Biodegradation and detoxification of bisphenol A with one newly-isolated strain *Bacillus* sp. GZB: Kinetics, mechanism and estrogenic transition

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ABSTRACT

A facultative anaerobic bacterial strain, *Bacillus* sp. GZB, was isolated and identified to effectively degrade bisphenol A (BPA) under anaerobic and aerobic conditions. Under anaerobic condition, Fe^{3+} can be used as an electron acceptor for *Bacillus* sp. GZB, while 5 mg L^{-1} BPA can be fully removed and 51% was mineralized under optimal aerobic conditions. Additionally, seven metabolites were identified by GC–MS, four of which were doubly confirmed by authentic standards (two synthesized) and three of four initial degradation intermediates were also quantified during BPA aerobic degradation. The evolution of 1-(4-hydroxyphenyl)ethanone showed a similar tendency with estrogenic activity changing during BPA biodegradation course, indicating its potential estrogenicity. The estrogenicity temporary increase first and decline ultimately during BPA degradation revealing the GZB can effectively detoxify BPA as well as its estrogenic intermediates. This was the first study to report a facultative anaerobic strain can degrade BPA with or without of oxygen.

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1. Introduction

Bisphenol A (BPA), one of the highest production volume chemicals (Burridge, 2003), is widely used as an intermediate in the synthesis of polycarbonate plastics, epoxy resins and flame retardants (Staples et al., 1998). As a frequently detected environment pollutant, BPA was not only found in the environmental samples, such as water, sludge and air (Khim et al., 2001; Zafra et al., 2003), but also in the biological samples as well as human tissues (Vandenberg et al., 2007). Unfortunately, more and more evidence revealed that BPA might cause reproductive toxicity because of its estrogenic activity (Hu et al., 2002). Although the affinity of BPA for estrogen receptors is five to six orders weaker than that of estradiol which was the most potent estrogen (Rehmann et al., 1999), it may still stimulate cellular responses and altered cell functions at very low concentration (Wetherill et al., 2007). Therefore, the study of BPA degradation and detoxification was essential to avoid the potential of reproductive disturbance.

Up until now, most BPA degradation studies were mainly focused on the oxidation reaction including photodegradation (Nomiyama et al., 2007; Ohko et al., 2001) and biodegradation (Kolvenbach et al., 2007; Yamanaka et al., 2007; Yim et al.,

2003). The organisms such as fungi (Kabiersch et al., 2011), plant-cultured cells (Saiyood et al., 2010) as well as bacteria (Kang and Kondo, 2002a,b; Lobos et al., 1992; Yamanaka et al., 2007) can all be used as biocatalysts for BPA biodegradation. However, all the biodegradations above were carried out under aerobic condition, and no reference reported the capability of both aerobic and anaerobic degradation of BPA by one facultative anaerobe. As far as was known, BPA is an important degradation intermediate of tetrabromobisphenol-A (TBBPA) from the reductive debromination (An et al., 2011a), and the possibility of BPA anaerobic degradation may offer an alternative way for TBBPA biodegradation since it is difficult to biodegrade BPA during TBBPA anaerobic debromination process (Ronen and Abeliovich, 2000). Thus, the isolation of one facultative anaerobe will be of interest for the biodegradation of BPA as well as TBBPA in water environments.

Moreover, not only BPA, as an estrogen disrupter, but also its degradation intermediates exhibited estrogenic activity (Nomiyama et al., 2007). For example, BPA could be metabolized into 4-methyl-2,4-bis(*p*-hydroxyphenyl)-pent-1-ene (MBP) by rat liver S9 fraction (Yoshihara et al., 2004), which showed a strong estrogenic activity to Japanese medaka fish *in vivo* (Ishibashi et al., 2005). Therefore, some researchers assessed the transitions of estrogenic activity during BPA photodegradation course (Nomiyama et al., 2007; Ohko et al., 2001), and one report found that the intermediates produced by the photo-oxidation of BPA exhibited

xeno-estrogen effects to aquatic wildlife (Nomiyama et al., 2007). The transition of estrogenic activity in BPA degradation by *Sphingomonas bisphenolicum* AO₁ also showed obvious temporary increase (Oshiman et al., 2007). Nevertheless, the disagreements still existed within the estrogenic variation in BPA degradation course, for instance, the transcriptional estrogenic activity decreased drastically during BPA photodegradation (Ohko et al., 2001), and similar decrease was also found in BPA biodegradation course (Ike et al., 2002).

Therefore, in this study, a facultative anaerobic bacterial strain was isolated to degrade and detoxify BPA with both anaerobic and aerobic conditions. The capability of BPA anaerobic degradation by the isolated strain was investigated at first. Furthermore, the BPA aerobic biodegradation condition was also optimized and the aerobic degradation intermediates were identified by GC–MS, and four important metabolites were also double confirmed, and three of them were quantified by their authentic standards, two of which were synthesized and characterized with NMR and GC–MS. In addition, the estrogenic activity transition during the BPA aerobic biodegradation was also investigated to evaluate the detoxify ability of the isolated strain.

2. Methods

2.1. Chemicals and growth medium

BPA (97%) and N,O–bis(trimethylsilyl)–trifluoroacetamide (BSTFA) (98%) were purchased from Acros Organics (New Jersey, USA). 1-(4-Hydroxyphenyl)ethanone (99%) was from J&K Chemical, and the estrogenic activity assay kit was obtained from the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. All other chemicals were of analytical grade and from Guangzhou Chemical Reagent Co. Inc., China. The detail recipe of growth medium (GM) and the mineral medium (MM) used for enrichment and isolation of the BPA-degrading strain can be found in the Supporting Information (SI).

2.2. Strain isolation and identification

The bacterial strain with BPA degradation activity was isolated from the sediment of a creek in an electronic-waste recycling site in Guiyu, China (An et al., 2011b). The MM contained BPA (range from 1 to 15 mg L⁻¹) as the sole carbon source and was used for the isolation of BPA degradation strain. The detailed isolation procedure was similar with TBBPA and described in detail in our previous study (An et al., 2011a; Zu et al., 2012). 16S rRNA gene sequence analysis, the physiological and biochemical determination were used for the bacterial strain identification. The specific experimental procedures are shown in the SI.

2.3. BPA biodegradation

The isolated strain was pre-cultured in Luria–Bertani (LB) medium and collected in the late logarithmic growth phase by centrifugation at 6000g for 3 min, then washed with MM twice. Unless otherwise specified, the BPA biodegradation experiments were performed by inoculating 20 mL of harvested cultures into 100 mL MM (in 250 mL shake flasks) containing 10 mg L⁻¹ BPA at 37 °C, pH 7.0, and 150 rpm for 96 h. To investigate the capability of BPA anaerobic degradation, three alternative electron acceptors such as 1.5 g L⁻¹ Na₂SO₄, 1.0 g L⁻¹ NaNO₃ and 5.0 g L⁻¹ amorphous Fe(III) (Arbeli et al., 2006) were separately added into the O₂-free degradation solution which was flushed with N₂ for 1 h, as well as the MM with O₂ as the control test. Amorphous Fe(III) was prepared according to the previous reference (Lovley and Phillips, 1986).

At different time intervals, BPA concentration was measured by using a high performance liquid chromatograph (Agilent 1200) equipped with a DAD detector set at the wavelength of 280 nm. The biodegradation metabolites were analyzed by gas chromatograph (Agilent 7890) coupled with a mass selective detector (GC-MSD, Agilent 5975C) with and without N,O-bis-(trimethylsilyl)- trifluoroacetamide (BSTFA) derivatization. The concentration of the mineralized product, CO_2, was detected by a GC coupled with a flame ionization detector (Shanghai, China). The detailed detection conditions for GC-MSD was shown in the SI, and other detailed conditions were all performed similar to our previous study (An et al., 2011a).

2.4. Estrogenic activity assay

During the biodegradation process, the estrogenicity transition at different five BPA initial concentrations (5, 10, 15, 20 and 30 mg L⁻¹) was detected by measuring the β-galactosidase activity of a recombination yeast cell. The experiment steps were all according to the operational procedure manual of the test kit. The β-galactosidase activity (*U*) was calculated as follows:

$$U = \frac{OD_{420} - OD'_{420}}{t \times V \times OD_{600}} \times D \tag{1}$$

where OD_{420} is the optical density after the colorable reaction at 420 nm, OD'_{420} is the OD_{420} of the blank control, OD_{600} is the optical density at 600 nm after the sample reacting with the yeast for 2 h, *t* is the reaction time, *V* (mL) is the volume of the yeast solution, and *D* is the dilution factor. 17β-estradiol (E2) was used as standard control, and six E2 concentrations with 10^{-11}, 5×10^{-10}, 10^{-10}, 5×10^{-9}, 10^{-9}, and 10^{-8} mol L⁻¹ were chosen to establish the calibration curve, and the dose response curves of these samples were obtained by fitting the data by Eq. (2) (Rehmann et al., 1999) as follows:

$$Y = \frac{A - D}{1 + (C/X)^B} + D \tag{2}$$

where *Y* represents the β-galactosidase activity, *X* is the estrogen concentration, *A* is the maximum β-galactosidase activity, *B* is the relative slope of the middle region of the regression curve, *C* is the estrogen concentration at half maximal response, and *D* is the minimum β-galactosidase activity.

2.5. Synthesis and characterization of 4-(2-hydroxypropan-2-yl)phenol and 4-(prop-1-en-2-yl) phenol

The synthesis route of 4-(2-hydroxypropan-2-yl)phenol showed as follows: 236 mg (9.69 mmol) of magnesium was put into the 50 mL flask fixed with reflux condensing tube, the catalyst iodine dissolved in diethyl ether was added with N₂ atmosphere. 5 mL of iodomethane (8.82 mmol, 549 μL) dissolved in diethyl ether was dropped slowly into sustain slight ebullition until the magnesium was totally dissolved with the drop-wise and stirred the solution. 1.10 mmol (150 mg) hydroxyacetophenone dissolved in 10 mL diethyl ether was added into the resultant solution, then 5 mL tetrahydrofuran was also added. The reaction mixture was stirred for 3 day at the room temperature, then saturated NH₄Cl was employed to quench the reaction and the pH value of solution was adjusted to 7.0–8.0. The diethyl ether and the saturated NaCl solution were used to extract and wash the solution twice, respectively. After rotary evaporation, the pale yellow colored crude product was obtained. Finally, the white solid product was obtained by the active carbon decoloration and column chromatography purification with the yield of 65.7%. The 1H NMR (300 MHz, DMSO-d6) result of prepared 4-(2-hydroxypropan-2-yl)phenol was shown as

follow: 9.13 (s, 1H), 7.23 (d, J = 8.40 Hz, 2H), 6.66 (d, J = 8.70 Hz, 2H), 4.79 (s, 1H), 1.37 (s, 6H). The detail spectra of NMR and GC-MSD were all shown in Fig. S1.

The 4-(prop-1-en-2-yl)phenol was also synthesized as follows (Chen et al., 2004): 6.0 g (26 mmol) 4,4'-(propane-2,2-diyl)diphenol was added into the 19 mL H_2SO_4 (98.3%) with stirring for 30 min at room temperature to fully dissolving. The resultant solution was added into 350 mL ice water and stirred further 12 h kept at 25 °C. The white solid was obtained after the sucking filtration and then dissolved into the diethyl ether. The filtrate was also extracted 3 times by 30 mL diethyl ether each time. The extract as well as white solid were combined, adjusted the pH value to 7.0 with 5% $NaHCO_3$ and dehydrated with anhydrous $MgSO_4$, then concentrated to obtain orange oily substance by the rotary evaporation. After that, NaOH was added as catalyst and the yellow crude fraction of 132–140 °C were collected during the vacuum distillation, then the ultimate product was purified by the column chromatography to yield 4-(prop-1-en-2-yl)phenol with the yield of 40.2%. The 1H NMR (300 MHz, $CDCl_3$) result was showed as follows: 7.37 (d, J = 8.70 Hz, 2H), 6.80 (d, J = 8.70 Hz, 2H), 5.28 (s, 1H), 4.99 (s, 1H), 4.77 (s, 1H), 2.12 (s, 3H). The detail spectra of NMR and GC–MS of prepared 4-(prop-1-en-2-yl)phenol were shown in Fig. S2.

3. Results and discussion

3.1. Properties of the BPA-degrading strain

A bacterial strain with BPA degradation activity was isolated by using BPA as the sole carbon source after two month incubation. Microscopic observation was used to ensure the purity of the culture of single strain, and the 16S rRNA sequence with 1464 bp length (GenBank accession number: HQ603747) was used to construct the phylogenetic tree to identify the genus of the strain. Neighbor–joining method was employed for the phylogenesis analysis after alignment with the related sequences from the GenBank database. The phylogenetic tree (Fig. 1) shows that this strain was clustered with a registered strain *Bacillus* sp. BSFA3-1, and shows high similarity to *Bacillus vallismortis* (99.7%) and *Bacillus subtilis* (99.5%). The physiological and biochemical characteristics shows that this strain is a Gram-positive facultative anaerobes, rod-shaped (0.68–0.66 × 1.0–2.0 μm) with peritrichous flagella (Table S1, Fig. S3), and with positive responses in motility, catalase

and oxidase tests as well as negative response for indol test. Therefore, all these phylogenetic and physiological data suggests that the isolated strain is belonged to the genus *Bacillus* and it was named as *Bacillus* sp. GZB.

3.2. BPA degradation with different electron acceptors

As a facultative anaerobic strain, the anaerobic degradation of BPA by *Bacillus* sp. GZB was investigated firstly with addition of alternative electron acceptors including NO_3^-, Fe^{3+} and SO_4^{2-} (Fig. 2), and the aerobic degradation of BPA by *Bacillus* sp. GZB was also compared with using O_2 as the electron acceptor. The results show that the concentration of BPA was almost no reduced by using NO_3^- and SO_4^{2-} as the electron acceptors, while an effective BPA degradation was obtained by adding Fe^{3+}, although the degradation rate was lower than that of using O_2 (Fig. 2). The slight inhibition by Fe^{3+} comparing with O_2 may due to the lower value of redox potential of Fe^{3+}/Fe^{2+} (770 mv) than that of O_2/H_2O (820 mv). These results show that Fe^{3+} could be used as an electron acceptor in anaerobic system to enhance the degradation of BPA by *Bacillus* sp. GZB, and this is the first report on a facultative anaerobe to be used in BPA biodegradation under anaerobic condition. No anaerobic degradation of BPA was detected with the addition of NO_3^- or SO_4^{2-} to the bacterial culture, indicating the selectivity of the electron acceptors by this facultative anaerobe *Bacillus* sp. GZB. The similar result was also found in a previous report and authors suspected that the inorganic ions NO_3^- and SO_4^{2-} might have potential toxicity to some bacterial strains (Arbeli et al., 2006).

3.3. The growth curve of Bacillus sp. GZB under aerobic condition

The optimized growth conditions are important parameters to obtain high biodegradation activity of bacterial cells. The initial pH value, temperature and shaking rate were chosen as the variable factors to investigate their effect on the growth of *Bacillus* sp. GZB. The results in Fig. S4 show that at all tested conditions, the lag and the stationary phases of *Bacillus* sp. GZB were about 4 and 18 h, respectively, and the highest OD_{600} was 0.488. The growth time will affect the biodegradation ability of the strains (Stasinakis et al., 2010), so the proper culture time was chosen at about 15 h which was close to the log phase of the strain. A slight effect on the growth of strain GZB was observed with the initial pH values range of 6.0–8.0 (Fig. S4a). At the stationary phase, the
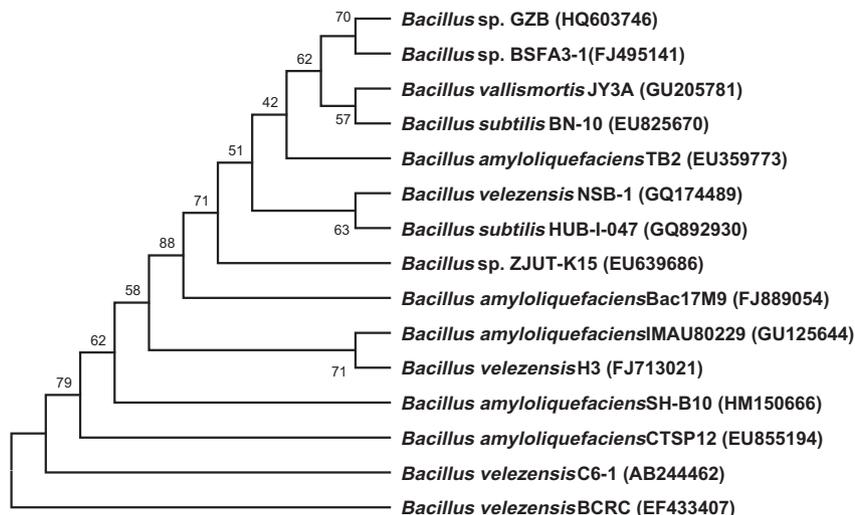

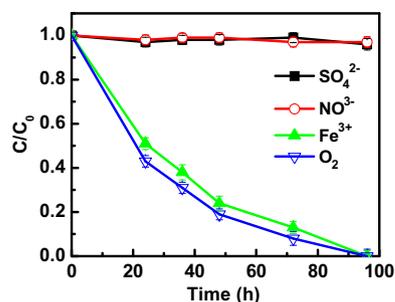**Fig. 1.** Phylogenetic tree based on 16S rRNA sequence analysis (1000 bootstrap for the confidence level).

Fig. 2. The effect of electron acceptors on BPA degradation by *Bacillus* sp. GZB (37 °C, pH 7.0, 150 rpm, BPA: 10 mg L^{-1}).

highest OD_{600} of 0.481 was achieved at pH 7.0, while the lowest OD_{600} value of 0.412 and the second lowest of 0.433 were obtained at pH 6.0 and 8.0, respectively, indicating that the isolated strain was preferred growing at the neutral pH. Fig. S4b shows that the OD_{600} was slightly increased from 0.437 to 0.470 at the stationary phase with the temperature increasing from 20 to 35 °C, and then a slight decrease occurred at 40 °C just following the value at 35 °C. This result is similar to a tribromophenol-degrading strain which was also belonging to the *Bacillus* sp. (Zu et al., 2012). The effect of shaking rate on the bacterial growth is also showed in Fig. S4c. With the increase of shaking rate from 60 to 150 rpm, the OD_{600} was raised moderately from 0.472 to the highest value of 0.488, and then followed with an obvious decrease when the shaking rate was increased to 200 rpm. The lowest OD_{600} of 0.409 was achieved at the shaking rate of 250 rpm, indicating that the excessive oxygen may inhibit the growth of this facultative anaerobic strain.

3.4. BPA biodegradation under aerobic condition

To the best of our knowledge, as a reductive debromination product of TBBPA, BPA cannot be mineralized at the same anaerobic condition in many previous reported systems (Ronen and Abeliovich, 2000; Voordeckers et al., 2002). Thus, to probe the possibility of the complete degradation of BPA, the aerobic degradation of BPA was also investigated by *Bacillus* sp. GZB with O_2 as the electron acceptor. Four affecting factors of BPA biodegradation including BPA concentrations, inoculum volumes, cultivation temperatures and pH values were optimized, respectively. Fig. 3a shows the effect of BPA concentrations (5, 10, 15, 20, and 30 mg L^{-1}) on the biodegradation efficiencies. It shows that after 96 h degradation, the biodegradation efficiencies were decreased from 92.9% to 66.3% with the increase of BPA concentrations. An inhibition effect was observed when the BPA concentration was higher than 20 mg L^{-1}, and much stronger at the initial degradation beginning time (0–24 h) when the substrate concentration was at 30 mg L^{-1}. This inhibition result agrees with the previous studies of BPA biodegradation due to its toxicity (Yamanaka et al., 2007; Zhang et al., 2007). In order to investigate the kinetics of BPA biodegradation, the dynamic equations were fitted and listed in Table S2. Results show that the aerobic biodegradation of BPA follows the pseudo-first-order kinetic model within the concentration range from 5 to 30 mg L^{-1}, and the corresponding degradation rate constants are decreased from 0.030 to 0.013 h^{-1}. While the half-lives of BPA biodegradation are increased from 22.6 to 52.7 h, which are almost the same as that (0.58–3.1 d) reported by Zhang et al. (2007), but significantly different from the results of 144 d reported by Fischer et al. (2010). Thus, it is concluded that *Bacillus* sp. GZB shows excellent performance in BPA degradation under aerobic condition.

The effect of the inoculum sizes on the BPA degradation efficiency was studied (Fig. 3b). The result shows that when the

inoculum size was higher than 20 mL, BPA could be completely degraded within 96 h, and the degradation efficiencies were increased from 80.6% to 100% with the inoculum size increased from 10 to 30 mL. Though the same 100% of the degradation efficiencies could be achieved at 30 and 40 mL inoculum size, the degradation rate was still higher with 40 mL than that of 30 mL, indicating that more bacterial cells can increase not only the efficiency of BPA degradation but also the degradation rate. This result is well agreed with previous studies, which showed a synergistic effect of the biomass amount on BPA degradation (Boonyaroj et al., 2012; Kang and Kondo, 2002a,b; Zhang et al., 2007).

As another important physical factor, temperature not only can affect the bacterial growth but also the BPA degradation activity. The results (Fig. 3c) show that the degradation efficiencies were obtained as 75.7%, 87.9%, 91.8%, 92.4% and 82.8% at the temperatures of 25, 30, 35, 37 and 42 °C, respectively. The highest degradation efficiency was achieved at 37 °C, which was similar to the previous results that higher temperature would promote the BPA degradation (Kang and Kondo, 2002a,b; Zhang et al., 2007). The decrease in the degradation efficiency of BPA at 40 °C was also obtained and be explained by the reason that the decrease is contributed to the evaporation of water in samples (Zhang et al., 2007). Furthermore, the optimal temperature of strain growth is at 37 °C, thus the bacterial growth will be inhibited at high temperature and subsequently affected the biodegradation activity when the temperature was further increased.

Fig. 3d also shows the effect of initial pH values on the degradation efficiencies of BPA. Five pH values, such as 6.5, 7.0, 8.0, 8.5 and 9.0 were chosen to determine the optimal pH value for biodegradation, and the corresponding degradation efficiencies were obtained as 87.5%, 100%, 100%, 97.7% and 81.6%, respectively. These results indicate that *Bacillus* sp. GZB can maintain high BPA degradation activity within the pH range of 7.0–8.5. Under weak acidic condition, it will reduce the hydrolysis of BPA, and subsequently influence the degradation efficiency of BPA because of the low solubility (An et al., 2011a). While under weak alkaline condition, it can increase the solubility of BPA, but it will inhibit the bacterial growth and subsequently reduce the enzyme activity of cultured bacteria for BPA degradation.

3.5. Metabolic mechanism of BPA under aerobic condition

To elucidate the BPA biodegradation pathways and metabolic mechanisms under aerobic condition, GC–MS was used to identify the degradation intermediates with or without BSTFA derivatization (Fig. S5). All obtained mass spectra of these possible intermediates were compared to the standard spectra of NIST Mass Spectral Library, and seven chemicals were tentatively identified as the intermediates of BPA biodegradation (Table 1): 4-(2-hydroxypropan-2-yl)phenol (A), 4-(prop-1-en-2-yl) phenol (B), 1-(4-hydroxyphenyl)ethanone (C), 4-hydroxybenzaldehyde (D), benzoic acid (E), 2-hydroxypropanoic acid (F) and 2-methylbutanoic acid (G), respectively. It should be noted that, intermediates (A), (F) and (G) were only detected with the BSTFA derivatization. To further confirm the accuracy of the identification, four key intermediates (A)–(D) were also double confirmed by their authentic standards, two of which (A and B) were synthesized by ourselves, and three initially produced intermediates (A), (B) and (C) were also qualified, respectively (Fig. S6). Results showed that the retention times of above mentioned four intermediates (A) (16.08 min, BSTFA derivatization), (B) (12.61 min), (C) (11.93 min) and (D) (11.48 min) were all completely agreed with that of the authentic standards (Figs. S7 and S8), which could further validate the accuracy of the identification results. Furthermore, the intermediates (A)–(D) were also verified in many other photocatalytic and biotransformation systems for BPA degradation (Fischer et al., 2010;
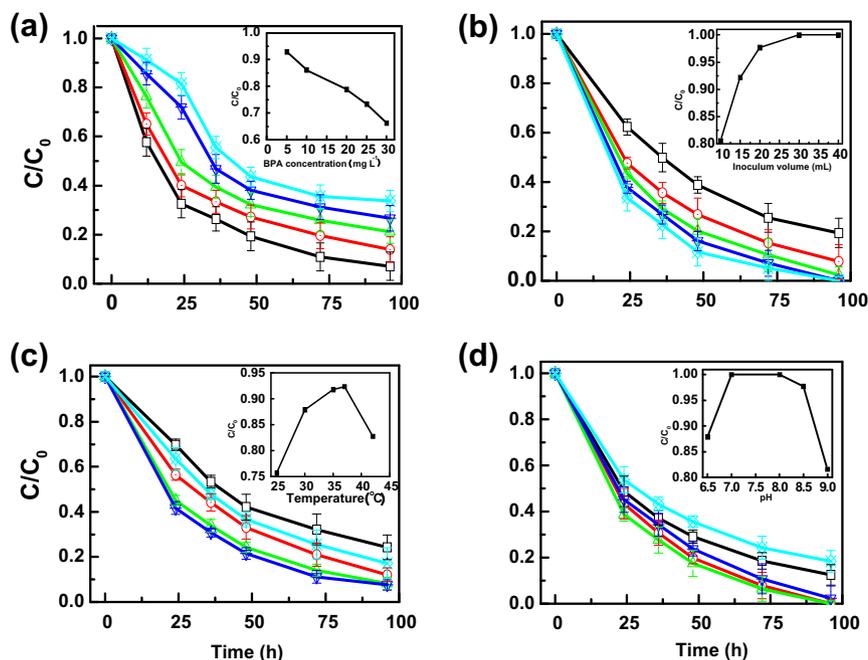
Fig. 3. The effect of (a) BPA concentrations (mg L-1): (□) 5; (○) 10; (△) 15; (▼) 20; (♦) 30; (b) inoculum volume (ml): (□) 10; (○) 15; (△) 20; (▼) 30; (♦) 40; (c) temperature: (□) 25 °C; (○) 30 °C; (△) 35 °C; (▼) 37 °C; (♦) 42 °C; and (d) pH value: (□) 6.5; (○) 7.0; (△) 8.0; (▼) 8.5; (♦) 9.0 on aerobic degradation efficiency of BPA. The error bar value presents the standard deviation of triplicates. Inset shows the curve of BPA biodegradation efficiency at 96 h on different (a) BPA concentration, (b) inoculum volume, (c) temperature and (d) pH value.

Table 1
The mass spectra of intermediates of BPA biodegradation identified with and without BSTFA derivatization.

Intermediates		Retention time (min)	m/z of observed fragment ions
A	4-(2-Hydroxypropan-2-yl)phenol (BSTFA derivatization)	16.08	296* [M$^+$], 281 [M$^+$-CH$_3$], 191 [281-TMS-O]
B	4-(Prop-1-en-2-yl) phenol	12.61	134* [M$^+$], 119 [M$^+$-CH$_3$], 107 [119-C$^+$],
C	1-(4-hydroxyphenyl)ethanone	11.93	136* [M$^+$], 121 [M$^+$-CH$_3$], 93 [121-CO]
D	4-Hydroxybenzaldehyde	11.48	122* [M$^+$], 93 [M$^+$-COH], 65 [93-CO]
E	Benzoic acid	8.22	122* [M$^+$], 105 [M$^+$-OH], 77 [105-CO]
F	2-Hydroxypropanoic acid (BSTFA derivatization)	6.67	234* [M$^+$], 219 [M$^+$-CH$_3$], 191 [219-CO]
G	2-Methylbutanoic acid (BSTFA derivatization)	4.60	174* [M$^+$], 159 [M$^+$-CH$_3$], 117 [159-CH$_3$CHCH$_2$]

Ike et al., 2002; Lobos et al., 1992; Nomiyama et al., 2007; Ohko et al., 2001; Sasaki et al., 2005; Zhang et al., 2007).

In addition, to further evaluate the extent of BPA biodegradation, the concentration transition trendy of the initial intermediates (A)–(C) detected during BPA degradation were also investigated (Fig. S9). With the decline of BPA concentration, both intermediate (A) and (B) firstly reached the highest amount at 24 h, and intermediate (C) was subsequently achieved the highest concentration at 48 h, indicating the formation of intermediates (A) and (B) may be produced earlier than that of intermediate (C). Furthermore, the accumulation of intermediate (A) was transient and only about 0.19 μM was detected at the first 24 h, while the concentration of intermediate (C) was higher than intermediate (B) during all the biodegradation course. From 24 to 96 h, the total amount calculated by BPA plus the three intermediates were 16.36, 14.92, 12.51, 8.06, and 3.06 μM, respectively. It can be found that the concentration amount of 16.36 μM was closest to the BPA initial concentration of 22 μM, indicating these three intermediates were the main products at the first 24 h. The difference between the initial BPA concentration of 22 μM and the detected concentration at five degradation time demonstrated the possibility of other subsequent intermediate formations.

According to the identified intermediates and the evolution curves, the pathways of BPA biodegradation are proposed (Fig. S10). The mechanism can be described as follows: one

benzene ring of BPA was first cleaved and hydroxylated to form intermediate (A) and p-benzenediol. It should be pointed out that p-benzenediol was found during all the degradation process due to its unstable property. The absence of this intermediate may be one main reason to the concentration difference between 22.0 μM BPA and 16.4 μM all intermediates. Also, intermediate (B) might either come from dehydration of intermediate (A), or directly from the cleavage of BPA, which was also frequently found in photocatalytic degradation of BPA pathway (Nomiyama et al., 2007). Similarly, intermediate (C) might be the daughter product from the oxidation of intermediate (A) as well as (B). Subsequently, product (D) was formed via the demethylation from intermediate (C), and then converted to benzoic acid (E). Small molecular products (F) and (G) were also detected only by the BSTFA derivatization and may produce from the cleavage of benzoic acid. The result of hydroxylation and oxidation pathways found in BPA biodegradation was well agreed with the previous patterns involved in both photocatalytic and biodegradation systems (Fischer et al., 2010; Nomiyama et al., 2007). The hydroxylation phenomenon might be due to the contribution from the cytochrome P450 monooxygenase during the biodegradation of BPA (Sasaki et al., 2005).

To further determine the aerobic biodegradation extent of BPA by strain *Bacillus* sp. GZB, the concentrations of the final mineralization product, CO_2, were also quantified and illustrated in Fig. 4. The dependence of the mineralization efficiencies on the degradation

time at 5 mg L^{-1} BPA showed that at the beginning hours (0–24 h), almost no CO_2 was produced. With the degradation time further going on, CO_2 was released gradually (24–72 h) and then was slow down after 72 h. Similar evolution trends were also obtained in other four BPA concentrations. Finally, after 96 h reaction, the mineralization efficiencies achieved 51%, 44%, 35%, 29% and 24% when the BPA concentrations were employed with 5, 10, 20, 25 and 30 mg L^{-1}, respectively. The decrease of total organic carbon (TOC) during BPA biodegradation by *Bacillus* sp. GZB was very closed to that reported previously, which showed that about 60% of the carbon in BPA could be mineralized into CO_2, and the other part of TOC was associated with the bacterial cells and soluble organic carbon remained in the medium (Lobos et al., 1992). Thus, from BPA mineralization results, it can be ultimately proposed that BPA and its metabolic intermediates would be oxidized into CO_2 and H_2O by this facultative anaerobic strain *Bacillus* sp. GZB.

3.6. Estrogenic activity evolution during BPA biodegradation

To evaluate the estrogenic activity evolution during the BPA aerobic biodegradation by *Bacillus* sp. GZB, the estrogenicity transition was investigated detailed with different initial concentration of BPA. The calibration curve of 17 β-estradiol fitted the sigmoidal response very well (R^2 = 0.9729) (Fig. S11), and the EC50 value was obtained as 0.98 nmol L^{-1}, which is closed to a previous results of 1.50 nmol L^{-1} (Rehmann et al., 1999), indicating the validation of the method by employing this estrogenic activity assay kit. For comparison, the estrogenic activity of BPA was also investigated (Fig. S12), and the result showed that the fitting curve was also one part of the sigmoidal curve (R^2 = 0.9980) within the concentration from 5 to 30 mg L^{-1} BPA. The calculated EC50 value of BPA was about 139 μmol L^{-1}, slightly higher than that reported 104 μmol L^{-1} by the reference (Rehmann et al., 1999). Compared to E2, the estrogenic activity of BPA was much lower than at five orders of magnitude.

The plot of the estrogenic activity during BPA biodegradation with different concentrations (5–30 mg L^{-1}) were also investigated (Fig. 5). The result shows that at initial 12 h degradation, the β-galactosidase activity was dependent on the initial BPA concentrations. From 12 to 24 h, the estrogenic activities of all degradation samples were increased quickly at first except that with BPA concentration of 5 mg L^{-1}, the enzyme activity was increased from 0.51 to 0.69 U with the increase of 24–60 h, and the highest value was achieved at 60 h. However, for other three concentrations of 10, 15 and 20 mg L^{-1}, the highest estrogenic activities were detected at 36 h and the values were obtained as 0.88, 0.95 and 1.07 U, respectively. The highest enzyme activity was increased to 1.27 U (at 48 h) when the BPA initial concentration was employed as 30 mg L^{-1}. After 60 h, the estrogenicities of all degradation concentrations (5–30 mg L^{-1}) were all decreased and the β-galactosidae activity on 96 h were obtained as 0.51, 0.61, 0.61, 0.65 and 0.80 U, respectively. This result indicated that *Bacillus*
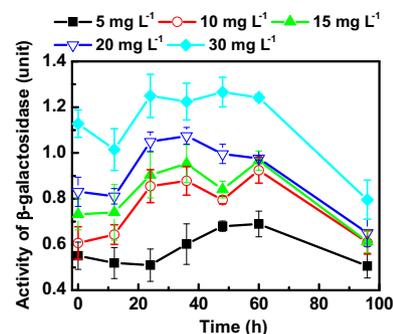
Fig. 5. The transition of the estrogenic activity during BPA biodegradation.

sp. GZB could effectively detoxify BPA within this concentrations range. Although the estrogenic activity decrease may be not so obvious at low BPA concentration due to the limitation of the toxicity detection method, it indeed validated the isolated strain possessing the high detoxification ability to BPA. Additionally, the trendy of the estrogenicity increase at first and then decrease during BPA biodegradation is well consisted with previously reported results (Oshiman et al., 2007). Another previous study showed that as one of BPA biodegradation intermediates, *p*-HAP (intermediate (C)), exhibited a slight estrogenicity (Ike et al., 2002). Thus, compared with the BPA estrogenic activity, with the increase of degradation time, the formed intermediate (C) showed a similar tendency to the estrogenicity transition during BPA biodegradation course, indicating this chemical was most likely to be the main contributor for the increased estrogenicity. In addition, the highest estrogen activities detected at different degradation time in the five tested BPA concentrations indicated that although p-HAP was the main source of the estrogenicity increase, the combined toxicity effect of all intermediates during the BPA biodegradation should not be ignored.

4. Conclusion

A BPA biodegradable facultative anaerobic bacterium, *Bacillus* sp. GZB, was isolated. It can use Fe^{3+} as an alternative electron acceptor in anaerobic system to degrade BPA, which provide a feasible for BPA bioremediation when degradation had to take under anaerobic condition. Under optimized aerobic conditions, the highest degradation and mineralization efficiencies were 100% and 51% at 5 mg L^{-1} BPA, respectively. BPA metabolic mechanism was also proposed tentatively. Estrogenicity evaluation shows that GZB possesses excellent detoxification ability to BPA. This was the first report that a facultative anaerobic bacterium was isolated for BPA bio-decontamination under either aerobic or anaerobic conditions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.03.067.
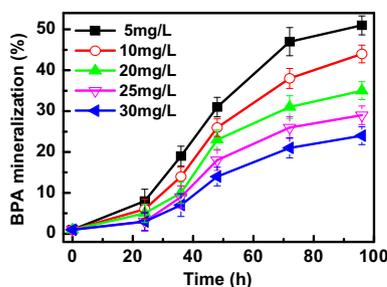
Fig. 4. BPA mineralization efficiencies with different BPA concentrations by *Bacillus* sp. GZB. The error bar value presents the standard deviation of triplicates.

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