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Simple and easy method for the determination of fungal growth and decolourative capacity in solid media

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Abstract

A technique was developed for studying the biodegradative ability of white rot fungi in different solid media. This technique enables the gravimetric determination of fungal growth (increase of biomass) and the spectrometric measurement of fungal decolourization ability (both by the determination of the production of the extracellular enzyme manganese-dependent peroxidase (MnP) and by the rate of decolourization of dyes). *Bjerkandera* sp., strain BOS55, was grown in different solid media. Its growth rate, decolourization of solophenil blue 2BL (azoic dye), neutral red (eurhodin dye), methyl green and crystal violet (triphenylmethane dyes) and the production of MnP were determined. Application of this technique enabled a spectrometric quantification of enzymatic activity. Assays indicate that greater amounts of MnP were present in agar plate cultures of *Bjerkandera* sp. than in liquid cultures.

Keywords: Rot fungi; Fungal growth; Decolorative capacity; Solid-media; Dye

1. Introduction

White rot fungi are the only organisms that can efficiently degrade polymeric components of plants such as lignin. They are directly responsible for the oxidative depolymerization of the aromatic macromolecule (Kirk and Farrell, 1987; Hammel et al., 1993). The low substrate specificity of their lignin degradation system (Tien and Kirk, 1983) suggests that white rot fungi could be used for the degradation of other complex compounds such as those present in wastewaters or polluted soils (Dott et al., 1995).

The effects of environmental factors on fungal growth and decolourization ability in solid media are often quantified by simple methods, as reported by Davidson et al. (1938), such as the measurement of the increase in diameter of the mycelial mat on a solid culture medium and the estimation of the decolourization rate in this medium using a calliper. Although this visual procedure is quick and easy to carry out, it provides unreliable data, which allows only semi-quantitative analysis of the enzymatic activity of the mycelium. This uncertainty is mainly due to the fact that the leading edges of the mycelial mat and decolourization halo are very diffuse (Trinci, 1971) and have an irregular shape.

We propose a simple analytical technique that would enable a quantitative determination of both fungal growth and decolourizing ability in solidified media such as agar as a routine test in screening programmes for white-rot fungi, which can be considered as potential candidates for the development of treatment systems of wastewater. This new technique was applied on different solidified media with different nutrient content (low or high nitrogen) and dyestuffs. The effect of the physical state of the media on fungal growth and decolourization ability was also determined in experiments carried out with a liquid N-limited medium (Tien and Kirk, 1988) and with the same medium solidified by the addition of agar.

2. Materials and methods

2.1. Microorganism

Bjerkandera sp., strain BOS55 (ATCC 90940), was maintained at 4°C on peptone yeast extract slants from

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which it was transferred to glucose malt extract plates (Mester and Field, 1997). The plates were incubated at 25°C for 5 to 6 days.

2.2. Media composition

Four different media were used, i.e. solid nutritive medium (SNM), liquid nutritive medium (LNM), solid N-limited medium (SKM) and liquid N-limited medium (LKM). The compositions of the four were: SNM (l^{-1}) - glucose 10 g, malt extract 3.5 g (27.5 mM N); agar 8 g; LNM—identical to SNM, but without agar; SKM (l^{-1}) - glucose 10 g, diammonium tartrate 0.2 g (2.2 mM NH_4^+-N), sodium acetate 3.3 g, BIII mineral solution 100 ml, thiamin 2 mg, agar 13 g; SKM—identical to SKM, but without agar.

2.3. Chemicals

Textile dyestuffs used were solophenil blue 2BL (azoic, C.I. 34210) from Ciba-Geigy, and methyl green (triphenylmethane, C.I. 42590), crystal violet (triaphenylmethane, C.I. 42555) and neutral red (eurhodin, C.I. 50040) from Aldrich.

2.4. Dye decolourization determinations

Dye decolourization was measured spectrometrically with a Shimadzu MultiSpec UV-VIS spectrometer (SM-1501) at the following wavelengths: methyl green, 635 nm; crystal violet, 580 nm; neutral red, 524.5 nm; and solophenil blue 2BL, 593 nm.

2.5. Enzyme determinations

Manganese-dependent peroxidase (MnP) activity was measured spectrometrically with a Shimadzu spectrometer (SM-1501) by the oxidation of 2,6-dimethoxyphenol (DMP) (Wariishi et al., 1992). It was assumed that two molecules of DMP were oxidized to produce one molecule of the dimeric product (Martinez et al., 1996). The reaction was initiated by the addition of 0.4 mM H_2O_2. One unit of enzyme activity (EU) is equivalent to 1 μmol of product formed min^{-1}, and the activities are reported as EU l^{-1}.

2.6. Dry mass determination

Mycelial mat was separated from culture fluid by filtration through tared glass microfibre filters (Schleider & Schuell GF50) and washed twice with 10 ml of hot distilled water. Fungal growth was determined gravimetrically after drying in an oven at 80°C for 8 h, cooling it to room temperature in a desiccator and weighing. The filters were then re-heated for 1 h, cooled, and weighed again until constant weight was reached.

2.7. Experimental assays

The fungi were grown in 20 ml of medium in loosely capped 250 ml flasks (i.d. 8.5 cm), which were inoculated with one 5-mm agar plug cut from the margin of colonies on glucose malt extract plates, and incubated at 26°C statically in darkness for 7 days. Flasks lacking inoculum were incubated in parallel under the same conditions as abiotic controls.

2.7.1. Influence of assay conditions

2.7.1.1. Influence of microwave radiation on dry mycelial mass For this assay abiotic and biotic controls were treated in parallel. The flasks, containing LKM or LNM were incubated for 7 days, and then 100 ml hot distilled was added to each. The flasks were then heated at high power (500 W) in a conventional microwave oven for approx 20 s, avoiding boiling, prior to dry mass determination (2.6). In biotic controls, after incubation 100 ml hot distilled water was added to the flasks, which were not microwave treated before mycelial dry mass determination.

2.7.1.2. Influence of agar medium on mycelial dry mass Twelve flasks containing SKM or SNM were incubated for 7 days. After incubation, hot distilled water was added to the flasks to give four-fold, five-fold and six-fold dilutions. These were orbitally shaken at 80 rpm for 1 min at room temperature (20–25°C), and then the whole system was heated in a microwave oven as above and then shaken again (for 2 min) in order to obtain a homogeneous solution, which was then filtered and the dry mass of mycelium was determined (2.6).

2.7.1.3. Influence on microwave radiation on dye colour and MnP activity In this assay, flasks containing LKM or LNM, supplemented with different dyes (90 μM) before incubation (time zero), were incubated for 7 days. A 2-ml aliquot for determination of dye colour and MnP activity was removed from each flask and centrifuged at 2000 rpm for 10 min. The remainder of the solution (18 ml) was heated as in 2.6 in a microwave oven, after which a 2-ml sample was removed and centrifuged as above.

2.7.1.4. Influence of blending procedure on MnP activity After incubation in parallel of abiotic and biotic control flasks containing LKM or LNM for 7 days, 25 ml distilled water was added to each and the contents were mixed in a Waring blender for 1 min. A 15-ml sample of the homogeneous suspension was centrifuged at 2000 rpm for 10 min prior to MnP activity determination. In the biotic control, 25 ml distilled water was added to each flask after incubation and the MnP activity was determined for a 2-ml aliquot of the contents, which were not blenderized.

2.7.2. Assay for fungal growth and biodegradative ability in different culture conditions

In these experiments flasks containing SNM, SKM or LKM were supplemented with different dyes and incubated as in 2.7.1.4.

After incubation of SNM and SKM flasks, 100 ml hot distilled water was added to each flask, which was then treated as in 2.7.1.2 to produce a homogeneous solution. A 2-ml sample from each homogenate was then centrifuged and used to determine the dye decolourization. The remaining solution was filtered as described previously and mycelial dry mass was determined as before. To parallel flasks, 40 ml distilled water was added after incubation. After mixing in a Waring blender for 1 min, 15 ml of the homogeneous suspension was centrifuged at 2000 rpm for 15 min and MnP activity of the supernatant was assayed.

In LKM experiments, the culture fluid was filtered after incubation and mycelial dry mass determined as before, as were dye decolourization and MnP activity of the filtrate.

2.7.3. Correlation between fungal biomass and MnP activity in SKM and LKM

Flasks containing 20 ml SKM or LKM supplemented with methyl green (45 μM) at zero time were incubated for 10 days. At intervals of 10 days, MnP activity and mycelial growth (determined as dry mass) were analysed.

2.8. Statistical procedures

All experiments were performed in quadruplicate. Experiments 2.7.2 and 2.7.3 were repeated in triplicate on separate days. Values are reported as means and standard deviation, statistical significance ($p < 0.05$) being confirmed using GraphPad Prism Software, version 4.0.

3. Results and discussion

Although the results are not shown, assays performed in the liquid media, LKM and LNM, to evaluate the effect of the microwave radiation on mycelial dry mass, dye colour and MnP activity showed that the microwave treatment had no effect on any parameter. Similar results were obtained for the MnP activity irrespective of whether the contents of the culture flasks had been blenderized or not.

In further experiments conducted with SKM and SNM to assess whether adhering agar could have contributed to the measured mycelial dry mass, no agar was observed to gel on the glass microfibre filter when the dilution of the microwaved contents of the culture flasks with hot distilled water was greater than five-fold. At lower dilutions, clogging of the glass microfibre filter was possible.

When grown on the agar medium SNM, *Bjerkandera sp.* showed a strong ability to decolourize solophenil blue 2BL, neutral red and methyl green dyes, although crystal violet was not visibly altered (Fig. 1). In the case of solophenil blue 2BL and neutral red, decolourization was related to the high biomass and MnP activity. On the other hand, biomass and MnP production were considerably lower in the presence of triphenylmethane dyes, methyl green and crystal violet. Any explanation of the degradation of methyl green cannot exclude the possible effect of other extracellular or even intracellular enzymes, e.g. ligninases produced during secondary metabolism (Ollika et al., 1993; Mester et al.,1996; Mester and Field, 1997). The observed lack of effect on crystal violet was probably due to the toxicity of the dye previously reported by Bumpus and Brock (1988).

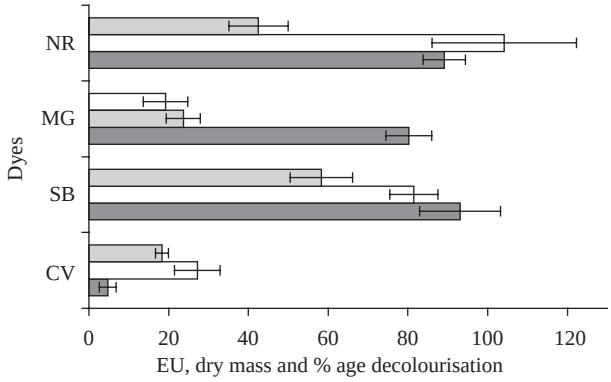

Fig. 1. Percentage of decolourization (cross-hatched bars) of crystal violet (CV), solophenil blue 2BL (SB), methyl green (MG) and neutral red (NR), MnP activity (EU l^{-1}, dotted bars) and mycelial dry mass (mg, clear bars) after incubation for 7 days on solid nutritive medium.

The effects of nitrogen limitation on the MnP production, decolourization and growth (as judged by dry biomass) are shown in Table 1. In the case of solophenil blue 2BL, the differences in the extent of decolourization can be attributed to differences in the culture media, i.e. low versus high nitrogen (Spadaro et al., 1992). For neutral red, methyl green and crystal violet, no significant differences in decolourization, MnP production and biomass were observed in SNM and SKM. These results are in agreement with Boyle et al. (1998), who reported no change in the biomass production and MnP activity of *Trametes versicolor* when the dye Poly R was decolourized in different agar media. In previous papers (Kaal et al., 1993; Mester et al., 1995; Kottermann et al., 1996) it was reported that the presence of Mn(II) in the liquid culture medium stimulated MnP production in *Bjerkandera sp.* When the organism was cultivated in Mn-deficient media, MnP activity was drastically lowered. This effect could be counteracted in the presence of organic N in the culture medium.

When the role of the physical nature of the culture medium, solid or liquid, on mycelial growth, MnP activity and decolourization was evaluated (Table 1), there were no significant differences in decolourization of any

Table 1
Effect of the medium composition on mycelial dry mass, MnP activity and percentage decolourization of dyes

Dye	Solid nutritive medium (SNM)			Solid N-limited medium (SKM)			Liquid N-limited medium (LKM)		
	MnP activity (EU l^{-1})	Decolourization (%)	Dry mass (mg)	MnP activity (EU l^{-1})	Decolourization (%)	Dry mass (mg)	MnP activity (EU l^{-1})	Decolourization (%)	Dry mass (mg)
CV	18.31 ± 2.10	4.72 ± 1.63	27.21 ± 5.70	17.44 ± 1.92	5.67 ± 1.39	36.55 ± 7.21	5.81 ± 6.32	3.43 ± 1.57	6.82 ± 2.60
MG	19.25 ± 5.78	80.24 ± 5.56	23.69 ± 4.27	15.46 ± 3.60	88.91 ± 6.60	28.19 ± 6.07	8.52 ± 2.44	97.77 ± 5.60	10.33 ± 2.91
NR	42.50 ± 5.29	89.09 ± 7.41	104.13 ± 18.10	46.32 ± 7.90	92.92 ± 9.09	92.03 ± 13.83	6.76 ± 1.90	84.29 ± 7.51	9.51 ± 1.63
SB	58.30 ± 10.18	93.08 ± 7.84	81.50 ± 6.02	43.76 ± 4.71	64.17 ± 7.56	72.40 ± 6.80	5.71 ± 1.66	72.47 ± 6.85	10.51 ± 1.02

CV: crystal violet; MG: methyl green; NR: neutral red; SB: solophenil blue 2BL.

of the dyes between the SKM and LKM cultures. This agrees with the findings concerning four azoic dyes and *Phanerochaete chrysosporium* and *Pleurotus sajorcaju* grown on a different mineral medium (supplemented with small amounts of yeast extract and thiamine) in both liquid and agar-gelled form (Chagas and Durrant, 2001).

Our results also show that a higher mycelial growth rate and higher MnP production occurred with SKM rather than with LKM. We assume that the physical state greatly affects biomass production. The fact that the higher MnP production observed in SKM had no effect on the degree of decolourization could be due to lower molecular mobility in solidified medium (Yonni et al., 2002). For example, for solophenil blue 2BL, the percentage of decolourization in SKM ($64.17 \pm 7.56\%$) and LKM ($72.47 \pm 6.85\%$) was broadly similar, although the corresponding MnP activity in SKM was, at 43.76 ± 4.716 EU l^{-1}, roughly eight times greater than in LKM (5.71 ± 1.66, EU l^{-1}).

When the action of *Bjerkandera sp.* on methyl green (MG) in LKM and SKM was monitored for 10 days in order to examine any relationship between fungal biomass and MnP enzymatic production, it was observed that under constant nutrient and external conditions enzyme production increased with biomass (Fig. 2). This suggests that the higher enzymatic activity recorded for the agar medium is probably due to the greater increase in fungal biomass on it than in the corresponding liquid medium.

To conclude, although other techniques which allow visual evaluation of fungal growth in solid media and decolourization of coloured substrates have been described (Davidson et al., 1938; Babich and Stotzky, 1977), none of them allows a quantitative evaluation of enzymatic activity. The results presented in this paper show that the technique developed, based on the use of solidified culture media, enabled the gravimetric determination of fungal growth (increase in dry biomass) and the spectrophotometric measurement of fungal biodegradative ability, in terms of both extracellular manganese-dependent peroxidase activity and decolourization of dyes.

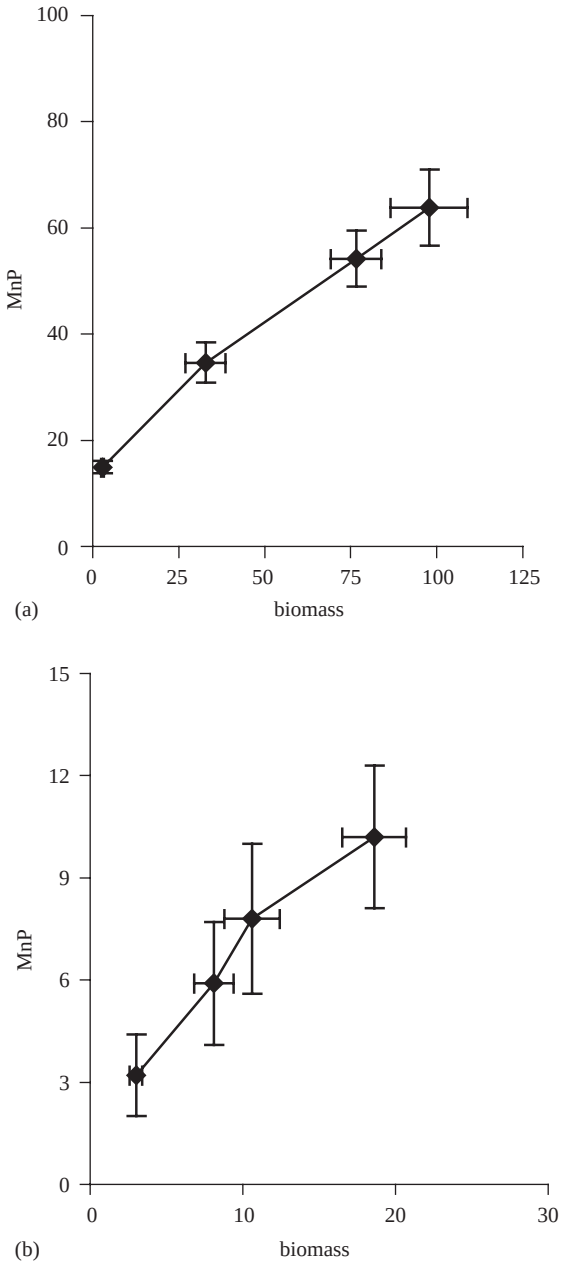

Fig. 2. Correlation between MnP titers (EU l^{-1}) and mycelial dry mass (mg) in mineral medium containing methyl green (45 μM): (a) solid N-limited medium (SKM), (b) liquid N-limited medium (LKM).

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