Bioremediation Journal
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/bbrm20

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R. González Matute [a b], D. Figlas [a b], G. Mockel [a] & N. Curvetto [a]

[a] Laboratory of Biotechnology of Edible and Medicinal Mushrooms, CERZOS (CONICET), Bahía Blanca, Argentina

[b] Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, Buenos Aires, Argentina

Available online: 29 Feb 2012

To cite this article: R. González Matute, D. Figlas, G. Mockel & N. Curvetto (2012): Degradation of Metsulfuron Methyl by Agaricus blazei Murrill Spent Compost Enzymes, Bioremediation Journal, 16:1, 31-37

To link to this article: http://dx.doi.org/10.1080/10889868.2011.628353

Bioremediation Journal, 16(1):31–37, 2012

ISSN: 1088-9868
DOI: 10.1080/10889868.2011.628353

Degradation of Metsulfuron Methyl by *Agaricus blazei* Murrill Spent Compost Enzymes

R. González Matute,[1,2] D. Figlas,[1,2] G. Mockel,[1] and N. Curvetto[1]
[1]Laboratory of Biotechnology of Edible and Medicinal Mushrooms, CERZOS (CONICET), Bahía Blanca, Argentina
[2]Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, Buenos Aires, Argentina

ABSTRACT Metsulfuron methyl (MM) is an herbicide used in cereal crops. The white rot mushroom *Agaricus blazei* Murrill is an important edible and medicinal mushroom reported to be a major laccase producer, a lignin-degrading enzyme with low substrate specificity. A search for assaying the potential use of *A. blazei* spent mushroom compost (SMC) as a remediation tool for cleaning MM polluted soils was carried out. A phytotoxic dose of this herbicide was separately incubated with two enzyme preparations obtained from the SMC after the second mushroom fruiting flush; the phytotoxicity of the resulting reaction mixtures was then assayed by using a plantlet growing test with *Brassica napus* L. Thus, the crude enzyme SMC extract preparation (I) or the partially purified enzyme SMC extract (II) and their dilutions, 1:10 and 1:100, were mixed with MM (5×10^{-3} ppm final concentration) and incubated at 25°C for 24, 48, 72, and 96 h. Plantlets separately exposed for 72 and 96 h to the resulting reaction mixtures between MM and those enzyme preparations showed a highly significant increase in their hypocotyl length with respect to plantlets exposed to MM alone. It was thus demonstrated the ability that complex enzyme fractions present in *A. blazei* SMC have to degrade MM during the right incubation time to compounds with no or lower phytotoxicity than this herbicide.

KEYWORDS *Agaricus brasiliensis*, bioremediation, phytotoxicity, lignin-modifying enzymes, white rot fungi, laccase, spent mushroom compost

INTRODUCTION

Metsulfuron methyl (MM) is a sulfonylurea developed as herbicide for the selective control of broadleaf weeds and some annual grasses. It is a systemic compound with foliar and soil activity that rapidly acts once it is absorbed by the plant. Metsulfuron methyl inhibits cell division in the shoots and roots of the plant (Samanta, Kole, and Chowdury 1999), and it is biologically active at low doses. It is typically applied on cereals at 4.0–20.0 g active ingredient per hectare (Hernández-Sevillano et al. 2001). Sulfonylureas have become the preferred herbicides by many plant growers due to their characteristics, i.e., wide application window (from preemergence until booting), smaller doses per hectare,

Address correspondence to R. González Matute, Laboratory of Biotechnology of Edible and Medicinal Mushrooms, CERZOS (CONICET), C.C. 738 8000, Bahía Blanca, Argentina. E-mail: rmatute@criba.edu.ar

wider spectrum of control, higher persistence, and low prices (Carriquiry and Ríos 2007). Particularly, MM possesses high biological activity and it is used to control a wide range of broadleaf weeds, being selective for crops such as rice, wheat, barley, soybean, and corn (Yu et al. 2005).

The accumulation of chemical products, such as some sulfonylureas, in cultivation soils causes environmental problems, even affecting sensitive crops in subsequent cultivation periods through the accumulation of low quantities of herbicide residuals, which are slow degrading or even nondegradable (Hernández-Sevillano et al. 1999). This persistence is favored by certain environmental conditions, such as highly alkaline soil, low temperature, scarce organic matter contents, heavy texture, and low permeability. A high pH in combination with a low content of organic matter increases the persistence of these compounds in the soils (Stork 1995; Hernández-Sevillano et al. 2001). As for its chemical stability to different pH, it was demonstrated that MM is stable at pH 7.0, without detectable hydrolysis at 25°C for 83 days, whereas at pH 5.0 and the same temperature, it had a half-life of 33 days (US Environmental Protection Agency 1989). Thus, it is necessary to allow for long periods of time to markedly diminish the phytotoxicity of this chemical before planting certain crops, e.g., 22 months for sunflowers, flax, corn, or safflower, and 10 months for sorghum (Smith 1986).

White rot fungi have evolved to feed themselves from lignocellulose in dead wood logs and for this purpose secrete a group of enzymes into them that are essential for the lignin degradation; different enzymes combine their actions to maximize the lignin mineralization (Pointing 2001). These enzymes are often denominated lignin-modifying enzymes and belong to different classes such as lignin peroxidase (LiP; E.C. 1.11.1.14), manganese-dependent peroxidase (MnP; E.C. 1.11.1.13), and a phenoloxidase named laccase (Lac; E.C. 1.10.3.2). Activity of independent MnP is also reported to exist in some white rot fungi (Vyas, Volc, and Šašek 1994).

The structural complexity and heterogeneity of lignin suggests that the lignin-modifying enzymes system is so nonspecific that it can degrade a variety of aromatics compounds, including polycyclic aromatics hydrocarbons (PAHs), some polychlorinated biphenyls (PCBs), and dioxins, DDT, herbicides, pesticides, tinctures, and many chlorinated phenols (Scheel et al. 2000; Cookson 1995). The possibility of using white rot fungi to remediate soils contaminated with hazardous compounds was previously explored by Kent, Lamar, and Glaser (1992), using pentachlorophenol as a model substrate, with positive results at both laboratory and field levels.

Basidiomycetes fungi are wood decomposers; one member of them is the Brazilian mushroom *Agaricus blazei* Murrill. This fungus is also known to be a white-rot fungus and an important edible and medicinal mushroom (Stijve, Amazonas, and Giller 2002). *Agaricus blazei* has been reported to be a major laccase producer (Ullrich et al. 2005). Most of the ligninolytic mushroom species produces at least one laccase isoform. Laccases are also dominant among the lignin-degrading enzymes in the soil environment (Baldrian 2006). Until now, over 100 laccase isoforms have been purified from mushrooms and characterized in terms of their biochemical and catalytic properties. As reported, these enzymes exhibit low substrate specificity and they oxidize different compounds through a mechanism of an electron, using molecular oxygen as electron acceptor. Hence they can act on various substrates, such as diphenols, polyphenols, different substituted phenols, diamines, aromatic amines, and even humic substances and inorganic compound such as iodine (Rodríguez and Toca 2006; Hamid and Rehman 2009).

Spent mushroom compost (SMC) is the residual compost waste generated by the mushroom cultivation industry. Rinker (2002) showed an extensive revision of the possible uses of SMC, e.g., for air, water and soil purification, bioremediation, greenhouse plants and flowers production, vegetables and fruit trees production, animal nutrition, soil conditioning through bio- and physicochemical modification of soils, red worm culture, plague and disease control, among others. Besides, the SMC is a good source for the extraction of economically important enzymes (Singh, Abdullah, and Vikineswary 2003).

The aim of this work was to demonstrate the potential application of SMC derived from the cultivation of *Agaricus blazei* Murrill in the remediation of soils polluted with MM, while demonstrating the absence of phytotoxicity of its breakdown products. Thus, a solution of a phytotoxic dose of MM mixture with crude enzyme preparations–obtained from *A. blazei* SMC after the second fruiting flush–was incubated, and the resulting reaction mixture was then assayed for phytotoxicity using *Brassica napus* L. plantlets in a plant growth test.

MATERIALS AND METHODS

Chemicals

Metsulfuron methyl (MM) 60% active ingredient (Trimet, Tamer S.A., Argentina).

Mushroom Strain and Cultivation

Agaricus blazei PL strain was obtained from Brasmicel, SP, Brazil.

For mushroom cultivation, spawn preparation was carried out following a standard procedure (Curvetto et al. 2004). The substrate composition was 50% sunflower seed hulls (*Helianthus annuus* L.), 41% wheat straw (*Triticum durum*, ca. 5 cm length), 4.5% wheat bran, with ammonium sulfate and urea (0.35% each), and calcium sulfate and calcium carbonate (1.9% each), on dry weight basis. The initial N concentration was ca. 1.2% and the C/N ratio 37 (according to Kopytowski Filho 2002). The composting system used was reported elsewhere (González Matute, Figlas, and Curvetto 2010).

After harvesting the second flush of mushrooms, the remaining substrate (SMC) was used to extract crude enzymes

Preparation of Enzyme Crude Extracts

The procedure used to obtain extracellular fungal enzymes from SMC residual from *A. blazei* cultivation was as follows: one portion of 3 g SMC was extracted with 30 ml 0.05 M phosphate buffer pH 7 containing 30 μl Triton X100, with mortar and pestle at 4°C for 1 min, then centrifuged at 1500 $\times$ *g* for 15 min in a laboratory centrifuge (Model 2036; Rolco S.R.L., Buenos Aires, Argentina). The residue was discarded and the supernatant was used to extract two additional 3-g portions of SMC. The last supernatant was then passed through a 0.2-μm filter (Millipore, MA, USA). The resulting mixture was the crude enzyme extract I and its protein concentration was 2.2 mg/ml, as determined by the Bradford method (Bradford 1976). This enzyme preparation I was immediately used for the first MM degradation trial and for preparing the crude enzyme preparation II by salting-out. To do this, the microfiltered enzyme protein fraction I was subjected to salting-out by adding ammonium sulfate to 3 M final content. After 15 min at 5°C, the protein pellet obtained by centrifugation was resuspended in 25 ml water and dialyzed against 2 L distilled water, with four changes. Protein concentration from this fraction II was 4.1 mg/ml.

These crude enzyme preparations and their dilutions (1:10 to 1:100) were assayed using the oil rape plantlet–growing test.

Laccase Activity

Since laccase is the main ligninolytic enzyme reported to be excreted by *A. blazei* during its cultivation, its activity was determined to track the presence of the ligninolytic enzyme complex present in the SMC after two mushroom flushes and to use the resulting crude enzyme preparation to demonstrate their ability to degrade MM.

Measurement of the laccase activity present in the crude enzyme preparations I and II was essentially made according to the protocol reported by Brum (2005), using a spectrophotometer (Metrolab 1600 plus; UV-Vis Metrolab S.A, Bernal, Argentina). Briefly, the composition of the enzyme reaction mixture (1 ml) was 0.90–0.99 ml of 0.1 M sodium acetate buffer (pH 5.0), 0.1–0.01 ml enzyme preparation, and 25 μl 20 mM syringaldazine (solubilized in methanol). Four tubes per sample, three replicates and a control (without syringaldazine addition), and a control for the reaction consisting of 1 ml buffer plus 25 μl 20 mM syringaldazine, were prepared. Control absorbances ($\lambda = 525$ nm) were read first, and then 25 μl syringaldazine (Sigma, MO, USA) were added to each of the three sample tubes, then were agitated in a vortex tube shaker for 10 s. The absorbance of the resulting solution was measured at the same wavelength after 1 min. The enzyme unit is defined as the quantity of enzyme that is able to oxidize 1 μmol substrate per minute, and laccase activity was given as U/g dry SMC.

Incubation of Metsulfuron Methyl with Crude Preparations of Ligninolytic Enzymes

The MM commercial product was used to prepare a 5×10^{-4} ppm MM stock solution in 0.05 M phosphate buffer pH 7; then for the incubation with SMC enzyme preparations, a 1:10 dilution of this stock solution was carried out to a final concentration of 5×10^{-3} ppm. The enzyme preparations (I and II) were previously adjusted to have the same laccase activity (245 mU/ml), and 1:10 and 1:100 dilutions of these normalized

preparations were incubated either with 5×10^{-3} ppm MM or just with buffer, at 25°C for 24, 48, 72, and 96 h.

A set of controls of the incubation of MM with the SMC enzymes under identical incubation conditions were carried out, in which the crude enzyme preparations were previously boiled for 30 min. For obtaining another set of controls, both crude enzyme preparations and the MM dilution were incubated separately during the same periods of time already mentioned. All these controls were also subjected to the *B. napus* plantlet bioassay.

Brassica napus L. Plantlet Growth Assay

The degradation of MM was evaluated using oil rape (*Brassica napu*s L.) as the plant indicator species to visualize the removal of the phytotoxicity effects by measuring the hypocotyl length as a valid plantlet growth parameter. Adequate controls treatments (boiled crude enzyme preparations incubated with MM, boiled crude enzyme preparations without MM, and 0.05 M phosphate buffer pH 7 alone) were also included besides the controls of crude enzyme preparations and MM dilution incubated separately. Magenta Boxes G7 (Sigma) were used as containers for seed germination and plantlet growth in a supporting medium containing 4 g of sterile perlite uniformly distributed on the container base. On this supporting material 20 ml of a plant nutrient medium, $\frac{1}{2}$-strength MS (Murashige and Skoog 1962), containing 70 ppm of active chlorine and 5 mM $CaCl_2$, was poured. The oil rape seeds were previously pregerminated for 24 h at 25°C in darkness in a 130 ppm commercial bleach water solution (8% active chlorine) for disinfection; 25 pregerminated seeds, with 2 mm average root length, were then used per Magenta box. After covering them with 2 g of sterile perlite layer, 30 ml of the corresponding treatment solutions as mentioned above prepared in the same basal nutrient medium ($n = 3$ per treatment) was added. The volume of these solutions was enough to overcome the imbibition matrix forces of the supporting medium, thus with a little excess of the treatment aqueous solution. In order to allow the germination of the seeds and the subsequent plantlet growth supported by own seed nutritional stores, containers were kept in darkness for 3 days, then were moved to a nursery room at 25°C, with 16 h photoperiod of photosynthetically active radiation with a photosynthetic photon density (PPD): 48 m^{-2} mol s^{-1}. Figure 1 shows this system. By day 7, 10 plantlets per treatment were randomly sampled, and the plantlet appearance and hypocotyl length were recorded.

Statistical Analysis

Results were analyzed by simple analysis of variance (ANOVA) and the mean multiple comparisons test of Tukey at a significance level of 1% was carried out.

RESULTS AND DISCUSSION

Laccase Activity

Laccase activity was 490 and 740 mU/ml for crude enzyme preparations I and II, respectively. In order to obtain the same laccase activity for all treatments, dilutions of the respective extracts were made to obtain an enzyme activity of 245 mU/ml.

Evaluation of Metsulfuron Methyl Degradation by SMC Enzymes by *Brassica napus* L. Plantlet Growth Test

A preliminary study on the oilseed rape seedling growth response to MM showed that hypocotyl length is an adequate indicator to statistically discriminate a wide range of concentrations of this herbicide when present on plant growing substrates. Using that parameter for the oilseed rape plantlet growth analysis, no significant plantlet growth differences with MM alone in the plant growing substrate, or with MM in the presence of the enzyme preparations (extract and its dilutions), could be observed, if previous to the incubation the later received a heat treatment (data not shown).

When the crude enzyme preparation was incubated together with MM for 72 and 96 h, a highly significant increase in the hypocotyl length was observed, in comparison with the growing response obtained for those times in the case of plantlets grown in the presence of MM alone. Otherwise, plant-growing substrate controls, containing just MM and buffer incubated for different periods of time, when used to analyze the growth response on *B. napus* hypocotyl length, showed no significant differences between them, thus suggesting that an inhibition in the herbicide activity after its reaction with SMC enzyme preparation was not due to a prolonged incubation at 25°C, but to the degrading enzyme activity (Figures 2 and 3).

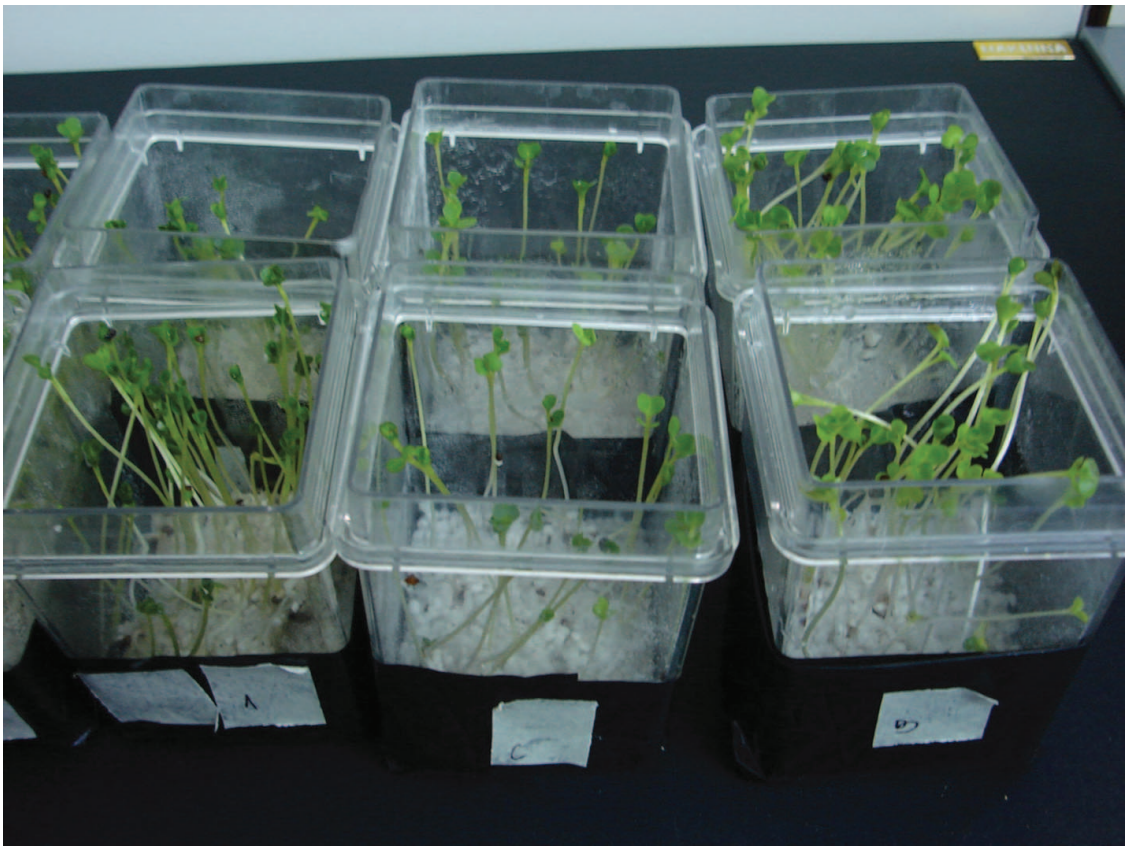

FIGURE 1 Plantlets of oil rape (*Brassica napus* L.) in a sterile perlite medium for the assessment of the metsulfuron methyl phytotoxicity removal effects of crude lignocellulolytic enzymes preparations obtained from the residual substrate of *A. blazei* cultivation by measuring the hypocotyl length (color figure available online).

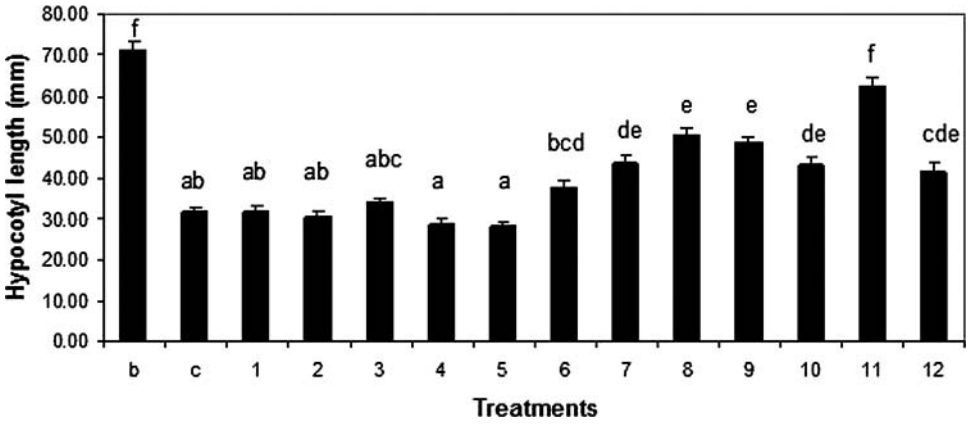

FIGURE 2 Hypocotyl length (mm) of oil rape plantlets (*Brassica napus* L.), grown in diluted MS basal nutritive medium (1:2) with the corresponding treatments of metsulfuron methyl (MM) incubated with the enzymatic extract II. b, blank of buffer; c, control of MM alone incubated 24 h; 1, extract II + MM 24 h incubation; 2, extract II diluted 1:10 + MM 24 h incubation; 3, extract II diluted 1:100 + MM 24 h incubation; 4, extract II + MM 48 h incubation; 5, extract II diluted 1:10 + MM 48 h incubation; 6, extract II diluted 1:100 + MM 48 h incubation; 7, extract II + MM 72 h incubation; 8, extract II diluted 1:10 + MM 72 h incubation; 9, extract II diluted 1:100 + MM 72 h incubation; 10, extract II + MM 96 h incubation; 11, extract II diluted 1:10 + MM 96 h incubation; 12, extract II diluted 1:100 + MM 96 h incubation. Different letters represent highly significant differences ($p < .01$) between treatments (Tukey's test). Hypocotyl length values (mm) corresponding to the controls of MM alone were 31.55, 30.31, 30.58, and 30.96 for 24, 48, 72, and 96 h of incubation, respectively.

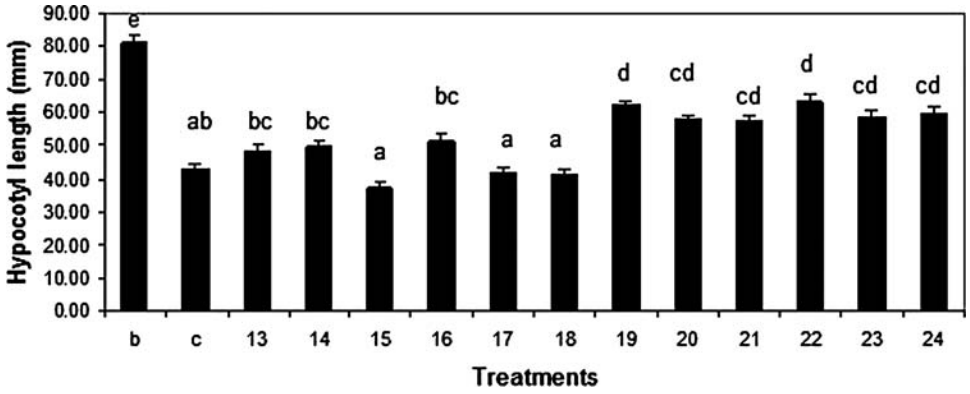

FIGURE 3 **Hypocotyl length (mm) of oil rape plantlets (*Brassica napus* L.), grown in diluted MS basal nutritive medium (1:2) with the corresponding treatments of metsulfuron methyl (MM) incubated with the enzymatic extract I. b, blank of buffer; c,control of MM alone incubated 24 h; 13, extract I + MM 24 h incubation; 14, extract I diluted 1:10 + MM 24 h incubation; 15, extract I diluted 1:100 + MM 24 h incubation; 16, extract I + MM 48 h incubation; 17, extract I diluted 1:10 + MM 48 h incubation; 18, extract I diluted 1:100 + MM 48 h incubation; 19, extract I + MM 72 h incubation; 20, extract I diluted 1:10 + MM 72 h incubation; 21, extract I diluted 1:100 + MM 72 h incubation; 22, extract I + MM 96 h incubation; 23, extract I diluted 1:10 + MM 96 h incubation; 24, extract I diluted 1:100 + MM 96 h incubation. Different letters represent highly significant differences ($p < .01$) between treatments (Tukey's test).**

Thus, a partial purification of the enzymatic crude extract may not be necessary for achieving an attenuation of MM phytotoxic effect.

Crude extracts of lignin-modifying enzymes from the residual substrate obtained after the second flush production of *A. blazei* mushrooms are able to decrease the phytotoxic effect of the herbicide metsulfuron methyl, as it was shown through the *B. napus* growth test, thus indicating a decrease in its content as well as the lack of phytotoxicity of its degradation product(s). Also, such property was preserved and even improved following a partial purification of the crude enzyme extract by salting-out, by being effective at a major dilution of it. However, the remotion of some limiting factor during this partial enzyme purification should not be discarded, since laccase activity was initially adjusted to the same value to make assays comparable when using SMC enzyme preparations I and II.

CONCLUSIONS

It can be concluded that low ratios of the *A. blazei* spent compost to metsulfuron methyl–polluted soils would be effective to degrade this herbicide in a relatively short time, which would also depend on the soil type and climatic factors, all of which should be deeply searched to assess for the application of this spent mushroom compost to clean polluted soils.

ACKNOWLEDGMENTS

This research was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC, La Plata, Argentina), and Universidad Nacional del Sur (UNS, Bahía Blanca, Argentina). The authors thank to Ricardo Devalis for his helpful technical assistance.

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