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Biphenyl degradation by *Paenibacillus* sp. PRNK-6: follows a characteristic lower metabolic pathway

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Abstract

The metabolic pathway involved in the assimilation of biphenyl in the facultative anaerobic bacterium *Paenibacillus* SP. PRNK-6 was studied, as the sole carbon source at an optimized concentration of 400 mgL^{-1}. The strain utilized 90.42% of biphenyl within 72 hours of incubation. The assimilation of biphenyl in the PRNK-6 strain began with the initiation of di-oxygenation, leading to the formation of cis-2,3-dihydro-2,3-dihydroxybiphenyl, which by undergoing dehydrogenation forms 2,3-dihydroxybiphenyl. The di-hydroxybiphenyl further upon ring cleavage at the meta position forms benzoic acid and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate. Further benzoic acid undergoes hydroxylation to give 4-hydroxybenzoic acid; a current novel metabolite of the study. The 4-hydroxybenzoic acid upon further hydroxylation and subsequent degradation of protocatechuate and catechol was obtained, as characterized by analyzing the metabolites using HPLC and GC-MS. A probable metabolic route was postulated by identifying metabolites using GC-MS and HPLC, the growth on pathway intermediates, and the specific activity of key enzymes.

Key words : *Paenibacillus* sp. PRNK-6, biphenyl, assimilation, metabolic pathway, ring cleavage.

Highlights

- The strain *Paenibacillus* sp. PRNK-6 strain was evaluated for biphenyl degradation.
- The strain PRNK-6 degraded 90.42% of biphenyl within 72 hours of incubation.
- The metabolites were characterized by HPLC and GC-MS.
- A potential metabolic pathway for the degradation of biphenyl in the PRNK-6 strain was proposed, which followed a lower benzoic acid pathway.

Biphenyl has been commonly employed as a biodegradable substitute for polychlorinated biphenyls (PCBs) in biodegradation research[20]. PCBs are a group of synthetic organic

chemicals that contain a biphenyl structure with 1–10 hydrogen atoms replaced by chlorine, resulting in up to 209 different congeners. Because of their chemical and physical properties, PCBs have been extensively produced[11] Thus large release of PCBs into the environment[14,19]. The relative volatility of PCBs contributes to their spread throughout the globe[5] where they accumulate in living organisms and increase in concentration at higher trophic levels in the food chain. Exposure to PCBs has been demonstrated to result in a wide variety of health effects[15] and are categorized as carcinogens. Because of their chemical stability, poor water solubility, and toxicity, PCBs are considered recalcitrant toxics[3]. Many reports are available on the degradation of biphenyl, where some reports fail to interpret the lower degradation pathway, which differs from organism to organism. The biphenyl degradation is initiated by forming a cis-dihyrodiol, which upon further dehydrogenation dihydroxy biphenyl is obtained by dehydrogenation[4,6]. Further upon ring cleavage 2-hydroxy-6- keto-6-phenylhexa-2,4-dienoic acid (HOPDA) is formed, which further undergoes cleavage to form benzoic acid and a few carbon compound[13]. Strategies for bioremediation of PCBs have been mainly focused on metagenomic studies on consortium[3] degradation but focusing on the pure culture, and there is still a need of the hour to know the dead-end pathways occurring in individual cultures, the degradation pathways of occurring down to the benzoic acid in biphenyl degradation pathways and enzymes responsible for the converting those to end products entering into the TCA cycle. The present study gives an understanding of the metabolic pathway of the biphenyl. In the lower biphenyl pathway, the benzoic acid has been reported by the formation of 4-hydroxybenzoic and by hydroxylation of benzoic acid is a unique pathway reported in this genus. In the current study, the metabolic versatility of the bacterium *Paenibacillus* sp. PRNK-6 was investigated analyzing its ability to degrade biphenyl as the sole source of carbon and energy, the metabolic pathway of biphenyl in PRNK-6 was deduced via metabolic intermediates analysis and utilization of metabolic intermediates and inducible enzymes involved in degradation.

Chemicals :

Biphenyl, 2,3-dihydroxybiphenyl, benzoic acid, protocatechuic acid (PCA), 4-hydroxybenzoic acid, catechol, gentisic acid, and *N*-Methyl-*N*- (trimethylsilyl) trifluoroacetamide (MSTFA,98.5%) were purchased from Sigma-Aldrich (Mumbai). A bovine serum album (BSA), anhydrous sodium sulfate was obtained from Himedia. The solvents, such as ethyl acetate, methanol, and acetone, were of HPLC grade. All the other chemicals were of the highest purity.

Media and cultural conditions :

All the experiments were performed in a mineral salt medium (MSM) with the following composition (gL^{-1}): K_2HPO_4 (6.3), KH_2PO_4 (1.8), NH_4NO_3 (1.0), $MgSO_4.7H_2O$ (0.1), $CaCl_2$ (0.1), $FeSO_4.7H_2O$ (0.1), $MnSO_4.4H_2O$ (0.1), and $Na_2MoO_4.2H_2O$ (0.006) at pH 7.0. All the components were dissolved in 1L of distilled water and pH was adjusted to 7. The media was autoclaved at 121°C and 15 psi for about 20 min. The biphenyl dissolved in acetone of respective

concentration was added to the flasks in hot conditions to vaporize acetone.1% culture was added and incubated at 180 rpm and 30 °C of the temperature under shaking conditions (Lab tech). The culture purity was checked by spreading on Luria-Bertani (LB) agar medium (BERTANI 1951).

Adaptation of bacterial cells for biphenyl degradation :

The previously isolated *Paenibacillus* sp. PRNK-6 strain in our laboratory (Reddy et al., 2018) was grown aerobically in 250 ml Erlenmeyer conical flasks containing 100 ml MSM. The flasks were supplemented with 100 mgL^{-1} of biphenyl dissolved in acetone as the sole source of carbon and energy. The flasks were then incubated for 120 hours at 30°C on a rotary shaker at 180 rpm. To obtain the effective biphenyl degrading culture for about 30 days, the subcultures were performed every 5 days. This well-adopted culture was used for further biphenyl degradation studies.

Optimization of concentration for biphenyl degradation :

To investigate the optimal degradation concentration by PRNK-6, the strain was cultivated in a 250 ml flask containing 100 ml of sterile MSM. Biphenyl (80–400 mg L^{-1}) was supplied to the flasks. As an inoculum, 2 ml of culture from mid-log phase developed cells (O.D-0.6) at 660 nm was used. The culture flasks were incubated for 120 hours at 30 °C with a shaking condition of 180 rpm.

Growth and utilization of biphenyl :

The utilization of biphenyl by PRNK-6 was studied by growing it in a 250-ml flask with 100 ml of sterile MSM and an optimized concentration of 400 mgL^{-1} of Biphenyl. The flasks were agitated in a shaking incubator at 30°C and 180 rpm. The biphenyl cells of the mid-log phase were used as inoculum (2%). Parallelly substrate controls were also incubated identically to assess the abiotic loss of the biphenyl.

The growth of PRNK-6 on biphenyl was monitored in terms of colony-forming units (CFU) and the residual concentration of biphenyl in the liquid medium was determined by using HPLC.

Analytical techniques :

Metabolite extraction :

To isolate the metabolites formed during the biphenyl degradation, PRNK-6 was grown in 100ml of MSM, containing acetone-dissolved biphenyl of 400 mg L^{-1} concentration at 30 °C and 180rpm for 36 h (mid-log phase). The culture was centrifuged (8000 rpm for 10 min) and cells were washed with phosphate buffer of 100 mM: pH 7.2 and resuspended in the same buffer. The washed cells were transferred to flasks containing buffer (100 ml) with 1500 mg L^{-1} of biphenyl. The flasks were incubated in a shaker at 180 rpm and a temperature of 30 °C. At every 24 h of incubation, the contents of the flasks were removed and used for the extraction of metabolites. The supernatant was extracted thrice the volume of ethyl acetate to extract a neutral fraction and to get an acidic fraction, the pH of the aqueous phase was converted to 2.0 with 2N HCl. All the fractions were pooled together, dried using anhydrous sodium sulfate, and

concentrated to 1 ml under vacuum (Heidolph Rotary Evaporator, Laborota 4000) and used for quantification by HPLC. A concentrated 1ml solution was filtered using 0.25um filter paper and directly used for HPLC analysis and GC-MS analysis involved derivatization using *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA)[16].

Metabolites analysis using HPLC *and* LC-MS *:*

The analysis of metabolites was carried out using High-performance liquid chromatography (HPLC). HPLC (waters-2489, 515 Binary pumps with C-18 Sunfire column 250mm × 4.6mm, particle size 5µm). The solvent system was degassed and vacuum filtered through 0.45 µm membrane filters. The detection wavelength was set at 254 nm with a mobile phase composed of a gradient solvent mixture comprising methanol and water (50-100%, v/v) at 1.0 ml min^{-1} flow rate for about 30 min of runtime.

GC-MS analyses were carried out using a Shimadzu GCMS-TQ8050 instrument. A DB-5 capillary column (J&W Scientific, Folson, CA, USA) measuring 30 m in length by 0.25 mm in internal diameter and 0.25 µm in film thickness was used for the GC separation. The GC was operated for one minute at 29 pressure using pulsed splitless injection, and the oven temperature gradient was set to rise from 50 °C to 200 °C for two minutes, hold for one minute, and then ramp to 15 °C/min before reaching 280 °C (18.07 min). The temperature at the inflow was 250 °C. Helium was employed as the carrier gas, and a constant flow rate of 1.2 mL/min was maintained. They made use of the electronic pressure control.

The GC-MS interface was maintained at 280 °C in temperature. The MS was run in electron impact (EI) ionization mode, with an electron energy of 70 eV. Between 50 and 400 amu (atom to mass unit) were scanned for suitable masses in chosen ion monitoring. Without standards, a GC-MS NIST library search was used to confirm the metabolites.

Enzyme assays of biphenyl degradation :

The PRNK-6 strain was grown in 100 ml mineral salt medium supplemented with 400 mg ml^{-1} of biphenyl. The cells were harvested in the mid-log growth phase by centrifugation at 8,000 rpm for 10 min, washed twice with 50mM phosphate buffer (pH 7.2), and re-suspended in the same buffer. The cells obtained were disrupted by sonication (6 cycles of 30 s each with 1 min off cycle for probe cooling) with a probe-type sonic oscillator (Sonics Vibra-cell). The cell suspension was kept in an ice bath during sonic treatments. The cell debris was removed by centrifugation at 12,000 rpm for 20 min. The cell supernatant obtained was used as a source of crude extracts.

The activity of Protocatechuate 3,4-dioxygenase was determined by the method of Mcdonald and Stanier[9]. The benzoate-4-hydroxylase activity was determined using the method outlined by McNamee and Don[10]. The activity of 4-Hydroxybenzoate hydroxylase was assessed as per the method described by Crawford[1]. The activity of gentisate 1,2-dioxygenase was assayed by Crawford's[1] method. The Catechol 1,2-dioxygenase by

Hegeman (1966) method and Catechol 2,3-dioxygenase was assayed according to the method of Nayak *et al*.,[12]. The specific activity of the enzyme was expressed as µmol of substrate oxidized or product formed min^{-1} mg^{-1} of protein under assay conditions. The protein concentration of the enzyme extracts was measured according to Lowry *et al*.[8] using BSA as a standard.

Growth of Paenibacillus sp. *PRNK 6 on metabolic intermediates of the biphenyl degradation pathway :*

Strain PRNK-6 was screened for the utilization of intermediates that occur in the biphenyl pathway as confirmed by HPLC and GC-MS, such as 2,3-dihydroxy biphenyl, benzoic acid, 4-hydroxybenzoic acid, catechol, and protocatechuic acid. The bacterium was grown in MSM (pH 7) with an appropriate carbon source (400mg l^{-1}) added to the flask. The culture was then incubated on a rotary shaker at 30°C (180 rpm). The growth PRNK-6 on the respective aromatic compounds was monitored by measuring the optical density at 660 nm.

Optimization of biphenyl concentration :

The optical density of the flasks with various biphenyl concentrations was evaluated. After 72 hours, the turbidity of every flask was higher, and the lag phase period was the shortest. Compared to the other flasks with lower biphenyl concentrations, the ones with the 400 mg L^{-1} concentration had the highest optical density after 72 hours. After 72 hours of incubation, the stationary phase is observed. Therefore, the optimal concentration for additional degradation research is the one with the maximum optical density. Fig. 1 displays the optical densities of PRNK-6 acquired for various concentrations of biphenyl with different incubation periods.

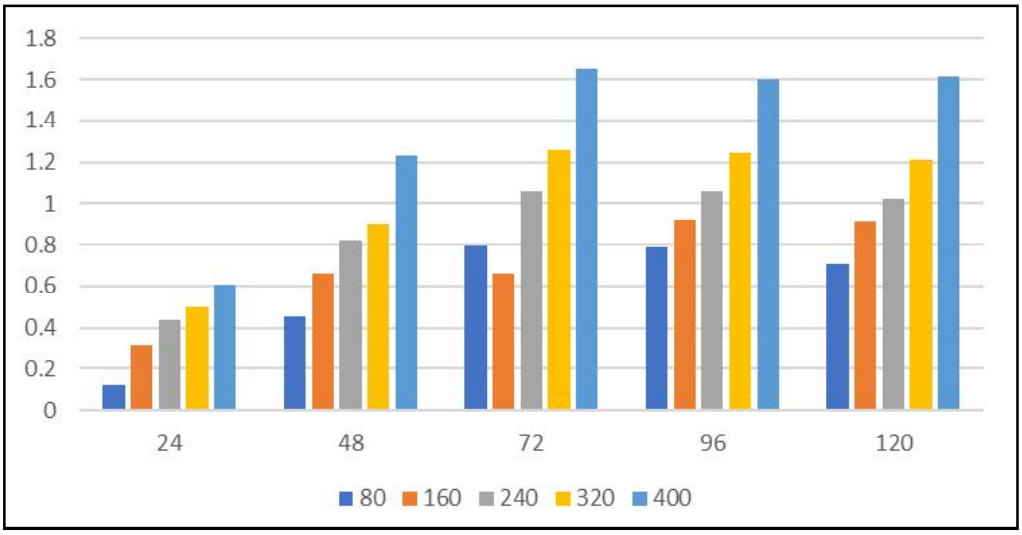


Fig. 1. The growth of PRNK-6 when grown on increasing concentrations of biphenyl.

Growth and utilization of biphenyl :

Paenibacillus sp. PRNK-6 was able to use biphenyl as a carbon source. Growth started immediately with no observed lag phase. Maximum growth occurred at 60 hours of incubation. A time-course study indicated a decrease in biphenyl concentration in the culture medium, accompanied by an increase in cell number (Fig. 2). The 90.42% of biphenyl (400 mg L^{-1}) was degraded within 72 hours of incubation. However, the degradation rate decreased significantly after that.

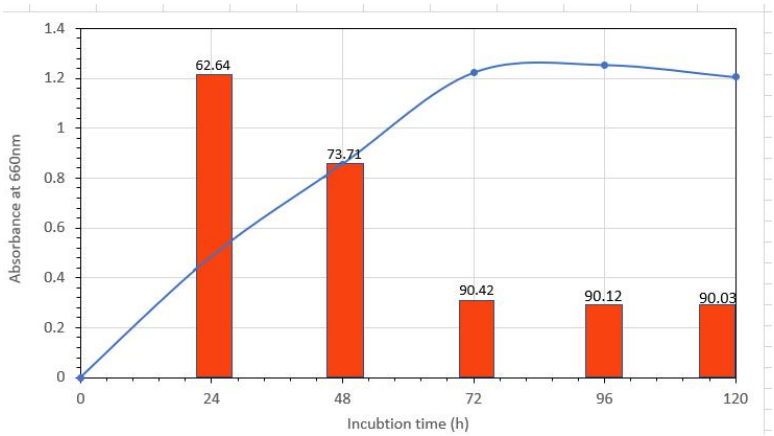


Fig. 2. Utilization of biphenyl by *Paenibacillus* sp. PRNK-6

Biphenyl metabolites characterization :

HPLC analysis of the metabolite extract showed 3 peaks, with identical retention times as the standards: Benzoic acid, 4-hydroxybenzoic acid, and Biphenyl (Fig. 3) and (Table-1).

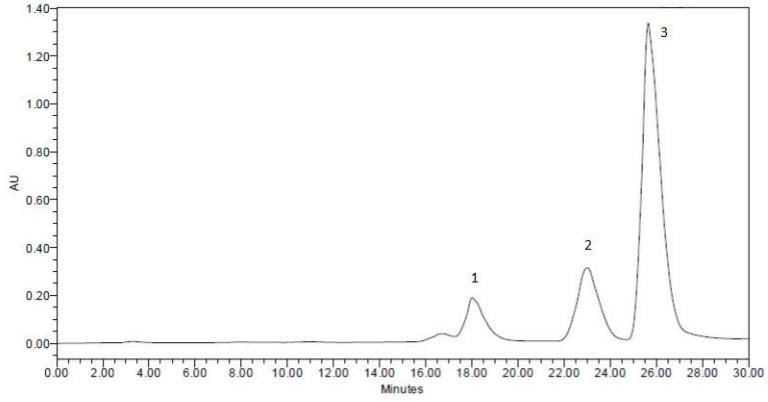


Fig. 3. High-performance liquid chromatography (HPLC) profile of the metabolite extract of the bacterium ***Paenibacillus* sp. PRNK-6** incubated with Biphenyl. 1: Benzoic acid, 2: 4-hydroxybenzoic acid and 3: Biphenyl.

Table-1. The retention time of the metabolite extract of the *Paenibacillus* sp. PRNK-6 culture incubated with biphenyl and the standard compounds.

Metabolite	Retention time (R_t) (min)		Compound
	Metabolite	Standard	
1	17.88	18.01	Benzoic acid
2	23.52	23.82	4-hydroxybenzoic acid
3	26.55	26.80	Biphenyl

The metabolite identities were further confirmed through GC-MS analysis. **Metabolite I** exhibited a base peak at m/z 332 (M+), with subsequent peaks at 154.08 (M^+-TMS+O_2×2), 129.07 (M^+-C_2H_2), and 65.04 (M^+-C_2H_5). These findings are consistent with the structure of 2,3-dihydroxybiphenyl. **Metabolite II** displayed the m/z peak at 259.1(M^+) followed by 219.01 (M^+-C_3H_4), 149.04 (M^+-TMS), 76.12. This fragmentation pattern corresponds to 2-Hydroxypenta-2,4-dienoate. The MS fragmentation pattern of **Metabolite III** clearly showed an m/z of 237, corresponding specifically to the TMS derivative of 4-hydroxybenzoic acid. Furthermore, distinct fragment ions were identified at m/z 223.06 (M^+-CH_3), 134.02 (M^+-C_3H_9O Si), 108.05 (M^+-Si), 77.04 (M^+-CH_2O), and 64.03 (M^+-CH) (Table-2).

Table-2. GC-MS metabolites formed during the biphenyl degradation by *Paenibacillus* sp. PRNK-6.

Meta-bolite	Retention time (min)	Identified metabolite	Mol. wt.	Major m/z (%) peaks*
I	8.14	2,3-dihydroxybiphenyl	186.21	332.14(M^+6.7), 154.08(100), 129.07(8.75), 65.04(7)
II	9.20	2-Hydroxypenta-2,4-dienoate	114.10	259.11(M^+100), 219.21(18.9), 149.04(3.3),76.12(2.6)
III	11.61	4-hydroxybenzoic acid	138.12	237.04(M^+-100), 223.06(90), 134.02(9.2),108.05(7.6), 77.04(76)

* TMS derivatized

Enzymatic studies :

The enzyme extract from PRNK-6, when grown on Biphenyl, 2,3-dihydroxybenzoic acid, benzoic acid, and catechol as a carbon source, demonstrated activity in enzymes such as 2,3-dihydroxybiphenyl dioxygenase, benzoate 4-hydroxylase, 4-hydroxybenzoate hydroxylase, and catechol 1,2-dioxygenase. However, it did not exhibit the activities of catechol 2,3-dioxygenase and gentisate 1,2-dioxygenase (Table-3). Additionally, the crude enzyme prepared from cells grown on glucose did not show the same enzyme activities as mentioned earlier.

Table-3. Specific activities of enzymes in cell-free extracts of *Paenibacillus* sp. PRNK-6 grown on Biphenyl,2,3-dihydroxy biphenyl, benzoic acid, and catechol.

Enzymes	Specific activity (μmol min^{-1} mg^{-1})			
	Biphenyl	2,3-Dihydroxy biphenyl	Benzoic acid	Catechol
2,3-Dihydroxybiphenyl 1,2-dioxygenase	0.65±0.05	0.68±0.07	0.58±0.05	0.48±0.03
Benzoate 4-hydroxylase	0.56±0.02	0.44±0.04	0.32±0.05	0.29±0.01
4-Hydroxybenzoate hydroxylase	0.46±0.05	0.42±0.02	0.39±0.03	0.36±0.08
Protocatechaute-3,4-dioxygenase	0.38±0.01	0.45±0.03	0.32±0.09	0.28±0.05
Catechol-1,2-dioxygease	0.28±0.03	0.26±0.09	0.24±0.07	0.25±0.03
Catechol,2,3-dioxygenase	ND	ND	ND	ND
Gentisate-1,2-dioxygenase	ND	ND	ND	ND

*ND not detected

Growth on metabolic intermediates of biphenyl pathway :

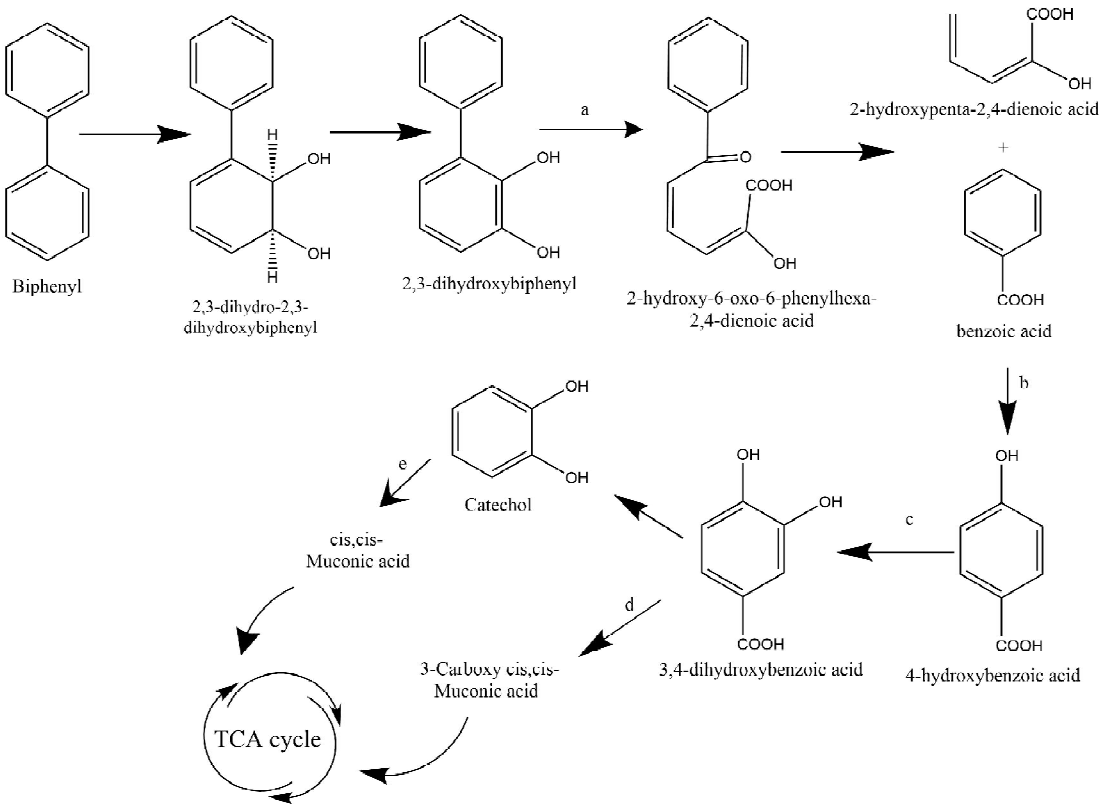
Apart from biphenyl, the strain PRNK-6 was able to utilize intermediates that occurred in the biphenyl degradation of the present study like benzoic acid, 4-dihydroxybenzoic acid protocatechuic acid, and catechol as the sole source of carbon and energy (Table-4).

Table-4. Growth of PRNK-6 on metabolic intermediates of biphenyl degradation

Substrate	O.D at 660nm
2,3-Dihydroxybiphenyl	+++
Benzoic acid	+++
4-Hydroxybenzoic acid	+++
Protocatechuate	+++
Catechol	++

Note: +++-excellent growth, ++-good growth

The metabolic versatility of microbes plays a crucial role in recycling matter and detoxifying harmful compounds[17]. *Paenibacillus* sp. is found to be involved in the degradation of many PAHs like fluorene, phenanthrene, pyrene, etc., and utilization of various aromatic hydrocarbons like phthalic acid, benzoic acid, protocatechuate, catechol, benzoic acid, 4-hydroxybenzoic acid showed the metabolic versatility and potential application of strain PRNK-6. The biodegradation of aromatic compounds is divided into two main steps the peripheral and the central pathways: microorganisms degrade diverse aromatic compounds via peripheral pathways into key common metabolic intermediates like benzoate, catechol protocatechuate[2,17]. The central pathways then cleave the aromatic ring and convert these into central metabolites such as succinyl-CoA, pyruvate, and acetyl CoA, which are used to grow the microbes. Thus, biodegradation forms an attractive strategy for the growth of microbes which limits the genetic burden which is essential for encoding the degradation of various natural as well as synthetic compounds. In the current study strain PRNK-6, the upper pathway transformed the biphenyl into multiple metabolic intermediates (benzoic acid,



a: 2,3-dihydroxybiphenyl dioxygenase, b: Benzoate-4-hydroxylase, c: 4-hydroxybenzoate hydroxylase, d: Protocatechuate-3,4-dioxygenase, e: Catechol 1,2 dioxygenase

Fig. 4. Putative pathway proposed for the catabolism of Biphenyl by PRNK-6.

di-hydroxybiphenyl, catechol, etc.,). The use of these intermediates in the central pathway has been thoroughly studied, including the benzoate pathway, hydroxyquinol pathway, and beta-ketoadipate pathway. In the present study, the dihydrodiol intermediate formed by the di-oxygenation of biphenyl undergoes dehydro-genation to form 2,3-dihydroxybiphenyl (**Metabolite-I**) which is confirmed by the results of GC-MS. The 2,3-dihydrobiphenyl upon ring cleavage at meta-position by extra-diol dioxygenase forms 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) a meta-cleavage product. Further benzoic acid and 2-hydroxypenta-2,4-dienoate (**Metabolite-II**) were the hydrolysis product of HOPDA. The HOPDA is a yellow color intermediate formed after 24 hours of incubation when analyzed using a UV-visible spectrophotometer at 434nm. The HOPDA further upon degradation forms benzoic acid. The presence of benzoic acid in the culture extracts of biphenyl-grown media HPLC and the activity of benzoate-4-hydroxylase in biphenyl,2,3-dihydroxybiphenyl, benzoic acid, and catechol confirms this conversion. Thus, formed benzoic acid gets

hydroxylated to form 4-hydroxybenzoic acid (**Metabolite -III)** by the action of enzyme 4-hydroxybenzoate hydroxylase and on further degradation forms protocatechuic acid and catechol sequentially[17]. The occurrence of 4-hydroxybenzoic acid, protocatechuic acid, and catechol in the metabolite extracts of biphenyl and their utilization of the same intermediates by strain PRNK-6 (Table-4) accounts for this sequential conversion. The terminal metabolite catechol upon ring cleavage by the action of catechol-1,2-dioxygenase, from the cell-free extracts of biphenyl, 2,3-dihydroxybiphenyl, benzoate, and catechol as the sole source of carbon and energy.

The present study shows the remarkable catabolic versatility of *Paenibacillus* sp. PRNK-6 in biphenyl degradation. The biphenyl degradation proceeds through initial di-oxygenation, forming di-hydroxybiphenyl, entering the benzoic acid pathway, and generating TCA cycle intermediates. An exciting highlight of this work is the unique conversion of benzoic acid, leading to the formation of 4-hydroxybenzoic acid and protocatechuate, representing a novel pathway for the facultative anaerobic bacteria, *Paenibacillus* sp. PRNK-6. The lower pathway down to the benzoic acid pathway elucidation is strongly supported by the analysis of metabolite extracts of biphenyl and the inducible enzymes responsible for catalysis, as well as the utilization of metabolic intermediates. Thus, the bacterium could be an ideal candidate for revitalizing environments contaminated with biphenyl/PCBs.

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