Chapter 9 -

Applications: Elimination of Estrogenic activity of Thermal paper by laccase from T. viride NFCC1-2745
9.1. Introduction

Thermal paper is a special fine paper that is coated with a chemical that changes color when exposed to heat. It is used in thermal printers and particularly in inexpensive or lightweight devices such as adding machines, cash registers, and credit card terminals. In thermal printing, BPA functions chemically as a developer and reacts with white or colorless dyes (color formers) in the presence of heat, converting them to a dark color. Presence of BPA and related bisphenols in thermal papers was very recently reported (Liao and Kannan 2011; Mendum et al. 2010; review by Schwartz et al. 2012) and remains a hot topic of further research because of its potential ill effects. BPA can transfer readily to skin in small amounts from these (Biedermann et al. 2010; Zalko et al. 2011). Holding of a receipt consisting of thermal printing paper for 5 seconds, can transfer roughly 1 μg BPA (0.2–6 μg) to the forefinger and the middle finger if the skin was rather dry and about ten times more if these fingers were wet or very greasy. This material can also contaminate recycled paper. Recycled paper used in household consumer products, such as paper towels, has been found to contain BPA (Vinggaard et al. 2000). In addition, the pulping of recycled thermal paper may introduce a chlorinated derivative BPA into effluent water streams (Hohne et al. 2008, Fukazawa 2001). BPA is also released to the environment when BPA-containing materials like certain plastic products (typically polycarbonate) or epoxy matrix break down due to surface damage with age, thermal,
chemical, or UV attack. BPA was recently detected in urine from the Indian population as well suggesting that Indians are exposed to BPA (Zhang et al. 2011).

The health hazards due to the exposure of Bisphenol A (BPA) from food cans, drinking water bottles and baby bottles has been widely reported as it can leach into food or drink. BPA an endocrine disrupting chemicals (EDC), has been shown to have developmental toxicity, carcinogenic effects, lower sperm count and infertile sperm and possible neurotoxicity (Lee et al. 2007; Murray 2007; Taylor et al. 2011; vom Saal and Hughes 2005; Zsarnovszky et al. 2006). BPA has been shown to be capable of crossing the placenta (Balakrishnan et al. 2010). Recently we have demonstrated the role of BPA as an inhibitor of human superoxide dismutase enzyme (Prasanth et al. 2012) and agonist of glucocorticoid receptor (Prasanth et al. 2010). Its damage to environment and organisms has caused an extensive concern. It has been reported that enzyme laccases secreted by certain microbes can oxidize BPA to other compounds and thereby detoxify it (Modaressi et al. 2005; Takao et al. 2004; Uchida et al. 2001).

9.1.1. Objectives of the study

The present study was focused to eliminate the endocrine disrupting properties of thermal paper with partially purified laccase.

The thermal paper used at the local Automated Teller Machine (ATM) counters of India were analyzed for the presence of BPA and
the capability of the paper to produce estrogenicity were assessed using a yeast two hybrid assay experimental system.

9.2. Materials and Methods

9.2.1. Chemicals

17-beta-estradiol (E2), and chlorophenol red- β -D-galactopyranoside (CPRG) (used as chromogen) was purchased from Sigma-Aldrich, Bangalore, India, and all other chemicals were of analytical grade and obtained from Himedia, Merck and SRL India Ltd. Solvents used for HPLC were of ultra pure (HPLC grade). Water used for conducting HPLC was obtained using a Millipore Q 300 system.

9.2.2. Thermal paper Sample

Sample thermal papers collected from Automated Teller Machine (ATM) counters were thoroughly cleaned using compressed air. BPA was extracted from thermal paper in methanol. Four thermal papers of size 8 x 11 centimeters (2 gram) were cut into pieces and of this was extracted in 100ml methanol by continuous stirring at room temperature for an hour. The extract was then concentrated to 10 ml and filtered using a 0.22 micron filter and stored for later use.

9.2.3. Analysis of BPA in Thermal papers

The methanolic extract of the thermal paper were analyzed for confirmation and quantifications of BPA using a Shimadzu -SPD-20A-
RP-HPLC system equipped with CBM-20A controller. The sample injection volume was 20 µl with a flow rate of 1.0 ml/min. Solvent system used was methanol and water in the ratio 7:3. The spectra were recorded using a Shimadzu - SPD-20A Photo Diode Array Detector at 280 nm. To detect the concentration of BPA in the samples, a reference standard of known concentrations of BPA from 0.025 mg- 0.25 mg was injected into the HPLC and concentration of the 30 fold diluted sample was extrapolated from the curves peak area.

9.2.4. Assay for Estrogenic Activity of Thermal papers

The estrogenic activity of the treated and untreated thermal paper extract was determined using the recombinant Yeast Estrogenic Screen (YES) obtained though the courtesy of John Sumpter (Brunel University, UK). In vitro cell cultures were performed adhering to Good Cell Culture Practice (GCCP) by Coecke, 2005. The recombinant yeast was *Saccharomyces cerevisiae* transformed with the human ER alpha gene, together with expression plasmids containing estrogen responsive elements and the lacZ reporter gene, encoding the enzyme beta-galactosidase. When the hER is bound to an estrogen-like compound, the receptor is co-expressed with the reporter gene lacZ, which codes for the enzyme β-galactosidase. This enzyme is secreted into the growth medium and catalyzes the transformation of the chromogenic substance chlorophenol red- β-D-galactopyranoside, which is subsequently measured colorimetrically in the medium. Handling of the yeast
culture, preparation of the growth medium and test procedure are described in detail elsewhere (Routledge and Sumpter, 1997). In brief, 20ul aliquots of the extract in methanol water were incubated with 200ul aliquots of the yeast assay medium containing recombinant yeast and CPRG, a chromogenic substrate of the beta-galactosidase reporter enzyme, in a 96-well polypropylene microtiter plate at 32°C. After 24–48 h, the absorbance of the red color due to the hydrolysis product of CPRG was read using a Bio-Rad 600 microplate reader at 540 nm. All assays were carried out at least in duplicate using a blank well containing the same amounts of the solvent and the yeast assay medium alone. The data were corrected for turbidity using the absorbance at 620 nm, and the values were calculated as follows: Net OD540 = (OD540 for test - OD620 for test) - (OD540 for blank - OD620 for blank).

9.2.5. Enzyme Source

The laccase producing salt and phenol tolerant fungi, Trichoderma viride Pers. NFCCI-2745, isolated from the coconut husk retting-pile in the estuarine waters of North Kerala India was used as a source for laccase. For purification of extracellular laccase, T. viride was cultivated on a defined medium with composition (in g l⁻¹): Glucose-10; Peptone- 1.0; Yeast extract – 0.5 using 0.3 mM copper to stimulate laccase formation.

Mycelia were separated by centrifugation (20 min; 10000 g) after 96 h cultivation when laccase activity reached its maximum. The laccase was precipitated from the culture filtrate, with ammonium
sulphate added to a final saturation of 20 to 60 %, incubated for 6 hrs at 4°C and afterwards centrifuged for 15 min at 10000 g. The pellet obtained by 60 % saturation which showed laccase activity was dissolved in 50 mM sodium acetate buffer of pH 4.0, repeatedly dialyzed against the same buffer and was applied to size exclusion chromatography column (45 cm X 2.5 cm) (Sephadex G100 (Fine); GE Healthcare Life sciences, India), pre-equilibrated with 50 mM sodium acetate buffer, pH 4 and eluted with the same buffer at a flow rate of 10.0 ml h⁻¹. Fractions containing laccase activity were pooled, concentrated using a 30 kDa ultrafiltration membrane (Millipore Amicon Ultra) and stored at -4°C until use.

9.2.6. Treatment of Thermal paper by laccase

In the first set partially purified laccase (1 U/ml) was incubated with ATM paper in such a way that the paper contains approximately 96 µg/ml BPA (1cm² paper), in the second set 0.5 mM ABTS (2, 2-azinobis-(3-ethylbenzthiazoline- 6-sulphonate) was added along with the purified enzyme as a mediator compound for laccase catalyzed BPA detoxification of ATM paper. Reactions were performed in 50 mM sodium acetate buffer, pH 4 for 6 hours at 60°C in dark. Reaction mixture with heat inactivated enzyme served as control.

The residues and metabolites of BPA were extracted with an equal volume of ethyl acetate. The organic phase was separated and evaporated and subsequently redissolved in 100 µl methanol for further analysis.
9.2.7. Estrogenic Activity of Treated Thermal paper and Estimation of Total phenolic compounds

Reduction in estrogenicity was assessed as described in the text 2.2.2. The reduction of phenolic compounds in the treated ATM paper was determined spectrophotometrically using Bray and Thorpe method (1954). Absorbance was measured at 725 nm. The amount of phenols in the reaction mixture was measured using a standard graph prepared from BPA.

9.3. Result and discussion

9.3.1. Analysis of BPA in Thermal papers

The thermal papers tested had quantifiable concentrations of BPA. The presence of BPA was confirmed as the sample retention time and peak corresponded to that of the standard BPA. Retention time was considered reasonably unique identifying characteristic of a given analyte.

Figures 9.1 show chromatogram in which x-axis is the retention time and the y-axis is a signal obtained by UV diode array detector corresponding to the amount of BPA in the sample. The peaks are characteristic of their identity, with a distribution around the mean position. The area inscribed by the peak is proportional to the amount of substance separated in the chromatographic system. The concentration of BPA in the sample was extrapolated from the curves peak area. The amount of BPA for a 1cm² receipt extracted in methanol was estimated to be 96 µg, which is approximately equal
to 6.65mg of BPA per gram thermal paper. (Figure. 9.2). The levels of BPA measured in the thermal papers are consistent with those expected from thermal paper patent literature. It is reported that there are three grades of thermal paper: one with full BPA content (9-19 mg/12 inches), low BPA content (1-3 mg/12 inches) and BPA free (< limit of detection (LOD)) (Mendum et al. 2010).

Figure. 9.1: HPLC chromatogram detection of BPA in Thermal Paper (BPA standard: Black; Methanolic extract of thermal paper: Blue)
9.3.2. Estrogenic Activity of the Methanolic extract of Thermal Paper

The estrogenic activity of the methanol extract of ATM using YES assay is shown in Figure 9.3b. The colour intensities produced by different samples is shown in Figure 9.3a. The maximum β-galactosidase activity i.e. absorbance value of 2.67 induced by 2.70 ng/ml of 17-beta estradiol was taken as 100%.
Figure 9.3 a. Photograph showing the color intensity produced by different samples in YES assay

A - Negative control (in the absence of estrogenic compound)

B - Positive control (in the presence of 100ul 0.2mg/ml BPA in 2 % ethanol solution

C- F – Different concentration of extracts of Thermal paper used of printing ATM receipts

Since both bus tickets and ATM receipts expressed comparable estrogenic activity, only ATM papers were used for further studies.
Figure. 9.3b: Estrogenic Activity of Thermal paper

Estrogenic activity of the ATM extract is expressed as the percentage of the β-galactosidase activity induced by 17-beta estradiol. The β-galactosidase activity produced by ATM extracts suggests that it is estrogenic. This may be due to the presence of BPA in the thermal paper. Since BPA in thermal paper exists as free, unreacted molecules, there is the potential for mobility and therefore human exposure during handling of receipt paper.

9.3.3. Treatment of Thermal Paper with Laccase

Laccase catalyzed oxidation of ATM paper produced water soluble and an insoluble compound that settled at the bottom of the glass vial. The residues and metabolites of BPA were extracted with an equal volume of ethyl acetate.
The organic phase was separated and evaporated and subsequently redissolved in 100 µl methanol for further analysis. The removal of the estrogenic property of the ATM extract by the partially purified laccase from Trichoderma sp NFCCI 2745 is represented in Figure 9.4.

The laccase helped in eliminating the estrogenic activity of the thermal paper by 20% after 1 h, 60% after 2 h, and 100% by 3 h. As shown in Figure 9.5, a reduction in the concentration of total phenolic compounds was observed after incubation with laccase.

While estrogenic activity of ATM paper was almost eliminated the reduction of phenolic compounds after treatment with laccase was reduced only up to 60%. This may be due to the detection of 4-isopropenylphenol (Takao et al. 2004), a water soluble compound of laccase catalyzed oxidation of BPA. NMR and electron-impact mass spectrum analyses reported earlier had showed that the insoluble products comprises of a BPA dimer, 5,5'-bis-[1-(4-hydroxy-phenyl)]-1-methyl-ethyl]-biphenyl-2,2'-diol (Uchida et al. 2001) and BPA oligomers (Takao et al. 2004).

Thus laccase may be removing the estrogenicity of thermal paper by oxidation and subsequent polymerization or degradation of BPA coated on to the paper as a colour developer.
Figure. 9.4: Elimination of Estrogenic Activity in Treated Thermal Paper

Figure. 9.5: Reduction of total phenol of treated Thermal paper
9.4. Conclusion

To our knowledge this is the first study assessing the estrogenic activity of thermal paper and elimination of its endocrine disrupting properties using partially purified laccase from an ascomycete fungus. The thermal papers tested had quantifiable concentrations of BPA. The results indicate that these papers can produce estrogen hormone like effect on experimental systems. It should be noted that on daily basis tons of such receipts are being dumped in the environment. Since BPA in thermal paper exists as free, unreacted molecules, there is the potential for mobility and therefore human exposure during handling of receipt paper. Estrogenic properties and phenolic contents of thermal paper were effectively removed from the reaction mixture within 3 h of incubation with the partially purified laccase. We propose the implementation of strategies for utilization of waste thermal paper as a cheap substrate for laccase production by microorganism for a safer and cleaner environment.