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Full Length Research Paper

Purification and characterization of a novel glucose oxidase-like melanoidin decolorizing enzyme from *Geotrichum* sp. No. 56

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A novel melanoidin decolorizing enzyme (MDE) produced by *Geotrichum* sp. No. 56, which exhibits decolorization activity against synthetic melanoidin and molasses containing wastewater, was purified and characterized. The purification process was performed using ammonium sulfate fractionation, DEAE-Cellulose and Sephadex G-150 column chromatography. The melanoidin decolorization activity of the MDE was improved up to 7.3 U mg^{-1}, the equivalent to 8.3-fold increase from the initial protein preparation, with an overall yield of 9.1%. The results of gel filtration and SDS-PAGE revealed that the purified MDE was a dimmer with 127.5 kDa, and both subunits were alike with 63.5 kDa each. The optimum pH and temperature for the purified MDE were 6.5 and 45°C, respectively. The MDE activity was highly specific for β-D-glucose, but completely inhibited by cysteine, iodoacetic acid, 2-mercaptoethanol, gluconic acid, $HgCl_2$ or $AgNO_3$. The MDE also decolorized the spent broth of monosodium glutamate fermentation. The *Geotrichum* sp. No. 56 producing MDE, which shares various characteristics with glucose oxidase (GOx), could be useful for decolorizing fermented wastewater.

Key words: Decolorization, *Geotrichum* sp., glucose oxidase, melanoidin, molasses wastewater.

INTRODUCTION

Wastewater produced by industrial microbial fermentation is a serious environmental problem. The media normally used by in industrial microbial fermentation contains molasses or starch as a carbon source and corn steep liquor or ammonia as a nitrogen source. After the interested products have been extracted, the spent fermentation broths contain high concentrations of organic compounds, such as carbohydrates, acids, amino acids, nitrogenous compounds, and a large amount of a dark brown pigment called melanoidin (Sirianuntapiboon et al., 1988), which is formed through a Maillard reaction between amino acids and carbohydrates. Adsorption, coagulation-flocculation, oxidation, filtration and electro-chemistry have been proposed to remove the pigments and reduce the chemical oxygen demand from the wastewater (Lin and Peng, 1996; Calabro et al., 1991; Tan et al., 2000). Although these physical and chemical methods have been proven effective, the operational costs are rather high and can produce unanticipated secondary pollutants, such as sludge or new toxic compounds, occur. Alternatively, biological degradation and decolorization may produce better results at lower cost, and produce little or no secondary pollution.

Different melanoidin decolorizing enzymes (MDEs) have been produced from various microbial strains, especially oxidoreductases and peroxidases which can catalyze degradation and decolorization of pigments in the fermentation spent broth. Glucose oxidase (β-D-Glucose: oxygen 1-oxidoreductase, EC 1.1.3.4, GOx), one of these MDEs, can catalyze the oxidation of β-D-glucose into D-glucono-δ-lactone, which then hydrolyzes to gluconic acid. In the GOx-catalyzed redox reaction, flavin adenine dinucleotide acts as an initial electron acceptor, and

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subsequently passes the electron to O_2, by which O_2 is reduced to H_2O_2. Due to the bactericidal property of H_2O_2 formation, GOx was originally mistaken as a penicillin-like antibiotic produced by *Penicillium notatum*, but later clarified as an enzyme (Coulthard et al., 1945). Bright and Appleby (1969) investigated the kinetic behavior and redox state of GOx from *P. notatum* and the current review of the general characteristics of GOx can be found in Bankar et al. (2009). It has been noted that GOx can be used for the treatment of industrial wastewater containing melanoidin (Aoshima et al., 1985; Sirianuntapiboon et al., 1988). Hydrogen peroxide had been shown to be able to decolorize and degrade melanoidins in molasses (Hayase et al., 1984), and GOx from mold can generate hydrogen peroxide through its catalytic action of glucose oxidation.

GOx is mostly found in fungal strains, such as genus *Aspergillus* (Hatzinikolaou et al., 1996), *Penicillium* (Simpson et al., 2007), *Saccharomyces cerevisiae* (Zakhartsev and Momeu, 2007) and *Botrytis cinerea* (Liu et al., 1998). However, GOx has not been reported produced from *Geotrichum* genus. In previous study, we reported the first isolation of a *Geotrichum* strain with potential decolorization ability (Hsieh and Su, 1999). In current research, we report that a novel GOx-like MDE from the *Geotrichum* strain was purified and characterized, and used to decolorize wastewater of monosodium glutamate (MSG) fermentation.

MATERIALS AND METHODS

Microbial strain and its maintenance

This study used *Geotrichum* sp. No. 56, which was isolated by our laboratory (Hsieh and Su, 1999). The mycelial strain was cultivated on a PDA agar plate at 30°C for 7 d. The well-grown mycelia were kept at 4°C and renewed every 2 months.

Preparation of synthetic melanoidin

One mole of glucose, one mole of amino acids and 0.5 mole of Na_2CO_3 were dissolved in one liter of distilled water and the solution was autoclaved at 121°C for 3 h to initiate the Mallard reaction. After adjusting the reaction mixture to pH 7.0, the solution was filtered through a 0.22 µm pore size membrane and lyophilized.

Production and preparation of crude MDE

For production of crude MDE, two mycelial disks (approximately 0.8 cm diameter each) from the plate were inoculated into Erlenmeyer flasks (500 ml) containing 100 ml of basal medium. The basal medium used here containing (per liter): 2 g melanoidin; 7.5 g sorbitol; 2 g NH_4Cl; 2 g peptone; 1 g KH_2PO_4; and 0.5 g $MgSO_4 \cdot 7H_2O$. The pH was adjusted to pH 6.0 prior to sterilization. The culture was incubated at 30°C on a rotary shaker at 125 rpm for 6 days.

The harvested mycelia were centrifuged, then washed twice and re-suspended in 0.1 mol l^{-1} phosphate buffer (pH 6.5), blended in a Warring blender for 2 min, and centrifuged at 12000 rpm for 40 min. The supernatant was used as the crude MDE extract for further experiments.

MDE activity and protein concentration

Following Ciucu and Patroescu (1984), the GOx activity of the MDE was determined at 50°C using benzoquinone as the substrate. The reaction mixture (4.9 ml) contained 0.95 ml of appropriately diluted enzyme solution, 2.0 ml of glucose (1 mol l^{-1}), phosphate buffer 0.1 mol l^{-1}, pH 6.5) and 1.0 ml of 0.1% benzoquinone. The oxidation of benzoquinone was detected by measuring the absorbance increase at 290 nm after 4 min using a spectrophotometer (UV-120-02, Shimadzu, Japan). One U of MDE activity was defined as releasing one µmole of hydroquinone per min and the enzyme-specific activity was in U per mg protein. All assays were carried out in triplicate for each sample. Protein concentration was determined after Lowry et al. (1951) using bovine serum albumin as the standard.

MDE isolation and purification

The crude MDE extract was used for enzyme purification. The extract was initially filtered though a whatman #1 filter to remove any insoluble particles. The protein in the filtrate was salted out with ammonium sulfate with 70% (w/v) saturation. The protein precipitate was collected by centrifugation at 8000 g for 30 min, dissolved in a phosphate buffer (50 mmol l^{-1}; pH 6.5) and dialyzed extensively against a 10 mmol l^{-1} phosphate buffer (pH 6.5). The $BaCl_2$ was used to determine any residual ammonium sulfate in the dialysis buffer.

The dialyzed protein (20 ml) was loaded onto a DEAE-cellulose column that had been pre-equilibrated with a 50 mmol l^{-1} phosphate buffer (pH 6.5). Unbound proteins were washed out with a 50 mmol l^{-1} phosphate buffer (pH 6.5) and the bound proteins were eluted using a 10 mmol l^{-1} phosphate buffer (pH 6.5) with a linear gradient of 0-0.8 mol l^{-1} NaCl. Each fraction of 5 ml was collected. The protein of each fraction was determined by measuring the absorbance at 280 nm. MDE activity of each fraction was determined as described previously.

The MDE rich fractions were pooled together and concentrated into 5 ml through an Amicon concentrator against a 10 kDa molecular weight cut-off membrane at 4°C. The concentrate was used for further purification on a Sephadex G-150 column. Proteins were eluted with the 10 mmol l^{-1} phosphate buffer (pH 6.5) and 3.5 ml fractions were collected. The MDE-rich fractions were pooled, concentrated, filtered through 0.22 µm membranes and kept at 4°C until use.

Molecular weight determination of MDE

The molecular weight of the intact MDE was determined by a Sephadex G-150 column using linear interpolation against a standard protein calibration curve. To make the calibration curve, commercial standard ferritin (440 kDa), catalase (232 kDa), alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were used.

The molecular weight of the denatured MDE was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following Weber and Osborn (1969), with 4% (w/v) stacking gel and 10% (w/v) resolving gel at 25 mA for 8 h. Stained and destained methods were carried out following Kitamura (1984). The molecular weight of the denatured MDE was determined by calculating the relative mobility of standard protein markers. The standard protein markers included myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa) and carbonic anhydrase (29 kDa) and trypsin inhibitor (21.5 kDa).

Table 1. Summary of the purification of melanoidin decolorizing enzyme from *Geotrichum* sp. No. 56.

Purification step	Total protein (mg)	Total activity of MDE* (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude MDE* extract	221.34	193.67	0.88	1	100
Salting out (70%, w/v)	57.51	82.87	1.44	1.65	42.79
DEAE-Cellulose	8.7	44.72	5.14	5.87	23.1
Sephadex G-150	2.41	17.57	7.3	8.33	9.07

* MDE: melanoidin decolorizing enzyme.

Effects of pH and temperature on MDE activity

The effect of pH on MDE activity was determined using the following buffer systems and pH ranges: 0.1 mol l^{-1} sodium acetate buffer, pH 3.5-5.5; 0.1 mol l^{-1} phosphate buffer, and pH 6.0-8.0; 0.1 mol l^{-1} glycine/NaOH buffer, pH 8.5-10.0. Ten µg of purified MDE was incubated within the above buffers for 5 min, after which the substrate was added into the reaction and the MDE activity was measured as described earlier. The effect of temperature on MDE activity was determined under optimum pH conditions.

Substrate specificity and the effect of inhibitors and metal ions

Substrate oxidizing activity of the purified MDE was determined using different sugars, including glucose, sorbitol, galactose, xylose, sorbose, mannitol, fructose, maltose, rhamnose, saccharose and arabinose. Concentrations of 40 mmol l^{-1}, 100 mmol l^{-1} and 300 mmol l^{-1} were tested for each sugar. The enzyme assay was performed as described above under conditions of optimum pH and temperature.

The effects of potential inhibitors were tested, including NaF (1 mmol l^{-1}), NaN_3 (1 mmol l^{-1}), erthylene-diamine tetraacetic acid (EDTA) (1 mmol l^{-1}), thiourea (1 mmol l^{-1}), cycteine (1 mmol l^{-1}), iodoacetic acid (IAA) (1 mmol l^{-1}), 2-mercaptoethanol (1 mmol l^{-1}), sodium dodecyl sulfate (SDS) (1 %), urea (3 mol l^{-1}), p-chloromercuribenzoate (PCMB) (0.1 mmol l^{-1}, 0.01 mmol l^{-1} and 0.001 mmol l^{-1}), gluconic acid (400 mmol l^{-1}) and H_2O_2 (0.1%). The effects of metal ions, including $NiSO_4$, $HgCl_2$, $AgNO_3$, $CdSO_4$, $FeSO_4$, $BaCl_2$, $CaCl_2$, $CuSO_4$, $ZnSO_4$, $MnSO_4$, $MgSO_4$ and $Pb(NO_3)_2$, were also determined. Concentrations of 0.1 mmol l^{-1} and 1 mmol l^{-1} were tested for each metal ion. Control groups were maintained without the MDE inhibitors and the metal ions.

Decolorization of melanoidin or MSG fermentation spent broth by purified MDE

The method for decolorizing melanoidin or MSG fermentation spent broth using purified MDE was described by Ohmomo et al. (1985). The color density of the synthetic melanoidin solution or MSG fermentation spent broth at 475 nm was first adjusted to 3.5. The decolorization reaction was set in an L-type tube, containing 0.5 ml 5% glucose, 0.2 ml synthetic melanoidin solution or fermentation spent broth, and 2.3 ml 0.25M phosphate buffer (pH 6.5). Following the addition of the purified MDE, the decolorization reaction was allowed to proceed at the optimum temperature on a rotary shaker at a shaking speed of 100 rpm for 20 h. Gel filtration chromatography of a metanoidin solution or spent broth on a Sephadex G-50 column was performed following Aoshima et al. (1985). Color density was measured at 475 nm.

Statistic analyses

The results represent the average values obtained in triplicate assays with variation of < 5%. The data were analyzed by ANOVA using the general regression models in Statistica 8.0 (Statsoft, Inc., Tulsa, OK) during the regression study.

RESULTS

Purification of MDE

Table 1 summarizes the steps for purifying MDE, including 70% saturated ammonium sulfate fractionation, and DEAE-Cellulose and Sephadex G-150 column chromatographies. The total enzyme activity of the cell-free extract and the final Sephadex G-150 column chromatography steps were respectively 193.67 U and 17.57 U, in which the recovery of total MDE activity was 9.07%. The specific activity of MDE in the purification steps of the cell-free extract and the final Sephadex G-150 column chromatography were respectively 0.87 U and 7.29 U mg^{-1}, in which the purity of MDE was improved 8.38-fold.

During the purification step of the DEAE-Cellulose column chromatography, the MDE activity was found in fractions 4-10 prior to the initiation of the linear gradient of 0.0-0.8 mol l^{-1} NaCl (Figure 1). The fractions with MDE activity were pooled and concentrated. The resulting 8.7 mg protein in 5 ml of 10 mmol l^{-1} phosphate buffer (pH 6.5) was subjected to Sephadex G-150 column chromatography. High MDE activity was found in fractions 7-11 (Figure 2). The fractions containing high MDE activity were pooled and concentrated. The procedure yielded 2.4 mg of purified enzyme from 100 ml of culture supernatant.

Determination of MDE molecular weight

As seen in Figure 3, the MDE had been purified to homogeneity as shown with a single protein band of approx. 63.5 kDa as determined by SDS-PAGE. To determine the molecular weight of the intact MDE, ferritin (440 kDa), catalase (232 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa) were used as standards. A straight calibration curve was obtained plotting the semi-logarithm

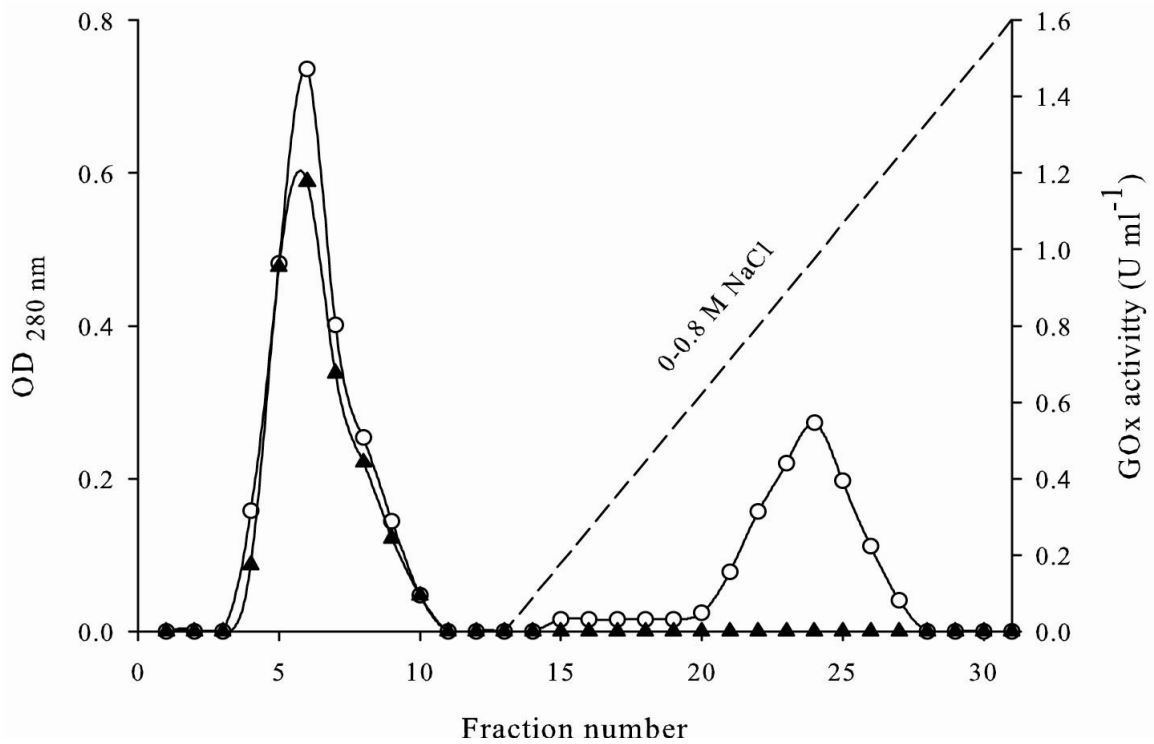

Figure 1. Ion-exchange chromatography on a DEAE-Cellulose column. The solid line with (○) represents the absorption of protein at 280 nm, and the solid line with (▲) represents the glucose oxidase activity of the melanoidin decolorizing enzyme from *Geotrichum* sp. No. 56. The dashed line indicates the gradient of NaCl concentration.

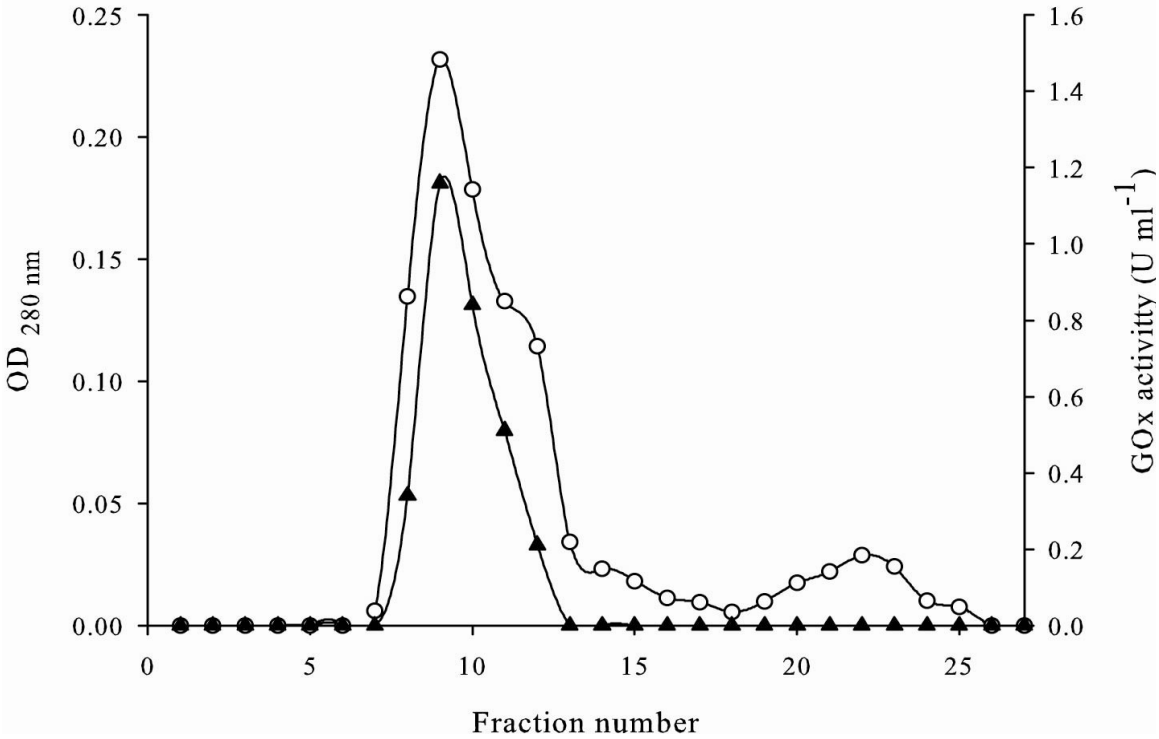

Figure 2. Gel filtration chromatography on a Sephadex G-150 column. The solid line with (○) represents the absorption of protein at 280 nm, and the solid line with (▲) represents the glucose oxidase activity of the melanoidin decolorizing enzyme from *Geotrichum* sp. No. 56.

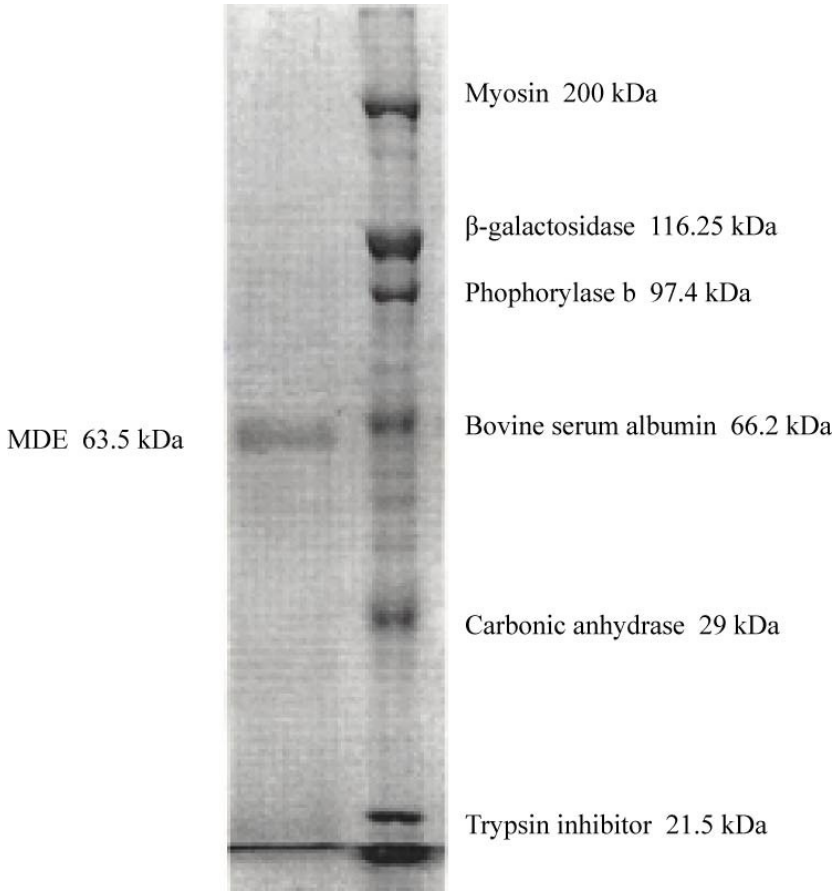

Figure 3. SDS-PAGE gel of the protein obtained from the purification of the melanoidin decolorizing enzyme from *Geotrichum* sp. No. 56. The right lane shows the protein markers; left lane shows a peak fraction with activity obtained by Sephadex G-150 chromatography.

of the molecular weights of the standards against the elution volume of Sephadex G-150 gel filtration. The respective chromatography volumes were 175.2, 125.0, 66.3, 43.2 and 15.9 ml with a regression equation of Y = 138.69X + 375.45 and a regression coefficient of R^2 = 0.992, where Y is the elution volumes of the standards, and X is the logarithm of the corresponding standard molecular weight (Figure 4). The elution volume of MDE under the same chromatography conditions was 83.5 ml. Therefore, the estimated molecular weight of MDE was 127.5 kDa.

Effect of pH and temperature

The pH profile for MDE activity against benzoquinone showed a peak of maximum activity at pH 6.5 (Figure 5). The optimum temperature of the purified MDE was determined at pH 6.5, and the maximum activity was observed at 45°C (Figure 6). The enzyme was stable at pH 6.5 as long as the temperature remained below 45°C. The enzyme activity diminished when the temperature rose above 45°C, and completely vanished when the temperature reached 80°C. The crude MDE extract was found to have a higher optimum temperature at 50°C (data not shown). However, no difference was found regarding the optimum pH before and after MDE purification.

Effect of sugars on the activity of purified MDE

The effects of various sugars on the activity of purified MDE are shown in Table 2. Glucose resulted in the most effective decolorization, while the other sugars (except for sorbose) had barely any decolorization effect when compared with the negative control without adding any sugar.

Effect of enzyme inhibitors on the activity of purified MDE

The effects of several putative inhibitors on MDE activity were examined (Table 3). The purified MDE was strongly

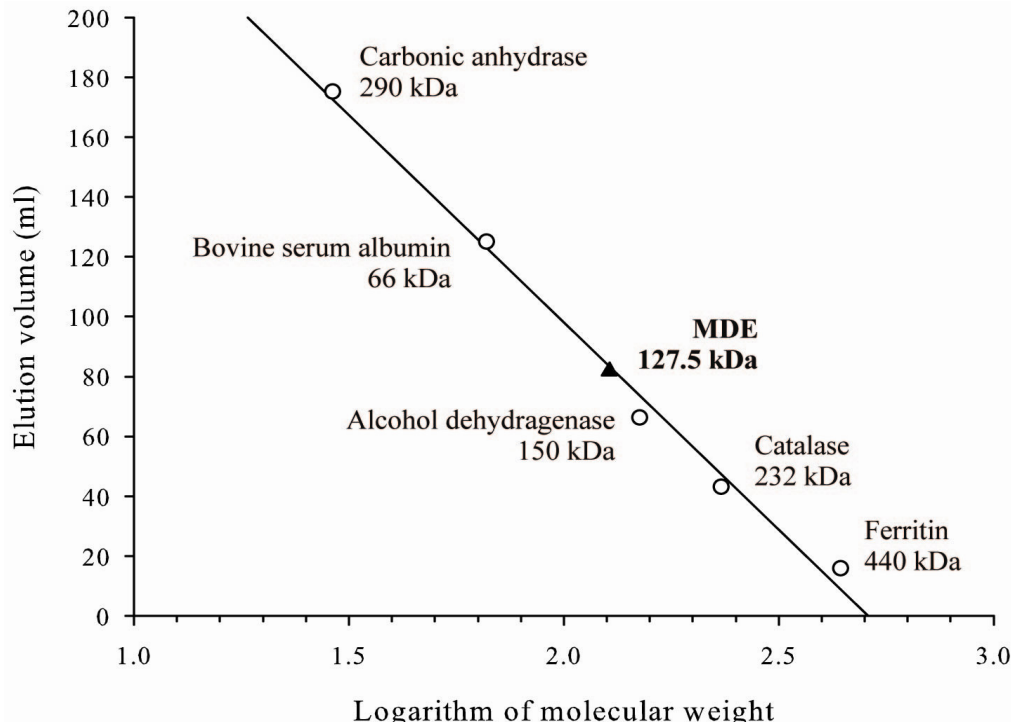

Figure 4. Estimation of molecular weight of the melanoidin decolorizing enzyme (MDE) by gel filtration on Sephadex G-150 column.

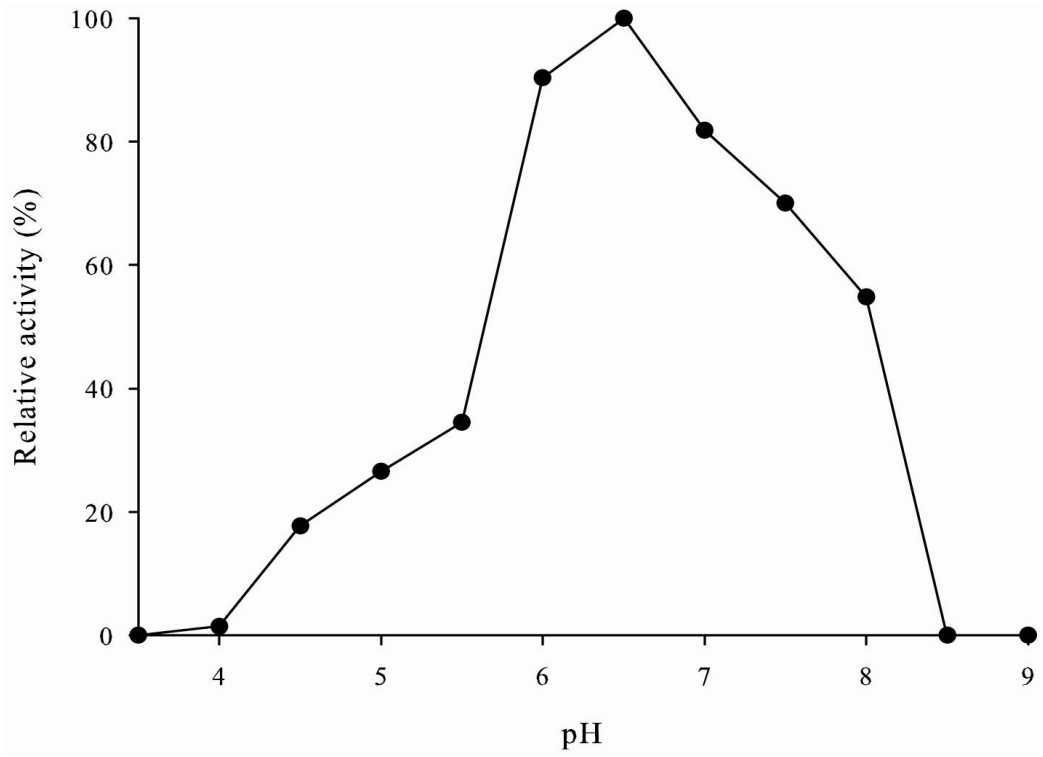

Figure 5. Effect of pH on enzyme activity of the purified melanoidin decolorizing enzyme. Enzyme activity of the purified melanoidin decolorizing enzyme was analyzed at each pH in 0.1 mol l^{-1} sodium acetate (pH 3.5-5.5), 0.1 mol l^{-1} sodium phosphate (pH 6.0-8.0) and 0.1 mol l^{-1} glycine/sodium hydroxide (pH 8.0-9.0) buffers. The relative activity was based on 100% activity in sodium phosphate buffer (pH 6.5) at 35°C.

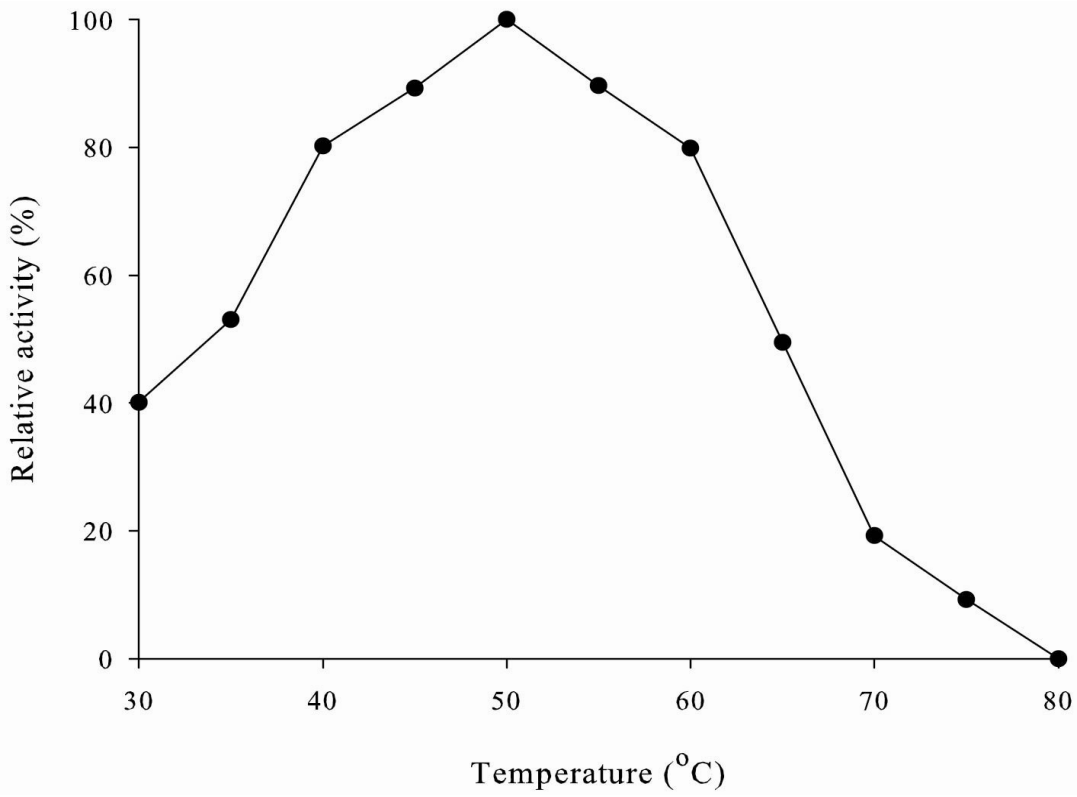

Figure 6. Effect of temperature on enzyme activity of the purified melanoidin decolorizing enzyme. The enzyme and melanoidin were incubated at various temperatures in 0.1 mol l^{-1} sodium phosphate (pH 6.5). Relative activity was based on 100% activity at 35°C (pH 6.5).

Table 2. Effect of sugars on the glucose oxidase activity of the purified melanoidin decolorizing enzyme from *Geotrichum* sp. No. 56.

Sugar	Relative activity* (%)		
	40 mmol l^{-1}	100 mmol l^{-1}	300 mmol l^{-1}
Glucose	95.3	98.8	100
Sorbitol	23.2	24.4	34.9
Galactose	33.7	33.7	51.2
Xylose	34.9	44.2	54.7
Sorbose	60.5	63.2	67.4
Mannitol	20.9	25.6	31.4
Fructose	34.9	51.2	53.5
Maltose	38.4	43.0	60.4
Rhamnose	29.1	30.0	31.4
Saccharose	38.4	39.8	40.7
Arabinose	25.6	34.9	43.0
None	27.9	-	-

*Relative activity was calculated from the glucose oxidase activity of the purified melanoidin decolorizing enzyme in the presence of various sugars in different concentrations against that in the presence of 1 mol l^{-1} glucose.

inhibited by 1 mmol l^{-1} cysteine, 1 mmol l^{-1} IAA, 1 mmol l^{-1} 2-mercaptoethanol, 3 mol l^{-1} urea, 0.4 mol l^{-1} gluconic acid and 0.1% H_2O_2, whereas the metal ion chelator EDTA showed only a 25.1% inhibitory effect and the phosphotase inhibitor NaF showed only a 6.2% inhibitory effect.

Table 3. Effect of enzyme inhibitors on the glucose oxidase activity of the purified melanoidin decolorizing enzyme from *Geotrichum* sp. No. 56.

Enzyme inhibitor	Concentration	Relative activity* (%)
None	-	100
NaF	10^{-3} mmol l^{-1}	93.8
NaN_3	10^{-3} mmol l^{-1}	76.9
EDTA†	10^{-3} mmol l^{-1}	74.9
Thiourea	10^{-3} mmol l^{-1}	94.9
Cysteine	10^{-3} mmol l^{-1}	0
IAA†	10^{-3} mmol l^{-1}	0
2-mercaptoethanol	10^{-3} mmol l^{-1}	0
SDS†	1%	49.2
Urea	3 mol l^{-1}	15.9
PCMB†	10^{-4} mmol l^{-1}	61.5
Gluconic acid	10^{-5} mmol l^{-1}	72.8
	10^{-6} mmol l^{-1}	100
	0.4 mol l^{-1}	0
	0.1%	38.5

*Relative activity was calculated from the glucose oxidase activity of the purified melanoidin decolorizing enzyme in the presence of 1 mol l^{-1} glucose and various enzyme inhibitors in different concentrations against that found in the presence of 1 mol l^{-1} glucose alone.
† Abbreviation: EDTA: ethylene diamine tetraacetic acid; SDS: sodium dodecyl sulfate; IAA: iodoacetic acid; PCMB: p-chloromercuribenzoate.

Table 4. Effect of metal ions on the glucose oxidase activity of the purified melanoidin decolorizing enzyme from Geotrichum sp. No. 56.

Enzyme inhibitor	Relative activity* (%)	
	1 mmol l^{-1}	0.1 mmol l^{-1}
None	100	-
$NiSO_4$	85.9	92.3
$HgCl_2$	0	0
$AgNO_3$	0	0
$CdSO_4$	31.5	89.9
$FeSO_4$	-	91.9
$BaCl_2$	73.2	91.9
$CaCl_2$	88.6	93.3
$CuSO_4$	74.5	-
$ZnSO_4$	81.2	92.6
$MnSO_4$	71.1	94.6
$MgSO_4$	100	100
$Pb(NO_3)_2$	79.9	96.6

*Relative activity was calculated from the glucose oxidase activity of the purified melanoidin decolorizing enzyme in the presence of 1 mol l^{-1} glucose and various metal ions in different concentrations against that found in the presence of 1 mol l^{-1} glucose alone.

Effect of metal ions on the activity of purified MDE

Among the metal ion tests, Hg^{2+} and Ag^{+} were the most effective inhibitors of MDE, causing 100% inhibition at concentrations of 0.1 and 1.0 mmol l^{-1}, respectively. The MDE was strongly inhibited by 1.0 mmol l^{-1} Cd^{2+} to an extent of 68.5%. The other metal ions also inhibited MDE activity at concentrations of either 0.1 or 1.0 mmol l^{-1}, yet at a reduced rate of below 28.9% (Table 4).

Synthetic melanoidin and MSG fermentation spent broth decolorization by purified MDE

Decolorization of synthetic melanoidin and MSG fermentation spent broth was investigated using the purified MDE as described in the Materials and Methods section. Gel filtration chromatograms of a synthetic melanoidin solution (Figure 7) or MSG fermentation spent broth (Figure 8) treated with purified MDE were respectively compared with those of the initial solution or spent broth. The large molecular weight components of melanoidin or spent broth were decolorized considerably and converted to low molecular weight components.

DISCUSSION

In the current study, *Geotrichum* sp. No. 56 produced a single MDE isoform in submerged fermentation. The produced MDE was purified approx. 8.38-fold (Table 1) to obtain a single protein band on SDS-PAGE with a molecular weight of approx. 63.5 kDa, as shown in

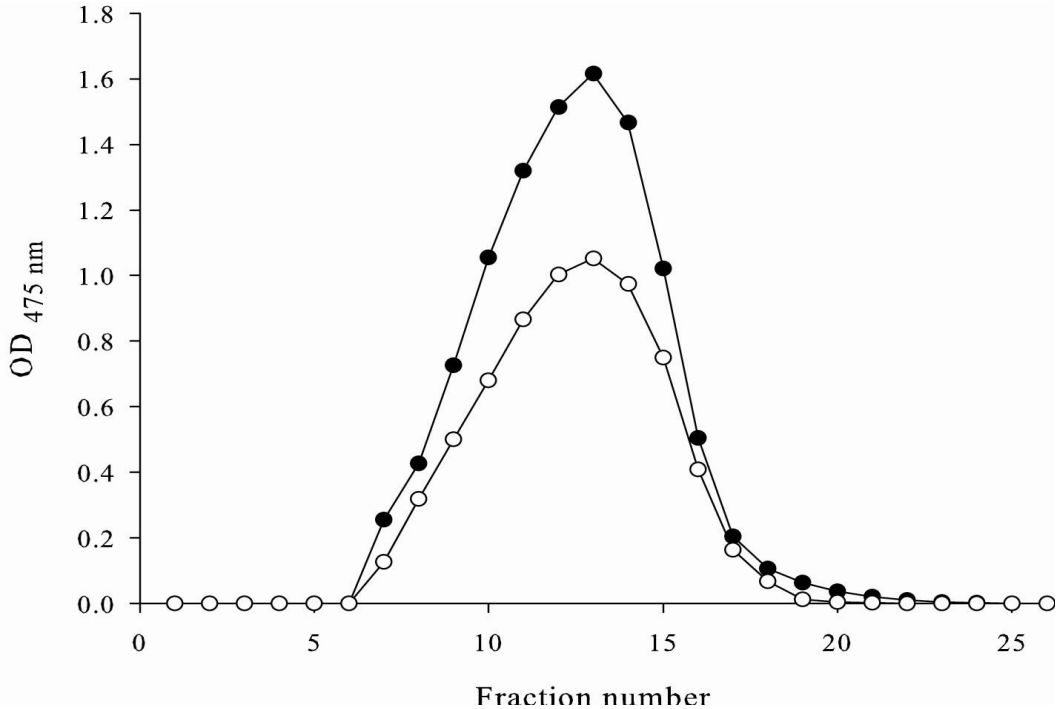

Figure 7. Chromatogram of synthetic melanoidin after treated with the purified melanoidin decolorizing enzyme on a Sephadex G-50 column. The solid lines with (●) and (○) respectively represent the color density of the synthetic melanoidin before and after being treated with the purified melanoidin decolorizing enzyme.

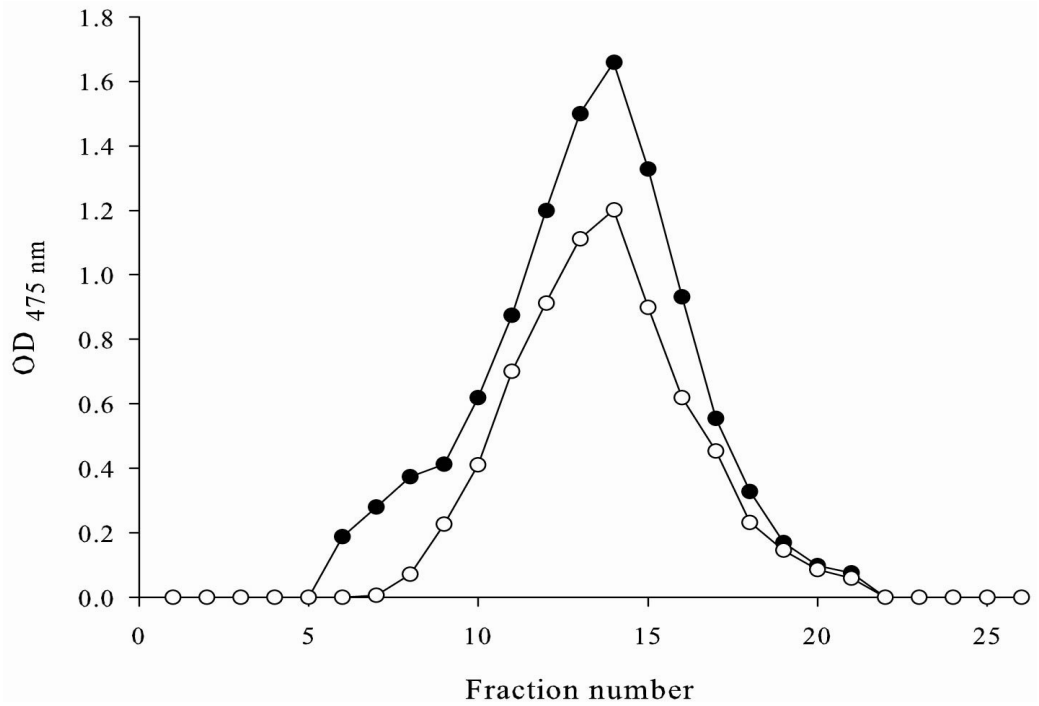

Figure 8. Chromatogram of monosodium glutamate fermentation spent broth after being treated with the purified melanoidin decolorizing enzyme on a Sephadex G-50 column. The solid lines with (●) and (○) represent the color density of monosodium glutamate fermentation spent broth before and after being treated with the purified melanoidin decolorizing enzyme.

Figure 3. However, Saphadex G-150 column chromatography demonstrated that the molecular weight of the intact MDE was in fact approx. 127.5 kDa (Figure 4). The results indicate that the MDE is actually a dimmer enzyme containing two subunits with the same molecular mass. The structural characteristics of this MDE are similar to those of GOx purified from other fungal strains described by Bankar et al. (2009), unless the molecular weight of this MDE is smaller relative to those of reported GOx. It has been reported that the fungal GOx is usually a dimmer enzyme with molecular weights ranging from approx. 130 to 175 kDa (Simpson et al., 2007; Kalisz et al., 1997), and consists of two identical polypeptide chain subunits covalently linked through disulfide bonds (Rando et al., 1997; Kalisz et al., 1997), though tetramer enzymes of GOx may also exist (Liu et al., 1998).

The purified MDE showed optimum activity at pH 6.5 (Figure 5). The optimum temperature of the enzyme was 45°C, above which the thermal stability decreased dramatically (Figure 6). This enzyme required the introduction of certain kinds of sugar to manifest decolorization activity. Table 2 shows that glucose, as opposed to sorbose or other sugars, was found to be most effective, which corresponds to GOx characteristics reported elsewhere. GOx from most fungi and yeasts have pH optima in the acidic to neutral range. For example, *A. niger* and *P. amagasakiense* show pH optima of 3.5 to 6.5 and 4.0 to 5.5, respectively (Nakamura and Fujiki, 1968). In contrast, the GOx obtained from *P.* sp CBS 120262 (Simpson et al., 2007), *P. funiculosum* 433 and *P. canescens* (Sukhacheva et al., 2004) show a slightly alkaline pH optima of 6 to 8.6. The lowest optimum temperature for GOx is reported to be 25-30°C from *P. funiculosum* 433 (Sukhacheva et al., 2004) with a highest optimum of 40 to 60°C from *A. niger* and *P. amagasakiense* ATCC 28686 (Kalisz et al., 1991).

Inhibition studies on several putative enzyme inhibitors revealed that NaF, NaN_3, EDTA, thiourea and p-chloromercuribenzoate did not effectively inhibit the purified MDE, whereas cysteine, IAA, 2-mercaptoethanol, urea, gluconic acid and H_2O_2 demonstrated a strong inhibition (> 50%) of MDE activity. The results indicate that the MDE may not be classified among phosphotases, cytochrom oxidases, metal containing enzymes, peroxidases or RNases but, rather, the intact protein molecule seems to be crucial for the MDE to maintain its decolorization activity. The tertiary structure of the MDE between two subunits is obviously maintained by disulfide bonds. Besides, the inhibition of MDE activity in the presence of gluconic acid or H_2O_2 suggests that the MDE is a kind of GOx. Its enzymatic activity is strongly suppressed by gluconic acid and H_2O_2 due to product-feedback inhibition. The MDE exhibited no enhanced enzymatic activity when the concentration of metal was raised from 0.1 to 1.0 mmol l^{-1} (Table 3). In addition, Hg^{2+}, Ag^{+} and Cd^{2+} significantly inhibited the enzymatic activity of the MDE. These results indicate that the MDE produced by *Geotrichum* sp. No. 56 can only decolorize the fermentation wastewater in the absence of Hg^{2+}, Ag^{+} and Cd^{2+}.

In this study, we observed that purified MDE catalyzed the decomposition of the large molecular weight part of melanoidin in both synthetic melanoidin and MSG fermentation wastewater. The decolorization ability of purified MDE from *Geotrichum* sp. No. 56 was evident, and its use may conceivably be extended to other industrial fermentation wastewater. Further investigations will focus on the catalytic mechanisms of this enzyme.

REFERENCES

Aoshima I, Tozawa Y, Ohmomo S, Ueda K (1985). Production of decolorizing activity for molasses pigment by *Coriolus versicolor* Ps4a. Agric. Biol. Chem., 49: 2041-2045.

Bankar SB, Bule MV, Singhal RS, Ananthanarayan L (2009). Glucose oxidase - an overview. Biotechnol. Adv., 27: 489-501.

Bright HJ, Appleby M (1969). The pH dependence of the individual steps in the glucose oxidase reaction. J. Biol. Chem., 244: 3625-3634.

Calabro V, Drioli E, Matera F (1991). Membrane distillation in the textile wastewater treatment. Desalination, 83: 209-224.

Ciucu A, Patroescu C (1984). Fast spectrometric method of determining the activity of glucose oxidase. Anal. Lett., 17: 1417-1427.

Coulthard CE, Michaelis R, Short WF, Sykes G, Skrimshire GEH, Standfast AFB, Birkinshaw JH, Raistrick H (1945). Notatin: an anti-bacterial glucose-aerodehydrogenase from *Penicillium notatum* westling and *Penicillium resticulosum* sp. nov. Biochem. J., 39: 24-36.

Hatzinikolaou DG, Hansen OC, Macris BJ, Tingey A, Kekos D, Goodenough P (1996). A new glucose oxidase from *Aspergillus niger* characterization and regulation studies of enzyme and gene. Appl. Microbiol. Biotechnol., 46: 371-381.

Hayase F, Kim SB, Kato H (1984). Decolorization and degradation products of the melanoidins by hydrogen peroxide. Agric. Biol. Chem., 48: 2711-2717.

Hsieh PC, Su YC (1999). The study of decolorization of melanoidin from amino acid fermentation waste water. J. Biomass Energy Soc. China, 18: 10-18.

Kalisz H, Hendle HJ, Schmid R (1997). Structural and biochemical properties of glycosylated and deglycosylated glucose oxidase from *Penicillium amagasakiense*. Appl. Microbiol. Biotechnol., 47: 502-507.

Kalisz HM, Hecht HJ, Schomburg D, Schmid RD (1991). Effects of carbohydrate depletion on the structure, stability and activity of glucose oxidase from *Aspergillus niger*. Biochim. Biophys. Acta, 1080: 138-142.

Kitamura K (1984). Biochemical characterization of lipoxygenase lacking mutants: L-1-less, L-2-less, and L-3-less soybeans. Agric. Biol. Chem., 48: 2339-2346.

Lin SH, Peng CF (1996). Continuous treatment of textile wastewater by combined coagulation, electrochemical oxidation and activated sludge. Wat. Res., 30: 587-592.

Liu S, Oeljeklaus B, Tudzynski B (1998). Purification and characterization of glucose oxidase of Botrytis cinerea. Physiol. Mol. Plant Pathol., 53: 123-132.

Lowry OM, Rosebrough MJ, Farr AL, Kandall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Nakamura S, Fujiki S (1968). Comparative studies on the glucose oxidases of *Aspergillus niger* and *Penicillium amagasakiense*. J. Biochem., 63: 51-58.

Ohmomo S, Aoshima I, Tozawa Y, Sakurada N, Ueda K (1985). Purification and some properties of melanoidin decolorizing enzymes, P-III and P-IV, from mycelia of *Coriolus versicolor* Ps4a. Agric. Biol. Chem., 49: 2047-2053.

Rando D, Kohring G, Giffhorn F (1997). Production, purification and characterization of glucose oxidase from a newly isolated strain of *Penicillium pinophilum*. Appl. Microbiol. Biotechnol., 48: 34-40.

Simpson C, Jordaan J, Gardiner NS, Whiteley C (2007). Isolation,

purification and characterization of a novel glucose oxidase from *Penicillium* sp. CBS 120262 optimally active at neutral pH. Protein Expr. Purif., 51: 260-266.
Sirianuntapiboon S, Somchai P, Ohmomo S, Atthasampunna P (1988). Screening of filamentous fungi having the ability to decolorize molasses pigments. Agric. Biol. Chem., 52: 387-392.
Sukhacheva MV, Davydova ME, Netrusov AI (2004). Production of *Penicillium funiculosum* 433 glucose oxidase and its properties. Appl. Biochem. Microbiol., 40: 25-29.
Tan BH, Teng TT, Omar AKM, (2000) Removal of dyes and industrial dye wastes by magnesium chloride. Wat. Res., 34: 597-601.
Weber K, Osborn M (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem., 244: 4406-4412.
Zakhartsev M, Momeu C (2007). Purification of glucose oxidase from complex fermentation medium using tandem chromatography. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 858: 151-158.