Manganese peroxidase production by *Phanerochaete chrysosporium* in different modes of fermentation

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Introduction

White-rot fungi are frequently employed as producers of ligninolytic enzymes. Particularly *Phanerochaete chrysosporium* is widely studied as the model microorganism for investigating several extracellular enzymes such as manganese peroxidase (MnP) and lignin peroxidase that are capable of degrading lignin (Reddy, 1995). Due to the important degradative potential of MnP, there is a general interest in producing this enzyme by fermentation processes. White-rot fungi are now widely used in bioremediation of polluted soils. On the other hand, it was suggested that MnP can be a potent biocontrol agent. However, the lack of delivery technology schemes and effective formulations limits the application of white-rot fungi.

Solid-state fermentation is accepted as one of the most economic process for ligninolytic enzyme production by fungi (Iwashita, 2002). Moreover, the production of lytic enzymes can be stimulated by the presence of cheap organic substances rich in ligno-cellulose when used in solid-state fermentations as substrate or support material. Agro-industrial wastes that are abundant at local level can be treated microbiologically by SSF and converted to value-added final products. Particularly in the Mediterranean region, residues and by-products derived from the olive oil production industry are abundant sources of organic matter with high fertilizing value.

On the other hand, immobilized (gel-entrapped) cells of soil-plant beneficial microorganisms are presenting a highly efficient approach to preparation and formulation of inoculants (Vassilev et al., 2001, 2005). Enhanced metabolic activity and stability and longer field persistence due to slow release of viable cells (sustained delivery), easier survival at adverse environmental factors, longer periods of storage, ease of transportation and application, are amongst the most advantageous characteristics of encapsulation-based formulations.

In this work, we test two different cultivation schemes of *P. chrysosporium* aimed at its further introduction into soil.

Materials and Methods

Microorganism. *Phanerochaete chrysosporium* was maintained on malt extract plates at 4^{o} C and incubated at 30^{o} C for 7 days before use to prepare the inoculum in fermentation experiments. Czapek-Dox liquid medium (1/2 strength) was used as medium for inoculum production where $NaNO_3$ was substituted by 1.5 g/l corn steep liquor.

Solid-state fermentation. The basal medium composition included (per flask) 15 g of solid dry olive wastes (DOW), previously dried in a 60^{o} C oven and then ground by mechanical blender

to pass a 2-mm pore screen. 20 ml of distilled water containing 0.5 g/l Tween 80, 0.5 g/l corn steep liquor, and 0.2 g/l $MgSO_4$ was added to each flask. The initial pH was adjusted to 5.0 before autoclaving. The sterilized (121^oC, 30 min) flasks were inoculated with homogenized 50-h *P. chrysosporium* culture, previously grown on medium for inoculum production, at a rate of 5 ml per flask. SSFs were performed at 37^o C for 20 days.

Experiment with gel-entrapped fungus. Gel-entrapment of the fungal biomass was performed as described by Loomis et al. (1997). Homogenized 50-h mycelium (80 ml) was mixed in 2% sodium alginate with 2.5 g of DOW powder obtained during the preparation of the medium for the SSF experiments. Medium solution contained the same components but DOW particles were replaced by 10 g glucose/l. Alginate beads (1.5 mm/d; 5 ml) were introduced into 50 ml medium/250-ml Erlenmeyer flask and medium was changed every 70 h.

Analytical methods. Samples of flasks were processed by analyzing 3 flasks for each time point. Phosphorus content was determined by the molybdo-vanado method described by Lachica et al. (1973). MnP activity was measured as described by Fujian et al. (2001). The reaction mixture contained 1.70 ml of sodium tartrate buffer (pH 7.0; 0.24 mol/l), 0.05 ml of $MnSO_4$ (0.40 mol/l), 0.25 ml of enzyme and 0.05 ml of H_2O_2 (0.016 mol/l). One unit of MnP activity was defined as 10% of Δ $OD_{240\ nm}$ increase/minute.

All experiments were carried out in triplicate. In some treatments, flasks were supplemented or not with 0.3 g per flask inorganic insoluble phosphate (Morocco rock phosphate; 12.8 % P; 1mm mesh) sterilized separately.

Results and Discussion

The results of this study showed that DOW-based medium provided necessary nutrients for MnP production by fungal culture of *P. chrysosporium* in conditions of SSF (Table 1). Optimizing several process parameters (data not shown in this work) such as solid:liquid ratio, temperature and pH resulted in enhanced enzyme production which reached 1100 U/l in the presence of insoluble inorganic phosphate source (rock phosphate, RP). Solubilization of RP added to the medium was achieved which was probably due to organic acid release by the fungus. It is well established that various carboxylic acids are produced by white-rot and brown-rot fungi which serve as organic chelators thus stabilizing the formation of Mn^{3+} (Hakala et al., 2005). The secretion of organic (mainly oxalic) acids is probably in equilibrium related to the production of H_2O_2. Milagres et al. (2002) demonstrated that acidic compounds present in fermentation broth of several of these fungi showed metal-chelating ability. In a separate (CAS) experiment, we confirmed this activity of *P. chrysosporium* which most likely stimulated RP dissolution (data not presented).

Treatment	Time (h)	MnP (U/l)	P_{sol} (mg/flask)
- RP	50	420±15	+
	100	981±23	+
	144	241±11	-
+RP	50	510±18	9.5±0.3
	100	1100±33	26.0±1.1
	144	290±8	2.8±0.1

T 37°C; solid liquid 1:2; pH 5.0

Table 1. Enzyme production and RP solubilization by *Phanerochaete chrysosporium* on dry olive wastes in conditions of solid-state fermentation

Similar fungal metabolic behaviour was observed when *P. chrysosporium* was encapsulated in alginate enriched with DOW powder (Table 2). In this case, however, MnP activity after the first batch cycle was sufficiently higher than the maximum values obtained in the SSF experiment independently of the presence of RP. It is also interesting to note the steady level of soluble P in the medium solution during the whole experiment and of the enzyme activity after the first batch. Although the absence of agro-waste in the medium, the DOW powder, used as filler in the alginate beads, stimulated the enzyme synthesis.

Treatment	Batch No	MnP (U/l)	P_{sol} (mg/flask)
- RP	1	380±11	+
	2	1230±36	+
	3	1211±41	+
+RP	1	690±10	19.8±1.0
	2	1450±27	18.5±0.3
	3	1310±43	20.1±0.7

T 37°C; pH 5.0; 1 batch, 72h

Table 2. Enzyme production and RP solubilization by alginate-entrapped *Phanerochaete chrysosporium*

Fig. 1 Biocontrol activity of *P. chrysosporium* cultivated 72-hour in conditions of solid-state fermentation (left dish) and gel-entrapped form (after the first batch; right dish) against *F. oxisporum* (red-coloured colonies)

Both modes of fermentation produced viable cultures of *P. chrysosporium* with efficient biocontrol activity which were capable of suppressing growth of *Fusarium oxisporum* although gel-entrapped culture grew faster and at the end of the studied period invaded the colony of the pathogenic fungus. Further studies are necessary to test the efficiency of these products after storage to determine their final economic parameters.

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