Effect of extraction conditions (temperature, pH and time) by cellulase on chemical properties of dried oyster mushroom (Pleurotus sajor-caju) extract

1,*, Nguyen, G.T.N. and 2Nguyen, T.M.

1Experimental-practical Area, An Giang University, Vietnam University Ho Chi Minh city, Vietnam
2Food Technology Department, Can Tho University, Vietnam

Abstract

Oyster mushroom (Pleurotus sajor-caju) is known to be highly nutritious food. However, the understanding of its quality and food products produced from it are still very limited. With the desire to increase the value of oyster mushroom and to create an intermediate product with high nutrients content for further processing, a cellulase enzyme-assisted extract of dried oyster mushroom was investigated. The effects of extraction conditions in terms of temperature (40, 45, 50, 55 °C), pH (4.5, 5.0, 5.5, 6.0) and extraction time (4, 6, 8, 10 hrs) on nutrients and bioactive compounds of extract were studied. The results showed that the highest extraction efficiency was obtained at 50 °C, pH 5.5 and 8 hrs of hydrolysis. The Brix of the extract was 2.53. In 100 g dry matter of extract, the content of saccharose, reducing sugar, total protein, amino acids, lysine, β-glucan, total phenolic and total flavonoid was 15.13 g, 19.41 g, 43.65 g, 7.96 g, 0.82 g, 10.63 mg, 62.60 mg TAE, 5.54 mg QE, respectively. The highest antioxidant capacity of the extract determined by Ferric reducing antioxidant power (FRAP assay) was 165.50 mM Fe²⁺/100 g dry matter and that measured by scavenging 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radicals was 69.32%.

1. Introduction

Oyster mushrooms (Pleurotus sajor-caju) have been identified as a vegetable in the food world. Carbohydrates and proteins are the main components, accounting for 70-90% of the dry weight of fruiting bodies. Protein and peptides are biologically active polymers of Pleurotus mushrooms that have anticancer and antiviral effects (Li et al., 2008). Polysaccharide and fibrin are considered “bio-reactivity modulators” with prebiotic potential to protect the intestinal microbiota of human (Jayachandran et al., 2017). Pleurotus mushrooms contain a higher concentration of oxidants than other commercial edible mushrooms (Mau et al., 2001) because of the presence of β-glucan which showed a positive effect on the precancerous lesion (Bobek and Galbavy, 2001). Besides that, the bioactive compounds present are the most antioxidant ingredients in all of Pleurotus spp. including flavonoid, phenolic acid with redox properties and act as a reducing agent, providing hydrogen, reducing free radicals, and single group oxygen (Barros et al., 2006; Michalak, 2006; Newell et al., 2010).

Extraction is a widely known process in which bioactive substances are separated from the structural part of the plant or mushroom (Chew et al., 2011). The extract contains higher nutritious content and higher bioavailability (Plaza and Turner, 2015). The extraction efficiency is reflected by the functionalities of components in the extract. However, extraction efficiency is dependent on the extraction method (Goli et al., 2004). The desirable extraction method allows the extraction of completely functional components without including any changes in their functionalities (Zuo et al., 2002). In this regard, extraction by an enzyme such as cellulase will be the best option due to its unique advantages. The cellulase is capable of degrading or disrupting cell walls, allowing release and extract effectively nutrients and bioactive compounds (Pinelo et al., 2005). The use of cellulase to extract apple peel increased the contents of phenolic and reducing sugar and improved antioxidant capacity in the extract. Approximately 60 mg of reducing sugar was obtained per gram of apple peel by cellulase whereas only 20 mg was archived in the non-enzyme extraction method. Enzyme-assisted extracts have been applied to extract antioxidant compounds from a variety of materials including pumpkin shell waste (Cucurbita moschata) (Wu et al., 2014), Agaricus blazei Murrill (Jia et al., 2013), rice bran (Kim and Lim, 2016). In addition, enzyme-assisted extraction showed lower energy...
consumption, faster extraction rate, higher efficiency and less solvent usage compared to traditional non-enzyme extraction methods (Puri et al., 2013).

Despite high nutritional values and abundance in materials of oyster mushrooms in Vietnam, the production of oyster mushrooms in Vietnam is very limited in terms of technology, productivity and product diversity. Therefore, the production of extract from dried oyster mushroom powder improves the value of oyster mushrooms and produces an intermediate product with high content of nutrients and bioactive substances, which can be used for further processing of many food products. The aim of study was to investigate the effects of extraction conditions of cellulase such as temperature, pH and extraction time from dried oyster mushroom on nutrients and bioactive compounds in the extract to find the optimal extraction conditions which allow the highest nutritional values of the extract to be obtained.

2. Materials and methods

2.1 Materials

Oyster mushrooms (Pleurotus sajor-caju) were supplied by An Giang University, Vietnam University Ho Chi Minh city (Vietnam). Fresh mushrooms were washed with water, drained and dried using a solar dryer until the moisture content of 11.32±0.05%.

Cellulase was bought from Regal Company (China). It is a brown powder and the enzyme activity was about 15,000 unit/g solid according to certification from Regal Company. Cellulase powder was diluted with distilled water to obtain the activity of 150 Unit/mL for experiments.

2.2 Experimental design

Dried oyster mushrooms were grounded and sieved through a 1 mm sieve. Approximately 5 g of the powder was mixed with 100 mL water and then adjusted pH of the mixture to 4.5, 5.0, 5.5 and 6.0 with 10% citric acid (Jaran et al., 2008), and 2 mL cellulase was added. The flasks were sealed with aluminium foil and incubated in a thermostatic bath (Memmert, Germany) at designated temperatures of 40, 45, 50 and 55°C for 4, 6, 8 and 10 hrs. After hydrolysing, extracts were filtered through a 1 mm sieve. Approximately 5 g of the powder was washed with water, drained and dried using a solar dryer until the moisture content of 11.32±0.05%.

2.3 Determination of saccharose and reducing sugar

Saccharose and reducing sugar contents (g/100 g dry matter) were measured by the DNS method (Miller, 1959) with some modifications. This method is based on the oxidation of the C=O group by 3,5-Dinitrosalicylic acid from yellow colour to orange-red in an alkaline medium. An aliquot (1 mL) of sample was put in a test tube and then added 2 mL of reagent DNS. The tubes of blank, solution of standard glucose and samples were put in boiling water for 10 mins. Next, 7 mL distilled water was added. The solution was analysed at an absorption of 575 nm. The concentration of saccharose and reducing sugar were based on a standard curve of glucose, 
\[ y = 23885x + 0.126 (R^2 = 0.9999), \]
where y is the absorbance and x is the concentration of the solution in the tube.

2.4 Measurement of colour

The colour of samples was measured using a Hunter Lab colourimeter (CR 400, Konica Minolta, Japan). The value a defines the red/green coordinate and the b value shows the yellow/blue coordinate. Chroma ΔC describes the change of colour during extraction (Maskan, 2001) and is calculated using equation 1:

\[ \Delta C = \sqrt{(a-a_0)^2 + (b-b_0)^2} \]

Where a₀ and b₀ are values of calibration with white ceramic plate; a and b are values of samples.

2.5 Determination of total protein

The total protein content (g/100 g dry matter) was measured by the Lowry method (Hartree, 1976) with some modifications. 0.1 mL of sample or standard was added with 0.1 mL of 2N NaOH in the tube and placed in boiling water for 10 mins. The tube was cooled to room temperature and then added with 1 mL of complex-forming reagent (including 2% (w/v) Na₂CO₃, 1% (w/v) CuSO₄·5H₂O and 2% (w/v) sodium potassium tartrate in the proportion of 100:1:1, respectively). Approximately 0.1 mL of Folin-Ciocalteu reagent was then added into the tube, vortexed and was left at room temperature for 30 mins. The Lowry method is based on the reaction of Cu⁺ with Folin-Ciocalteu reagent to react into an intense blue colour and measured with an absorbance of 750 nm. The concentration of total protein was based on the standard curve of protein, 
\[ y = 0.0041x + 0.0118 (R^2 = 0.9999), \]
where y is the absorbance and x is the concentration of the solution in the tube.

2.6 Determination of β-glucan

β-glucan content (mg/100 g dry matter) was analysed by the Phenol-Sulfuric method (John and Sons, 2001). 5 mL of sample was mixed with 20 mL of 96% ethanol and left to sit at 4°C for 24 hrs. The solution was filtered with Whatman number 5B filter paper. Approximately 2.5 mL of filtered solution was added with 2.5 mL of 1M NaOH for 1 hr at 60°C and later was left at room temperature. The mixture of 0.5 mL of 4% (w/v) phenol and 2.5 mL of 96% H₂SO₄ was mixed. The
absorption of the phenol and glucan was analysed at 490 nm. The concentration of β-glucan content was based on the standard curve of glucan, \( y = 2745.61x + 0.0003 \) \((R^2 = 0.9999)\), where \( y \) is the absorbance and \( x \) is the concentration of the solution in the tube.

2.7 Determination of total phenolic content

Total phenolic content (mg TAE/100 g dry matter) was determined using Folin-Ciocalteu reagent based on Singleton et al. (1999). Approximately 150 µL of the sample was mixed with 1200 µL of distilled water and 450 µL of 5% (w/v) Na$_2$CO$_3$ in a test tube. The mixture was mixed with 0.1 mL of Folin-Ciocalteu reagent and left at room temperature for 90 mins. Phenolics in the extract react with Folin-Ciocalteu to form a blue complex in the alkaline medium that is phosphomolybdenum complex. The concentration of total phenolic was calculated according to the standard tannic acid graph (TAE), \( y = 0.0021x + 0.0064 \) \((R^2 = 0.9999)\), where \( y \) is the absorbance and \( x \) is the concentration of the solution in the tube.

2.8 Determination of total flavonoid content

This assay was performed using the aluminium chloride colourimetric method described by Barros et al. (2008) with some modifications. The principle is related to AlCl$_3$ creating a stable acid complex with the C-4 keto groups and the hydroxyl C-3 or C-5 group of the flavon and flavanol. Approximately 100 µL of the sample was added to 1200 µL of distilled water and 30 µL of 5% (w/v) NaNO$_2$. After 5 mins, the mixture was mixed with 10% (w/v) AlCl$_3$.H$_2$O (60 µL). 200 µL of 1M NaOH and 110 µL of water were mixed into the solution. It was then analysed at 510 nm. The concentration of total flavonoid was calculated according to the standard quercetin graph (QE), \( y = 8.2634x + 0.0182 \) \((R^2 = 0.9999)\), where \( y \) is the absorbance and \( x \) is the concentration of the solution in the tube.

2.9 DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity

DPPH assay was measured using the method described by Molyneux (2004) with some modifications. 1.5 mL of sample was mixed with 1.5 mL of DPPH solution. This assay is based on the electron transfer that produces a purple solution in ethanol and was analysed at 517 nm. Inhibition of DPPH free radicals was calculated using equation 2:

\[
\text{Inhibition of DPPH radical} \% = 100 \times \frac{(A_{control} - A_{sample})}{A_{control}} \quad (2)
\]

Where \( A_{control} \) is the absorbance of the control and \( A_{sample} \) is the absorbance of the sample.

2.10 Determination of amino acids content

Amino acids content was measured using the Ninhydrin method (Friedman, 2004). Ninhydrin binds with amino acids and undergoes oxidation to release NH$_3$, CO$_2$ and hydrindantin. NH$_3$ reacts with another ninhydrin molecule and hydrindantin to produce the blue substance diketohydrindin (Ruhemann complex). 1 mL of sample was mixed with 0.5 mL of 0.2 M citrate buffer (pH 5.0) and 0.2 mL of 2% ninhydrin solution. After 30 mins, the mixture was measured at 570 nm. The concentration of amino protein was based on the standard curve of leucine, \( y = 614.820x + 0.002 \) \((R^2 = 0.9999)\), where \( y \) is the absorbance and \( x \) is the concentration of the solution in the tube.

2.11 Data analysis

Statistical analysis of the data was conducted using STATGRAPHICS Centurion 16.1 software. One-way analysis of variance (ANOVA) was used. Tukey’s multiple comparison test was employed to determine significant differences in treatment means at \( p<0.05 \). Microsoft Excel software was used for calculating and graphing.

3. Results and discussion

3.1 The influence of extraction conditions (temperature, pH and time) on the chemical compositions

The effect of temperature, pH and extraction time on chemical compositions is shown in Figure 1. The results showed that all investigated extraction parameters had a quadratic effect on the chemical compositions of the extract. When the temperature, pH and extraction time increased, the Brix, saccharose, total protein, reducing sugar and amino acids in the extract increased to an optimal value and then decreased. Specifically, when heating from 40 to 50°C, Brix, saccharose, reducing sugar and amino acids contents raised from 2.19; 5.30;
Temperature is one of the factors that greatly influence extraction by enzymes. When the temperature is high, the materials expand and the compounds in extract become more flexible and facilitating the extraction process. In addition, temperature greatly affects the catalytic activity of enzymes. Under the effect of extraction temperature, the reaction rate increases rapidly but to a certain extent, it will inhibit the extraction (Copeland, 2000). High temperature causes a gradual loss of enzyme activity along with protein inactivation (Peterson et al., 2007). Therefore, 50°C is the appropriate temperature for extraction by cellulase with the highest content of chemical compositions.

Furthermore, the extraction time also affects the content of the chemical compositions of the extract. The contents of reducing sugar, amino acids increased with increasing extraction time to an optimal value, then gradually decreased and reached the highest after 8 hrs of extraction. The Brix and saccharose contents tended to be similarly and gained the highest at 6 and 8 hrs of extraction (p≥0.05). The highest total protein content was observed from extraction time of 8 and 10 hrs.

The extraction time is also an important factor to determine the efficiency of the extraction process and energy cost. If the extraction time is short, the release of nutrients is small and the extraction process is not complete. By contrast, the energy is lost and the process is prolonged, the quality and quantity of nutrients will decrease with long extraction time. The enzymatic hydrolysis takes a minimum time for enzymes affecting the substrate. When the extraction time is extended, the extraction efficiency also increases. This can be explained by Fick’s second law of diffusion, as the extraction time increases, the more the nutrient content of material diffuses from the cell to the solvent as much (Cracolice and Peters, 2009). However, for a limited number of enzyme and substrate, at a given time, the substrate is almost completely metabolized (Landbo et al., 2007). Cellulase provides high hydrolytic efficiency, breaks down the cell wall structure and the hydrolysis reaction of this enzyme usually is strong in the early hrs.

Figure 1. The correlation of extraction conditions and the chemical compositions of oyster mushrooms extract (a) Brix, (b) saccharose, (c) reducing sugar, (d) total protein and (e) amino acids (one factor of which was fixed at the central point)
of the hydrolysis process. Prolonged hydrolysis may interfere with enzyme activity. Enzymes have a reversible and irreversible absorption capacity during hydrolysis that can lead to reduced enzyme activity (Martin et al., 1998). In this way, the time to extract oyster mushrooms by cellulase was 8 hrs.

Moreover, the contents of saccharose, total protein and amino acids contents of extract were the highest at pH 5.5. The Brix and reducing sugar contents of extract were high at pH 4.5 (Figure 1). Each enzyme will act at a certain pH range and the optimal pH can be found in the range of 4.5-5.5 for cellulase (Coral et al., 2002). Thus, pH 5.5 was chosen to carry out further studies.

3.2 The influence of extraction conditions (temperature, pH and time) on the bioactive compounds

Figure 2 shows the quadratic effect of temperature, pH and time of extract on the bioactive compounds. Similar to chemical compositions, total phenolic and total flavonoid, the contents of β-glucan and lysine increased to an optimal value and then slowed down when temperature increases, pH and time of extraction. In detail, when the extraction temperature increased from 40 to 50°C, the β-glucan content increased from 8.28 to 9.81 mg/100 g dry matter and lysine content increased from 0.63 to 0.74 g/100 g dry matter with increasing temperature from 40 to 45°C (Table 1). Similarly, β-glucan and lysine contents were highest after 8 hrs of extraction at pH 5.5 (Table 2 and 3). The cell wall of Pleurotus mushrooms is rich in non-starch polysaccharide, of which β-glucan is the most interesting functional component (Wang et al., 2001). Besides, lysine is one of the main essential amino acids found in oyster mushrooms. The cellulase breaks down the structure of the cell wall and releases the internal components, including β-glucan and lysine that make the extract increase the quality and therapeutic effect. The temperature also significantly affected the composition of bioactive compounds. The total phenolic content also tended to rise with the increase of extraction temperature. In contrast, the total flavonoid content decreased in increasing extraction temperature. Other studies showed that low molecular phenolic compounds were easily released from the cell wall of mushrooms after heat treatment, therefore, the total phenolic content increased (Jeong et al., 2004). However, as the temperature increased, the flavonoid compound in the extract was decomposed because of hydrolysis, intrinsic oxidation and polymerization (Simon et al., 1990). Besides that, the total phenolic and total flavonoid contents increased with rising extraction time to an optimal value, then gradually decreased and reached the highest after 8 hrs extraction. In addition, the efficiency of extracting bioactive substances will not increase after a certain period of extraction. The time of extraction depends on extraction conditions, such as solvent, extraction temperature, solvent/material ratio. Furthermore, the extraction time also depends on the properties of raw materials and the extracted compounds (Silva et al., 2007). When prolonging the extraction time, phenolic compounds of materials and extraction reached equilibrium, total phenolic content slowly increased. Moreover, these compounds may be oxidized by environmental factors (such as temperature, light, oxygen) (Naczk and Shahidi, 2004). Therefore, the time to extract oyster mushrooms by cellulase is 8 hrs. Also, the total phenolic and flavonoid contents increased with increasing pH and reached the highest value at pH 6.0.

Table 1. Statistical results of β-glucan and lysine contents of oyster mushrooms extract at different extraction temperature

| Temperature (°C) | β-glucan (mg/100 g Lysine (g/100 g dry | 40   | 8.28** | 0.63b |
|------------------|----------------------------------------|------|--------|-------|
| 45               | 9.37c                                 | 0.74d|
| 50               | 9.81d                                 | 0.71c|
| 55               | 8.55b                                 | 0.54a|

Level of significance ** **

Values are expressed as means of triplicate testing. Values with a different superscript in each column are statistically significantly different. ** indicates 99% statistically significant.

Moreover, the oxidation capacity of oyster mushrooms was significantly affected by the extracting factors. The influence of extraction temperature, pH and

Figure 2. The correlation of extraction conditions and the bioactive compounds of oyster mushrooms extract (a) Total phenolic, (b) Total flavonoid (one factor of which was fixed at the central point)
time to an oxidative capacity of the extract is shown in Figure 3. The extracting factors influenced the quadratic model on the antioxidant capacity of the extract [through DPPH radical scavenging (DPPH) and Ferric reducing antioxidant power (FRAP)]. As the temperature, pH and time of extraction increased, FRAP and DPPH increased and reached the highest values at 50°C for 8 hrs at pH 5.5. The antioxidant capacity of the extract decreased with continuing to increase extracting factors. All of the above showed that the chemical compositions and bioactive ingredients were found to be high when extracting at 50°C, pH 5.5 for 8 hrs. Therefore, it was reflected in the results found for DPPH and FRAP. At these extraction conditions, DPPH was 69.32% and FRAP was 165.50 mM Fe²⁺/100 g dry matter.

Moreover, the colour parameter is also important to evaluate the efficiency of the extraction. The more is the ΔC value, the brighter the colour of the extract. From Figure 4, the extracting factors also affected the colour of the extract through colours saturation ΔC. ΔC value was the highest at 50°C, pH 4.5 for 4 and 8 hrs of the extraction procedure. As aforementioned, reducing sugar and amino acids were the highest at 50°C for 8 hrs at pH 5.5. When increasing temperature, pH and extraction time, amino acids and reducing sugar decreased. This is because a non-enzymatic reaction that occurs between proteins or amino acids and reducing sugars during heating could be responsible for the formation of brown compounds (Nguyen and Schwartz, 1999). Therefore, the colour of the extract became darker with increased extraction temperature and prolonged extraction time.

In addition, a good correlation model requires a correlation coefficient (R²) to be higher than 0.8 (Guan and Yao, 2008). The analysis results indicated in Figures 1-4 showed that R² of the predictive models is quite high, they can be used to predict the effect of factors of cellulase extraction (temperature, pH and time) on chemical compositions, biological activities or antioxidant capacity of extract.

### 4. Conclusion

The study indicated that enzyme cellulase can be used to extract nutrients and bioactive compounds from the dried oyster mushroom. These compounds included saccharose, protein, reducing sugar, amino acids, phenolic, flavonoid, β-glucan and lysine which concurred that the
extracts have a high potential to be used as functional components in food products. The extract conditions of temperature, pH and hydrolysed time at which the highest quality of dried oyster mushroom extract in terms of chemical compositions and bioactive compounds was obtained was 50°C, 5.5 and 8 hrs, respectively. The obtained extract can be used to produce the concentrates or food components which can be applied to the products such as soy sauce and seasoning to increase nutrient contents.

Conflict of interest

The authors declare no conflict of interest.

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