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OPTIMIZATION OF ENZYME-MICROWAVE ASSISTED EXTRACTION, CHARACTERIZATION AND ANTIOXIDANT ACTIVITY OF POLYSACCHARIDE FROM *Ganoderma lucidum*

Optimasi Ekstraksi dengan bantuan Enzim dan Microwave, Karakterisasi dan Aktivitas Antioksidan Senyawa Polisakarida dari *Ganoderma lucidum*

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ABSTRACT
Enzyme-Microwave Assisted Extraction (EMAE) is a new process for extracting Ganoderma lucidum *polysaccharides (GLPs). Cellic® CTec2 was chosen as an enzyme that assists in microwave extraction. The four variables involved in this study were enzyme concentration (%), enzymatic reaction time (minutes), solvent-to-solid ratio (mL/g), and microwave extraction time (minutes). This study showed that the enzyme concentration and solvent-to-solid ratio had a significant effect on the response in the range studied. Yield extraction of polysaccharides from experiments conducted at optimum conditions showed good agreement with the predictions from the model. The EMAE method showed a higher polysaccharide extraction yield than hot water extraction (HWE) method. GLPs from EMAE method had antioxidant activity of 79.47 ± 0.71% (DPPH) and 0.884 ± 0.013 mM Fe^{2+}/L (FRAP), where these values were higher than those of the HWE method.*

Keywords: *Enzyme-microwave assisted extraction,* Ganoderma lucidum*, β-glucan, polysaccharides, response surface methodology*

ABSTRAK
Ekstraksi dengan bantuan enzim dan gelombang mikro menjadi proses baru untuk mengekstrak polisakarida dari *Ganoderma lucidum* (PGL). Cellic® CTec2 dipilih sebagai enzim yang membantu dalam ekstraksi gelombang mikro. Empat variabel yang terlibat dalam penelitian ini adalah konsentrasi enzim (%), waktu reaksi enzimatik (menit), rasio pelarut terhadap padatan (mL/g), dan waktu ekstraksi gelombang mikro (menit). Analisis statistik dari hasil percobaan menunjukkan bahwa konsentrasi enzim dan rasio pelarut terhadap padatan berpengaruh signifikan terhadap respons dalam rentang yang dipelajari. Rendemen ekstraksi polisakarida dari percobaan yang dilakukan pada kondisi optimum menunjukkan kesesuaian yang baik dengan prediksi dari model. Metode EMAE menunjukkan rendemen PGL yang lebih tinggi dibandingkan dengan metode HWE. PGL dari metode EMAE memiliki aktivitas antioksidan sebesar 79,47 ± 0,71% (DPPH) dan 0,884 ± 0,013 mM Fe^{2+}/L (FRAP), dimana nilai ini lebih tinggi dibandingkan dengan yang diperoleh dari metode HWE.

Kata Kunci: Ekstraksi berbantu enzim-gelombang mikro, *Ganoderma lucidum*, β-glukan, polisakarida, metode respons permukaan

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INTRODUCTION

Ganoderma lucidum, also called Ling-Zhi in China, Yeongji in Korea, and Reishi in Japan, has long been used as a medicinal treatment in many East-Asia countries. *G. lucidum* polysaccharides (GLPs) exhibits various activities that are beneficial for health. The biological activities of polysaccharides can be determined by their structural characteristics, including molecular weight, chemical components, glycosidic bonds, main chain lengths, polymerization, branching degrees, and three-dimensional conformations (Li et al. 2020). The major bioactive compounds consist of (1-3), (1-6)-α/β-glucans, glycoproteins, and water-soluble heteropolysaccharides (Ferreira et al. 2015). Researchers have reported *in vitro* and *in vivo* antioxidant activity of GLPs (Zhang et al. 2016, Alzorqi et al. 2017, Mustafin et al. 2022).

Hot water extraction (HWE) is the simplest method to extract polysaccharides from plants. However, degradation of the active compound could result in low bioactivity and low extraction yields because of long extraction times (Yin et al. 2018, Sakdasri et al. 2022). Currently, several techniques, such as microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE), and ultrasonic-assisted extraction (UAE) have been applied for the extraction of various bioactive compounds from microbes and plants (Alzorqi et al. 2017, Smiderle et al. 2017, Hwang et al. 2018, Düven et al. 2021).

Extraction using enzymes is a potential alternative method for isolating active plant compounds due to its high efficiency, low cost, low concentrations-needed, easy implementation, and good environmental compatibility. Mild operating conditions also prevent the active compounds from degradation (Kumar et al. 2020). Mainly, hydrolytic enzymes are commonly utilized to degrade cell wall constituents and accelerate the release of intracellular contents, which had been proved to be efficient for extracting polysaccharides by the previous literatures. For example, cellulase combined with papain and pectinase has been used to increase the yield of polysaccharide extraction from *Lentinus edodes* (Gu et al. 2023).

Microwave-assisted extraction (MAE) is one of the advanced techniques being considered to extract various active compounds (Le et al. 2019, Nana et al. 2021). MAE has several advantages related to reduction in cost, time of extraction, amount of solvent used, energy consumption and low CO_2 emission. Microwave is a non-contact heat source that generates heat energy via ionic conduction between solvents and dissolved ions, which ruptures the cell wall and assists in releasing active plant compounds. The synergic mass and heat action in the same direction can cause extraction efficiently (Gomez et al. 2020).

Nowadays, combining more than one extraction method is attracting the attention of researchers. This technique gives better results than a single extraction method (Ke and Chen 2016, Yin et al. 2018). This study used a combination of enzymes and microwaves (EMAE) to extract GLPs. Combining the two methods is expected to provide a synergistic effect so that the extraction process produces a higher yield. Optimizing the factors that influence EMAE is necessary in order to obtain extraction conditions that produce the highest yield of GLPs.

Statistical experimental design, such as response surface methodology (RSM), is valuable for optimizing processes involving multiple variables. In the RSM, Central Composite Design (CCD) is considered the most popular statistical experimental design. CCD involves a complete factorial design and can provide more accurate estimations (Bhattacharya 2021). Hence the objective of this study was to optimize the conditions of EMAE, such as enzyme concentration, enzymatic reaction time, solvent-to-solid ratio, and microwave extraction time using RSM-CCD, to maximize the yield of GLPs. Furthermore, physicochemical characteristics and antioxidant properties of the residue *G. lucidum* and polysaccharides extract were evaluated between the EMAE and HWE methods.

MATERIALS AND METHODS

Location and time

This study was conducted at the Laboratory for Biotechnology, Deputy for Research and Innovation Infrastructure-National Research and Innovation Agency (BRIN) from September 2022 to January 2023

Table 1. Proximate composition of *Ganoderma lucidum*

Chemical composition	Value (%)	Reference method
Moisture	8.67 ± 0.10	AOAC chapter 3, p. 40, 930.15
Ash	3.35 ± 0.01	AOAC chapter 3, p. 40, 930.10
Crude protein	14.13 ± 0.01	AOAC chapter 4, p. 74, 984.13
Fat	0.23 ± 0.01	AOAC chapter 32, p. 780, 920.85
Carbohydrates	30.37 ± 0.06	Calculation base
Dietary fiber	43.23 ± 0.04	AOAC chapter 3, p. 59, 930.10
Cellulose	5.33 ± 0.51	Calculation base
Hemicellulose	13.07 ± 1.46	Calculation base

Mean ± standard deviation (n = 3)

Raw material, Enzymes, and reagents

The dried *G. lucidum* fruiting bodies were obtained from local farmers in Godean Subdistrict, Sleman Regency, Special Region of Yogyakarta, Indonesia. The commercial enzymes used are proteolytic enzymes (Corolase® 7089 and Corolase ® 8000 were purchased from AB Enzymes GmbH, Germany); cellulolytic enzymes (Cellic® HTec2, Cellic® CTec2, and Viscozyme Cassava CL are produced by Novozymes, Denmark). 1,1-diphenyl-2-picrylhydrazyl (DPPH), galactose, arabinose, maltose, and glucose were obtained from TCI (Japan). Ascorbic acid and 2,4,6-tripyridyl-s-triazine (TPTZ) were products from Sigma-Aldrich Co. (St. Louis, USA). All other reagents employed were analytical grades.

Preparation and proximate analysis

G. lucidum fruiting bodies were ground with a grinder to obtain a powder. The proximate content of the mushroom (Table 1) was assessed using Association of Official Analytical Chemists standard techniques (AOAC, 1990). The moisture content is determined by measuring the loss of water content after drying at 105°C using a moisture analyzer, while the ash content, which is an inorganic residue, is determined by heating the sample at very high temperatures at 550°C in a furnace. The Kjeldahl method measured the protein content, while the total fat content was determined by the extraction method with n-hexane using a soxhlet apparatus. The residue of extraction was then heated in weak acid and base solutions. The remaining insoluble fiber was filtered and counted as crude fiber content. Carbohydrate was analyzed by difference method (100% subtracting with the content of moisture, ash, protein, fat, and crude fiber). *Ganoderma* powder was boiled in neutral detergent fibers (NDF) and acidic detergent fibers (ADF), then sample residues were calculated as NDF and ADF content. Lignin was determined by adding sulfuric acid to ADF residue. Further, cellulose content is determined by subtracting ADF from lignin, while hemicellulose is produced by subtracting NDF from cellulose and lignin.

Prior to the combination extraction experiment, *G. lucidum* powder was pre-extracted using 96% ethanol (w/v, 1:15) overnight to remove reducing sugars, fatty acids, phenols, and enzymes. The mushroom powder was separated from the alcohol and dried overnight in a 40°C oven to evaporate the remaining solvent. The defatted sample was then stored in the refrigerator for further experiments.

Enzyme selection experiments

Five types of commercial enzymes were tested to select the enzymes with the highest extraction yield for hydrolyzing *G. lucidum* cell walls. One gram of the defatted sample was suspended in 50 mL of distilled water with an optimum pH for each enzyme, as described in Table 2. Enzymatic hydrolysis was carried out using an enzyme concentration of 0.4%, a temperature of 50°C,

Table 2. Commercial enzymes were used and pH conditions of reaction

Commercial enzyme	Composition of the enzyme	pH solution
Corolase® 7089	Endoprotease	7,0
Corolase® 8000	Endoprotease	8,5
Cellic® CTec2	Cellulases, β-glucosidases and hemicellulases	5,0
Cellic® HTec2	Endoxylanases and cellulases	5,0
Viscozyme Cassava CL	Cellulases	5,0

and a reaction time of 60 minutes in a water bath shaker. Water without the addition of enzymes was used as a blank. After hydrolysis, the samples were centrifuged at 2000 x *g* for 15 min to separate the solution from the residue. Ethanol 96% was added to the solution with a ratio of 3:1 to precipitate the polysaccharides. The precipitate is dried under reduced pressure at 40°C and determine the extraction yield. All experiments were performed in triplicate.

Experiment design for EMAE

Design Expert (Trial Version 13, Stat-Ease Inc., Minneapolis, MN, USA) was used for experimental design, data analysis, and building models. Four independent variable factors, namely enzyme concentration (A), enzymatic reaction time (B), solvent-to-solid ratio (C), and microwave extraction time (D), were studied at five levels. Central composite design (CCD) was used to optimize the response (extraction results). Thirty experimental points were carried out randomly. The second-order general equation of the model showing the relationship between the independent (X) and dependent (y) variables is shown in equation according to Palanikumar (2021):

$$y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j \quad (1)$$

where y is extraction yield; X_i and X_j are the uncoded independent term; β_0 is the constant term, β_i is the linear term, β_{ii} is the quadratic term, and β_{ij} is the interaction term.

Enzyme-microwave assisted extraction (EMAE)

One gram of defatted sample was added to distilled water, followed by the addition of a selected enzyme solution at the desired concentration. After enzymatic treatment, the mixture was extracted by microwave extraction at 100 W (HT-WC1, Hento Co., Ltd., Henan, China). Variations of the independent variables, namely enzyme concentration, enzymatic reaction time, solvent-to-solid ratio, and microwave extraction time from each experiment, were carried out, as shown in Table 3. The extract was obtained by centrifuging the mixture at 2000 x *g* for 15 minutes to separate the residue.

Hot water extraction (HWE)

One gram of defatted sample was extracted twice with 50 mL of distilled water at 100°C for 2 hours. After centrifuging at 2000 x *g* for 15 min, the combined supernatant was concentrated to 50 mL in a rotary evaporator operated at 55°C and under reduced pressure. Experiments were performed in triplicate.

Precipitation and determination of extraction yield

The crude GLPs precipitate was obtained by adding three times the volume of 96% ethanol to extract and then stirring the mixture vigorously for one minute. To complete precipitation, the solution was stored overnight at 4°C. Centrifugation at 8000 x *g*, at 4°C, for 15 minutes, was carried out to isolate the precipitate, then dried under reduced pressure at 40°C. Extraction yield (%) is calculated as follows:

Extraction yield (%, w/w) =

$$\frac{\text{Weight of polysaccharides (g)}}{\text{Weight of } \textit{G.lucidum} \text{ powder (g)}} \times 100 \quad (1)$$

Analysis of polysaccharide content

The phenol-sulfuric acid method was used to measure the polysaccharide content of extracts adapted from Dubois et al. (1956). Crude GLPs were dissolved in 1.0 mL of distilled water and vortexed rapidly for 30 seconds. Then add 1 mL of 5% phenol solution and 5 mL of concentrated sulfuric acid. The solution was allowed to stand for 20 minutes in a water bath at room temperature and then

Table 3. Variables and levels for designing the CCD

Variables	Unit	-α	-1	0	+1	+α
(A) Enzyme concentration	%	0.4	0.8	1.2	1.6	2
(B) Enzymatic reaction time	min	2.5	5	7.5	10	12.5
(C) Solvent-to-solid ratio	mL/g	20	30	40	50	60
(D Microwave extraction time	min	5	10	15	20	25

α value = 2.00

the absorbance was measured at 490 nm (UV-Vis 2600, Shimadzu, Japan). The polysaccharide content was calculated using a calibration curve with glucose as standard.

Analysis of microstructure changes

Scanning electron microscopy (SEM) was used to observe the effect of extraction on the morphological surface of *G. lucidum*. The powder before and after extraction using HWE and EMAE methods was air-dried prior to SEM examination using Quanta 650 scanning electron microscope (Thermo Scientific, USA). The surface images of powder were taken using 10.0 kV of voltage in vacuum conditions and at 300x and 600x magnification.

Analysis of β-glucan content

β-glucan content of crude GLPs was determined using the Mushroom and Yeast β-glucan assay procedure by Megazyme (McCleary and Draga 2016). Briefly, total glucan was determined by hydrolyzing polysaccharides with exo-1,3-β-glucanase and β-glucosidase, while the α-glucan was hydrolyzed with amyloglucosidase plus invertase. Generated glucose from total and α-glucan procedures was quantified by adding glucose determination reagent (GOPOD). The absorbance of all solutions was measured at 510 nm against the blank. β-glucan content was quantified by subtracting α-glucan from total glucan. The results were calculated using Megazyme's Mega-CalcTM application. All glucans were given as g/10 g of crude GLPs.

Analysis of sugar composition

The crude GLPs sugar composition was analyzed using a procedure modified from previous studies (Jiang et al. 2022). Separation of sugar compounds was performed using an Aminex HPX-87H column 7.8 mm ID, 300 mm long (BioRad, CA, USA) using a Waters Alliance HPLC system with a refractive index detector (RI) (California, USA). Milli-Q water was used as diluent and 8 mM H_2SO_4 was used as mobile phase at a flow rate of 0.7 mL/min. The column and detector temperature was set at 35°C, and the detector sensitivity was 64. The total run time was 12 minutes, and the sample injection volume was 10 µL.

Analysis of antioxidant capacity

The DPPH radical scavenging activity was determined using the Blois method (1958). Two milliliters of crude GLPs solution with varying concentrations (0.2–2.0 mg/mL) were combined with an equal volume of 0.2 mM DPPH solution. Using a UV-Vis spectrophotometer (Shimadzu UV-2700, Japan), the absorbance at 517 nm was determined after vigorously mixing and keeping the reaction mixture for 30 mins at room temperature in a dark place. Ascorbic acid served as a standard. The crude GLPs DPPH scavenging rate was determined by applying the following equation:

$$\text{Scavenging rate (\%)} = \left[1 - \frac{(A_1 - A_2)}{A_0}\right] \times 100 \quad (2)$$

where A_0 was absorbance of DPPH and distilled water mixture, A_1 was absorbance of sample or standard, and A_2 was absorbance of sample and distilled water mixture.

Antioxidant activity was also determined using the ferric ion-reducing antioxidant power assay (FRAP) method described by Benzie and strain (1996) with slight modifications. Prior to use, fresh FRAP reagent was prepared by combining 300 mM acetate buffer pH 3.6, 10 mM TPTZ solution, and 20 mM $FeCl_3.6H_2O$ solution in the ratio (10:1:1) and heated at 37°C. 2850 µL of FRAP reagent was reacted with 150 µL of crude GLPs dissolved in distilled water at various concentrations (0.25-2.0 mg/mL) for 30 minutes in the dark. The absorbance of the solution was observed at 593 nm against a blank. Iron (II) sulfate heptahydrate (0.1–1.0 mM) was used to make a standard curve. The reducing power of the samples was evaluated using a standard curve ($y = 0.6459x - 0.0072$, $R^2 = 0.9993$), and the values obtained corresponded to the mmol Fe^{2+}/L of sample.

RESULTS AND DISCUSSION

Proximate composition

The proximate composition of *G. lucidum* based on dry weight is shown in Table 1. Moisture content 8.67 ± 0.10%. Dietary fiber and carbohydrates are the main components of this mushroom 43.23 ± 0.04% and 30.37 ± 0.06%, respectively. The fruit body of the mushroom is cork, tough and thick

Figure 1. *Ganoderma lucidum* fruiting body

with the total fat content is only 0.23%. The crude protein content is relatively high, about 14.13 ± 0.01%. The different proximate composition of *G. lucidum* was reported by Alzorqi et al. (2017) on mushrooms obtained from Tanjung Sepat, Selangor, Malaysia, with a carbohydrate content of 79.2%, 10.3% protein, 0.9% ash and 1.1% fat with almost the same water content, 8,5%. Several factors, including climate, nutrients, and geographical location, influence variations in the composition of the mushrooms.

Selection of enzyme

The study of enzyme selection focused on selecting the most efficient enzymes to break down the cell walls of *G. lucidum* and obtain the highest extraction yield. Figure 2 shows the percentage of extraction yield obtained from the enzymolysis treatment of samples by several commercial enzymes. Cellic® CTec2 produced the highest extraction yield compared to other enzymes. *G. lucidum* fruiting body has a solid woody tissue consisting of cellulose, hemicellulose, and lignin (Ma et al. 2018). Cellic® CTec2 is a second-generation designed for lignocellulosic biomass saccharification, which contains a high level of endo-β-1,4-glucanase and cellobiase activities. These enzymes will degrade cellulose and hemicellulose polymers into simple sugars and make the cell walls more porous,

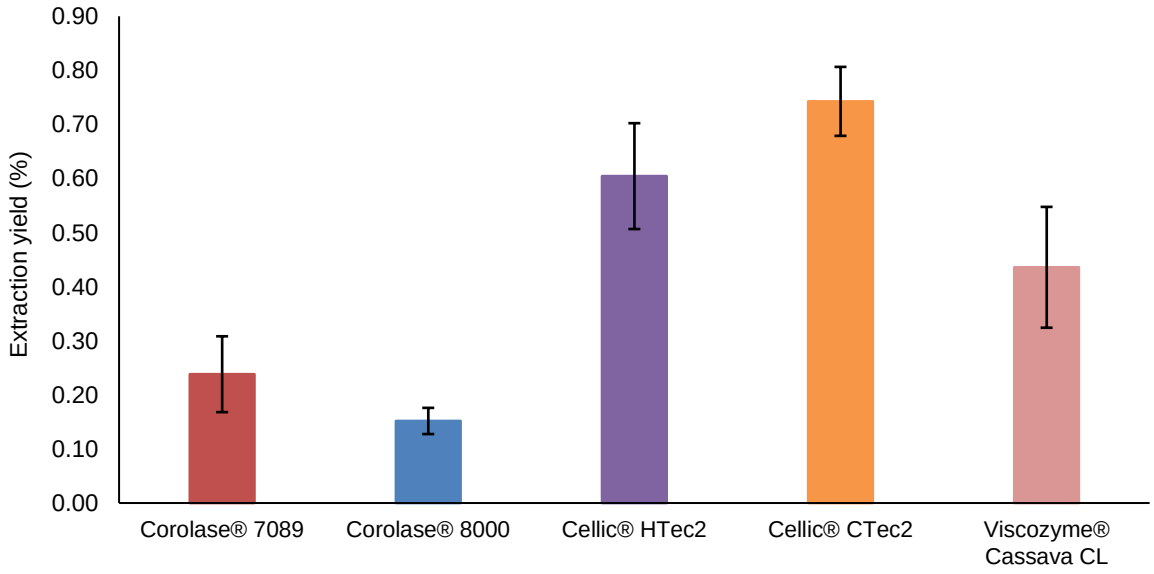

Figure 2. The percentage yield of GLPs obtained from several commercial enzymes

Table 4. CCD experimental design and response of EMAE

Run	Design				Extraction Yield (%)	
	A (%)	B (min)	C (mL/g)	D (min)	Experimental results	Predicted results
1	0	-α	0	0	1.11	1.14
2	-1	-1	+1	+1	1.13	1.00
3	+1	-1	+1	+1	1.05	1.17
4	+1	+1	-1	-1	0.78	0.88
5	+1	+1	+1	-1	1.66	1.64
6	0	0	0	0	1.19	1.26
7	0	0	0	0	1.24	1.26
8	-α	0	0	0	0.76	0.78
9	0	+α	0	0	1.20	1.20
10	0	0	0	0	1.07	1.26
11	0	0	0	+α	0.75	0.69
12	0	0	0	0	1.20	1.26
13	-1	+1	+1	-1	1.16	1.11
14	+α	0	0	0	1.35	1.35
15	+1	+1	-1	+1	1.02	0.97
16	+1	-1	+1	-1	1.64	1.51
17	+1	+1	+1	+1	1.39	1.37
18	0	0	0	0	1.38	1.26
19	0	0	0	-α	0.80	0.89
20	-1	+1	-1	-1	0.59	0.48
21	-1	+1	+1	+1	0.86	0.90
22	-1	-1	+1	-1	1.22	1.28
23	-1	-1	-1	+1	0.66	0.69
24	0	0	-α	0	0.40	0.37
25	+1	-1	-1	+1	0.72	0.73
26	0	0	+α	0	1.38	1.44
27	0	0	0	0	1.46	1.26
28	+1	-1	-1	-1	0.74	0.71
29	-1	+1	-1	+1	0.52	0.62
30	-1	-1	-1	-1	0.64	0.62

thereby increasing the extraction yield. Based on these results, Cellic® CTec2 was used as an enzyme combined with the MAE method to determine the optimum extraction conditions using RSM.

Statistical analysis

The extraction conditions were optimized using RSM and CCD to determine the influence of four parameters (enzyme concentration, enzyme reaction time, solvent-to-solid ratio, and microwave extraction time). CCD is suitable for estimating optimal conditions through a quadratic model approach requiring a minimum number of experiments, which could represent the numerical value of individual and interaction variables (Yang et al. 2020). Thirty runs are required to optimize the four variables for getting the optimum extraction yield. The response value of the experimental results and the model prediction value from various combinations of different variables, which are expressed in the coded variables, are shown in Table 4. The extraction results varied from 0.40 – 1.66 % (w/w). The correlation between the response variable and the test variable is represented by the second-order polynomial equation shown below:

$$y = 1{,}26 + 0{,}14\,A + 0{,}02\,B + 0{,}27\,C - 0{,}05\,D + 0{,}08\,AB - 0{,}03\,AC - 0{,}01\,AD - 0{,}01\,BC + 0{,}02\,BD - 0{,}09\,CD - 0{,}05\,A^2 - 0{,}02\,B^2 - 0{,}09\,C^2 - 0{,}12\,D^2 \quad (3)$$

where y is the extraction yield, and A, B, C, and D are the coded values for enzyme concentration, enzymatic reaction time, solvent-to-solid ratio, and microwave extraction time, respectively. The positive sign of the linear and quadratic terms indicates that they have a positive and encouraging effect on the efficiency of the extraction process. In contrast, the terms with a negative sign adversely influence the extraction process response.

Analysis of variance (ANOVA) was used to identify the effect of individual parameters as well as interactions between parameters on response (Table 5). A *P*-value of less than 0.05 is a requirement for the

Table 5. Summary of ANOVA for the response surface quadratic model

Source	DF	Sum of squares	Mean square	*F*-value	*p*-Value
Model	3.03	14	0.2167	15.46	< 0.0001*
Residual	0.2103	15	0.014		
Lack of fit	0.111	10	0.0111	0.5585	0.7975
Pure error	0.0993	5	0.0199		
Total	3.24	29			
A- Enzyme concentration	0.4817	1	0.4817	34.36	< 0.0001*
B- Enzymatic reaction time	0.0054	1	0.0054	0.3852	0.5442
C- Solvent-to-solid ratio	1.71	1	1.71	121.74	< 0.0001*
D- Microwave extraction time	0.058	1	0.058	4.14	0.06
AB	0.093	1	0.093	6.64	0.0211*
AC	0.0169	1	0.0169	1.21	0.2895
AD	0.0025	1	0.0025	0.1783	0.6788
BC	0.0009	1	0.0009	0.0642	0.8034
BD	0.0049	1	0.0049	0.3495	0.5632
CD	0.126	1	0.126	8.99	0.009*
A^2	0.0619	1	0.0619	4.41	0.053
B^2	0.0139	1	0.0139	0.9905	0.3354
C^2	0.216	1	0.216	15.41	0.0013*
D^2	0.3787	1	0.3787	27.01	0.0001*

R^2 = 0.9352; adjusted R^2 = 0.8747; Predicted R^2 = 0.7589; C.V. %=11.43; Std. Dev.= 0.1184; Mean = 1.04; Adeq Precision = 15.1639.
*model terms of significant.

model to be statistically significant. In this study, the *P*-value of the model is < 0.0001, indicating that the chosen model is appropriate and accurately predicted for the response. At the same time, the lack of fit has the *P*-value of 0.7975, indicating that the model has no abnormality from the residual diagnoses. Thus, the model was statistically adequate.

R^2 values were used to evaluate the model's quality (Jafari et al. 2017). The R^2 and adjusted R^2 values were 0.9352 and 0.8747, respectively. These results demonstrated that the model is adequate to evaluate and optimize the effects of EMAE variables on the extraction yield. The adjusted R^2 value is close to the predicted R^2 value (i.e., 0.8747 and 0.7589) the difference is less than 0.2, indicating that experimental data fitted well in the quadratic model. The adequate precision value must be greater than 4, which measures the signal-to-noise ratio. In this study, adequate precision was obtained at 15.1639.

Response surface analysis

Based on the *P*-values from Table 5, it can be concluded that variable C and variable A have a significant effect on the extraction yield, while other variables do not have a significant effect when evaluated individually. A greater influence is shown by variable C compared to A based on an F value which were 121.74 and 34.36, respectively. The other variables only have a weak effect on extraction yield. A significant effect of the quadratic variables was shown by variable D and variable C with *P*-values of 0,0013 and 0,0001, respectively. Based on the *F*-value, variable D (*F*-value 27.01) has a higher effect than variable C (*F*-value 15.41). In comparison, the interactive effects of variable B with variable C show the lowest *F*-value (0.0642) than the other interaction.

The interactive effects between two independent variables are shown in Figure 3 (a-f). Based on the *P*-value, significant interactions are shown by interactions between variable A and variable B as well as variable C and variable D, shown in Figure 3a and Figure 3f. Figure 3a shows the interaction effect between variable A and variable B. The extraction yield increased significantly with increasing enzyme concentration but changed only slightly with enzymatic reaction time. The interaction effect of these two variables resulted in an increasing significant extraction yield, as indicated in projection of response surface. Cellic® Ctec2 is an enzyme cocktail with a broad spectrum of lignocellulosic biomass hydrolyzing capabilities (López-Gutiérrez et al. 2021). *G. lucidum* has a high concentration of cellulose and hemicellulose. Therefore, increasing the

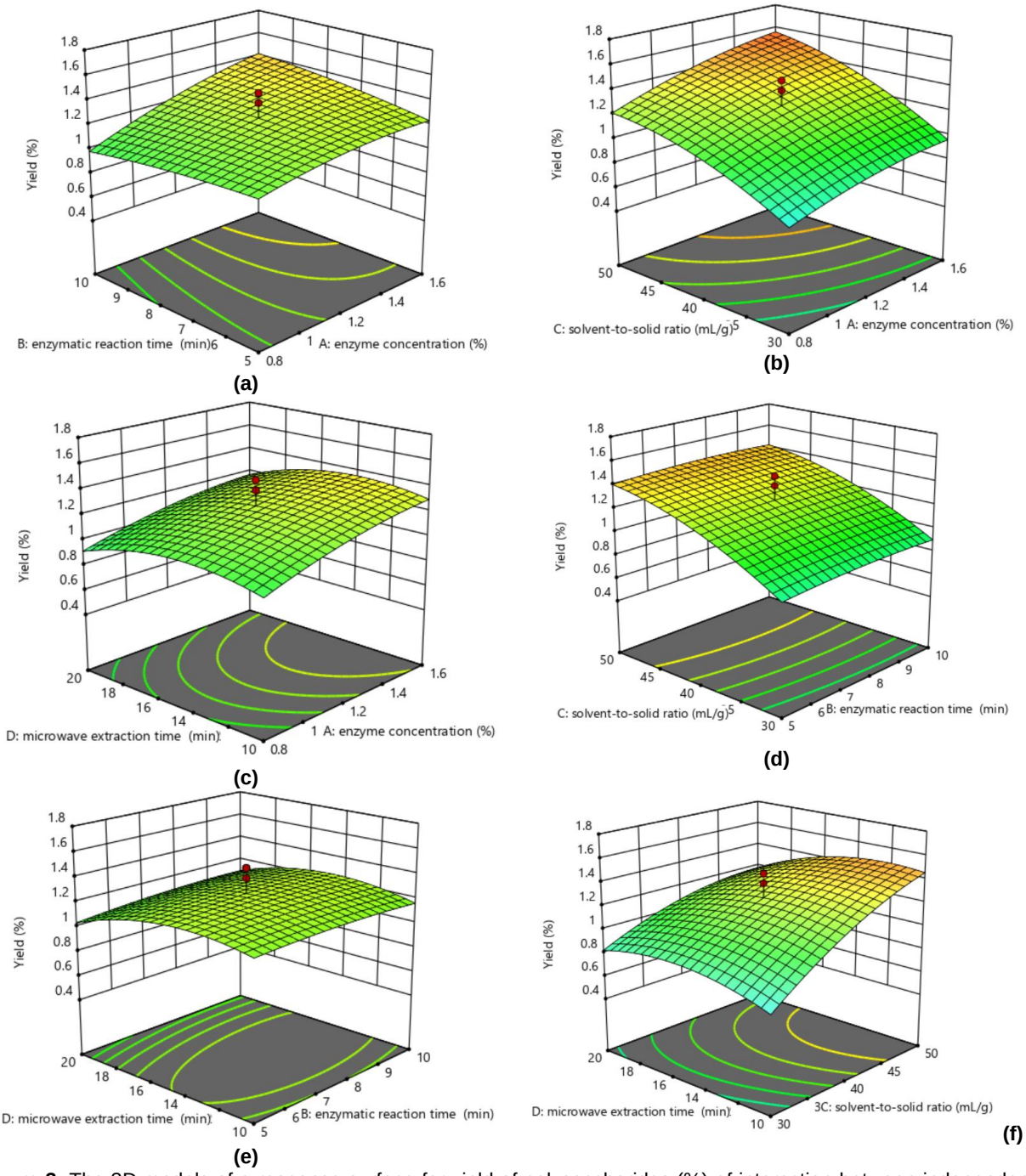

Figure 3. The 3D models of a response surface for yield of polysaccharides (%) of interaction between independent parameters. (a) enzyme concentration and enzymatic reaction time (b) enzyme concentration and solvent-to-solid ratio (c) enzyme concentration and microwave extraction time (d) enzymatic reaction time and solvent-to-solid ratio (e) enzymatic reaction time and microwave extraction time(f) solvent-to-solid ratio and microwave extraction time.

concentration of enzymes in the solvent will increase the potential to break the chains of polysaccharides into small molecules.

Different phenomena were observed in the interaction of variable D and variable A. Enzyme pre-treatment effectively destroys cell walls and shows a trend of increasing extraction results as the enzyme concentration increases to the highest concentration. Consequently, extraction yield only slightly increases when microwave extraction was applied. The interaction effect between these two variables only shows a slight effect on the extraction results as presented in Figure 3c. This is probably due to the majority of polysaccharides being extracted during the enzymatic process. Thus, when extracted using the microwave, only a small amount could be extracted.

Table 6. Comparison of GLPs results obtained from EMAE and HWE

Parameters	EMAE	HWE
Extraction time (min)	22	240
Ratio of water to raw material (mL/g)	50	100
Extraction yield (%)	1.62 ± 0.02	1.05 ± 0.03
Polysaccharide content (g/10 g)	3.24 ± 0.24	2.03 ± 0.03
Glucan content (g/10 g)		
Total glucan	0.90 ± 0.05	0.35 ± 0.05
α - glucan	0.20 ± 0.01	0.13 ± 0.02
β - glucan	0.70 ± 0.04	0.22 ± 0.03
Sugar composition (mol %)		
Maltose	22.22 ± 0.21	22.78 ± 0.20
Glucose	14.20 ± 0.02	12.53 ± 0.89
Galactose	60.92 ± 0.24	64.69 ± 0.70
Arabinose	2.66 ± 0.02	N.D

ND = not detected. All values are means ± standard deviation (n = 3)

Figure 3d illustrates the interaction between variables B and C. The solvent ratio is very influential on the extraction results. A higher solvent ratio results in a higher extraction yield because it increases the solubility of GLPs. The increase in variable B and its interaction with variable C did not significantly affect the extraction results.

Figure 3e represents the response contour of the interaction between variable B and variable D. These two variables do not show a significant effect, individually and in their interactions. At the same time, Figure 3f shows the interaction between variable C and variable D. The highest yield was obtained at a solvent-to-solid ratio of 50 mL/g and an extraction time of about 12 minutes. The mechanism of heat and mass transfer from the sample cell to the solvent in microwave-assisted extraction depends on the presence of water molecules. Microwave energy transfer to the heated solvent is via two mechanisms: dipole rotation and ionic conduction (Mao et al. 2021). These two variables show a significant interaction with the extraction yield.

Verification of models

Following a series of linear fitting and response surface analysis, the optimal extraction conditions of EMAE were as follows: at 1.6% enzyme concentration, 10 minutes enzymatic reaction, 50 mL/g solvent-to-solid ratio, and 12 minutes microwave extraction, the theoretical GLPs extraction yield was 1.66%. Later a verification of optimal conditions was carried out to assess the validity of the model. The actual extraction yield of GLPs from three replicates was 1.62 ± 0.02%. These results are quite close to the model's predicted value, which is equal to 1.66%, and only give a percentage of difference about 2.64%. These values indicate that the model could reflect the optimal conditions from the four variables.

Comparison of extraction results and polysaccharide content

A comparison of the extraction yield and the sugar composition of the GLPs obtained from HWE and EMAE methods is presented in Table 6. EMAE produced a higher extraction yield with a shorter extraction time than HWE. The extraction yield of EMAE is 54.29% higher than HWE. In addition, EMAE significantly reduces energy use, solvents, shortens extraction time, and increases the efficiency of the extraction process.

The polysaccharide content in crude GLPs from the EMAE method was obtained at 3.24 ± 0.24 g/10 g, which is higher than from the HWE method, which obtained 2.03 ± 0.03 g/10 g. The lower polysaccharide content observed by HWE may be due to prolonged exposure to high temperatures. These phenomena resulted in the decomposition of GLP composition. According to Alzorqi et al. (2017), the longer the extraction process, the lower the polysaccharides content and the higher the galacturonic acid content obtained in GLPs. Galacturonic acid is the oxidized form of galactose and glucose.

Microscopic of residue fiber

The surface morphologies *G. lucidum*, before and after extraction, are shown in Figure 4. The mushroom structure appears intact and compact before subjecting to any

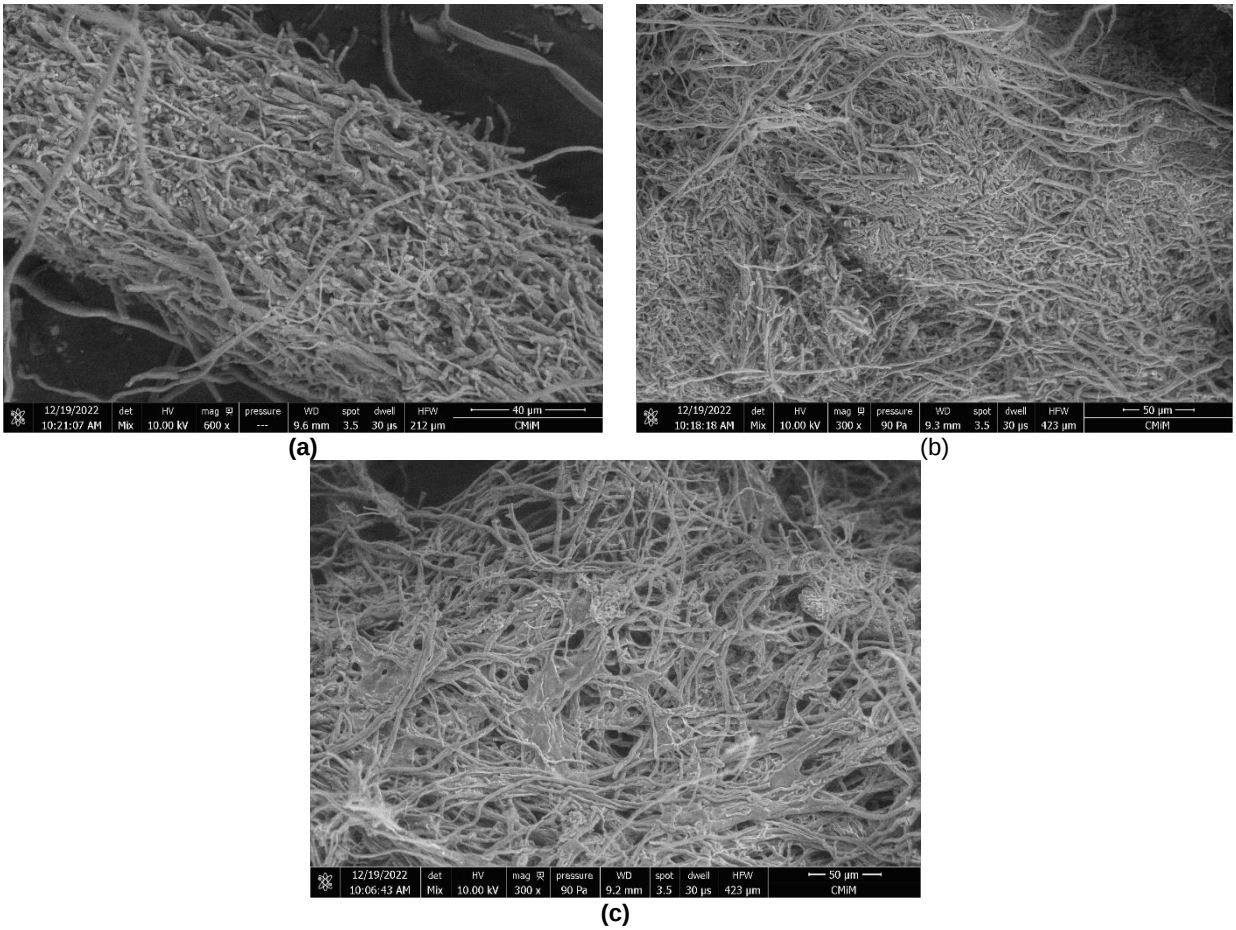

Figure 4. Scanning electron microscope images of (a) untreated G. lucidum sample, (b) sample after HWE (c) sample after EMAE.

treatment, as represented in Figure 4a. The surface morphologies of residue *G. lucidum* after treatment with HWE (Figure 4b) showed only a few slight ruptures. However, Figure 4c) shows that the surface morphologies of residue *G. lucidum* were greatly destroyed and porous after EMAE treatment. These results are consistent with reports by other researchers (Cheng et al. 2015, Chen et al. 2017, Yin et al. 2018, Lin et al. 2019).

In EMAE, the release of chemical compounds from the cell to the solvent occurs through two factors: firstly, the enzymatic treatment resulted in the breakdown of the cell wall to increase the release of chemical compounds from the cell to the solvent. Secondly, microwave irradiation generates heat due to the rotation of the H_2O dipole, which increases internal pressure in the material. Heat flow through the damaged cell wall occurs in the direction of mass transfer to increase the extraction yield (Zhang et al. 2013, Kumar et al. 2020).

Glucan content of GLPs

Because of the cohesiveness of *G. lucidum* wood tissue, glucan extraction is usually low. The use of enzymatic hydrolysis and microwave treatment is intended to solve this problem. EMAE significantly affected the glucan content in the crude GLPs, as shown in Table 6. The total content of glucan, α-glucan, and β-glucan in the crude GLPs from EMAE were 0.90 ± 0.05 g/10 g, 0.20 ± 0.01 g/10 g, and 0.70 ± 0.04 g/10 g, respectively. This value is higher than the crude GLPs from HWE, at 0.35 ± 0.05, 0.13 ± 0.02, and 0.22 ± 0.03 g/10 g. Cellic® CTec2 contains cellulase, β-glucosidase, and hemicellulases (Fang et al. 2015), it can degrade cellulose and hemicellulose components in the cell walls of *G. lucidum* and break polysaccharide glycosidic bonds into small molecules such as α or β-glucan. β-glucan in *G. lucidum* is also present in cells, so when cells are disrupted by microwaves, β-glucan from inside the cells is also released (Leong et al. 2021). Similar results were reported by Hwang et al. (2018).

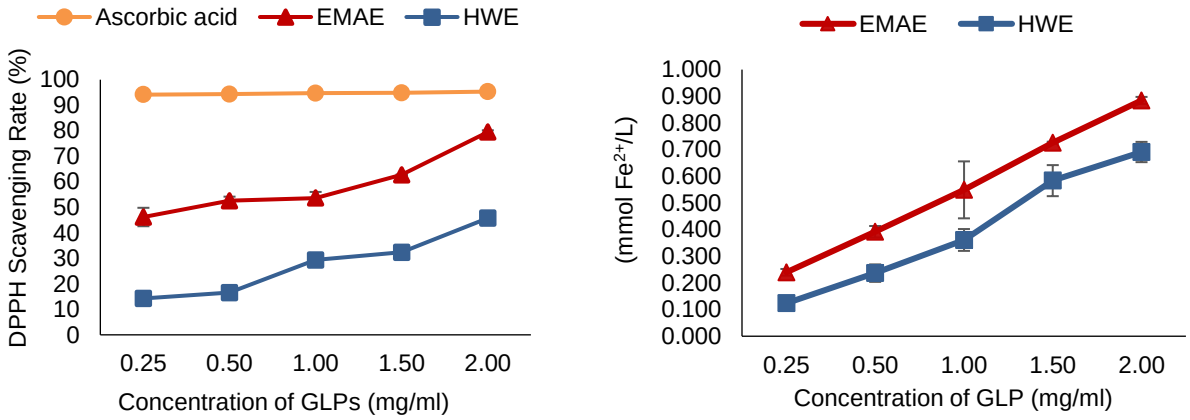

Figure 5. Antioxidant activities of GLPs from EMAE and HWE : (a) DPPH method (b) FRAP method

Sugar composition of GLPs

The chemical composition analysis intends to show the types of sugars that consist in GLPs. Crude GLPs mainly consists of a high mole percentage of galactose followed by maltose and glucose (Table 6). This finding is in line with previous studies on GLPs obtained by ultrasonic-assisted extraction (Alzorqi et al. 2017). The presence of these sugars refers to the high content of hemicellulose, cellulose, and glucan in the *G. lucidum* sample. The dominant content of galactose in the crude GLPs is due to the high concentration of hemicellulose and glucose confirms that GLPs are rich in glucan or cellulose polysaccharides. β-Glucan in mushrooms is comprised of glucose with β-(1-3) linkages and β-(1-6) linked branches. While cellulose is glucose molecules are linked together via 1-4 glycosidic bonds (Leong et al. 2021).

In addition, the presence of maltose in the extract is related to the content of α-glucan in GLPs. The GLPs obtained from EMAE and HWE contained galactose, maltose, and glucose, however, the arabinose content was only indicated by the EMAE method. By enzymatic hydrolysis, arabinose can be derived from biopolymers such as hemicellulose and pectin (Pol et al. 2020). Heteropolysaccharides consisting of maltose and arabinose were reported to be frequently present in polysaccharide extracts (Peng et al. 2015, Rjeibi et al. 2019, Wen et al. 2022). The sugar composition of polysaccharide extracts from mushrooms can vary depending on the growing environment which may contribute to differences in their bioactive activity.

Antioxidant assay

The DPPH and FRAP methods involve two components: antioxidants and oxidants. DPPH is stable organic nitrogen radical which acts as an oxidant like the Fe^{3+}-TPTZ complex. The two methods differ in the mechanism of their antioxidant mechanism. The FRAP test is based on transferring one electron to reduce metal ions, whereas DPPH is not only through donating electrons from antioxidants but also based on donating hydrogen radicals to free radicals (Munteanu and Apetrei 2021). The antioxidant activity test using the DPPH and FRAP methods of GLPs was carried out at various concentrations, as shown in Figure 5. The antioxidant activity of GLPs obtained from EMAE was higher than HWE for both methods. The antioxidant activity increased with increasing GLPs concentration (0.25 - 2.0 mg/ml).

In contrast, Kang et al. (2019) showed that: GLPs from HWE showed more DPPH radical-scavenging rate than GLPs from UAE. This is probably due to the ultrasonic waves destroying the high-molecular-weight polysaccharides and diminishing their antioxidant properties. Moreover, our results show the same phenomena as a study by Wei et al. (2019), polysaccharides extracted by MAE have higher antioxidant activity than those extracted by HWE. In addition, the result of FRAP assay agrees with other authors that the reduced power capacity from extracted eggplant peel by cellulase-assisted extraction was higher than the conventional solvent extraction (Amulya and ul Islam 2023).

Extraction of bioactive substances using HWE has disadvantages due to the degradation of their biological antioxidant because of extended exposure to high temperatures, resulting in decreased free radical scavenging activity (Kumar et al. 2020). The effect of long extraction time might degrade polysaccharides polymer structure and produce lower molecular weights. The GLPs molecular weight from the HWE method gives a lower value than the ultrasonic extraction method, which correlates with the lower antioxidant activity of the HWE method compared to the ultrasonic method (Alzorqi et al. 2017).

The EMAE method produced superior yield, chemical composition, and antioxidant activity in comparison to the HWE method. This study is expected to provide scientific data that may be employed as a reference to obtain a high yield of GLPs through a new method (EMAE), and the product could be implemented as antioxidant compounds.

CONCLUSION

EMAE is a better method to extract GLPs compared to the conventional HWE method and provides a higher extraction yield. In this research, RSM-CCD is used to optimize the extraction variables. The results showed that using 1.6% enzyme concentration, 10 minutes enzymatic reaction time, 50 mL/g solvent-to-solid ratio, and 12 minutes microwave extraction time were the best conditions to give the highest extraction yield, 1.62 ± 0.02%. The antioxidant activity, measured by the DPPH scavenging rate and the FRAP method of GLPs obtained from EMAE, was 79.47 ± 0.71% and 0.884 ± 0.013 mM Fe^{2+}/L, respectively. These values were higher than those obtained from the HWE method.

AUTHOR CONTRIBUTIONS

LWL sample preparation, data analysis, interpretation, and writing manuscript, SH data curation, review and editing manuscript, RY sample preparation, data analysis, SZ data curation, review and editing manuscript, AW supervision, data curation, review and editing manuscript. All authors approved the final manuscript.

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