Journal of Industrial Microbiology (1996) 17, 69–72

Production of laccase by *Botrytis cinerea* and fermentation studies with strain F226

MG Fortina[1], A Acquati[1], P Rossi[1], PL Manachini[1] and C Di Gennaro[2]

[1]*Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Sezione di Microbiologia Industriale, Università degli Studi di Milano, 20133 Milano;* [2]*Enichem Synthesis Spa, San Donato Milanese, Milano, Italy*

After induction, seven strains of *Botrytis cinerea* released into the culture broth considerable amounts of laccase in a brief production time. The set-up of a suitable production process was studied with a selected strain in a 10-L fermenter. The optimum fermentation conditions were a 3% inoculum with a high degree of sporulation, a simple medium containing 20 g L^{-1} of glucose and 2 g L^{-1} of yeast extract at pH 3.5, 2 g L^{-1} gallic acid as inducer, added after 2 days of growth, an agitation speed of 300 rpm, an aeration rate of 1.2 vvm and a temperature of 24°C. By optimizing the culture conditions, the enzyme activity reached 28 U ml^{-1} in 5 days with a specific activity of 560 U mg^{-1} protein. The best procedure to obtain a suitable crude enzyme preparation was concentration of the supernatant medium to 10% of the initial volume by ultrafiltration, followed by a fractional precipitation with ethanol. The optimum pH and temperature for laccase activity were 5.5 and 40°C, respectively, with syringaldazine as the substrate.

Keywords: laccase; *Botrytis cinerea*; scaling up; optimization

Introduction

Laccase (*p*-diphenol:O_2 oxidoreductase, E.C. 1.10.3.2.) is an enzyme which catalyzes the oxidation of phenolic substrates by molecular oxygen. This type of enzyme is widely distributed among microorganisms, principally in a variety of fungal strains [5,6,9,11–13,17,19].

For their specific catalytic activity, laccases could find application in the preparation of musts and wines and in fruit juice stabilization [4,10], since stabilization of the final product is one of the major problems in beverage production. Many innovative treatments, such as enzyme inhibitors, complexing agents and sulphate compounds, have been proposed for the removal of phenolics responsible for discoloration, haze and flavor changes. The possibility of using enzymatic treatments as a specific and mild technology for stabilizing beverages against discoloration and clouding represents an attractive alternative.

Comparative studies of fungal laccases have shown that these enzymes are similar in their catalytic activity on phenolic compounds, regardless of their origin, but differ markedly in their inducibility, number of enzyme forms, molecular weight and pH optimum [2].

Conditions favorable for laccase production have been studied [1,7,8,15] but the data reported concern shake flask production of laccase activity. Little information is available on the scale up of mycelial shake flask production in fermenters to obtain higher levels of enzyme activity.

This paper deals with the production of laccase activity in a 10-L stirred tank fermenter with a selected strain of *B. cinerea*. The most favorable conditions for crude enzyme recovery and the principal characteristics of the produced enzyme are reported.

Correspondence: MG Fortina, Sezione di Microbiologia Industriale, DISTAM, Via Celoria 2, 20133 Milano, Italy
Received 21 April 1995; accepted 30 May 1996

Materials and methods

Microorganism

Seven isolates of *Botrytis cinerea* were screened for laccase activity on malt agar plates in the presence of anthocyans. The isolates positive to the qualitative test were tested for quantitative production of laccase activity in flask cultures employing different media and in the presence of gallic acid as inducer.

One strain, F226, isolated from chestnut leaf and deposited in the Collection of the Section of Industrial Microbiology, DISTAM, Milan, Italy, yielded the best laccase production in synthetic media and therefore was selected for further studies. This strain was maintained on malt agar or PGY (potato-glucose-yeast extract) agar at 28°C and stored at 4°C.

Media

Medium A: potato (infusion of) 200 g L^{-1}, glucose 20 g L^{-1}, yeast extract 2 g L^{-1}, pH 5.0; medium B: glucose 20 g L^{-1}, yeast extract 2 g L^{-1}, pH 5.0; medium C: medium B at pH 3.5.

Tap water was used to make up the media. The copper content, ranging from 0.05 to 0.4 mg ml^{-1} was sufficient to guarantee an excess of this ion, as verified by experiments in which the addition of copper ion did not increase the laccase activity recovered.

Assay of laccase activity

Laccase activity was measured spectrophotometrically using 3 ml of 0.1 M syringaldazine in 0.05 M citrate-phosphate buffer (pH 5.5) [14]. The reaction was initiated by the addition of 0.15 ml of properly diluted enzyme solution

and the increase in absorbance was monitored at 525 nm. One unit of laccase was defined as the amount of enzyme required to cause a change in absorbance of 0.1 per min at 40°C. Similar conditions were used to test activity with guaiacol and catechol by their replacement with syringaldazine. In these cases substrate concentration and wavelength utilized were respectively: 0.001 M and 465 nm for guaiacol [7] and 0.02 M and 420 nm for catechol [4].

The concentration of protein in the enzyme preparations was determined according to Bradford [3], using bovine serum albumin as standard.

Dry weight determination

For determination of the dry weight of mycelia, 100 ml of the culture broth was centrifuged and the mycelium was washed twice with distilled water and dried to a constant weight at 105°C.

Fermentations

Fermentations were conducted with the selected strain *B. cinerea* F226. Batch fermentations were carried out in a 10-L (6-L working volume) glass jar (17 cm in diameter and 44 cm high) with a metal head fermenter (Cavallo Comm G, snc, Milan, Italy). The inoculum consisted of one culture grown in 750-ml flasks containing 150 ml of medium A. Production experiments were carried out using media B and C to which inducers were added at different concentrations (0.5, 1 or 2 g L^{-1}) and at different times. Tyraminium chloride, catechin, anthraquinone-2-sulphonic acid and gallic acid were used as inducers. With the exception of tyraminium, which is soluble in water, the compounds were dissolved in the smallest volume of ethanol and then sterilized by filtration. The effects of agitation speed and aeration rate on enzyme production were also studied.

Recovery of the crude enzyme

All steps were performed at 4°C unless otherwise stated. At the end of the fermentation period, the fungal culture was centrifuged at $10000 \times g$ for 20 min to remove mycelium and the supernatant medium was subjected to different methods of concentration: a) fractional precipitation with ammonium sulphate at 40, 60 and 80% saturation; b) fractional precipitation with ethanol; c) combination of ultrafiltration in a spiral cartridge (molecular weight cut-off 30 000) (Intersep Filtration Systems, Wokingham, Berkshire, UK) and ethanol precipitation.

Laccase characterization

The pH and temperature optima for laccase activity were determined employing syringaldazine as the substrate. The range of temperatures assayed was from 25 to 60°C and the range of pH from 3 to 6. Substrate specificity was determined by using catechol, guaiacol and syringaldazine at different pH values; the assays were conducted at 40°C.

Results and discussion

Laccase production by B. cinerea

When grown in shaken flasks incubated at 28°C on a rotary shaker at 150 rpm, on medium A supplemented with 2 g L^{-1} gallic acid, *B. cinerea* F226 produced appreciable levels of laccase (9.8 U ml^{-1}) in a brief period (5–7 days). Compared to other fungal species for which a longer production time (12–30 days) is required [2], this property is of some interest from the industrial point of view.

Laccase production was not associated with mycelial growth; maximum production of activity was observed in 5–7 days, whereas growth reached a maximum value (18 mg ml^{-1} dry mycelial weight) after 10–12 days.

Fermentation experiments

Starter cultures: Cells from PGY slants maintained at 28°C for 8–10 days were used to inoculate shake flask cultures. On this medium and after this incubation time, the *B. cinerea* culture showed a more extensive degree of sporulation than in other media. The mycelial mass so obtained was used to inoculate one 750-ml erlenmeyer flask containing 150 ml of medium, which gave a suitable inoculum for a 10-L fermenter.

Inoculum characteristics: We found a correlation between laccase production and the degree of sporulation of the inoculum. The most marked increase in activity occurred with inocula that were the most sporulated. However, during the preparation of the inoculum, we encountered difficulty in obtaining a good separation of spores from vegetative cells and in determining an exact concentration of the spores because of their tendency to remain aggregated. For this reason, we conducted optimization experiments using the culture broths as inoculum. Culture broths derived from slants with a high degree of sporulation and maintained on a rotary shaker at 28°C for 6–8 days yielded a good and reproducible inoculum with regard to production of laccase. The content of one culture flask, representing a 3% (v/v) inoculum was used to inoculate a 10-L fermenter.

Effect of medium composition: Initial experiments showed that *B. cinerea* strain F226 produced high laccase activity when grown in medium A. However, since the quality of commercial potatoes varies from batch to batch and the preparation of an infusion of potatoes for large volumes is not desirable, we conducted production experiments in which the medium was prepared without potato infusion (medium B). Comparable results of laccase production were obtained in the two media tested, and for this reason, potato infusion was excluded. Additional attempts to increase enzyme production were made studying the influence of glucose concentration in the medium. For this purpose, laccase production was evaluated in medium B prepared with carbon sources ranging from 2 to 20 g L^{-1}. The maximal level of laccase was obtained in the medium containing the highest glucose concentration.

The relative amount of laccase produced by *B. cinerea* strain F226 also depended on the pH of the medium. The laccase from *B. cinerea* F226 was stable in the pH range 3.5–5.0; little activity was detected when the medium was prepared at pH 6.5. At pH 3.5 the enzyme yield was 20% greater than in medium at pH 5.0. Moreover, when the pH was maintained at 3.5 a lower mycelial growth and a conse-

quent lower production of polysaccharidic compounds was observed, simplifying the subsequent steps of recovery of the crude enzyme. Thus, medium C was used for bulk laccase production by *B. cinerea* strain F226.

Effect of inducers: Attempts were made to determine the effect on enzyme yield of compounds employed for laccase induction in other fungi. No increase in enzyme yield above that in control cultures grown in the presence of gallic acid was found, and at the higher concentrations tested the compounds were inhibitory to enzyme production. Catechin was the most suitable of the inducers tested but at 1 g L^{-1} yielded 50% less laccase activity than the corresponding amount of gallic acid. Gallic acid was therefore the most effective. Thus, although its addition caused browning of the growth medium due to its oxidation, gallic acid was used as the inducer for the production of laccase by *B. cinerea* strain F226. The effect of gallic acid depended on the time of addition. Addition after 1 or 2 days was equally effective, but addition after 4 days resulted in much lower laccase formation; moreover, addition after 2 days apparently had some effect on production of other proteins, as an increase in specific activity was observed by a lowering of the measured total protein content. Optimal enzyme production was obtained using 2 g L^{-1} of inducer, *ca* 20% more activity than at 1 g L^{-1}.

Effect of temperature: For the fermentation process the temperature was lowered to 24°C to better control the mycelial growth to facilitate recovery of the crude enzyme.

Fermentation process

The optimum fermentation conditions for laccase production by *Botrytis cinerea* strain F226 were:

Inoculum: 3% v/v of a culture broth derived from an 8-day old PGY agar slant, grown in medium C, at 28°C for 7 days.

Fermentation: 10-L fermenter with working volume of 6 L; medium C; inducer: gallic acid 2 g L^{-1} added after 2 days of growth; cultivation temperature 24°C; aeration rate 1.2 vvm; agitation speed 300 rpm; pH automatically controlled at 3.5. When the fermentation was carried out under these conditions maximal laccase activity (28 U ml^{-1}) was obtained in 5 days, with a specific activity of 560 U mg^{-1} of protein. The dry weight of mycelia increased to 13 mg ml^{-1} in 5 days (Figure 1).

Recovery of the crude enzyme

At the end of the fermentation period the supernatant medium obtained by centrifugation of the cultures was viscous. We hypothesize that this viscosity was due to the presence of polysaccharides that were secreted into the medium with the enzyme. Different strains of *B. cinerea* produce a β-1,3-D-glucan exopolysaccharide (cinerean) some of which adheres to the mycelium forming a capsule while the remainder dissolves in the medium [18]. Several procedures were used to remove these contaminants during concentration of the supernatant.

Fractionation with ammonium sulphate allowed the removal of viscous matter at 60% saturation, but was not effective in precipitating the enzyme at 80% saturation, due to a low protein concentration (50 mg L^{-1}) in the medium.

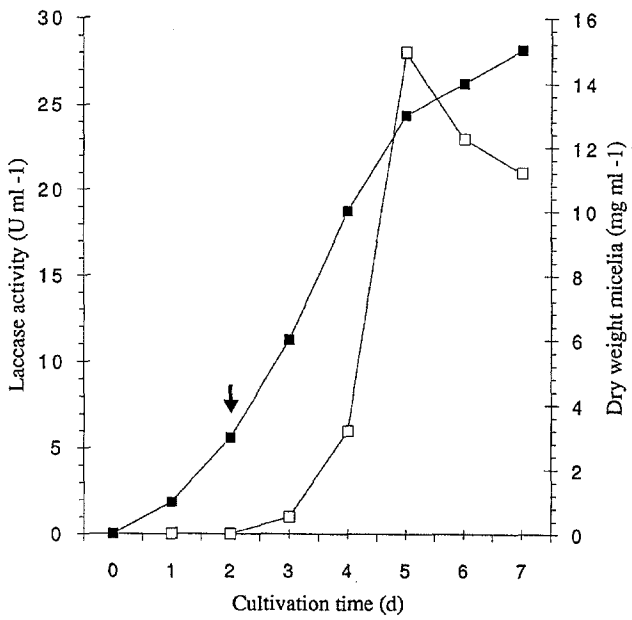

Figure 1 Time course of laccase production in fermenter by *Botrytis cinerea* strain F226. Symbols: ■, dry weight of mycelium; □, laccase activity; arrow, addition of gallic acid.

The possibility to obtain a suitable crude enzyme preparation quickly was tested by ethanol precipitation. The addition of 0.5 V of cold ethanol precipitated the gelatinous mass, after the mixture was allowed to stand at −20°C for 3 h. The supernatant phase was collected by centrifugation and was then supplemented with cold ethanol to a final concentration of 2 V and maintained at −20°C overnight. The precipitate, containing laccase activity, was dissolved in a minimal volume of citrate buffer at pH 3.5. The total recovery of enzyme was 85% with a 3-fold increase in specific activity.

To avoid the use of considerable amounts of ethanol, we carried out experiments in which the supernatant from culture broth was first concentrated to 1/10 of the initial volume by ultrafiltration with spiral cartridges (30000 M.W. cut-off) and then subjected to fractional precipitation with ethanol, as described above. The yield of the process was 78% with a 4-fold increase in specific activity. The last procedure was sufficiently fast and allowed us to obtain a crude enzyme preparation with high specific activity and free of polysaccharide compounds.

Principal properties of laccase activity

The pH optimum for the laccase activity with syringaldazine as substrate, was 5.5, with 60% residual activity at pH 3 and little or no activity above pH 6. The temperature optimum was 40°C, with 75% residual activity at 50°C and 5% at 60°C.

The substrate specificity of the crude enzyme preparation of *B. cinerea* strain F226 is shown in Table 1. All substrates tested are oxidized by the enzyme, but the pH optima for laccase activity differ depending on the substrate oxidized. This dependence was reported previously [16].

Table 1 Substrate specificity of laccase by *Botrytis cinerea* strain F226

Substrate	Substrate conc (M)	Laccase activity (%)	pH optimum
Syringaldazine	0.1	100	5.5
Catechol	0.02	67	5.0
Guaiacol	0.001	58	4.0

The laccase from strain F226 was stable from pH 3.5 to 5.0, and it was inactivated above pH 6. At pH 3.5 and at 4°C the enzyme retained 90% of its initial activity after 3 days, and 70% after 6 days. The enzyme remained completely stable at 40°C for 1 h at pH 3.5, while at pH 5.0 40% activity was lost. At 50°C the enzyme was unstable; after 1 h the residual activity was 10%.

Acknowledgements

This work was conducted with a contract on advanced biotechnologies in the National Research program of the Ministry of the University and Technological and Scientific Research and entrusted to Enichem Synthesis SpA, Palermo. The authors thank Dr Angelo Righetti of the Chemiferm (Livraga, Lodi) for his kind collaboration.

References

1 Assavanig A, B Amornkitticharoen, N Ekpaisal, V Meevootisom and TW Flegel. 1992. Isolation, characterization and function of laccase from *Triochoderma*. Appl Microbiol Biotechnol 38: 198–202.
2 Bollag J-M and A Leonowicz. 1984. Comparative studies of extracellular fungal laccases. Appl Environ Microbiol 48: 849–854.
3 Bradford MM. 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.
4 Cantarelli C, O Brenna, G Giovanelli and M Rossi. 1989. Beverage stabilization through enzymatic removal of phenolics. Food Biotechnol 3: 203–213.
5 Dubernet M, P Ribéreau-Gayon, HR Lerner, E Harel and AM Mayer. 1977. Purification and properties of laccase from *Botrytis cinerea*. Phytochemistry 16: 191–193.
6 Durrens P. 1981. The phenoloxidases of the ascomycete *Podospora anserina*: the three forms of the major laccase activity. Arch Microbiol 130: 121–124.
7 Fahraeus G, V Tullander and H Lyunggren. 1958. Production of high laccase yields in cultures of fungi. Physiol Plant 11: 631–643.
8 Froehner SC and K-E Eriksson. 1974. Induction of *Neurospora crassa* laccase with protein synthesis inhibitors. J Bacteriol 120: 450–457.
9 Froehner SC and K-E Eriksson. 1974. Purification and properties of *Neurospora crassa* laccase. J Bacteriol 120: 458–465.
10 Giovanelli G and G Ravasini. 1993. Apple juice stabilization by combined enzyme-membrane filtration process. Lebensm-Wiss u-Technol 26: 1–7.
11 Ishikawa H, WJ Schubert and FF Nord. 1963. Investigations on lignins and lignification. XXVII. The enzymic degradation of softwood lignin by white-rot fungi. Arch Biochem Biophys 100: 131–139.
12 Kurtz MB and SP Champe. 1982. Purification and characterization of the conidial laccase of *Aspergillus nidulans*. J Bacteriol 151: 1338–1345.
13 Leatham GF and MA Stahmann. 1981. Studies on the laccase of *Lentinus edodes*: specificity, localization and association with the development of fruiting bodies. J Gen Microbiol 125: 147–157.
14 Leonowicz A and K Grzywnowicz. 1981. Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate. Enzyme Microb Technol 3: 55–58.
15 Marbach I, E Harel and AM Mayer. 1983. Inducer and culture medium dependent properties of extracellular laccase from *Botrytis cinerea*. Phytochemistry 22: 1535–1538.
16 Mayer AM and E Harel. 1979. Polyphenol oxidases in plants. Phytochemistry 18: 193–215.
17 Sannia G, P Giardina, M Luna, M Rossi and V Buonocore. 1986. Laccase from *Pleurotus ostreatus*. Biotechnol Lett 8: 797–800.
18 Stahmann K-P, P Pielken K-L, Schimz and H Sahm. 1992. Degradation of extracellular β-(1,3)(1,6)-D-glucan by *Botrytis cinerea*. Appl Environ Microbiol 58: 3347–3354.
19 Von Hunolstein C, P Valenti, P Visca, G Antonini, L Nicolini and N Orsi. 1986. Production of laccases A and B by a mutant strain of *Trametes versicolor*. J Gen Appl Microbiol 32: 185–191.