Production of *Trametes versicolor* laccase by solid state fermentation using a fixed-bed bioreactor

Érica S. de Souza 1, Ivanete de L. Sampaio 2, Ana Karla de L. Freire 3, Babbyngtonn Khell S. da Silva 3, Agostinho da S. Sobrinho 3, Alita M. Lima 3 and João Vicente B. Souza 3*

1 Centro Universitário Luterano de Manaus, Manaus, Amazonas, Brazil. 2 Programa de Pós-graduação em Medicina Tropical, Universidade do Estado do Amazonas, Manaus, Amazonas, Brazil. 3 Instituto Nacional de Pesquisas da Amazônia (INPA), Av. André Araújo 2936, Bairro Aleixo, CEP 69060-001, Manaus, Amazonas, Brazil. *e-mail: joaovcentrebagasouza@yahoo.com

Received 30 November 2010, accepted 27 March 2011.

Abstract

The aim of this work was to investigate the production of *Trametes versicolor* laccase by SSF (solid state fermentation) using a fixed-bed bioreactor. The SSF optimization assays were carried out in Erlenmeyer flasks (250 mL) containing 12.5 g of dried wheat bran. The medium moisture (50, 60, 70, 80 and 90% w/w), inoculum (78, 240, 400, 560 and 720 mycelium plugs/g D.M.- dried matter) and the contents of glucose (0, 10, 30, 50 and 70 g/kg D.M.), yeast extract (24 and 48 g/kg D.M.) and CuSO4 (2.4 and 4.8 g/kg D.M.) were evaluated for laccase production. After this optimization an experiment in a fixed-bed bioreactor (250 mL) with 25 g D.M. of wheat bran was accomplished, and this reactor was aerated (1.5 L/min) with humidified air. The laccase activity was quantified using ABTS as substrate. The results of the experiments demonstrated that the moisture of 70% w/w and the inoculum of 240 mycelium plus/CPE 69060-001, Manaus, Amazonas, Brazil. *e-mail: joaovcentrebagasouza@yahoo.com

The SSF optimization assays were carried out in Erlenmeyer flasks (250 mL) containing 12.5 g of dried wheat bran. The medium moisture (50, 60, 70, 80 and 90% w/w), inoculum (78, 240, 400, 560 and 720 mycelium plugs/g D.M.- dried matter) and the contents of glucose (0, 10, 30, 50 and 70 g/kg D.M.), yeast extract (24 and 48 g/kg D.M.) and CuSO4 (2.4 and 4.8 g/kg D.M.) were evaluated for laccase production. After this optimization an experiment in a fixed-bed bioreactor (250 mL) with 25 g D.M. of wheat bran was accomplished, and this reactor was aerated (1.5 L/min) with humidified air. The laccase activity was quantified using ABTS as substrate. The results of the experiments demonstrated that the moisture of 70% w/w and the inoculum of 240 mycelium plus/CPE 69060-001, Manaus, Amazonas, Brazil. *e-mail: joaovcentrebagasouza@yahoo.com

The aim of this work was to investigate the production of *Trametes versicolor* laccase by SSF (solid state fermentation) using a fixed-bed bioreactor. Specifically, the influence of the content of glucose, yeast extract, CuSO4, inoculum size and medium moisture were investigated, and then the influence of a fixed-bed bioreactor was studied.

**Key words:** Laccase, *T. versicolor*, fixed-bed bioreactor.

Introduction

Laccases (benzenediol: oxygen oxidoeductases, EC 1.10.3.2) are multicopper enzymes capable of oxidising phenols and aromatic amines, reducing molecular oxygen to water, with the involvement of three types of copper centers which have different functions: type 1 (blue copper) catalyses the electron transfer from the substrate while type 2 and type 3 form a three-member cluster that collectively activates molecular oxygen 1.

These enzymes have industrial importance and are capable to delignify wood pulp, decolorize/detoxify effluents generated by the pulp and paper industry and degrade toxic environmental pollutants 2,3. The ligninolytic organisms like white-rot fungi are the best known laccases producers and the major sources of these enzymes 4,5.

Bioprocesses carried out using fungi have demonstrated that laccase can be produced by submerged (SbF) and solid state fermentation (SSF). SSF is defined as any fermentation process carried out on a solid material (employing either a natural support or an inert support) in absence of free flowing liquid 7. In recent years, SSF has received more and more interest from researchers, since several studies have demonstrated superior product yields and simplified downstream processing 8. The utilization of food-industrial wastes in SSF (cassava, pomace, bagasse, etc.) is also an alternative to reduce costs and reduce some pollution problems 1.

The wheat bran is a food-industrial waste that has demonstrated to be an adequate substrate for laccase production. This agriculture residue provides starch, cellulose, protein and inducers (as aromatic compounds) allowing the fungi development and enzyme production 9.

The selection of an adequate bioreactor is another important step in bioprocess to enzyme production. The most important factors to take into account to design a bioreactor operating in solid-state conditions are the parameters of the process and the nature of the support employed 10, 11. In addition, the cost and simplicity also deserve great attention. It is necessary to find an adequate combination support/bioreactor, which allows obtaining high productivity at low cost 1.

Although a large number of studies have been performed to investigate the production of laccase by *T. versicolor*, some scientific points should be clarified, such as viability of the SSF, the utilization of wheat bran as substrate, necessity of inducers, carbon and nitrogen sources and the efficiency of fixed bed reactor.

The aim of this work was to investigate the production of *Trametes versicolor* laccase by SSF (solid state fermentation) using a fixed-bed bioreactor. Specifically, the influence of the content of glucose, yeast extract, CuSO4, inoculum size and medium moisture were investigated, and then the influence of a fixed-bed bioreactor was studied.
**Materials and Methods**

**Microorganism:** *Trametes versicolor* (CCT 4521) was obtained from the fungi collection from the University of Campinas, São Paulo, Brazil. This strain was maintained on MEG agar slants (5 g/L malt extract, 10 g/L glucose, 20 g/L agar) at 4°C.

**Optimization of the Solid State Fermentation (SSF):** Univariate experiments were carried out for the optimization of laccase production. The SSF optimization assays were carried out in Erlenmeyer flasks (250 mL) containing 12.5 g of dried wheat bran. Uni-variated studies were carried out in order to optimize the content of: glucose (0-0.07 kg/kg), yeast extract (0-0.048 kg/kg), CuSO₄ (0-0.0048 kg/kg), inoculum (80-720 mycelium plugs/kg, 5 mm diameter mycelium discs obtained from MEG agar slants previously inoculated with *T. versicolor*, 7 days) and moisture (50-90%). The optimization experiments lasted 20 days and the samples for kinetics enzymatic analysis were collected every four days of bioprocess. The enzyme extraction was carried out using water (10 mL water and 1 g bioprocess biomass) under orbital agitation (100 rpm) per 30 min.

**Solid state fermentation in a fixed-bed bioreactor:** The optimized bioprocess was carried out with 25 g (dry matter) of wheat bran, 70% w/w of moisture and inoculum size of 240 mycelium plugs/kg. The bioreactor used in this study (Fig. 1) employs an internal tube (riser) of 19 cm height and 2 cm diameter. The working volume of this bioreactor was 250 mL and the temperature (25°C) was controlled by water circulation through the annular jacket surrounding the reaction zone. The reactor was aerated (1.5 L/min) with humidified air that was measured by a flow meter.

**Enzyme extraction/assay:** After fermentation, the fermented solids were removed from the reactor and transferred to a glass flask. Ten parts of water were added to each gram of fermented solids, the mixture was incubated in a rotary shaker (100 rpm) at 35°C for 20 min. The extract was obtained by filtration (gauze) followed by centrifugation (3000 rpm, 2 min) and the supernatant was used for enzymatic quantification. Laccase activity was determined by measuring the oxidation of 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) at 420 nm (ε = 3.6 x 10⁴ cm⁻¹M⁻¹) according to Master and Field. The reaction mixture contained 0.4 ml of 1 mM ABTS and 1.2 mL of 0.1 M glycine-HCl buffer pH 3.5 and 0.8 ml aliquots of appropriately diluted culture fluid. One laccase activity unit was defined, as amount of enzyme that oxidized 1 mmol ABTS per min. The activities were expressed in UI/kg (dry matter). All the values are the means of duplicate experiments.

**Results**

Fig. 2 demonstrates the results of the optimization experiments. The highest laccase production (19,341 UI/kg) was obtained in the medium without glucose, in the 20th day. The yeast extract and CuSO₄ content decreased the laccase production. The inoculum of 240 mycelium plugs/kg and the moisture of 70% were the best conditions for laccase production.

Fig. 2 presents the kinetic experiment for the laccase production carried out with the optimized conditions in the fixed-bed bioreactor. With the optimized conditions (moisture of 70%; inoculum of 240 mycelium plug/kg) and the aeration of 1.5 L/min it was achieved 18,917 UI/kg of laccase, in the 13th day. This highest production was observed after 312 h of fermentation, and productivity was 41.66 UI/L·h (RP= 18,917 UI/L/312h).

**Discussion**

The carbon sources play an important role in production of ligninolytic enzymes. In the present work the glucose caused inhibition in the laccase production. Mansur et al. demonstrated that glucose is not an option of carbohydrate of fast assimilation for laccase production. Therefore, new experiments of carbohydrate screening should be done in order to find a sugar that promotes a better *T. versicolor* growth and faster/highest laccase production.

According to Master and Field, the ligninolytic enzymes are produced during the secondary metabolism under conditions of limited nitrogen. The results observed in this study are consistent with these previous studies, since the yeast extract inhibited the laccase production. On the other hand, Kaal et al. reported that in *Pleurotus ostreatus*, a high concentration of nitrogen in the medium (glutamate as N source) slightly stimulated depolymerization of lignin compared to the N-limited medium. These data demonstrate that more studies are necessary in order to relate the lignin metabolism, ligninolytic enzymes production and nitrogen sources.

The data presented in Fig. 2 demonstrated that CuSO₄ content inhibits the laccase production. This result is opposite to what has been demonstrated in the experiments of submerged fermentation, however, even in these studies the copper content higher than 10 mM or the presence of the CuSO₄ in media with pH fewer than 4 decreased the fungal growth and enzyme production.

The best inoculum size observed in the present work was 240 inoculum plugs/kg. The lowest and the highest inoculum sizes caused a drastic decrease in laccase production. This strong relationship between inoculum size and enzyme production has been described.
in majority of the works of extracellular enzymes. Small inoculums delay microbial growth and the enzyme production while high inoculums cause microbial competition for substrate which leads to unwanted results.

As previously described, lignocellulosic residues can stimulate laccase production, this effect being attributed to their cellulose content. The C and N rich raw materials if mixed in appropriate ratio can serve as a complete source of nutrient pool without additional supplements. In the present work, the best condition for laccase production was the utilization of wheat bran as a single substrate. This result is appropriate, demonstrating that this T. versicolor bioprocess is robust and requires little nutritional interventions.

The fixed-bed bioreactor demonstrated to be adequate to the laccase production. This bioreactor promoted the production of similar amount of laccase than that was observed in Erlenmeyer flask, however, using more substrate. This is a good result because the main function of these reactors is to maintain the aeration ratio with the high biomass content that is necessary in the industry. The highest enzymatic activity obtained in this study was high comparing to other existing works.

Conclusions

From the results obtained, it can be concluded that the system wheat bran (without supplementation) and fixed-bed bioreactor was suitable for the production of laccase enzymes by T. versicolor under solid-state conditions. These promising results suggest the application of this system to industrial-scale operation in order to produce high amounts of laccase or other enzymes.

Acknowledgements

This work was supported by CNPq - Brazil.

References


Figure 2. Optimization of experiments carried out by solid state fermentation: a) influence of glucose content; b) influence of yeast extract and CuSO4 content; c) influence of inoculums size and d) influence of moisture content.

Figure 3. Kinetic experiment for the laccase production carried out with the optimized conditions in the fixed-bed bioreactor.


