Journal of Bioscience and Bioengineering
VOL. 127 No. 6, 732–737, 2019

www.elsevier.com/locate/jbiosc

Cometabolic degradation of bisphenol A by pure culture of *Ralstonia eutropha* and metabolic pathway analysis

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Received 27 August 2018; accepted 4 December 2018
Available online 29 December 2018

Bisphenol A (BPA) is a toxic compound emitting to the environment mainly by polycarbonate production facilities. In this research, BPA with the initial concentrations in the range of 1–40 mg l^{-1} was degraded by *Ralstonia eutropha*. The bacteria were unable to use BPA as the sole carbon source. Therefore, resting and growing cells of phenol-adapted *R. eutropha* were used for cometabolic biodegradation of BPA with phenol at the concentration of 100 mg l^{-1}. The optimum initial concentrations of BPA were 20 mg l^{-1} in both approaches of cometabolism. By using resting cells, BPA removal efficiency (RE) reached to 57%, however, RE decreased to 37% by growing cells in the presence of phenol. BPA-degrading activity was inhibited at BPA concentrations >20 mg l^{-1}. Liquid chromatography–mass spectrometry technique was used to identify some metabolic intermediates generated during BPA degradation process as 1,2-bis(4-hydroxyphenyl)-2-propanol, 4-(2-propanol)-phenol, 4-hydroxyacetophenone, 4-isopropenylphenol, and 4-hydroxybenzoic acid. Finally, metabolic pathways for BPA degradation were proposed in this study.

[**Key words:** Intermediate compounds; Metabolic pathway; Phenol; Specific degradation rate; Resting cells]

Bisphenol A (BPA) is commonly used for the production of some epoxy resins, different plastics, and polycarbonates. In 2015, global demand for BPA was about 7.7 million tons and is estimated to increase to 10.6 million tons by 2022 (1). BPA is extensively identified in rivers, sea, and soil as an abundant pollutant (2–4). Several methods, including biological methods, adsorption, filtration, advanced oxidation processes (AOPs), and photocatalytic degradation have been reported to remove BPA from aqueous solutions (5–8). However, biodegradation of BPA is very challenging as different researches have not reached an efficient BPA removal (9).

Cometabolism is a complex mechanism for the conversion of many recalcitrant and toxic compounds to less hazardous components in the environment. Cometabolism is an incidental biotransformation of a non-growth-substrate (NGS) as the target compound by the cells growing on a growth substrate (GS) or by resting cells (10).

Among the bacteria used in cometabolism, a few genera have shown the effective ability to degrade various compounds, such as *Sphingomonas* sp. (11), *Pseudomonas* sp. (12), *Pseudomonas fluorescens* (13), *Rhodococcus* sp. (14), *Nitrosomonas* sp. (15), *Pseudomonas putida* (16), and *Ralstonia eutropha* (17).

It is necessary to produce cofactors for enzymatic reactions, e.g., hydrogen donors for oxygenases, by the growth or resting cells during cometabolism. Therefore, NGSs with a similar structure to that of a GS can competitively attach to the enzymes binding sites due to the non-specific activity. For example, oxygenases, responsible for the oxidation of some aromatics, have shown non-specific activities toward other aromatic compounds (e.g., BPA) (18). Nzila (19) showed that many recalcitrant compounds are degraded by bacteria using GSs with similar structures to the target compounds. Therefore, chemical structures of both NGS and GS are important to find the bacteria capable of cometabolic degradation. Regarding BPA as an NGS for *R. eutropha*, another compound as a GS can be selected for the cometabolic degradation. The GS for *R. eutropha* can be one of some common organic compounds such as phenol, formaldehyde, and *p*-nitrophenol, that previously were used as the only source of carbon/energy for the microbial growth (20–22). As BPA and phenol have similar chemical structures and *R. eutropha* has shown the ability to degrade phenol, the enzymes involved in phenol biodegradation can probably degrade BPA as an NGS (23,24). In addition, it was recommended that phenol may be the best growth substrate for the cometabolic degradation, because of its lower toxicity than many other environmental pollutants (25). For instance, Fischer et al. (26) showed that by using phenol as the GS, the degradation rate of BPA could be significantly increased.

This study aims to evaluate the cometabolic BPA degradation by *R. eutropha* with phenol as a GS along with the characterization of biodegradation pathway by the detection of metabolic products accumulated in the culture during the removal of BPA.

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1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.12.001

MATERIALS AND METHODS

Chemicals and microbial culture All chemicals with analytical grade were obtained from Merck (Darmstadt, Germany). *R. eutropha* (PTCC 1615) was maintained at 4°C, on a medium including the following components (g l^{-1}): peptone, 2; yeast extract, 2; KH_2PO_4, 1; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.05; $(NH_4)_2SO_4$, 1; glucose, 3; agar, 15 (24). The pH was 7. Then, the medium was sterilized at 121°C in an autoclave. Subcultures were regularly prepared every month. The mineral salts medium (MSM) used for all phenol or BPA biodegradation, and cometabolic experiments contained (g l^{-1}): KH_2PO_4, 5; K_2HPO_4, 0.5; NH_4Cl, 2.5; $MgSO_4$, 0.2.

Experiments The preparation of inoculum and sterilization were described in detail elsewhere (24). The experiments were carried out in triplicates in conical flasks at 150 rpm and 30°C. A negligible change was observed in BPA concentration in a similar test with autoclaved biomass, indicating that physical adsorption of BPA is not significant under the operating conditions.

For resting cell experiments, the bacterial cells that previously used phenol at 100 mg l^{-1} were centrifuged (10 min at 3500 × *g*), and then re-suspended in a fresh medium containing BPA only. For growing cell experiments, both phenol and BPA were available to the bacterial cells initially. In these experiments, phenol concentration at 100 mg l^{-1} and different BPA concentrations in the range of 5–40 mg l^{-1} were present in the culture simultaneously. In both resting and growing cell experiments and for phenol biodegradation, the initial biomass concentration was 0.3 ± 0.01 g l^{-1}.

Analytical methods High-performance liquid chromatography (HPLC) (LC-6A, Shimadzu, Kyoto, Japan) equipped with a UV-VIS detector (SPD-6AV, Shimadzu) was used for the determination of BPA concentration. First, 1 ml of each sample was centrifuged in a refrigerated centrifuge (Combi 514R, Hanil Scientific, Gimpo, Korea) at 10,000 × *g* for 10 min. Then, 25 µl of supernatant was injected to the HPLC with a suitable column (ODS, 4.6 mm, 150 mm, 5 µm, Waters, Milford, MA, USA). A mobile phase consisted of acetonitrile, Milli-Q water, and acetic acid (37/63/0.1, v/v/v) was used at a flow rate of 1 ml min^{-1} for the elution of samples. The oven temperature and detection wavelength were set to 30°C and 280 nm, respectively.

A spectrophotometer (Unico-4802, Unico, Suite E Dayton, NJ, USA) at 765 nm was used to determine phenol concentration in the absence of BPA by Folin-Ciocalteau method (20). Also, Optical density of the solution at 600 nm (OD_{600}) was used for the determination of biomass concentration after providing a calibration curve between biomass dry weight at 105°C for 24 h and the related OD. For all experiments, OD_{600} of the solution was initially maintained at 0.42, corresponding to 0.3 g l^{-1} of biomass dry weight.

Liquid chromatography–mass spectrometry (LC–MS) with an XDB-C18 column (4.6 mm, 150 mm, 5 µm, Agilent, Santa Clara, CA, USA) was used as the analytical technique to elucidate the BPA degradation pathways through identifying the intermediate compounds. Electro spray (ES) mass spectrometer (Quattro micro API, Waters) was coupled with an LC (Alliance 2695, Waters) system. The injection volume and oven temperature were 25 µl and 40°C, respectively. The separation was performed with a C18 column using water-acetonitrile containing 0.1% formic acid as the mobile phase at a flow rate of 1 ml min^{-1}, starting at 70% water, then, changing to 90% acetonitrile in 15 min. The negative ion electrospray ionization mass spectrometric operating conditions were as follows: nitrogen gas temperature for de-solvation, 300°C; de-solvation gas flow, 250 l h^{-1}; capillary voltage, 4.5 kV; cone voltage, 35 V.

RESULTS AND DISCUSSION

BPA and phenol adaptation The adaptation of pure culture to BPA was evaluated up to the initial concentration of 40 mg l^{-1} with the initial biomass concentration of 0.3 g l^{-1} (Fig. 1). The bacteria could not use BPA solely. No significant BPA elimination was observed at different initial concentrations (removal efficiency, RE <5%). RE is represented by Eq. 1:

$$RE = \frac{S_0 - S_t}{S_0} \times 100 \quad (1)$$

where S_0 and S_t are initial and final substrate (BPA) concentrations (mg l^{-1}), respectively. The bacterium was also adapted to phenol through the step-wise increase of phenol concentration from 20 to 800 mg l^{-1} with an initial biomass concentration of 0.3 g l^{-1} (Fig. 2). After complete utilizing of phenol at each stage, the initial phenol concentration was increased to a new level. At the end of adaptation process to phenol, biomass concentration reached 1.5 ± 0.1 g l^{-1}. The final adapted biomass was used to determine phenol specific degradation rate (SDR) in a series of separate flasks

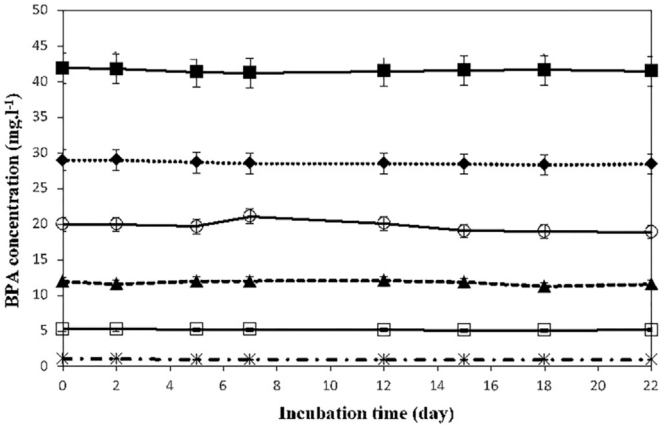

FIG. 1. Adaptation of *R. eutropha* to BPA at different initial concentrations.

containing different phenol concentrations from 20 to 800 mg l^{-1} with the initial biomass concentration of 0.3 g l^{-1}. The evolution of phenol concentration was monitored in each flask during the degradation by phenol-adapted bacteria to attain the SDR. In other words, SDR was calculated by dividing the degradation rate to the initial biomass (0.3 g l^{-1}) for each initial phenol concentration according to Eq. 2:

$$SDR = -\frac{dS}{dt}\frac{1}{X_0} \quad (2)$$

where S and X_0 represent the substrate concentration (mg l^{-1}) and initial concentration of biomass (g l^{-1}), respectively. Fig. 3 depicts the SDRs for the different phenol concentrations along with the required time of incubation for the complete removal of phenol. The maximum SDR for phenol was obtained about 97 $mg_{phenol} \cdot g_{biomass}^{-1} \cdot h^{-1}$ at the initial concentration of 100 mg l^{-1}. Hence, BPA degradation experiments by using phenol-adapted bacteria were carried out at initial phenol concentration of 100 mg l^{-1} by using resting and growing cells. The use of 100 mg l^{-1} phenol as the GS for the cometabolic degradation of ethyl mercaptan (18) and trichloroethylene (27) have been also reported previously.

BPA biodegradation by resting cells The results of BPA degradation at two different concentration ranges by phenol-

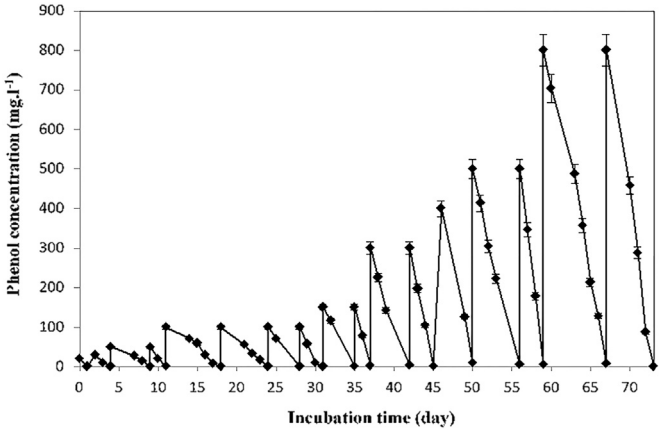

FIG. 2. Step-wise adaptation of *R. eutropha* to phenol with initial biomass concentration of 0.22 g l^{-1}. The peaks are related to the manual increase of phenol concentration in the culture.

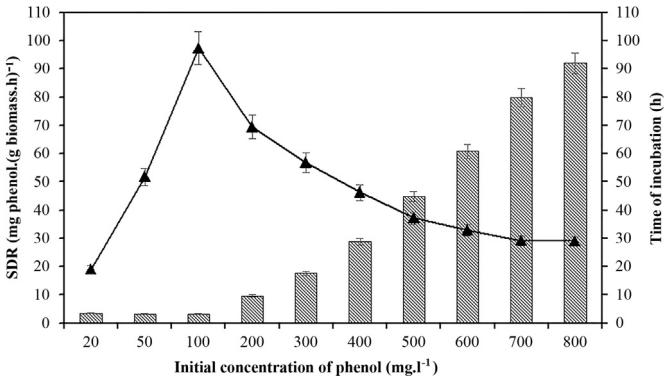

FIG. 3. Specific degradation rate (SDR) for various phenol concentrations (triangles) along with the required time of incubation for each sample to reach removal >99% (bars).

adapted cells in the absence of phenol (resting cells) are shown in Fig. 4. The results reveal that BPA could be partially degraded by using phenol as the GS, while there was no removal with un-adapted cells to phenol. It is expected that the ability of phenol-adapted cells to degrade BPA over un-adapted cells to phenol is related to the enzymes and cofactors induced by phenol (24). The phenol-adapted cells partially consumed BPA, but then the degradation was stopped. The highest BPA degradation were achieved 26%, 29%,40%, 57%, 31%, 25%, and 17% for the initial BPA concentrations of 5, 10, 15, 20, 30, 35, and 40 mg l^{-1}, respectively.

The accumulated metabolic compounds can inhibit BPA degradation after some operating days (28). However, some of the intermediate compounds can be degraded more simply than BPA in the presence of specific enzymes other than those enzymes involved in BPA degradation (29).

Zhang et al. (29) reported that BPA-degrading activity and cell growth of *Achromobacter xylosoxidans* B-16 were inhibited at the initial concentration 5, 10, 20, and 50 mg l^{-1} after 3 days of inoculation.

The use of phenol-utilizing bacterial culture of *R. eutropha* promoted BPA degradation. RE increased from 26% to 57% when initial BPA concentration increased from 5 to 20 mg l^{-1}. An additional increase in BPA concentration resulted in a decrease in RE, probably due to the substrate inhibition and toxicity at higher BPA concentrations. Therefore, 20 mg l^{-1} was the optimum concentration of BPA for biodegradation. The maximum SDR for BPA was achieved at 20 mg l^{-1} as 0.3 $mg_{BPA} \cdot g^{-1}_{biomass} \cdot h^{-1}$. A similar performance was reported for phenol and ethyl mercaptan by using *R. eutropha* cells (24). Li et al. (30) also observed substrate inhibition at BPA concentration >20 mg l^{-1} during BPA degradation by *Bacillus* sp. GZB after 96 h of incubation when the removal decreased from 92% to 66%. The inhibition during BPA biodegradation due to its toxicity was also reported in other studies (29,31).

BPA degradation by growing cells The results for BPA biodegradation in the presence of a constant initial concentration of phenol (100 mg l^{-1}) are shown in Fig. 5. Phenol was completely degraded on the first day of incubation due to the use of phenol-utilizing cells (data not shown). RE of BPA decreased in the presence of phenol in comparison with RE of BPA by resting cells (Fig. 4). After incubation for 16 days, the highest removals for BPA

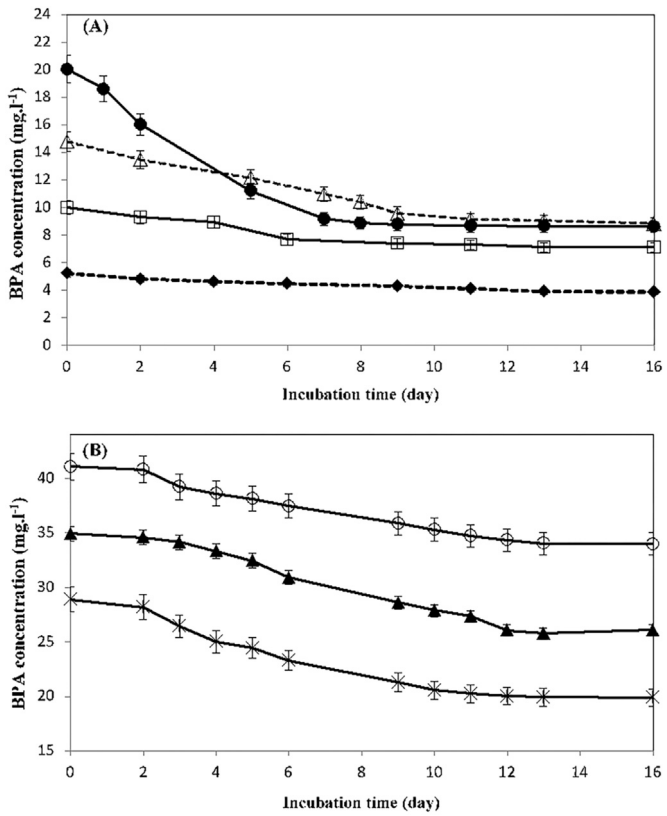

FIG. 4. Cometabolic degradation of BPA by resting cells. (A) BPA concentrations ≤20 mg l^{-1}. (B) BPA concentrations >20 mg l^{-1}.

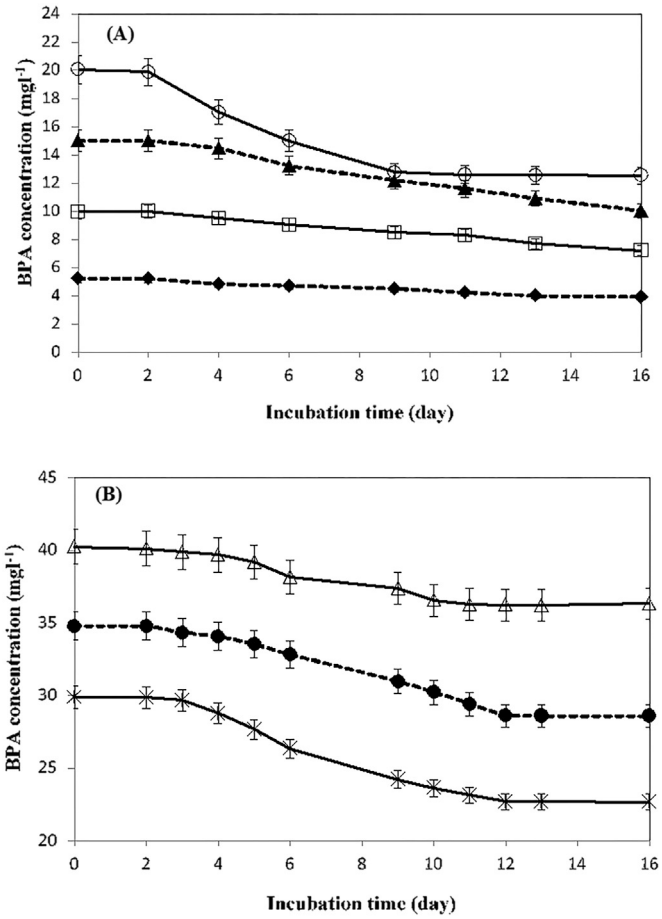

FIG. 5. Cometabolic degradation of BPA by growing cells. (A) BPA concentrations ≤20 mg l^{-1}. (B) BPA concentrations >20 mg l^{-1}.

TABLE 1. Results for BPA degradation by different pure cultures.

Microorganism	Operation type	Growth substrate	Initial optical density (OD_{600})	BPA initial concentration (mg l^{-1})	Operating time (day)	RE (%)	Ref.
Ralstonia eutropha	Resting cell	Phenol (100 mg l^{-1})	0.42	20	16	57	This study
	Growing cell	Phenol (100 mg l^{-1})	0.42	20	16	37	
Pseudomonas sp. and *Bacillus* sp.	Growing cell	Sodium glutamate (0.5%)		1000	12	81–85	45
Enterobacter gergoviae BYK-7	–	–	0.2	200	2	26	46
Achromobacter xylosoxidans B-16	–	–	0.317	20	3	25	29
Sphingomonas sp. BP-7 and *Pseudomonas* sp. BP-14	–	–	0.5	100	7	100	47

were as 24%, 28%, 33%, 37%, 24%, 18%, and 10% for the initial BPA concentrations of 5, 10, 15, 20, 30, 35, and 40 mg l^{-1}, respectively, in the presence of 100 mg l^{-1} phenol as the growing substrate. In this approach, also the BPA degradation rate increased with the increase in the initial BPA concentration. The maximum SDR for BPA was achieved at 20 mg l^{-1} as 0.05 $mg_{BPA} \cdot g^{-1}{}_{biomass} \cdot h^{-1}$.

Competition for the active sites of enzymes may happen when multiple substrates are concurrently available as the enzymes capable of catalyzing cometabolic reactions have active sites ready to accept a number of substrates. The rates of degradation for both compounds decrease when the affinities of enzymes for each substrate decrease during simultaneous degradation in a cometabolic process (18,32).

BPA cometabolic degradation with phenol resulted to RE of 90% and 33% for BPA degradation by resting and growing cells of *Cupriavidus basilensis* JF1, respectively (26). About 8-fold increase in the degradation capacity of cometabolic substrate (mg cometabolic substrate/mg biomass) was also reported for the removal of pentane, toluene, and benzene to boost cometabolic degradation of ethylene dibromide by resting cells (33). The higher transformation capacity of substrate by the use of resting cells in comparison to the use of growing cells was also observed in cometabolic biodegradation of c-DCE (14).

Table 1 compares the results for BPA degradation in this study with different bacteria that have been isolated to grow on BPA as the sole carbon source or degrade it cometabolically. It is evident that the degradation capacities of BPA by various pure strains are different. This difference may be due to the initial BPA concentration, degradation time, and the intrinsic capacity of various strains for degradation.

Metabolic intermediates and pathways Metabolites produced during BPA degradation (20 mg l^{-1}) by resting cells of *R. eutropha* were identified by LC–MS to clarify the BPA degradation pathway. In general, 1,2-bis(4-hydroxyphenyl)-2-propanol (MW= 244 g mol^{-1}), 4-(2-propanol)-phenol (MW= 152 g mol^{-1}), 4-hydroxyacetophenone (MW= 136 g mol^{-1}), 4-isopropenylphenol (MW= 134 g mol^{-1}), 4-hydroxybenzoic acid (MW= 138 g mol^{-1}) and a compound with MW of 216 g mol^{-1} were detected as the intermediates. For *Sphingomonas* sp. strain MV1, the main metabolic pathway (80%) is the BPA cleavage to generate *p*-hydroxyacetophenone and *p*-hydroxybenzaldehyde, with more degradation of both compounds to *p*-hydroxybenzoic acid (34). Also, two oxidation products were present in the culture with MWs of 454 g mol^{-1} and 680 g mol^{-1}, suggesting the formation of BPA dimers and trimers, respectively. Uchida et al. (35) similarly recognized dimers (MW = 454 g mol^{-1}), trimers (MW = 680 g mol^{-1}), and some other BPA polymerization products after degradation by *Trametes villosa*. In addition, production of BPA polymers was also reported in the BPA degradation by manganese peroxidase produced by *Stropharia coronilla*. According to LC–MS results and in line with previous findings (26,36), possible pathways for BPA biodegradation by *R. eutropha* are proposed in Fig. 6.

In pathway 1, oxygenases catalyze the oxidation of BPA to generate phenoxyl radical intermediates. These phenoxyl radicals

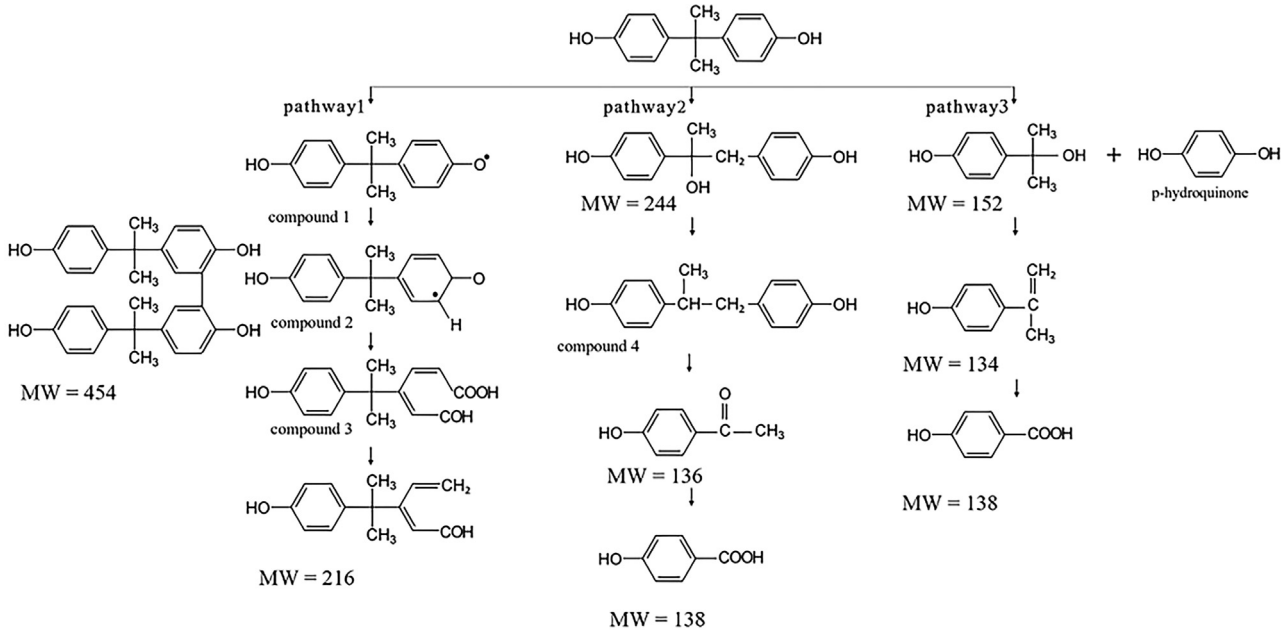

FIG. 6. Proposed possible pathway for the degradation of BPA by *R. eutropha*. The compounds with specified MWs were determined in LC–MS, other compounds were assumed to be produced.

react together to produce dimer and trimer forms of BPA with MWs of 454 g mol^{-1} and 680 g mol^{-1}, respectively. On the other hand, phenoxyl radicals can be eventually transformed to a compound with MW of 216 g mol^{-1} after oxidation and decarboxylation reactions (36).

In pathway 2, BPA is oxidized to 1,2-bis (4-hydroxyphenyl)-2-propanol, and then it is degraded to compound 4. Although compound 4 was not recognized in this study, it is assumed to be present in the pathway to get degraded to 4-hydroxyacetophenone and 4-hydroxybenzoic acid which were both identified (29,37).

It should be pointed out that some other bacteria can oxidize BPA following the proposed pathway 2, such as *Sphingomonas* sp. MV1 and *Pseudomonas paucimobilis* FJ-4 (9,38).

In pathway 3, it is suggested that a monooxygenase enzyme inserts an oxygen into the ring of BPA to cleave it to 4-(2-propanol)-phenol and *p*-hydroquinone. The compound of 4-(2-propanol)-phenol was detected, but *p*-hydroquinone was not detected in LC–MS. Generally, 4-isopropenylphenol is produced when a molecule of water is removed at the phenol residue of 4-(2-propanol)–phenol. It is expected that *p*-hydroquinone will be mineralized to carbon dioxide (26,37). Although *p*-hydroquinone was not identified in LC–MS, it is supposed to be present in the culture. Similar pathway was reported for *C. basilensis* JF1 and *Bacillus* sp. GZB (30). Moreover, 4-isopropenylphenol is converted via oxidation to 4-hydroxybenzoic acid through another pathway to get mineralized later (26).

Unfortunately, BPA is proved to be an endocrine disruptor due to the estrogenic activity (39–41). Furthermore, some BPA degradation intermediates demonstrated estrogenic activity (42). Therefore, the toxic effects especially estrogenicity of BPA and degradation intermediates have been assessed previously (30,42). Among the compounds detected in this study, 4-hydroxyacetophenone has lower estrogenic activity than BPA considering yeast two-hybrid and MCF-7 cell proliferation assays (43,44). Ike et al. (43) also reported that 4-hydroxybenzoic acid does not have any estrogenic activity.

Practical implications BPA is a toxic compound which emits to the environment mainly by polycarbonate production facilities. In this study, about 57% of BPA was degraded by using resting cells of *R. eutropha* and 37% removal was achieved in the presence of phenol, while no BPA degradation was observed when BPA was the only carbon source. As in polycarbonate production facilities, BPA is found together with phenol and some other aromatics, the idea of degradation of phenol and BPA co-metabolically is very interesting. Also, fungal treatment of wastewater by using extracellular enzymes is very difficult due to slower growth of fungal culture than bacterial culture. Therefore, bacterial treatment of wastewater containing recalcitrant compounds such as BPA is highly preferred particularly when another main pollutant such as phenol is removed simultaneously as the GS.

Conclusion Since *R. eutropha* could not use BPA as the sole carbon and energy source, a slight improvement in BPA degradation was achieved by cometabolic shaking batch cultures with phenol. In spite of using phenol-adapted *R. eutropha*, BPA was not degraded prior to the complete utilization of phenol. However, higher BPA degradation capacity was obtained when resting cells were used.

There are three possible pathways for BPA degradation by *R. eutropha*. BPA biodegradation led to the synthesis of intermediate compounds. Production of different metabolites in the culture may inhibit the related enzymes involved in further BPA degradation.

A phenol-utilizing bacterial culture of *R. eutropha* was effective for BPA degradation with the initial concentrations in the range of 5–40 mg l^{-1}. Identification of the probable toxicity level and estrogenicity of the metabolites of BPA transformation by *R. eutropha* are suggested for future research.

ACKNOWLEDGMENTS

The authors wish to thank Department of Pharmacology, Yazd University of Medical Sciences, Iran, for providing the lab facilities. Also, the authors acknowledge Research Division of Tarbiat Modares University (TMU) for the financial supports.

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