Among metal

ions, 100 mM of Zn^{2+} and 300 mM of Ca^{2+} ions maximally increased activity of MnP to 64.6 U/mL and 57.8 U/mL, respectively (Fig. 7C and 7D).

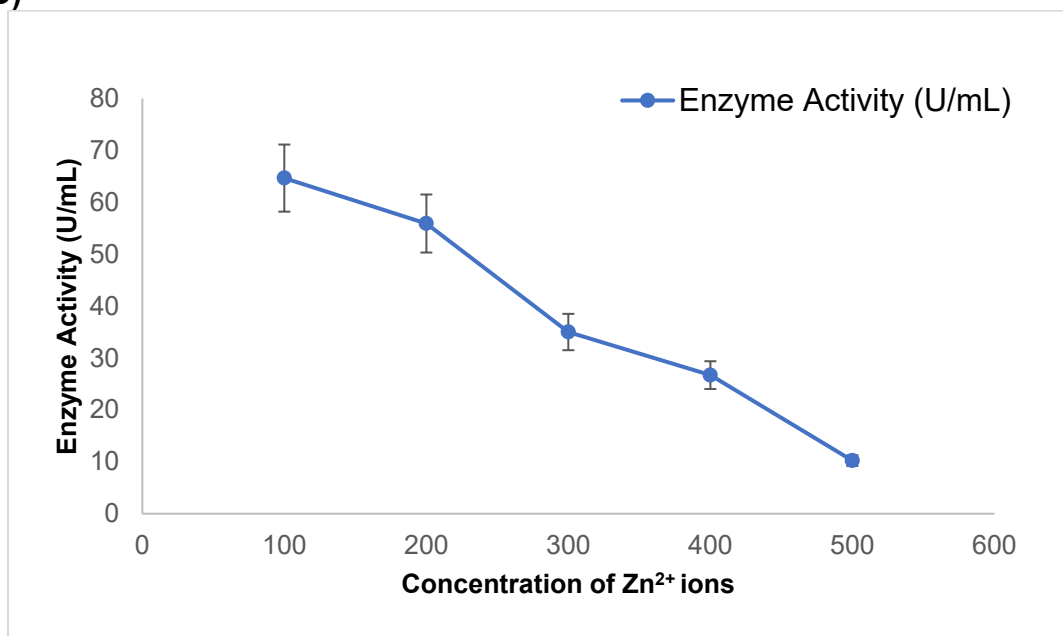
(7A)



(7B)



(7C)



(7D)

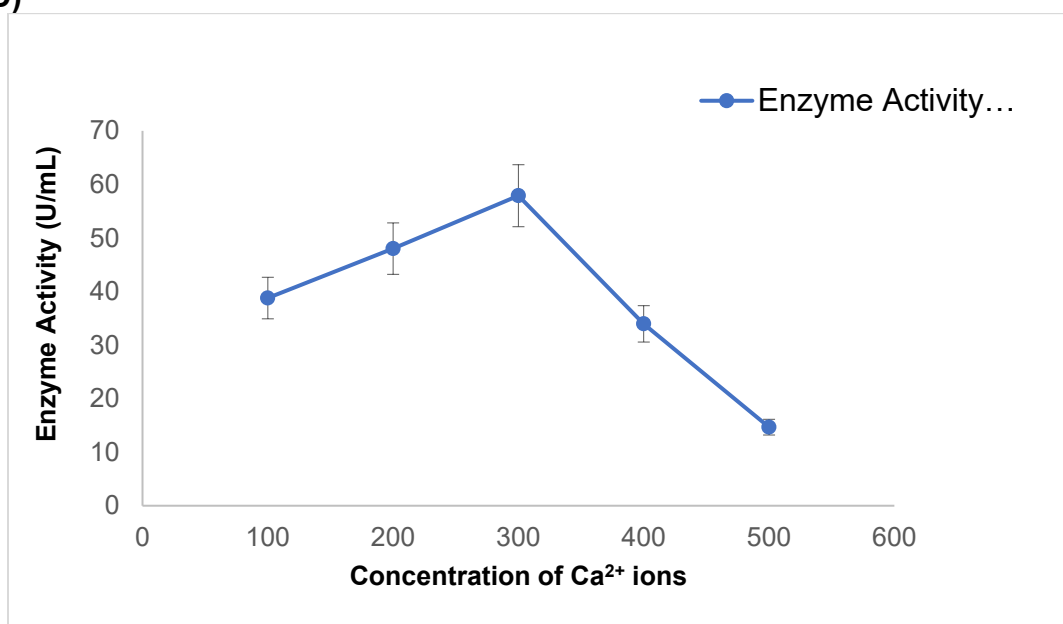


Fig. (7A). Effect of different parameters of SSF on MnP activity from *P. ostreatus*. The maximum activity was observed at 30 °C temperature, **(7B)**: Maximum activity of MnP was observed at pH 5, **(7C)**: Maximum activity of MnP was observed using 100 mM Zn²⁺ as metal ions, **(7D)**: The activity of MnP was decreased with Ca²⁺ as metal ions (300 mM) as compared to Zn²⁺ ions.

Confirmation of Substrate Degradation Through FTIR Analysis

The degradation of guaiacol as substrate by manganese peroxidase produced from *P. ostreatus* was confirmed through observation of change in the chemical structure of substrate using FTIR. The IR spectrum of control (without enzyme) showed a descending peak of -OH stretching at wavenumber of 3331.4 cm⁻¹, which confirmed the compulsory component of hydroxyl group as part of the guaiacol structure (Fig 8A). Another peak was

apparent at 1636.6 cm^{-1} (Fig 8A), which indicated -C=C stretching leading to confirmation of the presence of alkenes as a compulsory part of guaiacol structure in its aromatic ring.

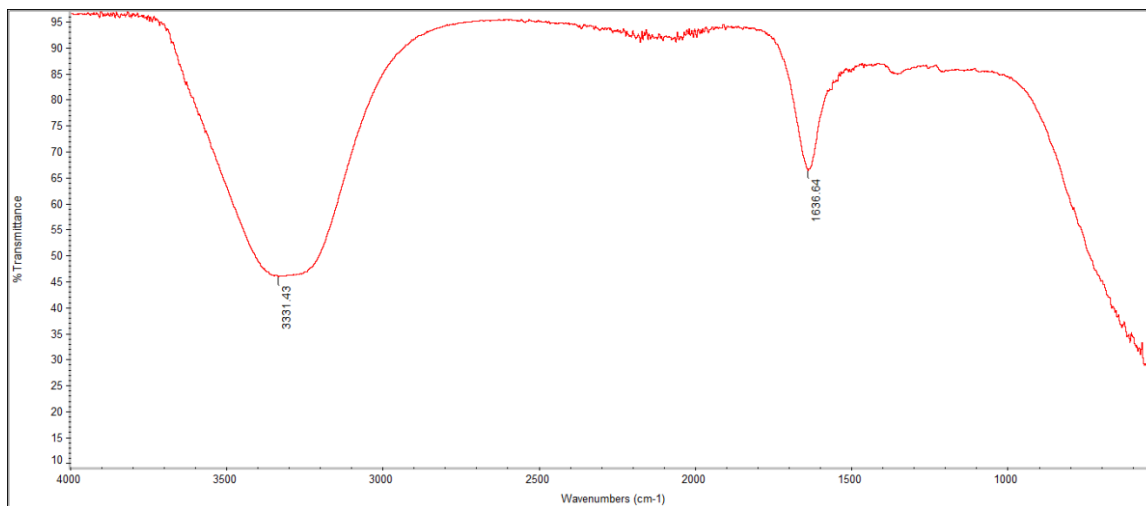


Fig. 8A. FTIR spectrum of substrate guaiacol showing characteristic peak of -C=O at 1636.6 cm^{-1} and -OH group at 3331.4 cm^{-1} , respectively, before its degradation by MnP

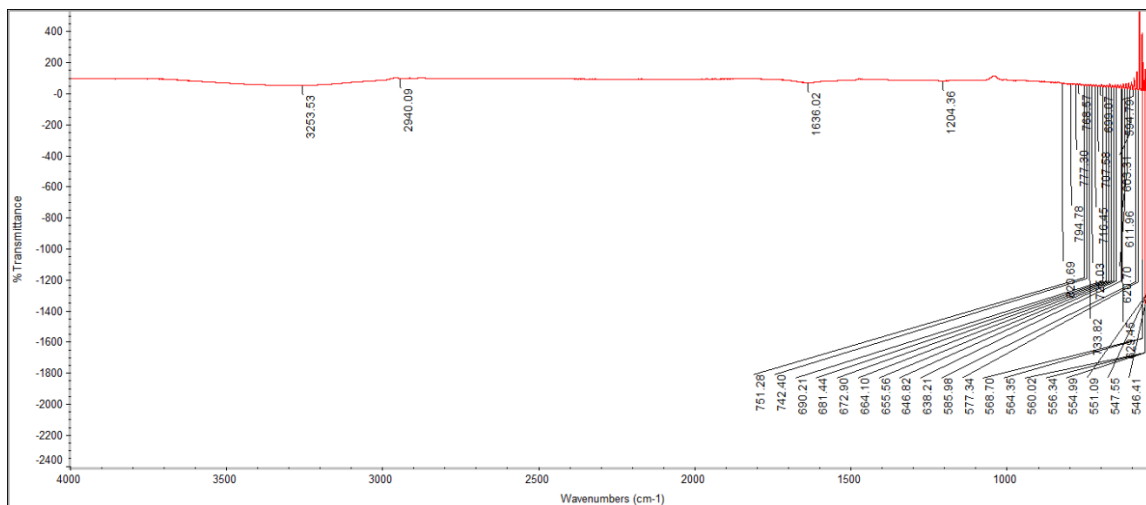


Fig. 8B. FTIR spectrum of substrate guaiacol indicating absence of characteristic peak of -C=O at 1636.6 cm^{-1} and -OH group at 3331.4 cm^{-1} , respectively, after substrate degradation by MnP from *P. ostreatus*

However, after treatment with MnP enzyme, both characteristic peaks of -OH group (3331.4 cm^{-1}) and -C=C- (1636.6 cm^{-1}) disappeared from the IR spectrum, confirming degradation of substrate (Fig. 8B). Hence, in addition to traditional methods of determination of substrate degradation through enzyme assay, FTIR analysis showed its value as a way to study and confirm the chemistry of substrates degradation by enzymes.

DISCUSSION

In the present study, an indigenous strain of *P. ostreatus* (WRF) was exploited to produce an economical, low cost, and effective MnP using RSM design through SSF. The problem of utilization of lignocellulosic wastes can be easily addressed through applications of enzymes in a natural and environment-friendly way. The PDA medium has been traditionally employed for the fungal growth (Pathania *et al.* 2018; Fernandes *et al.* 2024). However, better growth and sporulation of *P. ostreatus* were obtained with optimized synthetic nutrient agar media (SNA) containing calcium chloride, ammonium sulphate, potassium dihydrogen phosphate, and magnesium sulphate in addition to carbon and nitrogen sources. The additional nutrients of SNA culture media as compared to PDA medium additionally facilitated rapid fungal growth, which supports the maximum sporulation. The substrates were passed through milling and screening, in which raw substrates were converted to a suitable particle size. This approach was performed to encourage biomass usability and improve surface area. The peanut shells waste was found to be an ideal substrate to produce MnP from *P. ostreatus*. Similarly, other researchers have also employed biomass for enzyme production due to their easy availability and low cost (Hasan *et al.* 2023). The present study's RSM strategy was found to be more productive using peanut shell as raw substrate to achieve maximum enzyme activity as compared to other reported methods utilizing fungus *Ganoderma lucidum* (Nisa *et al.* 2014; Reddy *et al.* 2024). That biomass option successfully exhibited synergistic interactive effects of multiple parameters of SSF, which significantly reduced the time period for enzyme production.

RSM could be considered as a significant tool to enhance enzyme productions addressing various physical parameters such as pH, temperature, incubation time, substrate concentration, nitrogen/magnesium supplement, moisture content, and inoculum size. In addition, using 3-dimensional surface plots analysis can better reveal the regression analysis as these graphs present data through mesh and curves (Yasmeen *et al.* 2013).

Among the present findings, it was observed that there was strong and significant interaction among four variables (pH *vs.* substrate concentration, P-value 0.003) and (temperature *vs.* incubation time, P-value 0.000) during the first phase of RSM to produce MnP. However, the rest of the parameters exhibited non-significant interaction over enzyme production. In addition, during the second phase of RSM, significant interaction was again observed between inoculum *vs.* nitrogen supplement (P-value 0.005) and moisture content *vs.* magnesium sulphate (P-value 0.039). Hence, the role and effect of each parameter of fermentation on enzyme production can be easily accessed through RSM design. An inoculum size of 4.5 mL from *P. ostreatus* yielded better results to produce MnP, which is in agreement with others, who have also preferred using small inoculum sizes (Ramzan *et al.* 2013; Yasmeen *et al.* 2013). It is also suggested that high levels of moisture content might obstruct oxygen penetration by decreasing the spaces between substrate particles. It is also worth mentioning that magnesium sulphate seems to have a negligible impact on fungal growth for MnP production using SSF (Irshad and Asgher 2011; Yasmeen *et al.* 2013). In accordance with the current findings, values of 4 pH and 30 °C have also been reported by other researchers as optimized values for MnP production (Jiang *et al.* 2008; Oparaji and Eze 2023). Another study reports 5.0 g optimized substrate amount and pH 5 as optimum value for MnP production (Nisa *et al.* 2014). Hence, it is recommended to maintain a pH less than 5 for growth, sporulation, and enzymes production from white-rot fungi. Earlier studies suggest that using different substrates and

alterations in growth conditions of fungi affect production outcomes. It is also predicted that up to a specific limit, an increase in substrate concentration was directly proportional to MnP activity from *P. ostreatus*. Earlier studies also support the present strategy of optimizing multiple parameters simultaneously through RSM, as it was found that optimizing substrate concentration along with incubation period showed a significant synergistic effect, which enhanced enzyme activity (Shin *et al.* 2005; Elisashvili *et al.* 2009; Asgher *et al.* 2013). It was also revealed during present work that supplementing nitrogen in a lignocellulosic biomass enhanced MnP production through SSF, which is also supported in working with *S. commune* for enzyme production (Varshney *et al.* 2013). The 60% moisture content is analogous with outcomes reported earlier (Asgher *et al.* 2013), in which maximum MnP activity was observed by *S. commune* IBL-06; however, MgSO₄ seems to have a negligible impact on MnP production (Yasmeen *et al.* 2013).

In the current work, enzyme was precipitated at 70% saturation. It is predicted that a significant interaction takes place between protein and salt at 70% saturation. However, further addition of salt above 70% decreased protein solubility (Cai *et al.* 2010; Praveen *et al.* 2012; Rashid *et al.* 2018). Previously, MnP from white-rot fungi has been reported with maximum activity at 30 °C temperature.

It is also reported that MnP from *Schizophyllum commune* IBL-06 exhibited maximum activity at pH 5, MnP from *P. ostreatus* at pH 4, and MnP from *Lentinula edodes* at pH 4.5 (Boer *et al.* 2006; Cai *et al.* 2010; Asgher *et al.* 2013; Zeng *et al.* 2013; Qin *et al.* 2014; Bilal *et al.* 2015). Hence, it can be concluded that pH within range from 4 to 6 should be recommended as optimal for the MnP production from white-rot fungi. More enzyme activity was observed using magnesium ions as compared to calcium ions. The metal ions act as cofactors and can give rise to enhanced enzyme activities; however, higher concentrations reduce enzyme catalytic efficiencies (Asgher *et al.* 2013; Qin *et al.* 2014). In addition to traditional assays for determination of enzyme activities, FTIR spectroscopy was employed to observe covalent modifications in the chemical structure of substrate such as guaiacol after treatment with manganese peroxidase. The disappearance of functional group peaks of substrate clearly indicated their degradation by manganese peroxidase enzyme produced from *P. ostreatus*. Hence, it is suggested that FTIR spectroscopy could be employed for understanding degradation patterns and outputs of cellulosic substrates by enzymatic cleavage based on molecular vibrations among functional groups.

CONCLUSIONS

1. Manganese peroxidase (MnP) was hyper-produced from an indigenous strain of *Pleurotus ostreatus* using peanut shell as best substrate through response surface methodology (RSM) designing under solid state fermentation (SSF).
2. Peanut shell belongs to agro-industrial residues. Its exploitation during the present research led to the development of a cost-effective strategy for enzyme production with enhanced activity (74.7 U/mL) through optimization of various parameters including incubation period, pH, temperature, moisture content, inoculum size, substrate, substrate concentration, nitrogen, and magnesium sources.
3. Based on the present results it is recommended to scale up MnP production using RSM technique under SSF through exploitation of further indigenous fungal strains,

as it reduces the number of experiments and production periods for cost effective enzymes using agriculture waste as raw materials.

ACKNOWLEDGEMENTS

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Conflict of Interest

On the behalf of all authors, the corresponding author states that there is no conflict of interest.

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