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Antioxidant and free radical scavenging activities of pigments extracted from molasses alcohol wastewater

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Abstract

The pigments from molasses alcohol wastewater were extracted by the macroporous resin adsorption method. The antioxidant and free radical scavenging activities of these pigments were also investigated. The adsorptive characteristics of five macroporous resins including HPD-600, HPD-500, D301-R, NKA-II and D296-R were studied and the results showed that the macroporous resin HPD-600 was most appropriate for extracting the pigments from molasses alcohol wastewater. The antioxidant and free radical scavenging activities of pigments extracted from alcohol wastewater were evaluated using nitrate, hydroxyl radical, superoxide anion radical and 1,1-diphenyl-2-picrylhydrazyl (DPPH) *in vitro* model systems. The pigment extract exhibited a concentration-dependent radical scavenging activity in all the systems. Meanwhile, scavenging activity of pigment extract in the DPPH system was found to be significantly ($P < 0.05$) higher than that in other systems and the 50% inhibitory concentration (IC_{50} value) was about 0.07 mg/ml. The scavenging effect of pigment extract on superoxide anion radical was very weak with IC_{50} value greater than 10 mg/ml.

Keywords: Molasses alcohol wastewater; Pigment; Free radical; Antioxidant activity

1. Introduction

Molasses is one of the most important raw materials used in fermentation industries due to its low cost and wide availability (Chang & Yang, 1973; Underkofler & Hickely, 1954). The molasses obtained from a cane-based sugar mill is a rich resource as it is the main raw material in producing alcohol during fermentation process. Cane molasses contains around 2% of a dark brown pigment named melanoidins that impart the color of the alcohol wastewater (Kalavathi, Uma, & Subramanian, 2001). Melanoidins comprise low and high molecular weight polymers formed as one of the final products of Maillard reaction, which is a nonenzymatic browning reaction between the reaction of reducing sugars and amino compounds (Martins & van Boekel, 2004). Meanwhile, molasses alcohol wastewater from the above industries still contains melanoidin pigment, besides other colorants such as phenolics, caramel and melanin (Satyawali & Balakrishnan, 2007). Phenolic compounds are abundant in cane molasses wastewater (Godshall, 1999). Alcohol production from cane molasses can lead to large amounts of wastewater that cause serious environmental concern. It is characterized with extremely high chemical oxygen demand (COD), biochemical oxygen demand (BOD) and dark brown color (Satyawali & Balakrishnan, 2007). These pigments are poorly decolorized by the normal biological treatments such as activated sludge systems, aerated lagoons and anaerobic ponds (Chuang & Lai, 1978; Sirianuntapiboon, Zohsalam, & Ohmomo, 2004; Wedzicha & Kaputo, 1992). Various physicochemical treatments are also explored, such as activated carbon adsorption, membrane treatment and oxidation process, but the high cost makes these methods hard to be accepted (Satyawali & Balakrishnan, 2007). Therefore, it is significant to find a way to remove these pigments.

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Many researchers have reported that sugarcane extracts have good antioxidant activity (Nagai, Mizutani, Iwabe, Araki, & Suzuki, 2001; Saska & Chou, 2002). While melanoidins and phenolic compounds are also reported to be strong antioxidants (Pant & Adholeya, 2007; Tung, Wu, Kuo, & Chang, 2007; Xu, Tao, & Ao, 2007). Little information on the antioxidant activity of pigments from molasses alcohol wastewater is available. Therefore, the objective of this study was to extract and purify the pigments from molasses alcohol wastewater by macroporous resin and to evaluate their antioxidant and free radical activities.

2. Materials and methods

2.1. Preparation of pigment concentrate

Molasses alcohol wastewater was obtained from Jinguang sugar cane factory in Guangxi (China) which used cane molasses as the substrate. The following procedure was pretreated by centrifugation (8000*g* for 10 min) to remove the undissolved substances. Pigment concentrate was obtained by the further membrane filtration with molecular weight of 30,000 Da.

2.2. Adsorption of macroporous resin on pigment

Macroporous resin HPD-600, HPD-500, D301-R, NKA-II and D296-R (Changzhou chemical industry Co. Ltd., Hebei, China) were used as adsorbents. Their physical properties are listed in Table 1. The macroporous resin was extensively washed with abundant distilled water to remove salts and impurities. Prior to the adsorption experiments, aliquots of adsorbents were washed with ethanol and then the ethanol was thoroughly replaced with distilled water (Fu et al., 2005; Zhao, Chen, & Yao, 2007).

The adsorption tests of pigment from molasses alcohol wastewater on macroporous resins were performed as follows: Pigment concentrate was diluted 20 fold with distilled water and neutralized with lye. Five different macroporous resins (2.0 g) respectively in 250 ml of conical flask with a lid. Diluted pigment solution (100 ml) was added. Then the flasks were placed in a THZ-82A model incubator shaker (Yuejin medical instrument Co. Ltd., Shanghai, China) and shaken (130 rpm) for 2 h at 25 °C. Absorbance of the solution was determined by UV/Vis spectrophotometer (Dojin, Japan) at 560 nm wavelength using distilled water as the blank. Adsorption rate was calculated according to the following equation:

$$\mathrm{Ar}\ (\%) = \frac{A_0 - A_1}{A_0} \times 100\%$$

where Ar is the adsorption rate of test sample (%), A_0 is the absorbance of pigment solution before adsorption, A_1 is the absorbance of pigment solution after adsorption.

2.3. Desorption tests

The desorption processes were carried out as follows: 2.0 g of the adsorbed HPD-600 macroporous resin were first washed by distilled water and then desorbed with 100 ml ethanol–water solution (20%, 40%, 60% respectively) in the 250 ml of conical flask. The flasks were placed in a THZ-82A model incubator shaker (Yuejin medical instrument Co. Ltd., Shanghai, China) and shaken (130 rpm) for 2 h at 25 °C. Absorbance of solution was determined by UV/Vis spectrophotometer (Dojin, Japan). Desorption rate was calculated according to the following equation:

$$\mathrm{Dr}\ (\%) = \frac{A_2}{A_0 - A_1} \times 100\%$$

where Dr is the desorption rate of test sample (%), A_0 is the absorbance of pigment solution before adsorption, A_1 is the absorbance of pigment solution after adsorption. A_2 is the absorbance of pigment-ethanol solution after desorption.

2.4. Preparation of pigment extract

The pigment concentrate was first processed by macroporous resin adsorption, and then the effluent was concentrated in a rotary vacuum evaporator (Yarong biochemical Co. Ltd., Shanghai, China) at 45 °C after desorption. The powdery pigment extract was obtained by freeze-drying.

2.5. Determination of antioxidant activity

2.5.1. Nitrite scavenging activity

This assay was carried out as described by Saha, Lajis, and Israf (2004) and Zhang, Nie, Tao, and Ye (2002) with some modifications. The pigment extract was diluted with the distilled water to a suitable concentration for analysis. Three ml of pigment sample was put in the tube (10 ml), then 2 ml of citric acid buffer (pH 3.0) and 0.1 ml of 200 μg/ml $NaNO_2$ were added, respectively. Finally, water was added up to 10 ml. The mixture was immediately incubated for 60 min in the water bath at 37 °C. Then equal volume of Griess

Table 1
Physical properties of the tested macroporous resins

Types	Functional group	Surface area (m^2/g)	Average pore diameter (Å)	Particle diameter (mm)	Polarity
HPD-500	polystyrene	500–550	55–75	0.3–1.2	Polar
HPD-600	polystyrene	550–600	80	0.3–1.2	Polar
D301-R	Styrene	200–300	65–80	0.315–1.25	Strong-polar
NKA-II	Polystyrene	160–200	145–155	0.3–1.25	Polar
D296-R	Styrene	170–250	120–140	0.315–1.25	Middle-polar

reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethyline diamine hydrochloride, 2.5% H_3PO_4) was added to the above mixture. The absorbance was determined by UV/Vis spectrophotometer (Dojin, Japan) after 10 min at 538 nm. At the same time the control (without $NaNO_2$) and standard (without $NaNO_2$ and without pigment sample) were also measured. Vc and butylated hydroxyanisole (BHA) were used as the positive control compounds. $NaNO_2$ scavenging activity was calculated using the following equation:

$$\text{Sa } (\%) = \frac{OD_s - (OD_p - OD_c)}{OD_s} \times 100\%$$

where Sa is the $NaNO_2$ scavenging rate of tested sample (%), OD_s is the OD value of standard, OD_p is the OD value in the presence of tested sample, OD_c is the OD value of control.

2.5.2. Hydroxyl radical ($OH^{\cdot}$) scavenging activity

The hydroxyl radical scavenging activity was measured by the deoxyribose method (Siddhuraju & Becker, 2007). The reactions were performed in 10 mM phosphate buffer (pH 7.4), containing 2.8 mM deoxyribose, 2.8 mM H_2O_2, 25 μM $FeCl_3$, 100 μM EDTA and the extracted samples with different concentrations. The reaction was activated by adding ascorbic acid to a concentration of 100 μM and the reaction mixture was incubated for 1 h at 37 °C in a water bath. After incubation, the colour was developed by adding 1% thiobarbituric acid and 2.8% ice-cold trichloroacetic acid and heated in a boiling water bath (100 °C) for 20 min. The sample was cooled and the absorbance was measured at 532 nm. The control reaction mixture does not contain extracted sample. Vc and butylated hydroxyanisole (BHA) were used as the positive control compounds. Scavenging activity was calculated using the following equation:

$$\text{Sa } (\%) = \frac{A_s - A_b}{A_b} \times 100\%$$

where Sa is the scavenging activity of tested sample (%), A_s is the absorbance in the presence of the tested sample, A_b is the absorbance of the control.

2.5.3. Assay of superoxide radical (O_2^-) scavenging activity

Superoxide radical scavenging activity was measured by the pyrogallol autoxidation (Zhao, Yu, & Wang, 2003) with some modifications. Tris–HCl buffer (5.6 ml of 50 mM) (pH 8.2) and 0.2 ml tested samples were mixed in tubes with lids. Then the mixture was incubated for 10 min in the water bath at 25 °C. Meanwhile, 0.2 ml of 0.95 mM pyrogallol preheated at 25 °C was added immediately. The absorbance of sample and control were determined by UV/Vis spectrophotometer (Dojin, Japan) at 325 nm every 30 s. The curve was made based on the absorbance value. Vc was used as the positive control compounds. Scavenging activity was calculated using the following equation:

$$\text{Sa } (\%) = \frac{A_s - A_b}{A_b} \times 100\%$$

where Sa is the inhibition rate of tested sample (%), A_s is the absorbance of the tested sample, A_b is the absorbance of the control.

2.5.4. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay

The scavenging activity of pigment extract on 1,1-diphenyl-2-picrylhydrazyl (DPPH**.**) radicals (Sigma, analysis grade) was measured according to the method of Shimada, Fujikawa, Yahar, and Nakamura (1992). DPPH solutions (2 ml) in ethanol (2×10^{-4} mol/l) and 2 ml of tested samples with different concentrations were mixed in the tubes. The mixture was incubated for 60 min in the dark at 25 °C, and the decrease in absorbance at 517 nm was measured against ethanol using a UV/Vis spectrophotometer (Dojin, Japan). Methanol was used as the blank. Vc and butylated hydroxyanisole (BHA) were used as the positive control compounds. The scavenging activity of pigment on DPPH was calculated according to the following equation:

$$\text{Sa } (\%) = \left[1 - \frac{(A_i - A_j)}{A_0}\right] \times 100\%$$

where Sa is the scavenging activity of tested sample (%), A_i is the absorbance of 2 ml DPPH solution and 2 ml sample solution, A_j is the absorbance of 2 ml sample solution and 2 ml ethanol, A_0 is the absorbance of 2 ml DPPH solution and 2 ml sample solvent.

2.6. Determination of total phenolic compounds

The pigment extract was diluted with the stilled water, to a suitable concentration for analysis. Total phenolic content of pigment extract was assessed approximately by using the Folin–Ciocalteau phenol reagent method (Mathew & Abraham, 2006). Sample extract (200 ml) were added to 1.0 ml of Folin–Ciocalteau reagent and 0.8 ml of sodium carbonate (7.5% w/v), and the contents were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured in a UV–Vis Spectrophotometer. The total phenolic content was expressed as gallic acid equivalent (GAE) in milligrams per gram of sample, using a standard curve generated with gallic acid.

2.7. Statistical analysis

All experiments were conducted in triplicate and data are presented as means values ± standard deviation. A least significant difference (LSD) test with a confidence interval of 95% was used to compare the means. The inhibitory concentration 50% (IC_{50}) was calculated from concentration/effect regression line.

3. Results and discussion

3.1. Adsorption effect of macroporous resin on the pigment

Adsorption effect of five macroporous resins on molasses alcohol wastewater pigment is presented in Table 2.

Table 2
Adsorptive effect of five macroporous resins

Types of macroporous resin	HPD-600	HPD-500	D301-R	NKA-II	D296-R
Adsorption rate (%)	82.66 ± 0.26	54.46 ± 0.25	87.18 ± 0.31	44.57 ± 0.34	63.86 ± 0.18

Adsorptive abilities of HPD-600 and D301-R resin were significantly ($P < 0.05$) higher than those of other resins and their adsorptive rates were 82.66 ± 0.22% and 87.18 ± 0.13% respectively. NKA-II had the lowest adsorptive ability, which correlated with the capabilities of the resins and the chemical features of the adsorbed pigments. The matching of polarity between adsorbent and adsorbate is also an important factor affecting adsorption. The adsorption ability of macroporous resin depends on its hydrophobicity with adsorbate. Hence, macroporous resin HDP-600 with the high adsorptive effect and lower cost was selected to do the following experiments.

Adsorption is a prominent method for extracting the pigment from molasses alcohol wastewater. Activated carbon is a widely used adsorbent for the removal of organic pollutants from wastewater (Bernardo, Egashira, & Kawasaki, 1997; Chandra & Pandey, 2000). Another adsorbent that has been examined is the natural carbohydrate polymer chitosan. Lalov, Guerginov, Krysteva, and Fartsov (2000) studied the treatment of distillery wastewater using chitosan as an anion exchanger. But the relatively high cost restricts their usage. In comparison with these classical adsorbents, macroporous resin is more attractive alternatives because of their wide range of pore structures and physicochemical characteristics (Fu et al., 2005). It was used in this experiment because of its high chemical stability, excellent selectivity and lower cost.

3.2. Effect of concentration of ethanol solution on desorption

The desorption solvent of macroporous resin is generally ethanol solution with different concentrations because it can be recycled easily and has lower cost and has no toxicity on the samples. Different concentrations of ethanol solutions were used to perform desorption tests in order to find most suitable desorption solution. Fig. 1 shows the desorption rate of ethanol with different concentrations (20%, 40% and 60% respectively) on the macroporous resin. At first the desorption rate increased with the increasing time. But the desorption rate had slight increase with the increasing time after 30 min. The desorption rate increased obviously ($P < 0.05$) when increased the ethanol concentration from 20% to 40%. Ethanol solution (40%) showed the highest desorption rate (>98%) compared with those at other ethanol concentration. The matching of polarity between desorption solvent and adsorbate is an important factor affecting desorption. Therefore, 40% ethanol solution was selected as the desorption solvent.

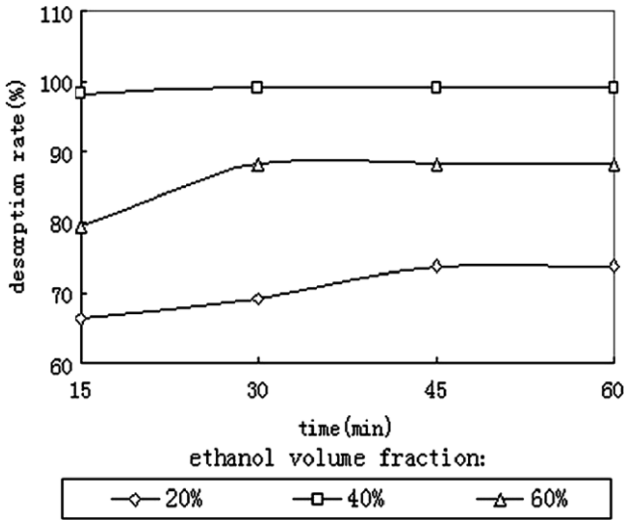

Fig. 1. Effect of ethanol concentration on desorption of macroporous resin.

3.3. Nitrite scavenging effect of the pigment extract

As shown in Fig. 2, the pigment extract exhibited a concentration-dependent anti-radical activity by inhibiting nitrite. The nitrite scavenging activity of pigment extract increased with the increasing concentration, but lower than those of the positive control compounds Vc and BHA at the same concentration. The nitrite scavenging activity of Vc and BHA only had slight increase with the increasing concentration (>0.5 mg/ml). The 50% inhibitory concentration (IC_{50} value) of the pigment extract on the nitrite is about 0.75 mg/ml, while those of BHA and Vc are about 0.16 mg/ml and 0.18 mg/ml respectively. It is reported that certain substance could scavenge nitrite because of their reductive ability. It can be noted by the following equation:

$$NO_2^- + 16H^+ + 12e^- = 2NH_4^+ + 4H_2O.$$

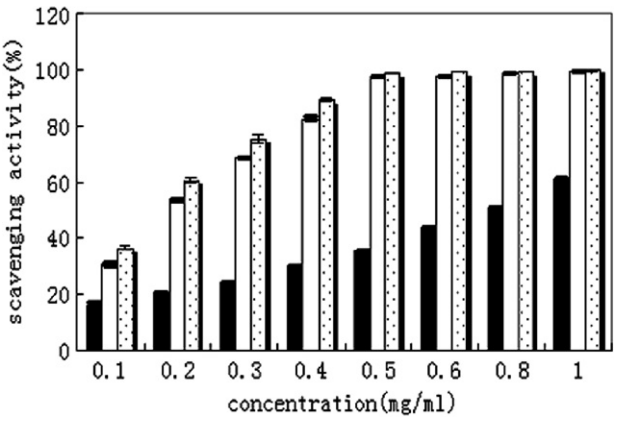

Fig. 2. Nitrite scavenging activity of pigment extract (black bar), ascorbic acid (white bar) and BHA (white spotted bar). Results are means ± SD of triplicate measurements.

Phenolic pigment from molasses alcohol wastewater has the reductive ability. This might be the reason that it can scavenge nitrite.

3.4. Hydroxyl radical scavenging effect of the pigment extract

Hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal ions, such as copper or iron. Hydroxyl radicals react with lipid, polypeptides, proteins, and DNA, especially thiamine and guanosine. When a hydroxyl radical reacts with aromatic compounds, it can add across a double bond, resulting in hydroxycyclo-hexadienyl radical. The resulting radical can undergo further reactions, such as reaction with oxygen, to give peroxyl radical, or decompose to phenoxyl-type radicals by water elimination (Lee, Koo, & Min, 2004).

The scavenging abilities of pigment extract on hydroxyl radical inhibition are shown in Fig. 3. The pigment extract had a scavenging activity on the hydroxyl radicals in a dose-dependent manner (0.25–1.25 mg/ml), which might be attributed to the combined effects of reducing power, donation of hydrogen atoms and scavenging of active oxygen. However, scavenging activity of pigment extract on hydroxyl radical is significantly ($P < 0.05$) lower than those of controls, Vc and BHA, at the same concentration. The IC_{50} value of the pigment extract is higher than 1 mg/ml, while those of Vc and BHA are about 0.75 mg/ml and 0.62 mg/ml respectively. The phenolic compounds of pigment extract might be responsible for hydroxyl radical scavenging activity.

3.5. Superoxide radical scavenging effect of the pigment extract

Superoxide anion is a reduced form of molecular oxygen created by receiving one electron, which is also an initial free radical formed from mitochondrial electron transport systems (Siddhuraju & Becker, 2007). Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive oxygen species. The superoxide radical is known to be produced *in vivo* and can result in the formation of H_2O_2 via dismutation reaction (Li, Li, & Zhou, 2007).

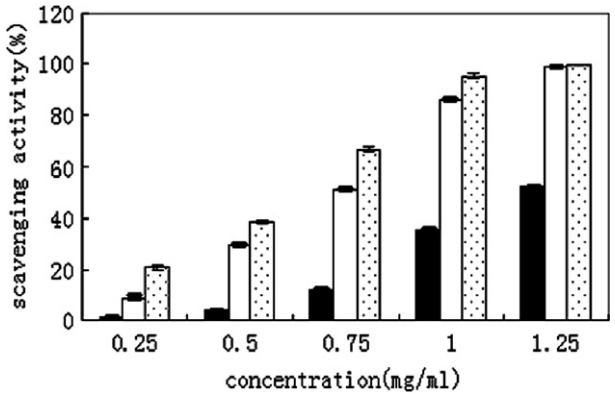

Fig. 3. Hydroxyl radical scavenging activity of pigment extract (black bar), ascorbic acid (white bar) and BHA (white spotted bar). Results are means ± SD of triplicate measurements.

Table 3
Scavenging activities of pigment extract and Vc on superoxide radical

Pigment concentration (mg/ml)	Scavenging activity (%)	Vc concentration (mg/ml)	Scavenging activity (%)
2	11.87 ± 0.33	0.2	37.18 ± 0.21
4	15.72 ± 0.16	0.6	85.73 ± 0.32
6	22.61 ± 0.21	1.0	98.13 ± 0.19
8	30.21 ± 0.18		
10	42.90 ± 0.25		

Scavenging activity of Vc on superoxide radical was higher than that of pigment extract (Table 3). To get the same absorbance value at the same time, the pigment extract required higher concentration. Pigment extract scavenged O_2^- in a dose-dependent fashion (scavenging activity was 42.9% at 10 mg/ml) (Table 3). The same kinetics suggested the presence of same scavenging mechanisms at different concentrations (data not shown). The 50% inhibitory concentration (IC_{50} value) of the pigment extract is higher than 10 mg/ml, however IC_{50} value of Vc is lower than 0.6 mg/ml, which shows that the pigment extract is not a strong superoxide radical scavenger.

3.6. DPPH radical scavenging effect of the pigment extract

DPPH is one kind of the compound that has a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (Tung et al., 2007; Yamaguchi, Takamura, Matoba, & Terao, 1998). It is well accepted that the DPPH radical scavenging by antioxidants is attributable to their hydrogen donating ability (Chen & Ho, 1995). This test system can be used for the primary characterization of the scavenging potential of compounds (Krings & Berger, 2001). The radical scavenging activity of pigment extracts from molasses alcohol wastewater was determined from the reduction in the optical absorbance at 517 nm due to scavenging stable DPPH free radical. Positive DPPH test suggested that the pigment extract was a good free radical scavengers.

Fig. 4 showed pigment extract had good DPPH scavenging activity when compared with standard Vc and had higher DPPH scavenging activity with the increasing concentration. The DPPH scavenging activity of pigment extract is lower than those of Vc and BHA at the same concentration, but their DPPH scavenging activities were almost same at certain concentration (about 0.2 mg/ml) to that of Vc and BHA. The 50% inhibitory concentration (IC_{50} value) of the pigment sample is about 0.07 mg/ml. The higher IC_{50} value is, the lower is the DPPH scavenging

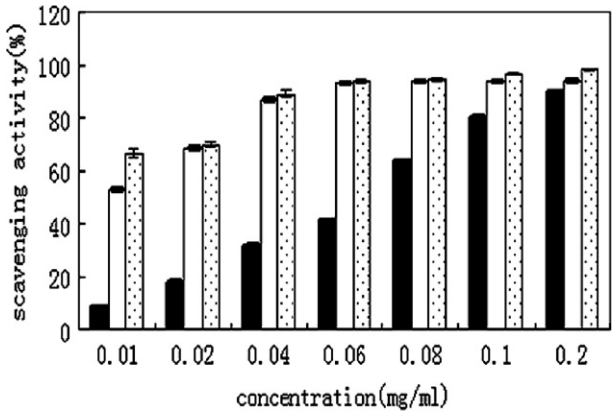

Fig. 4. DPPH radical scavenging activity of pigment extract (black bar), ascorbic acid (white bar) and BHA (white spotted bar). Results are means ± SD of triplicate measurements.

activity. The phenolics and products from Maillard reaction in pigment extract might be responsible for DPPH radical scavenging activity.

3.7. Total phenolic content

The total phenolic content of the pigment extract was estimated to be 146.65 ± 2.6 mg gallic acid equivalents/g of extract from triplicate measurements.

4. Conclusion

In conclusion, the results obtained in the present study indicated that the macroporous resin adsorption was a potential method for extracting the pigment from malasses alcohol wastewater and the pigment extract can effectively scavenge DPPH radical and nitrate under *in vitro* conditions. However, the pigment extract is not good superoxide radical scavenger. The methods adopted to monitor antioxidant activity may not be sufficient to make a valid and thorough judgment. Further specific studies on each component of pigment extract with regard to antioxidant activity in vivo are needed.

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