

Biodegradation of Endocrine Disruptor Bisphenol A by *Pseudomonas putida* Strain YC-AE1 Isolated from Polluted Soil, Guangdong, China

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Abstract

YC-AE1 is a Gram negative bacterial strain isolated from soil and identified as *Pseudomonas putida* by 16S rRNA and BIOLOG identification system. This strain found to have a high capacity to degrade the endocrine disruptor Bisphenol A (BPA). Response surface methodology using central composite design was used to statistically optimize the environmental factors during BPA degradation and the results obtained by significant model were 7.2, 30 °C and 2.5% for optimum initial pH, temperature and inoculum size, respectively. Prolonged incubation period with low NaCl concentration improve the biodegradation of BPA. Analysis of variance (ANOVA) showed high coefficient of determination, R^2 and Adj- R^2 which were 0.9979 and 0.9935, respectively. Substrate analysis found that, strain YC-AE1 could degrade a wide variety of bisphenol A-related pollutants such as bisphenol B, bisphenol F, bisphenol S, Dibutyl phthalate, Diethylhexyl phthalate and Diethyl phthalate in varying proportion. *Pseudomonas putida* YC-AE1 showed high ability to degrade a wide range of BPA concentrations (0.5-1000 mg l⁻¹) with completely degradation for 0.5- 500 mg l⁻¹ within 3 days. Metabolic intermediates detected in this study by HPLC-MS were identified as 4,4-dihydroxy- α -methylstilbene, p-hydroxybenzaldehyde, p-hydroxyacetophenone, 4-hydroxyphenylacetate, 4-hydroxyphenacyl alcohol, 2,2-bis(4-hydroxyphenyl)-1-propanol, 1,2-bis(4-hydroxyphenyl)-2-propanol and 2,2-bis(4-hydroxyphenyl) propanoate. This study reports *Pseudomonas putida* YC-AE1 as BPA biodegrader with high performance in degradation and tolerance to high BPA concentration. Moreover, it degrades BPA in a short time compared to other reported strains via two different degradation pathways.

1. Background

Xenobiotic endocrine disruptor compounds are environmental pollutants that have health and environmental harms, raising important concern (Atacag 2015; Wang et al. 2017). Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl) propane is a well-known endocrine disruptor that showing a highly estrogenic activity and acute toxicity (Alexander et al. 1988). It is an organic compound consisting of two phenolic rings connected by a carbon bridge with two methyl group attached to this bridge (Kang and Kondo 2006). The production of BPA has increased in the last few decades because of its widespread application in many industries (Ye et al. 2009) to reach the high global demand which was approximately 6.5 million tons in 2012 (Larsson et al. 2017; Zhou et al. 2015). BPA is used as a major component (as plasticizer) for various consumer products including detergents, phenol resins, epoxy resins, polycarbonates, polyesters, polyacrylates, plastic packing materials and lacquer coatings on food cans (Staples et al. 1998). There are a large numbers of studies that reported many damaging effects of BPA for both human and animal organs (Chouhan et al. 2014). Detectable concentrations of BPA were reported in amniotic fluid, fetal circulation and placental tissue of pregnant women (Yamada et al. 2002). Many studies reported that, exposure of aquatic animals to BPA cause reproductive toxicity like changing in sex hormone level which affected the development of ovary in rare minnow *Gobiocypris rarus* (Zhang et al. 2016), decreased sperm count, female biased sex ratio, and increased malformation and mortality in zebra fish (Chen et al. 2015). BPA was detected in a wide range of environment including landfill

leachates (Yamamoto et al. 2001), industrial wastewater (Lee and Peart 2000), source water and drinking water (Kleywegt et al. 2011), rivers, seas, and soils (Bolz et al. 2001; Kolpin et al. 2002; Staples et al. 1998; Yamauchi et al. 2003). Therefore, special attention has been given to BPA toxicity in these environments (Wang et al. 2017). The most common methods to remove pollutants from environment included photodegradation (Lee and Peart 2000), photoelectrocatalytic oxidation (Lee and Peart 2000), oxidation (Lee and Peart 2000) and biodegradation (Kang et al. 2006). Among all these methods, bacterial biodegradation of BPA was the most important one (Zhang et al. 2007) because of low costs and less disruption of the contaminated environment when compared to other cleanup methods. Many researchers reported several BPA-degrading bacteria and their degrading performance *Achromobacter xylosoxidans* strain B-16 (Zhang et al. 2007), *Pseudomonas* sp. and *Pseudomonas putida* strain (Kang and Kondo 2002), *Bacillus megaterium* Strain ISO-2 (Suyamud et al. 2018), *Sphingomonas bisphenolicum* strain A01 (Oshiman et al. 2007) and *Pseudomonas* sp and *Sphingomonas* sp (Larsson et al. 2017).

In industrial technology, the operating conditions should be optimized for maximum production. The optimization by one factor at time (OFAT) involves changing in one factor while other factors or variables at certain levels are fixed (Xu et al. 2003). This method of optimization is time consuming especially with large number of variables in addition to, ignoring the interactions between different variables. These disadvantages of single factor experiments can be overcome by using statistical optimization which allows all interactions of all variables and levels (Khambhaty et al. 2007; Kumar and Satyanarayana 2007).

In this study, a highly BPA tolerant strain with high BPA biodegradation capacity was isolated from soil sample beside one plastic factory in Guangdong province, China and identified as *Pseudomonas putida* YC-AE1, the environmental conditions affecting BPA degradation were studied using statistical methods and discussed. The ability of strain YC-AE1 to degrade other pollutants related to BPA was also studied. The metabolic intermediate compounds detected during the biodegradation process were identified and biodegradation pathways were proposed.

2. Methods

2.1. Chemicals and medium

The Bisphenols (BPA, BPB, BPF and BPS), DBP, DEHP and DEP used in this study were purchased from Shanghai Macklin Biochemical Co., Ltd. China, (all purities above 99%). Stock solutions (20000 mg l^{-1}) of all these substrates were prepared by dissolving it each individual in methanol (99 %). Other chemicals used were of analytical reagent grade, all solvents used were high performance liquid chromatography (HPLC) grade. Two growth media were used in this study: Luria-Bertani (LB) medium for growth and enrichment. It consisted of 10.0 g l^{-1} peptone, 5.0 g l^{-1} yeast extract, and 10.0 g l^{-1} NaCl. Trace Elements Medium (TEM) as described by (Nahurira et al. 2017) were used for isolation of BPA biodegradable strains and statistical optimization experiments.

2.2. Isolation, screening and culture conditions for high tolerant BPA degrading strain

Soil and water samples were collected from five different provinces in China beside plastic factories and these provinces were Heilongjiang, Hebei, Shandong, Guangdong and Anhui. Five grams or 5 ml of soil or water samples respectively, were separately added to 100 ml of TEM in 250ml Erlenmeyer flask and supplied with 100 mg l⁻¹ of BPA. The flasks were incubated in rotary shaker at 30 °C with 180 rpm. After 7 days, 5 ml of the culture (each flask) was transferred to fresh TEM with 200 mg l⁻¹ and incubated at the same condition mentioned above for 7 days. This process was repeated three more times with increasing BPA concentrations in each run to reach 500 mg l⁻¹ (Ren et al. 2016). The final cultures were spread on solid TEM containing 100 mg l⁻¹ BPA as sole source of carbon and incubated for 5 days at 30 °C, single colonies were picked and inoculated into TEM supplemented with 100 mg l⁻¹ BPA and incubated (30 °C and 180 rpm) to test the biodegradation. Uninoculated fresh culture of TEM with 100 mg l⁻¹ BPA was set as abiotic control. The residual concentrations of BPA were determined by using HPLC.

2.3. Identification of BPA degrading strain

The total genomic DNA of bacterial isolate was extracted by using Bacterial Genomic Extraction Kit (Takara, Japan). The 16S rRNA gene was amplified from genomic DNA by using two universal primers (27F: AGAGTTTGATCCTGGCTCAG, 1492R: AAGGAGGTGATCCAGCC). The PCR product was sequenced (Sangon Biotech Co., Ltd, Beijing, China) and obtained sequence was deposited to GenBank, and aligned to get the similar sequences by BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The phylogenetic tree was constructed using MEGA 5 software with a neighbor-joining algorithm. To confirm 16S rRNA identification and determining the biochemical tests of the bacterial isolate, Biolog tests (BIOLOG inc., USA), was accomplished and the results were analyzed by Microlog 3 (version 5.2) software.

2.4. Statistical optimization of BPA degradation by Response Surface Methodology (RSM)

RSM was conducted using factorial central composite design (CCD). CCD is one of the most common design used in RSM which has the same predictability in all directions from the center (Gao and Jiang 2006). Five experimental factors selected to design the experiment included pH (A), NaCl concentration (B), Incubation period (C), Inoculum size (D) and Incubation temperature (E) with 5 levels for each factor, generating 29 trials using Design-Expert software (version 8). The Reduced Cubic Model was fitted for biodegradation response analysis. The value of the dependent response was the mean of three replications. The detailed experimental designs of five parameters are shown in Table (1). In this experiment, TEM with BPA (200 mg l⁻¹) as sole source of carbon was used and inoculum was prepared by growing the bacterial strain on L.B medium to reach (O.D.₍₆₀₀₎ = 0.8), the bacterial cells were collected by centrifugation at 5000 rpm for 5 minutes and washed twice by fresh TEM and resuspended using the same original volume with TEM.

2.5. Substrate Utilization Test

To determine the ability of selected strain to degrade different organic pollutants other than BPA, the bacterial strain was inoculated (1% inoculum size) into TEM (15 ml) supplemented with 100 mg l⁻¹ of each of the following substrates: bisphenol B (BPB), bisphenol F (BPF), bisphenol S (BPS), Dibutyl phthalate (DBP), Diethylhexyl phthalate (DEHP) and Diethyl phthalate (DEP). The cultures were incubated in rotary shaker (30 °C and 180 rpm) for 72 hours. Cultures without inoculation were set as abiotic control. The residual concentrations of bisphenols (B, F, and S) and (DBP, DEHP) were measured by HPLC and gas chromatography respectively.

2.6. Detection of Intermediates

To determine the biodegradation intermediates, the selected bacterial strain was grown in TEM supplemented with BPA (200 mg l⁻¹) under optimal culture conditions. 100 ml of sample was taken from the culture at specific intervals as following (15, 20, 24, 28,32, 36 hours) and centrifuged at 10000 rpm for 10 minutes, the cell free supernatant was acidified (pH: 2–3) using HCl. The samples were extracted twice with same volume of ethyl acetate, organic layer separated and dried by Rotary evaporator (BUCHI, Switzerland) and re-suspended in 1 ml methanol (99%). The samples were subjected to high performance liquid chromatography-mass spectroscopy (HPLC-MS) to detect the intermediates.

2.7. Efficient BPA degradation at minimum and maximum concentrations

The concentration of BPA in natural polluted environments is very low compared with that always examined in vitro. Therefore, the ability of bacterial strain to degrade and utilize BPA at low and high concentrations is very important character especially in strains intended to use in environmental bioremediation. The high and low concentrations used were as follows (50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 mg l⁻¹) and (0.5, 1, 2, 4, 6, 8, 10 and 12 mg l⁻¹) respectively. The residual BPA concentration was determined after 3 days by HPLC and degradation rate was calculated.

2.8. Analysis methods

Cell growth was measured as optical density (OD) at 600nm using UV-Visible spectrophotometer (Thermo Scientific, USA). For detection of bisphenols, an Agilent 1200 system HPLC (Agilent, USA) with visible ultraviolet detector was used equipped with Eclipse XDB C18 column (4.6×150 mm×5 μm). The mobile phase was acetonitrile 85% and (water, 0.1% acetic acid) 15%, with flow rate 1 ml/minute and detecting wavelength 220 nm. The retention time was 1.55 minute and 2 μl for sample injection. For detection of DBP, DEHP and DEP, a GC system (GC–2010 SHIMADZU, Kyoto, Japan) with flame ionizing detector (FID) was used. Samples were extracted twice by equal volume of n-hexane and the hexan phase was separated and analyzed by GC (Zhang et al. 2016). The intermediate metabolites of BPA degradation were analyzed by (HPLC–MS, 1290, USA). The mobile phase was a mixture of (50:50%) of water and acetonitrile (with 0.1% formic acid for each one), running at a flow rate of 1 mL/min. The sample was injected (2 μL) as methanolic extract and scan mode with 3kV capillary voltage was used to analyze negative ionization. The dry gas flow was 5 L/min and the dry nitrogen was heated to 325 °C. Mass hunter (version A.02.00, Agilent, USA) was used to analyze and collect the data.

3. Results And Discussion

3.1. Isolation and identification of BPA degrading strain

YC-AE1 strain isolated from soil sample of Guangdong province showed highly degradation and tolerance to BPA. It is a rod-shaped, flagellated, Gram-negative bacterium. The amplified 16S rRNA gene sequence (1439 bp) was deposited in Gen Bank (accession number, MK318658) and related strains were obtained by BLAST. The similarity between YC-AE1 and others was detected by constructing a phylogenetic tree (Fig.1) and the most closely related one was *Pseudomonas putida* strain W30 with 99 % similarity. The BIOLOG tests was performed and showed consistent results with 16S rRNA (PROB = 97.2 %, SIM = 0.849, and DIST = 1.818 with *Pseudomonas putida*).. According to morphology, BIOLOG tests and 16S rRNA, our strain YC-AE1 identified as *Pseudomonas putida*.

3.2. Statistical optimization of BPA degradation using RSM

RSM is used to determine the combined effect of several variables and their interactions. A statistical optimization approach using CCD was used to study the linear, quadratic and interactive effects of various parameters on BPA degradation by *Pseudomonas putida* strain YC-AE1. The combined five factors with their levels and obtained experimental results are presented in Table (1), highest BPA degradation (95 %) was observed in result obtained by the design (run no. 21). The design was analyzed by regression analysis resulted in significant model with the following equation:

$$\text{BPA degradation} = 77.93957 + 14.45A + 12.65B + 22C + 11.425D + 4.45E + 1.38625AB + 1.48625AE + 2.71125BD + 17.65125BE + 11.9997A^2 + 3.59973B^2 + 8.27473C^2 + 9.68723D^2 + 17.5497E^2 + 7.42375A^2B + 21.6013A^2C + 8.23875A^2D + 18.7013A^2E + 13.8863AB^2$$

The F-value and probability value (p-value) are tools for evaluating the significance of each of the parameters in the model equation. The pattern of interactions between the variables is indicated by these coefficients. The larger F-value and the smaller P-value are an indication of the high significance of the corresponding coefficient (Khambhaty et al. 2007). Regression analysis for the biodegradation shown in Table (2), indicate that the model was highly significant with very low P-value (0.0001) and high F-value (229.53). As shown in Table 2, all linear and quadratic variables were highly significant with p value close to (0.00) indicating the model success. Two of variable interactions; pH and NaCl concentration (AB) and pH and incubation temperature (AE) were slightly non-significant with P-values 0.0944 and 0.0760 respectively, indicating the interactions were not favorable to the response while, the other two variables, NaCl concentration and inoculum size (BD) and NaCl concentration and incubation temperature (BE) were highly significant. The model fitness was expressed by coefficient of determination, R^2 and $Adj-R^2$ which were 0.9979 and 0.9935, respectively. The high value of R^2 (so close to 1) supports the accuracy of the model and demonstrates good correlation between actual and predicted values of BPA degradation. The significance of the model and high R^2 , support the model to predict responses.

Table 1: Experimental design (conditions and responses) for BPA degradation by *Pseudomonas putida* YC-AE1

Run	A	B	C	D	E	BPA degradation (%) ^a
	Initial pH (values)	NaCl Conc. (%)	Incubation period (hours)	Inoculum size (%)	Incubation temp. (°C)	
1	7.25 (0)	0.75 (0)	10 (-2)	2.5 (0)	31 (0)	2
2	7.25 (0)	1.5 (+2)	20 (0)	2.5 (0)	31 (0)	39.4
3	7.25 (0)	0.75 (0)	20 (0)	2.5 (0)	31 (0)	77
4	7.25 (0)	0.75 (0)	20 (0)	2.5 (0)	19 (-2)	17.8
5	6.13 (-1)	1.13 (+1)	15 (-1)	3.25 (+1)	37 (+1)	2.7
6	8.38 (+1)	0.38 (-1)	25 (+1)	1.75 (-1)	37 (+1)	1.7
7	5.00 (-2)	0.75 (0)	20 (0)	2.5 (0)	31 (0)	2.2
8	6.13 (-1)	1.13 (+1)	15 (-1)	1.75 (-1)	25 (-1)	7.8
9	8.38 (+1)	1.13 (+1)	15 (-1)	3.25 (+1)	25 (-1)	17.5
10	6.13 (-1)	0.38 (-1)	25 (+1)	1.75 (-1)	25 (-1)	81.7
11	6.13(-1)	1.13 (+1)	25 (+1)	1.75 (-1)	37 (+1)	0
12	6.13 (-1)	0.38 (-1)	15 (-1)	1.75 (-1)	37 (+1)	0
13	8.38(+1)	1.13 (+1)	25 (+1)	1.75 (-1)	25 (-1)	15
14	6.13 (-1)	1.13 (+1)	25 (+1)	3.25 (+1)	25 (-1)	6.4
15	7.25 (0)	0.75 (0)	20 (0)	2.5 (0)	31 (0)	77.2
16	7.25 (0)	0.75 (0)	30 (+2)	2.5 (0)	31 (0)	90
17	9.5 (+2)	0.75 (0)	20 (0)	2.5 (0)	31 (0)	60
18	8.38(+1)	0.38 (-1)	15 (-1)	3.25 (+1)	37 (+1)	7
19	7.25 (0)	0.75 (0)	20 (0)	4 (+2)	31 (0)	63.2
20	7.25 (0)	0 (-2)	20 (0)	2.5 (0)	31 (0)	90
21	8.38 (+1)	0.38 (-1)	25 (+1)	3.25 (+1)	25 (-1)	95
22	7.25 (0)	0.75 (0)	20 (0)	2.5 (0)	31 (0)	77.3
23	7.25 (0)	0.75 (0)	20 (0)	2.5 (0)	43 (+2)	0
24	7.25 (0)	0.75 (0)	20 (0)	1 (-2)	31 (0)	17.5
25	8.38 (+1)	1.13 (+1)	15 (-1)	1.75 (-1)	37 (+1)	0
26	8.38 (+1)	1.13 (+1)	25 (+1)	3.25 (+1)	37 (+1)	0
27	8.38 (+1)	0.38 (-1)	15 (-1)	1.75 (-1)	25 (-1)	78.3
28	6.13 (-1)	0.38 (-1)	25 (+1)	3.25 (+1)	37 (+1)	13.38
29	6.13 (-1)	0.38 (-1)	15 (-1)	3.25 (+1)	25 (-1)	93.5

For microbial effective process, it is necessary to monitor and control parameters that affect the process like, inoculum volume, salt concentration, pH, temperature, incubation period, etc. To determine the optimum values of BPA degradation conditions for obtaining highest and fastest degradation rate, three-dimensional (3-D) response surface curves were plotted as shown in Fig. 2. These results showed that an optimum was observed near the central value of pH, temperature and inoculum size. *Pseudomonas putida* YC-AE1 could grow and degrade BPA over a wide range of pH values. Maximum BPA degradation was observed at pH of 7.2 as shown in Fig. 2 (a-d) and our result is different from that obtained by (Ren et al. 2016; Zhang et al. 2007) which reported that maximum BPA degradation was shown at pH 7 by

Achromobacter xylosoxidans strain B-16 and *Arthrobacter* sp. YC-RL1. The biodegradation of BPA was highly affected by the incubation period, it can be easily observed this effect from high positive coefficient value (+22) of the variable as shown in Table (2). From Fig. 2 (a), prolonged incubation period was shown to increase the degradation of BPA by our strain YC-AE1. The inoculums used to be used in degradation medium must be in a healthy and active state moreover being of optimum size. Such conditions possibly minimize the length of log phase. Data illustrated in Fig. (2 c,e) showed that, the optimum inoculum size was 2.5 %. Lower inoculum volumes decrease the degradation efficiency of YC-AE and, this is may be due to low number of bacterial cells that decrease the amount of enzymes for biodegradation. Also, inoculum size higher than 2.5 % had negative effect on BPA degradation by YC-AE1, and this may be because the reduction of dissolved oxygen and increased competition towards nutrients (Ray and Mohapatra 2012). Zhang et al (2007) reported that, increasing the size of inoculum lead to increase the degradation of BPA by *Achromobacter xylosoxidans* strain B-16 isolated from compost leachate of municipal solid waste (Zhang et al. 2007).

Temperature is one of the most important parameters that affect any microbial process. The growth rate of microorganisms becomes slow below or above the optimum growth temperature because of a reduced rate of cellular production (Karthikeyan et al. 1996). There was a gradual increase in BPA degradation by YC-AE1 by increasing the temperature from 15–25 °C and the maximum degradation was observed between 25–30 °C. The degradation then decreased beyond increasing temperature degrees (Fig. 2-d). Fouda (2015) reported a relatively high optimum temperature (35–40 °C) for BPA biodegradation by *Klebsiella pneumoniae* J2 and *Enterobacter asburiae* L4 [35]. As shown in Table (2) the addition of NaCl to the degradation medium (TEM) has a reverse effect on BPA degradation, as described by a negative coefficient value (–12). Data presented in Fig. (2e, f) showed that, the biodegradation of BPA was greatly reduced by addition of NaCl to the medium component. Further incubation of strain YC-AE1 for another two days resulted in degradation of 70 % of BPA (Data not shown) and this result may be due to the effect of NaCl on the growth curve of our strain by elongating the lag phase and delaying the degradation process.

Table 2: Regression analysis for the biodegradation of BPA by *Pseudomonas putida* YC-AE1

Source	Coefficient	Sum of Squares	Degrees of freedom	Mean Square	F Value	P value Prob > F
Model		38370.26	19	2019.48	229.53	< 0.0001
pH, A	14.45	1670.42	1	1670.42	189.86	< 0.0001
NaCl Conc., B	-12.65	1280.18	1	1280.18	145.50	< 0.0001
Incubation period, C	22	3872	1	3872	440.09	< 0.0001
Inoculum size, D	11.425	1044.24	1	1044.24	118.68	< 0.0001
Incubation temp., E	-4.45	158.42	1	158.42	18.00	0.0022
AB	1.38625	30.74	1	30.74	3.49	0.0944
AE	-1.48625	35.34	1	35.34	4.01	0.0760
BD	-2.71125	117.61	1	117.61	13.36	0.0053
BE	17.65125	4985.06	1	4985.06	566.60	< 0.0001
A ²	-11.9997	3532.64	1	3532.64	401.52	< 0.0001
B ²	-3.59973	317.90	1	317.90	36.13	0.0002
C ²	-8.27473	1679.82	1	1679.82	190.92	< 0.0001
D ²	-9.68723	2302.26	1	2302.26	261.67	< 0.0001
E ²	-17.5497	7556.09	1	7556.09	858.82	< 0.0001
A ² B	-7.42375	293.93	1	293.93	33.40	0.0003
A ² C	-21.6013	2488.60	1	2488.60	282.85	< 0.0001
A ² D	-8.23875	362.01	1	362.01	41.14	0.0001
A ² E	-18.7013	1865.26	1	1865.26	212.00	< 0.0001
AB ²	-13.8863	1028.41	1	1028.41	116.89	< 0.0001
Residual		79.18	9	8.79		
Lack of Fit		79.13	7	11.30	484.50	0.0021
Pure Error		0.04	2	0.02		
Total		38449.44	28	R-squared =0.9979	Adj-R squared = 0.9935	

3.3. Substrate Utilization Test

The capability of *Pseudomonas putida* strain YC-AE1 to degrade six different organic pollutants (BPB, BPF, BPS, DBP, DEP and DEHP) was examined. Data presented in Fig. (3) showed that, the YC-AE1 has an ability to degrade all examined pollutants in varying proportions. *Pseudomonas putida* YC-AE1 showed high ability to degrade about 60 and 67 % of bisphenol B and F (100 mg l⁻¹) respectively, while lower degradation was observed in both bisphenol S, P and DEHP with about 30, 20 and 18% respectively. The lowest degradation ability was shown with DEP with degradation rate not more than 3%. Many Pollutants were reported to be degraded by *Pseudomonas sp* by researchers. For example, phenols (Basha et al. 2010), phenolics like pentachlorophenol [37] and Catechol [38].

3.4. Maximum and Minimum BPA Concentration for Efficient Biodegradation

Under normal environmental conditions, the concentration of environmental pollutants is always very low compared with that examined in vitro (Peng et al. 2014; Yamada et al. 2008) and these pose many problems for bioremediation process because, the concentration of pollutants is too low to sustain

microbial growth and ensure the accessibility to microbe and even more than that to induce metabolic genes (Ren et al. 2016). Therefore, determining the survival ability of *Pseudomonas putida* YC-AE1 against both high and low BPA concentrations is important. Strain YC-AE1 could survive in both conditions and remain active in all BPA concentrations. The degradation rates are demonstrated in Fig. 4 after 72 hours cultivation. The strain was able to degrade 100% of BPA (0.5–500 mg l⁻¹). However, when the BPA concentration increased (600, 700, 800, 900 and 1000 mg l⁻¹) the degradation rate decreased to (95, 90, 70, 60 and 7%), respectively. Further incubation of strain YC-AE1 for another two days resulted in complete degradation of BPA (600–1000 mg l⁻¹). This performance and ability of strain YC-AE1 to degrade and tolerate these extremely low and high concentrations is important for application in BPA bioremediation especially with fast degradation rate (200 mg l⁻¹ in 20 hours). This quality makes strain YC-AE1 a promising bacterium compared with other reported strains. Suyamud et al., (2018) reported *Bacillus megaterium* strain ISO–2, which can degrade 5 mg l⁻¹ of BPA within 72 hours on mineral salt medium supplemented with yeast extract as co-substrate (Suyamud et al. 2018). *Sphingomonas bisphenolicum* strain AO1 was reported to degrade 100 mg l⁻¹ BPA to undetectable level within 48 hours in minimum medium with 1 % glucose (Oshiman et al. 2007).

3.5. Metabolic intermediates and metabolic pathway

Detection of metabolic intermediates during BPA degradation with optimum conditions (200 mg l⁻¹ BPA, pH 7.2, 30 °C and 2.5% inoculum size) was performed. Based on the mass spectra (LC-MS) as shown in Fig. 5, eight compounds relating to BPA degradation were identified as following, BPA (m/z 227.1), 4,4-Dihydroxy-alpha-methylstilbene (m/z 225), *p*-hydroxybenzaldehyde (*p*-HBAL) (m/z 122), *p*-hydroxyacetophenone (*p*-HAP) (m/z 136), 4-hydroxyphenylacetate (HPA) and 4-Hydroxyphenylalcohol (same m/z, 152), 2,2-bis(4-hydroxyphenyl)–1-propanol and 1,2-bis (4-hydroxyphenyl)–2-propanol (same m/z, 244) and 2,2-bis (4-hydroxyphenyl) propanoate (m/z 258). These results have a good consistence with those reported (Eio et al. 2014).

Das et al. (2018) reported many different intermediate compounds detected during BPA degradation by recombinant laccase from *Bacillus* sp. GZB. Three different pathways were proposed based on 3 intermediates, Hydroxybenzaldehyde, *p*-hydroxybenzoic acid and *p*-hydroquinone (HQ) detected during biodegradation of BPA by *Achromobacter xylosoxidans* strain B–16 isolated from compost leachate (Zhang et al. 2007). Based on the intermediates obtained in our study using LC-MS and in light of literature (Das et al. 2018; Eio et al. 2014; Suyamud et al. 2018; Wang et al. 2017; Zhang et al. 2007), the proposed pathway for BPA biodegradation by strain YC-AE1 is represented in Fig 6. There were two different proposed pathways for BPA degradation by YC-AE1, both of them start by hydroxylation for BPA to form 1,2-Bis(4-hydroxyphenyl)–2-propanol and 2,2-Bis(4-hydroxyphenyl)–1-propanol in pathway (I) and (II), respectively. In pathway (I), 1,2-Bis(4-hydroxyphenyl)–2-propanol dehydrated to 4,4-Dihydroxy-alpha-methylstilbene (Eio et al. 2014). The previously mentioned compound was further oxidized to form *p*-HBAL and *p*-HAP. *p*-HAP was metabolized to HPA and then to HQ. Both of hydroxybenzoic acid (HBA) and HQ were assumed mineralized to carbon dioxide (CO₂) and bacterial biomass through benzoate

degradation pathway. HBA and HQ were not detected and presumed in the pathway. In pathway (II), 2,2-Bis(4-hydroxyphenyl)-1-propanol is metabolized to form 2,2-bis (4-hydroxyphenyl) propanoate and 2,3-Bis(4-hydroxyphenyl)-1,2-propanediol. The intermediate (2, 3-Bis (4-hydroxyphenyl)-1,2-propanediol) was further metabolized through many steps (as shown in Fig. 6) to form finally HBA which mineralized to CO₂ and bacterial biomass through benzoate degradation pathway.

4. Conclusion

This study has reported the ability of promising *pseudomonas putida* strain YC-AE1 isolated from Guangdong province, (China) to degrade BPA as sole source for carbon. This strain can degrade both of extremely high and low concentrations of BPA in very short time. Furthermore, our strain YC-AE1 can degrade many BPA-related pollutants. Analysis of intermediates showed two degradation pathways for BPA. These criteria recommend *pseudomonas putida* YC-AE1 as promising strain for natural remediation of environmental pollutant BPA.

Abbreviations

BPA: Bisphenol A TEM: Trace elements medium BPB: Bisphenol B

BPF: Bisphenol F BPS: Bisphenol S DBP: Dibutyl phthalate

DEHP: Diethylhexyl phthalate DEP: Diethyl phthalate

CCD: Central composite design *p*-HBAL: *p*-hydroxybenzaldehyde

p-HAP: *p*-hydroxyacetophenone HPA: Hydroxyphenylacetate

HQ: Hydroquinone HPLC: High performance liquid chromatography

RSM: Response Surface Methodology

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The nucleotide sequences of 16S rRNA gene were deposited to Gen Bank (NCBI) with accession number, MK318658.

Competing interests

Authors declare that they have no competing interests. Acknowledgements

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Authors' contributions

AE conducted the practical work for all experiments; YJ designed the statistical optimization experiments; JW analyzed the HPLC-MS results. YJ and JW contributed reagents/materials/analysis tools; AE and MAAK wrote the manuscript. NR, IK and YY revised the manuscript.

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Figures

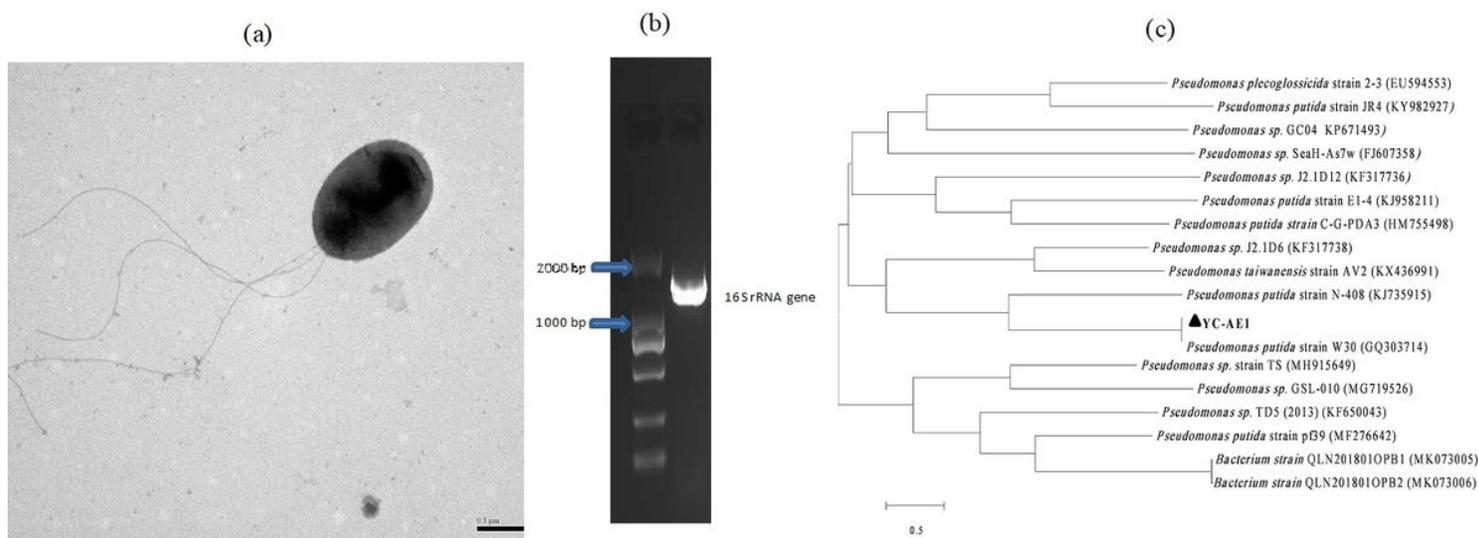


Figure 1

Fig. 1: Identification of YC-AE1, (a) TEM investigation showing cell shape and lophotrichous flagellation (b) amplified 16S rRNA gene (about 1500 bp) comparing with 2000 bp DNA ladder, (c) phylogenetic tree of *Pseudomonas putida* YC-AE1 with closely related strains.

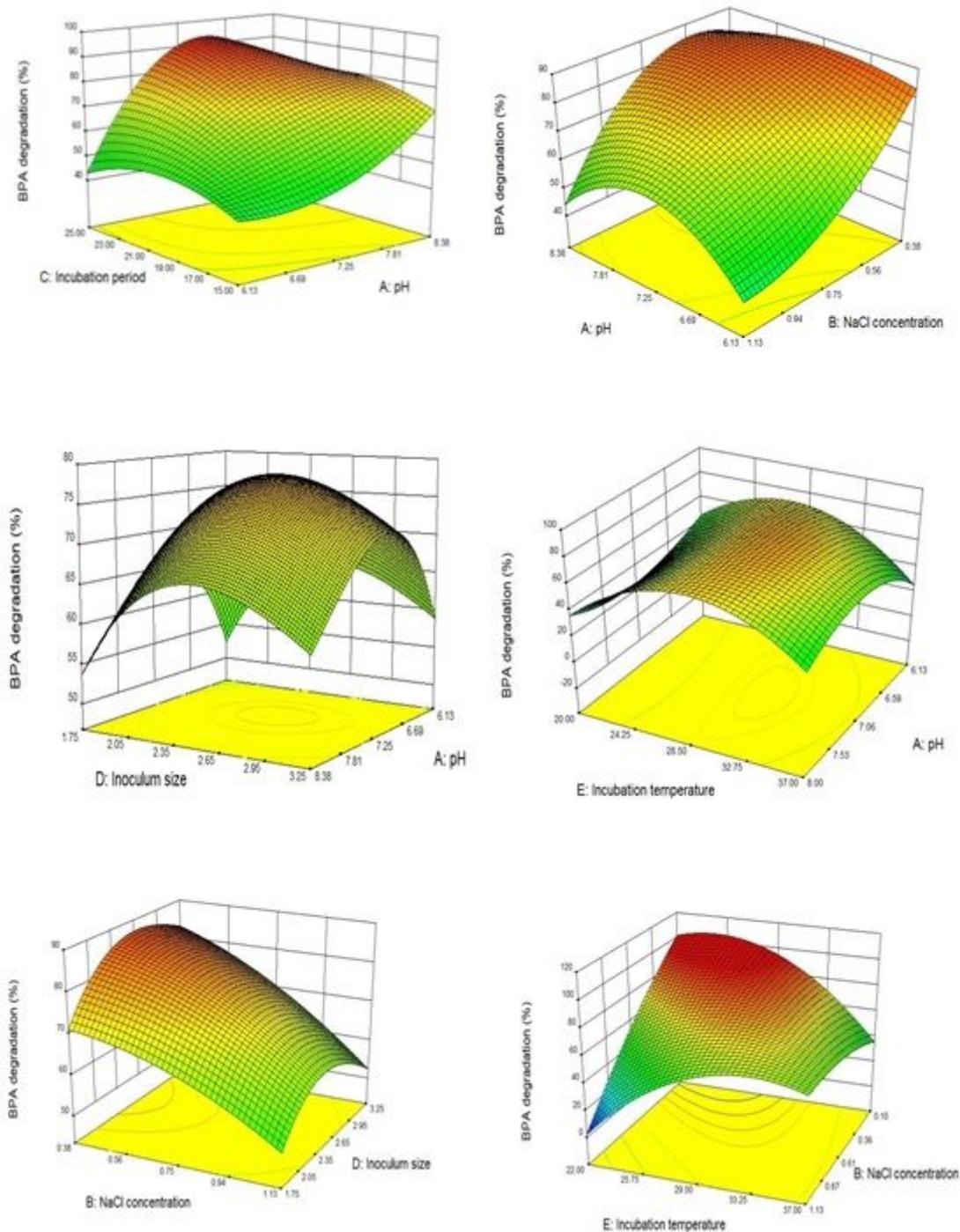


Figure 2

Fig. 2: Response surface plots showing the effect of incubation period and pH (a); pH and NaCl concentration (b); inoculum size and pH (c); incubation temperature and pH (d); NaCl concentration and inoculum size (e); incubation temperature and NaCl (f).

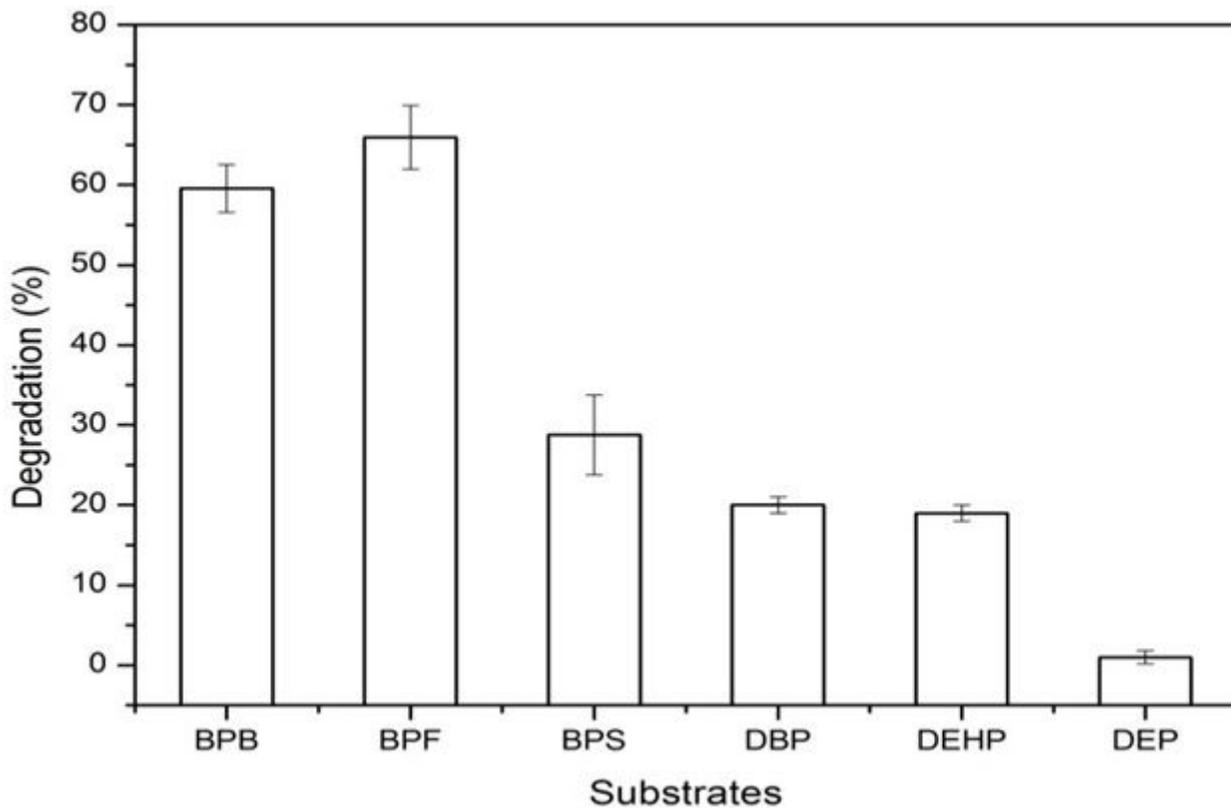


Figure 3

Fig. 3: Degradation efficiency for different pollutants (100 mg l⁻¹) by *Pseudomonas putida* YC-AE1. Error bars indicate the standard deviation obtained in three independent experiments.

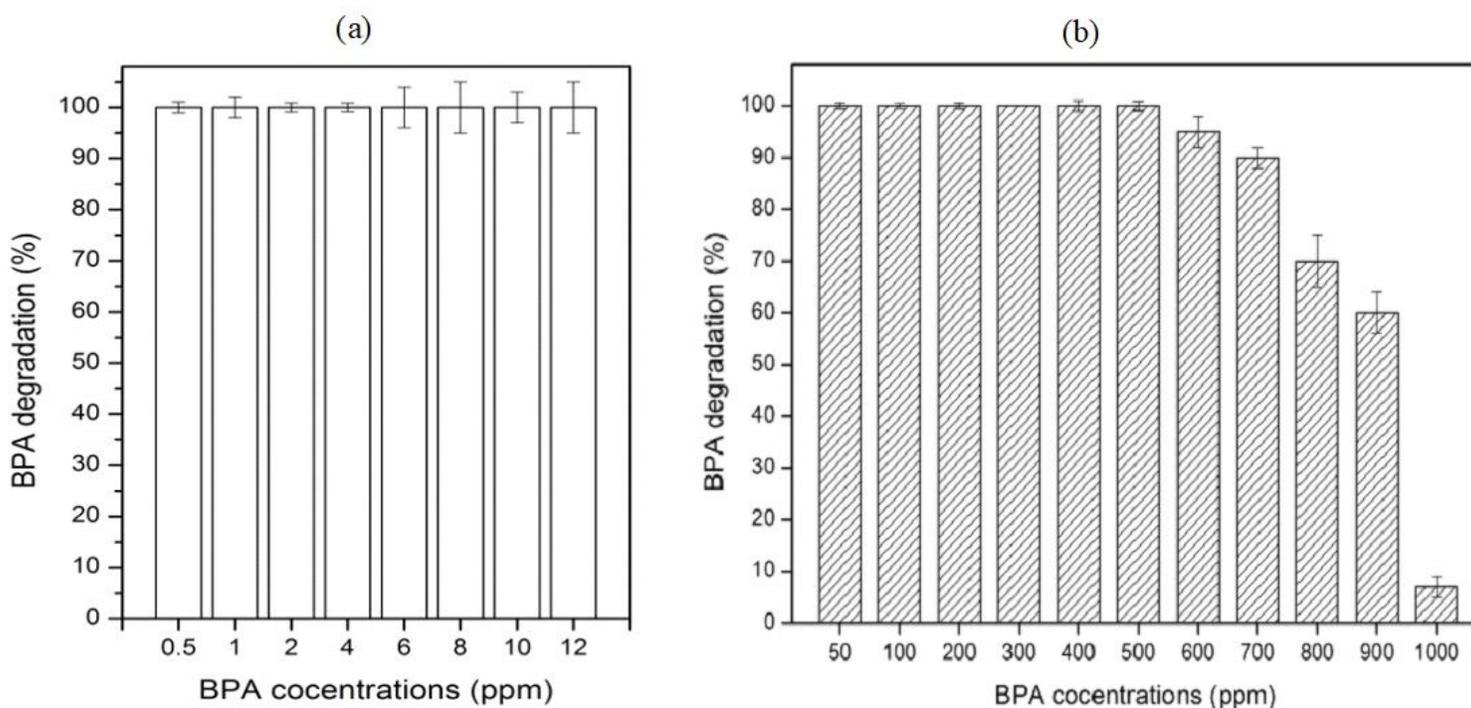


Figure 4

Fig. 4: (a) Minimum and (b) maximum degrading ability of BPA by *Pseudoonas putida* YC-AE1. Error bars indicate the standard deviation obtained in three independent experiments.

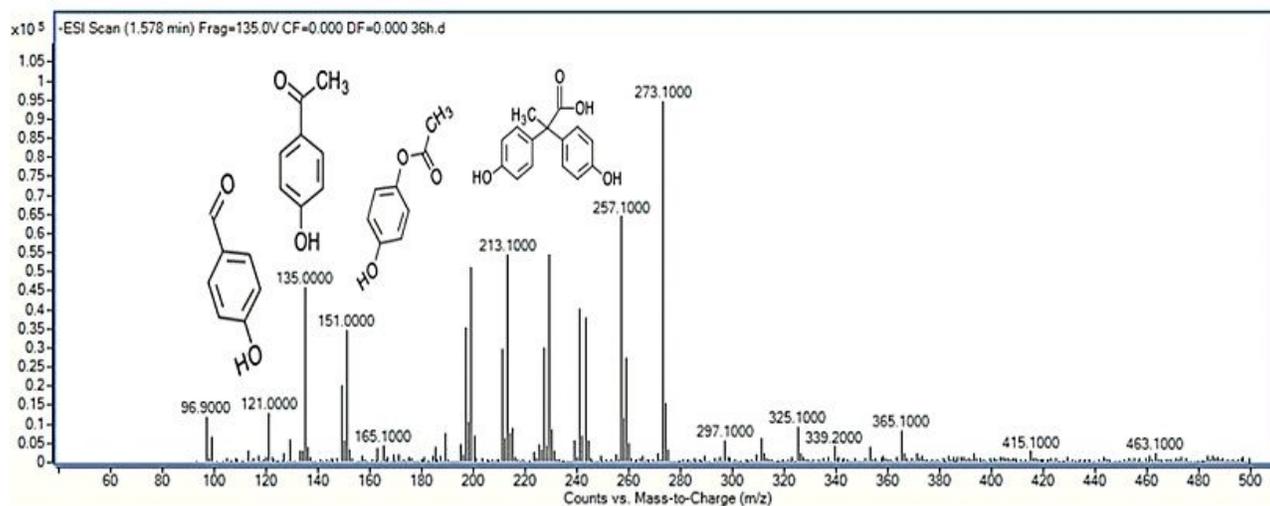
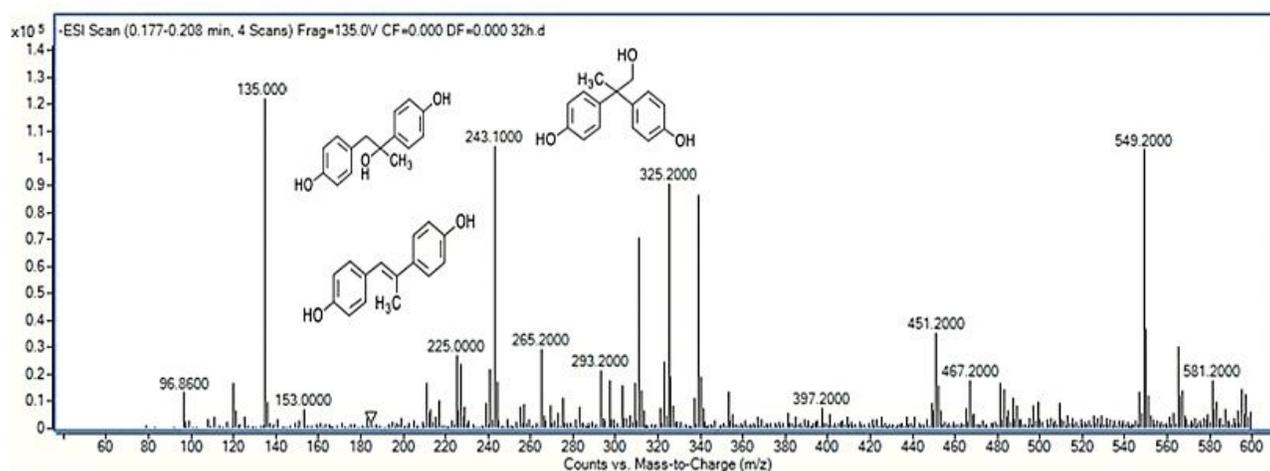
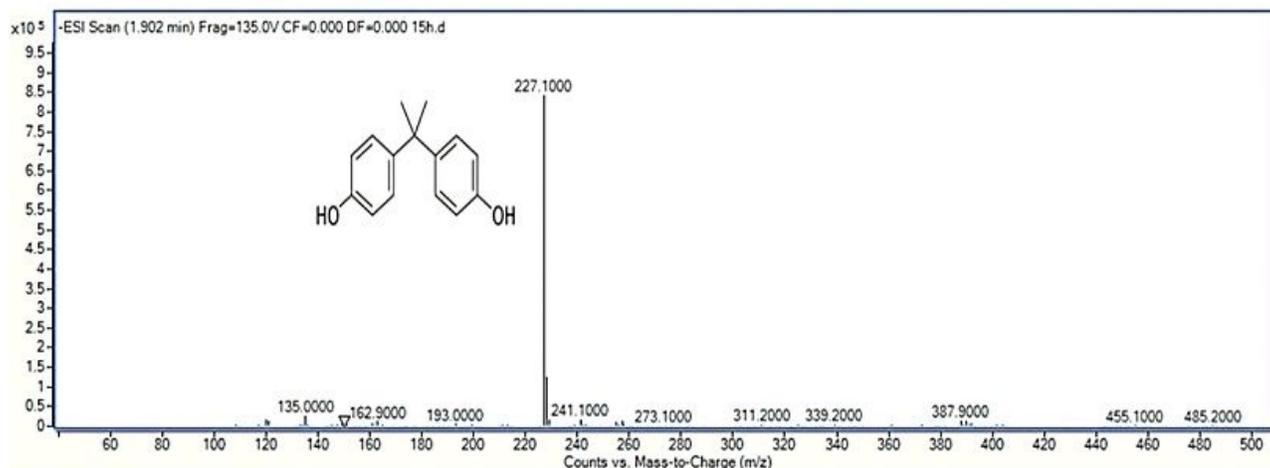


Figure 5

Fig. 5: Mass spectra from HPLC-MS during BPA degradation by *Pseudomonas putida* YC-AE1 and detected intermediate compounds, BPA (a), 4,4-Dihydroxy-alpha-methylstilbene, 1,2-bis (4-

hydroxyphenyl)-2-propanol and 2,2-bis(4-hydroxyphenyl)-1-propanol (b), 2,2-bis (4-hydroxyphenyl) propanoate, p-HBAL, p-HAP and HPA (C).

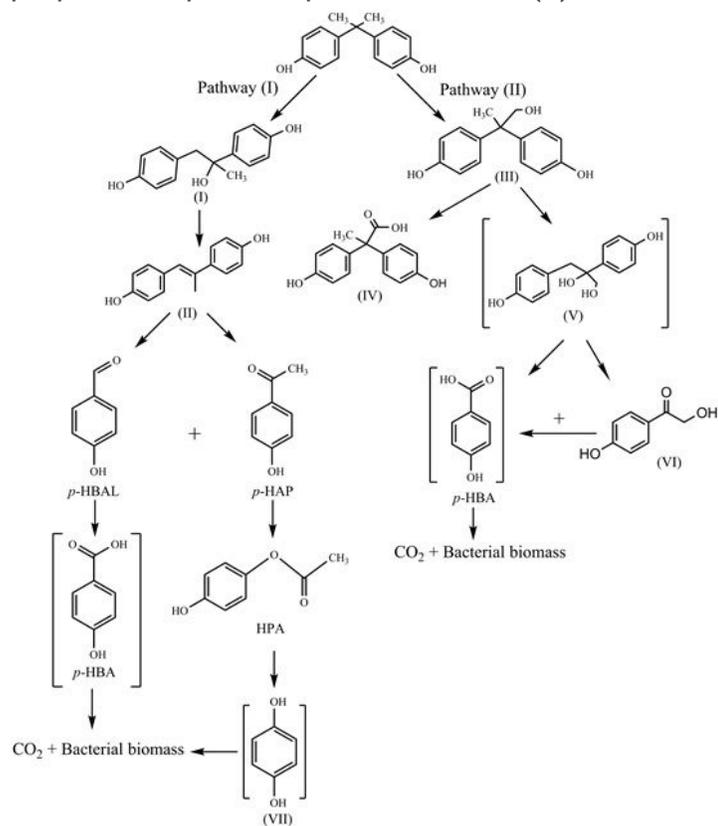


Figure 6

Fig. 6: Proposed pathway for BPA degradation by *Pseudomonas putida* YC-AE1, 1,2-bis(4-hydroxyphenyl)-2-propanol (I), 4,4-dihydroxy- α -methylstilbene (II), 2,2-bis(4-hydroxyphenyl)-1-propanol (III), 2,2-bis (4-hydroxyphenyl) propanoate (IV), 2,3-bis(4-hydroxyphenyl)-1,2-propanediol (V), 4-hydroxyphenacyl alcohol (VI), hydroquinone (HQ) (VII). The compounds in parentheses were not detected in our study but assumed to be produced.