LIGNOLYTIC ENZYMES PRODUCED BY *TRAMETES VILLOSA* CCB176 UNDER DIFFERENT CULTURE CONDITIONS

Renata Yamanaka[1]; Clarissa F. Soares[1]; Dácio R. Matheus[2]; Kátia M.G. Machado[1]*

[1]Universidade Católica de Santos, Santos, SP, Brasil; [2]Instituto de Botânica, São Paulo, SP, Brasil

ABSTRACT

The expression of the enzymatic system produced by basidiomycetous fungi, which is involved in the degradation of xenobiotics, mainly depends on culture conditions, especially of the culture medium composition. *Trametes villosa* is a strain with a proven biotechnological potential for the degradation of organochlorine compounds and for the decolorization of textile dyes. The influence of glucose concentration, addition of a vegetable oil-surfactant emulsion, nature of the surfactant and the presence of manganese and copper on the growth, pH and production of laccase, total peroxidase and manganese-dependent peroxidase activities were evaluated. In general, acidification of the medium was observed, with the pH reaching a value close to 3.5 within the first days of growth. Laccase was the main activity detected under the different conditions and was produced throughout the culture period of the fungus, irrespective of the growth phase. Supplementation of the medium with vegetable oil emulsified with a surfactant induced manganese-dependent peroxidase activity in *T. villosa*. Higher specific yields of laccase activity were obtained with the addition of copper.

Key words: laccase, metals, MnP, vegetable oil, surfactants

Lignocellulolytic basidiomycetous fungi are able to degrade a series of recalcitrant organic compounds, such as lignin and diverse classes of pollutants with little or no structural homology to lignin. The degradation of lignin and other recalcitrant compounds by basidiomycetes is a co-metabolic process and is mediated by the coordinated action of an enzymatic system and various low molecular mass metabolites. The expression of the enzymatic system involved in the degradation of xenobiotics has been shown to mainly depend on the culture conditions and composition of the culture medium, for example, the nature and concentration of carbon sources, nitrogen and certain metals (copper, manganese and calcium), pH, shaking and the addition of certain substances such as unsaturated fatty acids (1,11).

Bioremediation using basidiomycetous fungi is a promising method due to its low cost and possibility of complete mineralization of the pollutants. However, the difficulties in the implementation of a large-scale process demonstrate that the parameters established in the laboratory are not always adequate for application in bioreactors. Thus, the application of fungal bioremediation on a commercial scale does not only require the understanding of aspects related to process engineering, but also knowledge about basic aspects of fungal physiology in order to establish better conditions for growth and for the production and expression of the enzymatic system involved in the degradation of pollutants (28).

Trametes villosa is a strain with a proven biotechnological potential, which are able to mineralize pentachlorophenol and hexachlorobenzene in soil and to degrade reactive synthetic dyes (16-19). However, little is known about the physiology of these fungi. The lignicolous fungus *T. villosa* CCB176 was isolated from basidioma collected in a seasonal forest located in the municipality of Assis, interior of the State of São Paulo, Brazil (24). This fungus was being studied for the decontamination of soils contaminated with organochlorines in bioreactors with a capacity of 400 kg soil (20). The objective

*Corresponding Author. Curso de Ciências Biológicas, Centro de Ciências da Educação, Universidade Católica de Santos, Av. Conselheiro Nébias, 300, Santos, Cep: 11015-002, Brasil. Tel.: (+5513) 32055555. E-mail: katia@unisantos.br

of the present study was to identify the nutritional requirements for the growth and expression of the ligninolytic enzymatic system of *T. villosa* in terms of the concentration of glucose, copper and manganese, and to evaluate the effect of the addition of a vegetable oil-surfactant emulsion.

MATERIAL AND METHODS

Microorganism maintenance and culture conditions

Trametes villosa CCB176, obtained from the Basidiomycete Culture Collection (CCB), Institute of Botany, São Paulo, was maintained on potato-dextrose-agar (PDA) at 4ºC. The fungus was previously grown on plates with PDA at 25 ± 2ºC (5-7 days) until the mycelium occupied 3/4 of the medium surface Three fungal discs measuring 6 mm in diameter were removed to inoculate 250-mL flasks containing 50 mL basal medium. For enzyme production *T. villosa* was cultivated in basal medium (14) containing (per L): 1 mL thiamine HCl, 0.2298 g ammonium tartrate, 0.2 g KH_2PO_4, 0.05 g $MgSO_4.7H_2O$, 0.013 g $CaCl_2.2H_2O$, 1 mL mineral solution (3 g $MgSO_4$, 1 g NaCl, 100 mg $FeSO_4.7H_2O$, 100 mg $CoSO_4.H_2O$, 82 mg CaC_2, 100 mg $ZnSO_4$, 10 mg $CuSO_4.5H_2O$, 10 mg $AlK(SO_4)_2$, 10 mg H_3BO_3, 10 mg $NaMoO_4$), 0.5 mL vitamin supplement (2 mg biotin, 2 mg folic acid, 5 mg thiamine HCl, 10 mg pyridoxine, 5 mg nicotinic acid), and 10 mL 50 mM sodium acetate buffer, pH 4.7. The initial pH of the culture medium was 5.0. Glucose, vegetable oil (commercial soy oil), manganese sulfate and copper sulfate were added to get the following concentrations: glucose (5, 10, 20 and 30 g L^{-1}), vegetable oil (0.2, 0.6 and 1.0 g L^{-1}), $MnSO_4.H_2O$ (0, 50, 100 and 300 µM) and $CuSO_4.5H_2O$ (0, 0.5, 1, 2 and 3 mM). Vegetable oil was emulsified with Tween 20 or Renex (1:10, v/v). All supplements were added before the medium sterilization (121ºC, 20 min.). The cultures were incubated at room temperature (24 ± 2ºC) in a stationary manner. The content of the flasks was removed at different time intervals and filtered and the extract was used for the determination of pH, residual glucose and enzymatic activities. The tests were carried out in triplicate.

Biomass

Biomass was determined by dry mass at 60ºC for 24-48 hours, after washing with distilled water (23).

Determination of H_2O_2

The presence of H_2O_2 in the enzymatic extract was determined as described by Machado and Matheus (15) using horseradish peroxidase instead the H_2O_2 solution in total oxidation of ABTS assay, described below.

Glucose

Residual glucose was evaluated by the glucose oxidase colorimetric enzymatic method (Laborlab Kit).

Enzymatic Activities

Total oxidation of ABTS: 1 mL of the reaction mixture contained 0.25 mL 50 mM citrate-phosphate buffer, pH 4.0, 0.1 mL 5 mM 2,2-azinobis-(3-ethyl benzthiazoline-6-sulphonate) (ABTS), 0.6 mL of the enzymatic extract, and 0.05 mL 2 mM H_2O_2 (15). Absorbance was read at 420 nm for 10 min. One unit of enzymatic activity was defined as the amount of enzyme necessary to oxidize 1 µmol of substrate per liter per minute.

Laccase activity: was determined as described for Total oxidation of ABTS using destilled water instead H_2O_2. The specific yield of laccase activity was determined as enzymatic activity by mg of biomass.

Peroxidase activity: was calculated as the difference between the values obtained for total ABTS oxidation and laccase activity (15).

Manganese Peroxidase (MnP): was determined by fenol red oxidation at 610 nm. 2 mL of the reaction mixture contained 0.6 mL solution (0.2 M succinate buffer pH 4.5, 0,1 M sodium lactate and 0.5% bovine serum albumin), 0.1 mL 2 mM $MnSO_4$, 0.2 mL 0.1% fenol red, 1.0 mL of the enzymatic extract, and 0.1 mL 2 mM H_2O_2 (15).

RESULTS AND DISCUSSION

In the present study, aspects of the physiology of *Trametes villosa*, basidiomycete isolated from Brazilian ecosystem, were studied in order to provide data regarding the application of this fungus to the degradation of organic pollutants. The initial glucose concentration influenced the growth of the basidiomycete. Using 5 g glucose L^{-1}, the onset of the stationary growth phase of *T. villosa* was observed around day 15 and an increase in glucose concentration prolonged the exponential phase (Fig. 1). At initial concentrations of 10, 20 and 30 g glucose L^{-1}, the final biomass of *T. villosa* increased 1.7, 1.9 and 1.9 times (0.22 g, 0.25 g and 0.25 g), respectively, compared to the biomass obtained with 5 g glucose L^{-1} (0.13 g) at 31 days of culture. The results obtained for *T. villosa* were similar to those reported for other fungi. Dekker and Barbosa (6) observed an increase of about 50% in the biomass of *Botryosphaeria* sp. when the glucose concentration ranged from 10 to 30 mg mL^{-1}. Glucose exhaustion during the growth of *T. villosa* was only observed at initial concentrations of 5 and 10 g glucose L^{-1} (Figure 1). Glucose exhaustion was also observed during the growth of *Botryosphaeria* sp. and *Trametes versicolor* when initial glucose concentrations of 5 and 10 g L^{-1} were used (6,26).

Regardless of the culture condition, rapid acidification of the culture medium was observed within the first days of growth of *T. villosa*, with the pH reaching a value close to 3.5 at 7 days (Fig. 1). The pH remained close to this value throughout the
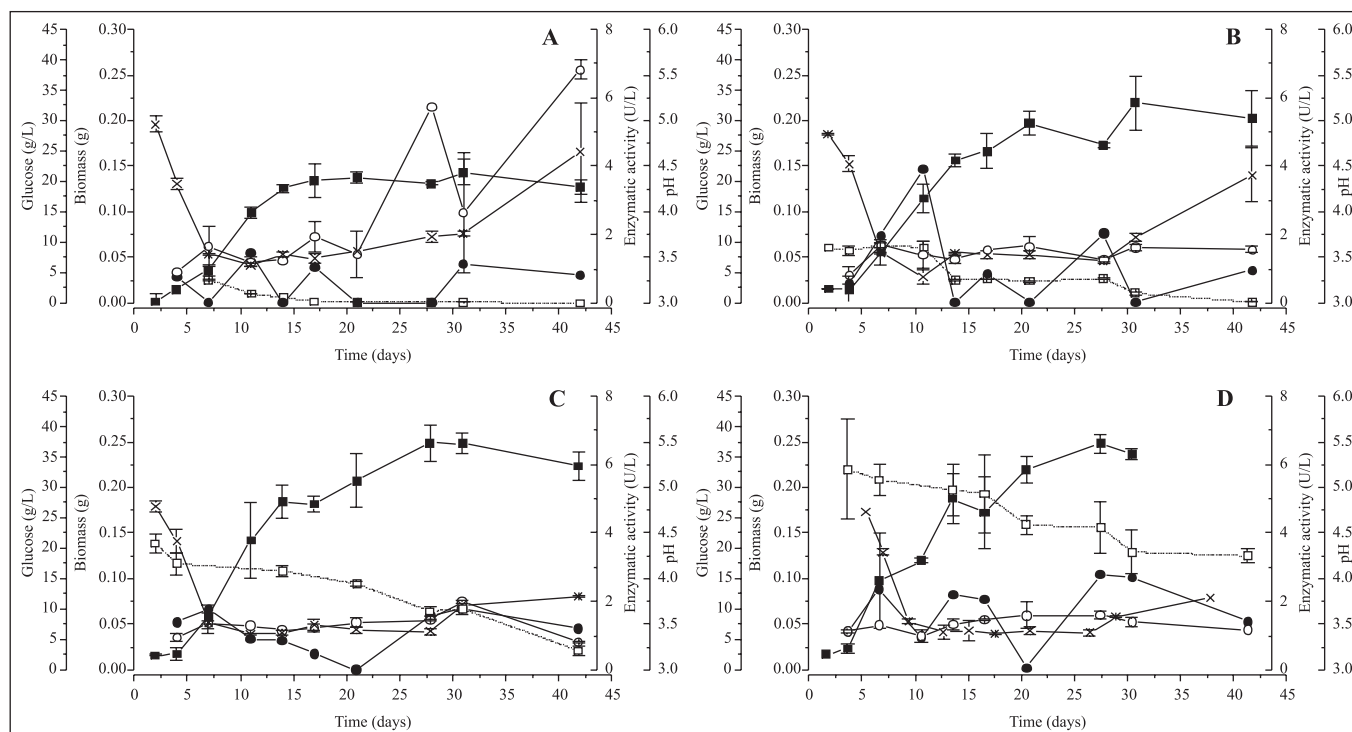
Figure 1. Growth of *Trametes villosa* CCB176 at different concentrations of glucose: (A) 5 g L^{-1}, (B) 10 g L^{-1}, (C) 20 g L^{-1} and (D) 30 g L^{-1}. Biomass (■), glucose (□), pH (x), peroxidase activity (●), laccase activity (○).

growth phase and the increase in medium pH coincided with the beginning of the stationary phase. In general, the pH optimum for the growth of basidiomycetous fungi is close to 4.5 (8,14). The association between acidification of the culture medium during the growth of basidiomycetes and the production of organic acids, as well as the increase in pH after glucose depletion, are well documented in the literature (6,8,27).

Irrespective of the initial glucose concentration, *T. villosa* produced laccase and peroxidase activities throughout the growth phase, in contrast to other basidiomycetes such as *P. chrysosporium* whose ligninolytic system is produced during secondary metabolism (14). An increase in the initial glucose concentration inhibited the synthesis of enzymes produced by *T. villosa* (Fig. 1) as showed for others basidiomycetes like *Botryosphaeria* sp., *T. pubescens* and *Cyathus bulleri* (6,8,21).

The addition of a vegetable oil-Tween 20 emulsion influenced the growth of *T. villosa*, but did not change the acidification process of the medium (Fig. 2). An increase in the concentration of the emulsion prolonged the growth phase of *T. villosa* and resulted in a larger final biomass (0.1; 0.7; 2.0 and 4.0 mg, at 0, 0.2, 0.6 and 1.0 g L^{-1} of emulsion, respectively). The stimulation of laccase activity produced by *T. villosa* was proportional to the concentration of the emulsion, with this activity being about 10 times higher at a concentration of 1.0 g L^{-1} than the laccase

activity obtained without emulsion (Fig. 2). Expressive stimulation of peroxidase activity was observed at a concentration of 0.6 g L^{-1}. Growth of *T. villosa* in the presence of the emulsion induced manganese-dependent peroxidase (MnP) activity, which was proportional to the concentration of the emulsion (Fig. 3). The nature of the surfactant used to emulsify the vegetable oil influenced the growth and production of ligninolytic enzymes by *T. villosa*. The use of Renex instead of Tween 20 inhibited the growth of *T. villosa* by 55% and resulted in 80, 60 and 100% inhibition of laccase, peroxidase and MnP activities, respectively, produced by this fungus. There are a large number of examples of the beneficial effect of detergents on the production of biotechnologically interesting compounds by microorganisms, without the underlying mechanism being completely understood (9). Jäger *et al.* (12) demonstrated for the first time that the addition of surfactants such as Tween 20, Tween 80 and CHAPS permitted the detection of ligninolytic activity produced by *P. chrysosporium* in submersed culture under shaking. Recently, Giese *et al.* (10) showed the inducing effect of Tween 20, 40, 60 and 80 on the production of laccase by the ascomycete *Botryosphaeria* sp.

In order to evaluate the enzymatic stimulation observed with the addition of emulsion, *T. villosa* was again cultured using 5 g L^{-1} glucose in the presence of 0.6 g L^{-1} emulsion and in the
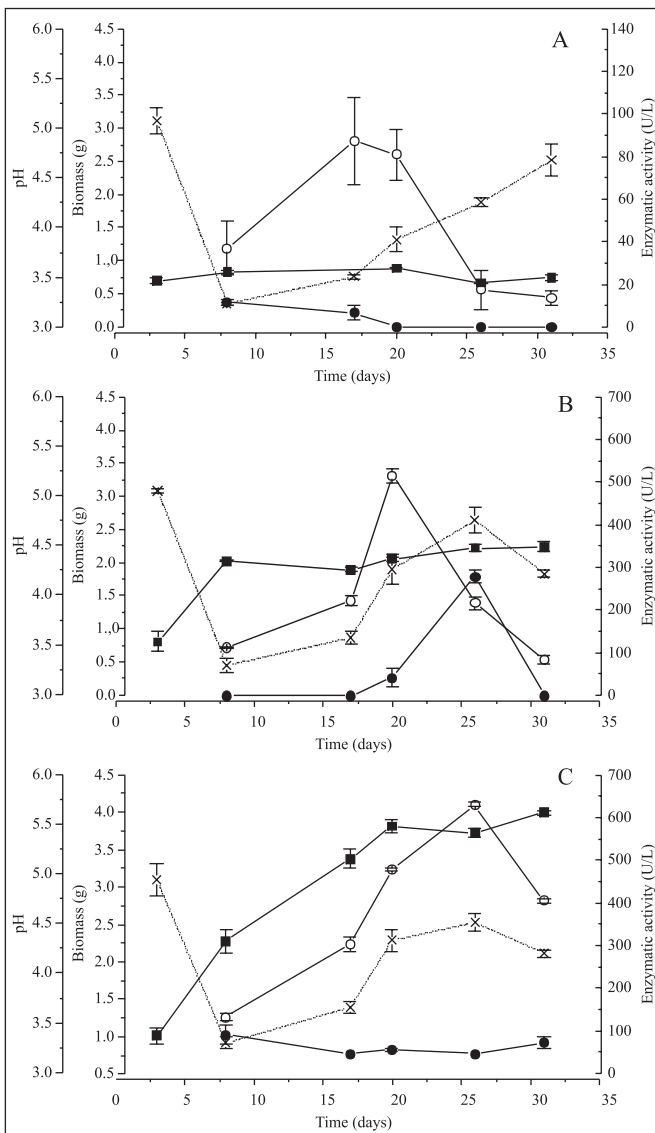
Figure 2. Growth of *Trametes villosa* CCB176 at 5 g L^{-1} of glucose with the addition of different concentration of vegetable oil emulsion with Tween 20: (A) 0.2 g L^{-1}, (B) 0.6 g L^{-1} and (C) 1.0 g L^{-1}. Biomass (■), pH (x), peroxidase activity (●), laccase activity (○).

Figure 3. Manganese peroxidase activity (MnP) produzed by *Trametes villosa* CCB176 at 5 g L^{-1} of glucose, in the absence (—) and at 0.2 g L^{-1} (■), 0.6 g L^{-1} (●) e 1.0 g L^{-1} (▲) of vegetable oil emulsion with Tween 20.

containing enzymatic extract obtained from fungi grown in the absence of emulsion, a finding demonstrating that the emulsion stimulated the production of enzymes during fungal growth and not enzymatic activity during the reaction as described for the process known as lipid peroxidation (11).

The initial Mn^{2+} concentration not influenced the growth of *T. villosa* (Fig. 4). An increase in Mn^{2+} concentration did not induce the production of MnP activity by this fungus and no significant stimulation of laccase activity was observed. Stimulation of peroxidase activity was observed at 22 days. Mn^{2+} is considered to be a mediator, inducer or substrate of MnP, but its role in the expression of ligninolytic enzymes by basidiomycetes is controversial. For many of these fungi such as *P. chrysosporium* and *P. ostreatus* the presence of this metal in the culture medium is important for the production of MnP (5,25). However, Mn^{2+} was not necessary for the production of MnP by *P. ostreatus* or *Bjerkandera* sp. BOS55 (13,22). In addition, the presence of Mn^{2+} may partially or totally stimulate or inhibit the production of the ligninolytic enzymes such as peroxidase and laccase as described for *P. chrysosporium*, *P. ostreatus* and *Bjerkandera* sp. (2,25).

An increase in copper concentration inhibited the growth of *T. villosa*. At 14 days, fungal growth was inhibited by 43% in the presence of 0.2 mM copper (Fig. 5). Higher copper concentrations (0.5; 0.8 and 1.0 mM) resulted in the complete inhibition of the growth of this fungus. Despite growth inhibition, the presence of copper stimulated the production of laccase and peroxidase activities. At 14 and 17 days of culture of *T. villosa*, 35 and 120 times higher laccase and peroxidase activities (731 U L^{-1} and 320 U L^{-1}) were observed when the fungus was

absence of emulsion and using only emulsion as a carbon source without the addition of glucose. The presence of glucose did not influence the final biomass, demonstrating the ability of *T. villosa* to use lipids as a single carbon source. However, the presence of glucose inhibited the production of laccase and MnP activity by about 90 and 50%, respectively. A repressing effect of glucose on the production of laccase activity has been described for *T. pubescens* (8). No MnP activity was detected when the emulsion was added directly to the reaction mixture
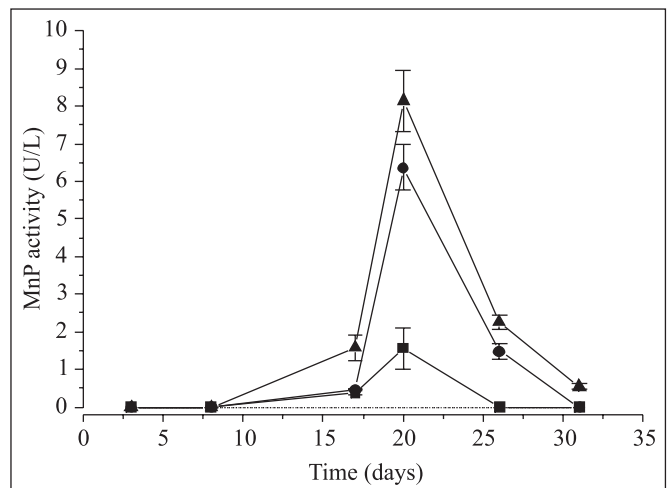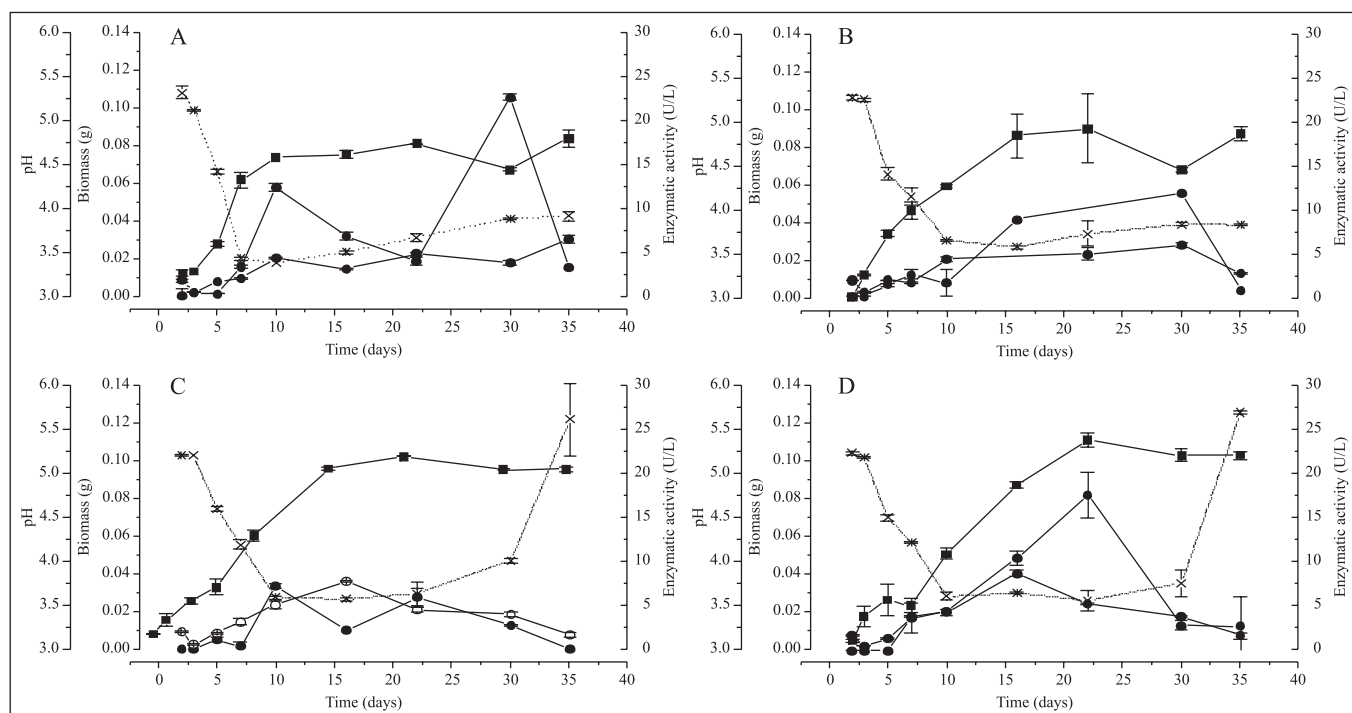
Figure 4. Growth of *Trametes villosa* CCB176 at different concentrations of manganese: (A) 0, (B) 50 µM, (C) 100 µM and (D) 300 µM de Mn^{2+}. Biomass (■), pH (x), peroxidase activity (●), laccase activity (○).

grown in the presence of 0.2 mM copper compared to the activities produced in the absence of the metal (Fig. 5). No enzymatic activities were detected at copper concentrations that completely inhibited the growth of the fungus. Growth inhibition and the stimulation or induction of laccase production by copper has been well documented in the literature, with the optimal concentration of this metal for the growth and production of laccase by basidiomycetous fungi being species specific (7,8). The growth of *Amanita muscaria* (7) was strongly inhibited in the presence of copper (5.0 - 25.0 mg L^{-1}), whereas *P. ostreatus* and *Trametes pubescens* were able to grow in the presence of 2 and 5 mM copper, respectively (1,8).

We calculated the specific yield of laccase activity (units laccase per gram biomass) under the different culture conditions (Table 1). Higher specific yields of laccase activity was obtained with the addition of copper. An increase in the initial glucose concentration resulted in a lower yield of laccase activity by the fungus. Addition of a vegetable oil-surfactant emulsion did not result in a significant increase of laccase yield in *T. villosa*.

CONCLUSION

The results obtained in the present paper reveal interferences of medium composition over the production of ligninolytic enzymes by *Trametes villosa* CCB176 and reinforce

Table 1. Specific yield of laccase activity (units laccase per gram biomass) produced by *Trametes villosa* CCB176 under different culture conditions.

Culture condition	Specific yield (U g^{-1})
Glucose (g L^{-1})	
5	61.0 (4) [a]
10	57.0 (4)
20	54.0 (4)
30	51.0 (4)
Vegetable oil emultion with Tween 20 (g L^{-1})	
0.2	106.0 (8)
0.6	110.0 (8)
1.0	121.0 (8)
Vegetable oil emultion with Renex (g L^{-1})	
0.6	9.0 (9)
Cobre (mM)	
0.2	4,627.0 (7)

a: Time (in days) of fungus growth.

the importance of comprehending the nutritional requirements of each species for the biotechnological application of basidiomycetes. The specific yields of laccase activity obtained
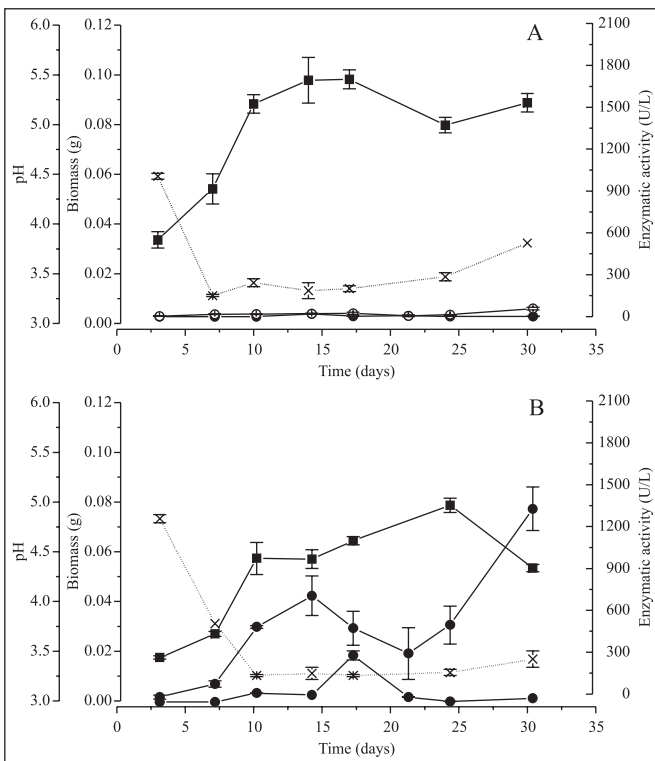
Figure 5. Growth of *Trametes villosa* CCB176 in absence (A) and at 0.2 mM de Cu^{2+} (B). Biomass (■), pH (x), peroxidase activity (●), laccase activity (○).

with the addition of copper show the possibility of optimizing laccase production, an enzyme of commercial interest, through the management of a parameter easy to control, even in industrial scale.

RESUMO

Enzimas ligninóliticas produzidas por *Trametes villosa* CCB176 em diferentes condições de cultivo

A expressão do sistema enzimático produzido por fungos basidiomicetos envolvido na degradação de xenobióticos é bastante dependente das condições de cultivo, principalmente da composição do meio de cultivo. *Trametes villosa* CCB176 é uma linhagem com comprovado potencial biotecnológico para degradação de compostos organoclorados e descoloração de corantes têxteis. Foi avaliada a influência da concentração de glicose, adição de emulsão de óleo vegetal e surfactante, natureza do surfactante e os metais manganês e cobre no crescimento, pH e na produção das atividades de lacase, de peroxidases totais e de peroxidase dependente de manganês. No geral, ocorreu acidificação do meio com pH atingindo valor próximo a 3,5, nos primeiros dias de crescimento. Lacase foi a principal atividade detectada nas diferentes condições e sua produção se deu durante todo o período de cultivo do fungo, independente da fase de crescimento. Suplementação do meio com óleo vegetal emulsificado com surfactante resultou em indução da atividade de peroxidase dependente de manganês produzida por *T. villosa*. Maiores valores de rendimento específico da atividade de lacase foram propiciados pelo cobre.

Palavras-chaves: lacase, metais, MnP, óleo vegetal, surfactante

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