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ORIGINAL PAPER

Isolation and characterization of alkalotolerant *Pseudomonas* sp. strain ISTDF1 for degradation of dibenzofuran

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Abstract An alkalotolerant *Pseudomonas* strain was enriched and isolated from effluent of the pulp and paper industry. This strain was able to degrade dibenzofuran and utilize it as a sole source of energy and carbon. The GC–MS based detection of various intermediary metabolites of biodegradation suggested the involvement of angular as well as lateral pathway of dibenzofuran biodegradation. The GC–MS based detection of various intermediary metabolites of biodegradation suggested the involvement of angular as well as lateral pathway of dibenzofuran biodegradation. This diverse dioxygenation property of the strain allowed it to utilize various recalcitrant chlorinated xenobiotics and PAHs compounds. This strain showed optimum utilization (~85%) of dibenzofuran (200 mg l^{-1}) within 36 h at pH 10 at 40°C. The growth of the strain was supported by a wide range of environmental conditions such as temperature, pH, and concentration of dibenzofuran, suggesting that it can be used for in situ bioremediation of dioxin-like compound.

Keywords Alkalotolerant · Biodegradation · Dibenzofuran · GC–MS · *Pseudomonas* sp. strain ISTDF1

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Introduction

Dioxins belong to a family of chlorinated biphenyl ethers that is known to be one of the most toxic groups of persistent organic pollutants (POPs), with carcinogenic, immunosuppressive, endocrine disruptive, and teratogenic properties [2, 9, 22]. Dioxins are formed as by-products of various industrial and municipal activities. Effluent from the pulp and paper industry is known to be one of the major sources for dioxin contamination [3, 19, 31]. Dioxins are recalcitrant to degradation because of their planar structure, strong sorption with soil, and their acute hydrophobic nature [24]. The highly alkaline nature of the effluent, which allows very limited microbial growth, limits the biodegradation process [11].

Therefore, it necessitates the search for alkalotolerant bacterial strains for biodegradation of dioxin. Dibenzofuran has been used as a model compound for the degradation of dioxin-like compounds. Many bacterial strains have already been isolated, characterized, and evaluated for degradation of dibenzofuran such as *Cunninghamella elegans* [5], *Pseudomonas* sp. strain HH69 [6], *Brevibacterium* sp. strain DPO 1361 [30], *Sphingomonas* sp. strain RW1 [35], *Pseudomonas aeruginosa* and *Xanthomonas maltophilia* [14], *Pseudomonas putida* PH-01 [10], and *Terrabacter* sp. strain YK3 [12].

Despite the prerequisite condition, no alkalotolerant bacterial strain has been well explored for in situ bioremediation of dioxin-like compounds in the effluent like that from pulp and paper. Dibenzofurans are metabolized through two different modes of initial dioxygenation: angular dioxygenation and lateral dioxygenation. In angular dioxygenation, *cis*-dihydroxylation takes place at the 4,4a-position of dibenzofuran and its carbon–oxygen (C–O) bond is broken, aerobically forming unstable phenolic hemiacetal

which results in the formation of 2,2′,3-trihydroxybiphenyl as the first stable intermediate metabolite [6, 8, 14, 25, 27, 28, 30, 35]. The cleavage of 2,2′,3-trihydroxybiphenyl leads to the formation of 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid. This intermediary metabolite was oxidized and the carbon–carbon bond was cleavaged and salicylic acid and 2-hydroxypenta-2,4-dienoic acid were formed [18]. Catechol was formed from the hydrolysis of salicylic acid and its further dihydroxylation leads to the formation of *cis*–muconoate that absorbed the tricorboxylic cycle [6]. In lateral dioxygenation, carbon–carbon (C–C) bonds are dihydroxylated at C1–C2 and C3–C4 positions of dibenzofuran and generate three dihydro-dihydroxy-dibenzofuran isomers as lateral dioxygenation.

Some naphthalene and biphenyl metabolizing-bacteria have been reported for the metabolism of dibenzofuran through both angular and lateral degradation pathways [23]. However, any detailed characterization of alkalotolerant bacteria for degradation of dibenzofuran are yet to be reported.

Therefore, the objectives of the present study were to isolate and characterize an alkalotolerant bacterium from the native bacterial community from pulp and paper industrial waste and investigate the degradation pathway, including the study of some factors affecting the biodegradation of dibenzofuran.

Materials and methods

Chemicals

Dibenzofuran and N, O-bis(trimethyl-silyl)trifluroacetamide (BSTFA) were procured from Sigma-Aldrich. All solvents and other chemicals (GC–MS grade) were purchased from Merck, India.

Sampling site and environmental sample

Sludge and sediment sample was collected from Century Pulp and Paper Mill, Lalkua, Nainital, Uttaranchal, India (29° 24′ N, 79° 28′ E). The effluent discharging canals in the premises of the industry were selected as sampling site.

Enrichment of microorganisms

A chemostat culture was set in a 2-l glass vessel, effective volume 1 l, with culture condition as stirring at 150 rpm; temperature at 30°C; and pH 10 in the mineral salt medium (MSM). The composition of MSM (g l^{-1}) was: Na_2CO_3, 4; $Na_2HPO_4{\cdot}2H_2O$, 7.8; KH_2PO_4, 6.8; $MgSO_4$, 0.2, ammonium ferric acetate, 0.01; $Ca(NO_3)_2.4H_2O$, 0.05; $NaNO_3$, 0.085, trace element solution with dibenzofuran (100 mg l^{-1}) as described [15, 33, 34]. The dibenzofuran was dissolved with DMSO (100 mM) in MSM [7]. The effluent (50 ml) of the pulp and paper industry containing bacterial cells populations was served as inoculums in the chemostat.

After of stabilization of growth pattern, 100 µl of the bacterial community was poured into an MSM agar plate with dibenzofuran (100 mg l^{-1}) as described by Thakur [33]. Only two types of bacterial colony had appeared after 12 h. Both bacterial strains were tested for dibenzofuran degradation from pH 6 to 11 in MSM. The culture medium was removed after 0, 6, 12, 18, 24, and 30 h for determining the growth pattern and utilization of dibenzofuran. The growth patterns of both bacterial strains were taken at O.D. at 595 nm with a spectrophotometer (Cary, 100 Bio, Varian Co, Australia) from pH 6 to 11 as described by Fortnagel et al. [6]. One of the two bacterial strains, 'ISTDF1', survived in the given pH range without losing its degradation ability of dibenzofuran, which indicated alkalotolerant ability. Therefore, this strain was selected for further study.

Morphological and biochemical properties

The morphologically distinct isolates were identified by morphological, physiological, and chemo-taxonomical properties in accordance with Bergy's Manual of Determinative Bacteriology [26]. For morphology of bacterial strain, bacterial colony was picked and fixed with buffer containing glutaraldehyde for scanning electron microscopy (SEM). After fixation, sample was dehydrated by gradually increasing ethanol percentage starting from 30, 50, and 90% for 10 min each and in absolute ethanol for 24 h. Samples were vacuum-dried followed by gold shadowing. The sample was observed under SEM.

Growth on recalcitrant aromatic pollutant

Bacterial growth was qualitatively tested over important recalcitrant chlorinated aromatic pollutant such as naphthalene, carbazole, dibenzothiophene, benzo(α)pyrene, fluorene, 2, 4-D and pentachlorophenol as a sole carbon source in MSM agar plates with 1 mM concentration at pH 10.

Identification of bacteria by 16S rRNA gene

The individual colony was picked up and purified, and cultured on mineral salt medium having dibenzofuran (1 mM) as the sole carbon source. Genomic DNA from the bacterial strain showing degradation potential was isolated with the Genome DNA Kit (Qiagen Inc., USA) as described by the manufacturer. The 16S rRNA gene was amplified from genomic DNA by using PCR with universal primers

5′-GAGAGTTTGATCCTGGCTCAG–3′ (forward) and 5′-CTACGGCT ACCTTGTTACGA-3′ (reverse) [30]. PCR amplification was done using primer with a DNA thermal cycler under the following conditions: 10–20 ng template DNA, 5 µl 10× reaction buffer, 2.5 U Taq DNA polymerase, 1 µM forward primer, 1 µM reverse primer, 200 µM of each dNTP and H_2O combined in a total volume of 50 µl. The tubes were incubated at 94°C for 5 min and then subjected to the following thermal cycling program: denaturation at 94°C for 2 min, primer annealing at 55°C for 2 min, and chain extension at 72°C for 2 min with an additional extension time of 10 min on the final cycle, for a total of 30 cycles. The amplified DNA was purified using Qiaquick PCR Purification Kit (Qiagen Inc., USA), adjusted to 200 ng μl^{-1} and cloned in the pDrive (Qiagen Inc., USA) and sent to the M/S Bangalore Genei for sequencing. Sequenced data was compared and analyzed with the existing database of GenBank, National Center for Biotechnology Information. A phylogenetic tree was drawn on the basis of the sequences. A bootstrap consensus tree (1,000 copies) was drawn by multiple sequence alignment with Neighbor-Joining method using software Mega, version 3.1 with different species of bacteria [20].

Utilization of dibenzofuran

All studies were carried out containing aerobic conditions and batch cultivation with initial inoculum 0.3 O.D. at 595 nm containing mineral salt medium and dibenzofuran (100 g l^{-1}) dissolved in dimethyl sulfoxide (100 mM) at pH 10 in a 500-ml flask. The culture supernatants (25 ml) were collected after different time intervals at 0, 6, 12, 18, 24, 30, and 36 h. The bacterial cells were removed by centrifugation at 8,000×*g* for 10 min. The culture medium was extracted with double volume ethyl acetate, followed by acidification with 6 N HCl to approximately pH 2.0 as described by Iida et al. [12]. The organic phase (extract) was separated by a separating funnel, and the extract was finally concentrated on a rotary evaporator. Ethyl acetate was evaporated and the residue was re-dissolved in 100 µl of acetonitrile. The concentration of dibenzofuran was identified by using gas chromatography (GC) (GC-Perkin-Elmer Instruments Model Auto System XL) equipped with a capillary column DB5 (dimension 0.25-mm film thickness × 0.25-mm internal diameter × 30 m in length). One microliter of each extract was analyzed by GC at condition (splitless mode; initial temperature 80°C for 1.5 min; temperature increased 80–230°C at a rate of 20°C min^{-1} and 230–250°C and kept it at 250°C for 4.5 min) [12]. The concentration was derived from a standard plot between peak area and concentration of dibenzofuran as shown with GC with flame ionizing detector (FID).

Elucidation of biodegradation pathways for dibenzofuran

Pathways were elucidated based on the intermediary metabolites. For metabolite identification, the extracted metabolites formed as described above after degradation of dibenzofuran were derivatized as described by Iida et al. [12]. In brief, derivatization of extracted metabolite was done by adding 10 µl of bis-(trimethylsilyl) trifluoroacetamide (BSTFA) and put in air-tight glass tubes of 3 ml at 70°C for 45 min. Metabolites were derivatized to prevent binding of free hydroxyl group of metabolites with the column.

Intermediate metabolites of dibenzofuran were identified by using gas chromatography-mass spectroscopy (GC-MS) (Varian) in the same condition as descried above. The metabolites were identified by fragmentation pattern and compared with the inbuilt standard chemical library system in GC-MS.

Statistical analysis

Normality test, equal variance test, and multiple comparisons versus control group (Holm-Sidak method) were performed for level of significance in biodegradation experiments using the statistical package SigmaPlot 11.0. All Samples were taken in triplicate and expressed as mean with standard deviation (±5%). For optimization experiments, single-factor ANOVA was performed in Microsoft™ Excel, 2003.

Results

Enrichment, isolation, and characterization of alkalotolerant bacterium

When the effluent was directly poured on at neutral pH on an LB agar plate with 10^{-3} serial dilution, 14 types of morphological different strains were found. Whereas, in DF-enrichment cultures (with added effluent), only two types of strain survived, designated as ‘ISTDF’ and ‘ISTDF1’. The bacterium ‘ISTDF1’ was found to have better degradation capability versus the bacterium ‘ISTDF’. Therefore, the former was selected for further analysis. The bacterium ‘ISTDF1’, showed substrate-dependent growth over dibenzofuran and it degraded dibenzofuran (100 mg) almost completely within 30 h at pH 10 and 40°C (Fig. 1).

Morphological, biochemical, and growth properties of alkalotolerant bacterium

The colony of bacterium ISTDF1 obtained from chemostat enriched at pH 10 was cultured on MSM containing dibenzofuran and its colony was found to be yellow in color, smooth, convex, and opaque. From biochemical testing, it

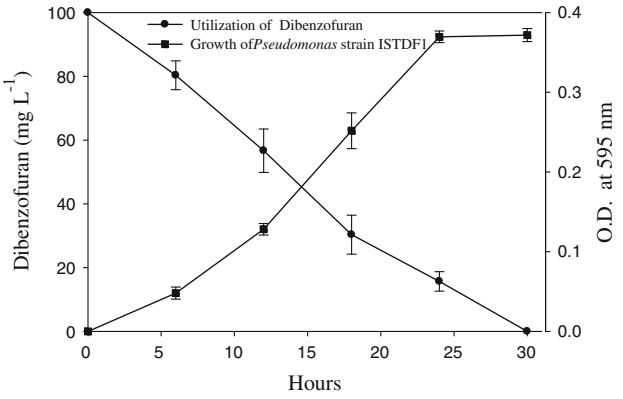

Fig. 1 Growth of *Pseudomonas* sp. strain ISTDF1 for degradation of dibenzofuran (100 mg l^{-1}) as the sole carbon source in mineral salt medium in shake flask culture at pH 10 and 40°C. Values are means of three replicates ± standard deviations

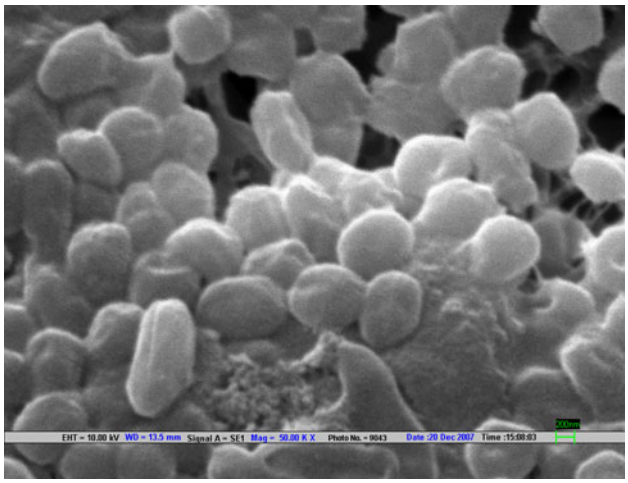

Fig. 2 Scanning electron micrograph (*SEM*) of *Pseudomonas* sp. strain ISTDF1. *Scale* represents 200 nm

was confirmed that this strain was Gram-negative, oxidase-positive, catalase-positive, indole-negative, urease-negative and citrate-positive. The size and shape of the cells was determined by scanning electron microscopy. The size of the bacterial cell was about 1 µm × 0.4 µm (Fig. 2). Cells were elongated and rod-shaped. This bacteria showed growth over important recalcitrant chlorinated aromatic compounds as well as some important PAHs such as naphthalene, carbazole, dibenzothiophene, benzo(α)pyrene, fluorene, 2, 4-D, pentachlorophenol as the sole carbon source with 1 mM concentration in MSM agar plates after 12 h as has been given (Table 1).

16S rDNA-based identification of dibenzofuran-degrading alkalotolerant bacterium

16S ribosomal gene sequence of bacterium ISTDF1 was compared with the sequence of already reported strains, and

Table 1 Comparative growth properties on different carbon sources by *Pseudomonas* sp. strain ISTDF1

Aromatic pollutants	Qualitative growth[a]
Naphthalene	++
Carbazole	++++
Dibenzothiophene	++++
Benzo(α)pyrene	+++
Fluorene	+++
Phthalate	++
2, 4 D	+++
Pentachlorophenol	+++

Symbol '+' indicates the number of colonies visibly apparent

[a] Qualitative assessment of the ability of strain ISTDF1 to grow on MSM agar plate with different aromatic pollutants with 1 mM as the sole carbon source after 12 h of incubation at 25°C

a phylogeny tree was constructed by MEGA 3.1 (Fig. 3). BLAST search (http://ncbi.nlm.nih.gov/blast.cgi) of the sequences of the present study showed maximum homology with *Pseudomonas aeruginosa* L-4 [accession number EU170480]. Finally, it was characterized and designated as *Pseudomonas* sp. strain ISTDF1. Sequence (accession number EU834943) was submitted to Genbank (N.C.B.I.). As the dendogram reflects, strain ISTDF1 showed its close relatedness with *Pseudomonas resinovorans* [accession number AB088750], a well-known dibenzofuran-degrading bacterium DBF-MAK [accession number AB086228].

Optimum growth condition of bacterium for biodegradation of dibenzofuran

The growth conditions were optimized with different parameters such as different concentration of dibenzofuran, pH, and temperature. Results indicated that the strain ISTDF1 was a mesophilic bacterium because it showed significant metabolic activity from temperature range of 25–40°C. The data of the study indicated that degradation activity gradually increased from 25 to 40°C but beyond 40°C, the degradation was drastically reduced. The maximum and optimal temperature was 40°C as strain ISTDF1 degraded dibenzofuran (100 mg l^{-1}) completely as a carbon source within different time intervals (Fig. 4).

The effect of pH was also identified as an influencing factor for the biodegradation process of dibenzofuran. The initial pH was set from 6 to 11. Strain ISTDF1 showed its maximum growth and degradation at pH 10 (Fig. 5). Degradation was gradually increased from pH 6 to 12. Beyond initial pH 10, strain ISTDF1 had shown a sharp decline in growth and hence the reduced degradation of dibenzofuran was detected. No bacterial growth and survival were observed lower than pH 6 and higher than pH 12.

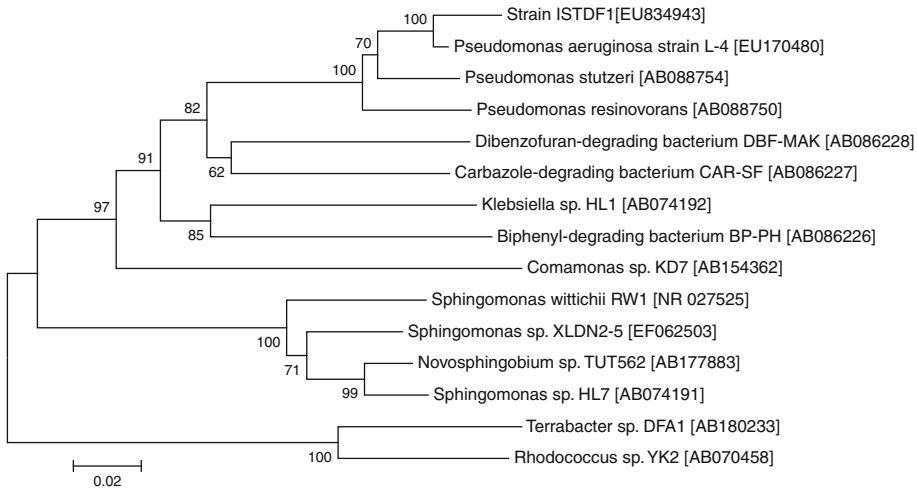

Fig. 3 16S rDNA-based phylogenetic identification of dibenzofuran-degrading alkalotolerant bacterium. The sequence of strain ISTDF1 is represented as strain ISTDF1 [EU834943], which characterized as *Pseudomonas* sp. strain ISTDF1

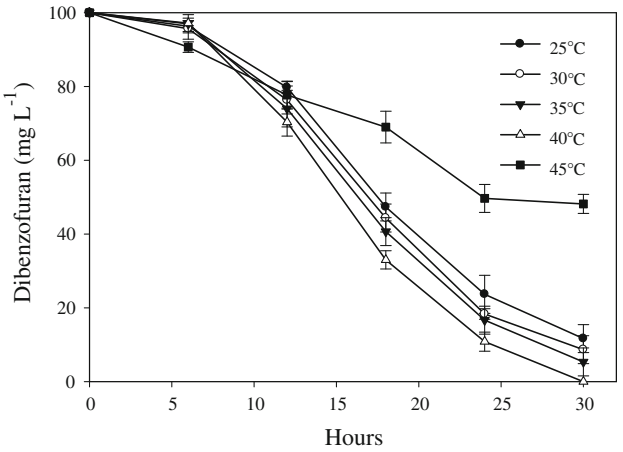

Fig. 4 Effect of temperature on biodegradation of DF by *Pseudomonas* sp. strain ISTDF1. The values are means of three replicates ± standard deviations

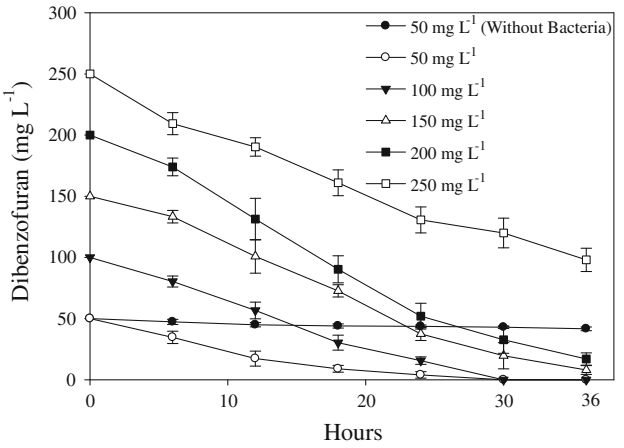

Fig. 6 Biodegradation of different concentrations of DF by *Pseudomonas* sp. strain ISTDF1. The values are means of three replicates ± standard deviations

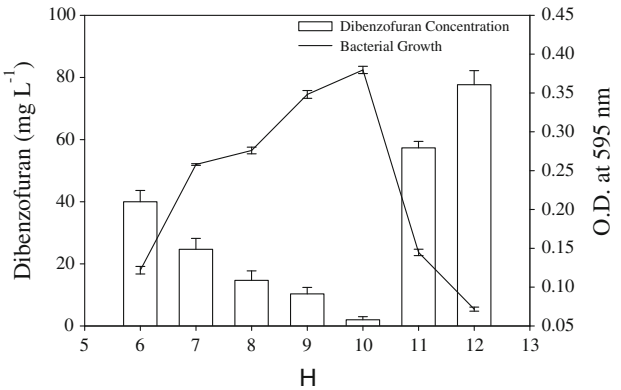

Fig. 5 Effect of pH on biodegradation of DF by *Pseudomonas* sp. strain ISTDF1. The values are means of three replicates ± standard deviations

Different concentrations of DF were tested in flask culture containing minimal salt medium for utilization potency of bacterium. Statistical analysis was attached as supplementary information. In this analysis, group comparison test indicated that in 150, 200, and 250 mg of dibenzofuran the p value was <0.001, whereas the p value was 0.04 for 100 mg. At $p < 0.050$, all treated samples were found to be significant. *Pseudomonas* sp. strain ISTDF1 showed maximum degradation at 85% of 200 mg l^{-1} DF within 36 h (Fig. 6). The variance of strain ISTDF1-treated dibenzofuran (200 mg) was found to be higher than 50, 100, 150, and 250 mg of dibenzofuran indicating the 200 mg was the optimum concentration for degradation. The F value (9.2) is greater than F_{crit} (2.4) and p value (9.91×10^{-06}) was very near zero, indicating the level of degradation of dibenzofuran by the strain ISTDF1 was significant.

The result of the study indicated utilization of most of the dibenzofuran in lower concentration of dibenzofuran (50 mg l^{-1}) at 24 h. Strain ISTDF1 took a longer time for degradation of dibenzofuran once the concentration was increased (more than 100 mg l^{-1}). In absence of bacterial inoculum, almost no degradation of dibenzofuran

(50 mg l^{-1}) was observed. Initially, the degradation rate was found to be higher with increasing concentration of dibenzofuran, which gradually declined with time. Strain ISTDF1 demonstrated an extended lag phase, resulting in slower degradation, probably because of the toxicity due to accumulated intermediary metabolites (data not shown).

Intermediate metabolites and proposed degradation pathway of dibenzofuran

The metabolites were extracted from MSM and elsewhere characterized by using gas chromatography-mass spectroscopy. Degradation of dibenzofuran was not observed after extraction and analysis of metabolites in the MSM without bacteria. The metabolites extracted and analyzed from culture broth of bacterial strains (*Pseudomonas* sp. strain ISTDF1) indicated several dominant peaks which were identified by fragmentation patterns generated by mass spectroscopy. Fragmentation patterns were matched with a built-in molecule library. The results indicated that dibenzofuran was degraded and produced many and diverse intermediary metabolites. These intermediary metabolites were identified by mass-to-charge fragmentation pattern and their relative intensities as identified and described earlier [6, 16, 20, 27].

Metabolites were identified as 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4 hexadienoic acid or 2′-OH-HOPDA (C) at RT 18.248 min., gentisic acid (E) at RT 8.945 min., catechol (F) at RT 6.643 min., 2-(1-carbonyl methylidine)-2,3-dihydrobenzofuranlidene at RT 24.941 min. and 2′hydroxy-4-(3′-oxo-3′*H*-benzofuran-2′-yliden)but-2-enoic acid or 3′-HOBB at RT 19.167 min as shown in Table 2. From various metabolites, 2-OH-HOPDA, gentisic acid, catechol are representative metabolites of angular dioxygenation, whereas 3′-HOBB and 2-(1-carbonyl methylidine)-2,3-dihydrobenzofuranlidene are signature metabolites of lateral metabolites (Table 2). Interestingly, this data indicated the involvement of both pathways in the same strain. On the basis of intermediary metabolites, pathways for degradation of dibenzofuran were proposed (Fig. 7).

Time-dependent formation of different metabolites was analyzed (Fig. 8). In the first 6 h, the signature metabolite for angular (C4–C4a hydroxylation) and lateral metabolites

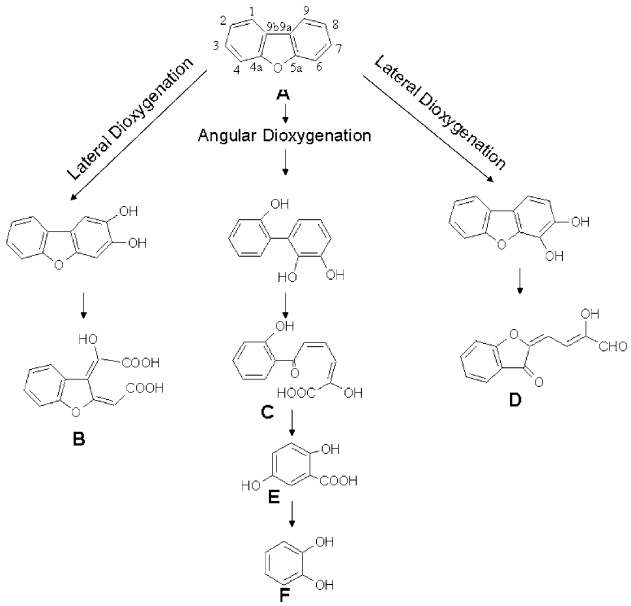

Fig. 7 Proposed metabolic pathway for degradation of dibenzofuran by *Pseudomonas* sp. strain ISTDF1

Table 2 Different metabolites detected in GC-MS after degradation of dibenzofuran by *Pseudomonas* sp. Strain ISTDF1 at pH 10 as indicated in Fig. 7

Metabolites	Compound	Retention time[c]	Molecular weight	Fragmentation pattern[d]	References
A	Dibenzofuran	10.115	168	168(100), 139(65), 94(25)	[27]
CC	2-OH-HOPDA[a]	18.248	450 (silylated)	450(15), 435(12) 407(7), 333(100), 257(17), 147(52), 73(40)	[16, 20]
E	Gentisic acid	8.945	370 (silylated)	370(32), 282(48), 194(14), 106(23), 73(100)	[6, 16]
F	Catechol	6.643	254 (silylated)	254(40), 166(75), 94(37), 73(100)	[27]
B	2-(1-carbonyl methylidine)-2, 3-dihydrobenzofuranlidene	24.941	464 (silylated)	464(12), 376(25), 259(100), 147(15), 73(30)	[20]
D	3′-HOBB[b]	19.167	376 (silylated)	376(10), 364(25), 333(10), 259(100), 203(25), 147(45)	[20]

[a] 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4 hexadienoic acid

[b] 2′hydroxy-4-(3′-oxo-3′*H*-benzofuran-2′-yliden)but-2-enoic acid

[c] In minutes

[d] Fragmentation pattern shown in terms of mass and charge ratio (*m/z*) with their relative intensity in percentage of different metabolites shown in '()' as detected by GC-MS

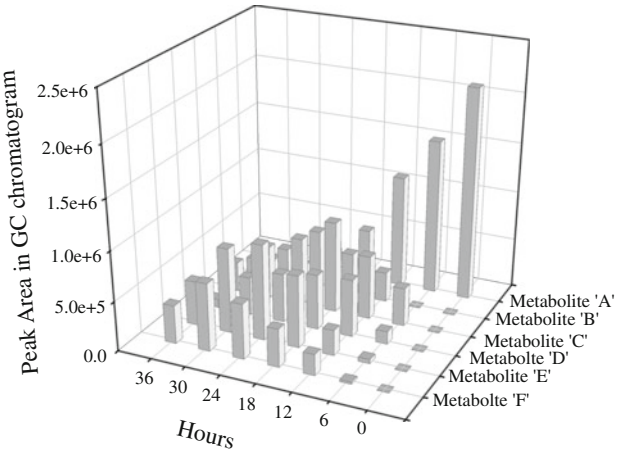

Fig. 8 Time-dependent formation of different intermediary metabolites (as characterized in Fig. 7) during degradation of dibenzofuran by *Pseudomonas* sp. strain ISTDF1

(C1–C2 hydroxylation) were detected to be 2′-OH-HOPDA and 3′-HOBB. At 12 -h formation 2-(1-carbonyl methylidine)-2,3-dihydrobenzofuranlidene was detected, which indicated the involvement of lateral degradation at C3–C4 hydroxylation in dibenzofuran. Various metabolites of lower pathways of degradation such as gentisic acid and catechol were also detected, which increase with time initially and decrease after 24 h, indicating the complete degradation of dibenzofuran.

Discussion

The pulp and paper industry is known as one of the important polluting sources for one of the most recalcitrant compounds—dioxin [4, 32]. Only a few bacteria have been reported so far that can grow directly on chlorinated dioxins such as *Sphingomonas* sp. strain. RW1 and recently reported *Pseudomonas veronii* PH-03 were already reported to grow on chlorinated dioxin. However, growth on 1,2,3,4-tetrachlorodibenzo-p-dioxin of strain PH-03 was shown to be poor, presumably due to the production of a toxic metabolite, 3,4,5,6-tetrachlorocatechol [10, 35].

One of the biggest challenges for in situ bioremediation of dioxin-like compounds is the highly alkaline nature of its effluent. As the effluent of the pulp and paper industry contains high organic load with toxic and recalcitrant compounds in alkaline condition, so it was very likely to get native bacterial strain/s which would be useful for dioxin degradation [13]. In the present study, an alkalotolerant bacterium was isolated after continuous enrichment having the capability to degrade dibenzofuran in alkaline condition.

For degradation of dioxin-like compounds, pH, temperature, and concentration of dibenzofuran are among most important abiotic parameters [1, 16, 17]. Therefore, we had taken these parameters for optimization after stabilizing the community at pH 10 as shown in Figs. 4, 5, and 6. The strain ISTDF1 showed the most statistically significant degradation of dibenzofuran (200 mg l^{-1}) in 36 h at pH 10 at 40°C, which is quite comparable with previously reported strains such as *Pseudomonas* sp. strain HH69 [6], *Sphingomonas* sp. strain RW1 [35], and *Terrabacter* sp. strain YK3 [12].

This strain also showed growth over important aromatic pollutants such as naphthalene, carbazole, dibenzothiophene, benzo(α)pyrene, fluorene, and halogenated pollutants such as 2, 4-D, pentachlorophenol, as shown in Table 1. This suggested that the strain might have a very robust system for degradation of aromatic pollutant, which needs to be explored. The biochemical properties (Gram-negative, oxidase-positive, catalase-positive, indole-negative, urease-negative, and citrate-positive) of elongated and rod-shaped strain ISTDF1 indicated the similarity with *Pseudomonas* sp. as shown in Fig. 2.

16S ribosomal DNA-based characterization of strain ISTDF1 appears to be closely related to *Pseudomonas* and showing close homology of 98% to *Pseudomonas aeruginosa* L-4 [accession number EU170480], which was already reported for biodegradation of aromatic pollutant. Strain ISTDF1 has also been shown to be closely related to previously known dibenzofuran degrading bacteria such as *Pseudomonas resinovorans* [AB088750], dibenzofuran-degrading bacterium DBF-MAK [AB086228], carbazole-degrading bacterium CAR-SF [AB086227], and biphenyl-degrading bacterium BP-PH [AB086226], as shown in Fig. 3. However, further characterization up to the species level of *Pseudomonas* sp. strain ISTDF1 will be done by fatty acid methyl ester (FAME) composition analysis. It is already known that *Pseudomonas* sp. are among the most diverse and can metabolize a wide range of xenobiotic compounds [29].

The catabolic pathway for degradation of dibenzofuran has been studied thoroughly in various microorganisms such as *Pseudomonas* sp. strain HH69 [6], *Sphingomonas* sp. strain RW1 [31], and *Terrabacter* sp. strain YK3 [10]. These bacterial strains have been reported to metabolize dibenzofuran as a carbon source and energy. Initial dioxygenation of dibenzofuran is the most important single step to destabilize the ring to lose its planner structure. In angular pathway, hydroxylation occurs at the carbon attached to the oxygen of the heterocycle (4, 4a position). Strain ISTDF1 metabolized dibenzofuran by formation of intermediate signature compound such as 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2, 4 hexadienoic acid, catechol and gentisic acid, whereas 2, 2′,3 trihydroxybiphenyl was not detected. This may have happened because of the accelerated

cleavage by bacterial enzymes because of its catechol moiety.

In the lateral pathway of strain ISTDF1, vicinal carbon is hydroxylated which is not attached to the oxygen and form three dihydroxydibenzofuran isomers. The dihydroxylated-dibenzofuran was hydrolyzed and cleaved into 2-(1-carbonyl methylidine)-2, 3-dihydrobenzofuranlidene, which was further hydroxylated and formed 2′hydroxy-4-(3′-oxo-3′*H*-benzofuran-2′-yliden)but-2-enoic acid. A biphenyl (BP)-utilizing *Pseudomonas putida* has already been reported to be capable of transforming dibenzofuran (DBF) via a lateral dioxygenation and *meta*-cleavage pathway. The ring cleavage product 2-hydroxy-4-(3′-oxo-3′*H*-benzofuran-2′-yliden)but-2-enoic acid (HOBB) was detected as a major metabolite [21]. *Pseudomonas* sp. strain ISTDF1 was also able to grow on naphthalene, benzo(α)pyrene, and nitrogen-containing carbazole, and sulfur-containing dibenzothiophene (as shown in Table 1) probably because of activation of the lateral dioxygenation pathway. It would be interesting to study the oxygenating enzyme system in strain ISTDF1 at both the molecular and biochemical levels, as it dioxygenated dibenzofuran by both modes as dibenzofuran 4,4a-dioxygenase (DFDO) from *Terrabacter* sp. strain DBF63 and as well as carbazole 1,9a-dioxygenase (CARDO) from *Pseudomonas resinovorans* strain CA10. CARDO and DFDO systems are distinctly different not only phylogenetically but also functionally as the preferred oxygenation reactions suggested that DFDO evolved from a polycyclic aromatic hydrocarbon dioxygenase while CARDO is a novel enzyme [31].

Thus, strain ISTDF1 degraded dibenzofuran through both the angular and lateral pathways of hydroxylation. Initially, the degradation rate was found to be higher with increasing concentration, which gradually declined with time (Figs. 6, 8). This might occur because of the toxic effects of accumulated concentration of intermediates such as catechol and gentisic acid [24]. Future direction for detailed investigation seems to be exciting. It would be interesting to study whether one or more enzyme/s are involved in hydroxylation of dibenzofuran and how its catabolic genes are regulated in such alkaline conditions as the effluent of the paper industry. To the best of our knowledge, this is the first study of alkalotolerant and mesophilic *Pseudomonas* to destabilize the dibenzofuran ring via diverse oxygenation with a wide substrate range, which can be very useful for in situ bioremediation of dioxin-like compounds.

Conclusions

In the present study we isolated an alkalotolerant and mesophilic *Pseudomonas* sp. strain ISTDF1 that can grow in a quite wide range of pH, from pH 6 to 10. Optimally, this strain showed ~85% degradation of dibenzofuran (200 mg l^{-1}) within 36 h at pH 10, which is quite comparable to previously reported strains. Its alkalotolerant nature, wide temperature tolerance (25–45°C), and accelerated and diverse metabolic pathway (lateral and angular oxygenation) of dibenzofuran and thus strain ISTDF1 can be useful for clean-up of areas contaminated with dioxins. The outcome can be enhanced for in situ bioremediation of dioxin-like compounds by taking more optimizing parameters in the light of microbial ecology and gene regulation of catabolic genes.

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References

1. Abraham WR, Nogales B, Golyshin PN, Pieper DH, Timmis KN (2002) Polychlorinated biphenyl-degrading microbial communities in soils and sediments. Curr Opin Microbiol 5:246–253. doi:10.1016/S1369-5274(02)00323-5
2. Adriaens P, Chang PR, Barkovskii AL (1996) Dechlorination of PCDD/F by organic and inorganic electron transfer molecule in reduced environment. Chemosphere 32:433–441. doi:10.1016/0045-6535(95)00231-6
3. Alcock RE, Jones KC (1996) Dioxins and furans in the environment: a review of trend data. Environ Sci Tech 30:3133–3143. doi:10.1021/es960306z
4. Bajpai P (2001) Microbial degradation of pollutants in pulp mill effluents. Adv Appl Microbiol 48:79–134. doi:10.1016/S0065-2164(01)48001-4
5. Cerniglia CE, Morgan JC, Gibson DT (1979) Bacterial and fungal oxidation of dibenzofuran. Biochem J 180:175–185
6. Fortnagel P, Harms H, Wittich R, Krohn S, Meyer H, Sinnwell V, Wilkes H, Francke W (1990) Metabolism of dibenzofuran by *Pseudomonas* sp strain HH69 and the mixed culture HH27. Appl Environ Microbiol 56:1148–1156
7. Gai Z, Yu B, Wang LLY, Ma C, Feng J, Deng Z, Xu P (2007) Cometabolic degradation of dibenzofuran and dibenzothiophene by a newly isolated carbazole-degrading *Sphingomonas* sp. strain. Appl Environ Microbiol 73:2832–2838
8. Harms H, Wilkes H, Wittich RM, Fortnagel P (1995) Metabolism of hydroxyl-dibenzofurans, methoxy-dibenzofurans, acetoxy-dibenzofurans, and nitro-dibenzofurans by *Sphingomonas* sp. Strain HH69. Appl Environ Microbiol 61:2499–2505
9. Hay A (1979) Dioxin: the 10-year battle that began with Agent Orange. Nature 278:108–109. doi:10.1038/278108b0
10. Hong HB, Chang YS, Choi SD, Park YH (2000) Degradation of dibenzofuran by *Pseudomonas putida* Ph-01. Wat Res 34:2404–2407. doi:10.1016/S0043-1354(00)00122-6
11. Hong HB, Nam IH, Murugesan K, Kim YM, Chang YS (2004) Biodegradation of dibenzo-p-dioxin, dibenzofuran, and chlorodibenzo-p- dioxins by *Pseudomonas veronii* PH-03. Biodegradation 15(5):303–313
12. Iida T, Mukouzaka Y, Nakamura K, Kudo T (2002) Plasmid-borne genes code for an angular dioxygenase involved in dibenzofuran

degradation by *Terrabacter* sp Strain YK3. Appl Environ Microbiol 68:3716–3723. doi:10.1128/AEM.68.8.3716-3723.2002
13. Ikeda K, Nakajima K, Yumoto I (1994) Isolation and characterization of a novel facultatively alkaliphilic bacterium *Corynebacterium* sp, grown on n-alkanes. Arch Microbiol 162:381–386. doi:10.1007/BF00282101
14. Ishiguro TY, Ohtake S, Nakayama Y, Inamori T, Amaga M, Soma M, Matsusita H (2000) Biodegradation of dibenzofuran and dioxins by *Pseudomonas aeruginosa* and *Xanthomonas maltophilia*. Environ Technol 21:1309–1316. doi:10.1080/09593332108618159
15. Jaiswal PK, Thakur IS (2007) Isolation and characterization of dibenzofuran-degrading *Serratia marcescens* from alkalophilic bacterial consortium of the chemostat. Curr Microbiol 55:447–454. doi:10.1007/s00284-007-9013-8
16. Jansen AM, Finster KW, Karlson U (2003) Degradation of carbazole, dibenzothiophene and dibenzofuran at low temperature by *Pseudomonas* sp. strain C3211. Environ Toxicol Chem 22:730–735. doi:10.1897/1551-5028(2003)022<0730:DOCDAD>2.0.CO;2
17. Kao CM, Chen SC, Liu JK, Wu MJ (2001) Evaluation of TCDD biodegradability under different redox conditions. Chemosphere 44:1447–1454. doi:10.1016/S0045-6535(00)00464-1
18. Kohler HPE, Schmid A, Maarel MV (1993) Metabolism of 2, 2′-dihydroxybiphenyl by *Pseudomonas* sp. Strain HBP: production and consumption of 2, 2′, 3-trihydroxybiphenyl. Appl Environ Microbiol 175:1621–1628
19. Kuehl DW, Butterworth BC, DeVita WM, Sauer CP (1987) Environmental contamination by polychlorinated dibenzo-p-dioxins and dibenzofurans associated with pulp and paper mill discharge. Biomed Environ Mass Spectrom 14:443–448. doi:10.1002/bms.1200140811
20. Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163. doi:10.1093/bib/5.2.150
21. Li Q, Wang X, Yin G, Gai Z, Tang H, Ma C, Deng Z, Xu P (2009) New metabolites in dibenzofuran cometabolic degradation by a biphenyl-cultivated *Pseudomonas* putida strain B6-2. Environ Sci Tech 43:8635–8642
22. Mandal P (2005) Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology. J Comp Physiol B 175:221–230. doi:10.1007/s00360-005-0483-3
23. Mohammadi M, Sylvestre M (2005) Resolving the profile of metabolites generated during oxidation of dibenzofuran and chlorodibenzofurans by the biphenyl catabolic pathway enzymes. Chem Biol 12:835–846. doi:10.1016/j.chembiol.2005.05.017
24. Nojiri H, Omori T (2002) Molecular bases of aerobic bacterial degradation of dioxins: involvement of angular dioxygenation. Biosci Biotechnol Biochem 66:2001–2016. doi:10.1271/bbb.66.2001
25. Omori T, Kasuga K, Nojiri H, Yamane H, Kodama T (1997) Cloning and characterization of the genes involved in the degradation of dibenzofuran by *Terrabacter* sp strain DBF63. J Ferment Bioeng 84:387–399. doi:10.1016/S0922-338X(97)81997-6
26. Palleroni NJ, Krieg NR, Holt JG (1984) Bergey's manual of systematic bacteriology. Williams and Wilkins, Baltimore, pp 141–198
27. Sato SI, Nam JW, Kasuga K, Nojiri H, Yamane H, Omori T (1997) Identification and characterization of genes encoding carbazol 1, 9a-dioxygenase in *Pseudomonas* sp Strain CA10. J Bacteriol 179:4850–4858
28. Schmid A, Rothe B, Altenbuchner J, Ludwig W, Engesser KH (1997) Characterization of three distinct extradiol dioxygenases involved in mineralization of dibenzofuran by *Terrabacter* sp strain DPO360. J Bacteriol 179:53–62
29. Spiers AJ, Buckling A, Rainey PB (2000) The causes of *Pseudomonas* diversity. Microbiology 146:2345–2350
30. Strubel V, Engesser KH, Fischer P, Knackmuss HJ (1991) 3-(2-Hydroxyphenyl) catechol as substrate for proximal meta ring cleavage in dibenzofuran degradation by *Brevibacterium* sp strain DPO 1361. J Bacteriol 173:1932–1937
31. Takagi T, Nojiri H, Yoshida T, Habe H, Omori T (2002) Detailed comparison between the substrate specificities of two angular dioxygenases, dibenzofuran 4, 4a-dioxygenase from *Terrabacter* sp. and carbazole 1, 9a-dioxygenase from *Pseudomonas resinovorans*. Biotechnol Lett 24:2099–2106
32. Thacker NP, Nitnaware C, Das SK, Devotta S (2007) Dioxin formation in pulp and paper mills of India. Environ Sci Pollut Res Int 14:225–226. doi:10.1065/espr2007.02.386
33. Thakur IS (1995) Structural and functional characterization of a stable, 4 chlorosalicylic acid degrading, bacterial community in a chemostat. World J Microbiol Biotechnol 11:643–645. doi:10.1007/BF00361007
34. Thakur IS, Verma PK, Upadhaya K (2002) Molecular cloning and characterization of pentachlorophenol-degrading monooxygenase genes of *Pseudomonas* sp. from the chemostat. Biochem Biophys Res Commun 290:770–774. doi:10.1006/bbrc.2001.6239
35. Wittich RM, Wilkes H, Sinnwell V, Francke W, Fortnagel P (1992) Metabolism of dibenzo-p-dioxin by *Sphingomonas* sp strain RW1. Appl Environ Microbiol 58:1005–1010