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Guaiacol-mediated oxidative degradation and polymerization of bisphenol A catalyzed by bitter gourd (*Momordica charantia*) peroxidase

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ABSTRACT

Peroxidase from bitter gourd was found to be highly effective in the oxidative degradation and polymerization of endocrine disrupting compound, bisphenol A. Bisphenol A was recalcitrant to the action of bitter gourd peroxidase. However, the oxidation of bisphenol A by bitter gourd peroxidase was enhanced remarkably in the presence of a redox mediator, guaiacol. Maximum oxidation of bisphenol A was observed in the presence of 0.3 mM guaiacol, 0.75 mM H_2O_2 and 0.32 U mL^{-1} bitter gourd peroxidase in the buffer of pH 7.0 at 40 °C. In batch process, 90% bisphenol A was removed by this enzyme in 4 h. FT-IR spectral analysis showed the presence of OH group (3308 cm^{-1}) and the benzene groups (1624 and 1224 cm^{-1}) on the bitter gourd peroxidase-oxidized product. 4-Isopropenylphenol was identified as one of the peroxidase-catalyzed oxidation products of bisphenol A by GC–MS and ^{1}H NMR spectral analysis.
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1. Introduction

Bisphenol A (BPA) is widely used in plastic industry as a plasticizer or a monomer for polycarbonate and epoxy resin. It has also been used as a stabilizing material or antioxidant for many types of plastics such as polyvinyl chloride [1]. The production and use of BPA is increasing due to high demand of such plastic wares in daily life. BPA has weak acute toxicity to aquatic organisms; LC_{50} or EC_{50} level for fish and invertebrates ranges from 1.1 to 10 mg L^{-1} [2]. Recently BPA has been reported to have estrogenic activity [3]. Patients treated with a bisphenol A diglycidyl ether (BADGE)-based dental sealant were found to have BPA in their saliva [4]. In this case, untreated BADGE in the sealant leached from the site of application and produced BPA on degradation. It has been reported that the level of BPA in atmosphere ranges from 2.9 to 3.6 μg M^{-3} [5]. However, in fresh and seawater samples, the level of this compound ranges from 0.010 to 0.268 μg L^{-1}. One of the most likely sources of BPA in environment is the leachate from hazardous waste landfill wherein BPA is found in very high concentration and very high frequency [6–8]. Moreover, the report of estrogenic behavior of BPA suggested that it might serve as an endocrine disrupter at environmental concentrations well below acute toxicity level [5].

In order to prevent the toxicity caused by BPA, its removal from polluted sites is necessary. Several chemical and physical methods have been developed for the treatment of such types of pollutants [9–11]. However, these methods were not successful due to certain inherent limitations [12]. Biological treatment of BPA resulted the formation of hazardous products which are not only toxic but some of them also have mutagenic and carcinogenic properties [2]. In view of these demerits of such treatment procedures, the use of enzymes has been suggested by some workers [13–16].

Application of peroxidase in various industrial and environmental remediation has already been described by a number of workers [17–19]. Lignin-modifying enzymes, mangnese peroxidase (MnP) and lignin peroxidase from white-rot basidiomycete, *Trametes versicolor*, have been investigated previously for the biodegradation of toxic compounds, including BPA [20]. Kim et al. [21] reported bisphenol polymerization using fungal, *Coprinus cinereus* peroxidase. It has been demonstrated that oxidative degradation and polymerization of BPA can be accomplished using a variety of enzymes including MnP [22], many plant peroxidases [8,23–27] and other enzymes [12,14,27].

In this work an attempt has been made to investigate the oxidative degradation and polymerization of BPA by bitter gourd peroxidase (BGP). The effect of different operational parameters such as concentration of H_2O_2, enzyme, redox mediator, pH, temperature and time on the oxidative degradation and polymerization of BPA has been examined. The BGP catalyzed products of BPA were

Abbreviations: BGP, bitter gourd peroxidase; HRP, horseradish peroxidase; HOBT, 1-hydroxybenzotriazole; VLA, violuric acid; VA, veratryl alcohol; ABTS, 2,2-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt; DMF, dimethylformamide; TP, turnip peroxidase; SBP, soybean peroxidase.

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also characterized by Fourier transform infra-red spectrum (FT-IR), gas chromatography–mass spectrometry (GC–MS) and ^{1}H nuclear magnetic resonance (^{1}H NMR).

2. Materials and methods

2.1. Materials

Bovine serum albumin, violuric acid (VLA) and *o*-dianisidine HCl were obtained from Sigma Chemical Co. (St. Louis, MO) USA. Ammonium sulfate, BPA, N-N-dimethylformamide (DMF), 1-hydroxybenzotrizole (HOBT) and guaiacol were purchased from SRL Chemicals, Pvt. Ltd. (Mumbai, India). Veratryl alcohol (VA) and syringaldehyde were purchased from Hi-Media Pvt. Ltd. (Mumbai, India). Bitter gourd was brought from local vegetable market. Other chemicals and reagents employed were of analytical grade and were used without any further purification.

2.2. Ammonium sulfate 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 layer of cheesecloth. The filtrate was then centrifuged at 10,000 × *g* on a Remi C-24 Cooling Centrifuge. The clear supernatant was subjected to salt fractionation by adding 20–80% (w/v) $(NH_4)_2SO_4$. This solution was stirred overnight at 4 °C to obtain maximum precipitation. The precipitate was collected by centrifugation at 10,000 × *g* on a Remi C-24 Cooling Centrifuge. The obtained precipitate was redissolved in 100 mM sodium phosphate buffer, pH 7.0 and dialyzed against the assay buffer [28,29].

2.3. Effect of redox mediators on BPA oxidation

The oxidation and removal of BPA (0.5 mM, 5.0 mL) was investigated in the presence of six different redox mediators; HOBT, VLA, VA, phenol, syringaldehyde and guaiacol. The molarity of each redox mediator was 0.3 mM. The oxidation of BPA was catalyzed by BGP (0.32 U mL^{-1}) in 100 mM sodium phosphate buffer, pH 7.0 in the presence of 0.75 mM H_2O_2 for 2 h at 40 °C.

2.4. Effect of enzyme concentration

BPA (0.5 mM, 5.0 mL) was incubated with increasing concentrations of BGP (0.01–0.4 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 3000 × *g* for 15 min. The decrease in absorbance at the respective $\lambda_{max\,(275\,nm)}$ was monitored. The percent oxidation was calculated by taking untreated BPA solution as control (100%).

2.5. Effect of H_2O_2

BPA (0.5 mM, 5.0 mL) was treated by BGP (0.32 U mL^{-1}) with increasing concentrations of H_2O_2 (0.05–1.60 mM) in the presence of 0.3 mM guaiacol in 100 mM sodium phosphate buffer, pH 7.0 for 2 h at 40 °C. The reaction was stopped by heating in a boiling water bath for 5 min. The insoluble product was removed by centrifugation at 3000 × *g* for 15 min.

2.6. Effect of guaiacol

The oxidation of BPA (0.5 mM, 5.0 mL) was performed in the presence of increasing concentrations of guaiacol (0.05–1.60 mM). The reaction mixture was treated by BGP (0.32 U mL^{-1}) in the presence of 0.75 mM H_2O_2 for 2 h at 40 °C. The reaction was stopped by heating the mixture in a boiling water bath for 5 min. The insoluble product was removed by centrifugation at 3000 × *g* for 15 min.

2.7. Effect of pH and temperature

BPA (0.5 mM, 5.0 mL) was treated by BGP (0.32 U mL^{-1}) in the buffers of different pH (2.0–10.0) in the presence of 0.75 mM H_2O_2 and 0.3 mM guaiacol for 2 h at 40 °C. The buffers used were glycine–HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0–8.0), and Tris–HCl (pH 9.0 and 10.0). The molarity of each buffer was 100 mM. Untreated BPA solution at each pH was taken as control (100%) for the calculation of percent oxidation.

BPA (0.5 mM, 5.0 mL) was treated with BGP (0.32 U mL^{-1}) in the presence of 0.75 mM H_2O_2 and 0.3 mM guaiacol in 100 mM sodium phosphate buffer, pH 7.0 at various temperatures (20–80 °C) for 2 h at 40 °C. The insoluble product was removed by centrifugation at 3000 × *g* for 15 min. Untreated BPA solution at 40 °C was taken as control (100%) for the calculation of percent oxidation.

2.8. Effect of time on the oxidative removal of BPA

BPA (0.5 mM, 5.0 mL) was treated by BGP (0.32 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 oxidation of BPA was monitored at the respective wavelength maxima (A_{275} nm). The percent oxidation was calculated by taking untreated BPA as control (100%).

2.9. Oxidation of BPA in batch process

BPA (0.5 mM, 500 mL) was treated by BGP (20 U) in batch process for varying times at 40 °C in the presence of 0.75 mM H_2O_2 and 0.3 mM guaiacol. The aliquots were taken out from the treated reaction mixture at different time intervals. The collected samples were heated in a boiling water bath for 5 min to stop the reaction. The insoluble product was removed by centrifugation at 3000 × *g* for 15 min. The percent oxidation was calculated by taking untreated BPA as control (100%).

2.10. FT-IR analysis

The oxidation product of BPA was monitored with INTERSPEC 2020 model FT-IR instrument, USA. The calibration was done by polystyrene film. The sample was injected by Hamiet 100 μL syringe in ATR box. The syringe was first washed by acetone and after that with distilled water. FT-IR analysis was done to monitor the functional groups present in the native compound and on the oxidative product.

2.11. GC–MS measurement

The GC–MS data were obtained on Shimadzu QP-2000 instrument at 70 eV and 250 °C. GC Column: ULBONHR-1, equivalent to OV-1, fused silica capillary: 0.25 mm × 50 M with film thickness 0.25 μm. An entry such as 60–5–5–250 means that the initial temperature was 60 °C for 5 min then heated at the rate of 5 °C min^{-1} to 250 °C. Carrier gas (helium) flow: 2 mL min^{-1} at Retention time 8.7.

2.12. ^{1}H NMR spectroscopy

^{1}HNMR spectra for treated sample were recorded on a Bruker DRX-500 spectrometer. Chemical shifts were reported in δ scale.

2.13. Measurement of peroxidase activity

The activity of peroxidase was estimated from the change in the optical density ($A_{460\,nm}$) in 100 mM sodium phosphate buffer, pH 7.0 at 40 °C by measuring the initial rate of oxidation of 6.0 mM *o*-dianisidine HCl in the presence of 18 mM H_2O_2 [19].

One unit (1.0 U) of peroxidase activity was defined as the amount of enzyme protein that catalyzes the oxidation of 1.0 μmol of *o*-dianisidine HCl per min at 40 °C into colored product (ε_m = 30,000 M^{-1} L^{-1}).

2.14. Estimation of protein

Protein concentration was determined using procedure described by Lowry et al. [31]. Bovine serum albumin was used as a standard protein.

3. Results and discussion

3.1. Oxidation of BPA in the presence of different redox mediators

Table 1 demonstrates the oxidation of BPA in the presence of six different redox mediators; HOBT, VLA, VA, phenol, syringaldehyde and guaiacol. The maximum oxidative degradation and removal of BPA was 66% in the presence of guaiacol, followed by 60, 56, 55, 46 and 45% in the presence of syringaldehyde, VA, phenol, HOBT and VLA, respectively.

3.2. Effect of H_2O_2 and guaiacol on BPA oxidation and removal

The effect of increasing concentrations of H_2O_2 (0.15–1.55 mM) on the oxidation of BPA by BGP has shown in Fig. 1. BPA removal was continuously increased with increasing concentrations of H_2O_2 and maximum loss of BPA from polluted water was seen in the presence of 0.75 mM H_2O_2. As the concentration of H_2O_2 was further increased, a slow decline in the BPA removal was observed. It has already been reported that excess H_2O_2 diminished the removal efficiency of phenolic compounds due to inactivation of the enzyme [32,33]. However; only a slight decrease in the removal efficiency of BPA was observed above 0.75 mM H_2O_2 concentration.

BGP catalyzed BPA removal was continuously enhanced with increasing concentrations of guaiacol. Maximum removal was obtained in the presence of 0.3 mM guaiacol. Further increase in the concentration of guaiacol resulted in decreased oxidation of BPA (Fig. 1). Several workers have demonstrated that the use of redox mediators has enhanced the rate of oxidative removal of aromatic compounds from polluted water by several fold but these mediators were required in very high concentrations, e.g., 5.7 mM VLA and 11.6 mM HOBT for laccase system [34–36], 1.0 mM HOBT for turnip peroxidase [37], 2.0 mM HOBT for BGP [36]. However, in this work we have reported a very low concentration of a redox mediator, 0.3 mM guaiacol, which could enhance the oxidation of BPA.

Table 1
Effect of various redox mediators on the oxidation and removal of BPA.

Redox mediator	BPA removal (%)
Phenol	55
Violuric acid	45
Veratyl alcohol	56
Syringaldehyde	60
Guaiacol	66
HOBT	46

The oxidation and removal of BPA (0.5 mM, 5.0 mL) was investigated in the presence of six different redox mediators, HOBT, VLA, VA, phenol, syringaldehyde and guaiacol. The molarity of each redox mediator was 0.3 mM. The oxidation and removal of BPA was catalyzed by BGP (0.32 U mL^{-1}) in 100 mM sodium phosphate buffer, pH 7.0 in the presence of 0.75 mM H_2O_2 for 2 h at 40 °C. Each value represents the mean for three independent experiments performed in duplicates, with average standard deviations, <5%.

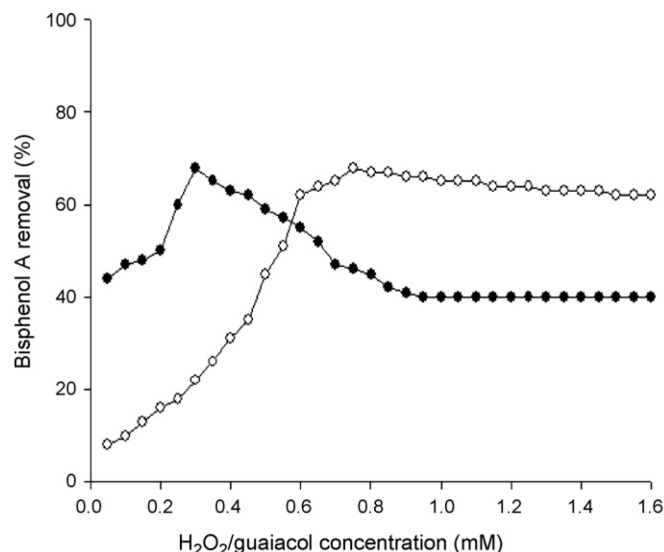


Fig. 1. Effect of H_2O_2 and guaiacol on BPA treatment. BPA (0.5 mM, 5.0 mL) was treated by BGP (0.32 U mL^{-1}) in the presence of varying concentrations of H_2O_2 (0.05–1.6 mM) and guaiacol (0.05–1.6 mM) in 100 mM sodium phosphate buffer, pH 7.0 for 2 h at 40 °C. The precipitate was removed by centrifugation at 3000 × *g* for 15 min. Percent removal was calculated by taking untreated BPA as a control (100%). Symbols indicate the effect of H_2O_2 (○) and guaiacol (●) on the treatment of BPA by BGP.

3.3. Effect of pH and temperature

The role of pH on the oxidation of BPA by BGP has been demonstrated in Fig. 2. BPA was maximally oxidized in the buffer of pH 7.0. Above and below this pH, oxidation of BPA was remarkably decreased. However, this observation showed that the oxidation of BPA was specifically pH dependent. A broad range of pH-optimum for the removal of phenols has been reported for many peroxidases. The maximum oxidation of BPA by soybean peroxidase and laccase was also reported at pH 7.0 [38,39].

The effect of temperature on the removal of BPA has been depicted in Fig. 3. The removal of this compound was maximum at 40 °C. Further increase in temperature resulted in declined

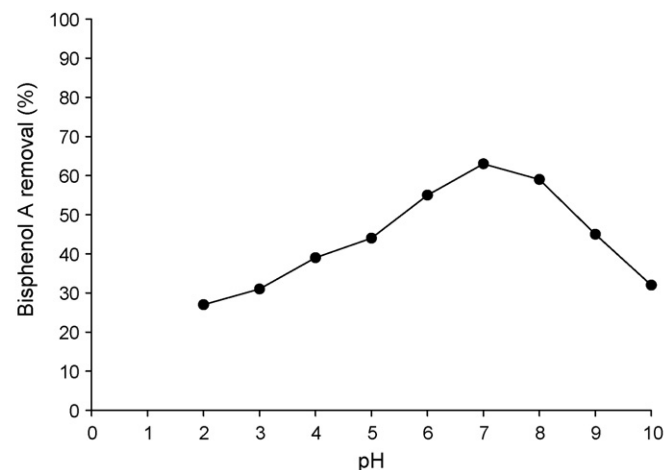


Fig. 2. Effect of pH on the treatment of BPA by BGP. BPA (0.5 mM, 5.0 mL) was treated by BGP in the buffer of different pH (2.0–10.0) in the presence of 0.75 mM H_2O_2 and 0.3 mM guaiacol for 2 h at 40 °C. The molarity of each buffer was 100 mM. The product was removed by centrifugation at 3000 × *g* for 15 min.

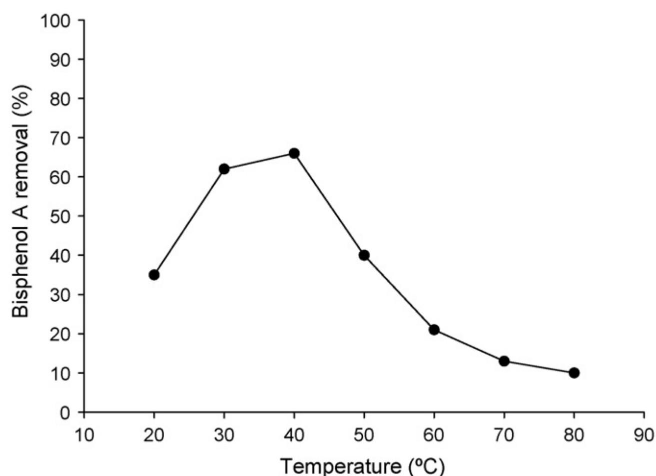


Fig. 3. Effect of temperature on the treatment of BPA by BGP. BPA (0.5 mM, 5.0 mL) was treated by BGP at different temperatures (20–80 °C) in the presence of 0.75 mM H_2O_2 and 0.3 mM guaiacol in 100 mM sodium phosphate buffer, pH 7.0 for 2 h. The product was removed by centrifugation at 3000 × *g* for 15 min.

oxidation of BPA. Some earlier investigators have also reported that the oxidation of alkylphenols, BPA, *p*-nonylphenol, and *p*-octylphenol by horseradish peroxidase (HRP) was maximum at 40 °C [30].

3.4. Effect of peroxidase concentration on the oxidation and removal of BPA

The removal of BPA was increased with increasing concentrations of BGP and its maximum removal was 67% by 0.32 U mL^{-1} of BGP (Table 2). Sakurai et al. [14] have described the removal of BPA from aqueous phase by using a culture broth of *C. cinereus*, as a source of peroxidase; however, the maximum oxidation of BPA was found in the presence of 5.0 U mL^{-1} of peroxidase under the optimized conditions (pH 7.0, 40 °C). In another study, it was reported that maximum removal of BPA was carried by 66.7 U mL^{-1} of HRP [30]. However, the maximum degradation of BPA was performed by a purified laccase (1.5 U mL^{-1}) from the basidiomycetes, *Trametes villosa* [15].

3.5. Effect of time on the oxidation and removal of BPA

The effect of time on the BPA removal by peroxidase-catalyzed polymerization has been shown in Table 3. The oxidation and removal of BPA was continuously increased with time. The maximum oxidation of BPA was observed after 2 h incubation. Further increase in time of incubation had no significant effect on the removal of BPA. Sakuyama et al. [30] have reported that only 10% of BPA was degraded or removed from polluted water after 60 min. The complete oxidation and removal of BPA from a peroxidase isolated from *C. cinereus* occurred after 2 h [14].

Table 2
Effect of enzyme concentration on the oxidation and removal of BPA.

Enzyme concentration (U mL^{-1})	BPA removal (%)
0.01	12
0.02	22
0.08	39
0.16	50
0.24	62
0.32	67
0.40	67

BPA (0.5 mM, 5.0 mL) was incubated with BGP (0.01–0.4 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 3000 × *g* for 15 min. Each value represents the mean for three independent experiments performed in duplicates, with average standard deviations, <5%.

3.6. Removal of BPA in batch process by BGP

Table 3 depicts the oxidation of BPA by BGP in a batch process. It was noticed that BGP could oxidize more than 90% BPA within 210 min of incubation. The oxidation of BPA was continuously increased with respect to time but after 210 min there was no further enhancement in the oxidation of BPA. The higher oxidative degradation and removal of BPA could be due to the formation of insoluble product [40].

3.7. FT-IR, GC–MS and ^1H NMR spectrum analysis

FT-IR spectra for BPA and its enzymatically oxidized products were recorded in the range of 1000–4000 nm. The IR spectrum for the oxidized BPA product showed an OH group at 3308 cm^{-1} like BPA, whereas it exhibited no oxo group. The oxidized product has shown the presence of a benzene groups at 1624 and 1224 cm^{-1}.

In GC–MS spectral analysis, one major compound showed *m*/*z* (relative intensity, % in parentheses) of 134 (M^+, 100), 119 (68.8), 94 (24.0), 77 (8.3) and 65 (10.0) leading to its identification as 4-isopropenylphenol (Fig. 4). Sakuyama et al. [30] have demonstrated that mass spectrum of HRP-catalyzed oxidation product of BPA was absolutely consistent with 4-isopropenylphenol. Actually, another peak at retention time (RT) 34.6 was observed instead of the peak at RT 8.7 when the oxidation product of BPA was hydrogenated and it was consistent with authentic 4-isopropenylphenol.

Fukuda et al. [15] have reported that BPA was converted into two types of compounds by laccase; one kind was high molecular weight compound that resulted probably by oxidative condensation, and another was identified as low molecular weight compound, 4-isopropenylphenol.

Oxidatively degraded and polymerized product of BPA was also identified by Bruker DRX-500 spectrometer (^1H NMR spectrum). The product had δ 1.639 (s, 3H, CH_3), 4.014 (m, 2H, $=CH_2$) 6.716–7.05 (m, 2H, ArH), 7.261–7.468 (m, 2H, ArH). On the basis of these results, the product was identified as 4-isopropenylphenol. The ^1H NMR spectral analysis of BPA catalyzed by crude enzyme preparation from potato has shown two products, where product A was identified as 4[1-(4-hydroxyephenyl)-1-methyl-ethyl]-benzene-1, 2-diol while product B was 4[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-benzene-1, 3-diol [12]. The possible reaction that might be

Table 3
Effect of time on the oxidation and removal of BPA and treatment in batch process.

Time course (min)	BPA removal (%)	
	Effect of time	In batch process
30	21	36
60	35	53
120	67	71
150	67	79
180	67	87
210	67	91
230	67	92
260	67	92
290	67	93

BPA (0.5 mM, 5.0 mL) was treated by BGP (0.32 U mL^{-1}) but 20 U of BGP was used in 500 mL of reaction mixture for the treatment of BPA (0.5 mM) in batch process. Collected samples from both reaction mixtures were incubated in 100 mM sodium phosphate buffer, pH 7.0 at 40 °C for various times in the presence of 0.75 mM H_2O_2 and 0.3 mM guaiacol. Samples were kept in a boiling water bath for 5 min to the reaction. Each value represents the mean for three independent experiments performed in duplicates, with average standard deviations, <5%.

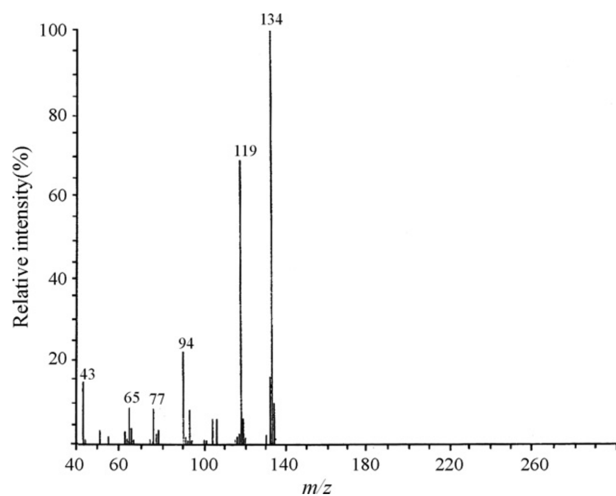
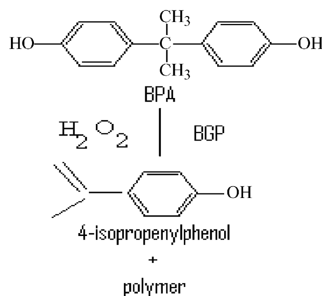


Fig. 4. GC–MS spectra of BGP-catalyzed products. The GC–MS data were obtained on Shimadzu QP-2000 instrument at 70 eV and 250 °C. GC column: ULBONHR-1, equivalent to OV-1, fused silica capillary: 0.25 mm × 50 M with film thickness: 0.25 μm. An entry such as 60–5–5–250 means that the initial temperature was 60 °C for 5 min then heated at the rate of 5 °C per min to 250 °C. Carrier gas (helium) flow: 2 mL per min at RT 8.7.

occurring due to BGP-catalyzed polymerization of BPA is illustrated below.



4. Conclusion

Various physical, chemical and biological methods have been used for the treatment of BPA from polluted water, but all these methods exhibit certain inherent limitations. Several enzymatic oxidation methods have also been reported by earlier workers; however, very few reports are available for the redox-mediated oxidative polymerization and degradation of BPA. Here, for the first time it has been shown that BGP can be efficiently used for the oxidative degradation and polymerization of BPA in the presence of various redox mediators. The treatment of BPA by BGP in the presence of redox mediator, guaiacol, produced insoluble aggregates within 2 h which could be easily removed simply by centrifugation or filtration. Thus, the peroxidase from bitter gourd has shown its potential in the remediation of hazardous aromatic pollutants. These findings suggested that the use of BGP could be extended to large-scale treatment of BPA and other related compounds by employing more effective and cheaper redox mediators. This as well as the scale up of enzymatic processes will be the subject of future study.

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