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Agaricus bisporus as a source of tyrosinase for phenol detection for future biosensor development

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Phenols are toxic compounds that are present in several industrial wastewaters, so their quantification has great environmental importance. In order to permit an analytical methodology for *in situ* monitoring, this work aims to study the application of *Agaricus bisporus* tissue as a source of tyrosinase and the optimum reaction conditions for the development of a phenol biosensor. Such an enzyme is a polyphenol oxidase that transforms many different phenolic compounds into quinones. Experiments with fungi tissue were performed to evaluate different sizes of tissue (0.5, 1.0 and 1.5 cm), different temperatures (23.5°C to 60°C), and different pH values (6, 7 and 8) to quantify analytically phenol content. Amongst the tested conditions, those that had presented larger efficiency in phenol oxidation were attained with the fungal tissue size of 1 cm, at pH 8.0, in the temperature range from 35°C to 45°C.

Keywords: mushroom tissue; biosensor; phenol; *Agaricus bisporus*; tyrosinase

Introduction

Phenolic compounds are extremely diverse and they have hazardous adverse effects [1], being considered one of the major pollutants present in wastewaters from several industrial activities: coal mining, petroleum refining, pharmaceutical production, steel and iron manufacturing, and leather tanning and finishing [2]. The US Environmental Protection Agency (EPA) lists 11 phenols as main pollutants. According to Article 34 from CONAMA 357/05 (The Brazilian Environmental National Council), concerning hazardous substances discharged into the aquatic environment, phenols in effluent should not exceed 0.5 ppm.

Phenolic compounds are produced as secondary metabolites by most plants, in which they probably act as natural antimicrobial agents, natural deterrents to grazing animals or inhibitors of pre-harvested seed germination. Furthermore, these compounds are also formed during the natural decomposition of humic substances, tannins, and lignins [3].

Several procedures have been developed for the quantitative determination of phenolic compounds, such as chromatographic and fluorimetric techniques, and spectrophotometric methods [4]. However, these techniques do not easily allow continuous monitoring in complex samples. They are expensive, time-consuming, need skilful operators, and sometimes require pre-concentration and extraction steps, increasing the risk of sample loss.

Biosensors are generally defined as an analytical device, incorporating biologically derived sensing elements, like enzymes, intimately associated with, or integrated within, a physicochemical transducer. They represent promising tools to supplement already existing techniques, due to their intrinsic characteristics such as selectivity, low cost, potential for miniaturization and for simple flow device construction in fast continuous monitoring [5]. In biosensors for phenol determination, the enzyme tyrosinase is commonly used as a biocomponent, which is isolated from mushrooms due to economic reasons [6].

Tyrosinases (monophenol, *o*-diphenol:oxygen oxidoreductase, EC 1.14.18.1) belong to a larger group of proteins named type-3 copper proteins. Tyrosinase has been widely distributed throughout the phylogenetic scale [7], catalyses the *o*-hydroxylation of monophenols (cresolase activity or monophenolase) and the subsequent oxidation of the resulting *o*-diphenols into reactive *o*-quinones (catecholase activity or diphenolase), using molecular oxygen. Subsequently, the *o*-quinones undergo non-enzymatic reactions with various nucleophiles, producing intermediates which spontaneously associate between each other forming dark brown pigments (Figure 1) [8–11].

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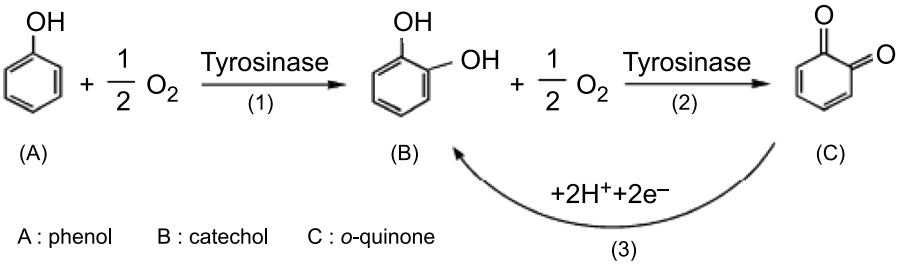

Figure 1. Simplified scheme of the reaction catalysed by the tyrosinase, where (1) represents the cresolase activity and (2) the catecolase activity.

This work aimed to study the possibility of using the tyrosinase naturally present in *Agaricus bisporus* mushroom tissue [12] to quantify the phenol contained in standardized samples. Some works have been published using mushroom tyrosinase as the biological component of a biosensor [6,10,11] and in phenol removal processes [13]. However, they do not use the enzyme in the tissue's natural immobilized form, i.e, *in natura* immobilization, which is the main objective of this work. *In natura* fungus was firstly used by Kameda *et al.* [14] in a phenol removal process (90% yield), demonstrating the viability of its utilization as a biocatalyst.

Materials and methods

Material

Fruit bodies of mushrooms (*A. bisporus*), usually known as champignon Paris mushrooms, used throughout this work were obtained from two local dealers: Cadeg and Cobal Humaitá (Rio de Janeiro – RJ, Brazil). In both places, mushroom batches used were always bought from the same retail outlet. Expiry dates of the mushrooms were equally taken into account.

Phenol determination

Phenol analysis was carried out using the standard methods for wastewater analysis with 4-amino-antipyrine (4AAP) [15]. Ammonium hydroxide (0.5M, 250 mL) was mixed with 10 mL of samples or blank (distilled water) and pH was adjusted to 7.9 ± 0.1 with potassium phosphate buffer. Subsequently, 100 µl of 4AAP solution (2% w/v) was added. Finally, 100 µl of potassium ferricyanide solution (8% w/v) was added to obtain the final mixture. Fifteen minutes later, the absorbance was measured at 500 nm. It is important to perform a solution clarification, each sample being previously filtered in a C18 PR-COLA cartridge for interference removal.

Phenol oxidation

The enzymatic reactions for phenol oxidation were carried out following some preliminary conditions. Mushroom tissue (5 g) was prepared in three sizes, 0.5, 1.0, and 1.5 cm, and put into an Erlenmeyer flask with a 250 mL aqueous phenol solution with an initial concentration of 10 ppm; media agitation at 200 rpm within an orbital agitator (Tecnal TE – 420) at 30°C. Grab samples of 7 mL were taken from the flasks as follows: with the first sample considered as 0 min, at intervals of 30 min up to 180 min. After each sample collection, the reaction was suspended by inactivating the enzyme through a heat shock (100°C) for 5 min.

All experiments were repeated five times and the graphics were developed with the averages of replicates and their deviations.

Enzymatic reactions

Size effect of the mushroom tissue

The capability for removing phenol was tested using different sizes of mushroom tissue. The enzymatic reactions were carried out with mushroom tissue of cubic shape, the sides of which measured 0.5 cm [16], 1.0 cm, and 1.5 cm. The conditions were kept the same as the tests detailed in *Phenol oxidation*. The mushroom was manually cut using a sterile bistoury. Gloves were used, so it is presumed that the tissues were free of undesirable substances that could be present on operator skin.

Temperature effect

The behaviour of tyrosinase in the mushroom tissue was verified by quantifying the phenol content at different temperatures: 23.5°C to 60°C. The assays were carried out with phenol solutions (250 mL) of 10 ppm and mushroom tissue of 1.0 cm at 200 rpm.

pH effect

The effect of pH on *in natura* tyrosinase catalysis was verified. Phenol samples had the following composition: initial concentration of 10 ppm (250 mL) prepared with sodium phosphate buffers in order to get different pH values (6.0, 7.0, and 8.0). The pH value was measured

directly through a calibrated pH-meter. The temperature of the assays was kept constant at 45°C. The size of the mushroom tissue was 1 cm and the agitation rate was 200 rpm.

Presence of phenol in mushroom tissue

Enzymatic reactions to verify phenol presence in mushroom tissue were carried out under the following conditions: 5 g of mushroom tissue size 1.0 cm were added to 250 mL of distilled water, free of phenol and chlorine, under an agitation speed at 200 rpm and temperature of 30°C. Phenol released from the mushroom tissue was quantified.

Phenol absorption in mushroom tissue

The analysis of phenol absorption in the mushroom tissue was verified through enzymatic reactions by comparing the results obtained by phenol extraction. Raw or preheated (previously boiled at 100°C for 5 min) tissues were used. The accomplished tests had the following conditions: 5 g of medium sized (1.0 cm) mushroom tissue were immersed in an aqueous phenolic solution (10 ppm), at 200 rpm and temperature of 30°C.

Results and discussion

Size of mushroom tissue

Three different sizes of mushroom tissue (0.5, 1.0, and 1.5 cm) were initially tested. The initial experiments carried out with the largest size (1.5 cm) showed a small variation in sample colour throughout 180 min. During the phenol quantification of the initial and final samples, this small colorimetric variation shows that little significant oxidation of phenol occurred. This method is based on reaction of phenolic compounds with potassium ferricyanide and 4-amino-antipyrine to form a red complex whose absorbance is measured at 500 nm. Due to insignificant variation between the initial and the final phenol concentration using the largest size of tissue, this size was discarded. Figure 2 shows the variation of the phenol concentration during the experiment course.

At zero-time, the curves show an initial concentration exceeding 10 ppm of phenol, meaning that the mushroom tissue itself can possibly be responsible for additional phenol content. In fact, experiments showed that phenol is present in the mushroom's composition, with an average concentration of 0.307 ppm (Figure 3).

The results herein showed that the best size of the fungus tissue is 1.0 cm. Phenol removal efficiency was approximately 64% of its initial content after a

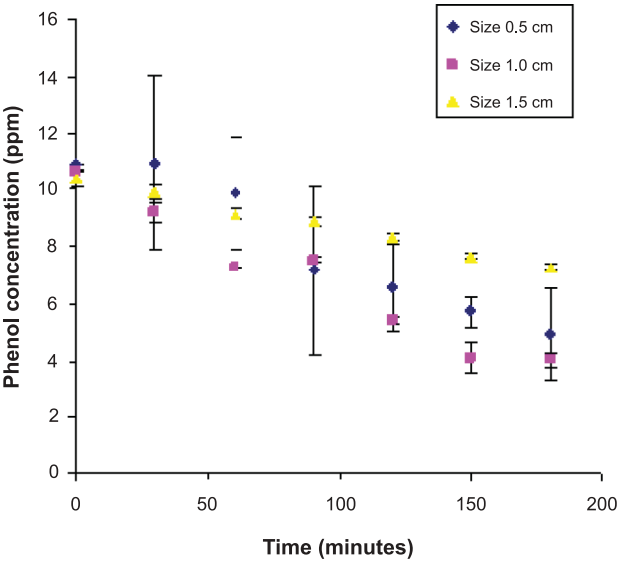

Figure 2. Phenol oxidation with mushroom tissue with sizes of 0.5, 1.0, and 1.5 cm, 30°C.

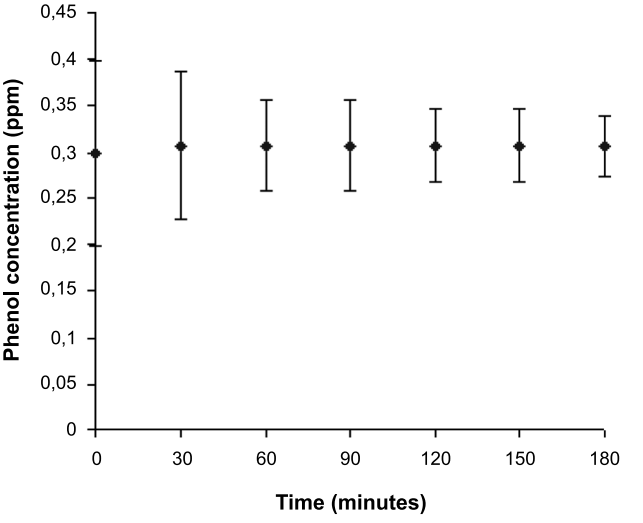

Figure 3. Tests to quantify the phenol concentration in the fungus tissue of size 1.0 cm, with homogeneity in initial point, over 180 min.

180-min running test. This result was particularly unexpected in comparison with the good results of phenol removal reported by Dec and Bollag [16] using vegetable tissue. Nevertheless, plants and fungi are different in morphology and structure, and therefore the medium size of mushroom tissue was chosen due to the good results on phenol removal compared with the results obtained by Dec and Bollag [16] using the smallest plant tissue with peroxidases (0.5 cm). On the other hand, due to a smaller surface–volume relationship, it could be expected that the tests herein described with fungus tissue (1.0 cm) would be less

efficient on removal of phenol when compared with the smallest size.

Temperature of the reaction

The choice of the best reaction temperature was accomplished through the behaviour of tyrosinase naturally entrapped in the mushroom tissue under different temperatures. The residual phenol was quantified at the end of each reaction at different temperatures.

The initial tests were accomplished at temperatures closest to room value (23.5°C, 28°C, 30°C, and 35°C), since to construct a biosensor high temperatures may not be appropriate due to the additional step required to heat samples in a specific apparatus being disadvantageous when *in situ* monitoring is desired. High temperatures may also cause physicochemical changes in other compounds present in real samples (industrial effluents) when the biosensor used is still developing.

Based on the results of these tests, and the work of Topçu *et al*. [10], additional tests were accomplished at higher temperatures, namely: 40°C, 45°C, 50°C, 55°C, and 60°C.

The average efficiency of phenol removal was evaluated for each studied temperature and the results are shown in Figure 4. The removal rates varied between 39.2% and 75.13%. These data are confirmed by Topçu *et al*. [10], where higher temperatures resulted in larger phenol removal rates.

Marín-Zamoura *et al*. [11] studied the effect of temperature on catalytic activity of immobilized tyrosinase of *A. bisporus* in the range 20–70°C and they obtained maximum enzymatic activity at 55°C. The response of the optical biosensor developed by Abdullah *et al*. [17], who used tyrosinase immobilized on chitosan, comparing the temperature range of 25°C–60°C showed that increasing temperature promotes the increase of enzyme activity, reaching maximum at 45°C. Campanella *et al*. [18], developing an enzyme sensor with tyrosinase for determination of phenol, analysed the influence of temperature on the activity of the instrument. When using both Tris and phosphate buffers, the sensitivity increased in the range below 40°C. However, although the temperature seemed to be best above 25°C, there were some problems such as: increased time for stabilization of the baseline and unavoidable excessive noise in the signals detected by the instrument. Furthermore, the reproducibility of the measurements decreased with increasing temperature and also, consequently, the rate of deactivation of the enzyme, shortening the life of the sensor. Therefore, 25°C was chosen as the temperature of activity of the enzyme sensor developed.

Considering such results, it is possible to observe that the largest removal rates occurred in the range 35°C–45°C. To choose the best condition for *in natura* immobilized tyrosinase catalysis, taking into account that the main objective of the work is to obtain adequate conditions for allowing, in future, the development of a phenol biosensor, it is believed that room temperature (24°C ± 1°C) could be an adequate temperature. This is because it possibly increases the biosensor stability and would not require the use of apparatus for heating the sample. Since a main objective of the work is to develop equipment for *in situ* monitoring, then lower interference influence, fast response, and instrument stability must be considered. Such conditions lead to as simple and lightweight a device as possible. Negative results for phenol absorption in the fungus tissue (Figure 5) were obtained, which is an interesting result since its

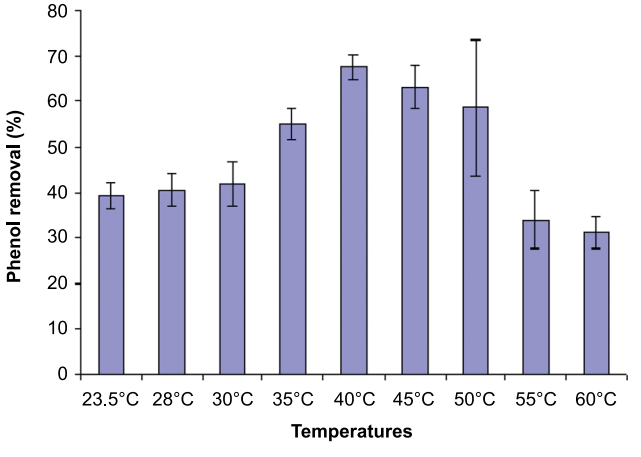

Figure 4. Comparison between the efficiency of action of tyrosinase at different temperatures tested over 3 h with fungus tissue 1.0 cm in size.

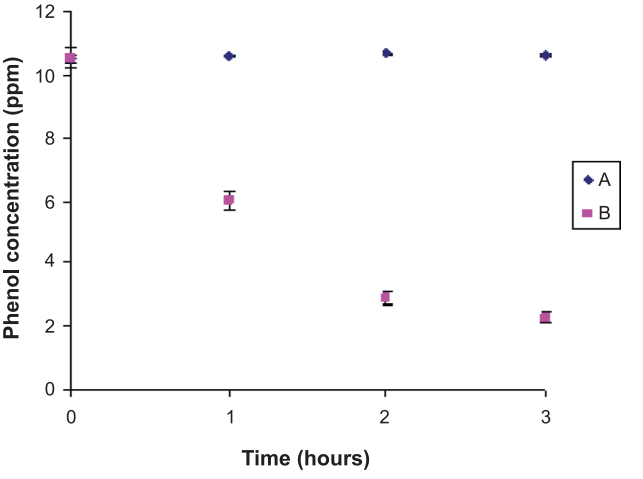

Figure 5. Test results of study of absorption of phenol in mushroom tissue 1.0 cm in size, where (A) represents the heat treatment procedure using the tissue and (B) no treatment.

reuse several times is desired as soon as the biosensor is mounted.

Reaction pH

The analysis of pH influence on phenol removal is presented in Figure 6. It was identified that the optimum pH is 8.0, as described by Topçu *et al*. [10].

The removal efficiency curves obtained at pH values of 6.0 and 7.0 showed some influences during the reaction, probably due to the phenol quantification method [15]. The addition of a significant amount of ammonium hydroxide to increase pH samples from 6.0 and 7.0 to 7.9 ± 0.1 may have affected the results and led to unstable profiles.

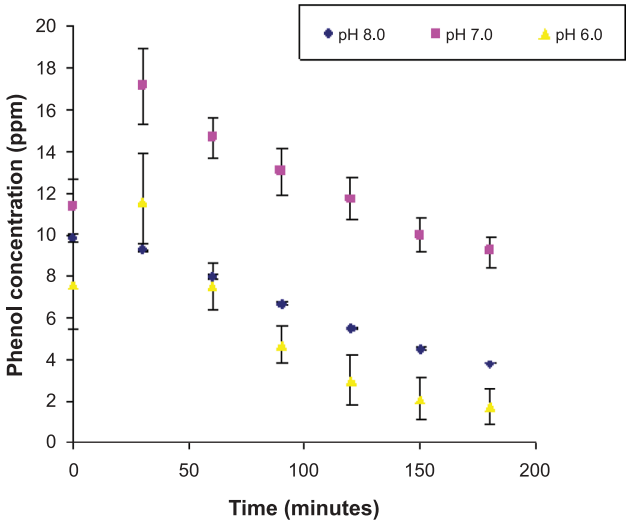

Figure 6. Comparison between phenol oxidation from the standard solution using sodium phosphate buffer containing different pH values, 45°C, and fungus tissue with size of 1.0 cm.

Conclusions

Being an excellent source of the enzyme tyrosinase, mushroom tissue (*A. bisporus*) presents itself as a possible biocomponent of an amperometric biosensor for detection of phenolic compounds. However, it is necessary to take into account that it presents phenol in its own composition, producing interference in the starting point (0 min) of the tests. In parallel, the possible absorption of phenol in the tissue did not occur.

The dimensions of the tissue, in a cube form near to 1.0 cm, promoted efficient phenol conversion, which indicates the possibility of the use of this tissue in a future biosensor. This is very useful since it is not necessary in this case to extract the enzyme. The experimental conditions of pH 8.0 and a temperature between 35°C and 45°C were established as adequate for the performance of the enzyme tyrosinase *in natura* immobilized. This fact is another important step for the future manufacture of a biosensor, since the results are in agreement with the literature.

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