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ORIGINAL ARTICLE

Laccase activity from the fungus *Trametes hirsuta* using an air-lift bioreactor

S. Rodríguez Couto[1,2], A. Rodríguez[1], R.R.M. Paterson[3], N. Lima[3] and J.A. Teixeira[3]

1 Department of Chemical Engineering, University of Vigo, Vigo, Spain
2 Department of Chemical Engineering, Rovira i Virgili University, Tarragona, Spain
3 Department of Biological Engineering, University of Minho, Braga, Portugal

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Correspondence
Susana Rodríguez Couto, Department of Chemical Engineering, Rovira i Virgili University, 43007 Tarragona, Spain. E-mail: susana.rodriguez@urv.net

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Abstract

Aim: To produce high laccase activities from the white-rot fungus *Trametes hirsuta* in an in-house air-lift bioreactor (ALB).
Methods and Results: *Trametes hirsuta* was grown in a 6-l ALB. A fed-batch strategy with glycerol as an addition resulted in maximum laccase activity of 19 400 U l^{-1}, which was the highest reported from the fungus.
Conclusion: The ALB configuration with additional glycerol resulted in high laccase activities.
Significance and Impact of the Study: This study provides useful information on how to produce high concentrations of laccase.

Introduction

Trametes hirsuta is a basidiomycete white-rot fungus (WRF) that causes wood decay. The term 'white-rot' refers to the wood material after selective degradation of lignin, which leaves the white cellulose exposed. Lignin is an extremely recalcitrant macromolecule. This phenolic macromolecule is the rate-limiting component in wood biodegradation and affords protection to other wood macromolecules and particularly cellulose, by the formation of chemical bonds. The fungus produces a range of lignin-degrading enzymes including laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2), which are part of the 'ligninases' complex of enzymes. The WRF can degrade lignin completely – a unique property in nature (Kirk and Fenn 1982). The enzymes are polyphenol oxidases that require O_2 to oxidize phenols, polyphenols and aromatic amines by one electron transfer resulting in the formation of reactive radicals. In addition, they are capable of oxidizing various nonphenolic substrates in the presence of redox mediators (Bourbonnais and Paice 1990) and to mineralize synthetic dyes (Rodríguez *et al.* 1999; Abadulla *et al.* 2000). Laccases specifically are versatile and active in a broad range of substrates. The versatility of laccase allows the biocatalyst to be suitable for several processes such as biopulping, biobleaching, treatment of industrial wastewater, textile dye decolouration and a wide range of other applications. Indeed, they could become one of the most important biocatalysts in fungal biotechnology (Schauer and Borriss 2004). The genus *Trametes* is known to be one of the most efficient lignin-degrading genera. *Trametes hirsuta*, in particular, is a promising candidate for the production of laccase (Abadulla *et al.* 2000), although *Tramates pubescens* is noted (Galhaup and Haltrich 2001).

The successful application of laccase requires the production of high amounts. However, a lot of work has been undertaken in small flask cultures (Collins and Dobson 1997; Kahraman and Yesilada 2001; Mougin *et al.* 2002), which is not conducive to mass production. Hess *et al.* (2002) found that laccase production by *Tramates multicolor* decreased considerably when the fungus was grown in a stirred-tank reactor (STB), presumably because of the damage to the mycelia caused by shear

stress. Fenice *et al.* (2003) also confirmed the depressing effect of agitation on laccase production. Thus, in order to achieve optimal production, the selection of an appropriate bioreactor configuration is critical.

Air-lift bioreactors (ALB) provide a low shear environment for enzyme production (Bonnarme and Jeffries 1990). They do not possess mechanical stirrers and so the risk of contamination is reduced, as is energy demand (Träger *et al.* 1989). In addition, they are uncomplicated, reliable and of low cost (Kiese *et al.* 1980). The use of a unique ALB to enable the production of high laccase activities is reported herein.

Materials and methods

Fungus

Trametes hirsuta (BT 2566) was obtained from Prof. Dr G.M. Gübitz, Institute for Environmental Biotechnology, Graz University of Technology, Austria, and was grown on potato dextrose agar (PDA) (Sigma Aldrich, St Louis, MO, USA) plates at 30°C for 10 days. Thereafter, the plates were maintained at 4°C.

Inoculum preparation

The composition of the culture medium was: 10 g l^{-1} glucose, 15 g l^{-1} yeast extract (Oxoid Ltd, Hampshire, UK), 0·9 g l^{-1} $(NH_4)_2SO_4$, 2 g l^{-1} KH_2PO_4, 0·5 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0·1 g l^{-1} $CaCl_2{\cdot}2H_2O$, 0·5 g l^{-1} KCl, 0·5 g l^{-1} thiamine in citrate-phosphate buffer (pH 4·5). Erlenmeyer flasks (250 ml) containing 100 ml of culture medium were inoculated with seven agar plugs (diameter 3 mm) from the fungus on PDA and incubated on an rotary shaker (70 rev min^{-1}, 30°C) for 7 days. Ten of these were then used as inoculum by pouring directly into the ALB.

Bioreactor configuration and operating conditions

The ALB employed was designed and constructed at the Department of Biological Engineering, University of Minho (Portugal), and made of Perspex (polymethyl methacrylate). The working volume was 6 l with a concentric draft tube. A full description of the ALB along with a diagram is provided in Domingues *et al.* (1999). The composition of the culture medium was similar to that for the inoculum. In addition, 1 mmol l^{-1} copper sulfate was added to the culture medium on the second day of cultivation to stimulate laccase production. Other nutrients were added during fermentation under sterile conditions (Table 1). The bioreactor was at room temperature, and filtered air was supplied at 1 vvm by means of a perforated plate at the bottom of the reactor, thus promoting efficient air diffusion throughout the reactor. pH was controlled at 4·5 throughout the fermentation by automatic addition of HCl (1 mmol l^{-1}). Samples were collected, centrifuged (8000 ***g***, 5 min) and analysed in triplicate. The values in the figure correspond to mean values of replicate experiments and had a standard deviation of <15% (Fig. 1). Growth of the fungus was assessed visually.

Table 1 Yield and activity increases in laccase after the addition of different carbon sources

Carbon	Addition time (days)	Yield increase (U l^{-1} 24 h^{-1})	$Laccase_{max}$ (U l^{-1})
Glucose	12	400	10 598
Cellulose	14	1870	12 468
Fructose	19	1417	7952
Glycerol	24	6634	19 394
Glycerol	33	2237	7551

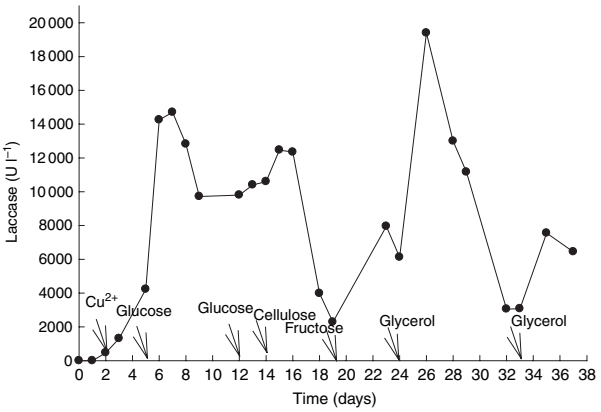

Figure 1 Laccase activity from *Trametes hirsuta* grown in the 6-l air-lift bioreactor.

Enzyme assay

The reagent 2,2′-azino-di-[3-ethyl-benzothiazolin-sulfonate] (ABTS) was used as a substrate for spectrophotometric determination of laccase activity (Niku-Paavola *et al.* 1990). One activity unit (U) was the amount of enzyme that oxidized 1 μmol of ABTS min^{-1} and the activities were expressed in U l^{-1}.

Results

There was a lag phase in growth for the first 24 h followed by a rapid increase in biomass until day 5 (Fig. 1). The biomass was maintained at a constant level after this period. Laccase activity was observed initially on day 2 (467 U l^{-1}) and increased rapidly to

Table 2 Maximum laccase activities obtained by other workers using bioreactors

Fungus	Type of reactor (working volume)	Type of cultivation	Inducer	Maximum laccase (U l^{-1})	Reference
Pycnoporus cinnabarinus	Packed bed (n.s.)	Immobilized on nylon cubes	–	270	Schliephake *et al.* (2000)
Trametes pubescens	Stirred tank (15 l)	Free cells	$CuSO_4$	61 900	Galhaup and Haltrich (2001)
Neurospora crassa	Capillary membrane	Immobilized on a membrane	–	10 000	Luke and Burton (2001)
T. multicolor	Stirred tank	Free cells	$CuSO_4$, glycerol	–	Hess *et al.* (2002)
Pleurotus ostreatus	Benchtop (3 l)	Free cells	OMW	65	Aggelis *et al.* (2003)
Irpex lacteus	Packed bed (27 ml)	Immobilized on PUF cubes	–	–	Kasinath *et al.* (2003)
Panus tigrinus	Stirred tank (2 l)	Free cells	OMW	4600	Fenice *et al.* (2003)
P. tigrinus	Air-lift (2·5 l)	Submerged fermentation	OMW	4300	Fenice *et al.* (2003)
P. tigrinus	Rotatory drum (1·3 l)	SSF on chopped maize stalks	OMW	1309	Fenice *et al.* (2003)
T. versicolor	Air-lift (2 l)	Free cells	Xylidine, Tween 80	1676	Rancaño *et al.* (2003)
T. versicolor	Immersion (2·5 l)	SSF on barley bran	Tween 80	600	Rodríguez Couto *et al.* (2003)
T. versicolor	Immersion (2·5 l)	SSF on nylon cubes	Tween 80	229	Rodríguez Couto *et al.* (2003)
T. versicolor	Expanded bed (0·3 l)	SSF on barley bran	Tween 80	600	Rodríguez Couto *et al.* (2003)
T. versicolor	Expanded-bed (0·3 l)	SSF on nylon cubes	Tween 80	126	Rodríguez Couto *et al.* (2003)
T. versicolor	Tray (1 l)	SSF on barley bran	Tween 80	3500	Rodríguez Couto *et al.* (2003)
T. versicolor	Tray (1 l)	SSF on nylon cubes	Tween 80	343	Rodríguez Couto *et al.* (2003)
T. versicolor	Fluidized (1·5 l)	Free cells	–	1685	Blánquez *et al.* (2004)
T. hirsuta	Fixed bed	Immobilized on stainless steel sponge	$CuSO_4$	2206	Rodríguez Couto *et al.* (2004a)
T. hirsuta	Immersion (0·5 l)	Immobilized on stainless steel sponge	$CuSO_4$	4892	Rodríguez Couto *et al.* (2004b)
T. hirsuta	Air-lift (2 l)	Immobilized into Ca-alginate	Veratryl alcohol	1043	Domínguez *et al.* (2005)
T. hirsuta	Immersion (0·5 l)	SSF on grape seeds	–	18 715	Rodríguez Couto *et al.* (2006)
T. hirsuta	Tray (0·2 l)	SSF on nylon sponge cubes	–	6898	Rodríguez Couto *et al.* (2006)
T. hirsuta	Tray (0·2 l)	SSF on grape seeds	–	12 877	Rodríguez Couto *et al.* (2006)
T. hirsuta	Air-lift (6 l)	Free cells	$CuSO_4$, glycerol	19 400	This work

SSF, solid-state fermentation; OMW, olive mill wastewater; PUF, polyurethane foam.

14 704 U l^{-1} on day 7 after the first addition of glucose. The activity decreased to approx. 10 000 U l^{-1} on day 9. A second addition of glucose on day 12 increased laccase to 10 598 U l^{-1}. Cellulose addition on day 14 increased activity to 12 468 U l^{-1} by day 15. However, on day 18, laccase activity decreased abruptly (3983 U l^{-1}) and the addition of fructose on day 19 increased the activity to 7952 U l^{-1}.

Glycerol was added on day 24, which increased laccase activity to the maximum of 19 394 U l^{-1}. Activity decreased subsequently until day 32. Another addition of glycerol on day 33 increased the value to 7551 U l^{-1}. The rates of increase in yields are provided in Table 1. Glycerol provided the most rapid increases of 6634 and 2237 U l^{-1} 24 h^{-1} for days 24 and 33 respectively.

Discussion

Growth was maximum after 5 days and there was a concomitant increase in laccase activity. The increase in enzyme activity was probably from the increase in biomass and not from the effect of the additional carbon sources. Highly significant increases in laccase were observed after the addition of glycerol in particular, especially on the first occasion. It is not known if the age of the culture was also a factor in the increase, and more work is required to determine the effect of culture age on laccase production. The absolute effect of fructose was unclear as activity was tested 4 days after addition and so an earlier effect may have been missed.

The effect of different carbon sources on the growth of the fungus was not determined. However, a concomitant increase in biomass within 24–48 h of the addition would not be expected as observed with laccase activity as it would take a longer time. Glycerol or fructose would take considerably longer to metabolize into biomass than glucose, and if any such increase were found, it would be reduced. Increases in laccase activity were over 100% in some cases, and this could not be matched with an equivalent increase in biomass especially in such a short period of time. Finally, addition of these compounds already has been demonstrated to stimulate specifically laccase production (Table 2) and so there is precedence in assuming that they are responsible for the observed effects. The sequential addition of the nutrients (copper, glucose, cellulose, fructose and glycerol) may have had an effect on laccase production. So, the results observed may be related to the combined effect of the various additions, which represents a unique feature of this protocol. However, individual additions require investigations in subsequent research.

Extraordinarily high laccase production was observed by Lomascolo *et al.* (2003) from *Pycnoporus cinnabarinus* with ethanol as the inducer. For example, 266 600 U l^{-1} were obtained. This was from small flasks and results are awaited for scale-up to indicate if an economic process is feasible. Previous work has indicated that growth in STB can be problematic (Hess *et al.* 2002). Values of more than 60 000 U l^{-1} were obtained for *T. pubescens* in a commercial STB and shake flasks (Galhaup and Haltrich 2001). However, yields for *T. hirsuta* were considerably lower in shake flasks (approx. 8000 U l^{-1}) and this fungus was not tested in the STB. It would be interesting to compare the yields from *T. pubescens* in the ALB described in the present report.

The results demonstrate that the ALB in the present report is suitable for achieving high laccase activities. In addition, glycerol was demonstrated to be effective in the same capacity. This agrees with the results reported by Hess *et al.* (2002), who found that glycerol was the most effective carbon source for laccase production by *T. multicolor* in shaken flasks cultures. However, Hou *et al.* (2004) demonstrated that glycerol and glucose led to similar laccase activities in static cultures of *Pleurotus ostreatus*. As mentioned, glycerol increased laccase significantly in the present work with *T. hirsuta*. Undoubtedly, laccase production is dependent on the microbial taxa employed.

The activities attained were due in part to the design of the reactor, which provided suitable production conditions including a low shear environment. Interestingly, Fenice *et al.* (2003) observed that the lag phase and the maximal production of laccase in an ALB were reduced compared with those observed from other configurations (Table 2) and this requires further investigation with the ALB described in the present report. It is noteworthy how many different growth conditions and fungi have been employed in the previous reports making comparisons difficult. Indeed a taxonomic revision of *Trametes* may be in order to define species concepts especially in terms of laccase production.

The ALB employed herein was suitable for the production of laccase by *T. hirsuta*, as high laccase activities were obtained for prolonged times without operational problems. This makes the system promising for its application to continuous operation, which may be advantageous. In view of these results, more studies to determine the optimal conditions are underway.

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