Fast Location and Isolation of Phenanthrene-degrading Halophilic Microbial Community and its Degradation Pathway

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Abstract

Microbial consortium WZ-4, which could degrade phenanthrene (PHE) as the main carbon and energy source, was isolated from the aerobic sludge of Weizhou wastewater treatment plant. Under the condition of high salinity (3%), the degradation of PHE (100 mg/L) was 87.76% in 7d. Its metabolites, genome sequence and biodegradation pathway were studied. The main metabolites include 1,2-dihydroxynaphthalene, 1-hydroxy-2-naphthalene, 5,6-benzocoumarin and phthalic acid. 12 PHE degrading enzyme genes appeared in the metagenome sequencing of WZ-4, and the genes involved in PHE degradation were included phdE, phdF, phdG, and pcaL. Based on the metabolites detected by GC-MS and the potential PHE-degrading genes identified by BLAST search, biodegradation pathway of PHE by WZ-4 was predicted.

1. Introduction

Among all kinds of petroleum compounds, polycyclic aromatic hydrocarbons (PAHs) composed from two or more fused aromatic rings had chemical stability and extremely difficult biodegradation[1]. As an common toxic carcinogenic pollutant to human and animals[2, 3], PAHs threatened human food safety through the accumulation in the food chain[4]. There were large number of PAHs utilizing microbes in high salinity oil polluted sea area and offshore oil field environment[5, 6]. However, few pure culture of extremely halophilic hydrocarbons degration microbes was isolated[7, 8].

Although a halophilic archaeal Phenanthrene (PHE) degrading strain MSNC 14 has been reported, but so far, a single PAH degrading strain adapted to high salinities had never been reported[9]. The informations about the ability of halophilic archaea degrade hydrocarbons in hypersaline environments were limited[9]. At present, few studies on degradation pathways of PHE by halophilic microbes were reported. And the degradation mechanism is still unclear. Therefore, how to improve the treatment effect of halophilic microbes on PAHs in high salinity environments was the bottleneck.

In this study, the effectiveness of halophilic bacterial consortium (WZ-4) to degrade PAHs was studied under a salinities up to 3% NaCl (w/v). Based on the metabolites detected by GC-MS and the potential PHE degrading genes identified by BLAST search, a PHE biodegradation pathway by WZ-4 was predicted. This study attempts to illuminate the mechanism of action of halophilic microbial consortium in the degradation of PHE.

2. Materials And Methods

2.1 Sample and material

Aerobic sludge was collected from activated sludge of an aerobic baffled reactor and sequence batch reactor (ABR-SBR) pond and stored in fridge at -20℃[10]. The oil field wastewater was collected from the water inlet of the plant and stored in fridge at 4℃. All the sample was provided by the Weizhou

wastewater treatment plant which belongs to Zhanjiang Branch of China National Offshore Oil Corp. (CNOOC), which is located in Beihai, Guangxi, China (109.15 E, 21.03 N).

Phenanthrene (purity, ≥ 98%), Chromatographic grade n-hexane, acetone and acetonitrile were purchased from Energy Chemical which belongs to Saen Chemical Technology (Shanghai) Co., Ltd, China. Other reagents were purchased from Sinopharm chemical reagent Beijing Co., Ltd., which are of analytical grade.

2.2 Microbes domestication and isolation

The original culturable microbial consortium was enriched from the activated sludge. The method was slightly modified according to Zhou and Huang[11, 12]. The high salinity beef extract peptone medium (composition in g/L: Beef extract, 3.0; Tryptone, 10.0; NaCl, 30.0; pH 7.0,salinity 3%) was prepared to enrich the microbial community. The activated sludge sample (5 mL) was transferred to a erlenmeyer flask (250 mL) containing sterilized medium (100 mL) and incubated at 35℃ and 150 rpm for 7 days, then centrifuged (4000 g, 10 min) and washed with sterilized normal saline for 3 times to obtain the microbial community. And then transferred to the prepared inorganic salt medium (composition in g/L: KH_2PO_4, 0.5; K_2HPO_4, 0.5; $(NH_4)_2SO_4$, 1.0; Na_2SO_4, 1.0; $CaCl_2$, 0.1; $MgSO_4$, 1.0; NaCl, 30.0; Yeast extract, 1.0; Sodium lactate, 1mL, pH 7.0). In each initial sterilized medium, 20 mg/L was added as the sole carbon source and energy. The inorganic salt medium was cultured at 35℃ and 150 rpm. After 7 days, the bacterial suspension (1 mL) was sampled and transferred to a new medium to continue domestication. The concentration of PHE in the next new medium gradually increased to 40, 60, 80 and 100 mg/mL.

The PHE were evenly distributed in n-hexane solvent, PHE (2 ml) was sprayed on the surface of the inorganic salt agar plate medium. After the solvent volatilized, the diluted of bacterial suspension (0.1 mL) was added and cultured at 35℃ for 2–3 days. The single strain that can grow with great difference in color and morphology were selected and keeped in tube cultures.

NEB Next® Ultra™ DNA Library Prep Kit for Illumina® was used to extract DNA from each sample and construct gene library. polymerase chain reaction (PCR) was performed to amplify 16S rRNA gene. Universal primer 27F (5'AGAGTTTGATCTGGCTCAG-3') and 1492R (5'CTACGGCTACCTTGTTACGA-3') was used to amplify the DNA fragment coding for 16S rRNA gene in PCR. The purified PCR products of each strain were used for DNA sequencing by PCR sequencer (ABI-2720, Applied Biosystems, USA). The sequence files were compared with the data in NCBI 16S database by NCBI blast program. 4 strains were isolated, which were all belongs to *Halomonas sp.*.

2.3 Degradation test of PHE

Using PHE as the sole carbon source and energy, 4 single strains and microbial consortium WZ-4 were enriched and cultured in inorganic salt medium at 35℃ and 150 rpm for 7d. And then the bacterial suspension was centrifuged (8000 g, 10 min), and the supernatant was extracted 3 times with an equal volume of solvent (acetone and n-hexane 1:1). The organic phase was extracted and dehydrated by

anhydrous Na_2SO_4. Than the organic phase was collected and placed in a dry-bath nitrogen gas blower for condensation, and continuously rinsed with acetonitrile. The organic phase was sampled, passed through a 0.22 μm nylon filter, and 1.5 mL was injected into the injection bottle.

The degradation process of PHE was determined by HPLC (Agilent 1260 infinity, Agilent Technologies, USA). The conditions of HPLC were as follows: column temperature was 25℃, mobile phase were acetonitrile and water (3:2), flow rate was 1 mL/min and the detection wavelength was 254 nm.

2.4 Intermediate metabolites determination

As experience group, microbial consortium WZ-4 was added into erlenmeyer flask with 30 mL inorganic salt medium and 100 mg/L PHE, and cultured at 35℃ and 150 rpm. 3 bottles added 30 mL inorganic salt medium and 100 mg/L PHE cultured at 35℃ and 150 rpm, as control group. Samples were taken continuously cultured for 0h-40h. Every two hours, 3 bottles sampled, then 0.1mL $HgCl_2$ was added to terminate the reaction and frozen. All samples were analyzed within 3d. Chromatographic grade n-hexane for solvent.

The intermediate metabolites in the degradation of PHE were determined by GC-MS (Clarus 600, Perkin Elmer, USA). The chromatographic conditions were as follows: the initial column temperature was 70℃, the retention time was 1.5 min, the temperature was raised to 250℃ and the retention time was 10 min. the injection temperature was 250℃, the mode of no split flow was adopted, and the chromatographic column was HP-5MS fused silica column (30 m × 250 μm × 0.25 μm). The mass spectrometry conditions were as follows: ion temperature 200℃, electron energy 70 eV, scanning range (M/z) 50–400, carrier gas helium, flow rate 1 mL/min.

2.5 DNA extraction and genome annotation

PowerSoil DNA extraction kit (MoBio Laboratoties Inc.) was used to extract macro DNA. The quality of the macro DNA sample was evaluated by agarose gel electrophoresis on a 1% agarose gel GelRedTM (Biotium Inc.). The Thermo Qubit 4.0 Fluorometer (Thermo Fisher Scientific Inc.) was used for DNA quantification. The establishment of gene library and high-throughput sequencing were commissioned by Shenggong Bioengineering (Shanghai) Co., Ltd. Prodigal was used to predict the ORF of long sequence contigs. Select genes greater than or equal to 100 bp to translate into protein sequences. Prodigal was used to predict the ORF of long sequence contigs. Genes with a length of 100bp are translated into protein sequences. Gene alignment between the obtained protein genes and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database were performed, using BLAST algorithm.

3. Results

3.1 Isolation and identification of PHE degrading bacterium

PHE degrading strains, marked as YA, YB, NY2 and NY3 were isolated, using PHE as a sole source of carbon and energy. These halophilic strains were culturable. The purified 16S rRNA gene PCR products of

each strain were sequenced and compared in GeneBank. These strains all belonged to *Halomonas sp.*. The comparison results was shown in Table 1.

Table 1
Strain identification.

Strain code	Identification results	Species
YA	*Halomonas* sp. PBN3(99.3%)	*Halomonas*
YB	*Halomonas salina* sp. CIFRI1(98.5%)	
NY2	*Halomonas anticariensis* sp. EA200(99.3%)	
NY3	*Halomonas anticariensis* sp. EA200(99.8%)	

The microbial consortium structures of the sampled aerobic sludge form Weizhou wastewater treatment plant (Fig. 1 WZ1, WZ2 and WZ3) and PHE domesticated sludge (Fig. 1P1) were determined by high-throughput sequencing. The results was shown in Fig. 1.

3.2 PHE degradation ability of bacterium

PHE were added into the culture medium of single strains (Y2, Y3, NY2 and NY3) and microbial consortium (WZ-4), and cultured for 7 days respectively. The degradation rates were shown in Fig. 2. The degradation rates of PHE on YA, YB, NY2, NY3 and WZ-4 was 38.11%, 58.24%, 75.86%, 83.90% and 87.04% respectively. Indicating the microbial community had better degradation rates then single strain.

3.3 Dynamic changes of metabolic intermediates and PHE

PHE (100 mg/L) was added into WZ-4 medium as the sole carbon source and cultured for 40h. Six kinds of intermediate products and their concentration changing trends were detected through HPLC. Comparing with the standard material, the concentration trend of PHE degradation was determined (Fig. 3 PHE). These results were shown in Fig. 3. According to the concentration of PHE and intermediate product (Fig. 3D), a lag phase about 5h after the inoculation of WZ-4 was presented. Between 4 ~ 32h, the concentration of intermediate products keeps changing and the concentration of PHE keeps decreasing. After 32h, the degradation rate of PHE tends to be stable (Fig. 3 PHE, close to 70%). Similar results from recent study have been reported that strain N4 could only nearly 80% of the PHE had been degraded in 8 days[13]. The concentration of intermediate product E (Fig. 3E) rises to the highest value (close to 45 mg/L), in 40h.

3.4 Structural analysis of metabolic intermediates by GC-MS

The metabolic intermediates produced during the degradation of PHE was analyzed by GC-MS. By comparing with the GC retention times of standard sample and retrieving with the mass spectrometry library and characteristic peaks in the reference[14], four metabolites were identified. Their corresponding mass spectra image were shown in Fig. 4. According to the above results, four metabolites (a), (b), (c)

and (d) were determined as 1-hydroxy-2-naphthoic acid, 1,2-dihydroxynaphthalene, 7,8-benzocoumarin and phthalic acid respectively.

3.5 Genome annotation

28737 genes regulating the pathway of carbon hydrocarbon metabolism were found, which indicating a strong biodegradation potential of WZ-4 in petroleum hydrocarbons. Gene alignment between the found genes and the KEGG database were performed, using BLAST algorithm. Gene annotation of possible metabolic pathways of WZ-4 were performed. Due to the high correlation with PHE degradation, genes related to carbohydrate metabolism in Fig. 5 were emphatically analyzed. Comparing with the KEGG database, genes related to phenanthrene degradation in the metagenomic group of WZ-4 were identified. Genes related to PHE degradation were shown in Table 2.

Table 2
Functions of various gene metabolitess in the degradation pathway of PHE.

Gene	Protein with the highest identity
phdE	3,4-Dihydrodihydroxyphenanthrene dehydrogenase
phdF	3,4-Dihydroxyphenanthrene dioxygenase
phdG	1-Hydroxy-2-naphthoic acid hydrolase
pcaG	Protocatechuic acid 3,4 dioxygenase
pcaL	4-Carboxymuconate decarboxylase
phtAa	Phthalic acid 3,4 dioxygenase large subunit
phtAc	Phthalic acid 3,4 dioxygenase ferredoxin subunit
catC	Adipic acid lactone D-isomerase
benA-xyL	Benzoic acid 1,2 dioxygenase
benB-xyL	Benzoic acid 1,2 dioxygenase β subunit
benC-xyL	Benzoic acid 1,2 dioxygenase reductase component
benD-xyL	Dihydroxycyclohexadiene carboxylic acid dehydrogenase

4. Discussion

4.1 Degradation of PHE by microbes

In this study, *Halomonas sp.* was the most abundant genus in PHE experement groups with the relative abundance of 18.68%. Strain identification showed that,YA, YB, NY2 and NY3 belongs to *Halomonas sp..* Degradation rates of PHE by YA, YB, NY2 and NY3 were 38.11%, 58.24%, 75.86% and 83.90%,

respectively. Different from YA and YB, NY2 and NY3 had obviously degradation rates of PHE (Fig. 2), and shown a homology with *Halomonas anticariensis sp.* (EA200),with a sequence similarity at 99.3% and 99.8% (Table 1).

Halomonas sp. was usually isolated from high salinity environment and has been proved to viable under different salinity conditions[15]. On the other hand, *Halomonas sp.* could be isolated from PAHs contaminated soil and degraded PAHs as the main carbon source. Govarthanan et al. identified strain RM as *Halomonas sp.*, effectively degraded 100 mg/L PHE (67.01%), pyrene (63.21%), naphthalene (60.12%) and benzoapyrene (58.00%) after 7 days of incubation[16]. *Halomonas sp.* and *Marinobacter sp.* could efficiently utilize PHE (90%) in a wide range of NaCl concentrations, from 1% to 17% (w/v)[17]. These results indicated that *Halomonas sp.* has a important role in degradation PHE.

These results showed that, upmentioned strains were potential hydrocarbon degrading bacterium, which could be used in PAHs degradation experiments. The results of PAHs degradation experiments showed, the degradation rates of PHE by microbial consortium WZ-4 were 87.04%, which were better than that of all the single bacteria. This result indicating a synergistic relationship between these 4 bacterium, which could increase the degradation depth of PHE. Previous studies reported similar phenomena, the degradation rate of PHE by 5 strains combined increased by 14%, compared with single strain[18]. PHE biodegradation using the microbial consortium was faster and reached higher degradation value[19] In addition, as the PAHs molecular complexity increased, the degradation rates decreasesed.

Based on the retention time in HPLC and molecular weight determined by GC-MS, intermediates metabolic A, B, C and D were inferred to be 1,2-dihydroxynaphthalene, phthalic acid, 1-hydroxy-2-naphthoic acid and 7,8-benzocoumarin. According to Figure 3, 7,8-benzocoumarin was produced 5 hours after inoculation, indicating that WZ-4 has a rapid response in degradation of PHE. Phthalic acid was formed at the 10th hour after inoculation, indicating that WZ-4 can decompose PHE to a high degree in a short time. In addition, at 35h after inoculation, the accumulation of intermediates metabolic E and the stagnation of PHE degradation rate occurred simultaneously. This is probably because a intermediates metabolic has substrate inhibition. This would be an important research direction to further improve the efficiency of WZ-4 degradation of PHE.

4.2 Degradation pathway of PHE by microbial consortium WZ-4

In the process of aerobic PHE degradation, the cracking of a benzene ring usually starts from the hydroxyl containing benzene ring[13]. According to previous studies, the biodegradation of PHE usually through the double hydroxylation of the C1-C2 pathway or C3-C4 pathway[20, 21]. In the C1-C2 pathway, dihydroxylation occured at C1 and C2 carbon sites. And then 1,2-dihydroxy-phenanthrene was cleaved to 2-hydroxy-1-naphthoic acid and 5,6-benzocoumarin[22]. As a secondary metabolite, 5,6-benzocoumarin was considered to be the final metabolite of this pathway, which will accumulate in a large amount during the

degradation process. On the C3-C4 pathway, dihydroxylation occurs at C3 and C4 carbon sites. And then 3,4-dihydroxyphenanthrene was cleaved to 1-hydroxy-2-naphthoic acid and naphthol[23]. In this study, 5,6-benzocoumarin was not detected in the culture medium by GC-MS (Fig. 6), which indicated that the initial oxidation of PHE did not through the C1-C2 pathway. However, 1-hydroxy-2-naphthoic acid and 7,8-benzocoumarin were detected (Fig. 6). Since 7,8-benzocoumarin was a reversible reaction product of 1-hydroxy-2-naphthoic acid, it indicated that PHE was degraded through the C3-C4 pathway.

Subsequently, 1-hydroxy-2-naphthoic acid produced in the previous reaction (C3-C4 pathway) was decomposed into 1,2-dihydroxynaphthalene after decarboxylation reaction[24]. According to previous studies, 1,2-dihydroxynaphthalene has two possible decomposition pathway: (1) The ortho benzene ring of 1,2-dihydroxynaphthalene could be decomposed into phthalic acid after decarboxylation reaction[25]. (2) 1,2-dihydroxynaphthalene could be cut off in the intermediate position and decomposed into salicylic acid and endup in tricarboxylic acid cycle[26, 27]. However, salicylic was not detected (Fig. 6), indicating the degradation of 1,2-dihydroxynaphthalene through the phthalic acid pathway.

Based on the results of genome annotation, potential PHE degradation genes were identified. Genes related to PHE degradation included 3,4-dihydroxy- phenanthrene dehydrogenase gene, 3,4-dihydroxyphenanthrene dioxygenase gene, 1-hydroxy-2-naphtholic acid hydrolase gene and 4-carboxymucate decarboxylase gene[28]. And downstream genes of PHE degradation were also identified, including protocatechuic acid 3,4 dioxygenase, benzoic acid 1,2 dioxygenase, adipic acid lactone D-Isomerase and dihydroxy-cyclohexanene carboxylic dehydrogenase. The discriminating of these genes indicated that WZ-4 could degraded PHE through the phthalic acid protocatechuic acid pathway.

In summary, the biodegradation process of PHE by WZ-4 was speculated as follows: (1) Under the catalysis of 3,4-dihydroxyphenanthrene dehydrogenase (*phdE*) and 3,4-dihydroxyphenanthrene dioxygenase (*phdF*), PHE was dihydroxylated at C3 and C4 carbon sites. (2) Under the catalysis of naphthoic acid hydrolase (*phdG*), the product was decomposed into 1-hydroxy-2-naphthoic acid. And then, 1-hydroxy-2-naphthoic acid was transformed into 1,2-dihydroxynaphthalene under the action of decarboxylase (pcaL). (3) 1,2-dihydroxynaphthalene was converted into 2-carboxystyrylic acid. And 2-carboxystyrylic acid was converted into 2-Carboxybenzaldehyde by hydrolysis acetal, and then oxidized to phthalic acid by oxidase. (4) Under the catalysis of phthalic acid 3,4 dioxygenase (*phtAa* and *phtAc*), the phthalic acid was hydroxylated and converted into 3,4-dihydroxyphthalic acid, and then converted into protocatechuic acid by the action of decarboxylase. (5) After the reaction of protocatechuic acid 3,4 dioxygenase (*pacG* and *pacH*), benzoic acid 1,2 dioxygenase (*benA-xyL*, *benB-xyL* and *benC-xyL*), Adipic acid lactone D-isomerase (*catC*) and dihydroxycyclohexadiene carboxylic acid dehydrogenase (*benD-xyL*), protocatechuic acid was converted to pyruvate and finally entered the tricarboxylic acid cycle to complete the degradation.

Conclusions

In this study, WZ-4, a microbial community that can effectively degrade phenanthrene, was located and separated from aerobic sludge through high-throughput sequencing technology. Based on the metabolic intermediates identified by GC-MS and the associated degradation genes revealed by genome sequencing and genomic annotation, the biodegradation pathway of WZ-4 was determined. In the upstream process of biodegradation of WZ-4, C3 and C4 carbon atoms undergo hydroxylation, and the downstream degradation process follows the phthalic acid pathway. The study of potential PHE degrading strains is of great significance and prospect for bioremediation of oil contaminated water and soil environment, which could be used for bioremediation of petroleum contaminated environment.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

All the authors declare that they have no conflicts of interest.

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Authors' contributions

P. Peng, L. Yuan and T. Shen designed and performed the experiments, Q. Li and C. Wang collected the data and performed data analysis; P. Peng and T. Shen contributed manuscript revision; P. Peng, L. Yuan and T. Shen suggested the results and discussion and also contributed to the Tables and Figures; Q. Xie Provided funding support and supervision. All authors have read and agreed to the published version of the manuscript.

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Figures

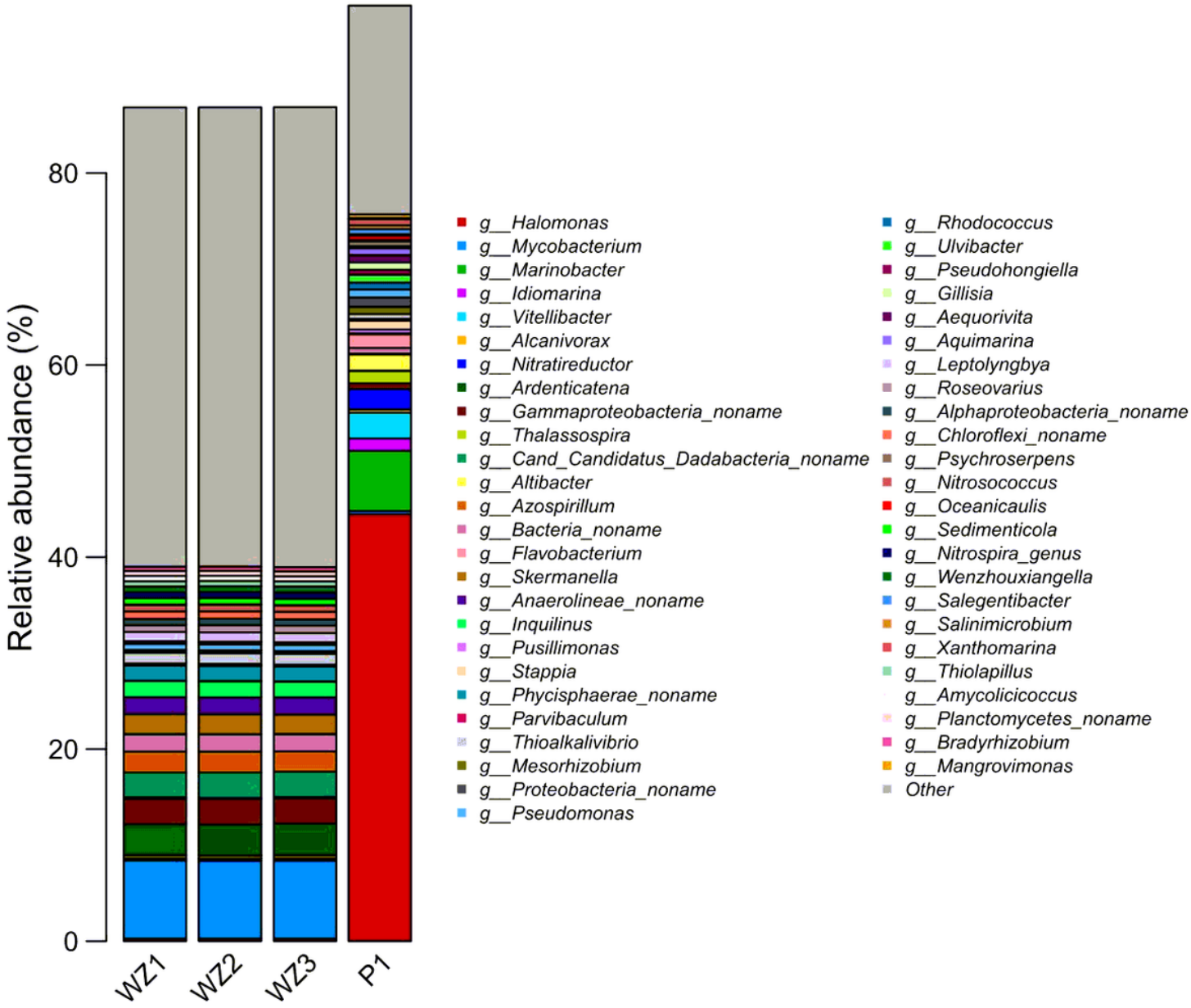


Figure 1

Microbial consortium of Weizhou sludge and PHE domesticated sludge.

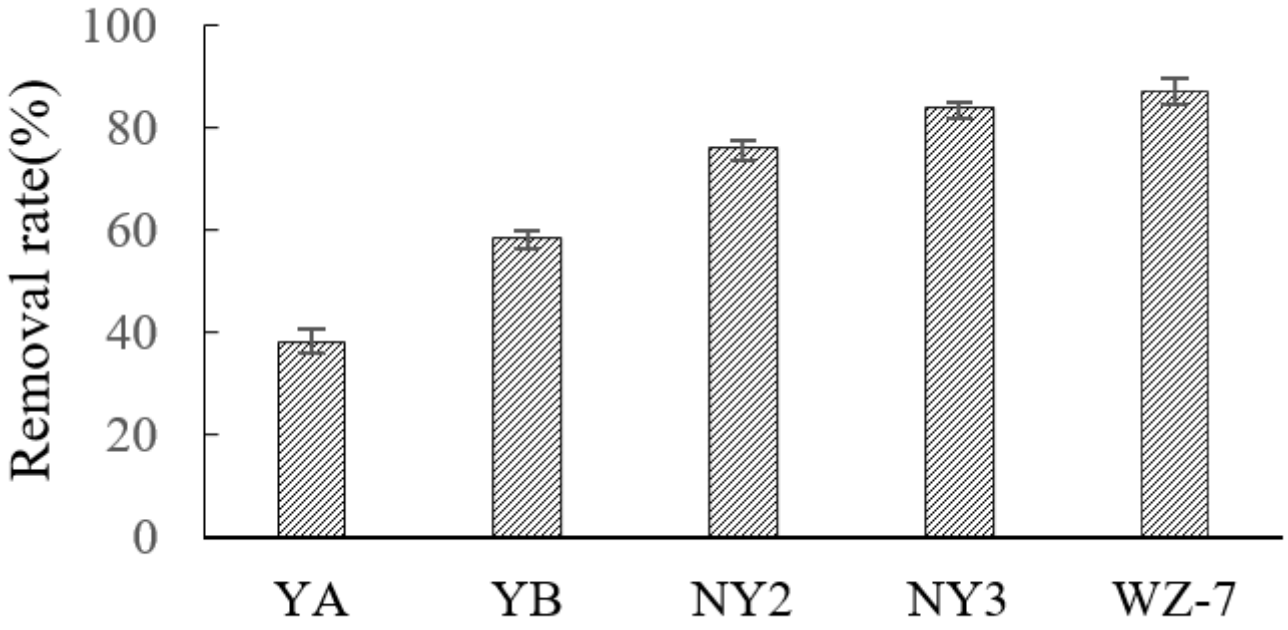


Figure 2

Degradation rates of single strains and WZ-4 on PHE.

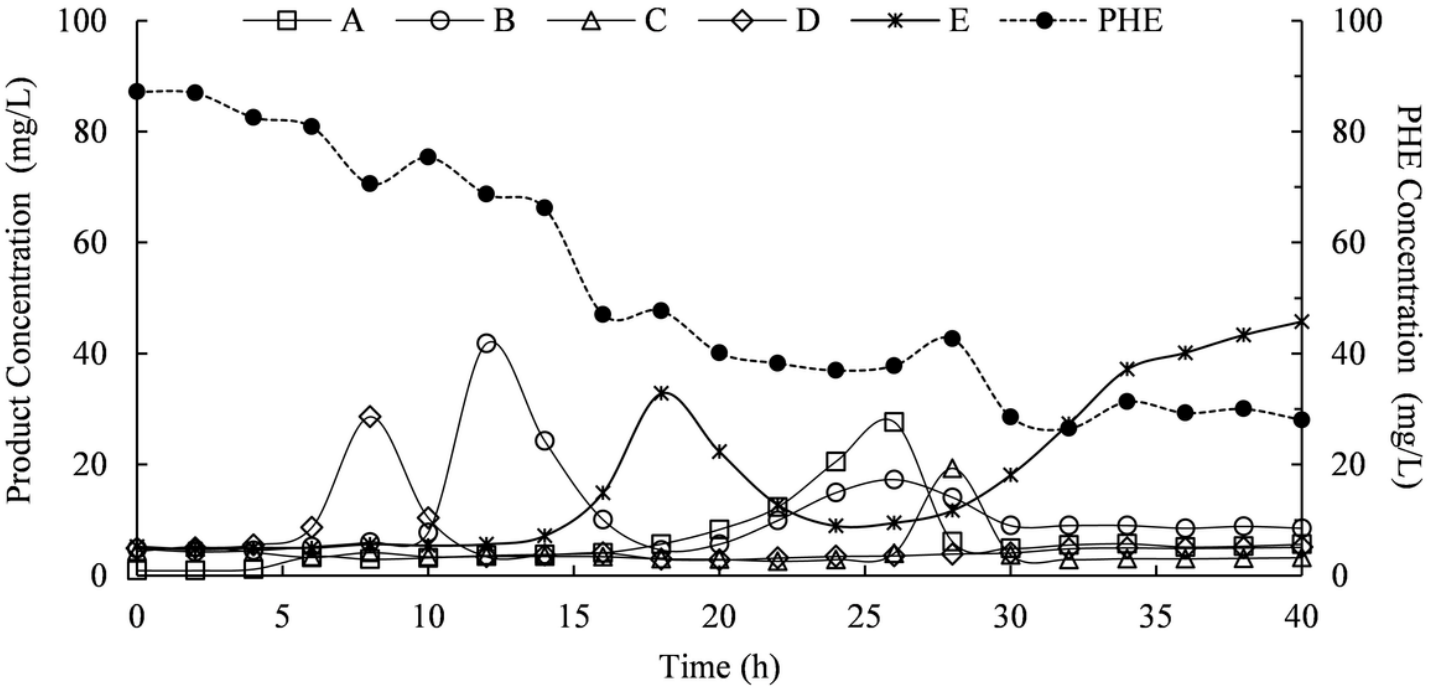


Figure 3

Concentration trends of PHE and intermediate products.

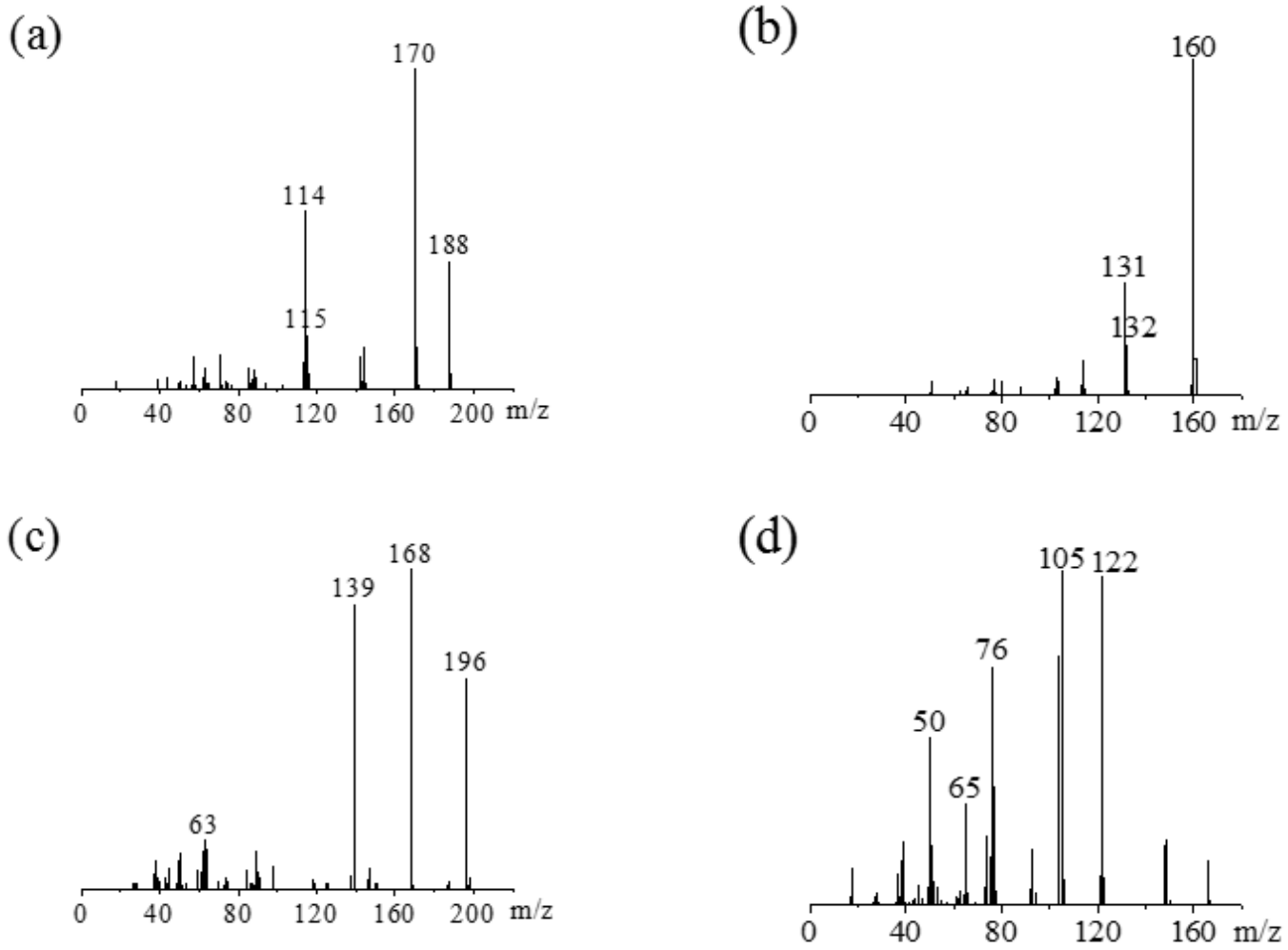


Figure 4

Mass spectra image of intermediate metabolites.

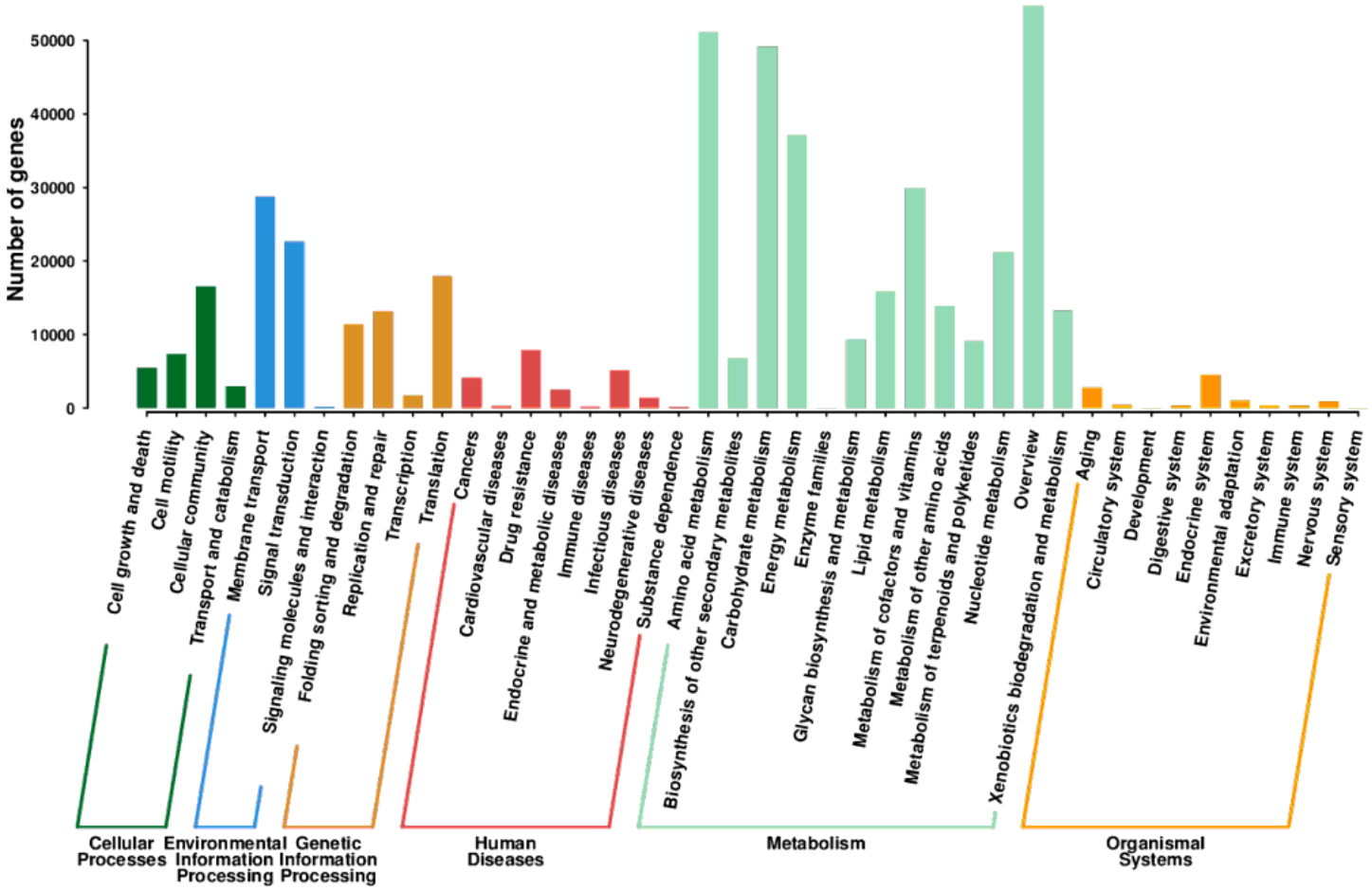
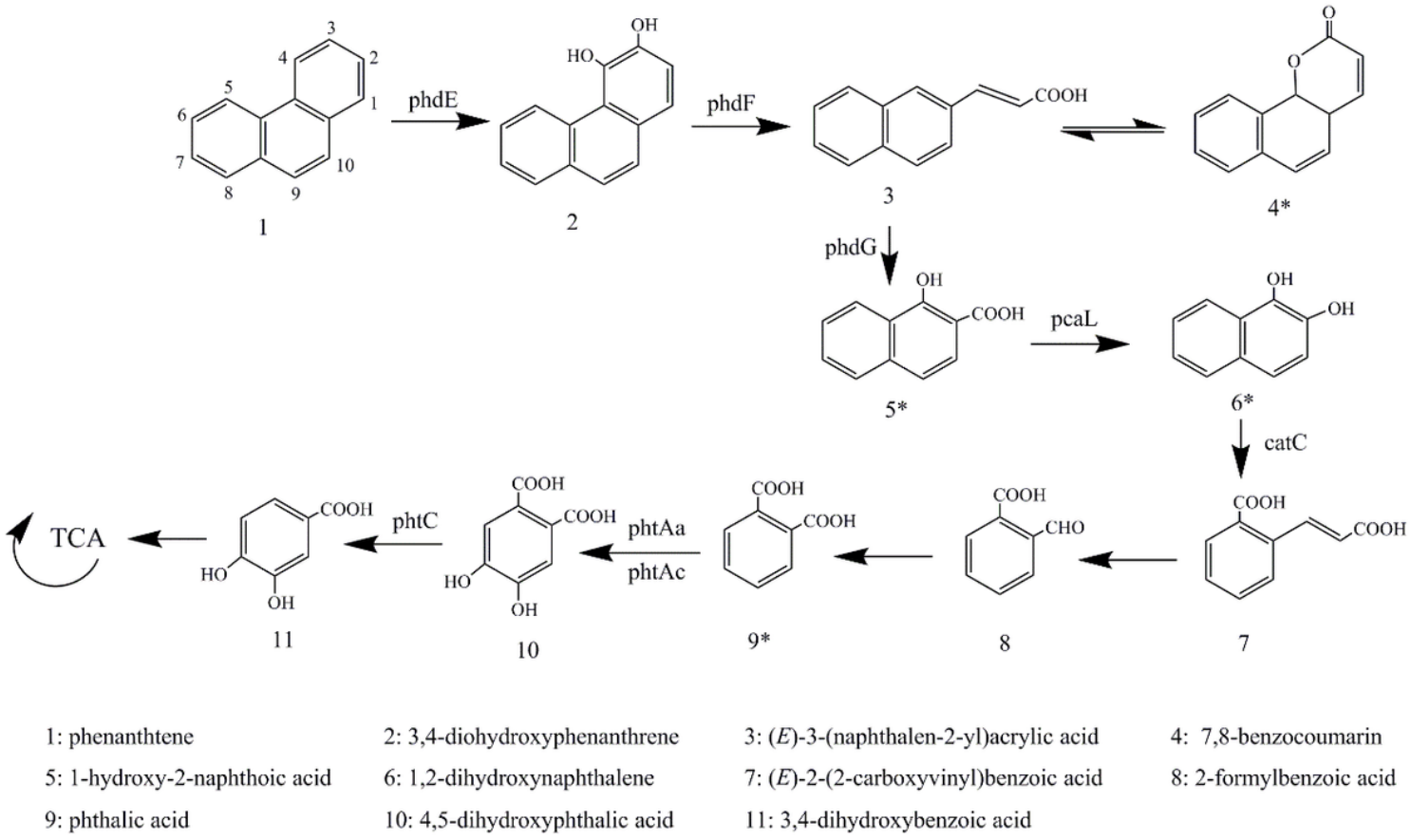


Figure 5

KEGG biological path classification statistics histogram.



1: phenanthtene
2: 3,4-diohydroxyphenanthrene
3: (*E*)-3-(naphthalen-2-yl)acrylic acid
4: 7,8-benzocoumarin
5: 1-hydroxy-2-naphthoic acid
6: 1,2-dihydroxynaphthalene
7: (*E*)-2-(2-carboxyvinyl)benzoic acid
8: 2-formylbenzoic acid
9: phthalic acid
10: 4,5-dihydroxyphthalic acid
11: 3,4-dihydroxybenzoic acid

Figure 6

Potential degradation pathways of PHE. The number under the compound with a * indicates that the compound was detected in this study.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstract.png