International Biodeterioration & Biodegradation 77 (2013) 106–113

Contents lists available at SciVerse ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Biodegradation of phenol using hairy roots of *Helianthus annuus* L.

Pamela Jha, Renitta Jobby, Subhash Kudale, Nikita Modi, Arohi Dhaneshwar, Neetin Desai*

Department of Biotechnology and Bioinformatics, Padmashree Dr. D.Y. Patil University, Sector 15/50, CBD Belapur, Navi Mumbai 400614 (M.S), India

ARTICLE INFO

Article history:
Received 15 March 2012
Received in revised form
1 November 2012
Accepted 1 November 2012
Available online 28 December 2012

Keywords:
Phytoremediation
L-proline
Peroxidases
Hydrogen peroxide
Cytotoxicity
Sunflower

ABSTRACT

Phenol is a common pollutant which is found in wastewater of many industries and removal of phenol from the industrial effluents is a major challenge. Recently, the use of hairy roots has been probed for the removal of phenol. In the present study, phenol at various concentrations (100–500 mg L^{-1}) was treated with hairy roots of *Helianthus annuus* (sunflower hairy roots, SHRs). SHRs removed 100 mg L^{-1} of phenol after 144 h of incubation. The effect of polyethylene glycol (PEG), L-proline and D-glucose on the rate of phenol removal was also studied. L-proline enhanced the removal efficiency of SHRs resulting in the removal of 100 mg L^{-1} of phenol after 24 h while PEG did not show any effect on removal. Peroxidase activity was induced after 24 h of phenol addition. Phenol metabolism to generate catechol as a major metabolite was confirmed using HPLC and GC–MS analyses. The detection of small amounts of *cis-cis* muconic acid and fumaric acid in the reaction medium suggests that these metabolites are produced from the ring cleavage of catechol. The phytotoxicity and cytotoxicity results suggest the non-toxic nature of the resulting phenol metabolites.

1. Introduction

Phenol has been extensively used as antiseptic and is used in the manufacture of pesticides, tinctures and Bakelite (Wagner and Nicell, 2002). Phenol is a characteristic pollutant in effluents of crude oil, coal conversion processes, paper mills and is also detected in river water (Singh et al., 2008). The extensive use of phenol has led to a wide spread contamination of soil, groundwater and has affected flora and fauna. The low volatility of phenol and its affinity for water makes consumption of contaminated water a great risk to humans (Prpich and Daugulis, 2005). The adverse effects of phenol on human health are well documented (Calabrese and Kenyon, 1991) with reports of death among adults on ingestion of phenol ranging from 1 to 32 g (Prpich and Daugulis, 2005). It causes blindness, hepatic damage, vomiting, nervous disorder and is also suspected to cause paralysis and cancer (Nair et al., 2008). Validating the adverse effects, phenol has been categorized as a priority contaminant by the U.S. Environmental Protection Agency (Sung et al., 2000) and labeled as a toxic compound by the Agency for Toxic Substances and Disease Registry (2003).

Several methods have been demonstrated for phenol removal such as precipitation, coagulation, ion exchange and ultra filtration. However, most of these methods are expensive and generate toxic by-products (Rengaraj et al., 2002). As an alternative approach, biodegradation promises to address the limitations of the earlier methods in removal of environmental pollutants like phenol (Environmental Protection Agency, 2004).

Biodegradation research has progressed in two directions: use of microbes and the emerging use of plants. While several microorganisms including bacteria (*Pseudomonas putida*), fungi (*Fusarium flocciferum*) and yeast (*Trichosporon cutaneum*) were shown to metabolize phenol (Basha et al., 2010), microbial treatment has also exhibited some limitations such as high cost of production of microbial culture, limited mobility and metabolic inhibition (Husain, 2010). In contrast phytoremediation which is an emerging plant-based remediation technology promises to be potentially low-cost, low-impact, and environmentally sound (Eapen and D'Souza, 2005).

Plant tissue cultures such as callus, cell suspensions, and hairy roots have been frequently used in phytoremediation research as model plant systems. Among these, hairy roots have the advantages of fast growth in hormone-free media, unlimited propagation in culture media and genetic stability providing a very good model system for studying detoxification of xenobiotics (Pletsch et al., 1999). Hairy roots have been used to check the plants ability to tolerate high levels of phenol (Araujo et al., 2002). They have been also used to remove phenol from aqueous solutions with high efficiency (Coniglio et al., 2008). However, it is also important to assess the toxicity of remediated solutions to establish the

* Corresponding author. Tel.: +91 22 39286175; fax: +91 22 39286197.
E-mail addresses: pamelajha@gmail.com (P. Jha), renitta7@gmail.com (R. Jobby), kudalesubhash@gmail.com (S. Kudale), neetindesai@gmail.com (N. Desai).

0964-8305/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibiod.2012.11.001

effectiveness of these treatments. In one such study, the toxicity of remediated phenol solutions obtained after using hairy roots of rapeseed and tomato has been assessed using the AMPHITOX bioassay (Paisio et al., 2010).

Sunflower (*Helianthus annuus* L.) is an annual plant belonging to the Asteraceae family and has been mostly used as an oil crop or an ornamental plant. Phytoremediation ability of its adventitious roots for phytoextraction of strontium and cesium (Adler, 1996) or degradation of PAHs (Hutchinson et al., 2003) has been explored. Also, hairy roots of *H. annuus* have been used for the phytoremediation of tetracycline and oxytetracycline (Gujarathi et al., 2005).

The objective of this study was to explore the biodegradation potential of *Helianthus* hairy roots for the removal of phenol.

2. Material and methods

2.1. Chemicals and tissue culture media

Phenol, PEG-6000, L-proline and D-glucose were purchased from SRL Chemicals, Mumbai. Potassium ferricyanide and 4-amino antipyrine were obtained from Hi-Media (Mumbai). All other chemicals used were of analytical grade.

2.2. Plant material

The seeds of ornamental variety of sunflower (*H. annuus* L.) were collected from local municipal gardens of Mumbai (India). Surface sterilization was carried out using 0.1% $HgCl_2$ for 5 min and washed 5 times with sterile distilled water. The seeds were then soaked in sterile distilled water for 18 h in dark and then aseptically germinated on half-strength MS (Murashige and Skoog, 1962) medium supplemented with 3% sucrose and 0.8% agar.

2.3. Bacterial strain and culture conditions

Agrobacterium rhizogenes (ATCC 15834) obtained from National Chemical Laboratory (Pune, India) was used for hairy root induction. The bacterial culture was revived and maintained on yeast extract (YE) agar. A single bacterial colony was inoculated in 50 ml of yeast extract broth (YEB). The culture was placed on a rotary shaker (100 rpm) at 27 °C for 19 h till the optical density reached 0.5 at 600 nm. The bacterial suspension was centrifuged at 5000 rpm for 10 min and the pellet was re-suspended in 10 ml liquid MS medium and used for co-cultivation of the explants.

2.4. Hairy root induction and PCR confirmation

Different parts of *in vitro* grown *Helianthus* seedlings including hypocotyls, epicotyls and cotyledonous leaves were pre-cultured for 2 days on MS media plates. The pre-cultured explants were co-cultivated with *Agrobacterium* suspension and MS broth for 30 min on a rotary shaker (100 rpm) in dark. After incubation, the explants were transferred on MS media plates for 3 days in dark and these explants were later transferred to MS media plates containing 400 mg L^{-1} cefotaxime. The explants were again subcultured on the same medium after a week. Cefotaxime concentration was then reduced in subsequent subcultures every week from 400 to 100 mg L^{-1} and finally cultures free of *A. rhizogenes* were transferred to MS media plates. The roots were then subcultured on MS media plates as well as MS broth every 4 weeks.

To confirm the transgenic nature and the presence of Ri T-DNA in hairy roots, PCR analysis was performed. DNA was extracted from non-transformed roots as a control and hairy roots using cetyltrimethyl ammonium bromide (CTAB) method (Khanuja et al., 1999). Primers used for the amplification reactions were from internal sequences of the *rolC* gene (0.5 kb). The primers were supplied by Eurofins, Bangalore. For *rolC* gene, the 5′ sequence ATG GCT GAA GAC GAC CTG TGT T and 3′ sequence TTA GCC GAT TGC AAA CTTGCT C were used (Oono et al., 1993). Amplification products were separated by electrophoresis on 1.2% agarose gel in 1X TBE buffer and stained with ethidium bromide and visualized under a UV-transilluminator.

2.5. Phenol removal assays

Hairy roots were exposed to varying concentrations of phenol (100–500 mg L^{-1}). A standard root inoculum of 0.8 g was incubated in 100 ml Erlenmeyer flasks containing a reaction mixture composed of 10 ml of MS medium, phenol solution and H_2O_2. The reaction mixtures were incubated at 25 ± 2 °C on a shaker (100 rpm). The effect of incubation time on phenol removal was monitored between 24 and 144 h. Every 24 h, 1 ml sample from each flask was withdrawn and used for the estimation of residual phenol through a colorimetric assay (Melo et al., 2005). Residual H_2O_2 in post-removal solutions (PRS) was evaluated using the procedure described by Sergiev et al. (1997).

Several controls were used to estimate other enzymatic or non-enzymatic mechanisms of phenol removal. Control 1: phenol solution (100 mg L^{-1}); control 2: phenol solution (100 mg L^{-1}) plus 5 mM H_2O_2. These controls were used for evaluation of phenol auto-oxidation, phenol loss by evaporation, as well as the possible reaction between both substrates. Control 3 contained 0.8 g of roots, which were incubated with 100 mg L^{-1} phenol solution, but without H_2O_2, in order to detect any enzymatic oxidation reactions other that those catalyzed by phenol oxidases or by peroxidases. Control 4, which is in fact the reactive flask, contained 0.8 g of roots, which were incubated with 100 mg L^{-1} phenol solution plus H_2O_2 (5 mM). Controls 5 and 6 were carried out with autoclaved roots (30 min, 121 °C), with and without H_2O_2 (5 mM), respectively, to estimate physical mechanisms of elimination. After incubation, in an orbital shaker (100 rpm) at 25 ± 2 °C for 144 h, roots were separated and residual phenol was estimated as described above.

The effect of addition of PEG-6000 (100 mg L^{-1}), D-glucose (1%) and L-proline (20 mM) to the reaction medium was evaluated and residual phenol was estimated as described above. The results were expressed as removal efficiency, which is defined as the percentage of pollutant removed from solution under established experimental conditions.

All the experiments were set up in triplicates and the observations were taken as average plus standard deviation.

2.6. Enzyme extraction and peroxidase assay

Hairy roots were macerated in liquid nitrogen and extracts were prepared with 0.1 M phosphate buffer (pH 7). The resulting cell homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C and the cell-free extract was assayed for peroxidase activity. One unit of peroxidase activity (U) represents the amount of enzyme catalyzing the oxidation of 1 μmol of guaiacol per 1 min at 25 °C. The activity was measured before the addition of phenol to the culture medium (0 h) and after addition of phenol at different intervals (24 h–144 h) (Singh et al., 2006).

2.7. Reuse studies in batch process

The use of SHRs was also studied in batch culture. The old medium with phenol (100 mg L^{-1}) was drained and replaced by fresh MS medium with phenol (100 mg L^{-1}) plus H_2O_2 (5 mM) every 6 days. The experiment was carried out for four cycles.