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Original Article

Unraveling polyphenol oxidase kinetics: a comparative analysis between *Agaricus bisporus* and *Terfezia leonis* mushrooms

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ABSTRACT

Enzymatic browning is a biochemical process catalyzed by polyphenol oxidase (PPO), employing endogenous phenolic compounds and molecular oxygen as substrates. This intriguing reaction leads to the formation of brown or black pigments, commonly referred to as melanins, specifically occurring on the surfaces of certain fruits and vegetables. This research focuses on investigating the kinetic parameters of PPO, also known as tyrosinase, extracted from *Agaricus bisporus* (Paris mushroom) and *Terfezia leonis* (Desert truffle). The study explores the effects of pH, temperature, and substrate concentration (L-tyrosine) on PPO activity from both sources. The results demonstrate that the activity of PPO from *Agaricus bisporus* reaches its maximum at pH 5 and a temperature of 45 °C, with inhibition observed in the presence of excess substrate. The kinetic parameters, Vmax and Km, for *Agaricus bisporus* PPO were determined to be 0.07587 ΔAbs.Min^(-1) and 0.1386 mmol.l^(-1), respectively. On the other hand, the activity of PPO from *Terfezia leonis* reaches its peak at pH 5 and a temperature of 40 °C, and the corresponding kinetic parameters are Vmax = 66.35 µM/min and Km = 0.17 mM. Enzymatic browning has been a subject of interest for numerous scientists who have explored various techniques to inhibit or eliminate the compounds responsible for the reaction, such as oxygen, copper, substrate, or the enzyme itself. Overall, this study provides valuable insights into the kinetic behavior of PPO from *Agaricus bisporus* and *Terfezia leonis*, shedding light on potential strategies for inhibiting enzymatic browning to preserve the visual appeal and quality of food.

KEYWORDS: polyphenol oxidase, tyrosinase, L-tyrosine, Paris mushroom, *Agaricus bisporus*, desert truffle, *Terfezia leonis*.

INTRODUCTION

Living organisms are dynamic entities, hosting a vast array of diverse biochemical reactions that occur under varying conditions. These intricate reactions are facilitated by biological macromolecules known as enzymes, which act as highly efficient biological catalysts, greatly accelerating numerous reactions within the organisms. However, one of

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the adverse reactions catalysed by enzymes is browning, which leads to undesirable changes in color, unpleasant odors, and altered taste in food [1]. Browning results from the oxidation of L-tyrosine, a process mainly catalyzed by the enzyme polyphenol oxidase also known as tyrosinase (EC 1.14.18.1), a copper-containing metallo-enzyme widely distributed in nature and found abundantly in most fruits and vegetables [1-3]. Tyrosinase exhibits dual activities in the presence of molecular oxygen; it hydroxylates monophenols to form o-diphenols, a phenomenon known as cresolase or monophenolase activity. These o-diphenols, in turn, undergo oxidation to produce o-quinones, termed catecholase or o-diphenoloxidase activity. The resulting o-quinones further polymerize to form pigments such as melanin, which can manifest as brown, red, or black hues [4].

While polyphenol oxidase (PPO) has been extensively studied and characterized from various fruit and plant sources, there has been limited research regarding PPO from Paris mushrooms. These mushrooms are naturally found in grasslands, fields, and meadows across Europe and North America, and the desert truffles, which are hypogeal mushrooms capable of establishing mycorrhizal associations with host plant roots, and are highly sought after by local populations in Algeria [5]. Therefore, our study aims to comprehensively understand the enzymatic activity of tyrosinase extracted and partially purified from both Paris mushrooms and desert truffles. Through our investigation into the enzymatic properties of PPO from these distinct sources, we aim to uncover valuable insights into the intriguing browning phenomenon. By doing so, we seek to explore potential applications in food preservation and other related fields, such as cosmetics, pharmaceuticals, and biotechnology. Understanding the behavior of tyrosinase from different origins can help us develop innovative strategies to control enzymatic browning in various food products, extend their shelf life, and maintain their visual appeal. Furthermore, these insights can be leveraged to devise novel approaches in cosmetic formulations, medicinal synthesis, and enzyme-based biotechnological processes.

MATERIALS AND METHODS

The *Agaricus bisporus* mushroom and *Terfezia leonis* truffle serve as valuable sources of polyphenol oxidase (PPO). To ensure their suitability for extraction, both mushrooms and truffles were washed thoroughly with distilled water to eliminate any soil residues. Subsequently, they were air-dried at room temperature and stored at 4 °C for later use. For the extraction process, 50 g of fresh peelings from the mushrooms and truffles were carefully washed with distilled water and left to dry naturally in the open air. These dried peelings were then ground for 5 minutes in a blender containing 100 ml of sodium phosphate buffer at pH 5.0 (0.05 M). After grinding, the resulting homogenate was filtered using a gauze, followed by manual pressing to obtain a homogeneous filtrate. This filtrate was then subjected to centrifugation at 4000 rpm for 10 minutes, resulting in the recovery of a supernatant (80 ml), which represents the crude extract of polyphenol oxidase (PPO).

To determine the protein concentration in the extract, the Biuret method was employed. Protein concentrations were calculated using linear interpolation, based on a standard range derived from bovine serum albumin. This assay provides valuable information on the protein content and aids in characterizing the PPO extracted from the *Agaricus bisporus* mushroom and *Terfezia leonis* truffle.

Determination of enzymatic activity

The cresolase activity of PPO is assessed by directly measuring the formation of o-quinones using spectrophotometry in the presence of oxygen. The measurement was performed at 475 nm and a temperature of 30 °C, utilizing L-tyrosine, a prominent endogenous mono phenolic substrate of fungi. After the lag phase (t), we determined the steady-state velocity (V0) by analyzing the linear portion of the reaction kinetics of the absorbance curve over time. The reaction volume in a quartz cuvette was 1 ml and comprises of 0.51 ml of 0.05 M sodium acetate buffer (pH 5.0), 40 µl of 20 mM SDS, 400 µl of 2 mM L-tyrosine in sodium acetate buffer, and 50 µl of the enzymatic extract from desert truffles. The change in absorbance was recorded every

10 seconds for 2 minutes following the addition of the enzyme extract.

Effect of pH on PPO activity

The investigation of pH's impact on the crude PPO extract was conducted using two types of buffers: sodium acetate buffer (0.05 M, pH 3.5 to 5.6) and sodium phosphate buffer (0.05 M, pH 6.0 to 8.0). PPO activity was measured at standard temperature conditions (30 °C), with a buffer solution volume of 0.51 ml, substrate volume of 400 µl at 2 mM, SDS volume of 40 µl at 20 mM, and enzyme volume of 50 µl.

Effect of temperature on PPO activity

To study the effect of temperature on PPO activity, the initial oxidation rate of tyrosine at 475 nm was measured at different temperatures: 30 °C, 40 °C, 50 °C, 60 °C, and 70 °C under standard measurement conditions. The reaction mixture consists of 400 µl of 2 mM tyrosine, 0.53 ml of 0.05 M sodium acetate buffer at pH 5.0, 40 µl of SDS, and 30 µl of the enzymatic extract. The change in absorbance was recorded every 10 seconds for 3 minutes after adding the enzymatic extract.

Effect of substrate concentration

Under standard conditions (0.05 M sodium acetate buffer, pH 5, 30 °C), the initial rates of L-tyrosine oxidation by PPO were measured for various substrate concentrations: 0.06, 0.1, 0.14, 0.2, 0.4, 0.8, 1, and 1.5 mM. The reaction medium consists of 400 µl of L-tyrosine at 2 mM, 0.51 ml of sodium acetate buffer, 40 µl of SDS, and 50 µl of the enzymatic extract.

RESULTS AND DISCUSSION

Investigation of the polyphenol oxidase activity of the enzyme preparation

Figure 1 illustrates the enzymatic oxidation of L-tyrosine to dopaquinone in the presence of PPO extracted from both mushrooms. The activity curve demonstrates a gradual increase, reaching a peak of 0.9583 IU/ml after six minutes of incubation. Notably, this oxidation process does not appear to be spontaneous, as evidenced by control tests conducted without the presence of enzymnes that showed no oxidation of L-tyrosine. Consequently, the results strongly suggest that the commercial enzyme is indeed active and

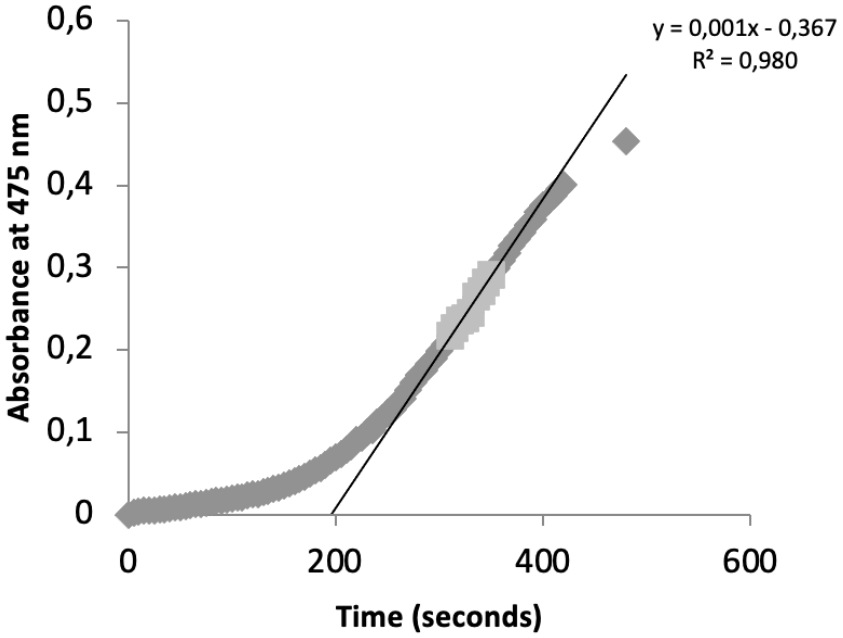

Figure 1. Variation of absorbance at 475 nm as a function of time during the oxidation reaction of L-tyrosine by tyrosinase.

responsible for catalyzing the conversion of L-tyrosine to dopaquinone.

Measurement of the volume and specific activities of the enzyme

Agaricus bisporus

Table 1 presents the specific and volume activities obtained under standard operating conditions ([L-tyrosine] = 2 mM, 0.05 M sodium acetate buffer at pH 5.00, temperature: 25 °C, reaction time: 5 minutes), along with the corresponding initial rate and protein concentration.

Terfezia leonis

Table 2 displays the volume and specific activities obtained under standard operating conditions ([L-tyrosine] = 2 mM, 0.05 M sodium acetate buffer at pH 5.00, temperature: 30 °C, reaction time: 2 minutes), along with the corresponding initial rate and protein concentration.

Influence of pH on the enzymatic activity of polyphenol oxidase

Enzymatic reactions are highly sensitive to variations in the pH of the surrounding medium since this factor directly impacts the ionization state of both the substrate and the enzyme molecule. The effect of pH is attributed to the participation of ionizable groups in substrate binding, catalytic processes, and the maintenance of the protein's secondary and tertiary structures. These interactions can either favor or impede enzymatic activity [6]. Consequently, when plotting enzyme activity against pH, a discernible trend emerges: the activity gradually increases until it reaches its peak at the optimal pH value. Subsequently, beyond this point, the enzymatic activity starts to decline. This pH-dependent pattern highlights the critical role of optimal pH conditions in facilitating the highest level of enzymatic efficiency.

Agaricus bisporus

The results presented in Figure 2 demonstrate a prominent peak, indicative of the maximum activity of tyrosinase at a pH of 5.00, aligning with the enzyme's optimum pH. Within the pH range of 5 to 6, the monophenolase activity exhibits notable stability, while beyond these values, the enzymatic activity gradually diminishes. This decline in enzyme activity can be attributed to changes in the degree of ionization of L-tyrosine and/or specific amino acid residues within the active site, leading to adverse interactions between PPO and L-tyrosine. These findings are consistent with previous literature reports, wherein the optimum pH of PPO from various plant sources such as apple, pear, peach, grape, banana, potato, green chili, and mushrooms typically falls between 5 and 7 [7].

Table 1. Volume and specific activities of tyrosinase under standard conditions.

Proteins concentration (g/l)	Initial speed (Abs/min)	Concentration activity (UI/ml)	Specific activity (UI/mg)
13.4766935 ± 2.2416	0.054 ± 0.0049	0.7546296 ± 0.031	0.05485 ± 0.0019

Table 2. Volume and specific activities of tyrosinase.

Proteins concentration (g/l)	Initial speed (Abs/min)	Concentration activity (UI/ml)	Specific activity (UI/mg)
21.1342 ± 2.26	0.16 ± 0.034	0.88 ± 0.19	0.04205 ± 0.0019

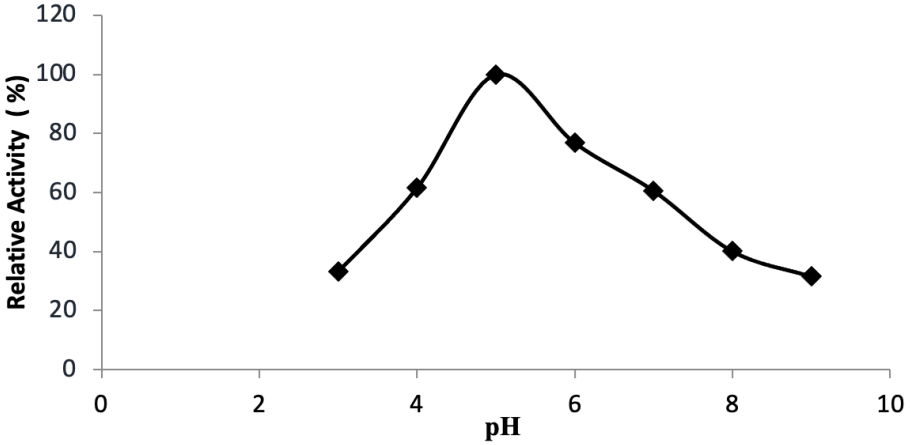

Figure 2. Effect of pH on PPO of Paris mushroom. ([Tyrosinase] = 2 mM; 0.05 M sodium acetate buffer solution-pH5; reaction time: 8 min; 25 °C).

The precise optimum pH of PPO can vary based on factors such as the enzyme's source, extraction method, and the specific substrate employed [3, 4, 8]. Optimal pH values are typically observed in the range of 4 to 7. Additionally, the type of buffer and enzyme purity also influence the optimum pH [3]. In light of our own results and those reported by other researchers, we have decided to continue our experiments using a 0.05 M-pH 5.00 sodium acetate buffer solution. This choice is warranted, as the pH of the medium significantly impacts the ionization state of numerous internal sites within the enzyme molecule, affecting its overall structure, substrate binding, and the reaction mechanism [9]. The selection of an appropriate buffer pH is crucial to ensure optimal conditions for studying the enzymatic activity of PPO effectively.

Terfezia leonis

According to Figure 3, the maximum enzymatic activity is observed at pH=5. Below and above this pH value, a decrease in activity is noticed. These findings are consistent with the research conducted by Gouzi [10], who also demonstrated that the cresolase activity of truffle PPO exhibits a broad optimum pH range, spanning from 4.5 to 8.0. Notably, a significant decrease in activity occurs above pH 8.0 and below pH 4.5. This wide pH optimum range observed in *Terfezia leonis* PPO is a notable characteristic, as most PPOs from various sources tend to have more restricted pH optimum ranges. Other studies have reported similar observations. Perez-Gilabert *et al*. [11] found that the PPO cresolase activity of *Terfezia claveryi* desert truffles has optimal activity at pH 4.5-5.5 when using L-tyrosine as the substrate. Moreover, it has been reported that the optimum pH values for the cresolase activities of the PPOs from Paris mushrooms (*Agaricus bisporus*) [12] and Izmir grapes (*Vitis vinifera L.*) [13] are 7 and 7.4, respectively, when employing tyrosine as the substrate.

In general, the optimum pH of PPO falls within the range of 4 to 7, but this value can be influenced by various factors such as the enzyme's source, extraction method, enzyme purity, buffer composition, substrate type, and the method used to measure enzymatic activity. The wide pH variability among different PPO sources underscores the significance of considering these factors when studying and utilizing PPO in various applications.

Influence of temperature on polyphenol oxidase activity

The impact of temperature on PPO activity has not received as much attention as the influence of pH [3, 14]. In this study, we conducted an investigation to examine the impact of temperature (ranging from 30 to 70 °C under standard conditions) on the initial oxidation rate of tyrosine at a concentration of 2 mM, catalyzed by polyphenol oxidase.

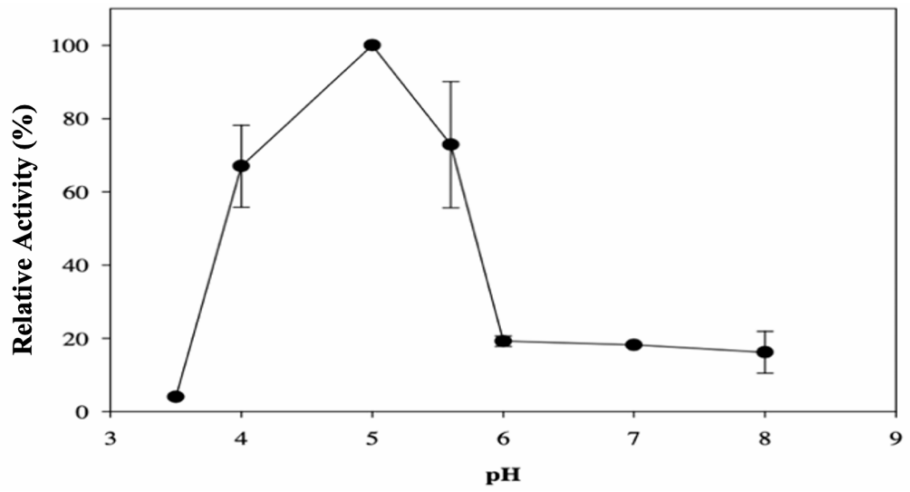

Figure 3. Effect of pH on the PPO activity of the desert truffle. ([Tyrosinase] = 2 mM (Sodium acetate buffer (0.05 M-pH [3.5 to 5.6]) and sodium phosphate buffer (0.05 M-pH [6.0 to 8.0]) reaction time: 8 min; 25 °C).

Agaricus bisporus

The graphical representation in Figure 4 exhibits an asymmetrical appearance, showcasing two distinct phases corresponding to different phenomena:

• The ascending phase: Within the temperature range of 20 to 45 °C, the enzymatic activity of tyrosinase increases with temperature. This elevation can be attributed to an augmented concentration of the activated complex as thermal energy is supplied to the reacting system. Notably, the enzymatic activity reaches approximately 1.055 IU/ml at 20 °C and 1.138 IU/ml at 45 °C. This segment of the curve represents the activation curve [15].

• The descending phase: Ranging from 45 to 70 °C, a decline in tyrosinase activity is observed, which can be attributed to the denaturation of the enzyme. In this phase, weak bonds, including hydrogen bonds, ionic bonds, and Van der Waals forces, are disrupted, leading to the loss of the enzyme's spatial configuration [14].

Under specified measurement conditions, a temperature of 45 °C, which corresponds to the maximum enzymatic activity (1.138 IU/ml), is identified as the optimum temperature for polyphenol oxidase. This temperature strikes a balance between promoting effective collisions and preserving most of the weak bonds that maintain the tertiary conformation of the protein [16]. Notably, Joslyn and Poyting [14] reported that tyrosinase exhibits maximum activity around 43 °C. The ascending segment of the relative activity curve as a function of reaction temperature (Figure 4) allows us to estimate the activation energy of the L-tyrosine oxidation reaction by tyrosinase. To accomplish this, we plotted the natural logarithm (denoted as Ln) of the initial hydrolysis rate against the inverse of the absolute temperature. As per Arrhenius' law, this should yield a straight line with a slope of '-Ea/R', where Ea represents the activation energy of the oxidation reaction, and R is the ideal gas constant. The activation energy often provides valuable insights into the chemical mechanism of the reaction at the enzyme level [9]. In this study, the thermal activation energy was determined to be 15.06 $KJ.mol^{-1}$. This result closely aligns with Gouzi's findings [17], who determined an activation energy of 18.6 $kJ.mol^{-1}$ for PPO derived from bananas.

Terfezia leonis

Based on Figure 5, it becomes evident that the activity of PPO is notably influenced by temperature changes. As the temperature gradually increases from 30 °C to 40 °C, the enzymatic activity of PPO shows a steady rise, reaching its maximum at 40 °C. However, beyond this point, a decrease in activity is observed, primarily attributed to the denaturation of the enzyme

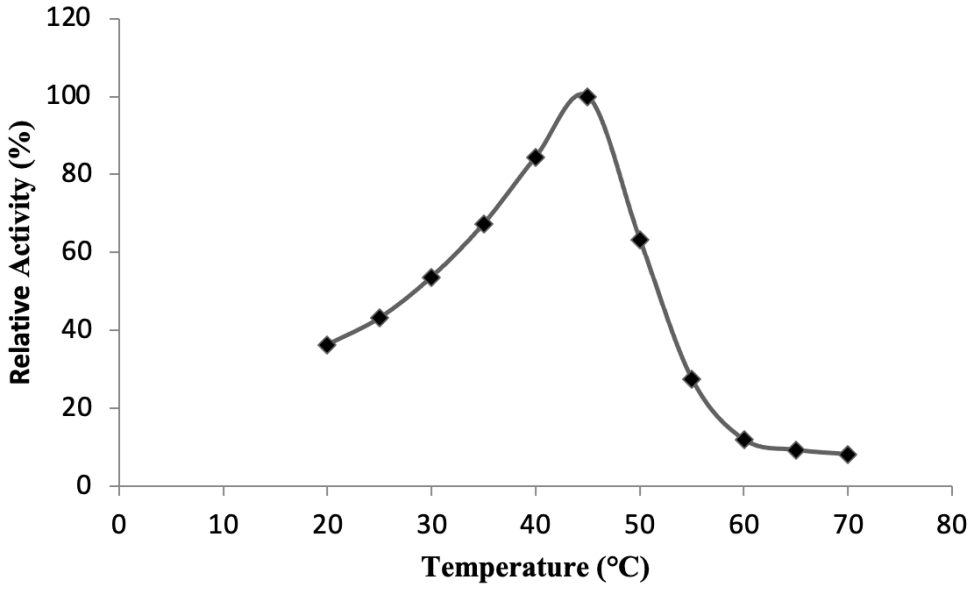

Figure 4. Effect of temperature on PPO enzymatic activity of Parish mushroom. ([Tyrosinase] = 2 mM; 0.05 M sodium acetate buffer-pH 5; reaction time 5 min; 25 °C).

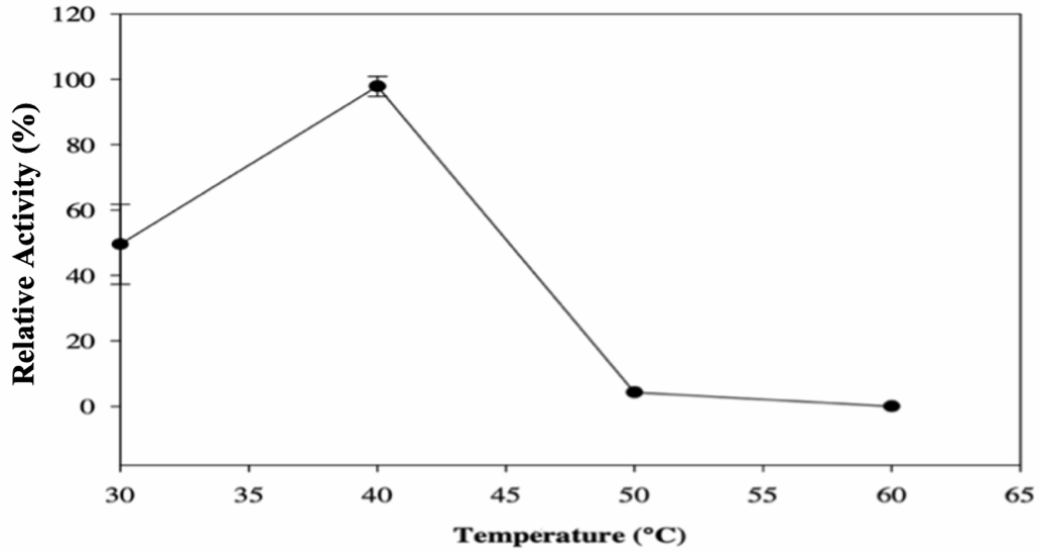

Figure 5. Effect of temperature on PPO enzymatic activity of desert truffle. ([Tyrosinase] = 2 mM; 0.05 M sodium acetate buffer-pH 5; reaction time 5 min; 25 ° C).

caused by excessive heat. The reduction in cresolase activity at higher temperatures is a consequence of structural alterations in the enzyme's secondary, tertiary, and quaternary structures. These findings closely resemble the results reported by Şimşek and Yemenicioğlu [18], who discovered that the monophenolase activity of tyrosinase from the edible mushroom *Agaricus bisporus* reaches its optimum at 40 °C. Moreover, the monophenolase activity of tyrosinase extracted from truffles (*Terfezia leonis*) appears to exhibit greater thermostability in comparison to the activity of PPO derived from edible mushrooms [8].

Determination of the kinetic parameters of polyphenol oxidase

Agaricus bisporus

To determine the kinetic parameters Km and Vmax of polyphenol oxidase at 25 °C and pH 5, we conducted experiments with varying substrate concentrations ranging from 0.2 to 2 mM. By measuring the rate of the L-tyrosine oxidation reaction catalyzed by tyrosinase at different substrate concentrations, we employed the Michaelis-Menten equation to evaluate Km and Vmax. For a more precise determination of these kinetic parameters, we employed the Lineweaver-Burk representation for non-saturating substrate concentrations. Subsequently, we expanded our analysis to include a broader range of substrate concentrations, from 0.2 mM to 6 mM, to gain a more comprehensive understanding. To better fit the experimental data, we employed a model that accounts for enzyme inhibition by excess substrate [19]. The curve Vin = f ([L-tyrosine]) displayed a deviation from the Michaelian model, which can be attributed to inhibition by excess substrate (see Figure 6). This model adjustment allows for a more accurate representation of the enzyme's behavior under varying substrate concentrations and sheds light on the interplay between enzyme activity and substrate concentration, contributing to a deeper understanding of polyphenol oxidase kinetics.

Based on the representation, the obtained values for the kinetic parameters are as follows:

Vmax = 0.072516316 ΔAbs/min;

Km = 0.113923132 mM;

Ks = 0.0676433951 mM;

r^2 = 0.999.

These precise values highlight the efficiency and accuracy of our experimental measurements, providing valuable insights into the enzymatic behavior under specific conditions. The high coefficient of determination (r^2 = 0.999) indicates a close fit between the experimental data and the model used, further affirming the reliability of our findings. These results contribute significantly to our understanding of polyphenol oxidase kinetics, particularly in relation to substrate interactions, and serve as a solid foundation for further research in this field.

Terfezia leonis

The kinetic parameters (Vmax and Km) for the oxidation of L-tyrosine by PPO were determined at 30 °C and pH 5. The results obtained reveal that the monophenolase activity of truffle PPO follows non-Michaelian allosteric kinetics. The analysis of

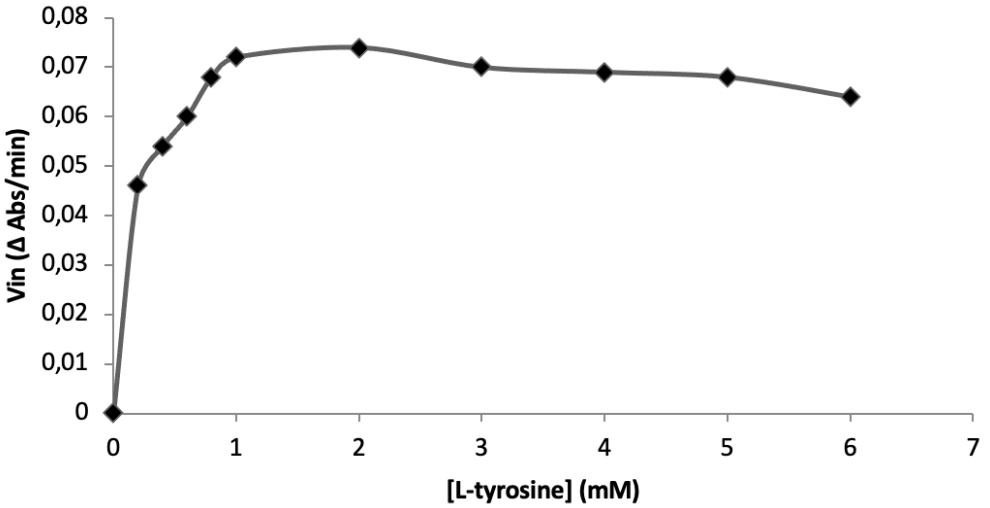

Figure 6. Effect of L-tyrosine concentration on the initial rate of the tyrosinase-catalyzed reaction (25 °C; 0.05 M sodium acetate buffer-pH 5.00).

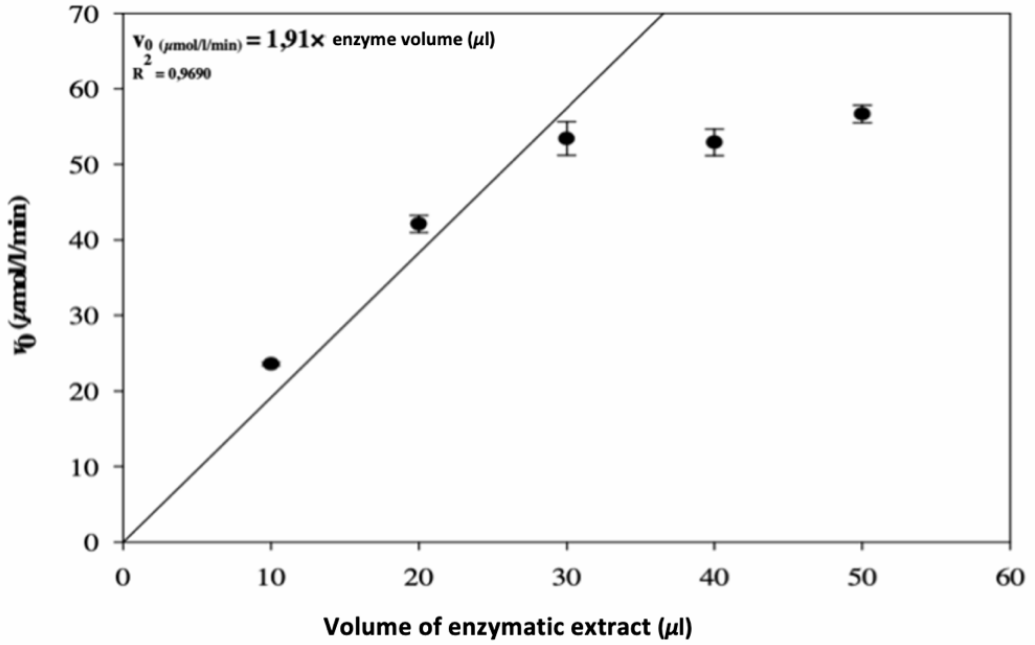

Figure 7. Initial rate of L-tyrosine oxidation as a function of PPO concentration of *Terfezia leonis*. (25 °C; 0.05 M sodium acetate buffer-pH 5.00).

the Hill graph yielded Km and Vmax values of 0.17 mM and 66.35 µM/min, respectively, with an r^2 value of 0.996, indicating a highly precise fit of the data.

To ascertain the kinetic parameters of PPO, it was essential to establish the enzyme concentration that would yield a linear response of the initial rate concerning the enzyme concentration. Figure 7 depicts the evolution of the initial rate of the L-tyrosine oxidation reaction by PPO at various enzyme concentrations. The initial rate of monophenolase activity exhibits a linear increase with the concentration of the enzyme in the reaction medium. This behavior aligns with observations made in monophenolase activities obtained from other sources [11, 20]. These findings also contribute to a deeper understanding of PPO kinetics and underscore the reliability of the obtained results for further investigations in this field.

CONCLUSION

Enzymes, as protein substances, play a pivotal role in facilitating and expediting biochemical reactions, and among them, polyphenol oxidase (PPO) holds significant importance in the browning process of mushrooms and various plant products. The objective of this study was to explore the kinetic properties of PPO extracted from Paris mushrooms and desert truffles. By investigating the effects of pH and temperature on tyrosinase activity, we identified an optimum pH of 5.00 and an optimum temperature of 45 °C, along with an activation energy of 15.06 $KJ.mol^{-1}$. Similarly, our investigation of the desert truffle *Terfezia leonis* revealed that its cresolase activity reaches the maximum potential at pH 5, with an optimum temperature of 40 °C. Furthermore, the impact of L-tyrosine concentration on enzyme activity exhibited non-Michaelian allosteric kinetics, yielding the maximum velocity (Vmax) and the Michaelis constant (Km) values of 66.35 µM/min and 0.17 mM, respectively. By gaining a deeper understanding of the mechanisms underlying enzymatic browning and exploring methods to mitigate its effects, researchers endeavor to enhance food quality, prolong shelf life, and maintain the appealing visual appearance of food products. The continuous progress in this field holds the promise of innovative solutions to minimize enzymatic browning in various food items, benefiting consumers and the food industry as a whole.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author upon request.

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This research received no external funding.

AUTHORS' CONTRIBUTIONS

All authors have contributed in the making of this article, and all authors have read and approved the version of the manuscript submitted for publication.

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None.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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