Peroxidase Catalyzed Decontamination of Water Polluted with 2,4-Dichlorophenols in Presence of Algae and Plant Materials

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ABSTRACT. Plants and algae were found useful in the decontamination of water polluted with chlorinated phenolic compounds. An artificial wastewater contaminated with up to 350 ppm herbicide 2,4-dichlorophenol (2,4-DCP), was successfully treated using horseradish, potato and *Ulva* sp. (sea lettuce), amended with H_2O_2 . Horseradish-mediated provided up to 99% removal of 2,4-DCP from model solution, while, purified horseradish peroxidase provided up to 95%. Due to the apparent ease of application, the use of plant material may present a breakthrough in the enzyme treatment of contaminated water.

Introduction

The high lipophilicity, low biodegradability and the persistence of organochlorine hydrocarbons introduce considerable problems to marine environment. Fish and biota exposed to such pollutants readily accumulate them in their fatty tissues. Numerous reports are found on organochlorine residues in the Egyptian coastal marine environment (El-Nabawi *et al.*, 1987; Abd-Allah *et al.*, 1992, Abd-Allah and Ali; 1994, and Abd-Allah, 1994).

Oxidoreductive enzymes such as peroxidase oxidize phenols to free radicals and/or quinones, which polymerize and form water insoluble oligomers. Horseradish peroxidase has not, however, been applied on an industrial scale, mainly due to the high cost of enzymatic treatment. It is demonstrated (Nakamoto & Machida, 1992 and Nicell *et al.*, 1993) that the reaction conditions can be optimized by application of various additives, such as gelatin or polyethene glycol, which prevent losses in enzymatic activity caused by adsorption of enzyme molecules on end-product polymers. The present study aims to test the usefulness of various small pieces of plants and algae (potato, sea lettuce and horseradish) compared to the purified horseradish peroxidase for decontamination of artificial polluted water with herbicide 2,4-DCP.

Materials and Methods

2,4-DCP, was purchased from Aldrich-Chemie GmbH (Sleinhein/Albuch, Germany). Horseradish peroxidase with a RZ (Reinheitsszahl) of 0.098 and activity of 102 units/mg of solid was purchased from Sigma Chemical Co. One unit of the enzyme was defined as the amount formed 10 g of purogallin from pyrogallol in 20 sec at pH 6 and 20°C. The studied plants were obtained from a local vegetable market, placed in soil bed before storage. Samples were taken from the soil immediately before each experiment, and washed with water. *Ulva* sp. was brought from Alexandria coast. Plant and algae were cut into small pieces, and thoroughly mixed.

Decontamination reaction

The model solution of 2,4-DCP in universal buffer was treated with horseradish peroxidase or the plants and algae pieces, and the reactions were initiated with the addition of H_2O_2 . The reaction mixture was incubated at ambient temperature in 2-L beakers (1-L samples). The buffers were composed of 0.2 M acetic acid, 0.2 M boric acid, and 0.2 M phosphoric acid and 1 M NaOH to provide pH range from 2 to 11. Samples with boiled enzyme and plant materials, or samples with enzymatic materials but without H_2O_2 served as controls. Specific reaction conditions (substrate concentration, enzyme activity, and amount of plants and algae, H_2O_2 concentration, number of pieces per unit weight, pH and incubation period) are detailed in legends of Figures 1 to 5 and Tables 1 and 2.

Analyses of reaction mixtures

The analyses was performed on High Performance Liquid Chromatography (HPLC) Gilson equipped with 20- μ l loop, UV detector operating at 280 nm. A 15 cm $\times$ 4.6 mm LC-18 column was used. The mobile phase, at a flow rate of 1.5 ml/min., was composed of an aqueous mixture, A (2% acetic acid, 0.018 mM ammonium acetate, pH 3.3), and organic mixture B (methanol, 2% acetic acid, 0.01 mM ammonium acetate), delivered at a ratio of 30% A to 70% B.

In a typical experiment aliquots (3-ml) were taken from the reaction mixtures at the specific incubation times immediately mixed with 0.9 ml of 99.7% acetic acid (pH 1.7) and centrifuged at 6000 G. The supernatants (1-ml) were filtered through Sep-Pack C18-cartridges and washed with 1:1 water/methanol to a final eluate volume of 10-ml. These eluates were analyzed for the remaining chlorophenol compounds.

Results and Discussion

Figure 1 demonstrates that the purified enzyme (9.5 units/ml + 20.4 mM H_2O_2) was very efficient, over 95% removal of most 2,4-DCP (initial concentration 5.2 mM) was achieved in a remarkable wide pH range (pH 3 to 10). As expected, the application of lower dosage of peroxidase (4.7 units/ml and 10.2 mM H_2O_2) resulted in less removal of 2,4-DCP. The effect of various amounts of peroxidase and H_2O_2 on the removal of 2,4-DCP was investigated at the optimal pH 5 (Fig. 2). The application of higher dosages of both peroxidase and H_2O_2 did not result in further removal of studied compound. The optimal dosage of 9.5 units/ml of peroxidase and 5.3 mM H_2O_2 was applied to 1-L

pH 5. 2,4-DCP removal was fast and the reaction was completed with 15 min of incubation (Fig. 3).

FIG. 1. Effect of pH on the transformation of 2,4-dichlorophenol (5.2 mM) by horseradish peroxidase in 2,4-D wastewater. The dosages of 4.7 and 9.5 units/mL for the enzyme were applied with 10.2 and 20.4 mM H_2O_2 , respectively. Incubations were performed for 2 h at 20°C (the SD ranged between 0.2% and 2.5%).

FIG. 2. Effect of the activity of horseradish peroxidase and the concentration of H_2O_2 on the transformation of 2,4-dichlorophenol (5.2 mM) in 2,4-D wastewater (the SD ranged between 0.1% and 2.7%).

Table 1 presents the results of experiments on the decontamination of the 2,4-DCP using potato, sea lettuce and horseradish (1.0 g of each chopped into 0.5 to 1-mm cubes per 5 ml of the reaction mixture, adjusted to pH 5). The addition of minced plants without H_2O_2 to the wastewater did not result in any transformation of 2,4-DCP, however, significant physical sorption of pollutants in the plant tissue was observed (44% to 78%). In the presence of H_2O_2 (2.65 to 21.2 mM), the reaction mixtures and the surfaces of the dissected plants changed color and precipitate of oligomerized chlorophenol

was formed. The percentage of 2,4-DCP removal increased with increasing amounts of H_2O_2 (Table 1), indicating that transformation of 2,4-DCP was caused by peroxidase activity. The maximum removal amounted 96% for potato, 90% of sea lettuce and 99.9% for horseradish. Purified enzyme at 9.5 units/ml with 20.4 mM H_2O_2 was very efficient, over 95% removal of most 2,4-DCP (initial concentration 5.2 mM) was achieved at pH 5.

FIG. 3. Transformation of 2,4-dichlorophenol (5.2 mM) by horseradish peroxidase (9.5 units/ml + 5.3 mM H_2O_2) in 2,4-D wastewater. Incubations were performed in a 1-L volume of the wastewater at pH 5 and 20°C (the SD ranged between 0.5% and 3.8%).

TABLE 1. Removal of 2,4-DCP (5.2 mM) from the model solution at pH 5 (2-hr incubation).

Addition of H_2O_2 (mM)	% Removal of 2,4-DCP		
	Horseradish	Potato	Sea lettuce
0	78.0	53.3	44.0
2.56	88.8	73.5	65.0
5.3	96.0	90.8	80.0
10.6	99.9	96.5	90.0
21.2	95.7	87.9	85.0

TABLE 2. Removal of 2,4-DCP (5.2 mM) from the model solution at pH 5.

Group	H_2O_2 mM	Peroxidase activity	% Removal of 2,4-DCP
I	1.3	2.3	46
II	10.2	4.7	70
III	20.4	9.5	95

The optimization studies with horseradish using buffered solutions of 2,4-DCP demonstrated that the extent of substrate transformation depended on the following factors: pH of the reaction mixture; piece size of the cut horseradish, the amount of horseradish; H_2O_2 and the duration of the incubation.

The effect of pH on the transformation of 2,4-DCP compounds was examined in the pH range between 2-11. Incubating 0.5 g of horseradish root cut into 0.5- to 1.0 mm cubes and 5.3 mM H_2O_2 for 2 h with 5 ml of 9 mM 2,4-DCP in universal buffer caused substantial substrates removal (90%) between pH 3 and 8 (Fig. 4). Most removal (96.4%) occurred at pH 6. For purified horseradish peroxidase (8.3 units/ml + 18.4 mM H_2O_2), the optimal pH was 5, and maximum removal (96% to 99%) was observed at pH 4 through 7 (Fig. 4).

Fig. 4. Effect of pH on the transformation of 2,4-dichlorophenol (9 mM) by cut horseradish (0.5 g/5 ml + 5.3 mM H_2O_2 and 0.2g/5 ml + 2.65 mM H_2O_2) and horseradish peroxidase (8.3 units/ml + 18.4 mM H_2O_2) in universal buffer (the SD ranged between 0.2% and 3.0%).

To determine the effect of the size of horseradish root pieces on the extent of the removal, buffered solutions (pH 6) of 2,4-DCP (9 mM) were incubated for 1 min by shaking with 0.5 g of the plant material cut into various size, H_2O_2 and 5.3 mM was added. The number of pieces in eight assays amounted to 1, 2, 4, 8, 16, 32, 64 and 128 per 0.5 g. In an additional assay, 0.5 g of mashed horseradish was tested. The results clearly indicated that the removal of 2,4-DCP was influenced by the size of horseradish parts. 2,4-DCP removal increased gradually with decreasing size of pieces with 65% removal (due to physical sorption in the absence of H_2O_2) ranged from 0.5 for one piece per 0.5 g to 24% for mashed horseradish.

The effect of the size of horseradish pieces on the 2,4-DCP removal at various incubation times is shown in Fig. 5. With 128 pieces per 0.5 g, the maximum removal (82.4%) was achieved after 30 min; whereas for 1 piece per 0.5 g, the maximum removal (71.3%) required 60 min.

FIG. 5. Time course of transformation of 2,4-dichlorophenol (9 mM) by horseradish cut into 1 part/0.5 g and 128 parts/0.5 g with 5.3 mM H_2O_2 (the SD ranged between 0.3% and 3.7%).

To examine the stability of the peroxidase contained in horseradish roots, 0.5 g portions of the plant material were cut into 128 pieces and were stored at room temperature for 0, 2, 8, 32, 128 days. Then sorted pieces were recycled seven times for 30 min with 5 ml samples of 9 mM solution of 2,4-DCP and 10.6 mM H_2O_2 in universal buffer at pH 6. The HPLC analysis of the reaction mixture showed (Fig. 5) that the cut horseradish roots gradually lose peroxidase activity during prolonged storage, but retained enough active enzyme to remove 60% of 2,4-DCP after 128 days of storage.

Conclusion

The results of the present study constitute another confirmation that almost complete decontamination of 2,4-DCP can be achieved by enzymatic treatment. Klivanov *et al.* (1980) reported a 97% removal of phenol from coal-conversion wastewater using horseradish peroxidase and H_2O_2 . In the present work, the same enzyme provided up to 99% removal of 2,4-DCP, (Fig. 3). As in other studies, (Klivanov *et al.*, 1983, Maloney *et al.*, 1986, Nicell *et al.*, 1993, and Wu *et al.*, 1993) 2,4-DCP transformation was extremely fast; a maximum removal could be achieved within less than 15 min. According to Nicell *et al.*, 1993, if peroxidase is used in excess, the number of catalytic turnovers is limited by the availability of substrate; whereas, excessive amount of H_2O_2 may cause partial inactivation of the enzyme.

As regards cost-effectiveness in using enzymes, this study demonstrated that minced horseradish roots and the other tested plants and algae are promising enzymatic agents for decontamination purposes (Table 1). Simplicity of application, short treatment times, and high efficiency at a wide pH range and stability of enzyme are the major fea-

tures that render horseradish suitable for pollution control. In fact, the common properties of unpurified plant and algae materials and immobilized enzymes suggest that the active enzyme is immobilized in the plant tissue. It is clear that peroxidase was catalyzed the decontamination of 2,4-DCP from water in the presence of H_2O_2 .

The application of plant technology to wastewater treatment can be performed either periodically in reactors with mechanical stirring, or continuously, through columns packed with the minced plant materials. Plants can also be applied directly to polluted natural aquifers; in the case of polluted groundwater, a "pump and treat" approach may be employed. The major reason that enzymatic treatment has not, as yet, been applied on an industrial scale is the huge volume of polluted environments demanding bioremediation. For instance, hundreds of thousands of liters of wastewater are generated daily from an average industrial site. Such extensive contamination makes the use of expensive free or immobilized enzymes economically impractical. Even the application of crude enzymes may be cost-prohibitive. In this situation, the use of minced plant materials that contain oxidoreductases may constitute an affordable and scientifically sound alternative.

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أنزيم البيروكسيد كحافز لإزالة التلوث من المياه الملوثة بالمركبات الفينولية المكلورة باستخدام النباتات والطحالب

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المستخلص . لما كانت للمركبات الفينولية المكلورة صفة المقاومة والبقاء في البيئة وخاصة البيئة البحرية لمدد طويلة ، فقد أدى هذا بالتالي لحدوث تراكم حيوي لهذه المركبات في الأحياء البحرية والتي أدت بدورها إما لحدوث أمراض مزمنة لهذه الأحياء أو يمتد هذا التأثير للموت الجماعي . واهتم هذا البحث بدراسة كفاءة استخدام كل من النباتات (الفجل الحار ، و البطاطس) وكذا الطحالب البحرية (خس البحر) في التخلص من الملوثات الفينولية المكلورة . وقد أخذ مثال واحد لهذه المركبات في هذه الدراسة وهو مبيد حشائش ومنظم للنمو في نفس الوقت ألا وهو (٢, ٤- ثنائي كلورو الفينول) . ولقد تم أخذ هذه النباتات والطحالب طازجة وتم تقطيعها واعتمدت التنقية على إنزيم البيروكسيديز والذي تم إضافته بصورة نقية وبتراكيز مختلفة لدراسة كفاءة هذا الإنزيم في تحويل مثل هذه المركبات لمركبات غير ضارة بالبيئة ، ولقد استخدم جهاز التحليل الكروماتوجرافي ذو التقنية العالية لقياس التغير في التراكيز المضافة من هذه المركبات وكان التركيز الذي بدأ به هو ٣٥٠ جزء في المليون . وتمت دراسة تأثير الرقم الهيدروجيني ، ومدة التعريض ، وحجم قطع النباتات والطحالب المقطعة ، وزمن التعرض . ولقد أكدت النتائج أن نبات الفجل الحار (٩٩, ٩٪) يليه البطاطس (٩٦٪) ذات كفاءة أعلى من خس البحر (٩٠٪) في تنقية المياه الملوثة من المركب تحت الدراسة . ولما كانت التنقية من الوجهة الصناعية التي تعتمد على التأثير الأنزيمي النقي غالية الثمن ، وكذا التنقية بالميكروبات تحتاج أزمان طويلة ، ففي هذا البحث يعطى تنقية إنزيمية منخفضة الثمن ويعتبر الإنزيم داخل النبات كأنه إنزيم نقي في حالة كبسلة ، وهذا يخفف من تكاليف التنقية .

ويعطى مؤشر طيب لزيادة البحوث في هذا المضمار لخفض نفقات التنقية ولاستخدام مواد نباتية غير ذات قيمة غذائية للإنسان ويفضل زيادة الدراسة على الطحالب غير الموسمية البحرية حتى يتسنى لنا تطبيقها من الوجهة الصناعية وعلى مئات الألوف من أطنان المياه الملوثة .