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Full Length Research Paper

Production and regulation of lignin degrading enzymes from *Lentinus squarrosulus* (mont.) Singer and *Psathyrella atroumbonata* Pegler

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The influence of metal ions on the production and regulation of ligninase and mycelia extension of two type Basidiomycetes (*Lentinus squarrosulus* and *Psathyrella atroumbonata*) cultivated on lignocellulose waste was investigated. Mn^{2+} and Ca^{2+} ions stimulated growth of both fungi and mycelia extension significantly. Ligninase production increased two to twelve fold under the influence of Mn^{2+} and Ca^{2+} ions at concentrations of 20 to 80 mM. Mg^{2+} and K^{+} ions did not stimulate growth and extension of fungal mycelia, rather fungal cultures became deactivated after six days. The importance of mycelia extension and enhanced enzyme production has biotechnological applications in wood and pulp, textile and tanning, as well as in oil industries.

Key words: Basidiomycetes, *Lentinus squarrosulus*, *Psathyrella atroumbonata*, ligninase, cofactors.

INTRODUCTION

The unique ability of white-rot basidiomycetes to degrade lignin has become a matter of high interest with the aim of developing environmentally sound biotechnological alternatives to reduce the cost of energy and chemicals, or lowering the environmental impact in pulp paper manufacturing.

Different culture conditions have been employed such as addition of tween detergents (Alexander et al., 1985), use of liquid (with or without agitation) and solid-state fermentations (Kirk et al., 1978; Leisola et. al., 1983). Other conditions involved the use of metal ions, particularly manganese Mn (II), cadmium, mercury, copper and organic acids as precursors (Gold, 1991; Baldrian et al., 2000) to enhance enzyme production and rapid degradability of substrates.

Lentinus squarrosulus and *Psathyrella atroumbonata* are cultivated edible mushrooms which can be exploited for their lignin-degrading properties (Fasidi and Kadiri, 1993). Fungal technology is vital for industrial products such as pharmaceuticals, flavours, organic acids and

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Table 1. Effect of metal ion on mycelia extension (mm) of *P. atroumbonata* at 32^{o}C at metal ions (mM) concentrations.

Metal ion	0	20 mM	40 mM	60 mM	80 mM
Mn^{2+}	20.60±1.17	33.83±1.50	48.44±1.79	52.32±1.86	77.85±2.28
Ca^{2+}	19.25±1.02	51.03±1.84	42.74±1.98	58.91±1.98	53.73±1.89
Mg^{+}	20.12±1.53	14.32±0.55	13.62±0.51	13.40±0.51	12.86±0.44
K^{+}	21.32±1.53	14.25±0.56	13.42±0.47	12.42±0.41	12.05±0.37

Values are means for three replicates.

Table 2. Effect of metal ion on mycelia extension (mm) of *L. squarrosulus* at 32^{o}C at metal ions (mM) concentrations.

Metal ion	0	20 mM	40 mM	60 mM	80 mM
Mn^{2+}	20.60±1.17	39.44±1.62	44.22±1.79	49.64±1.82	55.99±1.93
Ca^{2+}	19.85±1.02	47.46±1.77	50.64±1.83	56.71±1.94	51.40±1.85
Mg^{2+}	20.12±1.53	14.52±0.55	13.92±0.55	12.86±0.47	12.10±0.44
K^{+}	21.32±1.53	14.70±0.56	13.42±0.47	12.52±0.37	12.10±0.37

Values are means for three replicates.

enzymes, and fungi are also used in bioremediation technology (Wolter et al., 1997).

The aim of this research is to investigate the influence of metal ions as cofactors of enzyme production and mycelia extension in *L. squarrosulus* and *P. atroumbonata* through kinetic study and visual estimation of mycelia progress in solid-state fermentation condition.

MATERIALS AND METHODS

Fungal Cultivation

Cultures of *L. squarrosulus* and *P. atroumbonata* spores germinated previously (Wuyep, 2001) were maintained on malt extract agar incorporated with 0.0002% NaH_2PO_4, 0.001% $FeSO_4.7H_2O$, 2.0% thiamine hydrochloride, and 2% H_3BO_3.

Colonization of lignocellulosic waste

Shredded wood (particle size <2 mm) was mixed with distilled water (1:3 w/v) and allowed to soak over night in a refrigerator. 108 hollow glass tubes (length 280 mm, inner diameter 16 mm) were filled with 12 g of wet woodchips, spread horizontally to half of the glass tubes and sealed with cellulose stoppers. The tubes were autoclaved at 121^{o}C for 30 min. The wood chips in the hollow glass tubes were inoculated with an agar plug of *L. squarrosulus* and *P. atroumbonata* mycelia. The cultures were incubated at 32^{o}C and growth was monitored for 30 days. To determine the effect of metal ions of growth on fungi, 10 ml solution of 20, 40, 60 and 80 mM of Mn^{2+}, Ca^{2+}, Mg^{2+} and K^{+} (in their chloride forms) were added into the wood chip for 48 h to stabilize the medium before inoculation. Each experiment was done in triplicates. A visual estimate of the mycelial length as it colonized the wood chips from the inoculum point, toward the end of the glass tube was recorded every 48 h.

Extraction of crude enzymes

At the end of the experiment, colonized portions of the wood chips were carefully collected into 300 ml Erlenmeyer flasks. 150 ml of 25 mM sodium acetate buffer (pH 5.5) was added and the mixture was homogenized. The contents were later transferred into cheesecloth and then a vice grip which produce a force of 880 Nm^{2} capable of extruding most of the fluid out of the wood substrate was applied. The exudate was filtered, concentrated with ammonium sulphate to 60% saturation, and dialyzed overnight with 14 kD dialysis tubing against 25 mM sodium acetate (pH 5.5). The dialysate was used to measure ligninase activity using veratryl alcohol as described by Tien and Kirk (1984).

RESULTS

Average mycelia extension at different levels of metal ions which was monitored for 30 days for Mn^{2+} and Ca^{2+}, and six days for Mg^{2+} and K^{+} (because the culture became deactivated at the lag phase of growth) (Tables 1 and 2). The mycelia of *L. squarrosulus* and *P. atroumbonata* extended longer in the presence of Mn^{2+} and Ca^{2+} (20 to 80 mM). However, wood chips degradation in the presence of both fungi reached 35 - 50% with the highest values recorded at higher metal ion concentration 60 - 80mM for Mn^{2+}, and 40 - 60mM for Ca^{2+}. Wood chip degradation was significantly low, about 2 - 5%, in the presence of Mg^{2+} and K^{+}.

Table 3. Effect of Mn^{2+} ion on the activity (kinetic constants) of ligninase from *L. squarrosulus* and *P. atroumbonata* with veratryl alcohol as substrate.

Mn^{2+} (mM)	K_M /mM	Vmax/mM/min^{-1}	Vmax/K_M/min^{-1}
0	0.34[a], 0.42[b]	0.70 [a] , 0.47 [b]	2.05 [a] , 1.12 [b]
20	0.21, 0.32	1.11, 1.23	5.29, 3.84
40	0.18, 0.28	2.40, 1.56	13.33, 5.57
60	0.16, 0.21	2.80, 2.36	11.24, 17.50
80	0.14, 0.19	3.20, 2.84	2.86, 14.95

a = *L. squarrosulus*
b = *P. atroumbonata*
0 = control without exogenous Mn^{2+} ion as cofactor.

Table 4. Effect of Ca^{2+} ion on the activity (kinetic constants) of ligninase from *L. squarrosulus* and *P. atroumbonata* with veratryl alcohol as substrate.

Ca^{2+} (mM)	K_M /mM	Vmax/mM/min^{-1}	Vmax/K_M/min^{-1}
0	0.34[a], 0.36 [b]	0.59 [a], 0.44 [b]	1.69 [a], 1.22 [b]
20	0.21, 0.23	2.02, 1.56	9.62, 6.78
40	0.18, 0.21	2.41, 2.10	13.38, 10.50
60	0.14, 0.16	2.82, 2.25	20.14, 14.06
80	0.32, 0.31	2.90, 2.41	9.06, 7.77

a = *L. squarrosulus*
b = *P. atroumbonata*
0 = control without exogenous ca^{2+} ion as cofactor.

Ligninase activity was higher in wood chips amended with Mn^{2+}, which accounts for rapid mycelia progress (Table 3). The ligninase activity for *L. squarrosulus* by up to 11-fold at 80 mM. For *P. atroumbonata*, the ligninase activity at 80 mM Mn^{2+} increased 14-fold.

Ca^{2+} caused an increase of up to 20 -fold in ligninase activity in the culture of *L. squarrosulus* at 60 mM (Table 4). At 80 mM Ca^{2+}, there was a drop in ligninase activity. For *P. atroumbonata*, Ca^{2+} ion effect on ligninase activity followed a similar pattern with an increase 14-fold at 40mM, followed by a drop at 80 mM.

DISCUSSION

The inability of magnesium and potassium ions to support growth of both fungi may be attributed to nutrient composition of the culture which may determine the availability of these metal ions, and the lack of Mg^{2+}-ATPase and K^{+}-ATPase activity at alkaline pH. Magnesium and potassium ions uptake in fungi is slower at neutral pH (Comerford et al., 1985).

The rapid mycelia progress as observed in cultures supplemented with manganese and calcium can be attributed to the uptake of the metal ions and subsequent metabolism. Manganese plays an essential role in lignin biodegradation (Frederic and Gold, 1991). It acts as physiological effectors in cultures of white-rot basidomycetes (Hatakka et al., 1996). The ability of both fungi to grow on low and high concentrations of calcium corroborates the findings of Cooke and Whipps (1993) about improving extracellular enzymes of fungi. However, calcium is yet to be reported to play any role in the catalytic activity of ligninases except that it serves as protein structure stabilizers (Martinez, 2002).

In this work, we demonstrated that enhanced mycelia biomass production is promoted by manganese and calcium ions at high (<40mM) concentrations. This is advantageous to biodegradation and bioremediation technologies as mycelia of white-rot basidiomycetes can be introduced into such system when grown on lignocellulose material or immobilized on other matrix.

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