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Application of fly ash adsorbed peroxidase for the removal of bisphenol A in batch process and continuous reactor: Assessment of genotoxicity of its product

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ABSTRACT

In the present study peroxidase has been immobilized simply by adsorption on fly ash. On fly ash adsorbed nearly 1113 U of peroxidase activity per g. Comparative degradation of endocrine disrupter, bisphenol A has been performed by soluble and immobilized enzyme. Soluble and immobilized enzyme removed maximum bisphenol A in the presence of 0.3 mM guaiacol, a redox mediator, 0.75 mM H_2O_2 in sodium phosphate buffer, pH 7.0 at 40 °C. Degradation of bisphenol A in batch process was 61%, 100% and 100% at 20, 40 and 60 °C, respectively. Fly ash adsorbed peroxidase was more effective in the degradation of bisphenol A as compared to its free form. Immobilized enzyme catalyzed complete degradation of bisphenol A at 40 °C within 3.5 h. The oxidative degradation and polymerization of bisphenol A was also evaluated in the continuous bed-reactors at different flow rates. The removal of this compound was maximum at a flow rate of 20 mL h^{-1}. HPLC analysis showed two clear peaks, one related to bisphenol A and other related to its degradation product, 4-isopropenylphenol. Plasmid nicking and comet assays demonstrated that the product, 4-isopropenylphenol was significantly nontoxic.

1. Introduction

Bisphenol A (BPA) is one of the world's highest production volume chemicals, with more than 2 million metric tons produced worldwide in 2003 and its annual increase in demand was from 6% to 10% (Burridge, 2003). It is extensively used in epoxy resins lining food and beverage containers and as a monomer in polycarbonate plastics in many consumer products. Widespread and continuous exposure to BPA, primarily through food but also via drinking water, dental sealants, dermal exposure and inhalation of household dusts, is evident from the presence of detectable levels of BPA in more than 90% of the US population (Calafat et al., 2005; Vandenberg et al., 2007; Calafat et al., 2008). Studies on the health effects of BPA have focused on its estrogenic activity (Takeuchi et al., 2004) but the other reports have highlighted additional modes of its action, including liver damage (Nakagawa and Tayama, 2000; Elsby et al., 2001; Tyl et al., 2002; Bindhumol et al., 2003), disrupted pancreatic β-cell function (Ropero et al., 2008), thyroid hormone disruption (Moriyama et al., 2002) and obesity-promoting effects (Newbold et al., 2008). The potential for low dose effects has added to the controversy about possible hazards and whether currently recommended exposure thresholds require revision (vom-Saal and Hughes, 2005; vom-Saal et al., 2007; Goodman et al., 2006; Welshons et al., 2006). The US National Health and Nutrition Examination Survey 2003–2004 recently released the only large scale data on urinary BPA concentrations (Dekant and Volkel, 2008).

The presence of BPA in environment causes serious health problems. Therefore, prior to its final disposal in the environment its removal from polluted sites is necessary. Conventional chemical and physical procedures include electrochemical oxidation, membrane filtration, coagulation, flocculation, sorption, ion exchange, electrolysis, adsorption, advanced oxidation processes, chlorination, bleaching, ozonation, fenton oxidation, photocatalytic oxidation and chemical reduction for the treatment and removal of organic compounds including BPA from wastewaters has faced some unresolved problems such as the formation of hazardous by-products, incompleteness of purification, high costs, low efficiency and applicability to limited concentration range.

Recently biological procedures have attracted much attention of environmentalist but these procedures have their own limitations; such as hazards of radiations, toxicity of chemicals, high costs of production of microbial cultures, limited mobility and survival of cells in the soil, alternative carbon source, completeness of the indigenous populations and metabolic inhibition (Husain and Jan, 2000; Duran and Esposito, 2000). In view of these limitations, in recent years a great deal of research has been focused on developing processes in which peroxidases can be used to remove highly

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complex aromatic contaminants from toxic/polluted water (Husain, 2006; Husain and Husain, 2008; Husain et al., 2009).

Lignin-modifying enzymes, manganese peroxidase (MnP) and lignin peroxidase (LiP) from white-rot basidiomycete, *Trametes versicolor,* and different plant peroxidases already been employed for the biodegradation of toxic compounds, including BPA (Takamiya et al., 2008; Hong-Me and Nicell, 2008; Karim and Husain, 2009). In an earlier study it has been shown that bitter gourd peroxidase (BGP) catalyzed oxidative degradation and polymerization of BPA into an insoluble product and soluble 4-isopropenylphenol (4-IPP) (Karim and Husain, 2009).

In the present work an attempt has been made to standardize the treatment of BPA by soluble and immobilized BGP under various experimental conditions. Treatment of BPA by soluble and immobilized BGP was performed in batch process as well as in continuous bed-reactor. HPLC was used to evaluate remaining BPA and its oxidized product, 4-IPP. Toxicity of BPA and its oxidized soluble product, 4-IPP was examined by Comet and plasmid nicking assays.

2. Methods

2.1. Materials

Bovine serum albumin, ethidium bromide, phosphate buffered saline *(PBS)*, low melting point agraose (LMPA), Roswell Park Memorial Institute medium (RPMI) and *o*-dianisidine HCl were obtained from Sigma Chem. Co. (St. Louis, MO, USA). Guaiacol, glutaraldehyde, ethanolamine, glycerol, 4-isopropenylphenol, agarose, and bromophenol blue were procured from SRL Chemicals Co. (Mumbai, India). Fly ash was obtained from Thermal Power Plant, Kasimpur near Aligarh, India. Bitter gourd was purchased from the local vegetable market. Other chemicals and reagents employed were of analytical grade and were used without any further purification.

2.2. Ammonium sulphate fractionation of bitter gourd protein

Bitter gourd (50 g) was homogenized in 100 mL of 100 mM sodium phosphate buffer, pH 7.0. Homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 10,000*g* at 4 °C (Remi Cooling Centrifuge, Model C-24, India). The clear supernatant was subjected to salt fractionation by adding 10–80% (w/v) ammonium sulphate $(NH_4)_2SO_4$. This solution was stirred overnight at 4 °C to obtain maximum precipitate collected at 10,000*g*. The obtained precipitate was redissolved in 100 mM sodium phosphate buffer, pH 7.0 and dialyzed against the assay buffer (Karim and Husain, 2009).

2.3. Measurement of peroxidase activity and protein estimation

Peroxidase activity was measured by a change in optical density (A_{460} nm) by determining initial rate of oxidation of 6.0 mM *o*-dianisidine HCl in the presence of 18.0 mM H_2O_2 in 100 mM sodium phosphate buffer, pH 7.0 for 15 min at 40 °C (Karim and Husain, 2009). Immobilized BGP was continuously stirred for the entire duration of assay. The assay was highly reproducible with immobilized enzyme.

One unit (1.0 U) of peroxidase activity was described as the amount of enzyme that catalyzes the oxidation of 1.0 μmole of *o*-dianisidine HCl per min at 40 °C into colored product ($\varepsilon m = 30{,}000\ M^{-1}\ cm^{-1}$).

Protein was estimated as described by Lowry et al. (1951). Bovine serum albumin was as a standard.

2.4. Activation of fly ash (FA)

FA (10 g) was incubated at 120 °C overnight in an oven and next day it was suspended in 100 mL of distilled water and stirred for 1 h at room temperature. The fine particles present in the suspension were removed by decantation. This decantation process was repeated at least thrice (Satar and Husain, 2009).

2.5. Immobilization of BGP on fly ash

The binding of BGP on activated FA was performed by incubating 2130 U BGP g^{-1} of FA at 4 °C overnight in 100 mM sodium acetate buffer, pH 5.0. BGP adsorbed on fly ash was crosslinked by 0.5% glutaraldehyde for 1 h at 4 °C. Glutaraldehyde crosslinked FA adsorbed BGP (I-BGP) was finally suspended in 15 mL of 100 mM sodium acetate buffer, pH 5.0 and stored at 4 °C for further use (Satar and Husain, 2009).

2.6. Effect of enzyme concentration on removal of BPA

BPA (0.5 mM, 5.0 mL) was incubated with increasing concentrations of soluble and immoblized BGP (0.1–1.0 U mL^{-1}) in the presence of 0.3 mM guaiacol and 0.75 mM H_2O_2 in 100 mM sodium phosphate buffer, pH 7.0 for 2 h at 40 °C. The insoluble product was removed by centrifugation at 3000xg for 15 min. The decrease in absorbance at the specific $\lambda_{max(275nm)}$ was monitored. The percent oxidation was calculated by taking untreated BPA solution as control (100%).

2.7. Effect of time on the oxidative removal of BPA

BPA (0.5 mM, 5.0 mL) was treated by soluble and immobilized BGP (0.4 U mL^{-1}) in 100 mM sodium phosphate buffer, pH 7.0 at 40 °C for various time intervals in the presence of 0.75 mM H_2O_2 and 0.3 mM guaiacol. The reaction was stopped by heating in a boiling water bath for 5 min. After centrifugation the remaining BPA was monitored at the specific wavelength maxima (A_{275} nm). The percent oxidation was calculated by taking untreated BPA as control (100%).

2.8. Oxidation and removal of BPA in batch processes

BPA (0.5 mM, 250 mL) was treated by soluble and immobilized BGP (10 U) independently in batch processes for varying times at 20, 40 and 60 °C in the presence of 0.75 mM H_2O_2 and 0.3 mM guaiacol in sodium phosphate buffer, pH 7.0. Aliquots of 5.0 mL treated samples were removed at the indicated time intervals. The collected samples were heated in a boiling water bath for 5 min in order to stop the reaction. The insoluble product was removed by centrifugation at 3000*g* for 15 min. The percent removal was calculated by taking untreated BPA as control (100%).

2.9. Treatment of BPA polluted water in a continuous bed-reactor

Continuous bed-reactors were developed for the removal of BPA from polluted water. Two columns (16.0 × 1.5 cm) were independently filled with I-BGP (4575 U). BPA solution (0.5 mM) containing 0.3 mM guaiacol and 0.75 mM H_2O_2 was passed through these reactors at 40 °C. The flow rates of the columns were adjusted at 20 and 30 mL h^{-1}. The samples collected after every 5th d from each reactor were centrifuged and spectrophotometrically analyzed for remaining BPA.

2.10. HPLC analysis

HPLC was performed with a Waters Alliance 2690 Separations system coupled to a Waters 996 Photodiode Array Detector (200–650 nm). All samples were analyzed at 40 °C using a Luna 5 μ C18 (2), 2.0 mm inner diameter × 150 mm (Phenomenex, Torrance, Calif., U.S.A.). The mobile phase was a binary gradient of A (CH_3CN/H_2O = 1/99) and B (CH_3CN/H_2O = 99/1) with 0.02% formic acid. The initial LC condition was 35% B solution, whose state was held for 1 min. Solution B was ramped to 80% for 9 min, held for 7 min, then the mobile phase was returned to the initial conditions and the column was allowed to equilibriate for 15 min before the next injection.

2.11. Genotoxicity by plasmid nicking assay

Covalently closed circular pBR322 DNA (0.5 μg) was treated with the test samples (0.1 mM each of BPA and 4-IPP) in a total volume of 30 μl for 1 h. After the treatment, 8 μl of 5× tracking dye (40 mM EDTA, 0.05% bromophenol blue and 50 % (v/v) glycerol) was added and loaded on 1% (w/v) agarose gel. The gel was run at 50 mA for 2 h and stained with ethidium bromide (0.5 μg L^{-1}) for 30 min at room temperature. After washing, the gel was visualized on photodyne UV-Transilluminator (USA) and photographed.

2.12. Genotoxicity of the parent compound and the product by comet assay

2.12.1. Isolation and viability assessment of lymphocytes

Heparinized blood sample (2 mL) from single healthy donor was obtained by venupuncture and diluted suitably in $Ca^{2+}$and Mg^{2+} free PBS. Lymphocytes were isolated from blood using Histopaque 1077 (Sigma, USA) and the cells were finally suspended in RPMI 1640. The donor (first author) donated blood for all experiments.

The lymphocytes were checked for their viability before and after the reaction, using Trypan Blue Exclusion Test (Renner and Schmezer, 1993).

2.12.2. Evaluation of whole lymphocytes treated with the parent compound and the product, 4-IPP by standard comet assay

Lymphocytes isolated from 2 mL blood were diluted to the count of 10^5 cell mL^{-1} and suspended in RPMI 1640. Approximately 10,000 cells were mixed with 75 μL of pre-warmed LMPA in PBS and immediately applied to a frosted microscopic slide layered by 75 μL of 1% standard agarose in PBS. After that slides were transferred to another tank containing 0.4 M phosphate buffer, pH 7.5 for 10 min. These slides (10–20) were then transferred to 2 rectangular dishes (8 × 3 × 5 cm) containing 10^{-4} M solution of BPA and 4-IPP. All slides were incubated at 37 °C