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# Characterization of the biocatalysis of tyrosinase in selected organic solvent media using model phenolic substrates

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**Abstract**

Mushroom tyrosinase biocatalysis in selected organic solvent media, including dichloromethane and heptane, was investigated using phenolic model substrates including catechin, catechol, 4-methyl catechol, and *p*-cresol. $V_{max}$ values for tyrosinase biocatalysis in dichloromethane for 4-methyl catechol, catechol, and catechin were $5.07 \times 10^{-4}$, $6.03 \times 10^{-4}$ and $1.47 \times 10^{-3}$ δA/(μg protein s), respectively, while the $K_m$ values were 2.21, 2.36 and 2.52 mM, respectively. In heptane, tyrosinase showed $V_{max}$ values of $0.84 \times 10^{-4}$, $1.02 \times 10^{-4}$, and $1.22 \times 10^{-3}$ δA/(μg protein s) with *p*-cresol, catechol and catechin, respectively, with corresponding $K_m$ values of 1.07, 4.32 and 5.38 mM. The characterization of the oxidation products resulting from the reactions of tyrosinase with selected substrates was carried out by spectrophotometeric scanning, differential scanning calorimetry and pyrolysis/gas chromatography coupled to mass spectrometry. The results showed that the change in reaction medium resulted in the formation of oxidation products that differed with respect to their maxima absorbance, thermal parameters and wide range of pyrolysis residues.


*Keywords:* Tyrosinase; Biocatalysis; Organic solvent media; Oxidation products

## 1. Introduction

Tyrosinase is an enzyme that catalyzes both the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones [1]. Tyrosinase is a widely distributed copper-containing monooxygenase that possesses two distinct substrate-binding sites, one with high affinity for aromatic compounds, including phenolic substrates, and the other for metal-binding agents and oxygen [2]. Monophenols and *o*-diphenols have been reported to be the exclusive substrates of tyrosinase [2], a key enzyme in the biosynthesis of melanin and other polyphenolic compounds.

Tyrosinase biocatalysis in non-polar organic solvent medium as opposed to conventional aqueous buffer medium has numerous advantages, including increased solubility of hydrophobic substrates thereby allowing the potential use of certain substituted phenolic substrates, as well as improved stability of tyrosinase in water-immiscible organic solvents thereby facilitating specific oxidation reactions with water-insoluble organic substrates. In addition, the use of organic solvent media provides higher oxygen

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solubility, which in turn reduces the availability of the oxygen as a limiting factor [3]. Kazandjina and Klibanov [4] reported that polyphenol oxidase in chloroform was 10-times more active than in aqueous medium, which may be due to the increased solubility of oxygen and the phenolic substrates. Additional advantages of the use of organic solvent media also include the ability to shift the thermodynamic equilibrium of many enzymatic reactions towards the generation of the desired products, and easier recovery and reuse of enzymes due to their limited solubility [5].

This study is part of on-going research aimed at the optimization of tyrosinase biocatalysis in organic solvents [6–8]. The overall objective of this research is to generate novel biomolecules that could be used in pharmaceutical, cosmetic and food applications. The specific objectives of this work were to characterize tyrosinase activity in dichloromethane and heptane using catechin, catechol, 4-methyl catechol and *p*-cresol as model substrates.

## 2. Material and methods

### 2.1. Preparation of enzyme suspension

Commercially purified mushroom tyrosinase, possessing an oxidative activity of 3000–3400 units/0.85 mg protein/mg solid, was purchased from Sigma Chemicals Co. (St.-Louis, MO). One unit of enzyme activity was defined as the amount of enzyme that produced an increase of 0.001 in absorbance per minute at a defined wavelength, temperature and pH. The mushroom tyrosinase suspension was prepared in citrate–phosphate buffer solution (0.1 M) at pH 6.2 for the heptane and dichloroethane reactions, and at pH 6.6 and 6.0 for toluene and dichloromethane reactions, respectively.

### 2.2. Preparation of substrate solutions

Catechin, *p*-cresol, catechol and 4-methyl catechol were obtained from Sigma Chemicals Co. A stock solution (0.4 M) of catechin was prepared in methanol whereas those of *p*-cresol, catechol and 4-methyl catechol were prepared in ethanol. Homogenization of the stock solutions was performed by sonication using a Branson water-bath sonicator (Bransonic Corp., Banbury, CT).

### 2.3. Enzyme assay

The organic solvent reaction mixture, containing 5 μl stock solution (0.4 M) of one of the selected substrates, was incubated at 25 °C. The enzymatic reaction was initiated by adding the tyrosinase suspension. The reaction mixture was shaken continuously for 3 s to 2 min and the enzymatic reaction was then halted by the addition of 200 μl acetone. A blank trial, containing all the components except the tyrosinase suspension, was performed in tandem with the enzymatic assay. The enzyme activity in heptane, toluene, dichloromethane and dichloroethane, using catechin as substrate, was determined spectrophotometrically by measuring the color intensity of the oxidation products at 376, 379, 375 and 375 nm, respectively. The enzymatic activity in dichloromethane, using 4-methyl catechol and catechol as substrates, was determined by measuring the color intensity of the oxidation products at 384 and 376 nm, respectively, whereas that in heptane, with *p*-cresol and catechol as substrates, was measured at 330 and 372 nm, respectively.

### 2.4. Effect of protein concentration on tyrosinase activity

The effect of protein concentration on tyrosinase activity was determined by the addition of 18.3–64.2, 24.4–58.7, and 29.3–48.9 μg protein per 200 μl assay for the enzyme oxidations of catechin, catechol and 4-methyl catechol, respectively, in dichloromethane and 12.8–36.7, 3.9–32.4, and 29.3–73.3 μg protein per 200 μl assay for the oxidation of catechin, catechol and *p*-cresol, respectively, in heptane.

### 2.5. Effect of substrate concentration on tyrosinase activity

The effect of substrate concentration on tyrosinase activity in organic solvents was investigated using different substrates over a wide range of concentrations from 0.5 to 20.0, 2.0 to 20.0 and 2.0 to 12.0 mM for catechin, catechol and 4-methyl catechol, respectively, in dichloromethane, and 2.0 to 20.0, 1.0 to 5.0 and 2.0 to 12.0 mM for catechin, catechol and *p*-cresol, respectively, in heptane.

### 2.6. Characterization of tyrosinase biocatalysis oxidation products

#### 2.6.1. Purification of oxidation products by size-exclusion chromatography

Tyrosinase oxidation catalyzed products of catechin, catechol, 4-methyl catechol and *p*-cresol (Sigma Chemicals Co.) were concentrated either by vaporization of the organic solvent under a gentle stream of nitrogen or removal of aqueous medium by lyophilization. The oxidation products were then re-solubilized in deionized water and the resulting solution was filtered using a 0.22 μm PVDF syringe filter before being applied to a Trisacryle GF 05 M (Biosepra Inc.; Marlborough, MA) column (1.60 cm × 80 cm). The elution flow rate of acetone was 0.4 ml/min and fractions of 4 ml were collected and their absorbances were measured spectrophotometerically at their respective absorbance optima. Fractions were also characterized with respective to their $K_{av}$ values defined to be the solute behavior independent of these variables: $K_{av} = (V_e - V_o)/(V_t - V_o)$ where $K_{av}$ is available partition coefficient; $V_e$ elution volume of solute; $V_o$ column void volume; and $V_t$ total permeation volume of column. Oxidation products were subsequently lyophilized and stored under nitrogen at −80 °C for further analyses.

#### 2.6.2. Spectrophotometric scanning

Catechin, catechol, 4-methyl catechol, *p*-cresol and their respective purified oxidation products (0.2 mg) were dissolved in 1 ml methanol and subjected to spectrophotometric scanning (Beckman DU-650) over the range of 200–800 nm using methanol as a blank.

#### 2.6.3. Thermal analysis

Thermal analysis was performed using differential scanning calorimetry (DSC), (Mettler Toledo Inc., Greifensee, Switzerland). Catechin, catechol, 4-methyl catechol, *p*-cresol and their corresponding purified tyrosinase oxidation products (2 mg solid), formed by tyrosinase biocatalysis in selected reaction media, were sealed in separate aluminum pans. The sample pan and reference pan, which was empty, were put into a chamber heated at a temperature beginning at 10 °C and increasing at a rate of 10 °C/min to 250 °C. The power (energy per unit time) differential between the sample and reference was measured during the programmed heating and cooling periods.

#### 2.6.4. Pyrolysis gas chromatography/mass spectrometry analysis

The pyrolysis and GC/MS analyses of oxidation products were performed using a Hewlett-Packard Model 5890 Series gas chromatograph, which was coupled to a Series 5971 mass selective detector and interfaced to a CDS pyroprobe 2000 unit. A DB-5MS capillary column (60 m × 0.25 mm i.d. × 0.33 μm film thickness, Supelco Inc., Oakville, ON) was used. Catechin, catechol, 4-methyl catechol, *p*-cresol and their respective purified oxidation products (1.1 mg solid) were separately introduced into a quartz tube (0.3 mm thickness × 25.4 mm length), which was then plugged with quartz wool and inserted inside a coil probe; the pyroprobe interface temperature was set at 500 °C with a heating rate of 50 °C/s for a total heating time of 20 s, after which helium was applied at 60 psi for 2 min, and maintained at 1.5 ml/min for the rest of the separation run. The system operated in splitless mode. The temperature of the column was initially at −5 °C for 2 min, followed by an increase to 50 °C at a rate of 30 °C/min, and a subsequent increase at a rate of 8 °C/min to 250 °C, where it was held for 5 min so that the total separation time was 33.83 min. The capillary direct MS interface temperature was maintained at 280 °C and the ion source temperature was at 180 °C. The ionization voltage and the electron multiplier were at 70 eV and 2047 V, respectively. The mass range analyzed was at 30–350 amu and 2.2 scans/s. For product identification, the mass spectral library Wiley6n.L (G1034C Version C. 03.00, 1989–1994, Wiley, New York, NY) was used.

## 3. Results and discussion

### 3.1. Effect of protein concentration on tyrosinase activity

The effect of protein concentration on tyrosinase activity in the selected organic media was investigated using catechin, catechol, 4-methyl catechol and *p*-cresol as substrates. The results (not shown) indicated that the amounts of enzyme required for optimal activity were 45.8, 43.9 and 39.1 μg per 200 μl

assay for catechin, catechol, and 4-methyl catechol, respectively, in dichloromethane, whereas in heptane, the optimal concentration was 31.2, 4.3 and 34.2 μg per 200 μl assay for catechin, catechol and *p*-cresol, respectively.

There was a steady increase in the specific activity of tyrosinase with a concomitant increase in enzymatic protein concentration in dichloromethane; however, after maximal oxidative activity was reached, any further increases in enzymatic protein resulted in a gradual decrease in activity with 4-methyl catechol as substrate and a dramatic one with catechin and catechol. Similar results were obtained for tyrosinase biocatalysis in heptane. Kermasha and Tse [7] reported a similar trend using vanillin as substrate for tyrosinase biocatalysis in chloroform. These findings suggest that this phenomenon may be due to the aggregation of tyrosinase molecules in the organic solvent reaction media due to the limited solubility of the enzyme thereby resulting in less enzyme and substrate interaction [9,10].

### 3.2. Effect of substrate concentration on tyrosinase activity

The optimum concentration of catechin and 4-methyl catechol for tyrosinase activity was 10 mM, while that of catechol was 12 mM. In addition, the optimal substrate concentrations of *p*-cresol, catechol and catechin for enzyme activity in heptane was 8, 6 and 10 mM, respectively.

Table 1 shows that the $K_m$ values for tyrosinase activity in dichloromethane were 2.36, 2.21 and 2.52 mM with catechol, 4-methyl catechol, and catechin, respectively. These results indicate that tyrosinase demonstrated a similar affinity towards these substrates. Table 1 also indicates that the $K_m$ values for tyrosinase activity in heptane were approximately twice as high as those obtained in dichloromethane for catechol and catechin. However, *p*-cresol in heptane gave the lowest $K_m$ value for tyrosinase. These findings suggest that the differences in the tyrosinase affinity toward the selected substrates may be due to their various degrees of hydrophobicity, which influences their partitioning between the organic solvent used for biocatalysis and the enzyme active site. Moreover, the results suggest that this substrate effect may become more pronounced as solvent hydrophobicity is increased [11,12]. Butorn et al. [3] reported a similar trend for PPO biocatalysis in chloroform using *p*-substituted phenolic substrates. The $K_m$ values increased with a corresponding increase in substrate hydrophobicity due to a decrease in the amount of substrate available to the enzyme active site as a result of a greater degree of partitioning in chloroform.

Table 1
Kinetics of tyrosinase biocatalysis using selected substrates, including catechin, catechol, 4-methyl catechol and *p*-cresol in dichloromethane and heptane

| Reaction medium | Substrate | $V_{max}$[a] ($10^{-4}$) | $K_m$ (mM) | Catalytic efficiency[b] |
|---|---|---|---|---|
| Dichloromethane | Catechin | 14.7 | 2.52 | 5.83 |
| | Catechol | 6.03 | 2.36 | 2.56 |
| | 4-Methyl catechol | 5.07 | 2.21 | 2.29 |
| Heptane | Catechin | 12.2 | 5.38 | 2.26 |
| | Catechol | 1.02 | 4.32 | 0.24 |
| | *p*-Cresol | 0.84 | 1.07 | 0.79 |

[a] The maximal enzymatic reaction rate was defined as the change in absorbancy at a specific wavelength of 375, 376 and 384 nm for catechin, catechol and 4-methyl catechol, respectively, in dichloromethane and 376, 372 and 330 nm for catechin, catechol and *p*-cresol, respectively, in heptane, per μg enzymatic protein per second.

[b] The catalytic efficiency was defined as the ratio of $V_{max}$ to $K_m$, $\times 10^{-4}$.

However, the results (Table 1) show that the $V_{max}$ value for tyrosinase biocatalysis in dichloromethane was about 2.5-times higher with catechin than those obtained with catechol and 4-methyl catechol. In addition, Table 1 shows that the $V_{max}$ value for the tyrosinase-catechin biocatalysis was also much higher than those using catechol and *p*-cresol as substrates in heptane. Kermasha and Tse [7] reported that the difference in the reaction rate may be due to the availability of the *ortho* positions on the phenolic compounds for enzyme biocatalysis. The same author indicated that greater the availability of the *ortho* positions on the phenolic compounds, the higher the $V_{max}$ value.

The catalytic efficiency (Table 1), defined as the ratio $V_{max}/K_m$ [3] for tyrosinase biocatalysis, shows that the substrate conversion rate was two times higher with catechin compared to those obtained with catechol and 4-methyl catechol in dihcloromethane. The results also show that in heptane, the catalytic efficiency for tyrosinase biocatalysis was highest for

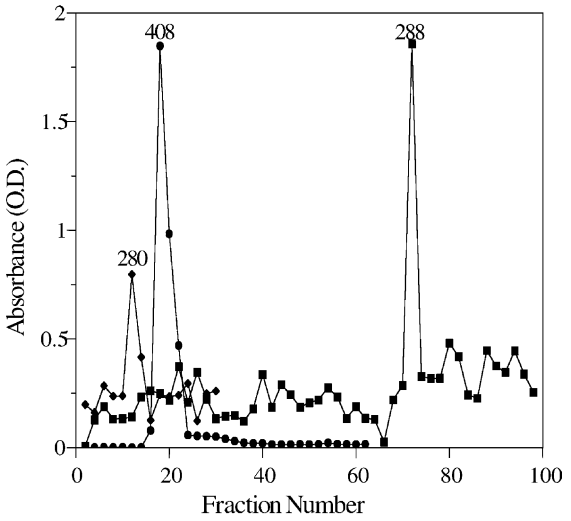


Fig. 1. Size-exclusion chromatography elution profile of tyrosinase, catechin and the corresponding enzyme oxidation catalyzed products in aqueous medium, with absorbance at (◆) 280 nm; (■) 288 nm and (●) 408 nm.

Table 2
$K_{av}$ values of catechin, catechol, 4-methyl catechol, *p*-cresol and their respective purified oxidation products

| Compound | $K_{av}$[e] | Compound | $K_{av}$[e] |
|---|---|---|---|
| Catechin | 1.85 | Catechol | 1.64 |
| CPA[a] | 0.43 | CLPA[b] | 0.41 |
| CPH[a] | 0.43 | CLPH[b] | 0.51 |
| CPT[a] | 0.41 | CLPDM[b] | 0.62 |
| CPDM[a] | 0.36 | | |
| CPDE[a] | 0.36 | | |
| 4-Methyl catechol | 1.38 | *p*-Cresol | 1.54 |
| 4MCPA[c] | 0.41 | *p*-CPA[d] | 0.35 |
| 4MCPDM[c] | 0.62 | *p*-CPH[d] | 0.51 |

[a] CPA, CPH, CPT, CPDM and CPDE refer to the enzyme oxidation catalyzed products of catechin in aqueous, heptane, toluene, dichloromethane and dichloroethane media, respectively.

[b] CLPA, CLPH and CLPDM refer to the enzyme oxidation catalyzed products of catechol in aqueous, heptane and dichloromethane media, respectively.

[c] 4MCPA and 4MCPDM refer to the enzyme oxidation catalyzed products of 4-methyl catechol in aqueous and dichloromethane media, respectively.

[d] *p*-CPA and *p*-CPH refer to the enzyme oxidation catalyzed products of *p*-cresol in aqueous and heptane media, respectively.

[e] $K_{av}$ was defined as the available partition coefficient, used to define solute behavior independent of these variables, and calculated as $K_{av} = (V_e - V_o)/(V_t - V_o)$ where $K_{av}$ is available partition coefficient; $V_e$ elution volume of solute; $V_o$ column void volume; and $V_t$ is total permeation volume of column.

catechin and 9.4 and 2.9 times lower for *p*-cresol and catechol, respectively. However, the catalytic efficiencies of all the model substrates in dichloromethane were higher than those in heptane. The overall experimental findings suggest that dichloromethane (log *P* 2.0) was more suitable for the biocatalytic oxidation of substrates than heptane (log *P* 4.0) due to the hydrophobicity of the organic solvent and the structural nature of the substrates. These results are also in agreement with those obtained by Estrada et al. [13] who reported that organic solvents with log *P* values between 2 and 2.5 supported higher PPO biocatalysis activity than those obtained using organic solvents with log *P* values higher than 2.5 due to the effect of solvent polarity on the conformation of the enzyme as well as the solubility of substrates and/or products.

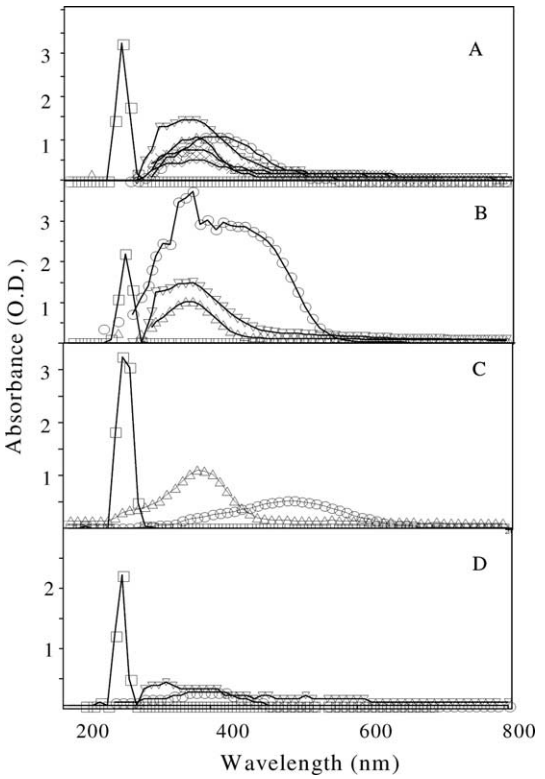


Fig. 2. Absorbances of substrates (----) and their respective enzyme oxidation catalyzed products in (○) aqueous; (▽) heptane; (Δ) dichloromethane; (□) toluene and (×) dichloroethane media using (A) catechin, (B) catechol, (C) 4-methyl catechol and (D) *p*-cresol as substrates.

### 3.3. Characterization of tyrosinase biocatalysis oxidation products

#### 3.3.1. Purification of oxidation products by size-exclusion chromatography

A typical elution profile of tyrosinase, catechin and the respective enzyme oxidation catalyzed products in an aqueous reaction medium is shown in Fig. 1. A similar elution profile was also obtained for tyrosinase biocatalysis in selected media using catechol, 4-methyl catechol and *p*-cresol as substrates. The results show the presence of three major peaks at 280, 288 and 408 nm, which correspond to tyrosinase, catechin and the respective oxidation products.

Table 2 shows the $K_{av}$ values obtained from the order of elution of substrates and their corresponding enzyme oxidation catalyzed products. Among the substrates examined, 4-methyl catechol was the fastest eluting compound as indicated by its $K_{av}$ value of 1.38, followed by *p*-cresol and catechol possessing $K_{av}$ values of 1.54 and 1.64, respectively; the results also show that catechin, the highest molecular weight substrate, eluted last as indicated by its relatively high $K_{av}$ value of 1.85. These findings suggest that the observed chromatographic behavior of the phenolic substrates on the Trisacryl GF05 gel was not only due to steric hindrance but also due to hydrogen bond interactions between the gel and the phenolic substrates [14,15].

Table 2 also shows that the enzyme oxidation catalyzed products of catechin, in the aqueous, heptane, toluene, dichloromethane and dichloroethane reaction media, showed similar $K_{av}$ values between 0.36 and 0.43; this suggests that oxidation products of similar molecular weight were formed by tyrosinase using catechin as substrate. However, the results indicate that tyrosinase oxidations of catechol, 4-methyl catechol and *p*-cresol resulted in the formation of oxidation products possessing different $K_{av}$ values ranging from 0.35 to 0.62, thereby suggesting the presence of polymers of different molecular weight. The results show that depending on the reaction medium used for tyrosinase oxidation, different products can be obtained.

Table 3
Thermal analysis data of catechin, catechol, 4-methyl catechol, *p*-cresol and their corresponding oxidation products

| Compound | Melting point (°C) | Crystallization point (°C) | $\Delta H$ (J/g) |
|---|---|---|---|
| Catechin | 93.5 & 153.0 | – | 30.6 & 104.3 |
| CPA[a] | 179.4 | – | 183.9 |
| CPH[a] | 132.9 | – | 154.0 |
| CPT[a] | – | 144.2 | −281.3 |
| CPDM[a] | – | 121.8 | −305.7 |
| CPDE[a] | – | 156.7 | −244.6 |
| Catechol | 102.2 | – | 202.1 |
| CLPA[b] | 184.7 | – | 32.0 |
| CLPH[b] | 65.4 & 88.8 | – | 10.1 & 3.9 |
| CLPT[b] | 87.0 | – | 19.2 |
| 4-Methyl catechol | 63.6 | – | 106.2 |
| 4MCPA[c] | 232.7 | – | 34.0 |
| 4MCPDM[c] | 26.3 | – | 5.9 |
| *p*-Cresol | 35.6 | – | 13.8 |
| *p*-CPA[d] | 78.6 & 223.8 | – | 29.8 & 18.9 |
| *p*-CPH[d] | 80.6 | – | 45.5 |

[a] CPA, CPH, CPT, CPDM and CPDE refer to the enzyme oxidation catalyzed products of catechin in aqueous, heptane, toluene, dichloromethane and dichloroethane media, respectively.

[b] CLPA, CLPH and CLPDM refer to the enzyme oxidation catalyzed products of catechol in aqueous, heptane and dichloromethane media, respectively.

[c] 4MCPA and 4MCPDM refer to the enzyme oxidation catalyzed products of 4-methyl catechol in aqueous and dichloromethane media, respectively.

[d] *p*-CPA and *p*-CPH refer to the enzyme oxidation catalyzed products of *p*-cresol in aqueous and heptane media, respectively.

*3.3.2. UV spectrophotometric scanning of oxidation products*

The maximum absorbancies ($\lambda_{max}$) of catechin, catechol, 4-methyl catechol, *p*-cresol and their respective enzyme products in the selected reaction media (Fig. 2) was determined. The results (Fig. 2A) show that the maximum absorbency ($\lambda_{max}$) of the oxidation products of catechin formed in heptane, toluene, dichloromethane and dichloroethane was at 376, 379, 375 and 375 nm, respectively. These results are in agreement with those reported by Guyot et al. [16] who indicated that PPO oxidation of catechin in aqueous medium yielded colored products with a $\lambda_{max}$ between 240 and 500 nm. Estrada et al. [13] reported that the enzyme oxidation products of catechin in organic media, including toluene, benzene and 1,1,1-trichloroethane, showed a $\lambda_{max}$ at 395 nm.

The results (Fig. 2B) also show that the $\lambda_{max}$ of catechol and its enzyme oxidation catalyzed products in aqueous, heptane and dichloromethane media was at 286, 383, 374 and 377 nm, respectively, whereas that (Fig. 2C) of 4-methyl catechol and its oxidation products in aqueous and dichloromethane media was at 287, 507 and 384 nm, respectively, and that (Fig. 2D) of *p*-cresol and its oxidation products in aqueous and heptane media was at 282, 399 and 342 nm, respectively. Richard-Forget et al. [17] reported that the enzymatic oxidation of 4-methyl catechol by apple PPO in aqueous medium resulted in oxidation products with a $\lambda_{max}$ at 280 nm. These findings suggest the formation of different oxidation products by tyrosinase biocatalysis in organic solvents in comparison to those obtained with the aqueous medium.

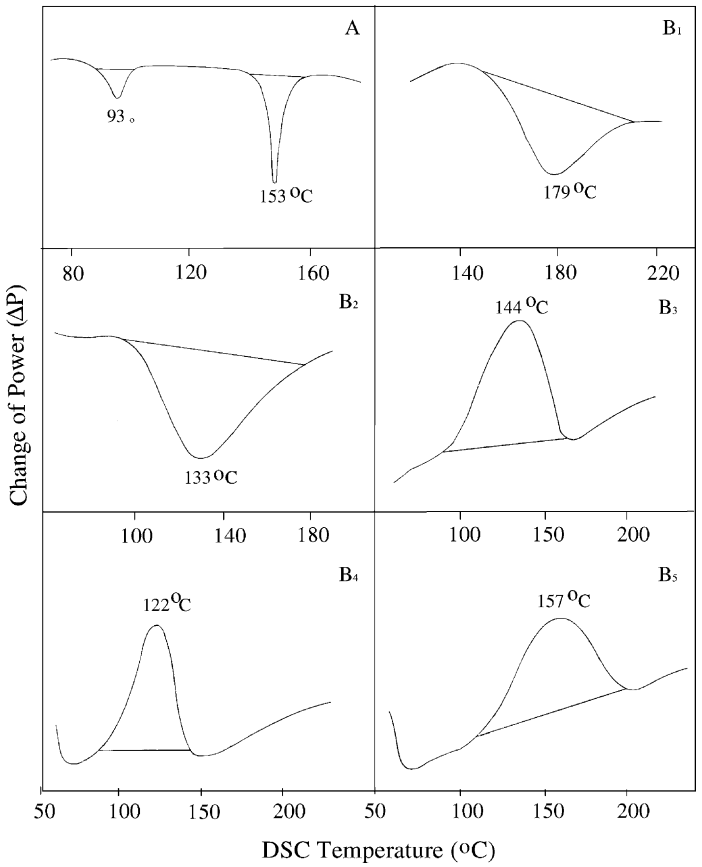


Fig. 3. Differential scanning calorimetry (DSC) curves of (A) catechin and ($B_1$) the tyrosinase-catalyzed oxidation products in aqueous; ($B_2$) heptane; ($B_3$) toluene; ($B_4$) dichloromethane and ($B_5$) dichloroethane media.

### 3.3.3. Thermal analysis of enzyme oxidation

The thermal analysis of catechin, catechol, 4-methyl catechol, *p*-cresol and their respective oxidation products (Table 3) was measured by DSC. Fig. 3 shows typical DSC curves obtained for catechin and the corresponding enzyme oxidation catalyzed products in selected reaction media. Similar DSC curves were obtained for catechol, 4-methyl catechol and *p*-cresol and their respective products. The results show that the DSC curve for catechin (Fig. 3A) indicated the occurrence of two melting points (Table 3) and corresponding endothermic $\Delta H$ values thereby suggesting that catechin undergoes an endothermic crystal phase transition at 93.5 °C to a corresponding stable crystalline form which in turn undergoes a melting transition at 153 °C [18]. Similar findings (Table 3) were obtained for the enzyme oxidation catalyzed products of catechol and *p*-cresol in the heptane and aqueous reaction media, respectively.

The results (Fig. 3$B_1$ and $B_2$) also show that the enzyme oxidation catalyzed products of catechin by tyrosinase activity in the aqueous and heptane media exhibited only one melting point and respective endothermic $\Delta H$ value (Table 3). Similar findings were obtained for the tyrosinase biocatalysis of catechol in the aqueous and toluene media, *p*-cresol in heptane and 4-methyl catechol in the aqueous and dichloromethane media thereby suggesting that these enzyme oxidation catalyzed products exist as only one crystalline form during the thermal treatment used.

However, Fig. 3$B_3$–$B_5$ also indicate that the oxidation products of the tyrosinase-catechin biocatalysis in toluene, dichloromethane and dichloroethane showed crystallization peaks and corresponding exothermic $\Delta H$ values (Table 3). These findings may be explained by Haines and Wilburn [18] who reported that when certain polymers are subjected to an increase in temperature, they melt before decomposing and then crystallize completely. Upon further heating, these polymers change to a more plastic material possessing an increased heat capacity, so that the polymer molecules have more freedom to move and subsequently rearrange into a more regular crystal structure, thereby exhibiting an exothermic $\Delta H$.

The overall results (Table 3) show that relatively higher melting points were obtained for the enzyme oxidation catalyzed products of the selected substrates in aqueous medium in comparison to those obtained for the oxidation products formed in organic solvents. In addition, the overall findings show that the biocatalysis of the selected substrates in different media indicated the presence of unique oxidation products with specific $\Delta H$ properties.

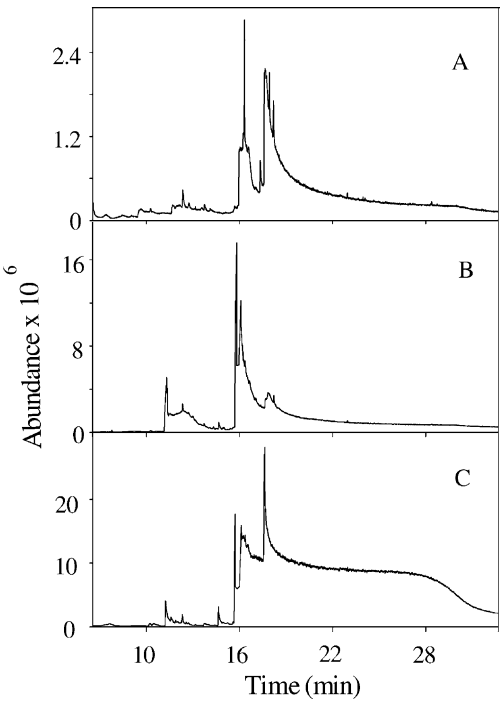


Fig. 4. Pyrograms of the tyrosinase-catalyzed oxidation products of catechol in the selected reaction media, including (A) aqueous; (B) heptane and (C) dichloromethane using the quartz tube at 500 °C for 2 s.

### 3.3.4. Pyrolysis-gas chromatography/mass spectrometry analysis of oxidation products

The characterization of tyrosinase biocatalysis products was investigated by pyrolysis-GC/MS. Fig. 4 shows a typical pyrogram of the tyrosinase-catalyzed oxidation products of catechol in the selected reaction media; similar results were obtained for the tyrosinase biocatalysis using catechin, 4-methyl catechol and *p*-cresol as substrates in the selected media. In addition, Tables 4 and 5 show the compounds identified by GC/MS possessing a quality higher than 80%.

The results (Table 4) show that only one major compound 10-methylnonadecane was formed by the pyrolysis of catechin. The results also show that the

pyrolysis of the enzyme oxidation catalyzed products of catechin, in aqueous medium, produced three major compounds while those produced in heptane and dichloroethane gave two major compounds. In contrast, the results show that more than 12 major peaks were detected from the pyrolysis of the oxidation products of the tyrosinase-catechin biocatalysis in toluene and dichloromethane. These overall findings suggest that depending on the reaction medium used, the tyrosinase biocatalysis of catechin resulted in the formation of different types and relative abundances of oxidation products.

In addition, Table 5 shows that two major pyrolysis compounds were obtained from the tyrosinase-catechol biocatalysis in aqueous medium; these products were also detected along with additional oxidation products of catechol in dichloromethane and heptane. Table 5 demonstrates that the enzyme oxidation catalyzed products of 4-methyl catechol in aqueous and dichloromethane media and of *p*-cresol in

Table 4
Major pyrolysis fragments of catechin and its oxidation products identified by GC/MS

| Compound | RT | Area (%) | Library/ID | Quality |
|---|---|---|---|---|
| Catechin | 33.23 | 20.29 | 10-Methylnonadecane | 80 |
| CPA[a] | 11.79 | 13.37 | Phenol | 90 |
| | 13.74 | 28.5 | 4-Methyl-phenol | 87 |
| | 18.3 | 5.75 | 2,4,7-Trimethylbenzofuran | 86 |
| CPH[a] | 21.16 | 38.17 | 2,4-bis(1,1-Dimethylethyl) phenol | 94 |
| | 28.79 | 7.37 | 2-*t*-Butyl-4-(dimethylbenzyl) phenol | 93 |
| CPT[a] | 7.12 | 33.75 | Acetic acid | 91 |
| | 8.93 | 1.31 | Hexamethyl-cyclotrisiloxane | 91 |
| | 9.27 | 2.83 | Hexanal | 90 |
| | 9.36 | 1.55 | 2,3,5-Trimethylfuran | 83 |
| | 9.96 | 2.69 | 2-Methyl-1H-pyrrole | 90 |
| | 10.62 | 5.40 | (*S*)-1,3-Butanediol | 90 |
| | 10.92 | 1.80 | 2-Heptanone | 91 |
| | 11.00 | 1.23 | 1,3,5,7-Cyclooctatetraene | 96 |
| | 11.50 | 1.97 | 2-Cyclopenten-1-one, 2-methyl- | 94 |
| | 12.69 | 6.38 | Phenol | 93 |
| | 12.88 | 1.94 | 2-Cyclopenten-1-one, 3-methyl- | 94 |
| | 13.54 | 1.04 | 1-Hexanol, 2-ethyl- | 86 |
| CPDM[a] | 9.27 | 4.15 | Hexanal | 86 |
| | 9.97 | 6.14 | 2-Methyl-1H-pyrrole | 91 |
| | 10.64 | 14.37 | (*S*)-1,3-Butanediol | 83 |
| | 10.92 | 4.48 | 2-Heptanone | 91 |
| | 11.50 | 3.71 | 2-Cyclopenten-1-one, 2-methyl- | 94 |
| | 12.89 | 11.41 | Phenol | 94 |
| | 12.68 | 4.90 | 2-Cyclopenten-1-one, 3-methyl- | 96 |
| | 13.99 | 2.14 | 2,4-Hexadiene, 3,4-dimethyl- | 83 |
| | 14.23 | 3.22 | Phenol, 2-methyl- | 97 |
| | 14.30 | 2.72 | 2,3-Dimethylcyclopent-2-en-1-one | 93 |
| | 14.73 | 8.03 | Phenol, 3-methyl- | 96 |
| | 15.11 | 3.32 | Mequinol | 87 |
| | 16.49 | 3.54 | Phenol, 2-ethyl- | 91 |
| | 19.28 | 3.75 | Indole | 91 |
| CPDE[a] | 21.02 | 4.86 | Phenol, 2,5-bis(1,1-dimethylethyl) | 90 |
| | 28.68 | 11.91 | Bicyclo [2.2.1] heptane-2-ol | 90 |

[a] CPA, CPH, CPT, CPDM and CPDE refer to the enzyme oxidation catalyzed products of catechin in the aqueous, heptane, toluene, dichloromethane and dichloroethane media, respectively.

Table 5
Major pyrolysis compounds of catechol, 4-methyl catechol, *p*-cresol and their oxidation products identified by GC/MS

| Compound | RT | Area (%) | Library/ID | Quality |
|---|---|---|---|---|
| Catechol | 20.39 | 100 | Ether | 86 |
| CLPA[a] | 17.66 | 28.3 | 1,2-Benzenediol | 86 |
| | 17.93 | 13.3 | 3-Methyl-1,3-bis(trimethylsiloxy) benzene | 80 |
| CLPH[a] | 15.80 | 18.8 | 1,2-Benzenediol | 91 |
| | 16.36 | 13.2 | 5,4′-Dimethoxy-2-methylbibenzyl | 83 |
| | 16.65 | 12.0 | Benzaldehyde | 86 |
| | 17.85 | 6.5 | 4-Ethyl-1,2-benzenediol | 91 |
| CLPDM[a] | 11.25 | 5.2 | 4-Methyl-phenol | 91 |
| | 14.66 | 2.9 | 1,3-Benzodioxol-2-one | 96 |
| | 15.71 | 11.4 | 1,2-Benzenediol | 91 |
| | 16.53 | 7.1 | 1,2-Benzenediol | 90 |
| | 17.63 | 36.8 | 1,2-Benzenediol | |
| 4-Methyl catechol | 17.59 | 33.1 | 3-Methyl-1,2-benzenediol | 91 |
| 4MCPA[b] | 17.6 | 35.0 | 4-Methyl-1,2-benzenediol | 91 |
| 4MCPDM[b] | 13.22 | 5.3 | 3-Methyl-phenol | 95 |
| | 15.72 | 7.4 | 4-Methyl-1,2-benzenediol | 91 |
| | 17.68 | 36.7 | 1,2-Benzenediol, 4-methyl- | 91 |
| *p*-Cresol | 13.24 | 100 | Phenol, 4-methyl- | 95 |
| *p*-CPH[c] | 11.89 | 2.3 | 4,4-Dimethyl-2-cyclopenten-1-one | 90 |
| | 13.28 | 6.9 | Phenol, 4-methyl- | 91 |
| | 17.54 | 12.3 | 1,2-Benzenediol, 3-methyl- | 87 |
| | 17.78 | 2.6 | Indole | 91 |

[a] CLPA, CLPH and CLPDM refer to the enzyme oxidation catalyzed products of catechol in aqueous, heptane and dichloromethane media, respectively.
[b] 4MCPA and 4MCPDM refer to the enzyme oxidation catalyzed products of 4-methyl catechol in aqueous and dichloromethane media, respectively.
[c] *p*-CPH refers to the enzyme oxidation catalyzed products of *p*-cresol in heptane.

heptane gave similar pyrolysis products. These overall findings indicate that there were some similarities in the pyrolysis of the oxidation products obtained from the tyrosinase biocatalysis of catechol, 4-methyl catechol and *p*-cresol in the selected organic solvents.

Kermasha and Tse [7] indicated that the conformation of the native tyrosinase in aqueous and chloroform media was different, one was predominately of the α-helix conformation while the other was mainly composed of the β-plate structure thereby suggesting the formation of different oxidation products depending on the media used. Ikeda et al. [19] also indicated that enzyme biocatalysis in a mixture of water–miscible organic solvent and buffer showed that the polymerization behavior was dependent on the solvent composition of the reaction medium. In addition, Kobayashi et al. [20] reported that the soybean peroxidase-catalyzed polymerization of phenol in aqueous methanol reaction medium showed that the presence of different proportions of the solvents strongly affected the yield, solubility, and molecular weight of the polymers.

## 4. Conclusion

The results gathered in this study show that mushroom tyrosinase showed a lower affinity towards catechin as substrate, but had a higher $V_{max}$ value and catalytic efficiency compared to that obtained with other substrates in both dichloromethane and heptane. Spectrophotometric scanning showed that the tyrosinase oxidation of phenolic substrate models in selected reaction media resulted in oxidation products with different maxima absorbancies. In addition, the results of differential scanning calorimetry

suggested that the oxidation products formed in the aqueous medium exhibited a higher thermal stability than those obtained in organic solvents. Moreover, the analysis of the pyrolysis residuals of the enzyme oxidation catalyzed products suggested that the structures of the polymer changed with the reaction media used for the biocatalysis.

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