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Purification of bioactive phenolics from *Phanerochaete chysosporium* biomass extract on selected macroporous resins

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Abstract. In this study, two different types of macroporous resins known as XAD-7HP and HP-20 were evaluated for the adsorption and desorption properties against bioactive phenolics extracted from Phanerochaete chrysosporium. From the previous static sorption studies, it was found that the adsorption capacity for both resins had has no significant difference. Then, the kinetic adsorption data were analyzed with both pseudo-first-order and pseudo-second-order equations and the later performed better. The adsorption isotherm data were fitted well by both Langmuir and Freundlich models. Meanwhile in desorption study, HP-20 and XAD-7HP gave 90.52% and 88.28% recoveries, respectively. Considering the desorption results of the macroporous resins, HP-20 and XAD-7HP were packed in chromatography column to further purify the phenolics. For dynamic adsorption, breakthrough capacity of HP-20 (0.522) was found to be higher than XAD-7HP (0.131). Different ethanol concentrations (30% to 50% (v/v)) were investigated at fixed flowrate (1 ml/min) on phenolics recovery from both types of resins. The highest recovery of bioactive phenolics was 94.3% using XAD-7HP resins at 50% (v/v) of ethanol. Only 77.1% of bioactive phenolics were recovered using HP-20 resin at the same experimental conditions. The purified extract subsequently was analyzed using HPLC. The results showed that three phenolics (gallic acid 3,4-dihydroxybenzoic acid and 4-hydroxybenzoic acid) were identified with higher concentrations as compared to non-purified extract. Finally, the purified extract was tested for scavenging activity against DPPH, and it showed that the activity increased significantly to 90.80% from 59.94% in non-purified extract.

1. Introduction

Phenolic compounds are known bioactive constituents from plants and fungi which have remarkable potential to reduce the risk of some health problems due to their antioxidant, antimutagenic, anti-inflammatory and antimicrobial properties [1]. Due to their ability to promote benefits for human health, they are object of great interest of pharmaceutical and food industries. However, crude phenolic compounds extracted from plant or fungi are not suitable for their applications in the pharmaceutical and food industries due to high levels of impurities as well as low concentration of bioactive compounds in the extracts. Therefore, it is essential to separate and purify the responsible

bioactive compounds (those with the observed biological activities) for in depth pharmacological research and effective applications in medical practice and dietry supplements.

Separation and purification methods of bioactive compounds have to be cost effective, scalable, economical feasible and also can minimize the oxidation, degradation or polymerization of the valuable compounds [2]. Adsorption method has been recognized as the most efficient way of purifying phenolic compounds [3]. Different types of resins have been proposed to purify phenolic compounds such as activated carbon, silica gel, polymeric, cation-exchange and macroporous resins. Activated carbon resins are superior to polymeric resins for phenol and flavonoid adsorption [4][5] but activated carbon resins are difficult to regenerate due to irreversible adsorption. Silica gel resins otherwise suffer low recovery of phenolic compounds because of poor adsorption capacity [6].

At present, macroporous resins have been used widely for the separation and purification of many phenolic compunds including licorice flavonoids [7], scutellarin [8], chlorogenic acid [9], genistein and apigenin [10]. Crude solvent extracts subjected to adsorption-desorption onto macroporous resins showed improvement in their antioxidant [11], immunosuppressive [12] and anti-inflammatory activities [7]. Macroporous resins are durable non-polar and polar polymers. The macroporous adsorption resins exhibit higher adsorption capacity not only because of their similar polarity with the target compounds, but also because of their higher surface area and larger average pore diameter. Because of their characteristics of fast adsorption rate, strong adsorption capacity and ease elution, macroporous resins are gradually regarded as favourable materials for separation and purification of bioactive compounds [13].

Phanerochaete chrysosporium is a filamentous basidiomycete white rot fungus that participates in the degradation process of complex woody materials. This fungus is the subject of many investigations due to its ability to mineralize lignin and other related molecules. It has great potential in many biotechnological applications including bioprotein production and bioremediation. To the best our knowledge, there is no report of the production and recovery of bioactive phenolic compounds from the mycelia biomass of this basidiomycete. Owing to the increasing interest in new natural sources of antioxidant and antimicrobial compounds, this is the first study on *Phanerochaete chrysosporium* mycelia biomass as potential source of bioactive compounds.

2. Materials and methods

2.1. Chemicals and reagents

The chemicals used were Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), 95% ethanol, distilled water, sodium hydroxide (NaOH), hydrochloric acid (HCl), potato dextrose agar, molasses, manganese II sulfate ($MnSO_4$), potassium dihydrogen phosphate (KH_2PO_4), di-potassium phosphate (K_2HPO_4), ammonium nitrate (NH_4NO_3), yeast extract, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH). All solvents for HPLC must be HPLC grade.

2.2. Resins pretreatment

Both macroporous resins (XAD-7HP and HP-20) were treated with 95% ethanol for 24 h and washed several times with deionized water before soaking in 5% (w/v) NaOH solution for 3 h. Then, the resins were washed using distilled water and soaked with 5% (w/v) HCl solution for another 3 h. Finally, the resins were washed with the distilled water thoroughly before pretreated with the ethanol for 12 h at 40 °C before usage.

2.3. Preparation of fungus

The fungal strain, *Phanerochaete chrysosprium* was obtained from the cell bank of Department of Chemical Engineering Technology, University Malaysia Perlis (UniMAP). The fungus was punched into round shapes with dimension of 5mm x 5mm using a cork borer from the petri dish and transferred onto a new Potato-Dextrose Agar (PDA) plate. The strain was incubated at 32°C for 5 days.

2.4. Preparation of inoculum and fermentation

The media used for cultivation of *Phanerochaete chrysosprium* consisted of 0.2% (w/v) of manganese II sulfate ($MnSO_4$), 0.2% (w/v) of potassium dihydrogen phosphate (KH_2PO_4), 1% (w/v) of ammonia nitrate (NH_4NO_3), and distilled water. The pH of the media was adjusted to pH 6. Then, the media was autoclaved at 121°C for 30 minutes. The spore suspension was harvested by washing the fungal plate with 20 ml of sterilized distilled water. About 2% (w/v) of spore suspension prepared was added into the culture media. The fermentation process was done in the incubator shaker at 30°C for 6 days and at 150 rpm to produce mycelial pellets.

2.5. Preparation of powdered biomass

Mycelial pellets were collected and filtered out by using Whatman No.1 filter paper and dried inside the oven at 40°C for overnight. The pellets were then scraped off from the aluminum foil and were grinded into fine powder using pestle and mortar. The powder was weighed in order to obtain the mass of the pellets produced.

2.6. Determination of total phenolic content

The total phenolic content (TPC) was determined using suggested method [14]. About 0.03 mL of sample (extract or standard) was added with 2.37 mL distilled water and 0.15 mL Folin-Ciocalteu reagent in 15 mL test tube. The mixture was vortexed for 1 min. Then, 0.45 mL of 20% (w/v) saturated sodium carbonate was added into the mixture. The mixture was incubated in water bath at 40°C for 30 min after vortexed for 1 min. The absorbance reading was measured at 750 nm. The total phenolic content was determined using the gallic acid standard curve and the result was expressed as mg of gallic acid equivalents per liter of sample (GAE $mg.L^{-1}$).

2.7. Aqueous two phase extraction

The phenolics were recovered using aqueous two-phase extraction system. The fine powder biomass was added into the solution contained ratio of 50% of 80 % (w/v) ethanol, 20% of 40% (w/v) salt (KH_2PO_4) and distilled water. The total mass of 14 g was used which comprised of 7 g of ethanol, 2.8 g of the salt and 0.028 g of the biomass powder and the distilled water. The extraction process was done in the centrifuge tube. The tube was centrifuged (3000 rpm, 25°C) for 10 minutes. Then, the tube was left for 2 hours at the room temperature. The ethanol phase was extracted out and placed in the oven at 40 °C overnight. The dried extract from this phase was used for the following study.

2.8. Dynamic adsorption

The glass columns (1 cm x 20 cm) was used in the dynamic adsorption and desorption experiments, which were wet packed with wet adsorbent HP-20 and XAD-7HP (2.5 g, dry weight) respectively at a BV (bed volume) of 10.0 ml. The dynamic breakthrough experiments was carried out at 298 K. Feed concentration was set constant throughout the experiment at 0.02 GAE mg/ml. Flow rate was fixed constant at elution of 1 mL/min. Samples elute were then collected for TPC detection in effluent [15][16]. A breakthrough curve was plotted and important points are calculated and analyze using Equation (1) till (4).

Total shaded area of breakthrough curve (t_t):

$$t_t = \int_0^{\infty} \left(1 - \frac{c}{c_0}\right) dt \quad (1)$$

t_u time equivalent to the usable capacity or the time at which the effluent concentration reaches its maximum permissible level, t_u is very close to t_b:

$$t_u = \int_0^{t_b} \left(1 - \frac{c}{c_0}\right) dt \quad (2)$$

where t_b is the break-point time.

Length of bed used up to break point (H_B):

$$H_B = \frac{t_u}{t_t} H_T \quad (3)$$

Length of unused bed (H_{UNB}):

$$H_{UNB} = (1 - \frac{t_u}{t_t}) H_T \quad (4)$$

where H_T is the total bed length.

2.9. Dynamic desorption

The column saturated with sample extract was washed with 2.0 BV of deionized water, and then eluted using different ethanol–water ratios (30:70 (v/v), 40:60 (v/v) and 50:50 (v/v)) at constant elution rate of 1mL/min. Eluents were collected for TPC determination [15][16].

2.10. Antioxidant activity

About 0.05 mL of extract sample was added with 0.15 mL of distilled water and 0.2 mL methanol. Then, 0.05 mL of 1mM DPPH in methanol solution was added to the mixture. The mixture was then incubated at 37℃ for 30 minutes. The absorbance was measured at 517 nm. Blank and control used for this method was DPPH solution without the sample extract and absolute ethanol respectively. The scavenging activity will be expressed as percentage of inhibition by using Equation (5):

Scavenging activity (%) = [(ΔA517of control - ΔA517of sample) / ΔA517of control) ×100] (5)

2.11. Identification and quantification of phenolics by using HPLC

The HPLC analysis was done according to the suggested method [17]. For quantification analysis, the standard curve of standard phenolic acids (3,4-dihydroxybenzoic acid, gallic acid and 4-hydroxybenzoic acid) was constructed from their concentration (0.5 mg/mL, 0.05 mg/mL, 0.005 mg/mL and 0.0005 mg/mL). The temperature of the column and the flow rate were set at 30 °C and 1.2 mL/min, respectively. The wavelength and the injection volume were fixed at 280 nm and 50 µL, respectively. The chromatographic separation was performed using C18 column. An isocratic solvent (82:18) of system of A (1.25% glacial acetic acid in water) and B (absolute methanol) were used as the mobile phase. The running time was 30 minutes for each injection. Prior to analysis, the sample was prepared by mixing with 1.8 mL of 1.25% glacial acetic acid in water and then was filtered by using 0.45µm prior to HPLC analysis.

2.12. Statistical analysis

All the measurements were done in triplicates. Analysis of variance (ANOVA) was used to determine the significant differences between the means ± standard deviations of the triplicates.

3. Results and discussion

3.1. Dynamic adsorption

The dynamic breakthrough curves of both resin XAD-7HP and HP-20 were performed based on the volume of injection liquid and the ratio of the outlet to inlet concentration of TPC (Cf/Co). For both resin, dynamic adsorption was proceed under the condition of constant flow rate of 1ml/min, initial concentration TPC of 0.02 GAE mg/mL crude extract and total bed volume of 10 BV packed in column (cross sectional area: 0.7854cm2, height: 12.7cm, mass of adsorbent: 2 g).

From figure 1, both resins have different breakthrough point and XAD-7HP reach breakthrough point earlier than HP-20. This may due to high bed height of HP-20 that creates a longer distance for mass transfer zone to reach outlet of column. Higher concentration of inlet will also shift breakthrough point to the left due to the fact that high feed concentration provides higher concentration gradient

causing a higher mass transfer or diffusion coefficient [18]. Thus, breakthrough point of HP-20 can be shift earlier by reducing bed height or increasing feed concentration.

The XAD-7HP resin shows lower maximum C/C_o value than HP-20. This may due to low flow rate of loading samples. Comparatively, at high flow rate, breakthrough point will be shifted earlier. This condition encountered by [19] where at high flow rate, time period to allow adsorption equilibrium to reach maximum is not sufficient, thus adsorbate elute out of column before equilibrium occurs. This will produce a higher value of C/C_o.

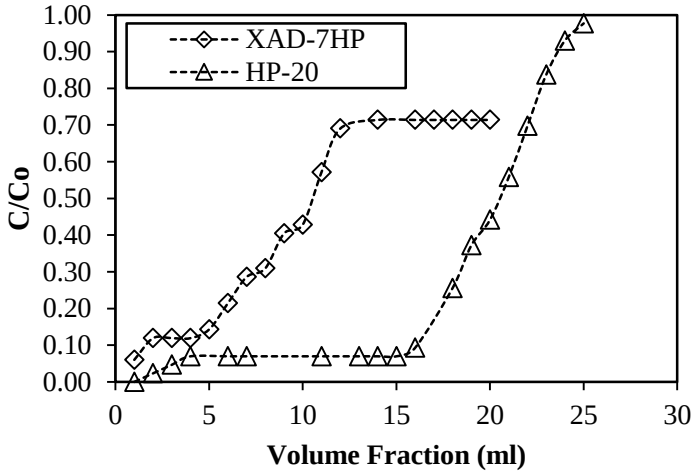

Figure 1. Breakthrough curves for both XAD-7HP and HP-20 resins.

From table 1, breakthrough capacity of HP-20 is much higher than XAD-7HP, which is 0.522 and 0.131 for respective resin. This shows that HP-20 has better ability to absorb phenolic compound upon breakpoint of column. This may assume that due to nature of HP-20 which is weak polar absorbent, which favours non-polar phenolic compound from sample extract. As for XAD-7HP, it shows a much lower breakthrough capacity compare to HP-20. This may due to low feed concentration where it provides low diffusion coefficient affecting the affinity of absorbate towards absorbent.

Table 1. Parameters of fixed bed adsorbents

Adsorbent	t_t (min)	t_u (min)	t_u/t_t	H_B (cm)	H_{UNB} (cm)
XAD-7HP	28.00	3.67	0.131	1.66	11.04
HP-20	29.17	15.24	0.522	6.64	6.07

Besides that, height of unused bed column of HP-20 packed bed is lower than that of XAD-7HP with the value of 6.605cm and 11.037cm respectively. Thus, HP-20 adsorbent-adsorbate system show higher column utilization due to its low solubility in solvent and high affinity towards resin. Therefore, in this experiment, HP-20 resin shows better ability in dynamic adsorption as compared to XAD-7HP.

3.2. Dynamic desorption

For dynamic desorption, column exhausted with sample extract was eluted with different ratios of ethanol-water solution at constant flow rate of 1mL/min. Each eluted fraction was collected to determine the total phenolic content and the results were plotted into desorption curves as shown in figure 2 and figure 3, respectively.

From figure 2, when ethanol concentration in eluent was reduced, elution time increased and serious peak broadening occurred [16]. Therefore, eluent suitable for XAD-7HP resin for desorption of phenolics was at 50:50 (v/v) of ethanol solution.

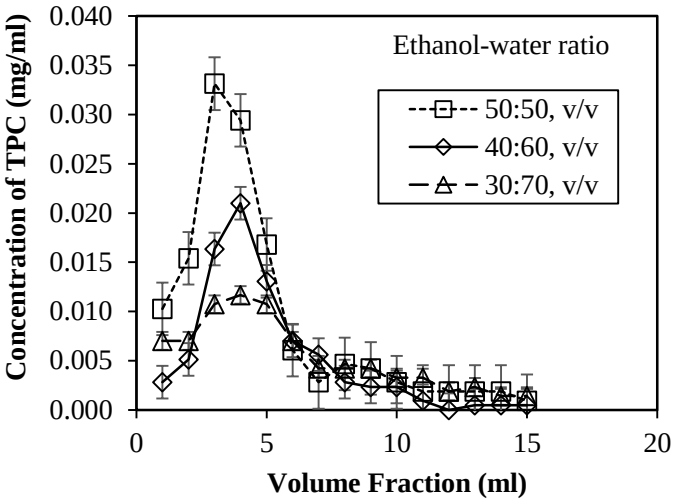

Figure 2. Desorption curve on XAD-7HP resin. Operating condition: Flow rate at 1ml/min; Feed concentration at 0.02 GAE mg.mL-1.

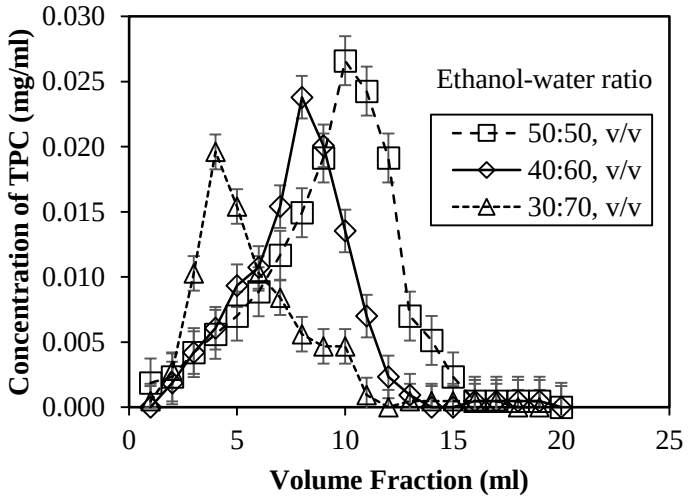

Figure 3. Desorption curve on HP-20 resin. Operating condition: Flow rate at 1ml/min; Feed concentration at 0.02 GAE mg.mL-1.

At this condition, column eluted the highest concentration of phenolic acid within a short elution period. However, the HP-20 resin desorption curve as shown in figure 3 shows that higher ethanol concentration in eluent produced broader peak followed by longer elution time. Therefore, for HP-20 resin, the optimum condition for desorption of phenolics was 30:70 (v/v) of ethanol solution.

Comparatively, both resins preferred different range of ethanol-water concentration in eluent. As XAD-7HP resin preferred higher ethanol concentration in eluent for desorption whereas HP-20 preferred lower ethanol concentration. This may due to XAD-7HP is a polar resin, to allow best desorption, a non-polar eluent is more prefer to reduce polar-polar interaction between adsorbent and eluent.

3.3. Antioxidant analysis

For antioxidant activity, DPPH assay is used to determine the scavenging potential of the antioxidant. The antioxidant activity related to the capability of the phenolic acids in donating the hydrogen and its electrons. The scavenging activity can be calculated by using the (equation 3). Purification from HP-20 has the highest value of the antioxidant activity which was 90.80 ± 0.22% as shown in table 2. The antioxidant activity usually related to the chemical structure of polyphenols, for example number of the hydroxyl group (OH) [20]. The antioxidant activity increased significantly after the purification process.

Table 2. Scavenging activity

Sample	Phenolics acids (GAE mg/mL)	Scavenging Activity (%)
Non-purified extract	0.0512	59.94 ± 0.16
Purified extract (XAD-7HP)	0.0279	80.49 ± 1.34
Purified extract (HP-20)	0.026	90.80 ± 0.22

From the result, it showed that XAD-7HP has the higher concentration of the phenolics acid compared to the HP-20. However, comparing with the scavenging activity, HP-20 has higher activity compared to the XAD-7HP. This may due structure of the phenolics acids as most of the phenolics that bind with the HP-20 contained the hydroxyl group (OH). Moreover, high value of DPPH scavenging activity may also resulted from the bulky or electron-donating substituents on the phenol ring, the meta-position of the electron-donating groups or meta-or ortho-position of hydrophobic groups and finally the ortho-position of the hydrogen-bond donor or the electron-donating groups [21]. Hence, it can be shown that most of the phenolics attached with HP-20 may arrange in the position that gave higher antioxidant activity.

3.4. HPLC analysis

From the HPLC analysis, all the samples contained three types of the phenolics acids; Gallic acid, 3, 4-dihydroxybenzoic acid and 4-hydroxybenzoic acid. Table 3 shows the different of concentration before and after the adsorption process. Both of the resins obtained higher concentration of the phenolics acids compared to the sample before the adsorption process, thus, showing that both of the resins can be used to purify the phenolics acids from the sample.

Table 3. Concentration of phenolics

Sample	Phenolic acids	Concentration (mg/mL)
Non-purified extract	Gallic Acid	0.0011
	3,4-Dihydroxybenzoic acid	0.0011
	4-Hydroxybenzoic acid	0.0031
Purified extract (XAD-7HP)	Gallic Acid	0.0073
	3,4-Dihydroxybenzoic Acid	0.0061
	4-Hydroxybenzoic acid	0.0095
Purified extract (HP-20)	Gallic Acid	0.0556
	3,4-Dihydroxybenzoic Acid	0.0493
	4-Hydroxybenzoic acid	0.0701

4. Conclusion

For dynamic adsorption, breakthrough capacity of HP-20 (0.522) was found to be higher than XAD-7HP (0.131). Different ethanol concentrations (30% to 50% (v/v)) were investigated at fixed flowrate (1 ml/min) on phenolics recovery from both types of resins. The highest recovery of bioactive phenolics was 94.3% using XAD-7HP resins at 50% (v/v) of ethanol. Only 77.1% of bioactive phenolics were recovered using HP-20 resin at the same experimental conditions. The purified extract subsequently was analyzed using HPLC. The results showed that three phenolics (gallic acid 3,4-dihydroxybenzoic acid and 4-hydroxybenzoic acid) were identified with higher concentrations as compared to non-purified extract. Finally, the purified extract was tested for scavenging activity against DPPH, and it showed that the activity increased significantly to 90.80% from 59.94% in non-purified extract.

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