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EXTRACTION OF BIOACTIVE COMPOUNDS AND SPORE POWDER COLLECTION FROM *Ganoderma lucidum*

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ABSTRACT

Ganoderma lucidum, commonly known as lingzhi mushroom, contains active compounds known as beta glucans which are polysaccharides with immune-boosting effects. The complex sugars found in Ganoderma lucidum may stop growth and prevent the spread of cancer cells. It is also rich in phenolic compounds, which prevent the oxidative damage induced by free radicals generated in body cells. The content of this paper including (i) optimization extracting process based on the experimental design of time (15-45 minutes) and temperature (70-130°C) and (ii) fermentation of G. lucidum spore with Lactobacillus plantarum for breaking spore wall from 24 to 72 hours. Spores with broken walls can release the effective ingredients. Response surface methodology was successfully applied to describe the relationship between polysaccharide, phenol compounds content and extraction temperature and time. The quadratic models for both polysaccharide and phenol compounds extraction were significant (R^2>0.95). From the results of numerical and graphical optimization methodology, the optimum extraction conditions were achieved at temperature of 130°C for 40-45 minutes. The optimum conditions (130°C, 40 min) were validated with actual polysaccharides and phenol compounds content of 667.5 mg/L and 631.25 mg GAE/L, respectively. The rate of spore wall breaking can become 52.38% after 48 hours of fermentation with Lactobacillus plantarum. The high antioxidant activity fungal spore powder was obtained.

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1 INTRODUCTION

Ganoderma lucidum has been considered as a rich source of natural antibiotics. Among different bioactive compounds of this mushroom (including triterpenoids, sterols, polysaccharides, tannin and fatty acids), polysaccharides and phenol compounds are considered active constituents and are reported to possess many beneficial effects, such as antitumor, cardiovascular, respiratory (Chang and Mshigeni, 2004). Djide *et al*. (2014) studied on the antibacterial activity of various extract of G. lucidum from Rain Tree against Staphylococcus aureus and Pseudomonas aeroginosa. A preliminary procedure for extraction of polysaccharides from G. lucidum involved solvent extraction and solvent adsorption (Sye, 1991). As a general procedure, there is a broad similarity in the various methods that have been developed to extract the polysaccharides from mushroom fruit-bodies, mycelium and liquid media. In addition, the bioactive substances in G. lucidum are protected by hard sporoderm and

these sporoderm need to be broken down in order to increase the absorption of bioactive substances (Jungjing *et al*., 2007). Some processes for making spores break down have been developed such as physical smashing, ultrasonic, high pressure and enzyme utilization. However, these methods require high cost and also decompose bioactive compounds (Fu *et al*., 2009). The mechanism of spore break down by lactic acid bacteria was investigated by Chaiyasut *et al*. (2010). The G.lucidum product in Vietnam today is tea bag. However, the disadvantages of this product is not fully utilized the bioactive substances, especially spores containing polysaccharides and triterpenoids were retained in the filter bag. Therefore, further assessment still need before they can be accepted for food and beverage industry. Processing of G. lucidum beverage containing high bioactive compounds will be able to create a marketable new product in Vietnam with quality assurance.

2 MATERIAL AND METHODS

2.1 Sample preparation and bioactive compounds extraction

The dried fruiting body of *G. lucidum* (Nấm Việt Biotechnology Joint Stock Company) was ground to pass through a 2 mm sieve and *G. lucidum* powder was harvested and its moisture content was 8.26%. A 40g of G. *lucidum* powder was suspended in 2 L of water and subjected in water bath for extraction at 70°C. The high temperature extractions (100-130°C) were carried out in autoclave. Extraction fractions were obtained by filtration and further centrifugation (3500 rpm in 5min), then it could be used for making instant beverage. The pellets which contained a multitude of spores were collected for fermentation and drying in order to produce the wall-broken spore powder.

2.2 Response Surface Methodology (RSM) Experimental Design

RSM was applied to find out the optimum extraction conditions for *G. lucidum* polysaccharides and phenol compounds. The extraction experiments were performed according to a central composite (CC) design with a two-factor and three levels (-1; 0; 1). The complete CC design comprised of twenty-two experiments in which design points (factorial and axial points) were duplicated, plus six center points (Table 1). Each experiment was performed in replicate and the average values were taken as the response. Statistical software Design-Expert version 8.0.7.180 (StatEase Inc., Minneapolis, USA) was used to code the variables and to establish the design matrix.

Table 1: Experimental matrices based on three levels central composite face-centered (CCFC) design

Runs	Code Factors		Actual Factors	
	X_1	X_2	Temperature (°C)	Time (min)
1	-1	-1	70	15
2	-1	-1	70	15
3	1	-1	130	15
4	1	-1	130	15
5	-1	1	70	45
6	-1	1	70	45
7	1	1	130	45
8	1	1	130	45
9	-1	0	70	30
10	-1	0	70	30
11	1	0	130	30
12	1	0	130	30
13	0	-1	100	15
14	0	-1	100	15
15	0	1	100	45
16	0	1	100	45
17	0	0	100	30
18	0	0	100	30
19	0	0	100	30
20	0	0	100	30
21	0	0	100	30
22	0	0	100	30

Models were fitted to a second-order polynomial equation (eq. 1).

$$Y = b_o + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i=1}^{k} b_{ij} X_i X_j + e \quad (1)$$

The b_o is Y-intercept, independent variables are extraction temperature (X_1, °C), extraction time (X_2, minute) and dependent variables (response variables) are total polysaccharides (Y_1, mg/L,) and phenol compounds (Y_2, mg of gallic acid equivalent per litter of extract).

2.3 Fermentation of G. lucidum with Lactobacillus plantarum

One mL spores of *G. lucidum* and two mL pasteurized broth media were fermented with *Lactobacillus plantarum* (*L. plantarum*) (Nguyễn Xuân Cương *et al*., 2013) 10^8 CFU/mL in incubator at 37°C (Chaiyasut *et al*., 2010). The samples were collected at 0, 24, 48 and 72 hours. The control sample is spores without *L. plantarum* at 37°C. The samples were then filtered using Whatman paper No.1. The spores, which remained on filter paper, were dried at 60°C for 3 hours. The efficiency (as percent) of spore breaking was determined as the ratio of the densities of broken spores and initial spores and multiplying by 100. The morphological change of the spores of *G. lucidum* during fermentation period was observed by light microscope.

2.4 Drying of spores powder

The effect of drying temperature to the antioxidant activity of spore powder was made. Spore powder was dried at temperatures ranging from 95 to 105°C until 2 to 3% moisture content. The antioxidant activity was measured.

2.5 Determination of total polysaccharides and total phenol composition

Total polysaccharides*:* for total polysaccharides determination, different glucose standard solutions (0.3, 0.25, 0.2, 0.15 and 0.1) mg/mL was prepared from glucose stock solution of 1 mg/mL. A quantity of 250 mg from the precipitate was dissolved in 50 mL hot water to get concentration of 5 mg/mL and subjected to the following methods for determination (Chia *et al*., 2009).

Total phenol: the total content of phenols in sample was determined by modified Folin-Ciocalteu colorimetric method using gallic acid as a standard (Lubica *et al*., 2007). The total phenolic content was expressed as mg of gallic acid equivalent per litter of extract (mgGAE/L).

Reducing power (RP) assay: the RP was determined according to the method of Oyaizu (1986). Each amount of spore powder (10 to 20 mg, 5 mg interval) was subjected in 2.5 mL of phosphate buffer (pH 6.6) and 2.5 mL of 1% $K_3[Fe(CN)_6]$. The mixture was vortexed and incubated at 50°C for 20 min. Aliquots of 10% (w/v) trichloroacetic acid (2.5 mL) were added to the mixture which was centrifuged (3000 rpm, 10 min). The supernatant (5 mL) was mixed with 5 mL of distilled water and 1 mL prepared of 0.1% $FeCl_3$. The absorbance was measured at 700 nm against a blank and ascorbic acid (AA) was used as the standard.

3 RESULT AND DISCUSSION

3.1 Optimisation of extraction of polysaccharides and phenol compounds from *G. Lucidum*

The regression equations in term of coded factors for response surface for total polysaccharide and phenol compounds extraction were obtained and described in equation 2 and 3, respectively.

$$Y_1 = 448.04 + 145.94\ X_1 + 57.67\ X_2 + 36.44\ X_1X_2 + 36.80\ X_1^2 - 47.82\ X_2^2 \quad (2)$$

$$Y_2 = 473.25 + 165.05\ X_1 + 73.22\ X_2 - 22.32\ X_1^2 - 54.39\ X_2^2 \quad (3)$$

The analysis of variance of regression equation for models of polysaccharide extraction is shown in Table 2.

Table 2: Analysis of variance (ANOVA) for the model of polysaccharide extraction

Source	Sum of Squares	df	MeanSquare	FValue	P-value Prob > F
Model	320857	5	64171.4	75.9789	< 0.0001
Residual	13513.52	16	844.5947		
Cor Total	334370.5	21			

R^2: 0.9596; Adj R^2: 0.947; Pred R^2: 0.9227; Adeq Precision: 26.831

It was observed that this regression model was highly significant (P<0.01) with an F-value of 75.98, implying a good fit between the predicted model and the experimental data. The low probability value (less than 0.0001) indicated model terms are very significant. In case of model

of polysaccharide extraction, terms of X_1, X_2, X_1X_2, X_1^2andX_2^2 were significant.

Results of the analysis of variance of the regression equation for phenolic compounds extraction are displayed in Table 3. The model F-value of 731.99 with a low probability value [(Prob>F)<0.0001)] indicated a high significance for the regression model. In this case, X_1, X_2, $X_1^2$and X_2^2 were significant model terms. The "Pred R^2" of 0.994 was in reasonable agreement with the "Adj R^2" of 0.993. Furthermore, the value of "Adeq Precision" of 84.103 which measured the signal to noise ratio indicated an adequate signal. Therefore, it could be used for theoretical prediction of the extraction of phenol compounds from *G. Lucidum.*

Table 3: Analysis of variance (ANOVA) of the regression equation for phenolic compounds extraction

Source	Sum of Squares	df	Mean square	F Value	p-value Prob > F
Model	413627.4	4	103406.9	731.9852	< 0.0001
Residual	2401.574	17	141.2691		
Cor Total	416029	21			

R^2: 0,9942; Adj R^2 : 0,9929; Pred R^2: 0,9896; Adeq Precision: 84,103

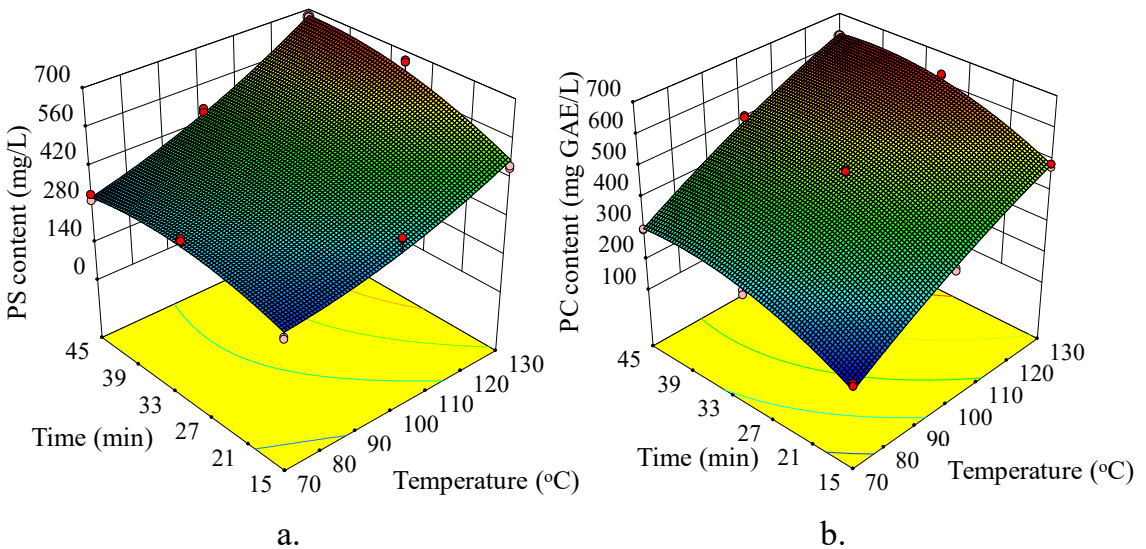

Fig. 1: The surface and contour plot of polysaccharides (mg/L of extract) (a) and phenolic compounds (mg GAE/L of extract) (b) as affected by extraction temperature and time

PS: polysaccharide, PC: phenolic compounds

In the plot of polysaccharides content against extraction temperature and time, this yield increased slightly in increasing extraction temperature from 70 to 100°C. However, it increased sharply at high extraction temperature (from 100 to 130°C). The influence of interaction effect of time and temperature was pronounced on polysaccharides yield, the highest yield was obtained at extraction temperature of 130°C for 40 to 45 minutes (Figure 1a).

Polysaccharide peptide is a protein-bound polysaccharide are covalently attached to the proteins by N- or O-linked forms and involved in many important cellular communication processes associated with cell adhesion, host-pathogen interaction, and immune responses (Wu and Wang, 2009). A high yield of polysaccharides was obtained with an increase in extraction temperature due to increasing the hydrolysis of polysaccharides (XuJie and Wei, 2008). Furthermore, at temperature of 120°C, hemicellulose was also extracted (Yu *et al*., 2008) and contributed to total polysaccharides yields. The results of Sood *et al.* (2013) showed that the relationship between extraction time, temperature and polysaccharides yield from *G. Lucidum* were also followed quadratic model. The optimum operating conditions for polysaccharides yield extraction (4.96%) was obtained about 7 hours at 100°C with using of 20 mL of 6% NaOH. Similarly, the yield of phenolic compounds increased with the increase of extraction time and temperature. It can be seen that linear and quadratic term was predominated;

however, an interaction between independent variables on the yield of phenolic compounds was not found (Figure 1b). The fruiting body of *G. Lucidum* was rich in total phenolics and phenolic acids such as p-hydroxybenzoic and p-coumaric acids (Heleno *et al.,* 2012).

By analyzing the effects of extraction conditions on the yield of polysaccharides and phenolic compounds, the optimum extraction conditions were obtained by graphical and numerical optimization using constraints for targeted variables as shown in Table 4. Multiple graphical optimizations were conducted by drawing the overlaid contour plot (Figure 2). In the plot, the optimum extraction conditions that satisfied the above constraints were described in yellow area. The result of the numerical optimization also confirmed that the most desirable conditions for both polysaccharides and phenol compounds extraction from *G. Lucidum* were achieved at temperature of 129-130°C and time of 40-45 minutes (Table 5).

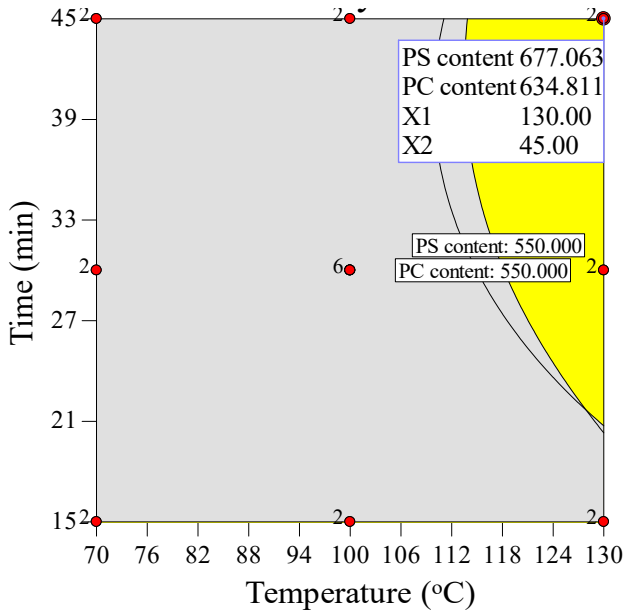

Fig. 2: Graphical optimization using overlaid counter plots for showing the optimum area (*PS: polysaccharide, PC: phenolic compounds)*

Table 4: Constraints for Targeted Variables

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
X_1: Temperature	is in range	70	130	1	1	3
X_2: Time	is in range	15	45	1	1	3
Y_1: PS content	maximize	550	671.2	1	1	3
Y_2: PC content	maximize	550	630.8	1	1	3

Note: PS: polysaccharide, PC: phenolic compounds

Table 5: Solution for optimum conditions of extraction of polysaccharides and phenolic compounds

Number	Temperature (°C)	Time (min)	PS content (mg/L)	PC content (mg GAE/L)	Desirability
1	130.00	45.00	677.063	634.811	1
2	129.79	44.77	675.306	634.515	1
3	129.83	40.89	672.540	639.805	1
4	129.96	43.02	676.113	638.407	1
5	129.72	41.55	672.606	639.001	1
6	129.44	44.95	672.283	632.656	1
7	129.71	42.25	673.357	638.353	1
8	129.53	44.70	673.095	633.624	1
9	129.46	43.39	672.186	635.85	1
10	129.86	44.53	675.843	635.293	1
11	129.65	43.18	673.599	636.902	1
12	129.98	42.68	676.031	638.947	1
13	129.95	40.66	673.073	640.331	1
14	129.45	42.78	671.625	636.648	1
15	129.79	43.30	674.901	637.314	1
16	129.99	44.18	676.928	636.56	1
17	129.69	41.32	672.025	639.02	1
18	129.42	43.27	671.758	635.865	1
19	129.56	41.97	671.82	638.015	1
20	129.68	42.12	672.98	638.361	1
21	129.91	40.23	671.996	640.267	1

Note: PS: polysaccharide, PC: phenolic compound

Verification of models

To verify the prediction of the model, the optimum conditions (130°C, 40 min) for extraction were applied. The yield of polysaccharide and phenolic compounds were obtained (667.5 mg/L and 631.25 mg GAE/L, respectively). The observed results were in agreement with the predicted value of polysaccharide yield of 677.063 mg/L and phenolic compounds of 634.811 mg GAE/L.

Breaking the spores wall of G. Lucidum by Lactobacillus plantarum

The spores of G. lucidum also contain a large amount of bioactive substances like the fruiting body of G. lucidum. The bioactivity of the spores may be higher than that of the fruiting body of G. lucidum (Min *et al*., 1998). The breaking of the spores of G. lucidum can improve the release of activity (Zhu *et al*., 2000). The shell of a Ganoderma lucidum spore is created with high-molecular weight chitin and it is usually difficult for the human digestive system to break down. The digestion of chitin normally requires symbiotic microbes that can ferment and break down the long-chained polymer. Lactobacillus plantarum had contributed to break the G. lucidum spores along the fermentation time. In the first 24 hours of fermentation, the rate of spore wall breaking achieved 38.1% (initially 0%). After 48 hours, this rate can become 52.38%, and then almost lightly increased after 72 hours of fermentation. As chitin is a component of the cell walls of fungi (cells contain 52.08 to 54.68% of chitin) (Jungjing *et al*., 2007), during fermentation by Lactobacillus plantarum, chitinases are generally found in organisms dissolve and digest the chitin of fungi (Brurberg *et al*., 1994). However, prolong the incubation period would not be obtained more soluble chitin membranes due to at the end of the process, the bacteria do not have more nutrients and incapable of producing enzymes.

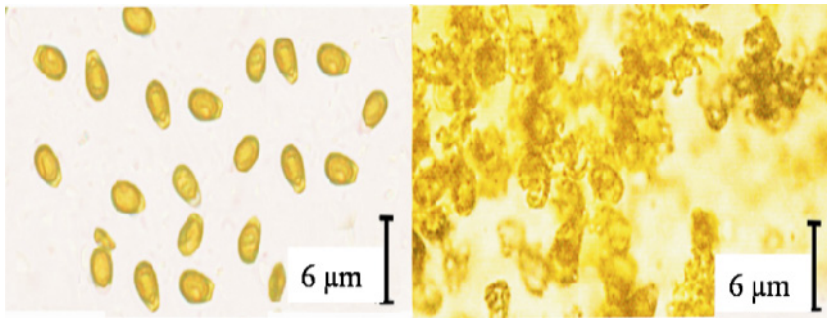

Fig. 3: Spores with unbroken walls and wall-broken spores

Figure 3 compare the structure of natural *G. lucidum* spores (left) with spores that have been processed using our wall-breaking technology (right). Spores with broken walls can release the effective ingredients and easily absorbed into the human body (Liu *et al*., 2002). The figure on the left displays a magnified picture of the intact spores.

3.2 Effects of drying temperature to antioxidant activity (described as RP) of *G. Lucidum* spore powder

It was observed that *G. lucidum* spore powder revealed high antioxidant activity, in some cases even higher than antioxidant property of ascorbic acid (Table 6). High RP of *G. lucidum* spore powder indicated their potential to provide electrons to reactive free radicals, in consequence converting them into more stable non-reactive species and finally terminate the free radical chain reaction (Zha *et al.,* 2009). The RP of *G. lucidum* spore powder increased with an increasing in drying temperature (from 95 to 105°C). The obtained results were in agreement with Madrau *et al.* (2009), concerning air-drying of apricots. They also suggested that antioxidant activity was enhanced during high temperature drying due to increasing antioxidant power of polyphenols at an intermediate state of oxidation, increase in reducing sugar and formation of Maillard reaction products, which have a great antioxidant activity. The product of spore powder (density of 1.38×10^9 CFU/g) remained light brown color and flavor characteristics of *G. lucidum* and could be soluble in hot water with light bitter taste.

Table 6: Reducing power of *G. Lucidum* spore powder (absorbance at 700 nm)

Temperature (°C)	Concentration (mg/mL)		
	10	15	20
95	1.32±0.19	2.48±0.19	3.07±0.25
100	2.14±0.19	2.93±0.10	3.70±0.18
105	2.66±0.08	3.06±0.15	3.67±0.11
AA	2.27±0.06	2.70±0.04	2.81±0.06

Each value was expressed as Mean ± Standard deviation (n = 3)

4 CONCLUSIONS

Response surface methodology was successfully applied to the extraction of *G. lucidum*. With the simple technique and low investment cost, the obtained results showed high feasibility and it could be applied for lingzhi musrhoom beverage processing. The fermentation with *Lactobacillus plantarum* has shown the efficiency in broken down of *G. lucidum* spore-wall. The high antioxidant activity (as RP) of wall-broken spore powder was obtained after drying.

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