Biotechnology Letters **24:** 1375–1377, 2002.
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Extracellular laccase activity in *Tetraploa aristata*

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Received 17 April 2002; Revisions requested 24 April 2002; Revisions received 11 June 2002; Accepted 11 June 2002

Key words: extracellular enzyme, laccase, *Tetraploa aristata*

Abstract

Tetraploa aristata CLPS 419 produced maximum extracellular laccase activity at over 9 mU ml^{-1} in shaking cultures supplemented with glucose and 3.5 mU ml^{-1} in sucrose-grown ones. Laccase activity did not exceed 0.7 mU ml^{-1} in stationary cultures with glucose and was not detected in similar cultures with sucrose or in ones grown on lignin.

Introduction

Laccases (*p*-diphenol: oxygen oxidoreductase; EC 1.10.3.2) are a phenol-oxidases group that are used for transforming and polymerising or degrading different aromatic pollutants causing environmental damage (Johannes & Majcherczyk 2000). They catalyze the direct oxidation of aromatic amines, a wide number of phenolic compounds, including lignin phenolic units, melanin precursors, chlorophenols, anthraquinone dyes and, to a certain extent, some polycyclic aromatic hydrocarbons (PAH), such as anthracene (Heinzkill & Messner 1997, Johannes & Majcherczyk 2000). However, different natural and synthetic compounds which are laccase substrates can act as electronic intermediates or mediators, extending the reactions catalyzed by laccases to the production of oxygen active species (Guillén *et al*. 2000), and oxidation of lignin non-phenolic units, azo and indigo dyes, and other PAH which cannot be oxidized by laccases on their own (Heinzkill & Messner 1997, Wong & Yu 1999, Johannes & Majcherczyk 2000).

One previous work (Saparrat *et al*. 2000) revealed the capacity of different *Hyphomycetes* isolated from Argentina to produce high extracellular laccase activities, higher than the values recorded for other ligninolytic *Basidiomycetes*. The aim of the present work was to report, for the first time, the occurrence of extracellular laccase activity in *Tetraploa aristata* Berkeley et Broome and optimize its production.

Materials and methods

Microorganism and growth

Tetraploa aristata (*Hyphomycetes*, *Deuteromycetes*) CLPS (Culture Collection of the Spegazzini Institute) 419 was isolated from crude oil-polluted organic matter in Santiago river (Buenos Aires province, Argentina). It was grown on a medium containing (per liter): 2 g ammonium tartrate, 0.5 g KCl, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 g yeast extract, 1 ml trace element solution (which contained per liter: 100 mg $B_4O_7Na_2 \cdot 10H_2O$, 70 mg $ZnSO_4 \cdot 7H_2O$, 50 mg $FeSO_4 \cdot 7H_2O$, 10 mg $CuSO_4 \cdot 5H_2O$, 10 mg $MnSO_4 \cdot 4H_2O$, and 10 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$), and 10 g glucose, sucrose or Kraft lignin. Cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml medium with 5% (v/v) mycelial suspension obtained from 14 day old stationary cultures, and incubated under agitation (in a rotatory shaker at 150 rpm) and in stationary conditions at 25 ± 1.5 °C. The mycelium was removed by centrifugation and the supernatant was assayed for enzyme activity. The lignin associated to the mycelium was dissolved by adding 10 ml 1 M NaOH to it. The growth of the organism

was estimated on the basis of mycelial biomass dry wt (mg 100 ml^{-1}).

Enzyme assays

Laccase activity was measured spectrophotometrically with 5 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma), in 100 mM acetate buffer, pH 5.5 (ϵ_{436} of the ABTS cation radical: 29 300 M^{-1} cm^{-1}). Peroxidase activity was assayed as laccase activity in presence of 3 mM H_2O_2. One enzymatic activity unit (U) was defined as the amount of enzyme that transforms 1 μmol substrate min^{-1}. Enzyme activity levels were analyzed statistically using the Tukey test.

Results and discussion

Tetraploa aristata grew only vegetatively – without development of conidia – under the culture conditions used in this work. The shaken cultures showed mycelial pellet formation and the stationary cultures had a velvet-like pellicle. The cultures grown on glucose and sucrose under agitation were a dark grey, while these cultures in stationary conditions were light grey to whitish. Dark-brown to black pigments from fungal cultures are due to the presence of melanins (Bell & Wheeler 1986). Previous work (Heinzkill & Messner 1997, Edens *et al.* 1999) has reported laccase in association with melanized structures. The presence of extracellular laccase activity in *Tetraploa aristata* darkened cultures would suggest a putative role of this enzyme in the melanization process and fungal resistance to crude-oil pollution. The cultures grown on lignin under the different conditions were not pigmented, however.

Tetraploa aristata had the highest growth in shaken cultures supplemented with glucose after 3 days. Growth with lignin was sparce (Figure 1). Extracellular laccase activity was highest in shaken cultures on glucose also after 3 days (Figure 2). In stationary cultures, activities of laccase did not exceed 0.7 mU ml^{-1} and were detected only in the presence of glucose (data not shown).

The differential production of laccase activity in shaken versus stationary cultures could be due to a difference in O_2 availability. Kanunfre & Zancan (1998) and Pointing *et al.* (2000) have reported higher activities of extracellular laccase in, respectively, *Thelephora terrestris* and *Pycnoporus sanguineus* (*Basidiomycetes*) shaken cultures when compared to static conditions.

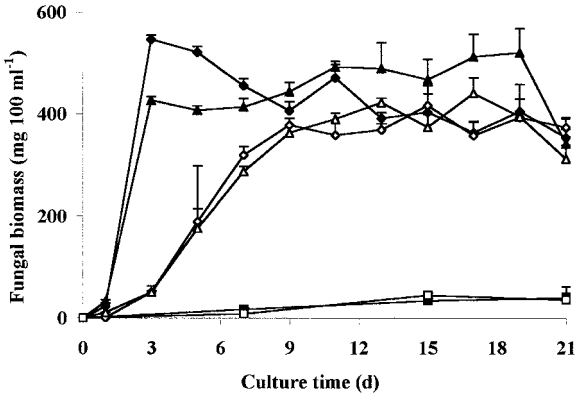

Fig. 1. Time course of fungal biomass of *Tetraploa aristata*. Cultures supplemented with 1% (w/v) of a determined C source under two different culture conditions: ♦, shaken with glucose; ◇, stationary with glucose; ▲, shaken with sucrose; △, stationary with sucrose; ■, shaken with Kraft lignin; □, stationary with Kraft lignin. All values are means for three replicate cultures; error bars represent 1 standard deviation from the mean.

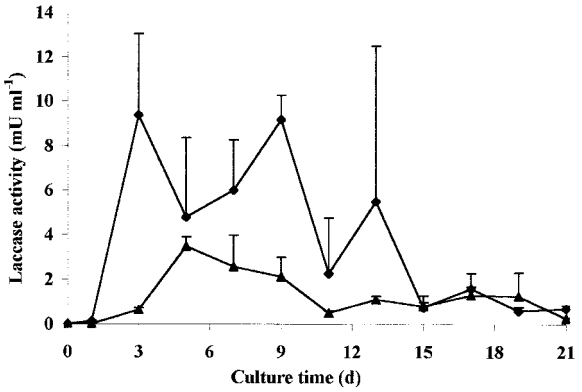

Fig. 2. Extracellular laccase activity of shaken cultures of *Tetraploa aristata* (CLPS 419) with 1% (w/v) glucose (♦) and sucrose (▲). All values are means for three replicate cultures; error bars represent 1 standard deviation from the mean.

Lignin cultures did not show extracellular laccase activity under any condition. H_2O_2 did not exert any effect on the oxidation of ABTS, indicating lack of extracellular peroxidase activity.

Conclusion

This is the first report of extracellular laccase production by *Tetraploa aristata* and highlights that shaken cultures with glucose give the highest laccase activities. These findings have implications in the culture conditions choice and design for the potential appli-

cation of *Tetraploa aristata* (CLPS 419) and their laccases in the biotechnology field.

Acknowledgements

The authors are indebted to Lic. A.M.M. Bucsinszky (CONICET) who is in charge of the culture collection at the Spegazzini Institute (Fac. Ciencias Naturales y Museo UNLP), and to Lic. H.A. Tournier (CIC) for their excellent technical assistance. M.C.N. Saparrat is a recipient of a scholarship from CONICET. M.N. Cabello is a researcher from CIC. A.M. Arambarri is a researcher from CONICET. This research was supported by Grants from CONICET and CIC, Argentina.

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