

Influence of dimethyl sulfoxide on extracellular enzyme production by *Pleurotus ostreatus*

Vishal Shah¹, Petr Baldrian², Ivana Eichlerova², Rachna Dave³, Datta Madamwar³, Frantisek Nerud² & Richard Gross^{4,*}

¹Department of Biology, Dowling College, New York 11769, USA

²Laboratory of Biochemistry of Wood-Rotting Fungi, Institute of Microbiology, ASCR, Vídeňská 1083, 14220, Praha 4, Czech Republic

³Post Graduate Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar 388 120, Gujarat, India

⁴NSF I/UCR Center for Biocatalysis and Bioprocessing of Macromolecules, Polytechnic University, Six Metrotech Center, Brooklyn, New York 11201, USA

*Author for correspondence (Fax: +1-718-260-3075; E-mail: rgross@poly.edu)

Received 13 December 2005; Revisions requested 20 December 2005; Revisions received 6 February 2006; Accepted 6 February 2006

Key words: DMSO, enzyme production, induction, white-rot fungi

Abstract

Dimethyl sulfoxide (DMSO) is commonly used as a co-solvent to dissolve poorly water-soluble biologically active agents to assess their biological activities such as for enzyme induction. The question addressed was whether DMSO can be assumed to be an inert co-solvent. The influence of DMSO on the production of extracellular enzymes by *Pleurotus ostreatus* was investigated. DMSO functioned as either an inducer or a repressor, depending on the enzyme studied. The production of laccase and endo-1,4- β -xylanase increased by 29 and 250%, respectively, in presence of DMSO. However, DMSO repressed the activities of manganese peroxidase, β -glucosidase, β -xylanase, and endo-1,4- β -glucanase by 30, 33, 99 and 16%, respectively. These results raise concerns about the interpretation of bioactivity measurements when DMSO is assumed to function as an inert co-solvent to solubilize water-insoluble molecules.

Introduction

As the global market for enzymes continues to expand, there is an increasing demand for novel inducers and suppressors of enzymes. Water-soluble inducers have been widely studied for enhancing the production of enzymes in bacterial and fungal systems (Moreira *et al.* 2001, Janas *et al.* 2002, Rajoka & Khan 2005). In contrast, less is known as to the induction activity of molecules with limited or very low water-solubility. Fatty acids, oils and surfactants are examples of amphiphilic molecules that have significant enzyme induction activities (Aster & Corrieu 1987) e.g. for P450s, lipases and other enzymes involved in

lipid biosynthesis (Perez-Esteban *et al.* 1997, Tanaka *et al.* 1999, Craft *et al.* 2003, de Maria *et al.* 2005).

Non-aqueous co-solvents are often used to dissolve water-insoluble molecules for studies of their activity as enzyme-inducers. Ideally, these solvents should be metabolically inert. In other words, they should exert neither a positive nor negative influence on cell growth or enzyme production. Currently, dimethyl sulfoxide (DMSO) and ethanol are most often used as solvents to dissolve water-insoluble molecules for biological activity studies. However, ethanol is an active agent for enzyme induction. Lee *et al.* (1999) reported that, when 40 g ethanol l⁻¹ was added as

a co-solvent to a medium containing 40 g l⁻¹ glucose as the carbon source, laccase production by *Trametes versicolor* increased by over 20 times. They also observed a similar stimulatory effect of ethanol on laccase production with the white rot fungi *Coriolus hirsutus* and *Grifola frondosa*. Despite the fact that DMSO is commonly used as a co-solvent to dissolve poorly water-soluble biologically active agents for their evaluation of enzyme induction activity, very little is currently known about its effect on enzymes expression. The present study begins this inquiry by evaluating the influence of DMSO on the extracellular production of ligno-cellulolytic enzymes of *Pleurotus ostreatus*. This fungus was selected as a model system because it is known to produce a plurality of enzymes at various stages of its growth cycle.

Materials and methods

Organism

Pleurotus ostreatus (Jacq.: Fr.) Kumm. CCBAS 473 was obtained from the CCBAS collection (Institute of Microbiology AS CR, Prague, Czech Republic).

Culture conditions

Static cultivations were performed in 100 ml Erlenmeyer flasks with 20 ml of N-limited CLN medium (Baldrian 2004) supplemented with 200 μ l filter-sterilized DMSO solution. The flasks were inoculated with two wort/agar plugs (10 mm diameter), cut from an actively growing part of a colony on a Petri dish and incubated at 27 °C for 21 days. Samples, 1 ml, were periodically withdrawn from the flasks for enzyme analysis.

Enzyme assays

Laccase (EC 1.10.3.2) activity was measured by monitoring the oxidation of ABTS in sodium tartrate buffer (100 mM, pH 4.5) (Bourbonnais & Paice 1990). The formation of green dye was followed spectrophotometrically. Activity of Mn-peroxidase (EC 1.11.1.13, MnP) was assayed

according to Ngo & Lenhoff (1980) in succinate lactate buffer (100 mM, pH 4.5). Briefly, 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino)benzoic acid (DMAB) were oxidatively coupled by the enzymes and formation of a purple indamine dye product was followed spectrophotometrically. The results were corrected by activities in test samples without manganese where manganese sulphate was substituted by EDTA to chelate Mn and other metal ions present in the extract.

Activity of endo-1,4- β -glucanase (EC 3.2.1.4), endo-1,4- β -xylanase (EC 3.2.1.8), and endo-1,4- β -mannanase (EC 3.2.1.78) were measured with azo-dye coupled carbohydrate substrates (carboxymethyl cellulose, birchwood xylan, and galactomannan, respectively, Megazyme, Ireland) following a literature method (Baldrian *et al.* 2005). Briefly, the reaction mixture contained 0.2 ml of 2% (w/v) dye-coupled substrate in 200 mM sodium acetate buffer (pH 5.0), and 0.2 ml sample. The reaction mixture was incubated at 40 °C for 20–60 min and the reaction was stopped by adding 1 ml of ethanol followed by 10 s vortexing and 10 min centrifugation at 10 000 \times g. The amount of released dye was measured at 595 nm and the enzyme activity was calculated according to standard curves. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μ mol of reducing sugars per min.

Activity of 1,4- β -glucosidase (EC 3.2.1.21) was assayed using *p*-nitrophenyl- β -D-cellobioside (PNPC, Sigma). The reaction mixture contained 0.16 ml of 1.2 M PNPC in 50 mM sodium acetate buffer (pH 5.0) and 0.04 ml sample. Reaction mixtures were incubated at 40 °C for 20–60 min. The reaction was stopped by adding 0.1 ml 0.5 M sodium carbonate, and absorbance was read at 400 nm. Enzyme activity was calculated using the molar extinction coefficient of *p*-nitrophenol (11600 M cm⁻¹). One unit of enzyme activity was defined as the amount of enzyme that releases 1 μ mol of *p*-nitrophenol per min. Activity of cellobiohydrolase (EC 3.2.1.91), 1,4- β -xylosidase (EC 3.2.1.37), and 1,4- β -mannosidase (EC 3.2.1.25) was assayed using *p*-nitrophenyl- β -D-glucoside, *p*-nitrophenyl- β -D-xyloside, and *p*-nitrophenyl- β -D-mannoside, respectively, using the method described above. All spectrophotometric measurements were performed in a microplate reader

(Sunrise, Tecan, Austria) or a UV-VIS spectrophotometer.

Results and discussion

Pleurotus ostreatus belongs to the group of white-rot fungi that, upon induction, produce lignin and cellulose degrading extra-cellular enzymes. The fungus generates laccase and manganese peroxidase to degrade lignin. Degradation of cellulose is carried out by endo- and exo-glucosidases and xylanases (Baldrian & Gabriel 2003, Baldrian *et al.* 2005). These enzymes are produced and exported from *P. ostreatus* into the medium in significant quantities. Thus, during fermentations of *P. ostreatus*, six enzyme activities were simultaneously monitored to investigate whether DMSO is inert or acts as an inducer or suppressor of enzyme production. All experiments were carried out in triplicate and standard deviation never exceeded 8%.

Figure 1 shows the activities of six lignocellulolytic enzymes by *P. ostreatus* cultivated in CLN medium in the presence and absence of 1% (v/v) DMSO. As shown in Figures 1a and b, in the absence of DMSO, lignolytic enzymes (laccase and manganese peroxidase) reach maximum production of 0.053 and 0.0048 U ml⁻¹ after 24 days of incubation. Adding DMSO to the production medium had a positive influence on laccase production by increasing the enzyme level to 0.068 U ml⁻¹. In contrast, DMSO represses MnP production by 30% during the same time period under identical incubation conditions. Similar selective induction/repression behavior is observed in the data obtained for cellulolytic enzymes (Figures 1c–f). Production of endo-1,4- β -glucanase(s) reached maximum levels at the end of the 10th day of incubation with 6.4 U ml⁻¹. Presence of DMSO reduced the production to 5.4 U ml⁻¹. However, on the 14th day of incubation, compared to 2.6 U ml⁻¹ of enzyme produced in the absence of DMSO, 6 U ml⁻¹ was measured in the medium amended with DMSO (> 2-fold increase, see Figure 1c). Figure 1d shows that DMSO acts as an inducer of endo-1,4- β -xylanase activity. In the absence of DMSO, *P. ostreatus* produces up to 4.1 U ml⁻¹ endo-1,4- β -xylanase activity as measured after 28 days of incubation.

By adding DMSO to the fermentation medium, 11 U ml⁻¹ was measured after 21 days, an increase of 2.5 fold. In the absence of DMSO, β -glucosidase levels were highest on the 10th and 28th day of incubations. However, when DMSO was present in the medium, 22 and 33% less enzyme levels were found on the respective days (Figure 1e). Of all the cellulolytic enzymes, the most dramatic repression of enzyme production by DMSO was observed for β -xylanase. By addition of DMSO to the medium the levels of β -xylanase in fermentations after 14 days of incubation decreased from 1.52 to 0.02 U ml⁻¹, a 99% reduction.

While DMSO influenced the extracellular enzyme production, there was no influence on the cell growth. The cell mass in the presence and absence of DMSO was unchanged as a function of the fermentation time.

Summary and conclusions

This study demonstrates that, in the presence of DMSO, the production of extracellular laccase and endo-1,4- β -xylanase by *P. ostreatus* is increased. In contrast, addition of DMSO to the medium resulted in decreased production of extracellular manganese peroxidase, β -glucosidase and β -xylanase. Endo-1,4- β -glucosidase activities were initially repressed by DMSO. However, upon further incubation, DMSO caused an increase in the level of endo-1,4- β -glucosidase formation. Therefore, DMSO functioned as either an inducer or repressor for the above extracellular enzymes produced by *P. ostreatus*. These results raise concerns about the interpretation of bioactivity measurements when DMSO is used as a co-solvent to solubilize water-insoluble molecules. We have shown the assumption that DMSO is an inert co-solvent may be incorrect for certain microbial enzyme producers. DMSO could influence the production by altering the membrane structure and thereby influencing the concentration of extracellular enzymes or influence the cellular machinery intracellularly.

Standard practice is to perform control experiments only with DMSO instead of with and without DMSO. If DMSO has a repressive effect on the enzyme under study, it might mute the

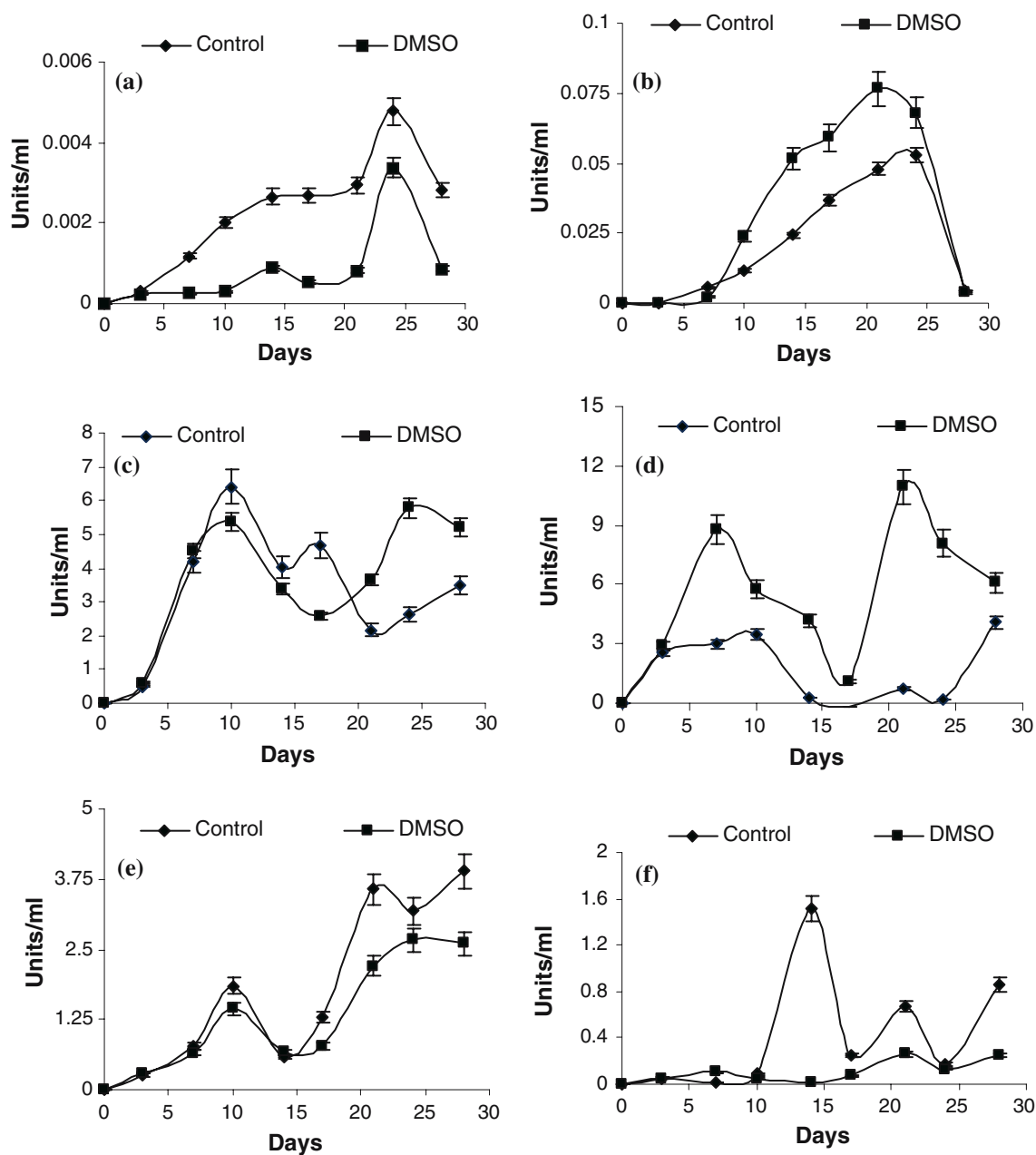


Fig. 1. Enzyme production (U ml^{-1}) in the presence (■) and absence (◆) of DMSO (1% v/v): (a) manganese peroxidase; (b) laccase; (c) endo-1,4- β -glucanase; (d) endo-1,4- β -xylanase; (e) β -glucosidase; (f) β -xylosidase.

effect of the candidate inducer. Similarly, if DMSO is a strong inducer of an enzyme, the combined effect of DMSO plus the candidate inducer may result in induction that is greater or less than that due to the lone effect of the candidate bioactive substance. Investigators may falsely conclude that a candidate molecule has a

stronger or weaker effect on enzyme induction when, in fact, this is due to unknown effects due to the dual-action of two biologically active molecules. Thus, DMSO should first be studied relative to DMSO-free medium to assess whether it modulates enzyme production. If it is found that DMSO is inactive, then it is reasonable to

continue with studies to determine the biological activity of candidate enzyme inducers using DMSO as a medium component. However, if DMSO functions as an inducer or suppressor of enzyme production, then any results obtained with two biologically active substances in the medium must be cautiously interpreted. Therefore, for any microbe-enzyme system under study, standard practice should be first to define the effect of DMSO on microbial physiology.

References

- Asther M, Corrieu G (1987) Effect of tween 80 and oleic acid on ligninase production by *Phanerochaete chrysosporium* INA-12. *Enzyme Microb. Technol.* **9**: 245–249.
- Baldrian P (2004) Increase of laccase activity during interspecific interactions of white-rot fungi. *FEMS Microbiol. Ecol.* **50**: 245–253.
- Baldrian P, Gabriel J (2003) Lignocellulose degradation by *Pleurotus ostreatus* in the presence of cadmium. *FEMS Microbiol. Lett.* **220**: 235–240.
- Baldrian P, Valášková V, Merhautová V, Gabriel J (2005) Degradation of lignocellulose by *Pleurotus ostreatus* in the presence of copper, manganese, lead and zinc. *Res. Microbiol.* **156**: 670–676.
- Bourbonnais R, Paice M (1990) Oxidation of non-phenolic substrates: an expanded role of laccase in lignin biodegradation. *FEBS Lett.* **267**: 99–102.
- Craft DL, Madduri KM, Eshoo M, Wilson CR (2003) Identification and characterization of the CYP52 family of *Candida tropicalis* ATCC 20336, important for the conversion of fatty acids and alkanes to alpha,omega-dicarboxylic acids. *Appl. Environ. Microbiol.* **69**: 5983–5991.
- de Maria PD, Sanchez-Montero JM, Alcantara AR, Valero F, Sinisterra JV (2005) Rational strategy for the production of new crude lipases from *Candida rugosa*. *Biotechnol. Lett.* **27**: 499–503.
- Janas P, Zdzislaw T, Mleko S (2002) New inducers for cellulases production by *Trichoderma reesei* m-7. *Electronic J. Polish Agric. Univ.* (Online) Available from <http://www.ejpau.media.pl/series/volume5/issue1/food/art-04.html> ISSN 1505-0297.
- Lee I-Y, Jung K-H, Lee C-H, Park Y-H (1999) Enhanced production of laccase in *Trametes vesicolor* by the addition of ethanol. *Biotechnol. Lett.* **21**: 965–968.
- Moreira FG, Lenartovicz V, de Souza CGM, Ramos EP, Peralta RM (2001) The use of α -methyl-D-glucoside, a synthetic analogue of maltose, as inducer of amylase by *Aspergillus* sp. in solid-state and submerged fermentations. *Braz. J. Microbiol.* **32**: 15–19.
- Ngo TT, Lenhoff HM (1980) A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. *Anal. Biochem.* **105**: 389–397.
- Perez-Esteban J, SanJose C, Jaspe A (1997) Lipase activity of *Pseudomonas fluorescens* in cold raw skim milk with different lipid supplements. *Folia Microbiol.* **42**: 345–348.
- Rajoka MI, Khan S (2005) Hyper-production of a thermotolerant β -xylosidase by a deoxy-D-glucose and cycloheximide resistant mutant derivative of *Kluyveromyces marxianus* PPY 125. *Electronic J. Biotechnol.* (Online) Available from <http://www.ejbiotechnology.info/content/vol8/issue2/index.html> ISSN: 0717-3458.
- Tanaka J, Sudo T, Ihara F, Nihira T, Yamada Y (1999) Increase production of lactonizing lipase (LipL) from *Pseudomonas* sp. strain 109 by lipids and detergents. *Biosci. Biotechnol. Biochem.* **63**: 900–904.