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Optimizing production of extracellular laccase from *Grammothele subargentea* CLPS no. 436 strain

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Summary

The production of extracellular laccase by the *Grammothele subargentea* CLPS no. 436 strain in liquid cultures grown on a carbon-limited basal medium was significantly enhanced when culture conditions, including the addition of $CuSO_4 \cdot 5H_2O$ or veratryl alcohol, were consecutively optimized. A laccase activity as high as 1954.5 mU ml^{-1} of liquid medium was obtained under optimum conditions, which corresponded to non-agitated cultures supplemented with 0.6 mM $CuSO_4 \cdot 5H_2O$. Veratryl alcohol at 1 mM was less effective than $CuSO_4 \cdot 5H_2O$ for increasing laccase activity levels; the supplementation of veratryl alcohol resulted only in maximum levels of 44 mU ml^{-1} in non-agitated cultures.

Introduction

Fungal laccases (*p*-diphenol: oxygen oxidoreductase; EC 1.10.3.2) belong to a group of phenol-oxidases that are currently under study, since they might be useful tools in the pulp and paper industry, bioremediation processes and other biotechnology applications (Yaropolov *et al.* 1994; Johannes & Majcherczyk 2000; Lund & Felby 2001). These enzymes catalyze the oxidation of aromatic amines, phenolic compounds including lignin phenolic units and chlorophenols, anthraquinone dyes and, to a certain extent, some polycyclic aromatic hydrocarbons (PAHs) (Heinzkill & Messner 1997; Johannes & Majcherczyk 2000). Several natural and synthetic compounds which are laccase substrates can act as mediators extending the reactions catalyzed by these enzymes. In this way, laccases are able to oxidize inaccessible substrates, lignin non-phenolic units, azo and indigo dyes, and other PAHs which cannot be oxidized by laccases on their own (Heinzkill & Messner 1997; Wong & Yu 1999; Johannes & Majcherczyk 2000; Lund & Felby 2001).

Grammothele subargentea (Speg.) Rajch. (*Aphyllophorales* Order, *Basidiomycetes*) is a white-rot fungus found mainly in temperate and tropical regions of America and in east Africa (Rajchenberg 1984). Saparrat *et al.* (2002b) found that *Grammothele subargentea* CLPS no. 436 strain showed the highest extracellular laccase activity among several other fungal species examined. This fungus has an outstanding ability to transform and degrade different components of *Eucalyptus globulus* Labill. wood, including lignin, as well as synthetic dyes (M. Saparrat, unpublished observations). In spite of the fact that there are numerous reports on laccases from different white-rot *Basidiomycetes*, there is scarce information regarding these enzymes from *G. subargentea* CLPS no. 436 and other strains of this group of fungi isolated from South America (Saparrat *et al.* 2002a, b), which are potential tools for biotechnological applications. This work examines different culture conditions for optimizing the production of laccase by *Grammothele subargentea* CLPS no. 436 in liquid cultures.

Materials and methods

Grammothele subargentea CLPS (Culture collection of the La Plata Spegazzini Institute) no. 436 was isolated from a fruiting-body collected from the trunk of an *Angiosperm* tree growing in the rain-forest of a subtropical area in the province of Misiones, Argentina. It was grown on the modified Czapek Dox liquid medium (basal medium) (Saparrat *et al.* 2002b). Thousand millilitre Erlenmeyer flasks with 200 ml sterile liquid medium were inoculated with a mycelial suspension (1%, v v^{-1}) as described in Saparrat *et al.* (2002b). Cultures were grown with and without agitation (150 rev. min^{-1}) at 25 ± 1.5 °C. The mycelium was removed from liquid cultures at different time intervals by centrifugation at 20,000 × *g* for 10 min at 4 °C. The

supernatant was collected to measure enzyme activity (expressed as mU ml^{-1} of culture medium) (see below), pH and reducing sugars. Reducing sugars were assayed by the Somogyi and Nelson method (Somogyi 1945). The effect of $CuSO_4 \cdot 5H_2O$ (at concentrations from 150 μM to 1.8 mM) and veratryl alcohol (1 mM) was tested by adding them on the third day of incubation to cultures grown on basal medium. The level of $CuSO_4$ in the basal medium was lower than 0.05 μM. Laccase activity was measured using either 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or 2,6-dimethoxyphenol (DMP) according to Saparrat *et al.* (2002a, b). Aryl-alcohol oxidase (AAO), lignin peroxidase (LiP), manganese-independent peroxidase (MIP) and manganese-dependent peroxidase (MnP) activities were estimated as described in Saparrat *et al.* (2002a, b). All the oxidation rates were determined at 25 °C. One activity unit (U) was defined as the amount of enzyme releasing 1 μmol reaction product min^{-1}. In all the experiments, measurements were carried out in three parallel cultures. The differences in laccase activity production ($P \leq 0.01$) among the treatments tested were assessed using one-way ANOVA with Tukey's honestly significant difference contrasts.

Results and discussion

G. subargentea CLPS no. 436 grew only vegetatively under the culture conditions used in this work. Agitated cultures grown on basal medium resulted in the formation of star-faced mycelial pellets of pale yellowish colour. Non-agitated cultures showed a velvet-like whitish mycelial surface. Agitated cultures were found in trophophase (growth phase) until the day 11 of incubation; the rest of the culture period took place under carbon-limited conditions (idiophase) (Figure 1a). Non-agitated cultures grew under unlimited-carbon conditions during 21 days of incubation (Figure 1b). Subsequently these cultures were found to be in idiophase (data not shown). The cultures of *G. subargentea* grown on basal medium with and without agitation, only revealed extracellular laccase activity. Although this enzyme's activity showed maximum levels of 3 mU ml^{-1} in agitated cultures and 13 mU ml^{-1} in non-agitated ones (Figure 1a and b), the differences were not statistically significant. In spite of the lack of detection of extracellular peroxidase activity in cultures of *G. subargentea*, low levels of extracellular MnP activity on phenol red were previously reported in liquid cultures of *G. subargentea* CLPS no. 436 strain grown on basal medium (Saparrat *et al.* 2002b). However, it was recently found that phenol red can also be oxidized by fungal laccases (Saparrat *et al.* 2002a). Therefore, it is possible that the oxidative activity on phenol red previously reported for the *G. subargentea* CLPS no. 436 strain corresponds to laccase activity.

The effect of either $CuSO_4$ (150 μM) or veratryl alcohol (1 mM) on the cultures of *G. subargentea* on

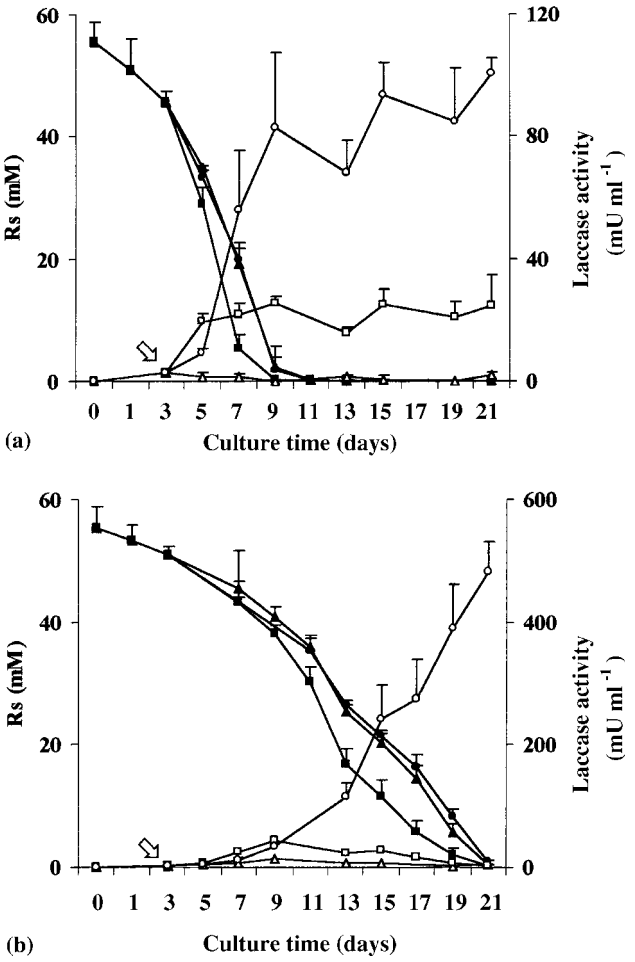

Figure 1. Time course of laccase activity (open symbols) and reducing sugars (Rs) (closed symbols) present in the extracellular fluid of *G. subargentea* CLPS no. 436 cultures grown with (a) and without (b) agitation on liquid basal medium (△) and in the presence of 150 μM $CuSO_4$ (○) or 1 mM veratryl alcohol (□). The arrow indicates the time of addition of the effector to the culture. Values are means of three replicate cultures; error bars represent 1 standard deviation from the mean. Laccase activity was determined with ABTS as substrate. Scale of laccase activity from agitated cultures is represented at a 5-fold lower level than the corresponding values from non-agitated cultures.

basal medium was analyzed. In spite of the orange-like colour of the agitated cultures supplemented with veratryl alcohol, all the inducer-supplemented cultures showed characteristics and kinetics of glucose consumption and pH evolution that were similar to cultures grown on basal medium. Extracellular laccase activity was increased by the addition of $CuSO_4$, both in agitated and non-agitated cultures, to maximum levels of 100 and 483 mU ml^{-1}, respectively. These values represent increments of 33.3- and 37.2-fold compared to cultures grown on basal medium. Several reports stated that copper is a strong laccase inducer among white-rot fungi like *Phanerochaete chrysosporium*, *Trametes versicolor* and *T. trogii* (Collins & Dobson 1997; Dittmer *et al.* 1997; Levin *et al.* 2002). In cultures supplemented with veratryl alcohol both in agitated and non-agitated conditions, laccase activity peaked at 25 and 44 mU ml^{-1}, respectively. These values of activity

represent increments of 8.3- and 3.4-fold compared to cultures grown on basal medium. Increases in the levels of laccase activity by the supplementation of veratryl alcohol to the cultures were previously reported in other white-rot *Basidiomycetes* (Heinzkill & Messner 1997; Lo *et al.* 2001). The supplementation of $CuSO_4$ to the cultures of *G. subargentea* resulted in peak laccase titres, which were significantly higher than those found in veratryl alcohol-supplemented cultures. The maximum levels of laccase activity on non-agitated cultures supplemented with $CuSO_4$ were significantly higher than those found in agitated ones. Veratryl alcohol was a less efficient inducer of laccase activity of *G. subargentea* than $CuSO_4$. In non-agitated cultures, the addition of veratryl alcohol resulted in peak laccase titres significantly higher than those found in cultures grown on basal medium ($P \leq 0.01$). However, the maximum levels of laccase activity found in agitated cultures supplemented with veratryl alcohol were not significantly different from those found in cultures grown on basal medium. In addition, extracellular AAO and peroxidase activities were not detected in cultures grown on basal medium supplemented with $CuSO_4$ or veratryl alcohol.

In order to investigate the effect of $CuSO_4$ levels on laccase production, the *G. subargentea* CLPS no. 436 strain was cultivated on liquid basal medium supplemented with different concentrations of $CuSO_4$. Figure 2 shows the time course of extracellular laccase activity in liquid cultures of the *G. subargentea* CLPS no. 436 strain containing different levels of $CuSO_4$ (0–1.8 mM) added on the third day of incubation. Extracellular laccase activity showed a peak titre after 20 days of cultivation; afterwards enzymatic activity levels decreased until 30 days. Although maximum laccase titres were not significantly different in the range of 0.6–1.2 mM $CuSO_4$ concentrations ($P \leq 0.01$), the highest titres were obtained with 0.6 mM $CuSO_4$ (1954.5 mU ml^{-1}). Concentrations of $CuSO_4$ below 0.6 mM resulted in low levels of laccase activity. Extremely high concentrations of $CuSO_4$ (1.5–1.8 mM) also resulted in significantly lower levels of laccase activity. Levin *et al.* (2002) have reported a 1 mM Cu concentration for maximal laccase production by liquid cultures of *Trametes trogii*. However, lower levels of Cu were found to result in optimal laccase production in liquid cultures of *Marasmius quercophilus* (Tagger *et al.* 1998) and *Pleurotus ostreatus* (Palmieri *et al.* 2000).

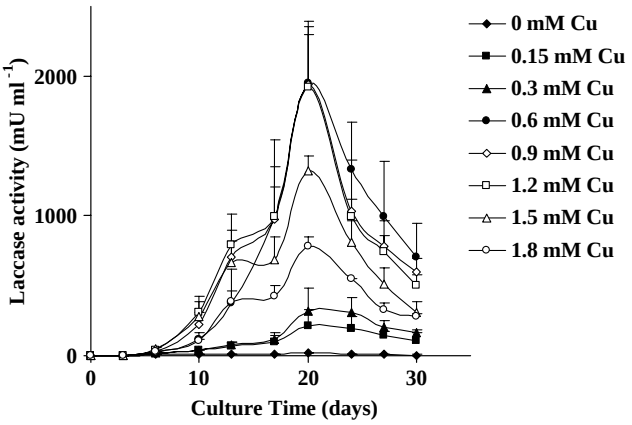

Figure 2. Time course of extracellular laccase activity from non-agitated liquid cultures of the *G. subargentea* CLPS no. 436 strain containing different levels of $CuSO_4$ (0–1.8 mM). The arrow indicates the time of addition of $CuSO_4$ to the culture. Values are means of three replicate cultures; error bars represent 1 standard deviation from the mean. Laccase activity was determined with DMP as substrate.

In this work, it was possible to substantially increase extracellular laccase activity production from *G. subargentea* CLPS no. 436 strain by consecutive optimization of the culture media and growth conditions. These findings make this process worthy of further investigation on a larger scale and have implications in the culture conditions choice and design for the potential application of *G. subargentea* (CLPS no. 436 strain) and its laccase activity in the biotechnology field.

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