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Image Analysis Technique as a Tool to Identify Morphological Changes in *Trametes versicolor* Pellets According to Exopolysaccharide or Laccase Production

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Abstract Image analysis technique was applied to identify morphological changes of pellets from white-rot fungus *Trametes versicolor* on agitated submerged cultures during the production of exopolysaccharide (EPS) or ligninolytic enzymes. Batch tests with four different experimental conditions were carried out. Two different culture media were used, namely yeast medium or *Trametes* defined medium and the addition of lignolytic inducers as xylidine or pulp and paper industrial effluent were evaluated. Laccase activity, EPS production, and final biomass contents were determined for batch assays and the pellets morphology was assessed by image analysis techniques. The obtained data allowed establishing the choice of the metabolic pathways according to the experimental conditions, either for laccase enzymatic production in the *Trametes* defined medium, or for EPS production in the rich Yeast Medium experiments. Furthermore, the image processing and analysis methodology allowed for a better comprehension of the physiological phenomena with respect to the corresponding pellets morphological stages.

Keywords Filamentous fungi · Pellets morphology · Image analysis · Enzyme activity · Exopolysaccharide production · Pulp and paper effluent

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Introduction

Bioresources, namely enzymes, microorganisms, and biological raw materials, are becoming progressively more important in industrial technology and biotechnology. Target solutions for the future in different areas such as energy supply and biofuels production, medical and pharmaceutical advances, new material proposals, and also new environmental applications are being built up by a wide range of bioprocess technologies.

Filamentous fungi are eukaryotic organisms with a lot of potential due to their different metabolisms that can be activated or repressed according to the conditions, namely inducer presence or environmental signals. A high number of important biotechnological commercial products have origins in fungi bioprocesses [1]. Penicillin G production has been one of the most important and studied bioprocesses [2, 3] for a long time, but progressively different enzymes for diverse industrial applications [4–6] as well as pharmacological target products obtained by genetically engineered specific strains are also being widely studied [7]. A lot of different products can be obtained using the same filamentous fungus with specific and different experimental culture conditions [8].

The knowledge of morphological form has been shown to have an important influence on the metabolism and productivity of the filamentous fungi [9, 10]. Image analysis has become a relevant tool for characterizing the morphology and growth of filamentous microorganisms and also to characterize the size and shape quantitatively [1, 11]. By this technique, it was possible to characterize the mycelium morphology and to estimate the viability by the morphology of the filamentous fungus *Trichoderma reesei* [12]. Measurement and simulation of morphological development [13] and definition of a mathematical model for apical growth, septation, and branching of *Streptomyces tendae* [14] were also made by means of image analysis.

Basidiomycetes white-rot fungi are responsible for the most extensive biodegradation of lignin, but a few studies relate metabolic products with morphology. They have a powerful extracellular enzymatic complex, able to depolymerize this aromatic biopolymer into lower molecular weight compounds, which can then be assimilated and used for growth and reproduction [15]. Higher fungi are heterotrophic and chemotrophic nonmotile organisms that produce enzymes as a way of surviving to environmental changes. These changes constitute one or more of a multitude of environmental signals that induce specific changes in growth and development patterns as a fungus response [16]. *T. versicolor* is a basidiomycete that has an efficient degradation capacity of lignin and produces three lignolytic enzymes, namely lignin peroxidase, manganese peroxidase and laccase. When other substrates are not available, the role of these enzymes on woods is the delignification of hemicelluloses by oxidative reactions in order to ensure survival of these carbon staffs. The biocatalytic potential of *T. versicolor* has been studied for different industrial applications such as bleaching of Kraft pulps [17, 18], effluents decolorization and biotreatment [19] and also to find out oxidation mechanisms with lignin models [20, 21]. This fungus is also reported to be a medicinal fungus producing an exopolysaccharide with antitumor activity when it is grown in specific experimental conditions [22]. When filamentous fungi are in liquid media, they grow on submerged cultures presenting either a nearly uniform suspension of hyphae (filamentous growth) which are freely dispersed in the medium or presenting pellet formation where mycelium grows in spherical aggregates presenting discrete pellets (pellets growth) [11, 23].

The objective of this work was to use image analysis techniques to study morphological pellet changes of *T. versicolor* under specific culture media conditions. The pellet morphological dependence of batch-stirred liquid submerged cultures during the production of laccase or during production of exopolyssacharide (EPS) was evaluated. For this purpose, a comparative study of

batch-submerged cultures with different culture media was carried out. This technique was applied to study morphological pellet changes under different metabolic pathways of *T. versicolor* based on its environment growth. The equivalent diameter, eccentricity, roundness, and solidity were used to evaluate morphological pellet changes.

Material and Methods

Microorganism and Kraft Effluent

White-rot fungus *Trametes versicolor* used was kindly given by the National Institute of Industrial Engineering and Technology (INETI, Portugal). The growth of mycelium was held on Petri plates at 28 °C using a medium described by Tien and Kirk [24].

The Kraft effluent was kindly provided by the pulp and paper company, Portucel—Empresa de Celulose e Papel de Portugal, SA. It was the final result of a primary effluent treatment with initial COD of 1,000 mg/L and color of 920 UPt.

Culture Medium Composition

Two different growth media were used:

Yeast malt extract medium (*YM*) [25]:
Glucose 10 g/L; malt extract 3 g/L; peptone 5 g/L; yeast extract 5 g/L.

Trametes defined medium (*TDM*) [26]:
Glucose 9 g/L, glutamine 0.78 g/L; NaCl 0.28 g/L; KH_2PO_4 0.68 g/L; $MgSO_4.7H_2O$ 0.25 g/L; thiamine 0.81 mg/L and 1 mL of element solution.

Element solution: $FeSO_4{\cdot}7H_2O$ 20 μmol/L; $CuSO_4{\cdot}5H_2O$ 2 μmol/L; $ZnCl_2$ 5 μmol/L; $MnSO_4{\cdot}H_2O$ 20 μmol/L; $CoCl_2{\cdot}6H_2O$ 6 μmol/L; $NiCl_2{\cdot}6H_2O$ 0.1 μmol/L and $(NH_4)_6MO_7O_{24}{\cdot}4H_2O$ 0.5 μmol/L.

Fermentation Processes for Laccase and EPS Production

Liquid batch tests were inoculated by transferring 70 mg of mycelium from Petri dishes to Erlenmeyer flasks, according to the methodology used by Tavares et al. [27], to 250 mL of YM or TDM media. After growing for 3 days at 28 °C and 180 rpm, mycelium from assays with TDM, for enzyme production, was sterile transferred to (a) flasks with TDM without glucose for carbon suppression and with xylidine addition (final concentration of 30 μM) for laccase induction and (b) flasks with 50 % (*v*/*v*) of TDM without glucose and with 50 % (*v*/*v*) of Kraft effluent. The YM medium was used for EPS production according to Tavares et al. [28]. All batch tests were carried out at 28 °C and with 180 rpm in an orbital incubator during 14 or 17 days. In all batch tests samples were taken for measuring laccase activity and for exopolyssacharide detection.

Laccase Activity Measurement

Laccase activity was evaluated spectrophotometrically by the method of Ander and Messner [29] using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate. The reaction mixture was carried out with 0.4 mM of ABTS in 50 mM citrate/100 mM phosphate at pH 4.5 at 40 °C in a total volume of 2.0 mL. Oxidation of ABTS was monitored through an

increase in absorbance at 420 nm (ε=36,000 M^{-1} cm^{-1}). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of substrate per min.

Exopolyssacharide Detection

The exopolysaccharide was detected in the culture medium by adding 5 mL of ethanol to a test tube with 2 mL of a sample of the fermented culture medium without biomass.

Biomass Determination

The final biomass concentration was determined by dry weight of fungal mycelium at the last day of all assays. The culture medium was vacuum filtered through a 0.45-μm glass microfiber filter (GF/C, Whatman, Oxon, UK). The biomass retained was washed with distilled water and dried at 100 °C to a constant weight.

Image Acquisition

For the pellets image acquisition, a volume of 200 μL of culture medium with filamentous fungus was placed on a slide and covered with a 20×20 mm cover slip for visualization and image acquisition in bright field microscopy. Around 20 images were acquired per sample to obtain representative information on the biomass content. All the bright field images were acquired in a Meiji stereomicroscope EMZ-5TR with magnifications ranging from ×10 to ×20, coupled to a DeltaPix camera DP-300. The images acquisition was performed in an eight-bit grayscale format (1,300×1,030 pixels through the commercial software DeltaPix Viewer LE, 2006). Figure 1 represents the final pellet images acquired during this study for each experiment.

Image Processing and Analysis Methodology

The image analysis and processing methodology consisted of two different programs: [1] one for the determination of the core morphological parameters and [2] a second for the determination of the filaments/core ratio. The first program was developed in Matlab 7.0 (MathWorks, Inc., Natick, USA) and the second in ImageJ 1.33U (National Institute of Health, USA). In order to obtain the binary images of the pellets from the original 256 gray level images, four main stages were employed as follows: image pretreatment, image segmentation, debris deletion, and image registering.

Image Pretreatment This step resides on the improvement of the 256 gray level original images. In this stage, a background image is used to eliminate the background light differences from the image, then a bottom hat filter is applied in order to obtain the boundaries of the aggregates and finally filtered by a wiener filter so that pixel sized differences can be softened.

Image Segmentation This stage consists primarily in the segmentation of the aggregates by an automatic threshold level. The first step resides on smoothing the image's histogram by an averaging filter. After this step the 256 gray level image is transformed into a binary image.

Debris Deletion Image filling and morphological operations such as erosion and reconstruction are used in order to identify and remove debris smaller than nine pixels. The objects cut off by the image boundaries are next removed.

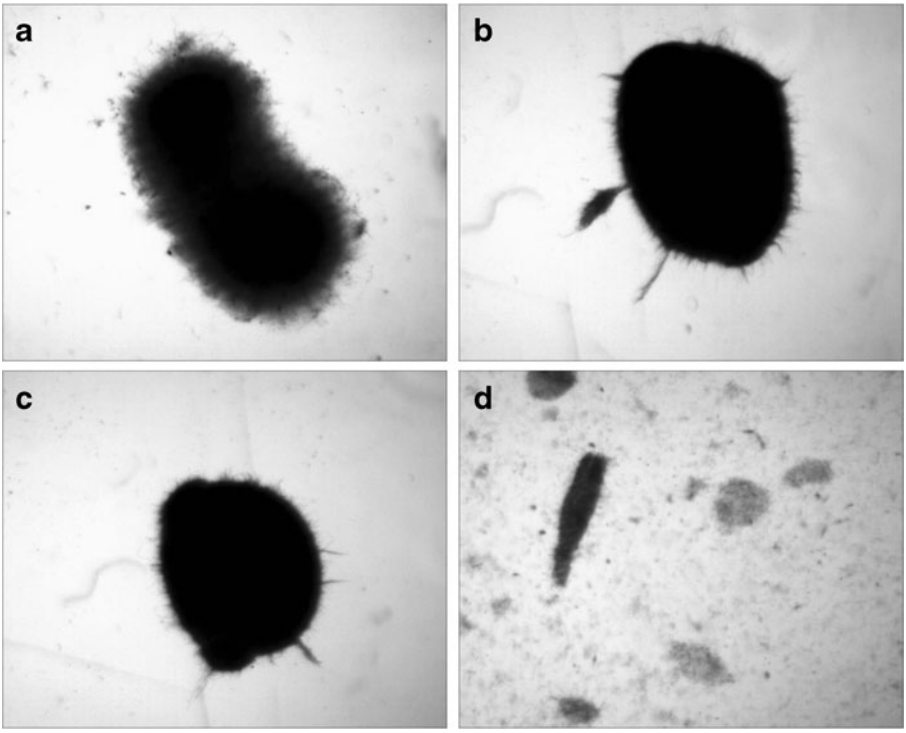

Fig. 1 Stereomicroscope images of final *T. versicolor* pellet grow in different culture media: **a** YM; **b** TDM; **c** TDM with effluent; **d** TDM with xylidine. Magnification: ×15 (**a**), ×20 (**b**), ×15 (**c**), and ×15 (**d**)

Registering The last stage resides on saving the final binary image of the pellets.

A second program was developed to determine the pellets core and allow for the determination of the pellets morphological parameters and the filaments/core ratio. The pellets core was obtained by a ten-pixel morphological opening, followed by a 1×1 median filtering. The resulting binary image is the core image, whereas the filaments image is obtained by the subtraction of the later image from the pellets binary image.

The determination of the pellets core morphological descriptors comprehended the equivalent diameter, eccentricity, roundness and solidity parameters [30]. The filaments/core ratio was simply determined as the quotient between the filaments area and the core area.

Results and Discussion

Image Analysis for *T. versicolor* Fermentation in Yeast Extract Malt Medium

YM medium is a rich medium with malt and yeast extract, peptone, and glucose. In a previous work, this medium was found to be optimal for EPS production by *T. versicolor* in submerged cultures [28]. In the YM batch experiments, the fungus metabolism for ligninolytic enzymes production was not stimulated, instead the metabolic pathway resulted in EPS production due to high sugars concentration. Such findings were already reported by Tavares et al. [28],

alongside with the EPS production [31, 32]. In addition, enzyme activity tests from submerged culture in YM medium did not provide any ligninolytic enzyme production during complete batch test. From Table 1, it can be seen that the EPS precipitation started after the tenth day of fermentation. Comparing all the culture media, the highest fungus biomass was obtained for this culture medium. Thus, the fungus metabolism was oriented for biomass growth and EPS production in this rich medium.

Pellets morphological results from image analysis showed that the pellets core presented good aggregation properties throughout all the experiment, with average values for eccentricity, roundness, and solidity of 0.56, 0.93, and 0.98, respectively (final values of 0.55, 0.92, and 0.98), as presented in Table 2. It was clear that the pellet morphology in a typical sample was very variable, so various image measurements were carried out to obtain significant statistical values for these parameters. It was also observed that filamentous extensions/core ratio kept constant (Table 2), presenting a value of 0.03. The equivalent diameter presented a slight decrease after 10 days of fermentation, but since the first days of the experiment up to the end, their values were among the largest pellets as the average 0.64 mm and final 0.62 mm values, with a maximum of 1.08 mm at day 10, probably due to the EPS production in this day since EPS is expelled to fermentation broth through hyphae surfaces. Figure 1 shows the acquired images to qualify the pellets from the *T. versicolor* through the different culture media. The pellet from Fig. 1a (YM medium) presented a dense and compact morphology, which was stable during all fermentation times according to the results explained above.

Comparing with the other culture media, the YM medium presented the largest pellets, with a core exhibiting good aggregation and mechanical properties throughout all the fermentation. Furthermore, the EPS production led to the lowest filamentous extensions/core ratio further emphasized as the experiment carried out. Certainly, these physical properties must be developed and related to a high viscosity or even adhesion capacity presented by the fungus hyphae surfaces due to the EPS production making the pellet more resistant and protected.

T. versicolor in this rich medium produced EPS and did not need to develop enzymatic syntheses of laccase, presenting no activity (Table 1), since it had enough monosaccharides as carbon source.

Image Analysis for *T. versicolor* Fermentation in *Trametes* Defined Medium Without Sugar

T. versicolor changes its metabolic pathway according to environmental signals and conditions. This nonrich medium was used without glucose, after the initial growth during 3 days, to stimulate enzymatic production [28] and to verify if simultaneously it presented any influence

Table 1 Results of *T. versicolor* fermentation for different culture media

Culture media	Final biomass concentration (g/L)	Maximum laccase activity (U/L)	EPS precipitation
YM	2.08 (17th day)[a]	–	Yes
TDM without sugar	1.20 (17th day)	339 (6th day)	No
TDM without sugar and with effluent 50 % (*v*/*v*)	0.84 (13th day)	229 (8th day)	No
TDM without sugar and with xylidine 30 μM	0.28 (14th day)	1754 (10th day)	No

[a] Text in parenthesis means the final day of fermentation for biomass quantification or day of fermentation for high laccase activity

Table 2 Structural and morphological parameters of *T. versicolor* fermentation with YM Medium

Day of fermentation	D_{eq} (mm)[a]	Ext/core	Eccentricity	Roundness	Solidity
3	0.68	0.04	0.50	0.92	0.98
6	0.69	0.03	0.56	0.92	0.97
10	1.08	0.03	0.57	0.93	0.97
12	0.62	0.03	0.59	0.95	0.98
14	0.57	0.03	0.47	0.94	0.99
17	0.62	0.03	0.55	0.92	0.98

[a] D_{eq} equivalent diameter

on the fungus morphology. In this batch test, maximum laccase activity of 339 U/L (Table 1) was attained on the sixth day of fermentation and no production of EPS was obtained.

Image analysis showed that the equivalent diameter vary during the entire assay (Table 3), presenting lower values than in the beginning (0.64 mm), with a final value of 0.44 mm. Figure 1b shows the acquired image of a representative pellet. This result was found to be in agreement with the total biomass at the end of the assays, which was much lower in this medium, 1.20 g/L (Table 1) than in the rich YM medium, 2.08 g/L. The pellets' morphological characterization (Table 3) showed an increase on the filamentous extensions/core ratio (from 0.04 to 0.06 with average 0.054) meaning that simultaneously with the hyphae, growth caused a small reduction in the core dimensions. Furthermore, the pellets core acquired better aggregation properties as proved by the decrease of the eccentricity values (final 0.70 and average 0.72) and increase of the roundness and solidity values (final 0.94 and 0.99 and average 0.90 and 0.97, respectively).

The growth of *T. versicolor* in a specific defined medium TDM (described for ligninolytic enzymes production) and simultaneously with carbon limitation situation, resulted in laccase production instead of EPS. Furthermore, the final biomass content was much lower in this medium due to carbon limitation, from third day of fermentation, when glucose was suppressed from the culture medium, resulting in the alternative metabolic pathway of growth involving the ligninolytic pathway. The pellets morphological analysis allowed establishing a strong hyphae growth during the experiment simultaneously with a retraction of the core size, which acquired better aggregation and mechanical properties.

Image Analysis for Fermentation in TDM with Effluent 50 % (*v*/*v*)

The effluent from pulp and paper industrial processes was also used as inducer to evaluate the morphology of *T. versicolor* during its fermentation for the production of laccase. It is also

Table 3 Structural and morphological parameters of *T. versicolor* fermentation with TDM medium

Day of fermentation	D_{eq} (mm)	Ext/core	Eccentricity	Roundness	Solidity
3	0.64	0.04	0.78	0.83	0.94
6	0.45	0.05	0.72	0.92	0.98
10	0.46	0.06	0.70	0.89	0.98
12	0.32	0.06	0.70	0.90	0.98
14	0.49	0.04	0.70	0.90	0.98
17	0.44	0.06	0.70	0.94	0.99

important to determine the morphological changes since this fungus is very used in the treatment of pulp and paper effluents. This experiment was carried out with the addition of pulp and paper effluent in the TDM without sugar. Once again, as glucose was suppressed from the third day of fermentation, the fungus performed a secondary metabolism for the production of laccase.

It was found that the introduction of the effluent only caused small differences in the fungus morphology with respect to the previous study with TDM medium, as shown in Fig. 1b–c and Table 4. The final biomass was lower than in TDM medium and a nearly proportional decrease on the maximum laccase activity, 229 U/L, was attained with a 2-day delay (Table 1). The lower biomass content was probably due to the negative effect of the effluent on the fungus growth. The average equivalent diameter presented a slight increase during the assay (Table 4), although among the lowest values (final 0.48 mm and average 0.45 mm), whereas the filamentous extensions/core ratio decreased throughout the experiment (from 0.08 to 0.03 with average 0.048). The morphological analysis of the pellets core showed that it has also acquired better aggregation properties with the behavior of eccentricity, roundness, and solidity somewhat similar to the previous TDM study (final 0.70, 0.90, and 0.98 and average 0.68, 0.90, and 0.97, respectively). These values indicate that the pellets have evolved to a more closed and less exposed structure that is in accordance to the significant drop of the filamentous extensions/core ratio (Table 4). The introduction of the effluent only caused slight differences in the fungus morphology with respect to the previous TDM medium, the most significant of which was the filamentous extensions/core ratio decrease. This fact could be linked to the increased effluent toxicity resulting furthermore in lower final biomass contents than in the experiment with TDM medium.

Image Analysis for Fermentation in TDM with Xylidine 30 μM

The addition of xylidine to the carbon-limited TDM medium was tested as this compound has been reported as a traditional inducer for ligninolytic enzyme production by *T. versicolor* [28, 33]. The analysis of Table 1 shows that xylidine indeed induced laccase production as it presented a very high laccase activity of 1,754 U/L. Furthermore, the small addition of xylidine in this experiment was responsible for an increase of 1,415 U/L (five times) in laccase activity. No production of EPS was found and after the 11th day the pellet disaggregation started probably due to a cellular destruction process.

The equivalent diameter increased steadily up to the 11th day (Table 5) with an average value of 0.59 mm and then, a sharp decrease took place to minute pellets with a final value of 0.16 mm, representing a severe disaggregation process (Fig. 1d), which is in accordance with the low final biomass concentration, 0.28 g/L. Furthermore, the filamentous extensions/core ratio increased throughout the experiment attaining the highest values with an average of 0.06 and a final value of 0.07. This high increase on the filamentous extensions/core ratio was

Table 4 Structural and morphological parameters of *T. versicolor* fermentation with TDM medium and with effluent

Day of fermentation	D_{eq} (mm)	Ext/core	Eccentricity	Roundness	Solidity
2	0.40	0.08	0.69	0.86	0.95
6	0.48	0.04	0.72	0.91	0.98
8	0.38	0.05	0.69	0.90	0.98
10	0.49	0.04	0.61	0.91	0.98
13	0.47	0.03	0.70	0.90	0.98

Table 5 Structural and morphological parameters of *T. versicolor* fermentation with TDM medium and with xylidine

Day of fermentation	D_{eq} (mm)	Ext/core	Eccentricity	Roundness	Solidity
3	0.43	0.05	0.53	0.96	0.99
7	0.53	0.04	0.54	0.96	0.98
9	0.70	0.07	0.54	0.95	0.98
11	0.71	0.05	0.53	0.94	0.98
14	0.16	0.07	0.49	0.89	0.97

probably associated to the higher production of laccase that was also found, in need to be excreted to the medium. Although the pellets morphological analysis showed that, in average, the pellets core presented good morphological conditions showing good aggregation properties (similar to those showed with YM medium), their evolution throughout the experiment was clearly towards more open and exposed structures. To illustrate these findings, the average values of eccentricity, roundness, and solidity were 0.53, 0.94, and 0.98 and their final values of 0.49, 0.89, and 0.97, respectively. The evolution towards these open and exposed structures was the sole among all the assays, and preconfigured the ability of these pellets to a disaggregation process.

The addition of xylidine to the carbon-limited TDM medium allowed for *T. versicolor* to undergo a strong secondary metabolism resulting in high laccase activity. Therefore, the presence of the laccase inducer xylidine resulted in much higher values of the laccase activity than the presented in the former studies. The higher production of this enzyme led to a steady increase of the filamentous extensions/core ratio throughout the experiment attaining the highest values of this study. Alongside this phenomenon, the pellets core evolution throughout the experiment led to larger, more open, and exposed structures up to day 11 where a severe disaggregation process took place. Furthermore, the extremely low values of the biomass contents at the 15th day clearly points to a surviving mechanism from the 11th day onwards. Comparing Fig. 1b–d it is clear that a decrease in size of the pellets occurred with the addition of xylidine to the culture medium. Additionally, xylidine caused significant changes in the morphology of the fungus forming aggregates with small eccentricity and at the same time, high laccase production. Pellets with small area could be beneficial to improve the mass transfer between the fungus and culture medium. This change on cell morphology probably facilitated the secretion of the laccase from the aggregated fungus. Nanou and Roukas [34] studied the morphology of the fungus *Blakeslea trispora* in response to the oxidative stress induced by butylated hydroxytoluene during carotene production. They found that the oxidative stress caused changes on the morphology of microorganism changing aggregates with large projected area to aggregates with small projected area.

Conclusions

The image analysis procedure allows evaluating the morphological changes of *T. versicolor* pellets according to the environmental stimulus from specific experimental conditions assayed to ligninolytic enzymatic production or to EPS production. Complementing the biomass content, laccase activity, and polymer precipitation study, the proposed image processing and analysis methodology allowed for a better comprehension of these physiological phenomena with respect to the corresponding pellets morphological stages. This methodology led to the conclusion that the morphology of *T. versicolor* pellets was strongly affected by the culture

medium and laccase inducers. The methodology herein used could be very useful for understanding the fungus metabolism behavior. This accurate and reliable technique opens new possibilities to study the relationship between the morphology, the physiological state, and the culture conditions of *T. versicolor* pellets and it could also be easily extended to other filamentous fungi.

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References

1. Grimm, L. H., Kelly, S., Krull, R., & Hempel, D. C. (2005). *Applied Microbiology and Biotechnology, 69*, 375–384.
2. Harris, D. M., van der Krogt, Z. A., van Gulik, W. M., van Dijken, J. P., & Pronk, J. T. (2007). *Applied and Environmental Microbiology, 73*, 5020–5502.
3. Samanidou, V. F., Evaggelopoulou, E. N., & Papadoyannis, I. N. (2006). *Journal of Separation Science, 29*, 1879–1908.
4. Liers, C., Ullrich, R., & Pecyna, M. (2007). *Enzyme and Microbial Technology, 41*, 785–793.
5. Manubens, A., Canessa, P., & Folch, C. (2007). *FEMS Microbiology Letters, 275*, 139–145.
6. Tavares, A. P. M., Coelho, M. A. Z., Coutinho, J. A. P., & Xavier, A. M. R. B. (2005). *Journal of Chemical Technology and Biotechnology, 80*, 669–676.
7. Schmidt, M., Babu, K. R., Khanna, N., Marten, S., & Rinas, U. (1999). *Journal of Biotechnology, 68*, 71–83.
8. Papagianni, M. (2004). *Biotechnology Advances, 22*, 189–259.
9. Wosten, H. A. B., Moukha, S. M., Sietsma, J. H., & Wessels, J. G. H. (1991). *Journal of General Microbiology, 137*, 2017–2023.
10. Agger, T., Spohr, A. B., Carlsen, M., & Nielsen, J. (1998). *Biotechnology and Bioengineering, 57*, 321–329.
11. Znidarsic, P., & Pavko, A. (2001). *Food Technology and Biotechnology, 39*, 237–252.
12. Lecault, V., Patel, N., & Thibault, J. (2007). *Biotechnology Progress, 23*, 734–740.
13. Yang, H., Reichl, U., King, R., & Gilles, E. D. (1992). *Biotechnology and Bioengineering, 39*, 44–48.
14. Yang, H., King, R., Reichl, U., & Gilles, E. D. (1992). *Biotechnology and Bioengineering, 39*, 49–58.
15. Pointing, S. B. (2001). *Applied Microbiology and Biotechnology, 57*, 20–33.
16. Saraiva, J. A., Tavares, A. P. M., & Xavier, A. M. R. B. (2012). *Applied Biochemistry and Biotechnology, 167*, 685–693.
17. Gamelas, J. A. F., Tavares, A. P. M., Evtuguin, D. V., & Xavier, A. M. R. B. (2005). *Journal of Molecular Catalysis B: Enzymatic, 33*, 57–64.
18. Tavares, A. P. M., Gamelas, J. A. F., Gaspar, A., Evtuguin, D. V., & Xavier, A. M. R. B. (2004). *Catalysis Communications, 5*, 485–489.
19. Xavier, A. M. R. B., Tavares, A. P. M., Agapito, M. S. M., & Evtuguin, D. V. (2008). *Journal of Chemical Technology and Biotechnology, 83*, 1602–1608.
20. Castro, A. I. R. P., Evtuguin, D. V., & Xavier, A. M. R. B. (2003). *Journal of Molecular Catalysis B: Enzymatic, 22*, 13–20.
21. Wong, D. (2009). *Applied Biochemistry and Biotechnology, 157*, 174–209.
22. Miyazaki, T., Yadomae, T., Sgiura, M., Ito, H., Fujii, K., Naruse, S., & Kunihisa, M. (1974). *Chemical and Pharmaceutical Bulletin, 22*, 1739–1742.
23. Carlile, M. J., & Watkinson, S. C. (1994). *The fungi*. London: Academic.
24. Tien, M., & Kirk, T. K. (1988). *Methods Enzyme, 161*, 238–247.
25. Kim, S. W., Hwang, H. J., Park, J. P., Cho, Y. J., Song, C. H. C. H., & Yun, J. W. (2002). *Letters in Applied Microbiology, 34*, 56–61.
26. Roy, B., & Archibald, F. (1993). *Applied and Environmental Microbiology, 59*, 1855–1863.
27. Tavares, A. P. M., Coelho, M. A. Z., Agapito, M. S. M., Coutinho, J. A. P., & Xavier, A. M. R. B. (2006). *Applied Biochemistry and Biotechnology, 134*, 233–248.

28. Tavares, A. P. M., Agapito, M. S. M., Coelho, M. A. Z., Silva, J. A. L., Barros-Timmons, A., Coutinho, J. A. P., & Xavier, A. M. R. B. (2005). *World Journal of Microbiology and Biotechnology, 21*, 1499–1507.
29. Ander, P., & Messner, K. (1998). *Biotechnology Techniques, 12*, 191–195.
30. Russ, C. R. (1995). *The image processing handbook*. Boca Raton, FL: CRC.
31. Sugiura, M., Ohno, H., Kunihisa, M., Hirata, F., & Ito, H. (1980). *Journal of Japanese History of Pharmacy, 30*, 503–513.
32. Ng, T. B. (1998). *Journal of General Pharmacy, 30*, 1–4.
33. Couto, S. R., Gundín, M., Lorenzo, M., & Sanromán, M. A. (2002). *Process Biochemistry, 38*, 249–255.
34. Nanou, K., & Roukas, T. (2010). *Applied Biochemistry and Biotechnology, 160*, 2415–2423.