BIOCATALYSIS OF TYROSINASE IN ORGANIC SOLVENT MEDIA USING PHENOLIC SUBstrate MODELS

by

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SHORT TITLE

TYROSINASE BIOCATALYSIS IN ORGANIC SOLVENT MEDIA
This thesis is dedicated to my parents, my sister Haiyu
and my husband Geng
ABSTRACT

M.Sc. Haihong Bao

The biocatalysis of tyrosinase was investigated in selected organic solvent media, using catechin as substrate. The results showed that the optimal enzymatic activity was obtained at pH 6.2, 6.6, 6.0 and 6.2 in heptane, toluene, dichloromethane and dichloroethane media, respectively. The kinetic studies indicated that the $K_m$ values were 5.38, 1.03, 2.52 and 4.03 mM, for the enzymatic reaction in heptane, toluene, dichloromethane and dichloroethane media, respectively, whereas the $V_{max}$ values were $12.2 \times 10^{-4}$, $3.3 \times 10^{-4}$, $14.7 \times 10^{-4}$ and $12.0 \times 10^{-4} \, \text{A} \, \mu \text{g} \, \text{protein}^{-1} \, \text{sec}^{-1}$, respectively. The results showed that the change in acetone concentration, used as co-solvent for the tyrosinase biocatalysis, from 5 to 30% (v/v) in the heptane medium resulted in a decrease of 4.3 to 96.7% in enzymatic activity. However, the presence of 12.5, 22.0 and 22.0% of acetone in the media of dichloromethane, dichloroethane and toluene resulted in a maximal increase in enzymatic activity of 42.6, 71.8 and 92.1%, respectively. Moreover, the biocatalysis of tyrosinase in dichloromethane and heptane reaction media, using model phenolic substrates was also investigated. The $K_m$ values for the tyrosinase biocatalysis in dichloromethane medium, using 4-methyl catechol, catechol and catechin as substrates, were 2.21, 2.36 and 2.52 mM, respectively, whereas the $V_{max}$ values were $5.1 \times 10^{-4}$, $6.0 \times 10^{-4}$ and $14.7 \times 10^{-4} \, \text{A} \, \mu \text{g} \, \text{protein}^{-1} \, \text{sec}^{-1}$, respectively. In addition, the $K_m$ values for tyrosinase biocatalysis in the heptane medium, using p-cresol, catechol and catechin as substrates, were 1.07, 4.32 and 5.38 mM, respectively, whereas the $V_{max}$ values were $0.8 \times 10^{-4}$, $1.0 \times 10^{-4}$ and $12.2 \times 10^{-3} \, \text{A} \, \mu \text{g} \, \text{protein}^{-1} \, \text{sec}^{-1}$, respectively. The characterization of the end products resulting from the tyrosinase biocatalysis, using selected substrates, was carried out by spectrophotometric scanning, differential scanning calorimetry and pyrolysis/gas chromatography coupled to mass spectrometry. The results showed that the change in reaction media resulted in the formation of end products that differed with respect to their maxima absorbance, thermal parameters and wide range of pyrolysis residuals.
RÉSUMÉ

M.Sc. Haihong Bao

La biocatalyse de la tyrosinase a été étudiée dans des milieux organiques sélectionnés, en utilisant la catéchine comme substrat. Les résultats ont montré que l'activité enzymatique optimale a été obtenue aux pHs 6.2, 6.6, 6.0 et 6.2 dans l'heptane, le toluène, le dichlorométhane et le dichloroéthane respectivement. De plus, les études cinétiques ont montré que les valeurs de $K_m$ sont de 5.38, 1.03, 2.52 et 4.03 mM, pour les réactions enzymatiques dans l'heptane, le toluène, le dichlorométhane et le dichloroéthane respectivement, alors que les valeurs de $V_{\text{max}}$ sont de $12.2 \times 10^4$, $3.3 \times 10^4$, $14.7 \times 10^4$ and $12.0 \times 10^4$ δA μg protein$^{-1}$ sec$^{-1}$, respectivement. Les résultats ont montré qu'une variation dans la concentration d'acétone, utilisé comme co-solvent lors de la biocatalyse de la tyrosinase, de 5 à 30% (v/v) dans l'heptane a abouti à une baisse de l'activité enzymatique de 4.3 à 96.7 %. Cependant, la présence de 12.5, 22.0 et 22.0% d'acétone dans les milieux de dichlorométhane, dichloroéthane et toluène, a abouti à une hausse maximale de l'activité enzymatique de 42.6, 71.8 et 92.1%, respectivement. De plus, la biocatalyse de la tyrosinase dans du dichlorométhane et de l'heptane a été étudiée, en utilisant des substrats modèles. Les valeurs de $K_m$ de la tyrosinase dans le dichlorométhane avec le 4-methyl catéchol, le catéchol et la catéchine comme substrats, ont été déterminées être de 2.21, 2.36 et 2.52 mM, respectivement, alors que les valeurs de $V_{\text{max}}$ ont été $5.1 \times 10^4$, $6.0 \times 10^4$ et $14.7 \times 10^4$ δA μg protein$^{-1}$ sec$^{-1}$, respectivement. De plus les valeurs de $K_m$ de la tyrosinase dans l'heptane avec le p-crésol, le catéchol et la catéchine comme substrats ont été de de 1.07, 4.32 et 5.38 mM, respectivement, alors que les valeurs de $V_{\text{max}}$ ont été de $0.8 \times 10^4$, $1.0 \times 10^4$ et $12.2 \times 10^3$ δA μg protein$^{-1}$ sec$^{-1}$, respectivement. La caractérisation des produits de réaction provenant de la biocatalyse de la tyrosinase avec des substrats sélectionnés a été étudiée par balayage spectrophotométrique, par calorimétrie à balayage différentiel et par pyrolyse/chromatographie en phase gazeuse couplée à la spectrométrie de masse. Les résultats ont montré que le changement de milieu de réactionnel a abouti à la formation de sous-produits différant par leur maximum d'absorbance, leurs paramètres thermiques et une large variété de résidus de pyrolyse.
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1. INTRODUCTION

Polyphenol oxidase (PPO) (EC 1.14.18.1) is a term used to describe a large number of related enzymes, including catecholase, diphenol oxidase, phenolase and tyrosinase (Lee and Whitaker, 1995). PPO is widely distributed in nature and generally occurs in plants and microorganisms. PPO catalyzes the hydroxylation of monophenols to produce diphenols and their subsequent oxidation to o-quinones (Oszmisnski and Lee, 1990). The o-quinones are unstable and highly reactive in aqueous media and can react with one another as well as with o-diphenols in the presence of O₂. In addition, o-quinones can also combine with amine, sulphydryl, phenol, indole and imidazole groups of amino acids, peptides and proteins to produce compounds of more intense color than that obtained from the polymerization of o-quinones alone (Cheynier et al., 1986; Rouet-Mayer, 1990).

The presence of PPO is responsible for the occurrence of the enzymatic browning reaction in injured or cut fruits and vegetables upon contact with oxygen. The rate of the enzymatic browning reaction depends on the activity of PPO present in food, the nature and content of phenolic compounds, the pH and temperature, as well as the presence of oxygen reducing substances and metal ions (Shahidi and Naczk, 1995). It has also been reported (Vámos-Vigyázó, 1981) that PPO preparations from different sources may exhibit the two consecutive oxidation reactions of hydroxylation and dehydrogenation at different ratios. Most of the PPO preparations, such as those from potatoes, apples, sugar beet leaves and mushrooms, possess both hydroxylation and dehydrogenation activities,
while those from tea leaf, banana, pear and sweet cherry have been reported to demonstrate only one.

For many years, the biocatalysis of PPO has been investigated using aqueous media (Richard-Forget et al., 1992; Kermasha et al., 1993a,b; Robert et al., 1995; Gauilard et al., 1997). However, PPO is limited in its use as a practical catalyst in aqueous media due to the instability of o-quinones in aqueous medium, which in turn inactivate the enzyme (Kazandjian and Klibanov, 1985). Recently, the use of organic solvents have been regarded as potential reaction media for PPO biocatalysis, particularly, for the production of natural stable pigments of selected color intensity due to limited o-quinone polymerization (Estrada et al., 1991).

The selection of the appropriate organic solvent as a reaction medium for enzyme biocatalysis depends on several factors such as solvent hydrophobicity, density, viscosity, surface tension, toxicity, flammability, waste disposal and cost (Dordick, 1989). Solvent hydrophobicity is represented as the logarithm of the partition coefficient (log P), where P is defined as the partitioning of a given solvent between water and 1-octanol in a two-phase system (Rekker and de Kort, 1979). Solvents with a log P value lower than 2 were reported not to be suitable for PPO biocatalysis, as they strongly distorted the essential water layer required to maintain the enzyme in its native catalytically active conformation. Solvents with a log P value between 2 and 2.5 showed higher enzymatic conversions since they did not interfere with the essential water coat surrounding the enzyme molecule in its active state (Zake et al., 1985; Lanne et al., 1987; Estrada et al., 1991).
Research work, carried out previously in our laboratory, was aimed at the optimization of the biocatalysis of tyrosinase in chloroform medium using catechin and vanillin as substrates (Tse et al., 1997). The results indicated that tyrosinase was more active in the chloroform medium than in the aqueous one.

In this research work, the biocatalysis of commercially purified mushroom tyrosinase in the selected organic solvent media, including heptane, toluene, dichloromethane and dichloroethane, using phenolic model substrates was investigated. The specific objectives of this research were:

1. To optimize the biocatalysis of tyrosinase, in selected organic solvent media, including heptane, toluene, dichloromethane and dichloroethane, using catechin as substrate.

2. To optimize the biocatalysis of tyrosinase in heptane and dichloromethane media, using selected phenolic substrate models, including catechin, catechol, 4-methyl catechol and p-cresol.

3. To purify the tyrosinase-catalyzed end products obtained, using catechin, catechol, 4-methyl catechol and p-cresol as substrates.

4. To characterize the enzymatically-catalyzed end products in terms of spectrophotometric scanning, differential scanning calorimetry and pyrolysis/gas chromatography coupled to mass spectrometry (Py/GC/MS).
Figure 1. The chemical structures of the phenolic substrate models
2. LITERATURE REVIEW

2.1. Polyphenol Oxidase (PPO)

Polyphenol oxidase (PPO) (EC 1.14.18.1) is a generic term for the group of enzymes that catalyzes the oxidation of phenol compounds to produce brown color on the cut surface of fruits and vegetables (Lee and Whitaker, 1995). PPO, also known as tyrosinase, phenolase, monophenol oxidase, cresolase and catecholase, is found in many plant tissues, some fungi and some higher animals, including insects and humans (Whitaker, 1995).

Tyrosinase is an enzyme, which can be purified from a commercial lyophilized powder prepared from edible mushrooms (Duckworth and Coleman, 1970). It is widely distributed in microorganisms, including bacteria and fungi, plants and animals (Janovitz-Klapp et al., 1990), where it is involved in the biosynthesis of melanin and other polyphenolic compounds (Lee and Whitaker, 1995).

2.1.1. Molecular Properties

The overall diversity of molecular weights of PPO is not known. However, Sherman et al. (1991) reported that the molecular weights of PPOs from four species of higher plants ranged from 33 to 200 kD or higher. The generally accepted molecular weight of mushroom PPO is 128 kD (Wong, 1995).

2.1.2. Substrate Binding Sites of PPO

There are two distinct substrate-binding sites on the enzyme, one with high affinity for aromatic compounds, including phenolic substrates, and the other for metal-
binding agents and oxygen; the latter site presumably interacts with the copper of the enzyme. Monophenols and o-diphenols have been considered as the exclusive substrates of PPO for a long time. The most important natural substrates of PPO in fruits and vegetables are catechin, cinnamic acid esters, 3,4-dihydroxy phenylalanine (DOPA) and tyrosine (Vámos-Vigyázo, 1981). In a recent study, the activity of PPO was studied with two new classes of substrates: aromatic amines and o-aminophenols, which are structural analogues of mono- and o-diphenols, respectively (Lee and Whitaker, 1995).

2.1.3. Determination of PPO Activity

PPO activity can be determined by measuring the rate of substrate consumption, or the rate of product formation. When determining the rate of substrate consumption, generally O$_2$ absorption is measured, either manometrically in a Warburg respirometer, or polarographically with an oxygen electrode (Vámos-Vigyázo, 1981). The rate of product formation can be determined spectrophotometrically by measuring the optical density of the colored compounds produced from the o-quinones (Espin et al., 1997).

2.2. PPO and Enzymatic Browning

2.2.1. Mechanism

PPO first catalyzes, in the presence of oxygen, the hydroxylation of monophenols at ortho position to o-diphenols and then the dehydrogenation of the o-diphenols in the presence of oxygen to o-quinones (Fig. 2). Non-enzymatic polymerization of the o-quinones by oxidative coupling then occurs by a series of oligomerisation and polymerization reactions with the subsequent formation of melanin or melanin-like compounds (Mayer, 1987; Lee and Whitaker, 1995; Boshoff et al., 1998).
First Reaction:

\[
\text{R} \quad \text{OH} + \quad \text{O}_2 \xrightarrow{\text{PPO}} \quad \text{R} \quad \text{OH} + \quad \text{H}_2\text{O}
\]

Second Reaction:

\[
\text{R} \quad \text{OH} + \quad \text{O}_2 \xrightarrow{\text{PPO}} \quad \text{R} \quad \text{OH} \quad + \quad \text{H}_2\text{O}
\]

**Figure 2. Mechanism of PPO Biocatalysis**

### 2.2.2. Inhibition of Enzymatic Browning

In some cases, enzymatic browning leads to major economic losses in some fresh fruits and vegetables, such as Irish potatoes, lettuce and other leafy vegetables, apples, bananas, grapes, peaches, strawberries, and tropical fresh fruits (Whitaker and Lee, 1995). Enzymatic browning also leads to off-flavors and losses in nutritional quality (Bajaj *et al.*, 1997). Enzymes may also be responsible for the dark discoloration or melanosis of shrimp and other crustacean species, thus reducing the market value of these products (Shahidi and Naczk, 1995).

The enzymatic browning reaction can be controlled by inactivation or inhibition of PPO activity, exclusion of oxygen, modification or lowering the phenolics content,
addition of reducing substances, interaction with the copper prosthetic group, as well as the reduction or trapping of quinones and even the removal of products of the browning reaction (Kermasha et al., 1993a; Kermasha et al., 1993b; Shahidi and Naczk, 1995). Cinnamic acid and benzoic acid were found to be effective in controlling enzymatic browning in apple juice, especially when used in combination with ascorbic acid. Carbon monoxide has also been proposed as an enzyme inhibitor for mushrooms (Sapers, 1993; Nursten et al., 1997).

2.2.3. Application of Enzymatic Browning

Enzymatic browning is desirable in products such as black tea, coffee, cocoa, Sultana raisins and prunes and black figs (Wong, 1995). Studies of o-diphenol oxidase (o-DPO) in tea showed that it plays an important role in the “fermentation” stage of tea manufacture. Where the major natural substrates, catechin, epicatechin and epicatechin gallate and their o-quinone oxidation products, were precursors of the more complex theaflavins and thearubigins. The beverage quality of coffee has also been shown to be related to the level of o-DPO in green coffee beans. In addition, o-DPO’s play a part in the development of the final color of processed cacao beans which contain large amounts of phenolic constituents such as epicatechin (Lee and Whitaker, 1995).

The melanin produced by PPO activity is also an excellent sun blocker when applied to skin, and some of the soluble pigments have found potential as food colorants (McCord and Kilara, 1983).
2.3. Biocatalysis in Organic Solvent Media

Water is a poor solvent for nearly all applications in industrial chemistry. Most of the organic compounds of commercial interest are very sparingly soluble and often unstable in aqueous solutions (Zakes and Russell, 1988). Chemists realized these limitations of aqueous-based biocatalysis and have hence replaced water with more suitable organic solvents a long time ago.

2.3.1. Physicochemical Characteristics of Enzymes in Organic Solvents

Enzymes exhibit their catalytic properties to the full extent only when they have a strictly defined (native) conformation, which involves complicated networks of both hydrogen bonds and electrostatic hydrophobic interactions. In order to combine these interactions to ensure the exact native conformation, the enzyme molecule should have a definite hydration shell (Khmelnitsky et al., 1988). Hydrophobic interactions play the dominant role in maintaining the catalytically active conformation of enzymes (Zaks and Klibanov, 1985), so the organic solvents used should possess the ability to maintain hydrophobic interactions to a sufficient degree.

In general, enzymes fall into two categories with respect to diffusion susceptibilities. For poorly active enzymes, high diffusion resistance does not affect enzyme kinetics, but for highly active enzymes, diffusion resistance is often rate limiting and in many cases severe enough to make the kinetic analysis of the reaction in organic media nearly impossible (Dordick, 1989). In fact, methods used to eliminate internal and external diffusion limitations in conventional heterogeneous chemical systems have been effectively applied to enzymatic catalysis in organic media. One such method is to spread
the enzyme onto materials with large surface areas such as glass beads (Kazandjian et al., 1985). Internal diffusions can also be optimized by modifying the solubility of the enzymes in organic solvents. External diffusions can be optimized by simply increasing agitation. Unlike aqueous or liquid-liquid biphasical systems, high agitation in monophasic organic solvent environments will not lead to shearing of the enzyme molecules, perhaps because the enzymes are insoluble and incapable of dissolving and becoming denatured at the analytical interface (Dordick, 1989).

2.3.2. Role of Water in Organic Solvent Media

Water plays a central role in enzymatic catalysis and is a ubiquitous component in all the biological processes such as biosynthesis, photosynthesis, metabolism and catabolism (Zaks and Russell, 1988). It has been correctly recognized for many years that water is absolutely essential for enzymatic catalysis. Not only does water participate in all non-covalent interactions, including electrostatic, hydrogen bonding, van der Waals and hydrophobic, which help maintain the catalytically active enzyme conformation (Dridick, 1989), but also it plays a crucial role in enzyme dynamics (Zaks and Russell, 1988). Generally, water is considered to maintain conformational flexibility in the protein molecules (Poole and Finney, 1983). Mechanistically, water’s role as a lubricant stems from its ability to form hydrogen bonds with the functional groups of the protein molecules, which were bound to each other, thereby “unlocking” the structure (Zaks and Klibanov, 1988a).

The interaction between organic solvents and the enzyme-bound water protects the activity of an enzyme so that physical disruption of the enzyme-bound water can
result in enzyme deactivation. This phenomenon is known as "water stripping" and appears to depict adequately the catalytic sensitivity of nearly all enzymes to organic media (Gorman and Dordick, 1992). Fortunately, it is difficult to completely dehydrate enzymes. The residual water remains tightly bound to the enzyme molecule and severely restricts enzyme mobility thereby resulting in increased hydrophobicity and a reducted ability to participate in hydrogen bonding (Wiggins et al., 1986).

Studies show that enzymes suspended in hydrophobic solvents require substantially less water for maximal activity than those suspended in hydrophilic ones. This phenomenon might be due to a difference in the distribution of water between the enzyme and the medium for the various solvents (Zaks and Klibanov, 1988a).

The optimal amount of water to be added depends on many factors. A small amount of water maintains enzyme hydration and avoids the stripping of water from the enzyme. However, an observed decrease in enzyme activity at water contents higher than 0.2 % at moderate temperatures indicate some instability of the enzyme at higher water contents (Esrada et al., 1993). Recent findings also indicate that the essential water can be substituted, to some degree, by other compounds, which compete for the interaction of water with the protein (Zaks and Klibanov, 1988a; Zaks and Klibanov, 1988b; Zaks and Russell, 1988).

2.3.3. Choice of Organic Solvent Media

There are several factors that affect the selection of the appropriate organic solvent as the reaction medium for an enzyme, including the compatibility of the solvent with the reaction of interest, as well as the reaction products. Polar products tend to
remain in the vicinity of the enzyme and can cause product inhibition or unwanted side reactions (Kazandjian et al., 1985). In addition, the solvent selected must be inert to the reaction. Additional factors, which may influence the choice of solvent include solvent density and viscosity, surface tension, toxicity, flammability, waste disposal and cost (Dordick, 1989).

2.3.3.1. Solvent Hydrophobicity

Hydrophobicity of the solvent is represented as the logarithm of the partition coefficient, \((\log P)\) where \(P\) is defined as the partitioning of a given solvent between water and 1-octanol in a two phase system (Rekker et al., 1979). Studies show that biocatalysis in organic solvent media is low in polar solvents having a \(\log P < 2\), since they strongly distort the essential water surrounding the enzyme, thereby inactivating or denaturing the biocatalyst. Biocatalysis is moderate in solvents having a \(\log P\) between 2 and 4, since the enzyme bound water is distorted so that the biological activity is affected to an extent that is rather unpredictable. Biocatalysis is high in apolar solvents having a \(\log P > 4\) since they do not distort the essential water coat, thereby leaving the biocatalyst in an active state. A correlation between polarity and activity can be seen, which parallels the ability of the organic solvents to distort the essential water layer that stabilizes the biocatalysts (Kazandjian et al., 1985; Lanne and Boeren, 1987; Dordick, 1989).

2.3.3.2. Solvent Dielectric Constant

The solvent dielectric constant is a good indicator of the power of solvents to strip away water bound to an enzyme. In general, solvents with high dielectric constants cause water desorption into the solvent; this phenomenon can be explained by the fact that
water is bound to the polar and charged residues of proteins through mainly electrostatic forces, so that solvent with a high dielectric constant will weakens these electrostatic forces and desorb water off the enzyme and into the solvent (Gorman and Dordick, 1992).

2.3.3.3. *Molar Solubility of Water Parameter*

The molar solubility of the water parameter, is also an accurate indicator of the water-stripping phenomenon. It should be noted that the molar solubility of the water parameter is valid only for water-immiscible solvents. In more polar solvents such as ethyl acetate and hexanol, the ability of the solvent to solubilize water is increased. Nonpolar solvents such as toluene and hexane are incapable of containing large amounts of soluble water and are, therefore, unable to strip away substantial amounts of water from enzymes. The molar solubility of the water parameter also accurately describes the variation in the fraction of water desorbed into alcoholic solvents such as hexanol and octanol (Gorman and Dordick, 1992).

2.3.3.4. *Polarity Index*

Polarity index is the guiding solvent parameter for enzyme stability in aqueous-organic co-solvent mixtures. Gupta *et al.* (1997) indicated that solvents with polarity indexes of 5.8 and above are "good" solvents for PPO biocatalysis; these solvents when used as co-solvents in aqueous-organic solvent media do not irreversibly denature the enzymes. The same authors also demonstrated that enzymes exposed to these solvents retain most of their activity even after 48hr of exposure, whereas solvents with polarity indexes of 5.1 denature the enzyme completely within 0-4 hr in most of the cases studied.
It appear that at higher concentrations of 50% and above, co-solvents effectively compete with the water layer around the enzyme.

2.3.4. Classification of Organic Solvent Systems

2.3.4.1. Liquid-Liquid Systems

A liquid-liquid system is a biphasic environment consisting of water and organic solvent (Carrea, 1984), in which the enzymes and hydrophilic cofactors are dissolved in the aqueous phase and the hydrophobic substrates are dissolved in the organic phase (Carrea, 1987). On stirring or shaking, substrates are transferred from the organic to the aqueous phase where they undergo an enzyme-catalyzed transformation into products and return to the organic phase (Carrea, 1984).

The biphasic systems can be divided into three groups. The first group includes very hydrophobic, water-immiscible solvents that evidently do not strip essential water from the enzyme even if exogenous water is not added. The second group is composed of organic solvents that are much less hydrophobic, although still not completely miscible with water; when no extra water is added, they apparently remove the essential water from the enzyme thereby inactivating it, unless they are presaturated with water. The third group involves hydrophilic, infinitely water-miscible solvents. Presumably the water molecules critical for the enzymatic activity are stripped away by these solvents regardless of whether any exogenous water is added (Kazandjian et al., 1986).

When using such a system, high yields require optimization of the system with the following parameters: nature of the solvent, organic phase/aqueous phase volume ratio, temperature, pH of the aqueous phase, rate of agitation, etc. (Khemelnitsky et al., 1988).
2.3.4.2. Liquid-Solid Systems

In liquid-solid systems, biocatalysts are present in an immobilized state. Immobilization is performed in an aqueous solution, either by adsorption onto a porous solid support, by covalent attachment to the surface of the solid support, by entrapment in polymer gels, or by covalently-binding to cross-linked protein membranes. An aqueous suspension of the solid support particles, containing the immobilized biocatalyst, is then introduced into the water-immiscible organic solvent to obtain a biphase liquid-solid reaction system (Klibanov et al., 1977).

The immobilized biocatalysts in these systems are therefore protected against unfavorable direct contact with the water/organic solvent interface. This system also provides a comparatively large water/organic solvent interface, which lowers the diffusion restraints during the transfer of the substrates and/or reaction products thereby increasing the rate of the overall process compared to unstirred liquid-liquid systems (Lilly et al., 1982).

2.3.4.3. Solid Enzymes Suspended in Organic Solvents

Some enzymes can function in the form of solid suspensions in organic solvents with low water content, displaying catalytic activity comparable with that observed in aqueous solution (Zake and Klibanov, 1984). The ability of enzymes to retain catalytic activity in a nonpolar medium can probably be explained by the formation of a layer of denatured protein on the surface of a solid enzyme particle, which protects deeper layers from unfavorable contact with the organic solvent. Suspensions of solid enzymes in organic solvents may be regarded as biphasic systems in which one phase is formed by an
organic solvent and the other is represented by a solid hydrated enzyme protected from contact with organic solvent by the layer of denatured protein. It is important to emphasize that this system can be successfully used only if the solvent is sufficiently hydrophobic (Khemelnitsky et al., 1988). Solid enzyme suspensions are advantageous when the biocatalytic process in organic media is carried out at high temperatures (Zake and Klibanov, 1984).

2.3.4.4. Micellar Systems

There are two main types of micelles, one consists of normal micelles designated as oil-in-water (O/W) suspensions existing in water at low concentrations of organic solvents, and the second is reversed micelles designated as water-in-oil (W/O) formed in organic solvents at a moderate water contents (Stenius, 1984). Micelle enzymology studies the biocatalysis by which enzymes are entrapped in hydrated reversed micelles of surfactants in organic solvents. Reversed micelles are formed spontaneously when surfactants are dissolved in nonpolar organic solvents (Khemelnitsky et al., 1988). The hydrophilic head groups associate to structures with polar cores, and the hydrophobic tails extend outward into the bulk organic solvent (Shield et al., 1986). Reversed micelles can entrap protein molecules (enzymes) into their inner cavities, as well as solubilize considerable amounts of water (up to 70 water molecules per surfactant molecule) (Khemelnitsky et al., 1988). These systems show promise for combining the advantages of organic- and aqueous-phase enzyme systems (Shield et al., 1986).

The most prevalent method of introducing enzymes into reversed micelles involves injecting an aqueous enzyme solution into the surfactant-containing organic
solvent, followed by agitation until an optically clear solution is formed. Phase transfer of the enzyme from a bulk aqueous solution into an organic/surfactant solution is the secondary means of forming enzyme-laden reversed micelle solutions. Enzymes can also be introduced into reversed micelles by agitating a reversed micelle solution over solid layer of enzymes (Shield et al., 1986).

2.3.5. Properties of Enzymes in Organic Media

2.3.5.1. pH Dependence

Since all enzymatic reactions in aqueous solutions are strongly pH-dependent, one of the most intriguing aspects of enzymatic catalysis in organic solvents is that enzymes exhibit 'pH memory'. Studies show that an enzyme 'remembers' the pH of the last aqueous solution in which it has been exposed to; this is due to the fact that at molecular level, the enzyme's ionogenic groups acquire the ionization states corresponding to that particular pH, which then remain in both the solid state and in organic solvents (Zaks and Russell, 1988). Zaks and Klibanov (1985) indicated that the catalytic activity of lipase in the organic medium depended to a great extend on the pH of the aqueous solution from which the enzyme was precipitated. Xu et al. (1996) also proposed that during lyophilization, the enzyme should maintain the protonation state of its ionogenic groups acquired at a certain pH, those for enzymatic activity. However, in the lyophilized enzyme extract, the \( pK_a \) values of the ionogenic groups should differ from those in water as the surrounding medium, consisting of vacuum or air, is distinct. Nevertheless, the protonation states remain unchanged because protons cannot be transferred by vacuum or (dry) air; in addition when the lyophilized enzyme is suspended in an aprotic solvent, such as acetonitrile, the protonation status of the ionogenic groups still does not change.
for the same reason; these ionogenic groups remain kinetically trapped in the "aqueous" protonation states giving rise to pH memory of enzymes in organic solvents. It therefore became a routine to prepare the enzyme suspension in buffer at the optimal pH for activity, followed by freeze-drying or solvent precipitation to concentrate the enzymatic extract before placing it in an organic solvent. Another alternative is to saturate a water-immiscible organic solvent with an aqueous buffer at the appropriate pH for enzyme activity, instead of water, prior to the addition of the enzyme powder (Klibanov, 1986).

2.3.5.2. Substrate Specificity

When water is replaced by organic solvent in the enzymatic reaction medium, the substrate specificity of enzyme shifts (Klibanov et al., 1977). Zaks and Klibanov (1986) have reported that suspending chymotrypsin and subtilisin in organic solvents significantly modified the substrate specificity.

Substrate specificity of enzymes stems from their ability to utilize the free energy of bonding with the substrate to facilitate the reaction; the net binding energy is the difference between the binding energies of the substrate with the enzyme and with water. Substrate specificity is therefore the difference obtained when water is replaced by organic media (Zaks and Klibanov, 1986). Another reason for the general shift in substrate specificity when enzymes are suspended in environments of lower water content is elimination of bulk water, which results in the rigidification of the enzyme structure and the inability to accommodate large substrates.

The use of organic solvent media instead of aqueous also affects the apparent $K_m$ values. In aqueous solutions, compounds partition into the hydrophobic active site,
whereas, in nonpolar organic media this partitioning is reduced and the apparent $K_m$ increases (Dordick, 1989). The hydrophobicity of the substrate is considered as an important part that affects the functioning of enzymes in organic solvents (Burton et al., 1993). In organic solvent media, the catalytic efficiency usually decreases as substrate hydrophobicity increases. This substrate effect, however, becomes more pronounced as solvent hydrophobicity is increased. (Ryu and Dordick, 1989).

2.3.5.3. Thermostability

A major problem associated with the industrial use of enzymes is their thermostability. Research showed that enzymes are extremely thermostable in water-restricted environments such as organic solvents, and can be stored at room temperature for months without loss in activity (Zaks and Russell, 1988).

Enzymes inactivate at high temperatures in aqueous media due to both the partial unfolding of the enzyme molecule and the covalent alterations in the primary structure of the molecule. In organic solvent media, dry enzyme extracts remain rigid and cannot undergo partial unfolding, but are still catalytically active (Dordick, 1989). An increase in the water content of dry enzyme extracts is thought to result in the loosening of the structure of the protein molecules, although it is accepted that water is also involved in the maintenance of noncovalent interactions in the enzyme (Ayala et al., 1986; Zaks and Kibinov, 1986).

2.3.6. Activation Effect

Almarsson et al. (1996) indicated that denaturing organic solvents, such as formamide and dimethyl sulfoxide, dramatically raise enzymatic activity in anhydrous
organic media. This phenomenon is most likely explained by the denature-induced enhancement of conformational flexibility of enzymes in anhydrous organic media. The same author also reported that the rate of transesterification, catalyzed by protease subtilisin Carlsberg in anhydrous acetonitrile medium, could be increased by more than 50% by the addition of 10 to 50% of formamide as co-solvent.

It was reported that lyophilization of subtilisin suspended in aqueous solution containing competitive inhibitors resulted in an enzyme which was up to 100-times more active than the enzyme lyophilized in the absence of such ligands. This phenomenon of ligand-induced “enzyme memory” also extends to the stability, affinity, and substrate specificity of subtilisin in organic solvents. This activation effect may be due to the percentage of ligands, which cause an activating conformational change in the subtilisin molecule. Even after removal of the ligand, the new conformation is retained due to the rigidity of the enzyme in the absence of water. (Russell and Klibanov, 1988).

2.3.7. Inhibition Effect

An interesting phenomenon is that the inhibition effects on enzyme activity in aqueous and organic solvent media are quite different. The results of Zaks and Klibanov (1988b) show that the effect of inhibitors on chymotrypsin in octane medium was exactly opposite to that in the aqueous medium; the enzyme inhibitors in the aqueous medium became less active in the octane medium and vice versa. An increase in the hydrophobicity of the inhibitors should no longer result in a higher affinity for the enzyme active site as hydrophobic interactions, the driving force of the enzyme-inhibition in water, will not exit in octane. In addition, the introduction of a carboxyl group should
enhance the inhibition, since the inhibitor will tend to “hide” from octane by partitioning into the active center of the enzyme.

2.4. PPO Biocatalysis in Organic Media

2.4.1. PPO Biocatalysis in Organic Media

Numerous advantages exist for performing the tyrosinase biocatalysis in nonpolar organic solvent media as opposed to conventional aqueous buffer media, including increased solubility of hydrophobic substrates, thereby allowing the potential use of certain substituted phenols as substrates, as well as improved stability of tyrosinase in water-immiscible organic solvents facilitating specific oxidation reactions with water-insoluble organic substrates (Zaks and Russell, 1988). In addition, the use of organic solvent media provides higher oxygen solubility, which in turn reduces the availability of the oxygen as a limiting factor (Burton et al., 1993). Kazandjina et al. (1985) reported that the biocatalysis of PPO in chloroform was 10-times greater than that obtained in aqueous medium, which maybe due to the increased solubility of O₂ and most phenolic substrates. The use of organic solvent media also makes it possible to shift the thermodynamic equilibrium of many enzymatic reactions to the desired products, while the limited solubility of enzymes in organic solvents allows easier recovery and reuse (Klibanov, 1986).

2.4.2. Thermostability of PPO in Organic Media

The effect of temperature on the activity of the immobilized PPO in organic solvent media was studied with organic solvents having log P ranging from 1.9 to 2.5. The results show that the activation energy of the reaction in a given solvent is
independent of the water content whereas the inactivation energies decreased as the water content decreased. The results also indicate the need for a certain amount of water to maintain the PPO activity at temperatures below 60 °C. On the other hand, there was decrease in PPO activity at water contents higher than 0.2 % at moderate temperatures due to the instability of PPO at higher water content (Esrada et al., 1993).

2.4.3. Substrate Specificity of PPO

The investigation of Burton et al. (1993) on PPO biocatalysis in chloroform medium showed that the hydrophobicity of phenol substrates affected their partitioning between the chloroform and the enzyme active site. An increase in substrate hydrophobicity resulted in a decrease in the amount of substrate available to the active site and an increase in the apparent \( K_m \) value. They also reported that PPO is known to be capable of hydroxylating molecules of considerable size in aqueous medium but in the chloroform medium, the accessibility of bulky substrates to the active site of the enzyme may have been restricted by the rigidity of the protein pocket of the enzyme (Burton et al., 1993).

2.4.4. Kinetics of PPO in Organic Solvent Media

A kinetic study of the activity of PPO in an organic system using a wide range of phenolic substrates shows a correlation between the efficiency of substrate transformation by PPO and the steric bulk of the \( p \)-substituent. Three parameters have been used in the study; these include the \( E_s \) values, which are indicative of steric size, the \( K_m \) values, which reflect the ability of an enzyme to bind a substrate, and the \( V_{\text{max}}/K_m \) values, the catalytic efficiency, which indicates the ability of the enzyme to transform the substrate.
An inverse relationship between $V_{max}/K_m$ and $E$ can be observed for the various substrates. For the $p$-alkylphenols, it was observed that as the steric size of the alkyl group increased from methyl to tert-butyl, the value of $V_{max}/K_m$ also decreased. Similarly, for the remaining phenolic substrates, taken in the order of fluoro-, methoxy-, chloro-, and bromo-, there was a decrease in the $V_{max}/K_m$ values as the steric size increased (Burton et al., 1993). In addition, Tse et al. (1997) indicated that tyrosinase was more active in the chloroform medium, using catechin and vanillin as substrate, than in the aqueous medium.

2.5. Characterization of PPO Biocatalysis

2.5.1. Purification of End Products

Concin et al. (1980) indicated that the retardation of phenols on size-exclusion columns could be related to the existence of hydrogen bonds between the solute and either the ether or the hydroxyl groups of the Sephadex LH 20. In addition, the same authors reported a relationship between the $K_{av}$ values and the number of free phenolic hydroxyl groups on the substrate. The results of Richard et al. (1991) also show that the chromatographic behaviors of phenols on Trisacryle GF05 was not only due to the steric hindrance but also due to the hydrogen bond interaction between the phenolic substrates and the amine and hydroxymethyl groups of Trisacryle GF05.

2.5.2. Characterization of End Products

Polymer characterization is currently one of the most dynamic fields in applied research and may be performed using many techniques, including thermal analysis, chromatography, and spectroscopy. Thermal analysis refers to a variety of techniques in
which a property of the sample is continuously measured as the sample is monitored through a predetermined temperature profile (Cheremisinoff, 1996). Knowledge of thermodynamic data on materials and mixtures provides the key for characterization. There is therefore a great deal of research in this field (Blond et al., 1996). GC is just one of several polymer characterization tools. Mass spectrometry (MS) is performed either following GC separation of the pyrolysis fragments or after receiving the fragments directly from the pyrolyzer. A segment of GC applications for polymer analysis has been developed in the field of analytical pyrolysis (Liebman et al., 1985).

2.5.2.1. Thermal Analysis

Thermal analysis can be used to characterize the physical and chemical properties of a system under conditions that simulate real world applications. It is not simply a sample composition technique. The various thermoanalytical techniques, and most particularly calorimetry, have been considerably developed in food science over the course of the last 20 years. Among the most common used techniques are thermal gravimetric analysis (TA) and differential scanning calorimetry (DSC).

In DSC, the measured energy differential corresponds to the heat content (enthalpy) or the specific heat of the sample over a certain temperature range. DSC is often used in conjunction with TA to determine if a reaction is endothermic, such as melting, vaporization and sublimation, or exothermic, such as oxidative degradation. It is also used to determine the glass transition temperature of polymers (Gedde, 1995).

Two types of DSC are recognized. One is power-compensated DSC, where the sample and reference are heated in separate, individual heaters, and the temperature
difference is kept close to zero, while the difference in electrical power needed to maintain equal temperatures \((\Delta P = d (\Delta Q)/dt)\) is measured. Another type is heat flux DSC, where the sample and reference are heated from the same source and the temperature difference \(\Delta T\) is measured. This signal is then converted to a power difference \(\Delta P\) using the calorimetric sensitivity (Haines and Wilburn, 1995).

A simple example of the relationship between “structure” and “properties” is the effect of an increase molecular weight of a polymer on its physical state; a progression occurs starting from an oily liquid to a soft viscoelastic solid, to a hard, glassy elastic solid. At high thermal energies, conformational changes via bond rotations are frequent on the time scale of typical processing operations and the polymer behaves as a liquid. At lower temperatures, the chains solidifies by either one of two mechanisms: by ordered molecular packing in a crystal lattices, crystallization, or by a gradual freezing-out of a long range of molecular motions, vitrification. These transformations define the principle theological regimes of mechanical behavior: the melt, the rubbery state, and the semicrystalline and glassy amorphous solid states, are accompanied by transitions in the thermodynamic properties at the glass transition temperature, the crystalline melting, and the crystallization temperature (Cheremisinoff, 1996).

The accuracy in the determination of the transition temperatures by DSC is dependent on several factors: Standardized samples geometry and amasses. The sample should be flat and have good thermal contact with the sample pan. The sample pan material should be ‘inert’. Thermal lag between sample and thermometer may be
corrected for by using the slope of the leading edge of the highly pure melting indium. Parallel processes should be inhibited (Gedde, 1995).

2.5.2.2. Pyrolysis / GC / MS Analysis

Analytical pyrolysis has achieved wide recognition for polymer characterization, particularly in the area of synthetic polymer structure determination. Materials of biological importance, such as proteins, enzymes, carbohydrates and microorganisms, are often intractable, thermally labile, and nonvolatile. Analytical pyrolysis is well-suited for the study of these biopolymers because of its ability to transform, by thermal fragmentation in the absence of oxygen, these complex polymers into smaller volatile molecules that are more amenable to analysis.

The analysis time required for analytical pyrolysis is primarily limited by the time required to analyze the volatile constituents resulting from thermal degradation. In contrast to the tedious and time consuming conventional chemical methods, the direct coupling of analytical pyrolysis to gas chromatography allows for the rapid volatilization, separation, and detection of characteristic fragments from biologically interesting compounds (Bayer et al., 1985). Py/GC/MS analysis reduces reaction characterization to a microscale level (Keyhani and Yaylayan, 1996).

A few of the more important variables that affect the composition of the resulting pyrolysates mixture include the amount of sample pyrolyzed, the nature of the pyrolyzer element surface, the mode and temperature characteristics of the pyrolysis step, and the type of atmosphere employed during the pyrolysis. The sample size may affect the nature of the reactions taking place during pyrolysis; for example, as the sample size increases,
the probability of secondary recombination reactions among pyrolysates may increase. Minor differences in temperature rise-time, the final pyrolysis temperature, the duration of pyrolysis, and the type of pyrolysis unit can produce dramatic differences in the pyrolysate distribution. The pyrolysis atmosphere may play a passive role; for example, although He and N₂ do not react chemically with pyrolysates, differences between programs using He and N₂ can be noted because of the different cooling effects of these inert gases. The carrier gas may also play a more active role; for example, H₂ can be used to hydrogenate unsaturated species (Bayer et al., 1985).

Different techniques of sample introduction, such as application of the sample on a ribbon probe (platinum filament) as a solution or as a solid in a quartz tube inserted inside the coil probe, can provide valuable information regarding the differences between the initial and the advanced pyrolysis products. On the ribbon probe, the pyrolysis products are swept away as soon as they formed by the carrier gas into the GC column, without undergoing further secondary reactions. On the other hand, the pyrolysis products produced in the quartz tube migrate from the sample to the hotter regions of the pyrolyzer, and this may initiate additional fragmentation and produce secondary products by further reactions (Huyghues-Despointes et al., 1994).
3. MATERIALS AND METHODS

3.1. Tyrosinase Bio catalysis in Organic Solvent Media

3.1.1. Preparation of Enzyme Suspension

Commercially purified mushroom tyrosinase (EC 1.14.18.1), possessing an oxidative activity of 3,000 - 3,400 units/mg solid, was purchased from Sigma Chemicals Co. (St-Louis, MO) and used throughout this study. The enzyme suspension was prepared in citrate-phosphate buffer solution (0.1 M) at a pH ranging from 4.0 to 7.0.

3.1.2. 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. The homogenization of the stock solutions was performed by sonication using a Branson water-bath sonicator (Branson Corp., Banbury, CT).

3.1.3. Enzyme Assay

The tyrosinase assay was carried out using selected organic solvent media (Fisher Scientific Co., Pittsburgh, PA) of different polarity including heptane (log P 4.0), toluene (log P 2.5), dichloromethane (log P 2.0), and dichloroethane (log P 2.0). All organic solvents used in this study were dried overnight with anhydrous sodium sulfate prior to the assays. The organic solvent reaction mixture (200 μl) contains 5 μl of substrate stock solution (0.4 mM) and 1 to 2.5 μl tyrosinase suspension. The enzymatic reaction was initiated by the addition of the tyrosinase suspension, prepared in citrate-phosphate buffer
solution (0.1 M, pH 6.0), to the organic solvent reaction mixture. The reaction mixture was shaken continuously for 3 sec to 2 min and the enzymatic reaction was then halted with the addition of 200 µl acetone. A blank trial, containing all components except tyrosinase suspension, was performed in tandem with the enzymatic assay. The enzyme activity in the reaction media of heptane, toluene, dichloromethane and dichloroethane, using catechin as substrate, was determined spectrophotometrically by measuring the color intensity of the end products at 376, 379, 375 and 375 nm, respectively. The enzymatic activity in the dichloromethane reaction medium, using 4-methyl catechol and catechol as substrates, was determined by measuring the color intensity of the end products at 384 and 376 nm, respectively, whereas that in the heptane reaction medium, with p-cresol and catechol as substrates, was measured at 330 and 372 nm, respectively. One unit of enzyme activity was defined as the amount of enzyme, which produced an increase of 0.001 in absorbance per minute at a defined wavelength, temperature and pH. The specific activity of tyrosinase was expressed as the change of the absorbency unit at a defined wavelength, per µg enzymatic protein per sec.

3.1.4. Effect of Protein Concentration on Tyrosinase Activity

The effect of protein concentration on tyrosinase activity in different organic solvent media, using catechin as a substrate, was determined by the addition of a wide range of enzymatic protein, 10.4 to 36.7, 39.8 to 70.5, 18.2 to 64.4 and 22.5 to 61.3 µg per 200 µl assay for the enzymatic oxidation in the heptane, toluene, dichloromethane and dichloroethane reaction media, respectively. The effect of protein concentration on tyrosinase activity was also investigated in the dichloromethane medium by the addition of a wide range of enzymatic protein, 24.4 to 58.7 and 29.3 to 48.9 µg per 200 µl assay.
using catechol and 4-methyl catechol as substrates, respectively. In addition, the enzymatic activity was also studied in the heptane medium with 3.9 to 32.4 and 29.3 to 73.3 μg per 200 μl assay for the oxidation of catechol and p-cresol, respectively.

3.1.5. Effect of pH on Tyrosinase Activity

The effect of pH on tyrosinase activity in the heptane, toluene, dichloromethane and dichloroethane reaction media was determined by preparing the tyrosinase-buffer suspension in phosphate-citrate buffer solutions (0.1 M) at a pH ranging from 4.6 to 7.0.

3.1.6. Effect of Temperature on Tyrosinase Activity

The effect of temperature on tyrosinase activity was determined by incubating the enzymatic reaction at a wide temperature range of 20 to 30, 15 to 30, 15 to 40 and 10 to 40°C for the reaction media of heptane, toluene, dichloromethane and dichloroethane, respectively.

3.1.7. Effect of Substrate Concentration on Tyrosinase Activity

The effect of substrate concentration on tyrosinase activity in different organic solvent media, using catechin as a substrate, was investigated at wide range of concentrations from 2 to 20, 2 to 12, 0.5 to 20 and 2 to 14 mM for the reaction media of heptane, toluene, dichloromethane and dichloroethane, respectively. The effect of substrate concentration was also studied in the dichloromethane medium with 2.0 to 20.0 and 2.0 to 12.0 mM of catechol and 4-methyl catechol, respectively. The enzymatic activity was also studied in the heptane medium with 1.0 to 5.0 and 2.0 to 12.0 mM of catechol and p-cresol, respectively.
3.1.8. **Effect of Acetone on Tyrosinase Activity**

The effect of acetone on the tyrosinase biocatalysis reaction was studied by using different concentrations of acetone as co-solvent in the enzymatic reaction mixture. The concentration of acetone used in the reaction media vary from 0 to 90% (v/v) for heptane, 0 to 60% (v/v) for toluene, 0 to 30% (v/v) for dichloromethane and 0 to 35% (v/v) for dichloroethane. The concentration of acetone as a co-solvent in the reaction medium was expressed as its percentage ratio (in \( \mu l \)) to the total assay volume (200 \( \mu l \)) (Tse *et al.*, 1997).

3.2. **Characterization of Tyrosinase Biocatalysis**

3.2.1. **Size-Exclusion Chromatography**

The tyrosinase-catalyzed end products of catechin, catechol, 4-methyl catechol and \( p \)-cresol (Sigma Chemicals Co.) were prepared in the selected reaction media using the optimal reaction conditions previously described. The end products were subsequently concentrated either by vaporization of the organic solvent under a gentle stream of nitrogen or removal of the buffer by lyophilization. The end products were then re-solubilized in Millipore water and the resulting solution (no more than 3 ml per injection) was filtered using a 0.22 \( \mu m \) PVDF syringe filter before being applied to the Trisacryle GF 05 M column (160 ml bed volume, 80 cm long), purchased from Biosepra Inc. (Marlborough, MA USA). The elution flow rate was 0.4 ml/min and fractions of 4 ml were collected and measured by spectrophotometer at 280, 288 and 408 nm, respectively. Fractions, containing the end products, were subsequently lyophilized and stored in a small sealed bottle under nitrogen at \(-20^\circ C\) for further analysis.
3.2.2. Spectrophotometric Scanning

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

3.2.3. Thermal Analysis

Thermal analysis was performed using DSC, purchased from Mettler Toledo Inc. (Greifensee, Switzerland). Catechin, catechol, 4-methyl catechol, p-cresol and their corresponding purified end products (2 mg solid), formed by tyrosinase biocatalysis in the 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.

3.2.4. Pyrolysis Gas Chromatography Mass Spectrometry Analysis

The analysis was 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 length x 0.25 mm I.D. x 0.33 μm film thickness, Supelco, Inc. Oakville, Ontario, Canada) was used. Catechin, catechol, 4-methyl catechol, p-cresol and their respective purified end products (1.1 mg solid) were each 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/sec for a total heating time of 20 sec, 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/sec. For product identification, the mass spectral library Wiley6n.L (G1034C Version C. 03.00, 1989-1994, John Wiley & Son, New York, NY, USA) was used.
4. RESULTS AND DISCUSSION

4.1. Tyrosinase Biocatalysis in Selected Organic Solvent Media

4.1.1. Effect of Enzymatic Protein Concentration on Tyrosinase Activity

Figure 3 shows the effect of enzymatic protein concentration on tyrosinase activity in the selected organic solvent media, using catechin as substrate. The results indicate that the optimal amount of enzymatic protein for tyrosinase biocatalysis was 31.4, 60.0, 45.8 and 53.1 µg per 200 µl reaction media of heptane, toluene, dichloromethane and dichloroethane, respectively. Figure 3 also shows that the initial addition of increasing amounts of enzymatic protein produced a concomitant increase in the specific activity of tyrosinase in all selected organic media; however, the results also show that after maximal tyrosinase activity was obtained, further increases in enzymatic protein resulted in a decrease in the specific activity. This phenomenon may be due to the limited solubility of the enzymatic protein in the selected organic solvents thereby resulting in the aggregation of enzyme molecules, and a subsequent reduction of enzyme and substrate interaction (Kazandjian et al., 1986; Kamat et al., 1995).

4.1.2. Effect of pH on Tyrosinase Activity

The effect of pH on tyrosinase activity (Fig. 4) was studied over a wide pH range. Figure 4 shows that the optimal pH for mushroom tyrosinase biocatalysis using catechin as substrate was 6.2 in the reaction media of heptane and dichloroethane, and 6.6 and 6.0 in those of toluene and dichloromethane, respectively. Kermasha et al. (1993) also reported that the optimal pH for mushroom tyrosinase activity was 6.0 using chlorogenic acid as substrate in aqueous solution. Guyot et al. (1995) investigated the PPO
Figure 3. The effect of enzymatic protein on tyrosinase activity in the reaction media of heptane (●—●), toluene (▲—▲), dichloromethane (■—■) and dichloroethane (◆—◆), using catechin as substrate, where the specific activity was defined as the change in absorbance at 376, 379, 375 and 375 nm, respectively, per μg protein per sec.
Figure 4. The effect of pH on tyrosinase activity in the reaction media of heptane (●-●), toluene (▲-▲), dichloromethane (■-■) and dichloroethane (◆-◆), using catechin as substrate, where the specific activity was defined as the change in absorbance at 376, 379, 375 and 375 nm, respectively, per μg protein per sec.
biocatalysis of catechin in aqueous solutions ranging from pH 3.0 to 7.0 and showed that reaction media with low pH values gave almost colorless solutions, whereas those with higher pH values yielded yellow solutions due to the presence of a higher concentration of colored pigments, reaching a maximum at pH 6.0; these results suggest that the color of the enzymatically-oxidized catechin could be due to the coupling of o-quinones, whose nature and relative abundance is influenced by the pH of the incubation medium.

The overall results, obtained in this study, also show that there were small shifts in pH ranging from 6.0 to 6.6 for maximal tyrosinase activity in the different organic solvent media. Similar findings were also obtained by Khamessan et al. (1995), who reported that the optimum pH for the hydrolytic activity of chlorophyllase was slightly affected by the type of organic solvent used in the reaction media. The same authors suggested that the slight shifts in pH values for optimal chlorophyllase activity from 7.0 to 8.0 may be due to the effect of the organic solvents used on the hydrogen ion dissociation of the enzyme. Zake et al. (1985) reported that enzymes maintain a 'pH memory' in organic solvent media so that their catalytic activity in organic reaction media is characteristic to that exhibited at their optimal pH in aqueous media; these findings suggest that in the organic solvent medium, the enzyme’s ionogenic groups maintain the corresponding ionization states acquired at the pH of the aqueous solution in which it was suspended before being added to the organic medium.

4.1.3. Effect of Temperature on Tyrosinase Activity

Figure 5 indicates that the optimal temperature for mushroom tyrosinase oxidative activity using catechin as substrate was 25 °C in the reaction media of heptane, toluene
Figure 5. The effect of temperature on tyrosinase activity in the reaction media of heptane (●—●), toluene (▲—▲), dichloromethane (■—■) and dichloroethane (◆—◆), using catechin as substrate, where the specific activity was defined as the change in absorbance at 376, 379, 375 and 375 nm, respectively, per µg protein per sec.
and dichloromethane, and 27.5 °C in that of dichloroethane. Estrada et al. (1991) also reported that the optimal temperature for the enzymatic oxidation of phenol in chloroform medium was 30 °C. The influence of temperature on the maximal reaction velocity for enzymatic activity in organic solvents having log P values ranging from 1.9 to 2.5 was studied by Estrada et al. (1993), who suggested that the optimal temperature for biocatalysis may be influenced by the intrinsic characteristics of the organic solvents such as boiling point, partition coefficient, interfacial tension at the aqueous and organic solvent interface, as well as the solubility of substrates and/or products.

The Arrhenius plot (Fig. 6) shows that the activation energy $E_a$ was 88.36, 42.78, 12.24 and 34.31 KJ/mol for the tyrosinase biocatalysis in the reaction media of heptane, toluene, dichloromethane and dichloroethane, respectively, while the corresponding temperature coefficient ($Q_{10}$) was 1.04, 1.86, 1.18 and 1.17. These results suggest that tyrosinase biocatalysis in the heptane reaction medium was more temperature dependent as indicated by its relatively high $E_a$ value of 88.36 KJ/mol; however, the tyrosinase oxidative activity in the reaction media of toluene, dichloroethane and especially dichloromethane was less influenced by changes in temperature in the range of 10 to 25 °C. The trend of these results is in agreement with those reported by Estrada et al. (1993) who indicated that the activation energy for the PPO biocatalysis of the immobilized mushroom enzymatic extract was 33.53, 29.17 and 32.40 KJ/mol in the reaction media of toluene, dichloromethane and dichloroethane, respectively, using p-cresol as substrate. The results obtained by Tse et al. (1997) also demonstrated that the $E_a$ values obtained for the tyrosinase oxidative activity in chloroform medium using catechin and
Figure 6. The Arrhenius plots for tyrosinase biocatalysis in the reaction media of heptane (●), toluene (▲), dichloromethane (■), and dichloroethane (◆), using catechin as substrate,
vanillin as substrates were 18.01 and 22.12KJ / mol, respectively, while the Q_{10} values were 1.91 and 1.88, respectively.

4.1.4. Kinetic Parameters of Tyrosinase Biocatalysis

Table 1 and Figure 7 show that tyrosinase possessed a higher affinity for catechin in the toluene and dichloromethane reaction media than that obtained in the reaction media of heptane and dichloroethane, as indicated by the respective K_{m} values of 1.03 and 2.52 in comparison to those of 5.38 and 4.03 mM. The catalytic efficiency, expressed as the V_{max} / K_{m} ratio (Burton et al., 1993), was also used as an indication of the ability of tyrosinase to convert catechin into the corresponding end products in the selected organic solvent media. Table 1 shows that the catalytic efficiency of tyrosinase in the reaction medium of dichloromethane was approximately twice as high as that obtained in the reaction media of heptane, toluene and dichloroethane. The overall findings show that maximal tyrosinase activity was demonstrated in the dichloromethane medium, followed by those of toluene and dichloroethane; however, the lowest tyrosinase biocatalysis was observed in the heptane reaction medium. These results are in agreement with those reported by Estrada et al. (1991) who stated that reaction media consisting of organic solvents with log P values between 2 and 2.5 showed higher PPO biocatalysis due to a higher conversion of substrate whereas those composed of organic solvents with log P value higher than 2.5 such as octanol (2.9), hexane (3.5) and heptane (4.0) showed very low PPO activity; the same authors suggested that this low enzymatic activity could be explained by the effect of solvent polarity on the conformation of the enzyme as well as on the solubility of the substrates and/or products.
Figure 7. The Lineweaver-Burk plots of tyrosinase biocatalysis using catechin as substrate in heptane (A), toluene (B), dichloromethane (C), or dichloroethane (D) reaction medium, without inhibitor (●—●), and with inhibitor (○—○). The initial reaction rate was definite as the change in absorbance at λ=376, 379, 375 and 375 nm for heptane, toluene, dichloromethane and dichloroethane reaction media, respectively, per µg protein per second.
Acetone concentration was expressed as the percentage of volume of acetone to the total reaction volume.

The catalytic efficiency was defined as the ratio of $\frac{A_{\max} \cdot \frac{\text{nm}}{2}}{K_{\text{m}}}$ to $A_{\max}$ or $\frac{A_{\max} \cdot \frac{\text{nm}}{2}}{K_{\text{m}}}$.

The maximal enzymatic reaction rate was defined as the change in absorbance at a specific wavelength per mg enzymatic protein per sec.

<table>
<thead>
<tr>
<th>Type of Inhibition</th>
<th>Efficiency $q$ (x10$^{-3}$) (M$^{-1}$)</th>
<th>Efficiency $q$ (%)</th>
<th>Medium Reacation</th>
<th>Reaction with acetone as inhibitor</th>
<th>Reaction without inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>mixed noncompetitive</td>
<td>0.99 4.63 30.0 3.0 4.03 120</td>
<td>1.24 4.24 2.00 2.2 2.57 14.7</td>
<td>Toluene</td>
<td>1.03 3.2 3.3 3.8 2.3 1.22</td>
<td>1.22 5.38 2.3 3.8 2.3 1.22</td>
</tr>
<tr>
<td>mixed noncompetitive</td>
<td>0.70 0.84 4.00 3.2 1.03 3.3</td>
<td>1.9 0.94 1.22 1.19 1.50 1.50</td>
<td>Heptane</td>
<td>1.22 5.38 2.3 3.8 2.3 1.22</td>
<td>1.22 5.38 2.3 3.8 2.3 1.22</td>
</tr>
<tr>
<td>competitive</td>
<td>0.70 0.84 4.00 3.2 1.03 3.3</td>
<td>1.9 0.94 1.22 1.19 1.50 1.50</td>
<td>Heptane</td>
<td>1.22 5.38 2.3 3.8 2.3 1.22</td>
<td>1.22 5.38 2.3 3.8 2.3 1.22</td>
</tr>
</tbody>
</table>

Table 1. Kinetic parameters of lysozyme biocatalysts in selected organic solvents media, using calcitin as substrate.
4.1.5. Effect of Acetone on Tyrosinase Activity

The effect of acetone as co-solvent on the tyrosinase-catechin oxidative reaction in the selected organic solvent media (Fig. 8) was investigated. The results show that the addition of 5 to 30% (v/v) acetone produced a 4.3 to 96.7% decrease in tyrosinase activity in the heptane reaction medium. In contrast, the results (Fig. 8) also indicate that the presence of 12.5, 22.0 and 22.0% acetone in the reaction media of dichloromethane, dichloroethane and toluene, respectively, produce a maximal increase of 42.6, 71.8 and 92.1%, respectively, in tyrosinase activity; however, the results indicate that additional increases in acetone concentration resulted in a subsequent inhibition of tyrosinase activity, due to the denaturing effect of acetone in removing the essential layer of water from the enzyme (Pool et al., 1983).

The overall findings show that lower concentrations of acetone were required to inhibit tyrosinase biocatalysis in the more hydrophobic medium of heptane (log $P = 4.0$) whereas higher concentrations were needed in the less hydrophobic reaction media of toluene (log $P = 2.5$), dichloromethane (log $P = 2.0$) and dichloroethane (log $P = 2.0$). These results suggest that the addition of acetone to the more hydrophilic reaction media of toluene, dichloromethane and dichloroethane produced an initial increase in tyrosinase activity due to the enhanced solubility of substrate and end products thereby allowing more substrate interaction and less end-product inhibition. These findings also suggest that the observed enzyme activation may be due to the “lubricating effect” of acetone on protein structure, thereby giving the enzyme molecule greater flexibility necessary for biocatalysis by reducing the intermolecular protein-protein contacts (Poole and Finnery, 1983). These results are in agreement with those reported by Almarsson et al.
Figure 8. The effect of acetone as co-solvent on tyrosinase activity in the reaction media of heptane (*--*), toluene (▲--▲), dichloromethane (■--■) and dichloroethane (♦--♦), using catechin as substrate. The percentage of activation or inhibition of tyrosinase activity was defined as the increase or decrease in enzymatic activity, respectively, relative to the initial value (without activator or inhibitor).
(1996) who indicated that the addition of a denaturing solvent, such as formamide and dimethyl sulfoxide, could actually dramatically increase enzymatic activity in anhydrous media due to enhanced conformational flexibility as a result of the weakening of intermolecular protein-protein contacts. The same authors reported that the rate of transesterification, catalyzed by protease subtilisin Carlsberg in anhydrous acetonitrile medium, could be increased by more than 50% with the addition of 10 to 50% of formamide as co-solvent.

The results (Table 1 and Fig. 7) also show that the addition of acetone to the heptane reaction medium showed a competitive inhibitory effect on tyrosinase activity as indicated by the $K_{\text{mapp}}$ value of 12.7 mM. This finding suggests that in the heptane reaction medium, acetone interacted with the active site of the enzyme in a similar fashion as the catechin substrate thereby disrupting the partitioning of catechin to the enzymatic active site; subsequently higher concentrations of substrate were required to obtain the $V_{\text{max}}$ of 11.9. The results (Table 1) also show that the addition of acetone to the toluene, dichloromethane and dichloroethane reaction media produced a mixed noncompetitive inhibitory effect on tyrosinase biocatalysis. These findings suggest that the inhibitory effect of acetone on tyrosinase activity in the more hydrophilic (log $P = 2.0-2.5$) reaction media of toluene, dichloromethane and dichloroethane is similar in that acetone competes with catechin to bind to the enzyme and distorts its conformation sufficiently to prevent proper positioning of its catalytic center, thereby rendering the enzymatic complex nonproductive upon interaction with the substrate; consequently, there is less bioconversion of catechin into the corresponding end products due to both
enzyme inactivation as well as lower substrate interaction in the presence of acetone (Segel et al., 1993).

The overall results also suggest that the difference in the inhibitory effect of acetone on tyrosinase activity in the heptane reaction medium in comparison to that obtained in the reaction media of toluene, dichloromethane and dichloroethane, may be due to the effect of solvent polarity on the conformation of enzyme. Tse et al. (1997) reported that Fourier Transform Infrared (FT-IR) spectroscopic analyses revealed that in the aqueous medium the native mushroom tyrosinase was predominately of the α-helical conformation while in the chloroform medium the enzyme was mainly composed of β-pleat structure.

4.2. Tyrosinase Biocatalysis Using Model Phenolic Substrate

4.2.1. Effect of Protein Concentration on Tyrosinase Activity

The effect of protein concentration on tyrosinase activity in the selected organic media of dichloromethane and heptane was investigated using catechin, catechol, 4-methyl catechol and p-cresol as substrates. The results (Fig. 9) show that the optimal amount of enzymatic protein for tyrosinase biocatalysis was 45.8, 43.9 and 39.1 μg per 200 μl assay for catechin, catechol, and 4-methyl catechol, respectively, in the dichloromethane medium, whereas in the heptane medium, the optimal concentration (Fig. 10) was 31.2, 4.3 and 34.2 μg per 200 μl assay for catechin, catechol and p-cresol, respectively.

Figure 9 also demonstrates that there was a constant increase in the specific activity of tyrosinase with a concomitant increase in enzymatic protein concentration in
Figure 9. Effect of enzymatic protein concentration on tyrosinase activity in the dichloromethane reaction medium, using catechin (■-----■), catechol (◆-----◆) and 4-methyl catechol (●-----●) as substrates, where the specific activity was defined as the change in absorbance, at 384, 376 and 379 nm, respectively, per µg protein per sec.
Figure 10. Effect of enzymatic protein concentration on tyrosinase activity in the heptane reaction medium, using catechin (■--■), catechol (♦----♦) and p-cresol (▲----▲) as substrates, where the specific activity was defined as the change in absorbance, at 330, 372 and 376 nm, respectively, per μg protein per sec.
the dichloromethane environment; however, after maximal oxidative PPO activity was reached, 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. Figure 10 also shows that similar results were obtained for the tyrosinase biocatalysis in the heptane reaction medium. Tse et al. (1997) reported a similar trend using vanillin as substrate for the tyrosinase biocatalysis in the chloroform medium. These findings suggest that this phenomenon may be due to the aggregation of tyrosinase molecules in the dichloromethane and heptane reaction media due to the limited solubility of the enzyme thereby resulting in less enzyme and substrate interaction (Halling, 1992; Kamat et al., 1995).

4.2.2. Effect of Substrate Concentration on Tyrosinase Activity

Figures 11 and 12 show that the tyrosinase biocatalysis, in the dichloromethane and heptane media with the selected substrates, followed Michaelis-Menten kinetics. Table 2 shows that the $K_m$ values for tyrosinase activity in the dichloromethane medium 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 2 also indicates that the $K_m$ values for tyrosinase activity in the heptane medium were approximately twice as high as those obtained in the dichloromethane environment for catechol and catechin as substrates; however, the use of $p$-cresol as substrate for tyrosinase activity in the heptane medium produced the lowest $K_m$ value for tyrosinase biocatalysis. These findings suggest that the differences in the tyrosinase affinity towards the selected substrates may be due to their various degrees of
Figure 11. The Lineweaver-Burk plots of tyrosinase biocatalysis in the dichloromethane reaction medium, using catechin (■—■), catechol (○—○) and 4-methyl-catechol (●—●) as substrates, where the specific activity was defined as the change in absorbance, at 384, 376 and 379 nm, respectively, per µg protein per sec.
Figure 12. The Lineweaver-Burk plots of tyrosinase biocatalysis in the heptane reaction medium, using catechin (■), catechol (●) and p-cresol (▲) as substrates, where the specific activity was defined as the change in absorbance, at 330, 372 and 376 nm, respectively, per µg protein per sec.
hydrophobicity, which may influence their partitioning between the selected organic solvent used for biocatalysis and the enzyme active site; moreover, the results suggest that this substrate effect may became more pronounced as solvent hydrophobicity is increased (Zake et al., 1986; Ryu et al., 1989). Butorn et al. (1993) reported a similar trend for PPO biocatalysis in the chloroform medium 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 the chloroform medium.

However, the results (Table 2) show that the $V_{max}$ value for the tyrosinase biocatalysis in the dichloromethane medium was about 2.5-times higher with catechin than those obtained with catechol and 4-methyl catechol. In addition, Table 2 shows that the $V_{max}$ value for the tyrosinase-catechin biocatalysis was 12-times higher in heptane reaction medium than that obtained using catechol and $p$-cresol as substrates. Tse et al. (1997) 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 the greater the availability of the ortho positions on the phenolic compounds the higher the $V_{max}$ value.

The catalytic efficiency (Table 2), defined as the ratio $V_{max}/K_m$ (Burton et al., 1993) for the tyrosinase biocatalysis, shows that the substrate conversion rate was 2-times higher with catechin compared to that obtained with catechol and 4-methyl catechol as substrates in the dichloromethane medium. The results also show that in the heptane reaction mixture, the catalytic efficiency for the tyrosinase biocatalysis was highest for
Table 2. Kinetic parameters of tyrosinase biocatalysis in dichloromethane and heptane reaction media, using model phenolic substrates.

<table>
<thead>
<tr>
<th>Reaction medium</th>
<th>Substrate</th>
<th>$V_{\text{max}}^a$</th>
<th>$K_m$</th>
<th>Catalytic efficiency$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$(10^{-4})$</td>
<td>(mM)</td>
<td></td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>Catechin</td>
<td>14.7</td>
<td>2.52</td>
<td>5.83</td>
</tr>
<tr>
<td></td>
<td>Catechol</td>
<td>6.03</td>
<td>2.36</td>
<td>2.56</td>
</tr>
<tr>
<td></td>
<td>4-Methyl catechol</td>
<td>5.07</td>
<td>2.21</td>
<td>2.29</td>
</tr>
<tr>
<td>Heptane</td>
<td>Catechin</td>
<td>12.2</td>
<td>5.38</td>
<td>2.26</td>
</tr>
<tr>
<td></td>
<td>Catechol</td>
<td>1.02</td>
<td>4.32</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>$p$-Cresol</td>
<td>0.84</td>
<td>1.07</td>
<td>0.79</td>
</tr>
</tbody>
</table>

$^a$ The maximal enzymatic reaction rate was defined as the change in the absorbance unit at a specific wavelength per μg enzymatic protein per sec.

$^b$ The catalytic efficiency was defined as the ratio of $V_{\text{max}}$ to $K_m$, x10^{-4}.
catechin and 9.4 and 2.9 times lower for \( p \)-cresol and catechol, respectively. However, the overall results show that the catalytic efficiencies of all the model substrates were higher in the dichloromethane medium than those obtained in heptane medium. The overall experimental finding suggest that the dichloromethane reaction medium (\( \log P \) 2.0) was more suitable for the biocatalysis of the selected substrates than that of 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. (1991) who reported that organic solvents with \( \log P \) values between 2 and 2.5 showed higher PPO biocatalysis activity than that 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.

Previous results obtained in our lab (Tse et al., 1997; Madani and Kermasha, 1999) showed that the \( V_{\max} \) values of tyrosinase biocatalysis in aqueous medium, using catechin, catechol, 4-methyl catechol and \( p \)-cresol as substrates, were 4.2\( \times \)10\(^{-3} \), 2.3\( \times \)10\(^{-3} \), 2.14\( \times \)10\(^{-3} \) and 7.1\( \times \)10\(^{-4} \) \( \delta A \) \( \mu g \) protein\(^{-1} \) sec\(^{-1} \), respectively, whereas the \( K_m \) values were 0.49, 0.42, 0.40 and 0.25 mM, respectively. In comparison to the kinetic parameters obtained for tyrosinase activity in the selected organic solvent media, the results show that tyrosinase is less active in the heptane and dichloromethane media than that observed in the aqueous medium. Zake and Klibanov (1988b) also obtained similar results for proteases \( \alpha \)-chymotrypsin and subtilisin whose activities were \( 10^4 \)-\( 10^5 \)-times lower in anhydrous octane than in water; the two enzymes remained less active in most organic solvents. Klibanov (1997) indicated that there were several reasons for the occurrence of lower enzymatic activity in organic solvent media; these included diffusional limitations,
active center blockage, conformational change, unfavorable energetic of substrate desolvation, transition state destabilization, reduced conformational mobility and suboptimal pH situation. The same author also stated that none of these factors was insurmountable, indeed, ‘aqueous-like’ activities of enzymes in nonaqueous solvent media have been reported by Zake and Klibanov (1988a) by optimizing the water activity in the reaction medium.

4.3. Characterization

4.3.1. Purification of End Product by Size-Exclusion Chromatography

A typical elution profile of tyrosinase, catechin and the respective enzymatically-catalyzed end products in an aqueous reaction medium is shown in Figure 13; a similar elution profile was also obtained for the tyrosinase biocatalysis in the 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 end products. In addition, Table 3 shows the $K_a$ values obtained from the respective order of elution of the substrates and the corresponding enzymatically-catalyzed end products. The results (Table 3) show that among the selected substrates, 4-methyl catechol was the fastest eluting compound as indicated by its $K_a$ value of 1.38, followed by p-cresol and catechol possessing $K_a$ 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 $K_a$ 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 (Richard et al., 1991); the same authors
Figure 13. Size-exclusion chromatography elution profile of tyrosinase, catechin and the corresponding enzymatically-catalyzed end products in aqueous medium, with absorbance at 280 (●—●), 288 (■—■) and 408 (○—○) nm.
Table 3. $K_{av}$ values of catechin, catechol, 4-methyl catechol, $p$-cresol and their respective purified end products.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_{av}$</th>
<th>Compound</th>
<th>$K_{av}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>1.85</td>
<td>Catechol</td>
<td>1.64</td>
</tr>
<tr>
<td>CPA $^a$</td>
<td>0.43</td>
<td>CLPA $^b$</td>
<td>0.41</td>
</tr>
<tr>
<td>CPH $^a$</td>
<td>0.43</td>
<td>CLPH $^b$</td>
<td>0.51</td>
</tr>
<tr>
<td>CPT $^a$</td>
<td>0.41</td>
<td>CLPDM $^b$</td>
<td>0.62</td>
</tr>
<tr>
<td>CPDM $^a$</td>
<td>0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPDE $^a$</td>
<td>0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Methyl Catechol</td>
<td>1.38</td>
<td>$p$-Cresol</td>
<td>1.54</td>
</tr>
<tr>
<td>4MCPA $^c$</td>
<td>0.41</td>
<td>$p$-CPA $^d$</td>
<td>0.35</td>
</tr>
<tr>
<td>4MCPDM $^c$</td>
<td>0.62</td>
<td>$p$-CPH $^d$</td>
<td>0.51</td>
</tr>
</tbody>
</table>

$^a$ CPA, CPH, CPT, CPDM and CPDE refer to the enzymatically-catalyzed end products of catechin in aqueous, heptane, toluene, dichloromethane and dichloroethane media, respectively.

$^b$ CLPA, CLPH and CLPDM refer to the enzymatically-catalyzed end products of catechol in aqueous, heptane and dichloromethane media, respectively.

$^c$ 4MCPA and 4MCPDM refer to the enzymatically-catalyzed end products of 4-methyl catechol in aqueous and dichloromethane media, respectively.

$^d$ $p$-CPA and $p$-CPH refer to the enzymatically-catalyzed end products of $p$-cresol in aqueous and heptane media, respectively.
indicated that the chemical structure of Trisacryl GF05 includes the presence of one secondary amine group and three primary hydroxymethyl groups thereby suggesting that hydrogen bonding occurred between this matrix and the phenolic substrates. Concin et al. (1980) also indicated that there was a relationship between the $K_{av}$ values and the number of free phenolic hydroxyl groups on the substrate, using the Sephadex LH 20 gel.

Table 3 also shows that the enzymatically-catalyzed end products of catechin, in the aqueous, heptane, toluene, dichloromethane and dichloroethane reaction media, showed similar $K_{av}$ values between 0.36 to 0.43; these findings suggest the formation of end products of similar molecular weight by tyrosinase activity, using catechin as substrate. However, Table 3 also shows that the tyrosinase biocatalysis of catechol, 4-methyl catechol and $p$-cresol in the selected reaction media resulted in the formation of end products possessing different $K_{av}$ values ranging from 0.35 to 0.62, thereby suggesting the presence of polymers of different molecular weight. The overall results show that depending on the reaction medium used for tyrosinase biocatalysis, different end products can be obtained using the selected substrates.

4.3.2. UV Spectrophotometric Scanning

The maximum absorbency ($\lambda_{\text{max}}$) of catechin, catechol, 4-methyl catechol, $p$-cresol and their respective enzymatically-catalyzed end products in the selected reaction media (Fig. 14) was determined. The results (Fig. 14A) show that the maximum absorbency ($\lambda_{\text{max}}$) of catechin and its corresponding end products formed in the aqueous, heptane, toluene, dichloromethane and dichloroethane media was at 288, 408, 376, 379, 375 and 375 nm, respectively. These results are in agreement with those reported by
Guyot et al. (1995) who indicated that the PPO biocatalysis of catechin in aqueous medium yielded colored products with a $\lambda_{\text{max}}$ between 240 and 500 nm. In addition, Estrada et al. (1991) reported that the enzymatically-catalyzed end products of catechin in organic media, including toluene, benzene and 1,1,1-trichloroethane, showed a $\lambda_{\text{max}}$ at 395 nm.

The results (Fig. 14B) also show that the $\lambda_{\text{max}}$ of catechol and its enzymatically-catalyzed end products in aqueous, heptane and dichloromethane media was at 286, 383, 374 and 377 nm, respectively, whereas that (Fig. 14C) of 4-methyl catechol and its end products in aqueous and dichloromethane media was at 287, 507 and 384 nm, respectively, and that (Fig. 14D) of $p$-cresol and its end products in aqueous and heptane media was at 282, 399 and 342 nm, respectively. Richard-Forget et al. (1992) reported the enzymatic oxidation of 4-methyl catechol by apple PPO activity in aqueous medium resulted in end products with a $\lambda_{\text{max}}$ at 280 nm.

The overall results show that the selected substrates all exhibited a $\lambda_{\text{max}}$ between 282 – 288 nm. The overall findings also indicate that the enzymatically-catalyzed end products of catechin, catechol, 4-methyl catechol and $p$-cresol in the aqueous medium showed slightly higher $\lambda_{\text{max}}$ values of 408, 383, 507 and 399 nm, respectively, in comparison to the $\lambda_{\text{max}}$ values of 342 and 374 to 384 nm obtained using the selected organic reaction media; these findings suggest the formation of different end-products by tyrosinase biocatalysis in the organic solvent media in comparison to those obtained with the aqueous medium.
Figure 14. Scanning profiles of substrates (□—□) and their respective enzymatically-catalyzed end products in aqueous (○—○), heptane (△—△), dichloromethane (△—△), toluene (◇—◇) and dichloroethane (X—X) media while using catechin (A), catechol (B), 4-methyl catechol (C) and p-cresol (D) as substrates.
4.3.3. Thermal Analysis

The thermal analysis of catechin, catechol, 4-methyl catechol, p-cresol and their respective end products (Table 4) was measured by DSC. Figure 15 shows typical DSC curves obtained for catechin and the corresponding enzymatically-catalyzed end products in the selected reaction media; similar DSC curves were obtained for catechol, 4-methyl catechol and p-cresol and their respective end products. The results show that the DSC curve for catechin (Fig. 15A) indicated the occurrence of two melting points at 93.5 and 153.0 °C with a corresponding heat of reaction (ΔH) of 30.6 and 104.3 J/g, respectively (Table 4); these findings suggest 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 (Haines et al., 1995). However, the results (Table 4) indicated the presence of only one melting point in the DSC curves for catechol, 4-methyl catechol and p-cresol at 102.2, 63.6 and 35.6 °C, respectively, thereby suggesting that these substrates exist only one crystalline form during the thermal treatment used.

The results (Fig 15B1 and 15B2) also show that the enzymatically catalyzed end products of catechin by tyrosinase activity in the aqueous and heptane media exhibited only one melting point at 179.4 and 132.9 °C, respectively, with corresponding endothermic ΔH of 183.9 and 154.0 J/g (Table 4); similar findings were obtained for the tyrosinase biocatalysis of catechol in aqueous and toluene media, of p-cresol in heptane media and of 4-methyl catechol in aqueous and dichloromethane media. In addition, the findings (Table 4) indicate that two melting points were obtained for the enzymatically-catalyzed end products of catechol and p-cresol in the heptane and aqueous reaction media, respectively. These results are in agreement with the theory of Haines et al.
(1995) who stated that some polymer samples may show multiple melting endotherms, due to different portions of the sample having crystallized at different temperatures during the thermal treatment.

However, Figures 15B3-15B5 also indicate that the end products of the tyrosinase-catechin biocatalysis in the toluene, dichloromethane and dichloroethane media showed a crystallization peak at 144.2, 121.8, and 156.7 °C, respectively, with a corresponding exothermic ΔH of 281.2, 305.7 and 244.6 J/g (Table 4). These findings may be explained by Haines et al. (1995) who reported that when certain polymers are subjected to an increase in temperature, they melt before decomposing and then crystallize completely, whereas, other polymers remain as irregular, amorphous solids or glass; upon further heating, these polymers change to a more plastic material which possesses 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 ΔH. The overall results (Table 4) show that relatively higher melting points were obtained for the enzymatically-catalyzed end products of the selected substrates in aqueous medium in comparison to those obtained for the end products formed in organic solvent media. In addition, the overall findings show that the biocatalysis of the selected substrates in different media indicated the presence of unique end products with specific ΔH properties.

4.3.4. Pyrolysis Gas Chromatography Analysis

The characterization of the tyrosinase biocatalysis with model phenolic substrates in the selected reaction media was investigated by pyrolysis gas
Figure 15. Differential scanning calorimetry (DSC) curves of catechin (A) and the tyrosinase-catalyzed end products in aqueous (B1), heptane (B2), toluene (B3), dichloromethane (B4) and dichloroethane (B5) media.
Table 4. Thermal analysis data of catechin, catechol, 4-methyl catechol, p-cresol and their corresponding end products.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Melting Point (°C)</th>
<th>Crystallization Point (°C)</th>
<th>ΔH (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>93.5 &amp; 153.0</td>
<td>-</td>
<td>30.6 &amp; 104.3</td>
</tr>
<tr>
<td>CPA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>179.4</td>
<td>-</td>
<td>183.9</td>
</tr>
<tr>
<td>CPH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>132.9</td>
<td>-</td>
<td>154.0</td>
</tr>
<tr>
<td>CPT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>144.2</td>
<td>-281.3</td>
</tr>
<tr>
<td>CPDM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>121.8</td>
<td>-305.7</td>
</tr>
<tr>
<td>CPDE&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>156.7</td>
<td>-244.6</td>
</tr>
<tr>
<td>Catechol</td>
<td>102.2</td>
<td>-</td>
<td>202.1</td>
</tr>
<tr>
<td>CLPA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>184.7</td>
<td>-</td>
<td>32.0</td>
</tr>
<tr>
<td>CLPH&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65.4 &amp; 88.8</td>
<td>-</td>
<td>10.1 &amp; 3.9</td>
</tr>
<tr>
<td>CLPT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>87.0</td>
<td>-</td>
<td>19.2</td>
</tr>
<tr>
<td>4-M Catechol</td>
<td>63.6</td>
<td>-</td>
<td>106.2</td>
</tr>
<tr>
<td>4MCPA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>232.7</td>
<td>-</td>
<td>34.0</td>
</tr>
<tr>
<td>4MCPDM&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26.3</td>
<td>-</td>
<td>5.9</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>35.6</td>
<td>-</td>
<td>13.8</td>
</tr>
<tr>
<td>p-CPA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>78.6 &amp; 223.8</td>
<td>-</td>
<td>29.8 &amp; 18.9</td>
</tr>
<tr>
<td>p-CPH&lt;sup&gt;d&lt;/sup&gt;</td>
<td>80.6</td>
<td>-</td>
<td>45.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> CPA, CPH, CPT, CPDM and CPDE refer to the enzymatically-catalyzed end products of catechin in aqueous, heptane, toluene, dichloromethane and dichloroethane media, respectively.

<sup>b</sup> CLPA, CLPH and CLPDM refer to the enzymatically-catalyzed end products of catechol in aqueous, heptane and dichloromethane media, respectively.

<sup>c</sup> 4MCPA and 4MCPDM refer to the enzymatically-catalyzed end products of 4-methyl catechol in aqueous and dichloromethane media, respectively.

<sup>d</sup> p-CPA and p-CPH refer to the enzymatically-catalyzed end products of p-cresol in aqueous and heptane media, respectively.
chromatography, Figure 16 shows a typical pyrogram of the tyrosinase-catalyzed end 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 5 and 6 show the compounds identified by GC/MS possessing a quality higher than 80%.

The results (Table 5) show that only one major compound 10-methylnonadecane was formed by the pyrolysis of catechin. The results also show that the pyrolysis of the enzymatically-catalyzed end products of catechin, in aqueous medium, produced three major compounds including phenol, 4-methyl-phenol and 2,4,7-trimethylbenzofuran, while those produced in the reaction media of heptane and dichloroethane gave two major compounds, including 2,4-bis (1,1-dimethylethyl) phenol and 2-t-butyl-4- (1,1-dimethylbenzyl) phenol, as well as 2,5-bis (1,1-dimethylethyl) phenol and bicyclo [2,2,1] heptan-2-ol, respectively. In contrast, the results show that more than 12 major peaks were detected from the pyrolysis of the end products of the tyrosinase-catechin biocatalysis in the reaction media of toluene and dichloromethane; moreover, Table 3 shows that hexanal, 2-methyl-1h-pyrrole, (S)-1,3-butanediol, 2-heptanone, 2-methyl-2-cyclopenten-1-one and phenol were present from the pyrolysis of the end products formed in both the toluene and dichloromethane media. 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 abundance of end products.

In addition, Table 6 shows that two major pyrolysis compounds, including 3-methyl-1, 2-benzenediol and 1, 3-bis (trimethylsiloxy) benzene were obtained from the
Figure 16. Pyrograms of the tyrosinase-catalyzed end products of catechol in the selected reaction media, including aqueous (A), heptane (B) and dichloromethane (C), using the quartz tube at 500 °C for 2 sec.
Table 5. Major pyrolysis fragments of catechin and its end products identified by GC/MS.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT</th>
<th>Area (%)</th>
<th>Library / ID</th>
<th>Quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>33.23</td>
<td>20.29</td>
<td>10-methylnonadecane</td>
<td>80</td>
</tr>
<tr>
<td>CPA</td>
<td>11.79</td>
<td>13.37</td>
<td>phenol</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>13.74</td>
<td>28.5</td>
<td>4-methyl-phenol</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>18.3</td>
<td>5.75</td>
<td>2,4,7-trimethylbenzofuran</td>
<td>86</td>
</tr>
<tr>
<td>CPH</td>
<td>21.16</td>
<td>38.17</td>
<td>2,4-bis (1,1-dimethyl) phenol</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>28.79</td>
<td>7.37</td>
<td>2-t-butyl-4- (dimethylbenzyl)</td>
<td>93</td>
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<tr>
<td>CPT</td>
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<td>33.75</td>
<td>Acetic acid</td>
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</tr>
<tr>
<td></td>
<td>8.93</td>
<td>1.31</td>
<td>hexamethyl-cyclotrisiloxane</td>
<td>91</td>
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<tr>
<td></td>
<td>9.27</td>
<td>2.83</td>
<td>hexanal</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>9.36</td>
<td>1.55</td>
<td>2,3,5-trimethylfuran</td>
<td>83</td>
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<tr>
<td></td>
<td>9.96</td>
<td>2.69</td>
<td>2-methyl-1h-pyrrole</td>
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</tr>
<tr>
<td></td>
<td>10.62</td>
<td>5.40</td>
<td>(S)- 1,3-butanediol</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>10.92</td>
<td>1.80</td>
<td>2-heptanone</td>
<td>91</td>
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<tr>
<td></td>
<td>11.00</td>
<td>1.23</td>
<td>1,3,5,7-cyclooctatetraene</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>11.50</td>
<td>1.97</td>
<td>2-cyclopenten-1-one, 2-methyl-phenol</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>12.69</td>
<td>6.38</td>
<td>2-heptanone</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>12.88</td>
<td>1.94</td>
<td>2-cyclopenten-1-one, 3-methyl-</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>13.54</td>
<td>1.04</td>
<td>1-hexanol, 2-ethyl-</td>
<td>86</td>
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<tr>
<td>CPDM</td>
<td>9.27</td>
<td>4.15</td>
<td>hexanal</td>
<td>86</td>
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<td>9.97</td>
<td>6.14</td>
<td>2-methyl-1h-pyrrole</td>
<td>91</td>
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<td>10.64</td>
<td>14.37</td>
<td>(S)- 1,3-butanediol</td>
<td>83</td>
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<td></td>
<td>10.92</td>
<td>4.48</td>
<td>2-heptanone</td>
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</tr>
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<td></td>
<td>11.50</td>
<td>3.71</td>
<td>2-cyclopenten-1-one, 2-methyl-phenol</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>12.89</td>
<td>11.41</td>
<td>2-cyclopenten-1-one, 3-methyl-</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>12.68</td>
<td>4.90</td>
<td>2,4-hexadiene, 3,4-dimethyl-phenol, 2-methyl-</td>
<td>97</td>
</tr>
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<td>13.99</td>
<td>2.14</td>
<td>2,3-dimethylcyclopent-2-en-1-one</td>
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</tr>
<tr>
<td></td>
<td>14.23</td>
<td>3.22</td>
<td>phenol, 3-methyl-</td>
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<td></td>
<td>14.30</td>
<td>2.72</td>
<td>phenol, 2-ethyl-</td>
<td>87</td>
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<td>14.73</td>
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<td>mequinol</td>
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<td></td>
<td>15.11</td>
<td>3.32</td>
<td>phenol, 2-ethyl-</td>
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</tr>
<tr>
<td></td>
<td>16.49</td>
<td>3.54</td>
<td>indol</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>19.28</td>
<td>3.75</td>
<td></td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>28.68</td>
<td>11.91</td>
<td>bicyclo [2.2.1] heptane-2-ol</td>
<td>90</td>
</tr>
</tbody>
</table>

CPA, CPH, CPT, CPDM and CPDE refer to the enzymatically-catalyzed end products of catechin in the aqueous, heptane, toluene, dichloromethane and dichloroethane media, respectively.
Table 6. Major pyrolysis compounds of catechol, 4-methyl catechol, \( p \)-cresol and their end products identified by GC/MS.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT</th>
<th>Area (%)</th>
<th>Library / ID</th>
<th>Quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>20.39</td>
<td>100</td>
<td>ether</td>
<td>86</td>
</tr>
<tr>
<td>CLPA</td>
<td>17.66</td>
<td>28.3</td>
<td>1,2-benzenediol, 3-methyl-ether</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>17.93</td>
<td>13.3</td>
<td>1,3-bis (trimethylsiloxy) benzene</td>
<td>80</td>
</tr>
<tr>
<td>CLPH</td>
<td>15.80</td>
<td>18.8</td>
<td>1,2-benzenediol</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>16.36</td>
<td>13.2</td>
<td>5,4'-dimethoxy-2-methylbibenzyl</td>
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<td></td>
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<td>12.0</td>
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<tr>
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<td>17.85</td>
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<tr>
<td>CLPDM</td>
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<td>5.2</td>
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<td>15.71</td>
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<td>16.53</td>
<td>7.1</td>
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<td></td>
<td>17.63</td>
<td>36.8</td>
<td>1,2-benzenediol, 3-methyl-ether</td>
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<td>4MCPDM</td>
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<td>5.3</td>
<td>phenol, 4-methyl-</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>15.72</td>
<td>7.4</td>
<td>1,2-benzenediol</td>
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<tr>
<td></td>
<td>17.68</td>
<td>36.7</td>
<td>1,2-Benzenediol, 4-methyl-ether</td>
<td>91</td>
</tr>
<tr>
<td>( p )-Cresol</td>
<td>13.24</td>
<td>100</td>
<td>phenol, 4-methyl-</td>
<td>95</td>
</tr>
<tr>
<td>( p )-CPH</td>
<td>11.89</td>
<td>2.3</td>
<td>4,4-dimethyl-2-cyclopeten-1-one</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>13.28</td>
<td>6.9</td>
<td>phenol, 4-methyl-</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>17.54</td>
<td>12.3</td>
<td>1,2-benzenediol, 3-methyl-</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>17.78</td>
<td>2.6</td>
<td>indole</td>
<td>91</td>
</tr>
</tbody>
</table>

\( a \) CLPA, CLPH and CLPDM refer to the enzymatically-catalyzed end products of catechol in aqueous, heptane and dichloromethane media, respectively.

\( b \) 4MCPA and 4MCPDM refer to the enzymatically-catalyzed end products of 4-methyl catechol in aqueous and dichloromethane media, respectively.

\( c \) \( p \)-CPH refers to the enzymatically-catalyzed end products of \( p \)-cresol in heptane media.
tyrosinase-catechol biocatalysis in aqueous medium; the former was also detected along with phenol, 1, 3-benzodioxol-2-one, and 1, 2-benzenediol from the pyrolysis of the end products of catechol enzymatically-catalyzed in the dichloromethane medium. Moreover, the results show that 1, 2-benzenediol was also detected from the pyrolysis of the end products of catechol in the heptane medium; other major degradation compounds included 5,4'-dimethoxy-2-methylbibenzyl, 4-ethyl-benzaldehyde and 4-methyl-1,2-benzenediol.

Table 6 demonstrates that the enzymatically-catalyzed end products of 4-methyl catechol in the aqueous medium also gave 3-methyl-1,2-benzenediol as a pyrolysis product while those formed in the dichloromethane medium gave similar pyrolysis products, including 1,2-benzenediol and 4-methyl-1, 2-benzenediol. The findings also show that the pyrolysis of the end products of p-cresol resulting from the tyrosinase biocatalysis in the heptane medium also showed the presence of 3-methy 1-1, 2-benzenediol as well as 4, 4-dimethyl-2-cyclopenten-1-one, 4-methyl-phenol and indole. These overall findings indicate that there were some similarities in the pyrolysis of the end products obtained from the tyrosinase biocatalysis of catechol, 4-methyl catechol and p-cresol in the selected organic solvent media.

Tse et al. (1997) 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; the same author also suggested this change in conformation may produce differences in the enzymatic activity exhibited in the various selected reaction media by the formation of
different end products. Ikeda et al. (1996) also indicated that the study of 2,6-dimethylphenol polymerization by 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. (1998) 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.
5. CONCLUSION

The results gathered in this study indicated that tyrosinase demonstrated a high catalytic activity in the dichloromethane medium, using catechin as substrate, in comparison to that obtained in the toluene, heptane and dichloroethane media. The results also confirmed the trend of the kinetics data obtained in this study is in agreement with that reported previously in our laboratory as well as that reported in literature.

Although the biocatalysis of tyrosinase in the dichloromethane and heptane media showed a lower affinity towards catechin as substrate, a higher $V_{\text{max}}$ value and catalytic efficiency were obtained compared to those observed with catechol, 4-methyl catechol and $p$-cresol, as substrates.

The spectrophotometric scanning showed that the tyrosinase oxidation of phenolic substrate models in selected reaction media resulted in end products with different maxima absorbance. In addition, the results of differential scanning calorimetry suggested that the end products formed in aqueous medium exhibited a higher thermal stability than those obtained in organic solvent media did. Moreover, the analysis of the pyrolysis residuals of the enzymatically-catalyzed end products suggested that the structures of the polymer changed with the change of reaction media, used for the biocatalysis.
6. REFERENCES


