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Effect of mannan oligosaccharide elicitor and ferulic acid on enhancement of laccases production in liquid cultures of basidiomycetes

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Abstract

The effects of mannan oligosaccharides preparation (MO), as elicitor, and ferulic acid inducer for enhancement in laccases production in liquid cultures of three strains of basidiomycetes, *Pycnoporus sanguineus*, *Coriolopsis polyzona* and *Pleurotus ostreatus* was studied using a full factorial 3^2 experimental design. MO, either individually or combined with ferulic acid, enhanced laccases levels in the three different strains of the white-rot fungi. The enhancement of laccases production was species specific with the highest increase in liquid cultures of *P. sanguineus* (88-fold) followed by *P. ostreatus* (3-fold) and *C. polyzona* (2-fold). Separate additions of 75 mg/l of MO to the cultures of *P. sanguineus* and *P. ostreatus* caused the increase while a combined addition of 150 mg/l of MO and 1 mM ferulic acid resulted in the optimal production of laccases in the cultures of *C. polyzona*.

Keywords: Basidiomycetes; Mannan oligosaccharides; Ferulic acid; Laccases; Response surface methodology

1. Introduction

White-rot fungi, a heterogeneous group of lignin-degrading basidiomycetes, have received considerable attention for their bioremediation potential [1–3]. They have the capability of degrading lignin due to their extracellular non-specific and non-stereoselective enzyme system composed by laccases (EC 1.10.3.2), lignin peroxidases (LiP, EC 1.11.1.14) and manganese peroxidases (MnP, EC 1.11.1.13), which function together with peroxide-producing oxidases and secondary metabolites [4,5]. The same unique non-specific mechanisms that give these fungi the ability to degrade lignin, also allows them to degrade a wide range of pollutants, among them polycyclic aromatic hydrocarbons, chlorinated phenols, polychlorinated biphenyls, dioxins, pesticides, explosives and dyes [1,3,6]. Because the key enzymes are extracellular, white-rot fungi can degrade many hazardous environmental pollutants, as they do not require preconditioning to a particular pollutant. This makes them useful for biotechnological applications [4,5]. Recently however, there has been growing interest in studying laccases enzymes of a wider array of white-rot fungi, not only from the standpoint of comparative biology but also with the expectation of finding better systems for use in various biotechnological applications [7].

Potential exploitation of enzymes to replace or reduce the use of hazardous chemicals in industrial processes such as pulp and paper, textile and leather industry has attracted attention in recent years due to the public concern and governmental regulations. In the pulp and paper industry, for example, enzymatic applications at different parts of the manufacturing process have proved successful. The use of xylanases has helped in reduction of the need for chlorine and lipases have proved effective in controlling the accumulation of pitch. Enzymes have great potential in degrading contaminants in industrial waste streams and laccases with their extensive properties can play important role in reducing the toxicity of industrial wastewaters. However,

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laccases production on an industrial scale presents a problem due to low titres and high costs. Optimisation of media and culture conditions has been one approach for improvement in laccases production. The small amounts of laccases produced constitutively in basidiomycetes, however, can be considerably enhanced by the presence of a wide variety of inducing substances, mainly aromatic or phenolic compounds related to lignin or lignin derivatives such as ferulic acid, 2,5-xylidine, *p*-anisidine and veratryl alcohol. The successful biotechnological application of laccases requires production of high amounts of the enzyme at low operation cost; so far only obtained in flask cultures [8,9].

The application of statistical methodologies is helpful in finding the effect and interactions between the physiological factors that play a role in biotechnological process such as microbial enzyme production. The use of different statistical designs for medium optimisation has been recently employed for lysozyme, xylanase, amylase and laccases production by fungal cultures [10–16]. Statistical optimisation methods can take into account the interactions of variables in generating a process response [17,18]. Thayer et al. [19] used response surface designs to optimise media and process conditions rapidly. Parra et al. [10] used an orthogonal design to optimise 13 medium components to enhance squalestatin production. Bull et al. [20] used a Plackett–Burman design to screen 20 different culture media components to identify those important for cell growth and protein production.

An inexpensive and economically viable approach for enhancement of secondary metabolites production in fungi has been through the use of oligosaccharides as elicitors [21]. Oligosaccharides such as OG (oligoguluronate), OM (oligomannuronate), MO (mannan oligosaccharides) and pectin oligosaccharide, as elicitors, enhance the production of penicillin G in *Penicillium chrysogenum* P2 cultures [21] and increase the levels of chrysogenin in *P. chrysogenum* NRRL 1951 [22] and *P. chrysogenum* MUCL 30168 [23]. Locust bean gum-derived mannan oligosaccharides were shown to be the most generic and potent elicitors investigated in filamentous fungi [21]. Although the effects of several medium ingredients have been reported, there are no published work on the synergistic effect of elicitors and inducers in the production of laccases.

Therefore, the aim of this study was to apply a full factorial 3^2 design and response surface methodology to investigate the interaction between the levels of mannan oligosaccharides (elicitor) and ferulic acid (inducer) on laccases production by three strains of white-rot fungi, *Pycnoporus sanguineus* (*P. sanguineus*), *Corylopsis polyzona* (*C. polyzona*) and *Pleurotus ostreatus* (*P. ostreatus*) in submerged fermentation.

2. Materials and methods

2.1. Organism and their maintenance

P. sanguineus MUCL 38531 and *C. polyzona* MUCL 38443, and *P. ostreatus* IT01 strains were used in this study. The master cultures were kept at −80 °C. The cultures were activated on potato dextrose agar plates and grown for 2 weeks at 28 °C.

Table 1
Medium composition designed for each strain from based on previous studies

	Potato dextrose (g/l)	Glucose (g/l)	Lactose (g/l)	Yeast extract (g/l)
Pycnoporus sanguineus[a]	5	0	15	1
Coriolopsis polyzona[b]	5	0	15	5
Pleurotus ostreatus[c]	25	7	15	5

[a] $CuSO_4$ 75 μM and pH 5.0.
[b] $CuSO_4$ 225 μM and pH 4.0.
[c] $CuSO_4$ 225 μM and pH 5.0.

2.2. Media preparation

Based on previous studies (data not included) the best medium for laccases production designed for each strain was used to evaluate the effect of ferulic acid and mannan oligosaccharide additions on laccases production. Table 1 shows the optimal medium designed for high laccases activity for each strain.

2.3. Mannan oligosaccharide preparation

The preparation of mannan oligosaccharide elicitors from locust bean gum (*Ceratonia siliqua*) was performed as described previously [24]. The preparation contained oligosaccharides with the degree of polymerisation of 5–8.

2.4. Fermentation conditions

Actively growing 8-day-old colonies of *P. sanguineus*, *C. polyzona* and *P. ostreatus* on potato yeast agar (PDA) were used to inoculate 250-ml Erlenmeyer flasks with 50 ml of the medium designed for each strain. The flasks were inoculated with three 10 mm plugs and incubated in an orbital shaker (2 cm throw) at 150 rpm and 28 °C for 26 days.

2.5. Addition of ferulic acid and MO

Different additions corresponding to different MO and ferulic acid combinations were prepared according to a full factorial experimental design. MO was added at 24 h and ferulic acid at 72 h. The factors and levels are coded as positive (+1), zero (0) and negative (−1) for the higher, middle and lower levels of the variables used in the experiment (Table 2).

2.6. Laccases assay

Samples were taken from the flasks every 48 h and centrifuged at 13,000 rpm and 4 °C for 15 min. Laccases activity of the cell-free broth was determined using ABTS as substrate. The assay mixture contained 200 μl of 2.5 mM ABTS (dissolved in 100 mM sodium tartrate buffer pH 3), 50 μl of supernatant and 950 μl 100 mM sodium tartrate buffer at pH 3. The product formation rate from enzymatic oxidation of ABTS was measured spectrophotometrically at 415 nm with an extinction coefficient $\varepsilon = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$. The unit activity of laccases was expressed as 1 μM of product formed per min. The enzymatic reactions were carried out at room temperature.

Table 2
Concentrations and levels for different combinations of factors to be investigated

Factor	Levels		
	−1	0	+1
MO (mg/l)	0	75	150
Ferulic acid (mM)	0	0.5	1

The oligosaccharide MO was added at 24 h and the inducer at 72 h.