Evaluation of *Corynebacterium variabilis* Sh42 as a degrader for different poly aromatic compounds

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Abstract: *Corynebacterium variabilis* sp. Sh42 is used to investigate the biodegradation potentials and metabolic pathways of different poly aromatic compounds (PACs) in batch flasks. Effects of PACs size, molecular weight, alkylation and their presence individually or in mixture on biodegradation potentials of Sh42 were studied; Naphthalene (Nap) as a model compound for di-aromatic ring; Antheracene (Ant) and Phenantherene (Phe) as model compounds for tri-aromatic ring; while Pyrene (Pyr) as a model compound for four-aromatic ring compounds were used as representatives for different PAHs. Dibenzothiophene (DBT), 4-methyldibenzothiophene (4-MDBT) and 4,6-dimethyldibenzothiophene (4,6-DMDBT) were taken as representative models for PASHs compounds. While, 2-hydroxybiphenyl (2-HBP) and 2, 2'-bihydroxybiphenyl (2, 2'-BHBP) were taken as models for phenolic compounds. The experimental results show that biodegradation rate decrease with increase ring size, alkylation's group within homologous series and Sh42 has the highest capability to biodegradation of toxic phenolic compounds either in single (BD% ≈ 90%) or mixed substrates cultures (BD% ≈ 48%). To ensure detoxification and mineralization of these toxic PACs; metabolic pathways of representative model compounds (Pyr, DBT and 2,2'-BHBP) were elucidated by GC/MS analysis which confirmed that, Sh42 completely metabolized all representative compounds to CO_2 and H_2O. [Journal of American Science. 2010;6(11):343-356]. (ISSN: 1545-1003).

Keywords: Polynuclear aromatic compounds, Biodegradation, Metabolic pathways.

1. Introduction

Contamination by poly aromatic compounds (PACs); i.e. polynuclear aromatic hydrocarbons (PAHs) and poly aromatic sulfur heterocycles (PASHs) are of great environmental concern because of their toxic, mutagenic and carcinogenic properties (Hirano et al., 2004 and Perugini et al., 2007). These PAHs and PASHs are actually persistent compounds in the environment. Nevertheless, various bacteria and fungi are reported for their ability to degrade different PAHs and PASHs (Cheung and Kinkle, 2001; El-Gendy, 2006; Valentin et al., 2007; El-Gendy and Abo-State, 2008 and Seo et al., 2009).

Phenol and phenolic compounds are hazardous pollutants that can be found in waste waters from oil refineries, petrochemical plants, coal gasification plants, coking plants and dyes industry (Martínková et al., 2009). Phenolic compounds are known to affect microbial growth and degradation activities even in low concentrations (Vincenza and Liliana, 2007).

Dibenzothiophene (DBT) which is a typical PASH in crude oil is used as a model compound for biodesulfurization and biodegradation studies of

PASHs (Chen et al., 2008). Biodegradation of DBT can be classified into three independent categories, among which; complete mineralization, Kodama and the sulfur-specific cleavage, i.e. 4S pathway, which is the most extensively studied (Xu et al., 2006). In "4S" pathway, DBT is first oxidized to DBT sulfoxide, then DBT sulfone and finally to sulfinate, followed by hydrolytic cleavage to free sulfur product, 2-HBP or 2, 2'-BHBP and subsequently release of sulfite or sulfate (Chen et al., 2008). 2-HBP and 2,2'-BHBP were reported to be furtherly metabolized to benzoic acid and salicylic acid, respectively in complete mineralization pathway (Kohler et al., 1988 and El-Gendy, 2004). Previously mentioned hydroxylated biphenyls are reported to be also produced through the bio-transformation reactions precede via hydroxylation of the aromatic rings in biodegradation of different studied PACs (Kohler et al., 1993 and Sondossi et al., 2004). Some previous studies have indicated that the final metabolite 2-HBP or 2,2'-BHBP of DBT biodegradation via 4S pathway could inhibit the microbial growth and DBT biodegradation (Lee et al., 1995 and Chen et al., 2008). Consequently, knowledge of microbial metabolism and environmental fate of these compounds is desired because they are by-products which have been identified as contaminants in almost every component of the global ecosystem and because they constitute a severe environmental hazard because of their high toxicity. Thus obtaining of microbial isolates with the ability to utilize several PACs is of

great interest from the stand point of understanding the principles of PACs utilization and their use in bioremediation technologies.

It is rather unusual for environments to be polluted by a single poly aromatic compound (PACs) (Tang et al., 2005; Seo et al., 2009 and Wei et al., 2009).

Therefore, the aim of this study is to investigate the potentiality of *Corynebacterium variabilis* sp. Sh42 to metabolize different PACs as single or mixture substrates; Naphthalene (Nap) as a model compound for di-aromatic ring; Antheracene (Ant) and Phenantherene (Phe) as model compounds for tri-aromatic ring; while Pyrene (Pyr) as a model compound for four-aromatic ring compounds were used as representatives for different PAHs. Dibenzothiophene (DBT), 4-methyldibenzothiophene (4-MDBT) and 4,6-dimethyldibenzothiophene (4,6-DMDBT) were taken as representative models for PASHs compounds. While, 2-hydroxybiphenyl (2-HBP) and 2,2'-bihydroxybiphenyl (2,2'-BHBP) were taken as models for phenolic compounds. Also, elucidation of the possible metabolic pathways for a model compound representative to each PACs group has been done to ensure the metabolism of these toxic compounds to non-toxic ones.

2. Material and Methods

Chemical reagents

All 9 PACs and other chemical reagents employed in this study were of analytical grade and purchased from Sigma Chemical Company, USA.

Acetonitrile (Ace) and Water (W) used for HPLC analysis were of HPLC grade and purchased from Aldrich.

Microorganism

Corynebacterium variabilis sp. Sh42 was isolated from hydrocarbon polluted waster sample collected from El-Lessan Area of Damietta River Nile Branch in Egypt during year 2008 for its ability to degrade and tolerate high concentration of 2, 2'-BHBP.

Media:

Tryptone glucose yeast extracts (TGY)

This media was used for preparation of inocula and monitoring of total viable count, TCFU (cells/mL) and was prepared according to Benson (1994).

Basal salts medium (BSM)

This media was used for studying BD capabilities of *C. variabilis* sp. Sh42 and was prepared according to Piddington et al. (1995).

Analytical tools:

Bacterial growth was monitored by count the total viable colony (cells/mL) on TGY-agar plates.

High Performance Liquid Chromatographic (HPLC) analysis

Liquid-liquid extraction for quantitative analysis of residual PACs was carried out by using ethyl acetate as the extractant. After the extraction, ethyl acetate layer was analyzed using HPLC model Waters 600E equipped with a UV detector model Waters 2487 (set at 254 nm) and C18 reversed phase column (4.6x250 mm, 300^{o}A, 5µ). The mobile phase was Ace: W (40:60 v/v), and the flow rate was 1 mL/minute, injection volume 2 µL. Standard curves were established for each of the studied PACs from 5 to 1000 mg/L.

Gas Chromatography/Mass Spectroscopy (GC/MS) analysis

GC/MS Perkin Elmer Clarus 500 with mass-selective detector was used to monitor the BD of mixture of PACs; the tested mixtures of nine poly aromatic compounds (PACs) were analyzed using GC/MS (system-1) to quantify percent removal. Identification of expected pathway for metabolism of different studied PACs (phenolic, PAHs and PASHs compounds) by *C. variabilis* sp. Sh42 GC/MS (system-2) was used (Table 1).

Preparation of samples

For GC/MS analysis, ethyl-acetate extracts of the studied mixture were dehydrated over anhydrous Na_2SO_4 and concentrated by evaporation at 60 ^{o}C before injection.

The GC/MS was equipped with HP-1 column, polymethyl siloxane (capillary 60 m x 0.32 mm x I.D. 0.25 µm film thickness), mass-selective detector with ionization mode, E.I. ev 70, source temperature 250 ^{o}C; the carrier gas was helium with flow rate 1.5 mL/minute. Sample size was 1 µL.

Preparation of inoculum

Cells were incubated at 30 ^{o}C in TGY broth medium for 24 hours in a shaking incubator (150 rpm). Cells were harvested by centrifugation at 5000 rpm for 15 minutes and then washed three times with BSM then re-suspended in BSM free of any C-source to be used as inoculum.

Table (1): Programmable methods

programmable method	System (1)	System (2)
Injector temperature	Start from 60 ^{o}C held (0.1 minute) to 250 ^{o}C at fixed rate 200 ^{o}C/minute. Split (1:50)	Start from 80 ^{o}C held (0.1 minute) to 250 ^{o}C at fixed rate 200 ^{o}C/minute. Split (1:50)
Oven temperature	Start from 60 ^{o}C held for 1 minute, followed by 7 ^{o}C/minute ramp rate to 200 ^{o}C held to 5 minute, followed by 4 ^{o}C/minute ramp rate to a final temperature 280 ^{o}C which was held to another 15 minutes.	Start from 50 ^{o}C held for 1 minute, followed by 8 ^{o}C/minute ramp rate to a final temperature 280 ^{o}C which was held to another 15 minutes.
Run Time	50 minutes	60 minutes
	Standard calibration for mixture of the studied 9 PACs was established for their identification and quantification.	Possible structure assignments of metabolities were confirmed using NIST library and/or from the available literature data.

Identification of biodegradation pathway of different PACs using *C. variabilis* sp. Sh42

This was done using GC/MS analysis system (2) of the ethyl acetate extract of different PACs compounds; Pyrene as a model for PAHs; DBT as a model for PASHs and 2,2'-BHBP as a model for phenolic compounds after incubation at 30 ^{o}C for 10 days in shaking incubator (150 rpm).

Study of the biodegradation capabilities of *Corynebacterium Variabilis* sp. Sh42 on different individual PACs

This was used for studying the ability of Sh42 to utilize different PACs as sole source of carbon and energy for growth. Washed cells were inoculated into BSM that contained 1000 mg/L of each of the studied PACs and mixture of all. The growth and concentration of PACs were monitored at prescribed time intervals of (1- 4 weeks). Changes in pH values were also recorded. All steps were done in duplicates.

3. Results and discussion

Biodegradation capabilities of *Corynebacterium Variabilis* sp. Sh42 on different PACs:

Losses due to abiotic processes were calculated; recorded average ranged between ≈ 6%, 4% and 2.6 for phenolic, PAHs and PASHs compounds after 4 weeks, respectively. Any observed loss exceeding these values in the inoculated flasks can be attributed to biodegradation processes.

The experimental results show that pH decrease with incubation period up to 3 weeks for all cultures but increase with further incubation period. This decrease in pH might be due to the formation of acidic metabolites from biodegradation of these PACs in the cultures while the increase of pH again might be due to the further degradation of the acidic metabolites or due to the production of intermediates that might increase the pH.

Generally, Figures (1 and 2) show that the maximum growth and biodegradation (BD) potential were observed after 2 weeks for all representative model compounds of PACs in single substrate cultures, it was observed that there was no difference in growth and BD% within further incubation period. While in mixed substrates cultures, it showed good increasing growth and BD% throughout the whole incubation period reaching its maximum at the end of incubation period.

This observation is in agreement with other reports where, the removal of PACs was directly related to the cell density and growth potential. Hong et al. (2008) reported that; the removal of Phe and Flu in mixture by *Nitzschia* sp. increase by increasing its cell density in the medium.

It was observed that, the average rate of biodegradation can be ranked in the following decreasing order; phenolic > PAHs > PASHs in either single or mixed substrates cultures at the end of incubation period. This might be attributed to the enrichment of *C. variabilis* sp. Sh42 on phenolic compounds showing higher BD efficiency and high resistance of hetero-PAH (PASHs) to biodegradation which have the lowest aqueous solubility.

It is obvious that phenolic compounds and Nap show nearly similar and highest BD% in single substrate cultures with average BD% ≈ 90% after 4 weeks. This might be due to the high capability of Sh42 to utilize phenolic compounds as it was enriched and isolated on 2, 2'-BHBP in addition to the high evaporation and assimilation rate of Nap than other PACs compounds or due to the higher specificity of Sh42 towards Nap biodegradation. While in mixed substrates culture only Nap show the highest biodegradation efficiency (BD% of 100% after 1week of incubation). This might be due to the

high capabilities of Sh42 to utilize Nap over phenolic compounds or the mixture might have enhanced the metabolic rate of Nap over phenolic compounds.

Also, the BD rate of 2,2'-BHBP is higher than that of 2-HBP (Figures 1 and 2) in both single and mixed substrate cultures which might be due to the enrichment and isolation of Sh42 on 2,2'-HBP, therefore, it would have higher capabilities towards 2,2'-BHBP biodegradation than 2-HBP, the higher toxicity of 2-HBP than 2,2'-BHBP or the difference in activity of enzymatic system required for biodegradation of 2,2'-BHBP and 2-HBP. Hiraoka et al. (2002) reported that; monohydroxylated biphenyl (2-HBP or 3-HBP) inhibited cell division of biphenyl biodegrader strain *Comamonas testosteroni* TK102, but the effect was not observed with 2,3-dihydroxybiphenyl which confirm that 2-HBP was more toxic than 2,2'-BHBP.

Generally, Figures (1 and 2) show that, the biodegradation efficiency for all tested PAHs compounds follow the same trend in either single or mixed substrate cultures which can be ranked in the following decreasing order; Nap > Ant ≈ Phe > Pyr. This observation is in agreement with other reports where low molecular weights PAHs are more biodegradable than high molecular weights PAHs. The same trend was observed For PASHs compounds; where the biodegradation efficiency for all tested PASHs compounds can be ranked in the following decreasing order; DBT > 4-MDBT > 4,6-DMDBT.

Hong et al. (2008) reported that; the molecular weight, water solubility and lipophobicity of a compound would affect its bioaccumulation and degradation by microorganisms. Yu et al. (2005) reported that; PAHs with low molecular weights such as 2-rings, naphthalene and 3- rings, phenanthrene and anthracene are more susceptible to bacterial degradation and more extensive than high molecular weights PAHs having more than 3-rings, pyrene.

On the other hand, by comparing the results in Tables (2 and 3) and represented in Figure (3) it is observed that; there are high significant differences in biodegradation efficiencies of all tested 9 PACs in single and mixed substrates cultures. Where, BD% for each substrate in a mixture is lower than that in single substrate culture except for Nap which shows no significant difference.

The average BD% recorded ≈ 90%, 70% and 64% for phenolic, PAHs and PASHs in single culture after 4 weeks, respectively while in mixed substrate culture the BD% reached ≈ 48%, 42% and 31%, respectively. This might be due to one or all of the following reasons;

First, the interactions between PACs in mixture might influence biodegradation rate and led to negative or positive effect on its biodegradation efficiency and this agrees with Stringfellow and Attken (1995); Yuan et al. (2000); Johnsen et al. (2002) and Wei et al. (2009). Wei et al. (2009) reported that, there were inhibitory effects from interactions between the three PAHs mixture (Phe, Pyr and Flu) on *Mycobacterium* sp. MEBIC 5140 which led to a negative effect on the biodegradation of PAHs.

Second, different studies in culture media have shown that hetero-PAHs (NSO-PAHs) can have a significant inhibiting effect on the biodegradation of PAHs and mono aromatic hydrocarbons (Arcangeli and Arvin, 1995; Dyreborg et al., 1996 and Lantz et al., 1997). Meyer and Steinhart (2000) reported that; degradation of two- to five-ring PAHs was inhibited by the presence of hetero-PAHs, whereas degradation of just some hetero-PAHs was inhibited by the presence of PAHs.

Third, there may be another reason for the decrease in biodegradation of different studied PACs in a mixture reported by Bastiaens (1998) and Herwijnen et al. (2003); the toxic effect caused by produced metabolites during biodegradation processes.

Fourth, the order of biodegradability of single substrates in complex mixtures is determined by their polarity and bioavailability. Consequently, as the Molecular weight of PAHs and number of alkyl group of PASHs increase the aqueous solubility and bioavailability decrease and therefore exhibits a protective function against biodegradation.

There is no observed significant difference between growth potential for *C. variabilis* sp. Sh42 in single or mixed substrate cultures of PACs, this might indicate that mixtures of PACs may not have any significant effect on cell growth but might have a significant effect on the biodegradation enzymatic system for *C. variabilis* sp. Sh42. Where, the negative effects might be due to competitive inhibition of multiple substrates or other means, retarding the degradation of one substrate in the presence of another. Similar or identical enzyme systems may catalyze the degradation of compound(s) which are structurally similar (Bauer and Capone, 1988).

Hong et al. (2008) reported that, when two or more PACs are present together, one PAC has the capacity to influence the rate and extent of biodegradation of the other.

Seo et al. (2009) reported that, alkylated and hetero PACs are more resistant to be biodegraded than the parent PACs where, alkyl- and hetero-PACs are among common substituted PACs and have substantial toxicities. The ability of PACs dioxygenase to remove the substitutions is currently

the subject of debate and probably requires additional steps to be removed. Also, their presence may inhibit proper orientation and accessibility of the PACs into dioxygenases.

In general, our results are in agreement with other reports where, the rate of polycyclic aromatic compound biodegradation in the mixed substrates culture decreases with increasing ring size and within a homologous series, decreases with increasing alkylation (Neff, 1979; Douglas et al., 1994; Elmendorf et al., 1994).

Fedorak and Westlake (1983) and (1984) reported that; microbial degradation of organic sulfur compounds in Prudhoe Bay crude oil revealed that the order of susceptibility of the sulfur heterocycles in homologous series was; DBT > C1-DBTs > C2-DBTs > C3-DBTs.

Nagata et al. (1978) reported the degradation of crude oil by *Corynebacterium* sp. isolated from sea water in the harbor of Kobe showing good biodegradation capabilities on diaromatic and polyaromatic hydrocarobons than monoaromatic ones.

In general, *C. variabilis* sp. Sh42 expressed the highest ability to utilize PACs mixture especially the toxic phenolic compounds and because of its metabolic versatility, this bacterium has been thought to be a potential candidate for bioremediation of PACs-contaminated areas.

Metabolic pathway study:

Study of 2, 2'-BHBP metabolic pathway using *Corynebacterium variabilis* sp. Sh42

Data obtained from GC/MS analysis system (2) of ethyl-acetate extracte of 2, 2'-BHBP cultures with *Corynebacterium variabilis* sp. Sh42 suggesting the biodegradation pathway illusterated in Figure (4) which is a meta-cleavage pathway. This pathway is similar to that reported by Kohler et al. (1993) and Sondossi et al. (2004) for biodegradation of 2,2'-BHBP with *Pseudomonas* sp. Strain HB1 and *Comamonas testostero*ne B-356, respectively. The obtained suggested pathway in this study suggesting that aromatic ring of 2, 2'-BHBP is degraded via a site-specific monooxygenase that hydroxylates aromatic compounds at the C-3 position where there is a hydroxyl group at C-2 and alkyl or phenyl rest at C-1. Interestingly, 2, 2', 3-trihydroxybiphenyl also serves as a substrate for the monooxygenase activity producing 2, 2', 3, 3'-tetrahydroxybiphenyl. Therefore, the monooxygenase also hydroxylates the C-3' postion of 2, 2', 3- trihydroxybiphenyl. This finding provides additional evidence for the previously suggested relaxed specificity of the monooxygenase with respect to the molecular rest at the C-1 position of the aromatic backbone structure reported by Kohler et al. (1988).

There was a yellow colouration observed in phenolic cultures inoculated with Sh42 which might be due to the production of 2,2',3,3'-tetrhydroxybiphenyl.

Kohler et al. (1993) reported the formation of yellow meta-cleavage compounds from 2,2',3-trihydroxybiphenyl (2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid) and 2,2',3,3'-tetrahydroxybiphenyl (2-hydroxy-6-(2,3-dihydroxyphenyl)-6-oxo-2,4-hexadienoic acid) which did not remain stable in an aqueous solution. They also reported the formation of yellow meta-celavage metabolite 2-hydroxymuconic semialdehyde from catechol produced from salicylate monooxygenases of salicylic acid produced through the biodegradation of 2,2',3-trihydroxybiphenyl which was produced from monooxygenase of 2,2'-bihydroxybiphenyl.

The proposed pathway for the metabolism of 2,2'-bihydroxybiphenyl presented in Figure (4) indicate that the first intermediate; 2,2',3-trihydroxybiphenyl, may be metabolized via two different routes. On one hand, it serves as a substrate to the extradiol ring cleavage dioxygenase, and on the other hand, it can be turned over by the monooxygenase.

The conversion of produced catechol and pyrogallol to 2-hydroxymuconic semialdehyde and 2-hydroxymuconic acid, respectively; indicate that the extradiol ring cleavage dioxygenase activity from strain Sh42 is a broad-spectrum meta-cleavage dioxygenase because it is able to turn over various 2,2',3-trihydrox- and 2,2',3,3'-tetrahydroxybiphenyl, catechol and pyrogallol.

Study of DBT metabolic pathway using *Corynebacterium variabilis* sp. Sh42

Data obtained from GC/MS analysis system (2) of ethyl acetate extracte of DBT cultures inoculated with *Corynebacterium variabilis* sp. Sh42 suggest the biodegradation pathway illusterated in Figure (5) which is complete mineralization pathway. Where DBT was first oxidized through 4S-pathway to DBT-sulfoxide, DBT-sulfone then to 2'-HBP-2-sulfinic acid and 2'-HBP-2-sulfonic acid which leads to the production of 2-HBP and 2,2'-BHBP, respectively and which were furtherly degraded through meta-cleavage pathway as illustrated before. 2-HBP can be also degraded by dioxygenation of vicinal ortho-meta carbons of the un-substituted ring producing 2,2'-BHBP and then 2,2',3-trihydroxybiphenyl which is furtherly degraded through meta-cleavage pathway as discussed before.

Sondossi et al. (2004) reported that *Comamonas testosterone* B-356 is able to metabolize

monohydroxybiphenyls through the biphenyl catabolic pathway leading to the production of benzoic acid and 2-hydroxypentanoate.

Omori et al. (1992) reported that, the soil isolate, *Corynebacterium* sp. strain SY1, utilized DBT and a wide range of organic and inorganic sulfur compounds as sole source of sulfur, such as DBT sulfone, dimethyl sulfide, dimethyl sulfoxide, dimethyl sulfone, CS_2, FeS_2 and even elemental sulfur. Strain SY1; metabolize DBT to DBT sulfoxide and DBT sulfone and 2-HBP, which subsequently nitrate to produce at least two different hydroxynitobiphenyls during cultivation.

Also, Constanti et al. (1996) reported that, *Corynebacterium* sp. MC401 and Corynebacterium sp. MC402 isolated from a coal mine area by enrichment culture with DBT. Both cultures were able to use DBT, $DBTO_2$ as sole source of sulfur for growth. These compounds were metabolized to 2-HBP and sulfate.

Study of Pyrene metabolic pathway using *Corynebacterium variabilis* sp. Sh42

Data obtained from GC/MS analysis system (2) of ethyl acetate extracte of Pyrene cultures inoculated with *Corynebacterium variabilis* sp. Sh42 suggest the proposed Pyrene biodegradation pathway illustrated in Figure (6) which also involve phenanthrene, naphthalene and o-phethalate degradation pathways.

This pathway was similar to that proposed by Liang et al. (2006) for biodegradation of Pyrene by *Mycobacterium* sp. Strain KMS. According to the literature review, at least 15 enzymes are involved in the degradation of Pyrene and the o-phethalate degradation from phenanthrene, with some enzymes being common to the degradation of both PAHs. Pyrene is first oxidized in the K region by a dioxygenase to form cis-4, 5-pyrene-dihydrodiol, which is rearomatized to form 4, 5-dihydroxy-pyrene by dihrdrodiol dehydrogenase. 4, 5-Dihydroxy-pyrene is subsequently cleaved to yield phenanthrene-4, 5-dicarboxylic acid by intradiol dioxygenase, followed by loss of a carboxyl group by decarboxylase and 4-phenanthroic acid is formed. Oxidation of 4-phenanthroic acid by ring-hydroxylating dioxygenase produces 3,4-phenanthrene dihydrodiol-4-carboxylic acid, which is further transformed to 3,4-dihydroxyphenanthrene by dehydrogenase/decarboxylase. Once 3,4-dihydroxyphenanthrene is formed, it enters the phenanthrene degradation pathway (Krivobok et al., 2003). Where it is metabolized to 1-hydroxy-2-naphthoic acid and then mineralized through two different pathways (Figure 6), in one pathway, 1-hydroxy-2-naphthoic acid is oxidized to 1, 2-dihydroxy naphthalene, which is furtherly metabolized via salicylic acid. In the other pathway (Figure 6), 1-hydroxy-2-naphthoic acid undergoes ring cleavage and furtherly metabolize via o-phthalic acid and protocatchuic acid. Similar pathway was reported by Pinyakong et al. (2000) for biodegradation of Phenanthrene by *Sphingomonas* sp. P2. It has been demonstrated that a common set of enzymes is responsible for the conversion of Phe to 1-hydroxy-2-naphthoic acid as well as that of naphthalene to salicylic acid (Yang et al., 1994).

Due to the known reported toxicity of pyrene-4,5-dione, it is important to identify this metabolite and determine its fate during Pyrene metabolism. It was observed as an end product in some gram negative cultures (Kazunga and Aitken, 2000) and may result in an increase in toxicity during in situ bioremediation (Guthrie et al., 2003).

Identification of Pyrene-4, 5-dione in the GC/MS chromatogram of the studied culture *Corynebacterium variabilis* sp. Sh42 might indicate the presence of dioxygenase gene in the obtained bacterial isolate *Corynebacterium variabilis* sp. Sh42. As according to Khan et al. (2001), Pyrene-4,5-dione was identified to be Pyrene metabolite in the phagemid clone My6-pBK-CMV, which contain a dioxygenase gene when it was incubated with Pyrene.

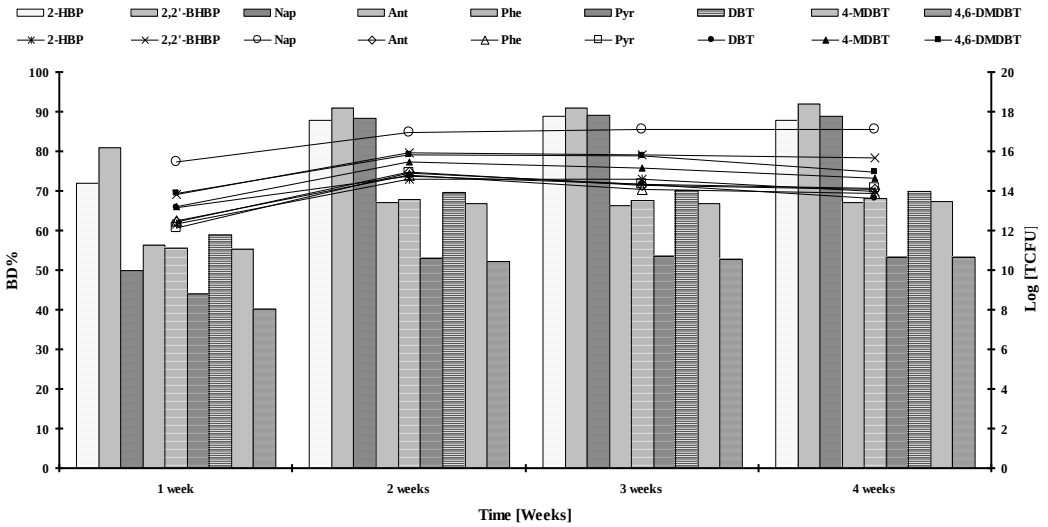
The quinone, Pyrene-4, 5-dione can be also formed following the non-enzymatic autoxidation of 4, 5-dihydroxypyrene.

Pyrene-4,5-dione may be reduced back to 4,5-dihydroxypyrene by quinone reductase (PQR), as reported for *Mycobacterium* sp.(Kim et al., 2004 and Liang et al., 2006). This might explain the abundance of 4, 5-dihydroxypyrene and pyrene-4, 5-dione peaks, respectively in the GC/MS chromatogram. The presence of the quinone, pyrene-4,5-dione might explain the observed pink coloration occurred in the cultures.

4. Conclusion

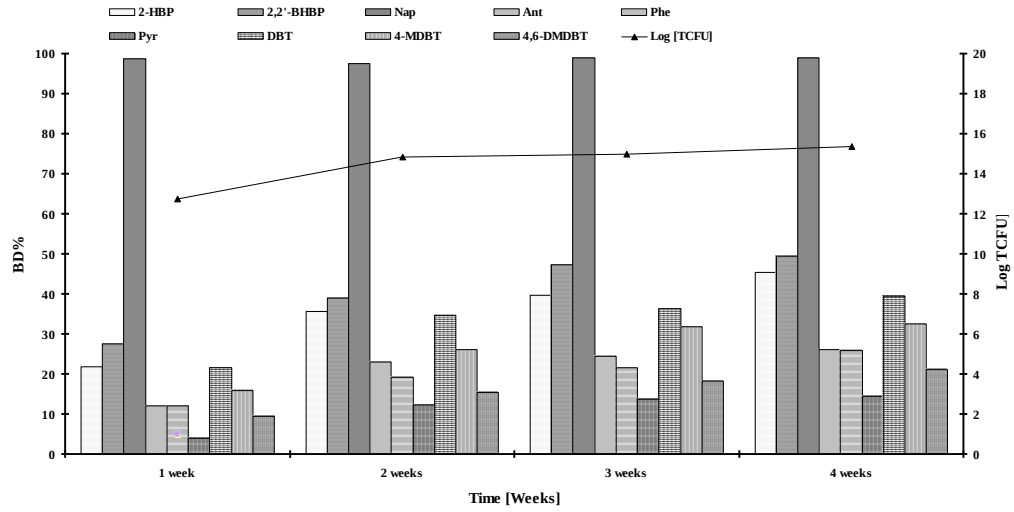
Corynbacterium variabilis Sh42 isolated from El-Lessan Area of Damietta River Nile Branch in Egypt has a high capability to metabolism of different PACs (phenolic, PAHs and PASHs) and utilizes them as a carbon and energy source for its growth either in single or mixed substrates cultures. While the biodegradation rate decreased in mixed substrate culture than single substrate cultures for all PACs, this might attribute to inhibition effect of metabolic enzymatic system. Also, BD% decrease with increasing ring size, within homologous series and BD potentials can be ranked in the following decreasing order; phenolic > PAHs > PASHs. In addition, *C. variabilis* Sh42 has capability to completely metabolism of 2,2'-BHBP through meta-

cleavage pathway, DBT through 4S pathway and pyrene which also involve phenanthrene, naphthalene and o-phethalate metabolic pathways.



Legend: columns represented BD% while lines represented growth potentials.

Figure (1): Effect of different incubation periods on growth potential and PACs BD efficiency of *Corynebacterium variabilis* sp. Sh42 in single substrate cultures.



Legend: columns represented BD% while lines represented growth potentials.

Figure (2): Effect of different incubation periods on growth potential and PACs BD efficiency of *Corynebacterium variabilis* sp. Sh42 in mixed substrate cultures.

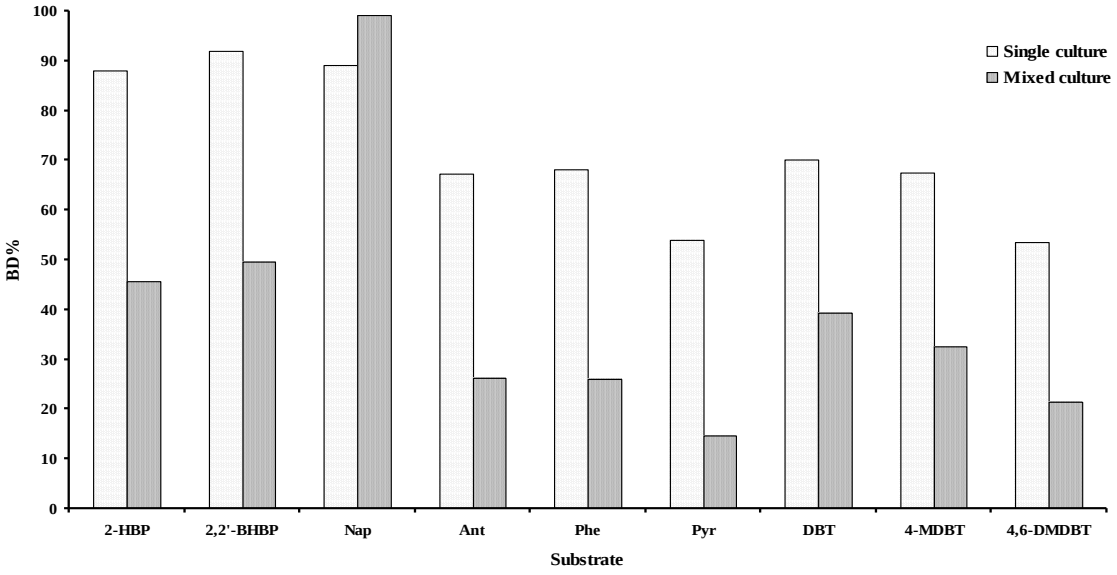


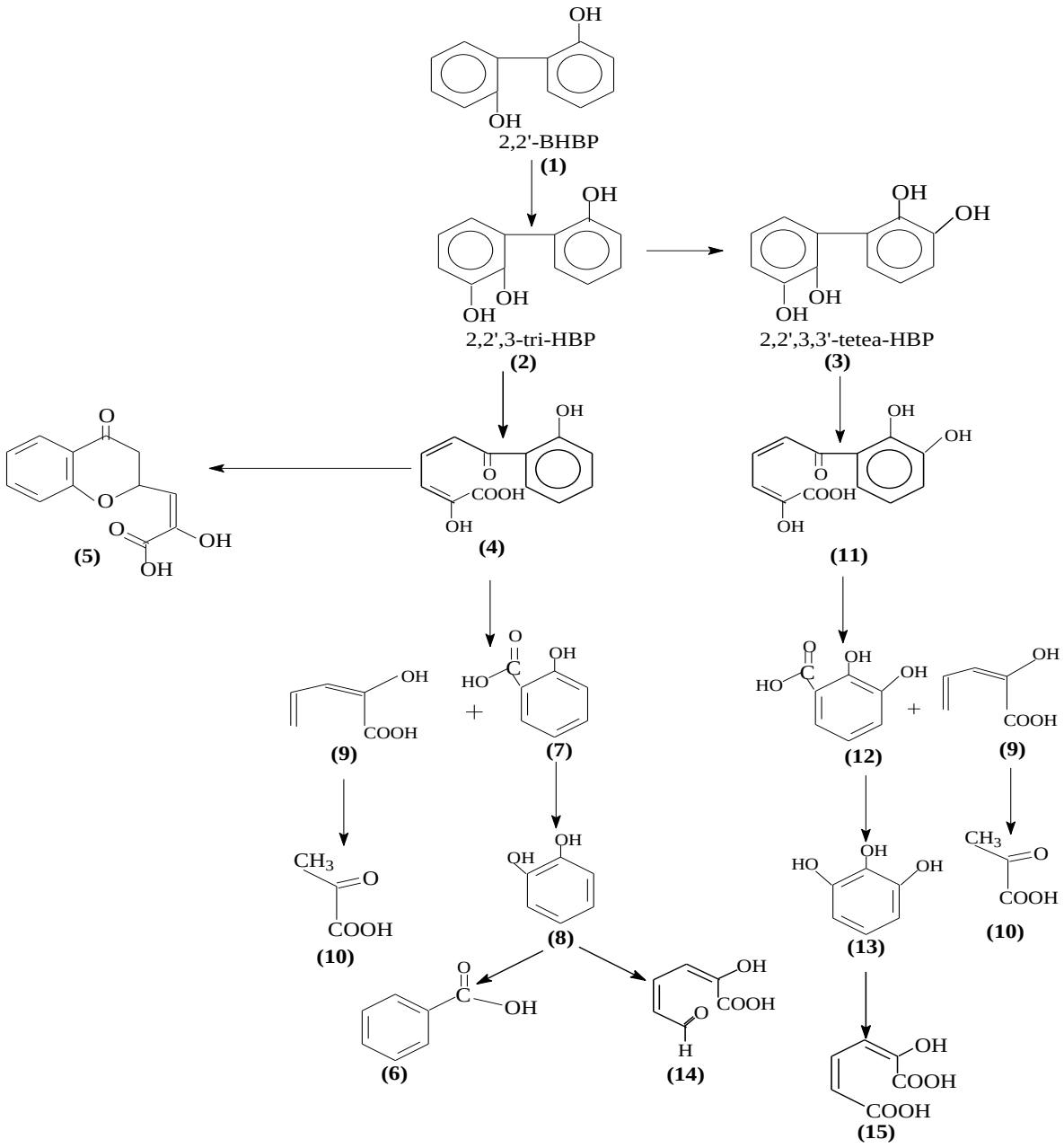
Figure (3): Comparison between biodegradation efficiencies of different PACs in single and mixed substrate cultures.

Table (2): The biodegradation percent (BD %) of different PACs compounds in single and mixed substrate cultures after different incubation periods.

Period [weeks]	1 week		2 weeks		3 weeks		4 weeks	
	BD%							
Culture	Single	Mixed	Single	Mixed	Single	Mixed	Single	Mixed
2-HBP	72	21.9	88	35.8	89	39.7	88	45.6
2,2'-BHBP	81	27.7	91	39.1	91	47.3	92	49.6
Nap	49.9	97.5	88.4	98.9	89	99.1	89	99.2
Ant	56.3	12.1	65.5	23.2	67	24.5	67.3	26.2
Phe	55.7	12.2	67	19.4	67.8	21.8	68.1	25.9
Pyr	44	4.1	53	12.3	53	13.9	54	14.7
DBT	58.9	21.8	69.7	34.7	70	36.3	70	39.4
4-MDBT	55.4	16	66.8	26.3	67	32	67.4	32.6
4,6-DMDBT	40.3	9.5	52.2	15.4	53	18.2	53.5	21.3

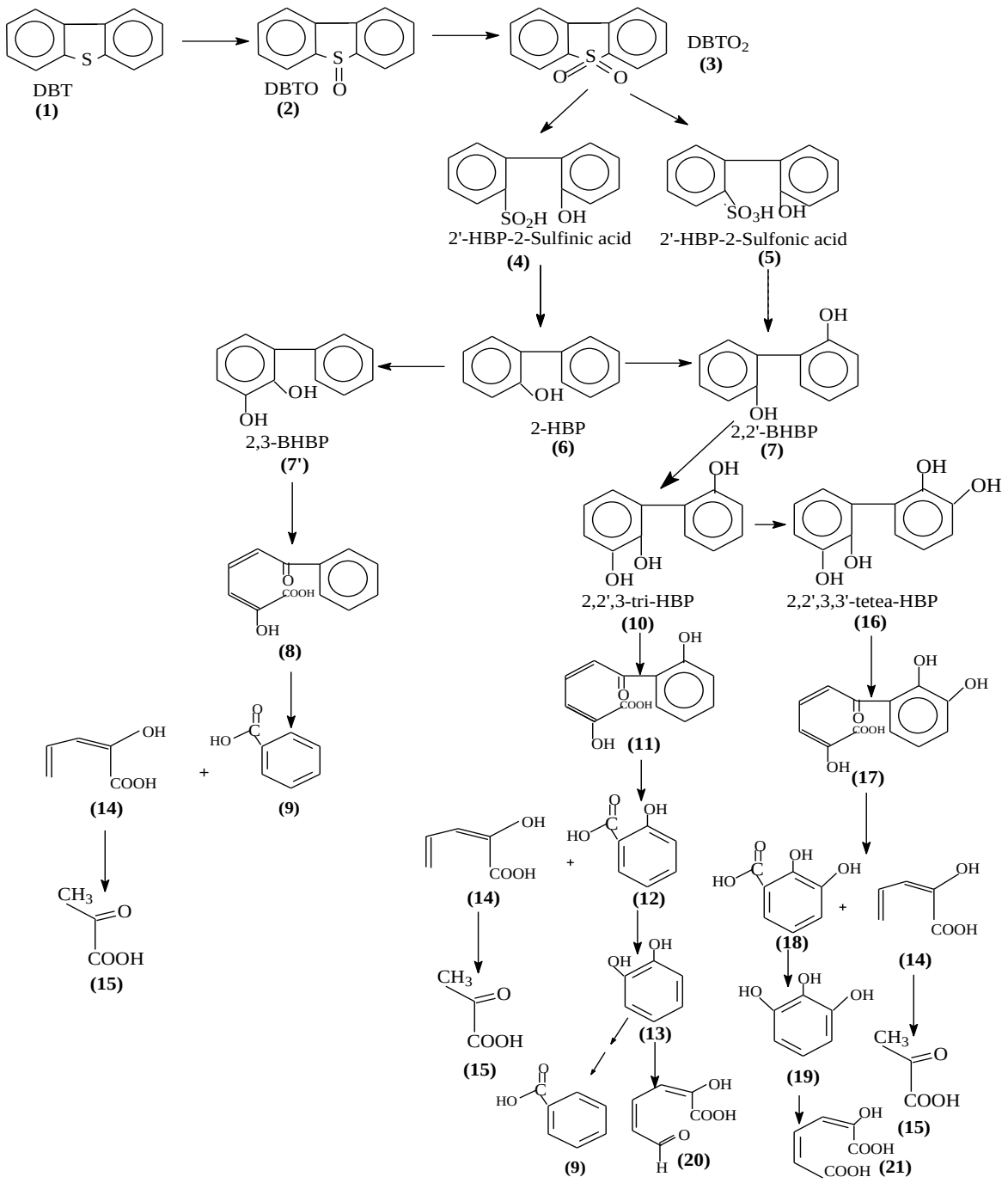
Table (3): The average biodegradation percent (average BD %) of different PACs groups in single and mixed substrate cultures after different incubation periods.

Compounds	Phenolic		PAHs		PASHs	
Time [weeks]	BD Average%					
	Single	Mixed	Single	Mixed	Single	Mixed
1 week	76.5	24.83	51.5	29.31	51.53	15.73
2 weeks	89	37.41	69.15	38.11	62.9	25.47
3 weeks	90	43.48	69.3	39.83	63.37	28.84
4 weeks	90	47.61	69.48	41.47	63.6	31.09



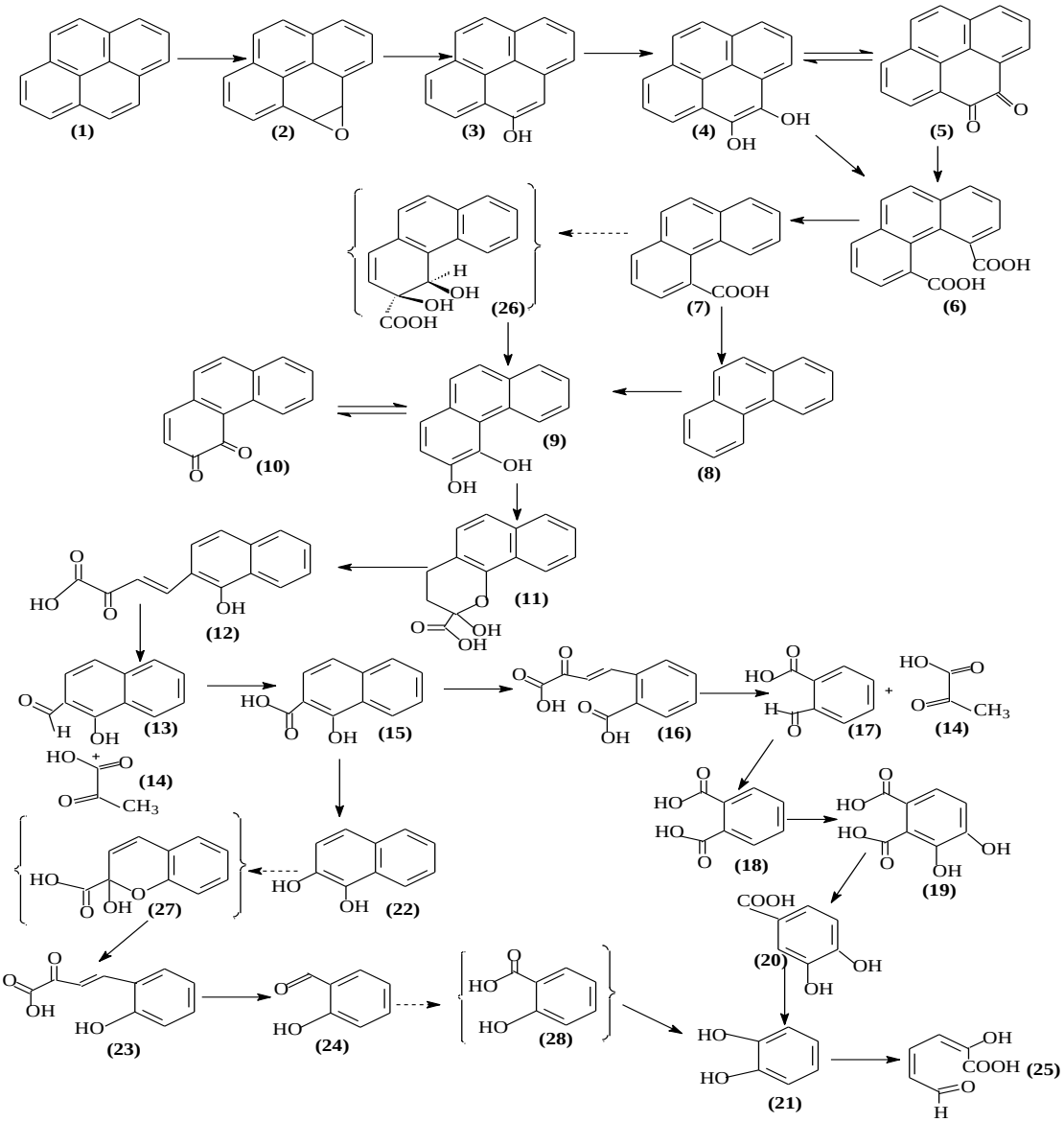
Legend: **1**, 2,2'-bihydroxybiphenyl; **2,** 2,2',3-trihydroxybiphenyl; **3**, 2,2',3,3'-tetrahydroxybiphenyl; **4**, 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid; **5**, 3-(chroman-4-on-2-yl)pyruvate; **6**, benzoic acid; **7**, salicylic acid; **8**, catechol; **9**, 2-hydroxy-2,4-pentadienoic acid; **10,** pyruvic acid; **11**, 2-hydroxy-6-(2,3-dihydroxyphenyl)-6-oxo-2,4-hexadienoic acid; **12**, 2,3-dihydroxybenzoic acid; **13,** pyrogallol; **14**, 2-hydroxymuconic semialdehyde; **15**, 2-hydroxymuconic acid.

Figure (4): Proposed pathway for the metabolism of 2,2'-bihydroxybiphenyl by *Corynebacterium variabilis* sp. Sh42.



Legend: **1**, dibenzothiophene (DBT); **2,** DBT-sulfoxide; **3**, DBT-sulfone; **4**,2-hydroxybiphenyl sulfinic acid; **5**, 2-hydroxybiphenyl sulfuric acid; **6**,2-hydroxybiphenyl; **7**, 2,2'-bihydroxybiphenyl; **7'**, 2,3-bihydroxybiphenyl; **8**, 2-hydroxy-6-oxo-phenylhexa-2,4-dienoic acid;**9**, benzoic acid; **10,** 2,2',3-trihydroxybiphenyl; **11**, 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid; **12**, salicylic acid; **13,** catechol; **14**, 2-hydroxy-2,4-pentadienoic acid; **15**, pyruvic acid; **16**, 2,2',3,3'-tetrahydroxybipheny; **17**, 2-hydroxy-6-(2,3-dihydroxyphenyl)-6-oxo-2,4-hexadienoic acid; **18**, 2,3-dihydroxybenzoic acid; **19**, pyrogallol; **20**, 2-hydroxymuconic semialdehyde; **21**, 2-hydroxymuconic acid.

Figure (5): Proposed pathway for the metabolism of dibenzothiophene by *Corynebacterium variabilis* sp. Sh42.



Legend: 1, Pyrene; 2, Pyrene-4,5-oxide; 3, Pyrene-5-ol; 4, Pyrene-4,5-diol; 5, Pyrene-4,5-dione; 6, Phenanthrene-4,5-dicarboxylic acid; 7, Phenanthrene-4-carboxylic acid; 8, Phenanthrene; 9, 3,4-dihydroxyphenanthrenr; 10, Phenanthrene-3,4-dione; 11, 2-hydroxy-2H-benzo[h]chromene-2-carboxylic acid; 12, *Trans*-4-(1'-hydroxynaphth-2'-y1)-2-oxobut-3-enoic acid; 13, 1-hydroxy-2-naphthaldehyde; 14, Pyruvic acid; 15, 1-hydroxy-2-naphthoic acid; 16, *Trans*-2'-carboxybenzapyruvic acid; 17, 2-carboxybenzaldehyde; 18, Phthalic acid; 19, 3,4-dihydroxyphthalic acid; 20, 3,4-dihydroxybenzoic acid; 21, Catechol; 22, 1,2-dihydroxynaphthalene; 23, *Trans*-o-hydroxybenzylidenepyruvic acid (tHBPA); 24, Salicylaldehyde; 25, 2-hydroxymuconic semialdehyde; 26, 3,4-dihydroxy-3,4-dihydro-phenanthrene-4-carboxylic acid; 27, 2-hydroxychromene-2-carboxylate (HCCA); 28, Salicylic acid.

Figure (6): Proposed pathway for the metabolism of Pyrene by *Corynebacterium variabilis* sp. Sh42.

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Abbreviation

2,2'-BHBP	2,2'-bihydroxybiphenyl
2-HBP	2-hydroxybiphenyl
4,6-DMDBT	4,6-dimethyldibenzothiophene
4-MDBT	4-methyldibenzothiophene
Ace	Acetonitrile
Ant	Anthracene
BD	Biodegradation
BSM	Basal salt medium
DBT	Dibenzothiophene
Flu	Fluoranthene
Nap	Naphthalene
PACs	Poly aromatic compounds
PAHs	Poly aromatic hydrocarbons
PASHs	Poly aromatic sulfur heterocyclic
Phe	Phenanthrene
Pyr	Pyrene
TCFU	Total colony forming unit
TGY	Tryptone glucose yeast extract
W	Water

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