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Phenol removal using *Brassica juncea* hairy roots: Role of inherent peroxidase and H_2O_2

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Abstract

Removal of phenol, a major pollutant in aqueous effluents was studied using plant hairy root cultures. Among four different species of hairy roots tested, *Brassica juncea* showed the highest potential for phenol removal. The effect of phenol concentration and reuse in a batch system was studied using *B. juncea* hairy root cultures. Unlike most of the studies reported earlier, phenol removal by the hairy roots was seen to take place without the need for addition of external hydrogen peroxide (H_2O_2). To understand the mechanism of phenol removal, levels of peroxidase and phenol oxidase were monitored in the hairy roots. Peroxidase activity in the roots was enhanced when exposed to phenol, while phenol oxidase remained constant. Since peroxidase has a pre-requisite for H_2O_2, the levels of H_2O_2 were monitored for its in situ synthesis. H_2O_2 levels were seen to increase in the presence of phenol. Thus, a mechanism wherein hairy roots also produce H_2O_2 besides peroxidase, as a protection strategy of plant against xenobiotic stress is plausible.

Keywords: Hairy root culture; *Brassica juncea*; Phenol; Hydrogen peroxide; Peroxidases; Phytoremediation

1. Introduction

Phenols are major pollutants (Wentz, 1989) in aqueous effluents from coal conversion processes and in waste waters from production of fungicides, herbicides and insecticides and can cause major risk to human health (Sharma et al., 1997). Thus, they need to be removed from the industrial waste water. Current methods for removal of phenols include solvent extraction, adsorption on activated carbon, chemical oxidation and also microbial degradation (Klibanov et al., 1983). In our laboratory, we have carried out extensive work on phenol degradation using acclimatized activated sludge (Joshi and D'Souza, 1999; Israni et al., 2002; Melo et al., 2005). Although conventional methods are effective, they also pose certain limitations (Klibanov et al., 1983).

Phytoremediation, the use of green plants to extract, sequester and detoxify pollutants has recently been recognized as a cheap and ecofriendly alternate tech-

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nology that can be tried out for the remediation of organic contaminants (Cunningham et al., 1995; Macek et al., 2000; Eapen and D'Souza, 2005). Hairy root cultures are obtained by genetic transformation of plant cells by a soil bacterium, *Agrobacterium rhizogenes*. Due to their fast growth, unlimited propagation in culture media, genetic stability, growth on hormone-free media without microbial interruptions allowing to distinguish plant metabolism without interaction between plant and microbial communities in the rhizosphere, hairy roots have proved to be a very good model system for studying detoxification of xenobiotics (Pletsch et al., 1999). Various herbicides and pesticides (Komosa and Sandermann, 1992), polyaromatic hydrocarbon-PAH's, PCB's (Pradhan et al., 1998; Mackova et al., 1996), non-agricultural xenobiotics such as TCE, TNT, GNT (Brigmone et al., 1998) and other chlorinated compounds (Langebartels and Harms, 1984; Salt et al., 1998) are known to be transformed/degraded into non-toxic compounds by plants.

Phenol remediation through enzyme-catalyzed polymerization has been recognized as an alternate detoxification method (Bollag et al., 1988; Tatsumi et al., 1996). Involvement of peroxidase in oxidative polymerization of phenol in the presence of hydrogen peroxide (H_2O_2) has been demonstrated (Maloney et al., 1986; Yu et al., 1994; Roper et al., 1996). This method has an advantage over the conventional methods, as it is not affected if the phenol concentration in the effluent is either high or low. Besides, one can also use materials which are a source of this enzyme. Hence plant pieces (Dec and Bollag, 1994) and recently hairy roots of *Daucus carota* and *Brassica napus* (Araujo et al., 2002; Agostini et al., 2003) producing peroxidase have been used and shown to remove phenol or its derivatives from solutions.

In the present study, results on phenol removal from solutions using hairy roots of *Brassica juncea* without the need for external addition of H_2O_2 and the principle underlying the possible mode of action are presented.

2. Materials and methods

2.1. Plant material and induction of hairy roots

Indian mustard (*B. juncea* L. (Czern and Coss), beet root (*Beta vulgaris* L.), white radish (*Raphanus sativus* L.) and neem (*Azadirachta indica* L. Juss) seeds were aseptically germinated on Murashige and Skoog's (MS) basal medium (Murashige and Skoog, 1962) supplemented with 0.8% agar. The seeds were sterilized with 70% ethanol for 30 s followed by 0.1% mercuric chloride for 5 min. The sterilized seeds were washed five times with sterile water and germinated on MS medium.

Agrobacterium rhizogenes strain 9402 (INRA, France) cultured on YMB medium (Hooykass et al., 1977) was used for infection of *B. juncea*, *A. indica*, *R. sativus* and *B. vulgaris*. Two-week old seedlings were used for infection. The hypocotyl or epicotyl was wounded with a needle and 48 h old *A. rhizogenes* was applied on the wound. Two to 3 weeks after infection, the hairy roots induced at the site of infection were isolated singly and cultured on MS medium supplemented with cefotaxime at 500 mg l^{-1} and solidified with gelrite (0.25%). A single fast growing clone from each plant species was used for the experiments. After two passages on MS medium supplemented with 250 mg l^{-1} cefotaxime, the hairy roots were transferred to 50 ml MS liquid medium in 250 ml flasks and kept on a gyratory shaker at 50 rpm. Two-week old hairy root cultures each one initiated from a single clone from four different plant species were used for studies on phenol remediation.

2.2. Confirmation of transformation

For confirmation of transformation, DNA was isolated from hairy roots of all four clones using Dellaporta's method (Dellaporta et al., 1983) and used for amplification. For amplification, primers were for ORF-13 coding sequence which included (+) 5′ CAG CTT CTA AAT GTG GAG GCC and (−) 5′ CTT TGC CGA TTG CCA GTA TGG C. These primers amplified a 498 bp domain. For amplification of *mas* 1′ sequence, primers used were (+) 5′ CGG TCT AAA TGA AAC CGG CAA ACG and (−) 5′ GGC AGA TGT CTA TCG CTC GCA CTC C and they amplified a 970 bp domain. For amplification of *virB*10 sequence primers used were (+) 5′ CAA TCC CGA TCA AGT CGT GCT C and (−) 5′ AGA CGC CAA CCT CGT GAA ACC G, which defined a 644 bp domain. DNA amplification was performed on an Eppendorf thermal cycler in 25 μl reaction mixture consisting of 25 ng template, 2.5 μl 10× Taq polymerase buffer [(3-Tris