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Study of Mangan Peroxidase (Mnp) Enzymes from *Pleurotus ostreatus* Produced using Coffee Grounds and Rice Bran as Substrates through Response Surface Methodology

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Abstract

This study aimed to optimize the production of Manganese Peroxidase (MnP) enzyme from *Pleurotus ostreatus* using agricultural waste substrates such as coffee grounds (CG) and rice bran (RB) through Response Surface Methodology (RSM) approach. Optimization was conducted using a Central Composite Design (CCD) with three main variables, namely $MnSO_4$ concentration (0.5-1.5) mM, incubation temperature (25-35) °C, and reaction time (20-30) min. The enzyme fractionation process was carried out by ammonium sulfate saturation (0-20%), (20-40%), (40-60%), followed by dialysis, and SDS-PAGE analysis. The results showed that the 0-20% fraction of CG:RB (50:50) substrate had the highest enzyme activity, which was 20.588 ± 0.141 U/mL, with the highest specific activity of 76.967 ± 0.325 U/mg. The highest protein content was found in CG:RB (0:100) substrate at 0.825 ± 0.016 mg/mL. However, the enzyme activity was lower due to the presence of non-enzymatic proteins and natural inhibitors. This study successfully demonstrated that the substrate combination of CG:RB (50:50) provided optimal results for MnP production. The Response Surface Methodology proved effective in optimizing process variables, providing great potential for biotechnology applications using agricultural waste as an environmentally friendly raw material.

Keywords: Pleurotus ostreatus, Manganese Peroxidase, Coffee Dregs, Rice Bran, RSM, SDS-PAGE

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Abstrak (Indonesian)

Penelitian ini bertujuan untuk mengoptimasi produksi enzim Mangan Peroksidase (MnP) dari *Pleurotus ostreatus* menggunakan substrat limbah pertanian berupa ampas kopi (AK) dan dedak padi (DP) menggunakan pendekatan *Response Surface Methodology* (RSM). Optimasi dilakukan menggunakan Central Composite Design (CCD) tiga variabel utama, yaitu konsentrasi $MnSO_4$ (0,5-1,5) mM, suhu inkubasi (25-35)°C, dan waktu reaksi (20-30)menit. Proses fraksinasi enzim dilakukan dengan penjenuhan amonium sulfat (0-20%), (20-40%), (40-60%), diikuti dengan dialisis, dan analisis SDS-PAGE. Hasil penelitian menunjukkan bahwa fraksi 0-20% substrat AK:DP (50:50) memiliki aktivitas enzim tertinggi, yaitu 20,588 ± 0,141 U/mL, dengan aktivitas spesifik tertinggi 76,967 ± 0,325 U/mg. Kandungan protein tertinggi ditemukan pada substrat AK:DP (0:100) yaitu 0,825 ± 0,016 mg/mL. Namun, aktivitas enzimnya lebih rendah karena adanya protein non-enzimatik dan inhibitor alami. Penelitian ini berhasil menunjukkan bahwa kombinasi substrat AK:DP (50:50) memberikan hasil yang optimal untuk produksi MnP. *Response Surface Methodology* terbukti efektif dalam mengoptimalkan variabel proses, sehingga memberikan potensi yang besar untuk aplikasi bioteknologi dengan menggunakan limbah pertanian sebagai bahan baku yang ramah lingkungan.

Kata Kunci: Pleurotus ostreatus, Mangan Peroksidase, Ampas Kopi, Dedak Padi, RSM, SDS-PAGE

INTRODUCTION

Coffee is one of the most popular beverages in the world today, only 0.2% of the coffee beans are used as beverages or consumed and the remaining 99.8% become coffee grounds (SCG) [1]. Currently, SCG is disposed of in landfills or as compost in mixed municipal waste. Their toxicity to plants and aquatic organisms makes them harmful to the environment and toxic compounds such as caffeine, tannins and polyphenols can poison aquatic ecosystems. However, burning and decomposing SCG leads to the generation of more carbon dioxide, atmospheric particles and other greenhouse gases [2]. Coffee waste has recently been widely considered for cultivation as a growing medium for mushrooms, especially the *Pleurotus genus*. In addition, SCG is a residue that contains lipids, polysaccharides, and proteins, making it attractive for chemical processing to produce high-value products [3].

Pleurotus ostreatus, also known as oyster mushrooms, have higher protein levels than other protein sources, such as beans or soybeans. They are also high in inorganic minerals and low in fat. White weathering fungi are oyster mushrooms that produce ligninolytic and extracellular enzymes such as manganese peroxidase (MnP), have the ability to adapt, remove aromatic compounds, and are able to degrade organic, inorganic contaminants through catalysis with extracellular ligninolytic enzymes such as MnP [4].

In this study, Response Surface Methodology (RSM) was used. To optimize the production of MnP enzyme from *P. ostreatus* based on variations in temperature, incubation time, and substrate concentration. The RSM method is used because it is able to analyze the simultaneous influence of various variables more efficiently than conventional approaches. The use of RSM enables the reduction of the number of experiments, identification of interactions between variables, as well as determination of optimal conditions for enhancing enzyme activity. Thus, RSM becomes a very useful method in enzymatic research, including for industrial and biotechnological applications [5].

Using this method, the effect of temperature, substrate concentration, and incubation time on the production and activity of lignocellulosic enzyme, manganese peroxidase (MnP), will be studied using RSM optimization method. These indicators can affect the stability, growth, and expression of enzymes to increase sustainable energy that is environmentally friendly.

MATERIALS AND METHODS

Materials and instruments

The tools used in this study were tools commonly used in biochemical laboratories, namely incubators, freezers (LG), analytical scales (O'Haus), centrifugators and UV-Vis spectrophotometers, a set of SDS-PAGE apparatus. The materials used were coffee grounds (CG), *P. ostreatus* strain (F0), *rice bran* (RB), PDA (Potato Dextrose Agar), corn kernels, ABTS or (2,2′ - azinobis - 3 - ethyl benzothiazoline – 6 - sulfonate), H_2O_2, acetic acid, CH_3COONa, $MnSO_4$ substrate, sodium acetate buffer, BSA (bovine serum albumin), distilled water, cellophane membrane, Lowry reagent, phosphate buffer, NaOH, and sodium potassium tartrate. Stacking gel (4%) and separating gel (10% or 15%), sample loading buffer, Tris-Glycine-SDS buffer, pH 8.3 Coomassie Brilliant Blue R-250.

Sterilization and preparation of PDA media

Materials (such as PDA media) and apparatus (such as glassware) were sterilized using autoclave at 121^{o}C, 15 psi, for 15 minutes *P. ostreatus* culture (F0) was cultured on PDA media for 7 days with a modified method [6]. To generate the inoculum (F1), seeds were prepared in glass bottles along with uncontaminated maize seeds as substrate. Then, the seeds were incubated for two weeks at 25-35°C.

Substrate preparation and inoculation

Coffee grounds (CG) and rice bran (RB) were soaked in 1% $CaMg(CO_3)_2$ solution for 7 days. After 24 hours the cleaned CG and RB were mixed with variations of CG:RB (100:0)%, CG:RB (75:25)%, CG:RB (50:50)%, CG:RB (25:75)%, and CG:RB (0:100)%. The sterilized substrate was inoculated with *P. ostreatus* inoculum (F1) and then the substrate was incubated at 30°C with 70-80% humidity. After seven days on each day, the diameter of fungal colonies on PDA media was measured and for 23 days, the length of mycelium was measured on CG and RB substrates using a caliper [7].

Enzyme extraction

Cultured *P. ostreotus* was added phosphate buffer (pH 7.2) in a ratio of 1:3 (b/v) and then was centrifuged at 5000 rpm 4^{o}C for 15 minutes [8]. The enzyme crude extract was purified using ammonium sulfate through the salting out method. There are three saturation levels (0-20%, 20-40%, and 40-60%). Dialysis is performed in 0.02 M phosphate buffer (pH 6) at 5-10°C for one day [9].

Determination of protein content and activity test

The amount of protein in the crude enzyme extract was calculated using the Lowry technique using BSA standards. 0.1 ml solution was taken from each concentrated enzyme fraction and 5 ml of Lowry D reagent (Copper reagent) was added to each sample. Then, shaken and allowed to stand for 10 minutes at room temperature, added 0.5 ml of Lowry reagent E (Folin-Ciocalteau reagent), incubated with shaking for 30 minutes at room temperature. Absorbance of samples were measured at $\lambda = 600$ nm [10].

Mangan (IV) Sulphate ($MnSO_4$) solution was prepared as a substrate with a concentration of 1 mM. ABTS was prepared with a concentration of 0.5 mM, 0.1 M sodium acetate buffer pH 4.5-, and 1-mm hydrogen peroxide. 160 µL $MnSO_4$ solution was taken as much as, 100 µL enzyme extract, 1.64 ml Na-acetate buffer pH 4.5, 50 µL H_2O_2 1 mM were added into, and 50 µL ABTS 0.5 mM. The mixture was incubated 50 µL for 25 minutes, 50 µL to allow the MnP enzyme to oxidize $MnSO_4$. 2M NaOH was added when the reaction was to be stopped by adding 40 µL. Finally, the absorbance of the solution was measured at $\lambda = 420$ nm [11]. A measure of the catalytic efficiency of the enzyme called enzyme specific activity was measured as enzyme activity per unit mass of protein (U/mg). A higher level of specific activity indicates that the enzyme sample is purer [12].

SDS-PAGE analysis

The separating gel is prepared in advance with 15% acrylamide or bis-acrylamide, and a layer of stacking gel of 4-15% to ensure protein denaturation, the sample protein was then mixed with loading buffer in a 50:50 ratio and heated using a water bath at 95°C for five minutes. Marker proteins were loaded into different wells with 10-20 µL protein sample. Electrophoresis was carried out with a voltage of 80 V until the protein passed through the gel stacking and continued with a voltage of 120 V with protein separation in the gel separation. Electrophoresis is completed, for the gel staining process is soaked using Coomassie Brilliant Blue solution for 30 minutes to 1 hour. The gel is then rinsed using a stain remover solution until the protein bands are clearly visible [13].

RSM optimization Analysis

To evaluate and optimize the effect of independent variables on a particular response, Response Surface Methodology (RSM) using a Central Composite Design (CCD) experimental design. In this study, three variables of $MnSO_4$ concentration (0.5, 1, 1.5 mM), incubation temperature (25, 30, 35°C), and incubation time (20, 25, 30 minutes) were analyzed at three levels (bottom, middle, top points) with $\alpha = 1$. To improve reliability, the experimental design incorporated twenty combinations including repetition at the midpoint. Software such as Design-Expert 13 was used to analyze the data using multivariate regression. To generate ideal parameters, this program was used as well. To ensure the accuracy of the results, each experiment was conducted in triplicate [14].

RESULTS AND DISCUSSION

Mushroom growth on PDA

P. ostreatus grew significantly on PDA media for seven days. On day-0, the colony diameter increased from 0.7 cm on the 0 day to 9 cm on the 7th day, as shown in **Figure** 1. Measurements with two averaged diagonals ensure accurate and effective results without over-measurement [15]. These growth curves were very useful used as a standard reference to compare other media, such as coffee grounds substrate.

(a) (b) (c)

Figure 1. Growth of *P. ostreatus*on on PDA media for (a). 1 days, (b). 5 days, (c). 7 days.

The three phases of the logistic pattern of the mycelium growth curve, Lag phase (day 0 to day 1) showed slow initial growth (D = 0.7 to 2.1 cm), indicating mycelium adaptation to PDA media. Log phase was occurred from the 1st to 5th day. The growth significantly increased which measured based up on the diameter, for example unincreased number from 3.8 to 7.8 cm were observed on x to y day. This indicated that media or nutrient of PDA was utilized for growth. At the end of Stationary phase (day-6 to day-7): due to lack of space and nutrients, growth begins to slow down as it approaches the physical limit of the medium (9 cm).

In previous research on the growth of *P. ostreatus* mushrooms on PDA media has several growth phases which on days 0-2 lag phase (adjustment) mycelium adapts, days 3-7 log phase (exponential) mycelium grows fast and spreads, days 8-12 stationary phase (maximum growth) mycelium fills the petri dish, and day 13 and above the death phase (decreased growth) nutrients run out, growth slows down so that mycelium can die [16].

Over 23 days, different growth patterns were observed on CG (Coffee Dreg Waste) and RB (Rice Bran) media with different ratios. Due to the high

lignocellulose content and the amount of carbon, nitrogen, and phenolic compounds, which are essential nutrients for fungal growth, CG:RB (50:50) medium was most suitable for use (Figure 2). The production of manganese peroxidase (MnP) enzyme in *Pleurotus ostreatus* fungus usually occurs from log phase (exponential) to stationary phase. This is because MnP functions to catalyze the de-polymerization of plant lignin as part of the ligninolytic enzyme complex, which aids fungal growth on lignin-rich substrates [17].

CG substrate is added to RB media to support optimal growth because it can increase the overall nutrient content of the media, accelerate growth and increase mycelium colonization rate [18], while RB media alone has a fairly slow growth because it is not suitable as a single media due to pH instability, poor physical structure, and nutrient imbalance. For media that has the slowest growth and is a less optimal substrate is CG (100) due to the high content of inhibitors including phenolic acids (chlorogenic acid, caffeine, and tannins) in coffee grounds which can inhibit the operation of fungal enzymes at the initial colonization stage [2]. On all three substrates, the mycelium growth pattern followed a logistic curve: lag phase (day 0 to 2), log phase with exponential growth (day 3 to 15), fastest growth on CG:RB (50:50) and CG:RB (25:75), and stationary phase (after day 20) due to lack of nutrients and limited space.

Extraction and fractionation

For enzyme extraction, 50 grams of fungus and 50 ml phosphate buffer media at pH 7.2 were mixed in a ratio of 50:50 (b/v), the initial supernatant obtained was ± 85 ml after the centrifugation process. To maintain the activity and stability of the lignocellulolytic enzyme, phosphate buffer at pH 7.2 was chosen because it is close to the ideal physiological pH [19].

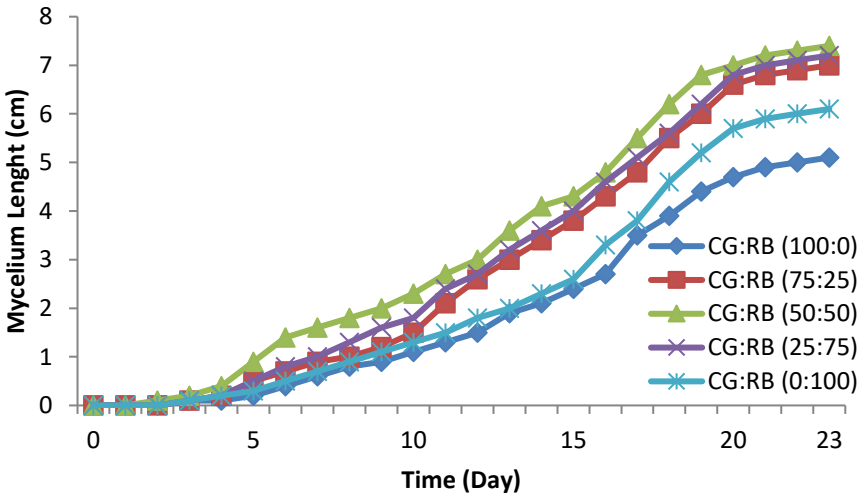

Figure 2. Mushroom growth curve on coffee grounds (CG) and rice bran (RB) media.

Table 1. Extraction and fractionation results

Type	CG (100) mL		CG:RB (75:25) mL		CG:RB (50:50) mL		CG:RB (25:75) mL		RB (100) mL	
	A1	B1	A2	B2	A3	B3	A4	B4	A5	B5
Crude enzyme extract	10	10	10	10	10	10	10	10	10	10
Fraction 0-20% + Buffer	11.3	11	11	12	12.5	12	13.3	13	13.5	13.2
Fraction 20-40%+ Buffer	7	7	8	9	10	9.7	9.5	10	7	7.5
Fraction 40-60%+ Buffer	1	1	2	3	3.7	4	3	2.6	2	1
Initial supernatant	±85	±85	±85	±85	±85	±85	±85	±85	±85	±85

Using ammonium sulfate, fractionation was carried out in three stages this is shown in **Table** 1. where saturation is 0-20%, with a high fraction volume yield of 11-13.5 ml due to high protein solubility and starting to precipitate. For protein saturation of 20-40%, medium solubility proteins precipitated with a volume yield of 7-10 ml. However, 40-60% saturation of proteins with these characteristics remain solubility and there is precipitation only that the amount of precipitation has a small volume of 1-4 ml [20]. Because the salt ions compete with water molecules, so that the precipitation process requires a decrease in

protein solubility. As a result, the protein is denatured only partially and precipitates [21]

Protein content

The linear relationship between absorbance (600 nm) and protein concentration (ppm) is depicted by the BSA standard curve in **Figure** 3 which has a linear equation (y = 0.001x + 0.048). This is done from the chemical reaction between Cu^{2+} ions and peptide groups on proteins. Then, the Folin-Ciocalteu reagent is reduced by aromatic amino acid residues such as tryptophan and tyrosine [22]. The Lowry method is used because it has a high sensitivity in detecting proteins.

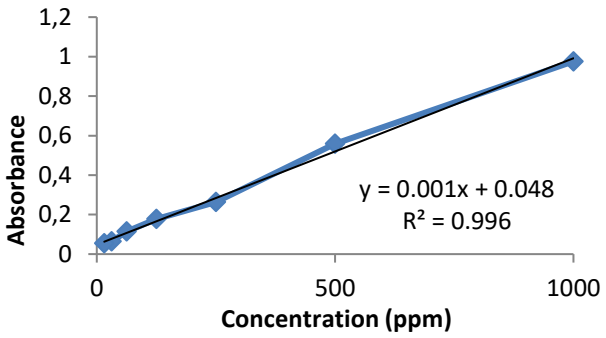

Figure 3. BSA standard curve.

In **Figure** 4, CG:RB (100:0) yielded 343-563 ppm of protein in the crude extract; most of the others were precipitated between 0-20%; 20-40%; and 40-60%. The protein content of the crude extract (701-733) ppm was shown by the mixed fraction CG:RB (75:25), which indicates the amount of protein dissolved in the substrate. After saturation conditions are reached, solutes that cannot enter the solution will directly form precipitates between 0-20%; 20-40%; and 40-60%. Significant protein levels were found in the mixed fractions of 0-20%; 20-40%; and 40-60%. CG:RB (50:50) showed the highest protein levels (764-827) ppm, CG:RB (25:75) showed the highest protein levels (789-801) ppm, and CG:RB (0:100) showed the highest protein levels (813-836) ppm. This indicates that the combination of substrates increases protein complexity (**Table** 1**).** Ammonium sulfate added to a protein solution causes the protein to precipitate through a "salting out" process. This occurs because the ammonium sulfate ions cause an increase in the ionic strength of the solution, which in turn reduces the solubility of the proteins, so that the more soluble proteins will precipitate out [23]. During dialysis, certain proteins that dissolve at higher ion concentrations may remain in the supernatant. As a result, the total protein concentration measured after dialysis decreases [24].

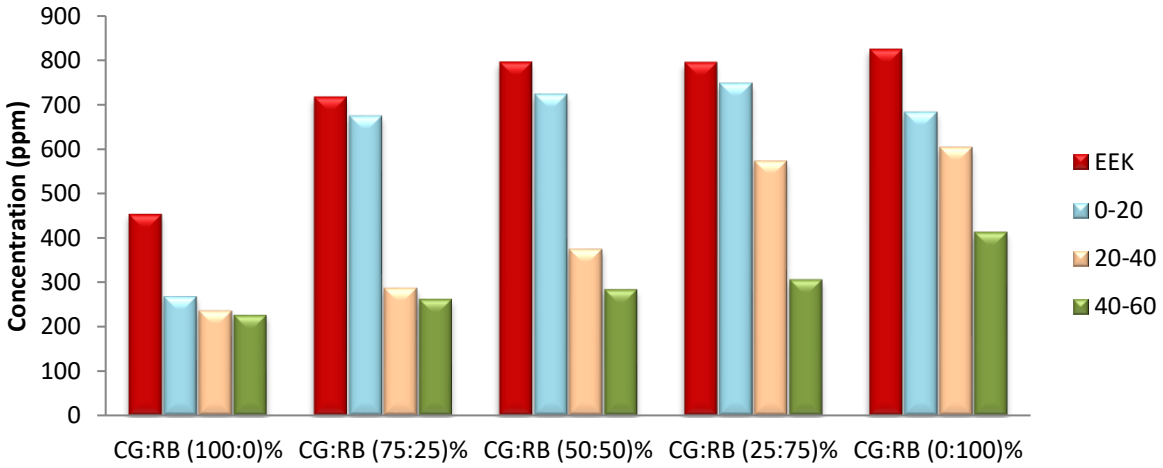

Figure 4. Graph of protein concentration on each substrate.

Result of activity test and specific activity of the MnP enzyme

Manganese Peroxidase (MnP) belongs to the oxidoreductase family, specifically acting on peroxides as acceptors (peroxidases), is an extracellular hemeprotein that catalyzes the O_2-dependent H_2 oxidation of lignin derivative-based polymers. This enzyme is the most common lignin degrader and has great potential application in agriculture for the degradation of some cellulose, hemicellulose and lignin, etc. [17].

In CG:RB (50:50) and CG:RB (75:25) substrates, the highest activity was found in the 0-20% and 20-40% fractions, with 20.588 ± 0.141 U/mL and 19.922 ± 0.015 U/mL, respectively (**Table** 2), indicating enzymatic dominance of active proteins. Increasing the

temperature to an idle point can increase enzyme activity [25]. In contrast, CG:RB (0:100) and CG:RB (75:25) mixtures had high protein content of 0.825 ± 0.016 mg/mL and 0.795 ± 0.008 mg/mL, respectively, but lower enzyme activity, due to other proteins in the sample, such as structural proteins or other enzymes that did not contribute to the measured activity or the presence of high inhibitors such as caffeine [26]. With a fraction of 0-20%, precipitation with ammonium sulfate proved effective in separating MnP from other proteins or contaminants in the cellular mixture. This is a key target for optimizing enzyme activity.

CG:RB (50:50) 0-20% and CG:RB (100:0) 20-40% fractions had the highest specific activities, with values of 76.967 ± 0.325 U/mg and 69.389 ± 1.827 U/mg, respectively. CG:RB (100:0) EEK and CG:RB (75:25) EEK substrates had the lowest specific activities, which were 18.511 ± 1.174 U/mg and 20.444 ± 0.473 U/mg, respectively (**Table** 2). Low specific activity may indicate that most of the proteins in the sample are active enzymes, while high specific activity may indicate that most of the proteins in the sample are inactive or non-enzymatic enzymes [27].

MnP enzymes generally have a molecular weight of about 40-50 kDa [28]. In **Figure** 5 a band with a certain intensity in the range of 40-46 kDa, indicating that CG:RB (0:100), CG:RB (75:25), and CG:RB (50:50) as substrates favored the production of MnP enzyme. Partial purification of each fraction increased the specific activity of the enzyme, as shown in the study presented in **Table** 2. This increase indicates that the enzyme produced has a higher level of purity. The fraction with the highest specific activity had twice the purity level of the crude extract. In addition, the 0-20% CG:RB (50:50) fraction had a lower protein content than the crude extract due to the successful separation process of removing non-enzyme proteins that had been mixed with the enzyme previously [29]. This finding is reinforced by the results of enzyme analysis using SDS-PAGE electrophoresis shown in Figure 5.

RSM optimization analysis of laccase enzyme

Using coffee grounds waste and rice bran as substrates, RSM was used to optimize the production of MnP enzyme of Pleurotus ostreatus fungus. This method has the highest specific activity, which allows evaluation of various variables, such as substrate concentration, temperature, and reaction time on CG:RB (50:50) substrate in 0-20% fraction (**Table** 2). This makes it possible to find the most effective areas to support increased enzyme activity.

Table 2. Results of enzyme activity and specific activity of the enzyme (MnP)

Substrate	Sample Type	Enzyme Activity (U/mL)	Protein Content (mg/mL)	Specific activity (U/mg)
CG:RB 100:0	crude extract	14.699 ± 0.110	0.453 ± 0.155	18.511 ± 1.174
	fraction 0-20%	19.566 ± 0.078	0.323 ± 0.012	27.086 ± 0.559
	fraction 20-40%	16.344 ± 0.675	0.236 ± 0.003	69.389 ± 1.827
	fraction 40-60%	13.833 ± 0.424	0.226 ± 0.003	61.337 ± 0.919
CG:RB 75:25	crude extract	16.255 ± 0.550	0.795 ± 0.008	20.444 ± 0.473
	fraction 0-20%	17.355 ± 0.251	0.748 ± 0.002	23.218 ± 0.402
	fraction 20-40%	14.266 ± 0.597	0.287 ± 0.0007	49.798 ± 2.206
	fraction 40-60%	17.877 ± 0.141	0.262 ± 0.003	68.375 ± 1.465
CG:RB 50:50	crude extract	20,292 ± 0.627	0.716 ± 0.044	47.854 ± 17.819
	fraction 0-20%	20,588 ± 0.141	0.268 ± 0.007	76.967 ± 0.325
	fraction 20-40%	15.266 ± 0.063	0.375 ± 0.003	40.768 ± 0.552
	fraction 40-60%	15.322 ± 1.021	0.284 ± 0.002	53.936 ± 3.059
CG:RB 25:75	crude extract	17,944 ± 0.235	0.717 ± 0.022	25.034 ± 0.461
	fraction 0-20%	19,777 ± 1.131	0.674 ± 0.012	29.333 ± 1.124
	fraction 20-40%	19.922 ± 0.015	0.573 ± 0.001	34.768 ± 0.113
	fraction 40-60%	13.544 ± 0.298	0.306 ± 0.011	44.311 ± 2.613
CG:RB 0:100	crude extract	18.333 ± 0.471	0.825 ± 0.016	22.234 ± 0.133
	fraction 0-20%	18.333 ± 0.314	0.683 ± 0.019	26.866 ± 0.291
	fraction 20-40%	14.355 ± 0.628	0.604 ± 0.038	23.782 ± 0.462
	fraction 40-60%	11.866 ± 0.597	0.413 ± 0.013	28.759 ± 0.510

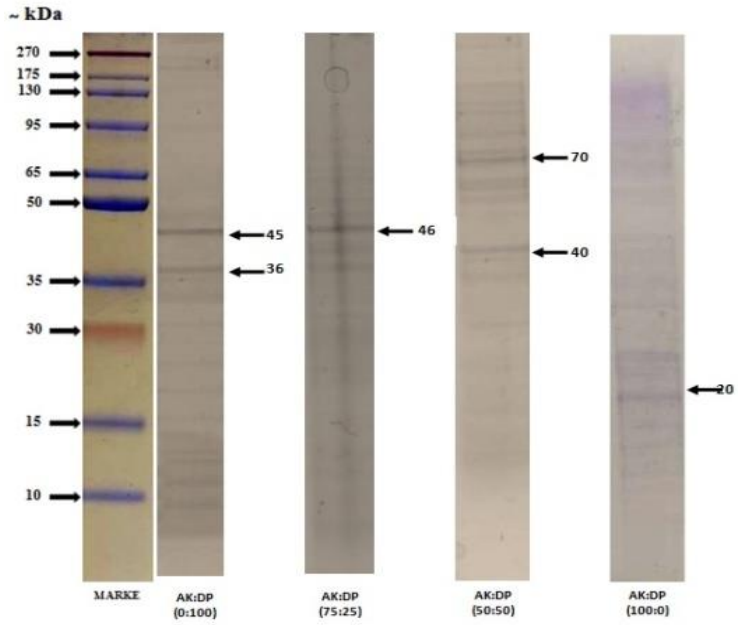

Figure 5. Results of analysis with SDS-PAGE.

Table 3. Running results of MnP activity with central composite design (CCD) (*n = 3)*

Concentration MnO_4 (mM)	Temperature (^{0} C)	time (minute)	Actual Activity (U/mL)	Predicted Activity (U/mL)
1.5	25	20	8.14	8.51
0.5	25	20	7.61	6.19
1	25	25	11.4	11.86
1.5	25	30	9.52	9.27
0.5	25	30	6.32	7.14
1	30	20	9.58	12.57
1	30	25	16.71	15.79
1	30	25	16.71	15.79
1	30	25	16.71	15.79
1	30	25	16.71	15.79
1	30	25	16.71	15.79
0.5	30	25	12.04	14.12
1	30	25	16.71	15.79
1.5	30	25	15.93	16.59
1	30	30	12	11.74
0.5	35	20	12.44	12
1.5	35	20	16.33	14.82
1	35	25	13.96	16.23
0.5	35	30	10.63	9.57
1.5	35	30	11.48	12.20

According to the results of the analysis performed on the Sequential Model Sum of Squares (**Table** 5), the quadratic model is the most suitable model to explain how process variables interact with enzyme activity. The highly significant p value (0.0006) and the increase in Adjusted R^2 (0.7499) and Predicted R^2 (-0.0302) compared to other models indicate that this choice is the right one [30]. The results of ANOVA

analysis on the quadratic model showed that several factors significantly affected the enzyme activity. (**Table** 5) shows that MnO_4 concentration (A), which has a p value of 0.0512, temperature (B), which has a p value of 0.0029, and squared MnO_4 concentration (A2) which has a p value of 0.6856, are the main actors, respectively.

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The following is the prediction model obtained to calculate the prediction of enzyme activity:

$$\text{Activity (U/mL)} = 4.96859 \times (A) + 8.22527 \times (B) + 5.42698 \times (C) + 0.05050 \times (A^2) - 0.01850 \times (C^2) - 0.03375 \times (B^2) - 1.77455 \times (AC) - 0.06995 \times (AB) - 0.14555 \times (BC)$$

Contour plots and response surface plots (3D) of the Response Surface Methodology (RSM) analysis results provide a better picture of how the process variables affect the MnP enzyme activity. In **Figure** 5 explains that the response surface plot influences the interaction factors on the MnP activity results. (1) the interaction between MnO_4 temperature-concentration and enzyme activity, (2) the interaction between MnO_4^- concentration-incubation time and enzyme activity, and (3) the interaction between MnO_4^- concentration and temperature on MnP activity. The concentration of MnO_4^- plays a significant role in increasing the activity of MnP, where the maximum activity of MnP is achieved at a temperature of 33-35°C, an incubation time of 24-28 minutes, and a concentration of MnO_4^- of about 1.5 mM. If the temperature or incubation time is too low, the enzyme activity is not optimal.

Table 4. ANOVA test results central composite design (CCD)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	205.57	9	22.84	7.33	0.0023	significant
A-Concentrate MnO_4 (mM)	15.28	1	15.28	4.90	0.0512	
B-Temperature (°C)	47.74	1	47.74	15.32	0.0029	
C-Incubation time (min)	1.72	1	1.72	0.5528	0.4743	
AB	0.1275	1	0.1275	0.0409	0.8437	
AC	0.0171	1	0.0171	0.0055	0.9424	
BC	5.70	1	5.70	1.83	0.2062	
A^2	0.5412	1	0.5412	0.1737	0.6856	
B^2	8.41	1	8.41	2.70	0.1314	
C^2	36.41	1	36.41	11.69	0.0066	
Residual	31.16	10	3.12			
Lack of Fit	31.16	5	6.23			
Pure Error	0.0000	5	0.0000			
Cor Total	236.72	19				

Table 5. Sequential model sum of squares response activity

Source	Sequential p values	P-value Lack of Suitability	Adjusted R^2	Predicted R^2	
Linear	0.1535	-	0.1373	-0.1723	
2FI	0.9263	-	-0.0258	-2.8211	
Quadratic	**0.0006**	-	**0.7499**	**-0.0302**	**Suggested**
Cubic	0.3930	-	0.7702	-88.1472	**Aliased**

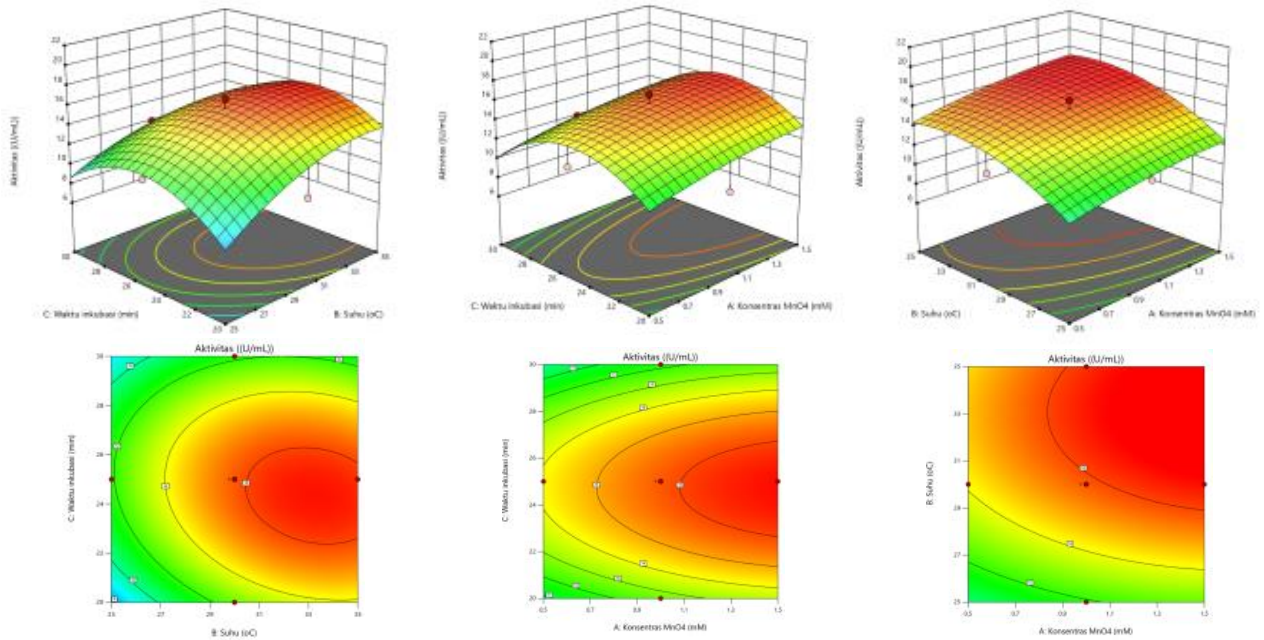

Figure 6. Contour plot of response surface methodology (RSM) analysis results

The interactive effect on enzyme activity is shown by the relationship between the two variables on the response surface plot (**Figure** 6**)**. Variable one remains constant. The relationship between the variables is not linear, according to the curved surface, according to the chosen quadratic model. The quadratic model can capture the complexity of the interaction between the variables in this system. This is because the peak of the response surface finds the optimal combination of process variables resulting in maximum enzyme activity. Adjusted R^2, a metric that takes into account many variables in the model, provides a more conservative estimate of accuracy compared to the usual R^2, as shown by the Adjusted R^2 value of 0.7499 (**Table** 5). This indicates that the quadratic model can explain 74.99% of the data variability. This high value indicates that the model can clearly describe the relationship between independent variables such as MnO_4 concentration, temperature, and incubation time and the response, namely enzyme activity. The predicted activity validation data, calculated using the model equation, is compared with the actual activity data below (**Table** 6).

Table 6. Prediction results of the laccase enzyme model.

Actual Activity (U/mL)	Predicted Activity (U/mL)
15.87 ± 0.07	15.85
14.71 ± 0.11	14.70
11.41 ± 0.12	11.40
Mean Squared Error (MSE) = 0.0633	R^2 Value = 0.99

With a Mean Squared Error (MSE) value of 0.0633 and an R^2 value of 0.99, this validation shows that the results are consistent [30]. This indicates that, based on the process variables, the model has a good ability to predict the enzyme activity of MnP

CONCLUSION

By using coffee grounds and rice bran as substrates, this study successfully optimized the production of *Pleurotus ostreatus* MnP enzyme. *Response Surface Methodology* (RSM) was used to achieve this goal. The quadratic model proved effective with a value of $R^2 = 0.7$ which showed high accuracy in predicting enzyme activity. The highest enzyme activity was obtained in the 0-20% fraction of CG:RB (50:50) substrate with a value of 20.588 ± 0.141 U/mL, having a high protein content found in the cassava extract of CG:RB (0:100) substrate of 0.825 ± 0.016 mg/mL while the highest specific activity was found in the 0-20% fraction of CG:RB (50:50) substrate of 76.967 ± 0.325 U/mg . Temperature and MnO_4 concentration really affect the enzyme activity. With good model validation ($R^2 = 0.99$), the contour plot and response surface showed the process interaction variables on the enzyme activity results.

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