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ScienceDirect
Dyes and Pigments 75 (2007) 32–37
DYES and PIGMENTS
www.elsevier.com/locate/dyepig

Banana skin: A novel waste for laccase production by *Trametes pubescens* under solid-state conditions. Application to synthetic dye decolouration

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Received 23 February 2006; received in revised form 6 May 2006; accepted 8 May 2006
Available online 30 June 2006

Abstract

In this paper, we investigated the potential of banana skin as a support-substrate for the production of extracellular laccase by the white-rot fungus *Trametes pubescens* CBS 696.94. Laccase showed a maximum activity of 1570 U/l. In addition, we assessed the degrading ability of the extracellular liquid obtained. For this, we performed the *in vitro* decolouration of two structurally different dyes such as the anthraquinonic dye Remazol Brilliant Blue R (RBBR) and the triphenylmethane dye Methyl Green (MG). The former was decolourised about 57% in 4 h, whereas the latter presented a lower decolouration rate (40.9% in 4 h). Interestingly, RBBR decolouration was considerably higher than that attained by a commercial laccase (23.2% in 4 h), whereas MG decolouration (46% in 4 h) was very similar for both laccases. This shows the high potential of *T. pubescens* laccase for synthetic dye decolouration, especially for anthraquinonic dyes.

Keywords: Banana skin; Laccase; Methyl Green; Remazol Brilliant Blue R; Solid-state fermentation; *Trametes pubescens*

1. Introduction

Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) have been the subject of continuous study since the end of the 19th century. The genus *Trametes*, which belongs to the white-rot fungi, is assumed to be one of the main producers. Among them, *Trametes pubescens* has been described as a promising laccase producer [1]. The biotechnological importance of this enzyme lies in its ability to oxidise both phenolic and non-phenolic lignin-related compounds [2,3] as well as highly recalcitrant environmental pollutants [4,5].

Solid-state fermentation (SSF) is defined as any fermentation process occurring in absence or near absence of free liquid, using an inert substrate or a natural substrate as a solid support [6]. The former only functions as an attachment place for the microorganism, whereas the latter also acts as a carbon source, which considerably reduces the production costs [7]. SSF is advantageous in obtaining concentrated metabolites and subsequent purification procedures are economical [8]. In SSF, the microorganisms grow under conditions close to their natural habitat. This may allow them to produce certain enzymes and metabolites, which usually would not be produced or would only be produced at a low yield in submerged cultivation [6].

The selection of an adequate support for performing SSF is essential, since the success of the process depends on it. The most important factors to take into account are particle size, porosity and chemical composition. In addition to this, availability and cost are also criteria of great importance. In recent years, there has been an increasing trend towards the utilisation of organic wastes such as residues from the agricultural, forestry and alimentary industries as raw materials to produce value-added products by SSF technique [9]. The use of such wastes, besides providing alternative substrates, helps to solve

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doi:10.1016/j.dyepig.2006.05.021

environmental problems, which are caused by their disposal. Furthermore, most of these wastes contain lignin or/and cellulose and hemicellulose, which act as inducers of the ligninolytic activities. Moreover, most of them are rich in sugars, which make the whole process much more economical. All these make them very suitable as raw materials for the production of secondary metabolites of industrial significance by microorganisms. In particular, the present study focuses on laccase production by *T. pubescens.*

Banana skin has been selected to perform the present study due to its high content in carbohydrates, which due to their organic nature are easily metabolised by microorganisms, and it has the physical integrity to serve as a supporting material. In addition, its content in ascorbic acid exerts an inhibitory effect against bacteria [10]. Moreover, the banana processing industry generates a huge amount of solid wastes, which are dumped in landfills, rivers, oceans and unregulated dumping grounds. Therefore, their reutilisation would help to diminish the pollution problems caused by their disposal.

The goal of the present paper was to investigate the potential of banana skin as a support-substrate for the production of laccase by *T. pubescens* under SSF conditions, since the utilisation of such a support would mean an important reduction in production costs. It is very interesting to find new ways of producing laccase with higher activities at lower cost due to the enormous potential that this enzyme offers for the development of efficient biotechnology processes (biopulping, biobleaching, treatment of wastewater, etc.). To the best of our knowledge, this is the first report on laccase production using banana skin as a support-substrate.

2. Materials and methods

2.1. Microorganism

T. pubescens (CBS 696.94) was maintained on malt extract agar (MEA) plates at 4 °C and sub-cultured every three months.

2.2. Waste material

Chopped banana (*Musa cavendishii*) skins (particle size 7.5 mm × 7.5 mm), purchased at a local market, were used as support-substrates for laccase production by *T. pubescens* under SSF conditions. Table 1 shows the composition of the banana skin.

Banana skins were pre-treated as follows: they were first soaked for an hour in 30 ml of KOH 83.17 mM (10 g of fresh support) to neutralise organic acids [11]. Then, they were thoroughly washed with distilled water and dried at moderate temperature. Prior to use, the skins were autoclaved at 121 °C for 20 min.

2.3. Culture conditions

The composition of the culture medium was prepared according to Rodríguez Couto et al. [12] except that glucose was at a concentration of 2 g/l. The cultures were performed in cotton-plugged Erlenmeyer flasks (250 ml) containing 7 g of chopped banana skins and 20 ml of culture medium. Inoculation was carried out directly in the Erlenmeyer flasks. Three agar plugs (diameter, 7 mm), from an actively growing fungus on MEA, per Erlenmeyer were used as inoculums. The Erlenmeyer flasks were incubated statically under an air atmosphere at 30 °C and in complete darkness.

Table 1
Chemical composition (%dry matter) of the banana skin [10]

Compound (g per 100 g)	
Dry matter	14.08
Crude protein	7.87
Crude fat	11.60
Crude fibre	7.68
Total ash	13.44
Carbohydrates	59.51
Moisture	78.4
Mineral and ascorbic acid content (mg per 100 g)	
Calcium	7
Sodium	34
Phosphorus	40
Potassium	44
Iron	0.93
Magnesium	26
Sulphur	12
Ascorbic acid	18

2.4. Analytical determinations

Laccase activity was determined spectrophotometrically as described by Niku-Paavola et al. [13] with ABTS (2,2′-azino-di-[3-ethyl-benzo-thiazolin-sulphonate]) as a substrate. One activity unit was defined as the amount of enzyme that oxidised 1 μmol ABTS per minute. The activities were expressed in U/l.

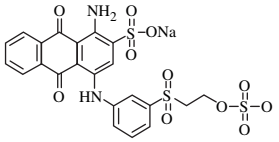
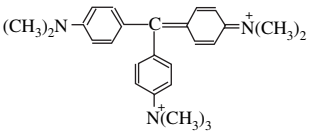
2.5. Decolouration studies

The dyes used were Remazol Brilliant Blue R (RBBR), purchased from Sigma Aldrich (St. Louis, MO, USA) and Methyl Green (MG), purchased from Merck (Germany). The characteristics of the dyes are summarised in Table 2. Stock solutions (0.1% w/v in water) were stored in the dark at room temperature.

Culture broth from banana skin cultures of *T. pubescens*, collected on day 14, and a commercial laccase (EC 1.10.3.2), supplied from Novo Nordisk (Denmark), were used for dye decolouration experiments.

The reaction was carried out directly in the spectrophotometer cuvette and the reaction mixture (final volume 1.5 ml) consisted of an aqueous solution of dye and extracellular liquid or commercial laccase (300 U/l, final concentration) in succinic buffer (pH 4.5). Dye concentrations were selected in order to obtain around 1.3 absorbance units at the maximum wavelength in the visible spectrum (0.133 g/l for RBBR and 0.033 g/l for MG, final concentration). All the reactions were incubated at room temperature, in static conditions and in complete darkness.

Table 2
Characteristics of the dyes employed

Dye	Class	λ_{max} (nm)	CI number	CI name	Structure
Remazol Brilliant Blue R	Anthraquinonic	595	61 200	Reactive Blue 19	
Methyl Green	Triphenylmethane	630	42 585	Basic Blue 20	

CI = colour index.

The residual dye concentration was measured spectrophotometrically and was associated with the decrease in the absorbance at the peak of maximum visible wavelength (595 nm for RBBR and 630 nm for MG). Dye decolouration was expressed in terms of percentage. A control test containing the same amount of a heat-denatured laccase was performed in parallel. The assays were done twice, the experimental error being below 10%.

2.6. Microscopic examination

Banana skin samples were fixed with 6% glutaraldehyde in phosphate buffer 0.1 M for 4 h. Afterwards, the samples were washed twice with phosphate buffer 0.1 mM for 15 min at 4 °C. Then, the samples were post-fixed with osmium tetroxide 0.1 M at 4 °C. Following fixation, the samples were washed twice with phosphate buffer 0.1 mM for 15 min. After that the samples were dehydrated through an ethanol–amylacetate series to pure amylacetate and critical point dried using CO_2 as the transition liquid. The dried samples were mounted on aluminum stubs, sputter coated with gold (20 nm) and examined with a Jeol 6400 scanning electron microscope (SEM) at 15 kV, belonging to SRCiT (Scientific and Technical Services) of the Rovira i Virgili University (Tarragona, Spain).

2.7. Mathematical analysis of the SEM images

SEM images were analysed using MATLAB 6.5 (MathWorks Inc., Natick, MA). The relief of the SEM images was obtained by a cross-section analysis of the gray-scale information. Discrete Fourier Transformation (DFT) was applied to the cross lines of SEM images in order to obtain the frequency information according to the following equation:

$$X(k) = \sum_{n=1}^{N} x(n) e^{(2j\pi(k-1)(n-1)/N)}, \quad 1 \le k \le \frac{N}{2} \tag{1}$$

where

N is the number of pixels of one line of the SEM images
j is the imaginary unit
$x(n)$ is the normalised gray-scale value of each nth-pixel
$X(k)$ is the kth armonic component of the signal

3. Results and discussion

3.1. Laccase production

As shown in Fig. 1, laccase production began on the 3rd day (63 U/l) and, then, it sharply increased up to a maximum activity of nearly 1600 U/l at the end of the cultivation. No other ligninolytic activities were detected in the extracellular culture. This is very interesting for the subsequent application of this enzymatic complex to the biotechnological processes, since the purification stage would be more economical.

3.2. Microscopic examination

The most important characteristics that influence adhesive behaviour of filamentous fungi to the support are hydrophobicity and surface charge. Fig. 2 shows SEM microphotographs of banana skin with (A) and without (B) fungus. It can be

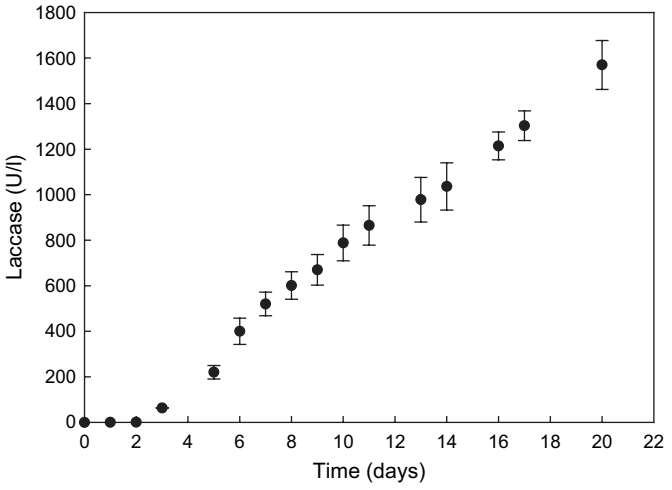


Fig. 1. Laccase production by solid-state cultures of *T. pubescens* grown on banana skins.

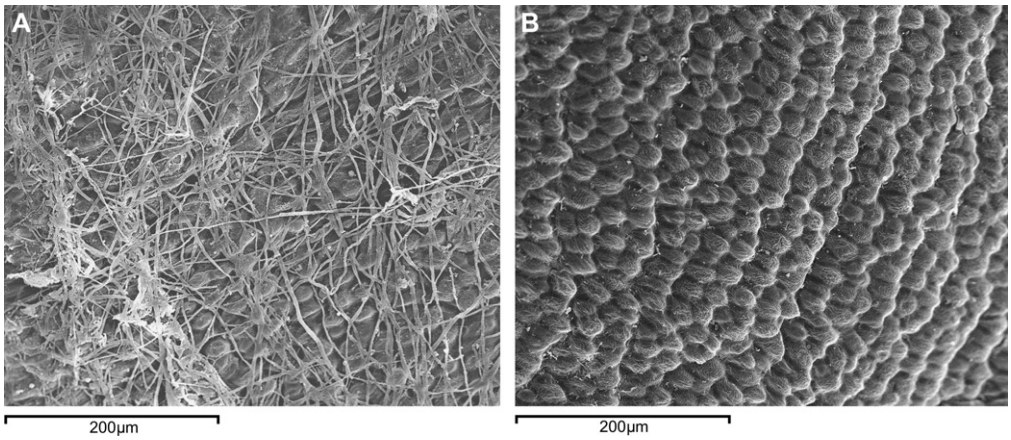


Fig. 2. SEM microphotographs of banana skin: (A) with fungus; (B) without fungus.

observed that the fungus grew well attached to the banana skin. This is due to the high hydrophobicity of the banana skin, which eases the attachment of the fungus to the carrier [14]. Therefore, banana skin is very suitable as an attachment place for filamentous fungi. This together with its high content in carbohydrates (Table 1) makes banana skin very suitable as a support-substrate to perform solid-state processes.

SEM images were analysed by software techniques in order to obtain information about the position of the fungus on the banana skin cells. Dotted line in Fig. 3 represents the cross-section of the banana skin cells, a cyclic shape can be appreciated and represents the position of each cell. Higher values correspond to the upper part of the cells; meanwhile, lower values correspond to the space between cells. Solid line represents the cross-section of the banana skin with fungus on it. More frequent peaks can be noticed as an indicator of the tubular structure of the fungus. Also, big covered areas show places where the fungus agglomerates. The growth of the fungus is not regular and does not cover symmetrically the surface of the cells; it is concentrated in different places where carbon source is surely more accessible. It also presents tubular structures that make clusters and grow irregularly, not following a specific parameter.

Fig. 4 presents a Fourier frequency analysis of the same SEM images, where low frequency peaks correspond to the size of the cells and high frequency peaks correspond to the tubular structure of the fungus and the roughness of the cells. Fourier analysis shows the mean size of the banana skin cells through the vertical and the horizontal axes (17 and 25 µm, respectively).

3.3. Decolouration studies

The ability of white-rot fungi to decolourise synthetic dyes has been widely studied, particularly with *Phanerochaete chrysosporium* and *Trametes versicolor* [15]. In the present study, we assessed the ability of the extracellular fluid from

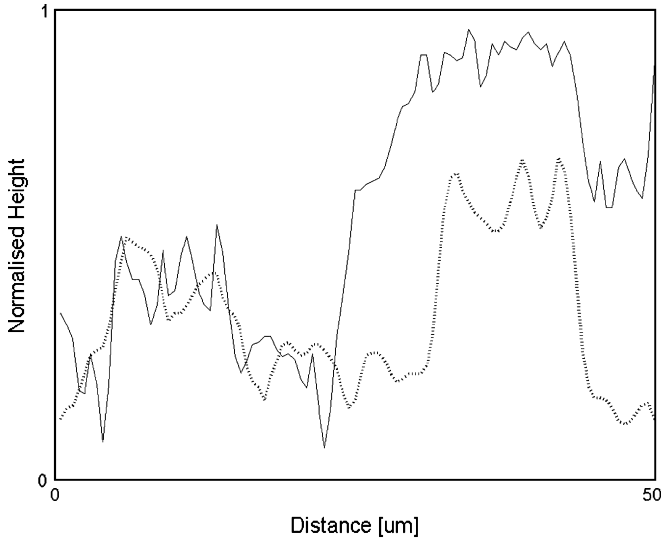


Fig. 3. Cross-sections of banana skin with (solid line) and without fungus (dotted line).

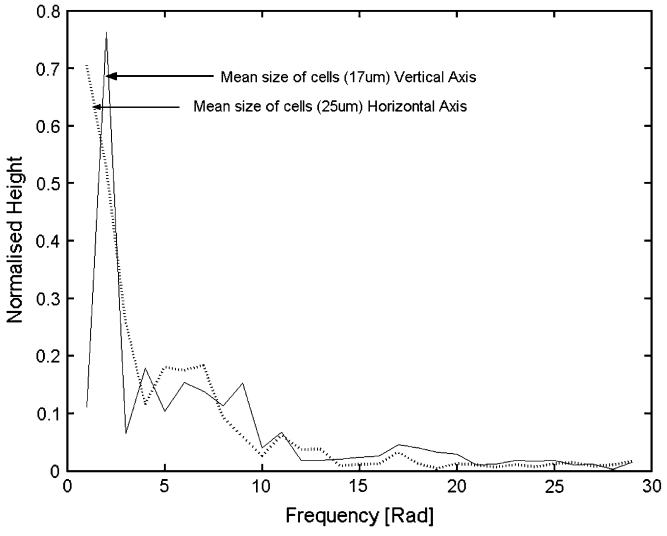


Fig. 4. Fourier frequency analysis of the cross-section of the banana skin with fungus. Vertical axis analysis (solid line) and horizontal axis analysis (dotted line). The two peaks, 25 and 17 µm, correspond to the second and third armonics, respectively, of the Discrete Fourier Transformation (DFT) with $N = 50$ (1 pixel = 1 µm) extracted from information in Fig. 3 (cross-section of banana skin with fungus). The DFT equations used can be found in the following link: http://astronomy.swin.edu.au/~pbourke/other/dft/.

T. pubescens, a little studied white-rot fungus, to decolourise two structurally different synthetic dyes (RBBR and MG). The decolourisation of type model dyes is a simple method to assess the aromatic degrading capability of ligninolytic enzymes [16].

As it can be seen in Fig. 5, the decolouration rate obtained was very different in each case. Thus, RBBR showed a degree of decolouration about 57% after 4 h of treatment. However, from here onwards decolouration proceeded very slowly and it reached a value of 84.5% in 21 h. This could be due to either enzyme inhibition by some products generated in the decolouration process or substrate inhibition. These results differ from those found by Soares et al. [17], who reported that the addition of a redox mediator was necessary for RBBR decolouration by a laccase from a genetically modified *Aspergillus* microorganism. The discrepancy between our results and those from Soares [17] could be due to the difference in fungal species from which the laccase was obtained. Also, the redox potential of laccases varies depending on the laccase source [18], which could also dictate the need of a redox mediator for the decolouration of a particular dye to occur.

MG was decolourised about 41% in 4 h reaching a decolouration of 96.4% after 21 h. So, the decolouration rate was slower than that of RBBR but the final decolouration was higher (Fig. 5). It has been reported that highly substituted triphenylmethane dyes required longer time to be decolourised or could only be decolourised to a certain extent [19].

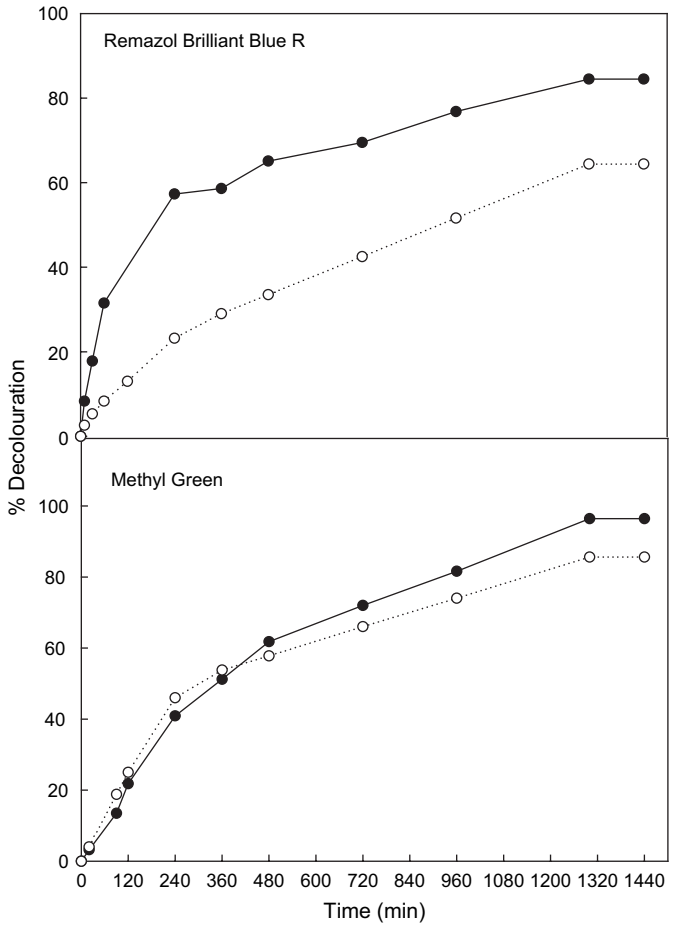


Fig. 5. Profile of dye decolouration attained: (●) extracellular liquid from banana skin cultures of *T. pubescens*; (○) commercial laccase.

When a commercial laccase was used, RBBR decolouration was significantly lower than that attained by laccase from banana skin cultures of *T. pubescens*. On the other hand, MG decolouration was very similar for both laccases (Fig. 5).

It was observed that from 24 h of incubation onwards decolouration did not increase (data not shown). This could be due to enzyme inhibition by some products generated in the decolouration process.

Since equal doses (300 U/l) of laccases were used in the decolouration process, the difference in the decolouration efficiency of the two laccases was most likely due to the difference in laccase isoenzymes produced by the different strains as well as due to the difference in specificities to different dyes of diverse structures [20]. In addition, as commented above, it could also be due to the difference in the redox potential of laccases from different microorganisms.

The fact that *T. pubescens* was able to decolourise the dye RBBR with no mediator addition is very interesting, since this dye is frequently used as a starting material in the production of polymeric dyes. Also, it represents an important class of often toxic and recalcitrant organopollutants [21].

4. Conclusions

The results clearly showed the enormous potential of banana skin as a support-substrate for the production of laccase by *T. pubescens* under solid-state conditions. In addition, the laccase produced presented a highly decolourising ability, especially for anthraquinonic dyes. This makes laccase from this fungus very attractive for further investigations as well as for its application to different biotechnology areas. More studies in order to optimise the culture conditions and the decolouration process are underway in our laboratory.

Acknowledgements

SRC and JLTH are *Ramón y Cajal Senior Research Fellows*. Therefore, the authors thank the Spanish Ministry of Education and Science for promoting the *Ramón y Cajal Programme*. JFO is supported by a predoctoral fellowship from the Rovira i Virgili University.

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