



Growth kinetics of *Pseudomonas aeruginosa* RS1 on fluorene and dibenzothiophene, concomitant degradation kinetics and uptake mechanism

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Received: 18 December 2020 / Accepted: 12 March 2021 / Published online: 26 March 2021
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Abstract

The current study illustrates the growth kinetics of an efficient PAH and heterocyclic PAH degrading bacterial strain, *Pseudomonas aeruginosa* RS1 on fluorene (FLU) and dibenzothiophene (DBT) over the concentration 25–500 mg L⁻¹ and their concomitant degradation kinetics. The specific growth rate (μ) was found to lie within the range of 0.32–0.57 day⁻¹ for FLU and 0.24–0.45 day⁻¹ for DBT. The specific substrate utilization rate (q) of FLU and DBT over the log growth phase was between 0.01 and 0.14 mg FLU mg VSS⁻¹ day⁻¹ for FLU and between 0.01 and 0.18 mg DBT mg VSS⁻¹ day⁻¹ for DBT, respectively. The μ and q values varied within a narrow range for both FLU and DBT and they did not follow any specific trend. Dissolution together with direct interfacial uptake was the possible uptake mechanism for both FLU and DBT. The q values over the log growth phase depicts the specific substrate transformation rates. Kirby-Bauer disc diffusion studies performed using an *E. coli* strain indicated accumulation of some toxic intermediates of FLU and DBT during their degradation. Decrease in TOC and toxicity towards the end of the degradation experiments indicates further utilization of the intermediates.

Keywords Fluorene · Dibenzothiophene · Total organic carbon · Surface tension · Contact angle

Introduction

Fluorene (FLU) and dibenzothiophene (DBT) are constituents of crude oils, shale oils and creosote, and DBT often co-exists with polycyclic aromatic hydrocarbons (PAH) in the environment. Spillage and seepage of fossil fuels, industrial wastewater discharge, stormwater runoff and volcanic eruptions are the important sources of FLU and DBT in surface water (oceans, rivers and lakes), groundwater and industrial wastewater (Salam et al. 2017). DBT has been reported to be present at 0.06–12.34 ng L⁻¹ in surface water, 0.03–15 μ g L⁻¹ in groundwater and 0.18 μ g L⁻¹ to less than 20 μ g L⁻¹ in coking wastewater (Shi et al. 2015). On the other hand, FLU has been reported to be present at 0.04–1173 ng L⁻¹ in surface water, 0.001–610 μ g L⁻¹ in

groundwater and at 0.04–12.5 mg L⁻¹ in various wastewaters (sewage, pulp mill and coking wastewaters) (Shi et al. 2015).

FLU is a model PAH compound and is among the 16 PAHs on the list of priority pollutants prescribed by the US Environmental Protection Agency (Ping et al. 2017). It is formed during fossil fuel combustion and can be frequently found in areas contaminated by oil spills, vehicular exhausts, waste incineration products, industrial effluents (mostly from coal and oil industries) and smoked food (Jamal and Pugazhendi 2018). FLU and its derivatives constitute approximately 7.6% of the total PAHs present in creosote (Mojiri et al. 2019). FLU has been reported to be toxic towards fish and algae. Although it was identified as non-genotoxic based on Ames Assay, it was found to be a point carcinogen based on studies with various plants, microalgae, and planktons (Mojiri et al. 2019; Sonwani et al. 2019).

DBT is a 3-ring sulfur heterocyclic PAH (PASH) compound, which is among the 13 most common heterocyclic PAHs in creosote. It is not genotoxic, but it has a high tendency to bioaccumulate in biota present in contaminated environments and is reported to be one of the most persistent organic pollutants (Li et al. 2019). Toxicity of DBT has been

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tested using various acute toxicity assays. EC_{50} ($mg\ L^{-1}$) values of DBT with respect to *Vibrio fischeri*, *Pseudomonas fluorescens* and *Daphnia magna* have been reported to be 0.12–0.2 (30 min exposure), 0.13 (48 h exposure) and 0.2 (48 h exposure), respectively (Li et al. 2019). DBT has shown mutagenicity and toxicity in various test organisms. However, very little human exposure data are available for DBT.

Physical methods are ineffective in removing FLU and DBT from industrial wastewater (Mojiri et al. 2019). Most of the chemical treatment processes either transfer such hazardous compounds to a separate phase or transform them to intermediates and complete mineralization is not always achieved (Mishra et al. 2016). Highly toxic and polar end products are also formed in some of the chemical processes. On the other hand, production of environmentally benign end products in biological treatment encourages its application for the removal of these compounds (Borah et al. 2019).

Low aqueous solubility of both FLU ($1.69\ mg\ L^{-1}$ at $25\ ^\circ C$) and DBT ($1.47\ mg\ L^{-1}$ at $25\ ^\circ C$) makes them less bioavailable and hence, they become more environmentally persistent and often appear in the solid state in the environment (Li et al. 2019). Degradation rate of these compounds may vary depending upon the bacterial species, initial substrate concentration, bioavailability of the compounds and other laboratory test conditions. There are two kinds of bacterial uptake mechanisms for these compounds. Some bacteria produce exopolysaccharides and form biofilm on the surface of the solid crystals. Such bacteria may be capable of direct interfacial uptake of the compounds. Yet other bacteria are capable of producing biosurfactants to enhance the mass transfer of compounds from the solid to the aqueous phase and thus enhance their uptake (Jasmine 2016).

Several bacterial species capable of degrading DBT under aerobic condition (as sole source of carbon and/or sulfur) have been reported in the literature, such as *Microbacterium*, *Gordonia*, *Rhodococcus*, *Shewanella*, *Sphingomonas*, *Achromobacter*, *Stenotrophomonas*, *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Corynebacterium* and *Brevibacterium* (Mojiri et al. 2019; Papizadeh et al. 2011). DBT degradation efficiency by the bacterial species varied between 40 and 100% for incubation time varying from 6 h to 13 days and initial concentration varying over the range of $14\text{--}553\ mg\ L^{-1}$. Several bacterial strains, such as *Pseudomonas cepacia* F297, *Arthrobacter* sp. strain F101, *Pseudomonas* sp. strain F274, *Pseudomonas vesicularis*, *Janibacter* sp. strain YY-1 and *Ochrobactrum* sp. VA1 have been reported to aerobically degrade FLU with degradation efficiency varying from more than 90 to 100% over 1 to 14 days incubation period and initial concentration ranging between 3 and $1000\ mg\ L^{-1}$ (Mojiri et al. 2019; Rabodonirina et al. 2019).

Earlier studies demonstrated the capability of *Pseudomonas aeruginosa* RS1 to degrade a nitrogen containing

heterocyclic PAH, carbazole (CBZ) in mineral media (Ghosh and Mukherji 2020a, 2018) and to degrade CBZ, FLU and DBT in refinery wastewater (Ghosh and Mukherji 2020b). It could also grow well on oil extracted from oily sludge (Jasmine and Mukherji 2015). The major objective of the present study was to elucidate the growth kinetics of the bacterial strain on FLU and DBT at initial concentration varying from 25 to $500\ mg\ L^{-1}$ and to assess the degradation kinetics. Other objectives were to explore the uptake mechanisms of FLU and DBT, investigate the toxic effects of the metabolites of the compounds generated during their degradation and estimate the protein concentration in the culture supernatants and cell extracts during bacterial growth on FLU and DBT. This study for the first time presents a comprehensive investigation on degradation of both FLU and DBT when applied as sole substrates at initial concentration varying from 25 to $500\ mg\ L^{-1}$ by a bacterial strain, *P. aeruginosa* RS1, their uptake mechanism and identifies the toxic effects of metabolites on *Escherichia coli*.

Methods

Reagents and materials

Fluorene (98%) and dibenzothiophene (98%+) were obtained from Sigma Aldrich Chemicals Pvt. Ltd., St. Louis, USA and Thermo Fisher Scientific, India, respectively. Dichloromethane and acetonitrile (HPLC grade) were purchased from Merck, India. Inorganic salts for preparing the mineral media were procured from Finar Ltd. (India), Loba Chemie (India), Merck (India), Ranbaxy (India), and Thomas Baker (India). Coomassie Brilliant Blue G-250, bovine serum albumin and EDTA solution were purchased from Thermo Fisher Scientific (India), Himedia (India) and Merck (India), respectively.

Media composition, culture conditions and experimental protocol

Pseudomonas aeruginosa RS1 (MTCC 25391) was isolated from oily sludge procured from a Mumbai-based oil refinery in India. It has the capability to efficiently degrade a nitrogen containing heterocyclic PAH, CBZ (Ghosh and Mukherji 2020a, 2018) and a four-ring PAH pyrene (Ghosh et al. 2014) in mineral media and could also degrade CBZ, FLU and DBT in refinery wastewater matrix (Ghosh and Mukherji 2020b) and other diverse hydrocarbons across various groups (Dasgupta et al. 2018).

Nutrient broth grown cells were centrifuged at 8000 rpm for 10 min and the harvested cells were washed using 2% phosphate buffer saline (PBS) and the final absorbance (OD_{600}) was adjusted to 1.8. This culture was subsequently

added (3% v/v) in sterilized Erlenmeyer flasks (500 ml) containing FLU or DBT (each at 250 mg L⁻¹) and 50 mL mineral media and was incubated at 37 °C and 120 rpm within a rotary shaker. The composition of the media was as reported elsewhere (Ghosh and Mukherji 2018). Multiple subculturing was conducted in order to acclimatize the culture to FLU and DBT. An earlier biodegradation study on the nitrogen containing heterocyclic PAH, CBZ revealed that acclimatization of the bacterial strain *P. aeruginosa* RS1 to CBZ improved its degradation (initial concentration 100 mg L⁻¹) from 7.08 ± 0.68% to 38 ± 1.2%. The method of preparing the stock solutions of FLU or DBT and the subculturing technique was the same as discussed by Ghosh and Mukherji (2018).

Bacterial growth and degradation experiments were conducted on FLU and DBT in batch mode at initial concentrations, 25, 50, 100, 200, 300, 400 and 500 mg L⁻¹ and the batch cultures were incubated at 37 °C and 120 rpm. Concentration in excess of aqueous solubility of FLU and DBT were used since aqueous solubility of these substrates are very low (FLU: 1.69 mg L⁻¹ at 25 °C and DBT: 1.47 mg L⁻¹ at 25 °C) and microbial growth cannot be supported at such low substrate concentration (Ghosh et al. 2014). The inoculum of *P. aeruginosa* RS1 used was acclimatized to the corresponding substrate. The bacterial growth studies were conducted over 14 days in duplicate flasks and sample aliquots (0.5 mL) were collected daily and serially diluted and spread on nutrient agar plates for enumerating the number concentration of the cells in terms of colony-forming unit (CFU). The degradation experiments were also performed in duplicate flasks over 14 days and at every 2 days interval a pair of flasks were sacrificed for quantifying the residual substrate concentration. Dichloromethane (DCM) was used to extract the residual substrate by liquid–liquid extraction. The DCM extract was evaporated and the residue was subsequently dissolved in acetonitrile. FLU and DBT in the acetonitrile solution were analysed using HPLC (410 Prostar Binary LC with 500 MS IT Photodiode Array Detectors, Varian Inc., USA). A reverse-phase Pinnacle II C18 HPLC column (Restek Corporation, Bellefonte, PA, USA) with particle size 4 µm (250 mm × 4.6 mm ID) was used for separating the analytes. The method details for extracting FLU and DBT were same as that reported for carbazole by Ghosh and Mukherji (2018). The composition of the mobile phase was maintained at 95:5 (v/v) acetonitrile:water and the flow rate was 0.5 mL/min. Both FLU and DBT were detected using a PDA detector at 254 nm wavelength.

Modelling of bacterial growth kinetics

Growth of *P. aeruginosa* RS1 on FLU and DBT was fitted using logistic (Eq. 1) and Gompertz (Eq. 2) models (Tjørve and Tjørve 2017).

$$N = \frac{N_{\max}}{1 + e^{\left[\ln\left(\frac{N_{\max}}{N_0} - 1\right) - \mu t\right]}} \quad (1)$$

$$N = N_{\infty} \cdot e^{-e^{-k_g(t-t_c)}} \quad (2)$$

where N is the number concentration (CFU/mL) of bacteria at any time (t), N_{\max} represents the maximum value of N , N_0 is the initial value of N (at $t=0$), N_{∞} is the upper asymptotic value of N , k_g is the rate coefficient of bacterial growth and t_c represents the time corresponding to the point of inflection. The linearized logistic and Gompertz models (Eqs. 3, 4, respectively) were used for fitting the data.

$$\ln \left[\frac{N(N_{\max} - N_0)}{N_0(N_{\max} - N)} \right] = \mu t \quad (3)$$

$$\ln \left[\ln \left(\frac{N_0}{N} \right) \right] = -k_g(t - t_c) \quad (4)$$

Linear regression was performed to relate the absorbance of the *P. aeruginosa* culture grown on nutrient broth to the number concentration of bacterial cells (CFU/mL) and biomass concentration (TSS and VSS in mg L⁻¹). These equations were subsequently used for quantification of the biomass produced during growth on FLU and DBT provided as sole substrate in mineral media. The slope of the linear portion of the plot between $\ln(N/N_0)$ and time obtained from the bacterial growth data on FLU and DBT was used for determining specific growth rate (μ).

Modelling of degradation kinetics of FLU and DBT and calculation of yield

Maximum biodegradation rate (MBR) and specific substrate utilization rate (q) during bacterial growth on FLU and DBT as sole substrate were determined using the substrate degradation data and performing linear regression over the log and stationary growth phases (Eq. 5) were plotted against the initial concentration of the substrates. Linear regression was performed over the log and stationary growth phases of the bacterial culture in order to calculate MBR and q (Eq. 5).

$$q = \frac{\text{MBR}}{X_{\text{av}}} = \frac{(S_1 - S_2)}{X_{\text{av}}} \quad (5)$$

where S_1 and S_2 represent the slopes of abiotic and the biotic decay profiles (mg substrate L⁻¹ day⁻¹), and X_{av} denotes the average biomass concentration (mg L⁻¹) calculated over the log growth phase of the bacterial culture (volatile suspended solids, mg L⁻¹).

The yield coefficient (Y , CFU/mg FLU or DBT) was determined over the linear segment of the log growth phase

of the bacterial culture on FLU and DBT (details of calculation of Y are given in supplementary information, SI).

Measurement of TOC during degradation of FLU and DBT

The TOC value of the culture filtrate of *P. aeruginosa* RS1 grown on FLU and DBT (100 mg L^{-1}) as sole substrates was determined using a TOC analyzer (TOC VCSH, Shimadzu, Japan). Accumulation of polar metabolites in the culture broth over time was indicated by increase in TOC. The TOC in uninoculated controls was also determined over time to depict the contribution of dissolved FLU and DBT.

Determination of acute toxicity by zone inhibition test

Nutrient broth grown cells of *Escherichia coli* MTCC 443 were used for conducting zone inhibition studies on the samples collected during the degradation of FLU and DBT. Biodegradation experiments on FLU and DBT were set up in duplicate Erlenmeyer flasks at $150 \text{ mg-TOC L}^{-1}$ concentration of individual substrate for 14 days. At every 2 days, a pair of flasks were sacrificed by filtering the culture broths through $0.2 \mu\text{m}$ filters. Kirby–Bauer disc diffusion study was performed using the culture filtrate (Ghosh and Mukherji 2018; Jasmine 2016).

Determination of protein concentration

Bradford assay was used for quantifying protein concentration in the culture supernatant and in the cell extract during degradation of FLU and DBT (for $10\text{--}100 \mu\text{g}$ protein concentration). Protein concentration profiles were determined for 100 mg L^{-1} of FLU and DBT during their degradation in optimized media using FLU and DBT acclimatized *P. aeruginosa* RS1 cells, respectively, when each substrate was applied at 100 mg L^{-1} initial concentration. The protocol for sample preparation and measurement of protein concentration are described elsewhere (Ghosh and Mukherji 2020a).

Elucidation of FLU and DBT uptake mechanism

Biodegradation experiments were conducted on FLU and DBT at 100 mg L^{-1} initial concentration. A Surface Tensiometer (Model 3S, GBX, France) was employed to determine the surface tension of the cell-free culture broth obtained during FLU and DBT degradation experiments. For each sample collected, the bacterial cells were first removed from the culture broth by centrifugation at $12,000 \text{ rpm}$ and this was followed by filtration of the supernatant through a $0.2 \mu\text{m}$ membrane filters. The details of the protocols

followed for preparing samples for measuring contact angles can be found elsewhere (Ghosh and Mukherji 2018).

Contact angle (which is a measure of Cell surface hydrophobicity) of FLU and DBT (100 mg L^{-1}) grown cells (collected from mid-log, end-log and decay phases of bacterial growth) of *P. aeruginosa* RS1 was determined using bacterial mats prepared on filters. Contact angle measurement was performed using the sessile drop technique using a Digidrop Goniometer (Model DS, GBX, France) equipped with a digital camera (Chakraborty et al. 2010). The details of the protocol for sample preparation for measuring the contact angle on a bacterial cell mat can be found elsewhere (Chakraborty et al. 2010; Ghosh and Mukherji 2018).

Results and discussion

Modelling of bacterial growth and degradation kinetics on fluorene and dibenzothiophene

Growth of *P. aeruginosa* RS1 on 25 mg L^{-1} fluorene (FLU) and dibenzothiophene (DBT) and FLU and DBT degradation profiles are shown in Fig. 1a, b for FLU and DBT, respectively. The growth and degradation profiles were also obtained at other concentrations up to 500 mg L^{-1} and are shown in SI. The specific growth rates of the bacteria (at varying initial concentrations of FLU and DBT) were first determined from the linear portion of the growth profile in the log growth phase. This was followed by fitting the linearized logistic and Gompertz models (up to the end of log growth phase) and the model parameters were compared. The logistic model and the Gompertz model fits for bacterial growth data on 25 mg L^{-1} FLU and 25 mg L^{-1} DBT are shown in SI. Similar model fits for bacterial growth on FLU and DBT applied at other concentrations were also performed. Better fits were obtained using the logistic model and its parameter values were closer to those determined from the linear portion of the log growth phase. Table 1 summarizes the model parameter values along with the effective yield values (Y , CFU/mg substrate) of the strain at each initial concentration of FLU and DBT. The Y values were determined based on the linear portion of the bacterial growth and FLU/DBT degradation profiles at varying initial concentrations of FLU and DBT. A regression equation relating $\log N$ (CFU/mL) and VSS (g L^{-1}) developed using nutrient broth grown *P. aeruginosa* RS1 was used to calculate biomass concentration at different stages of FLU and DBT degradation (SI).

Degradation of FLU (D , %) after 14 days of incubation were (mean \pm SD): 80 ± 9 , 68 ± 6 , 52 ± 10 , 47 ± 4 , 43 ± 1 , 40 ± 5 and 39 ± 4 for initial FLU concentration (S , mg L^{-1}) of 25, 50, 100, 200, 300, 400 and 500 mg L^{-1} , respectively. Whereas, DBT degradation (D , %) after 14 days of

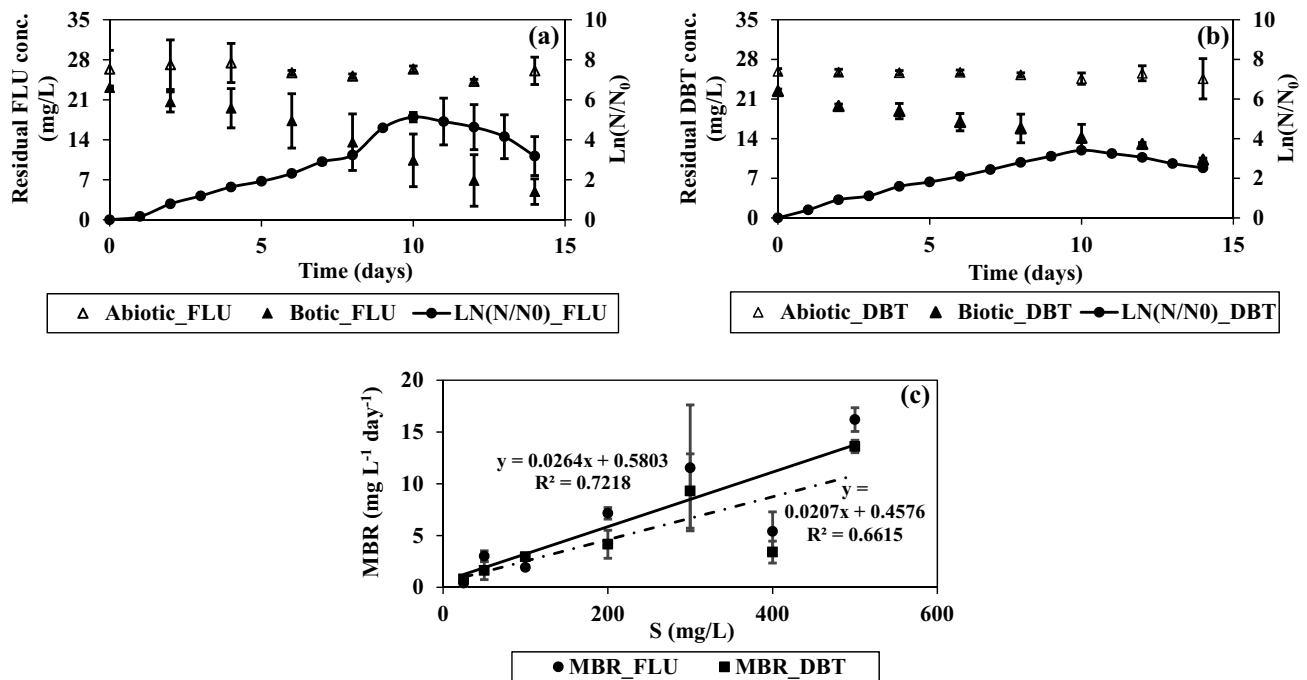


Fig. 1 Growth and degradation profile of *P. aeruginosa* RS1 on **a** 25 mg L⁻¹ FLU and **b** 25 mg L⁻¹ DBT, and **c** variation in MBR of FLU and DBT over 25 to 500 mg L⁻¹ initial concentration and their linear fits (continuous line for FLU and dashed line for DBT)

incubation were (mean \pm SD): 59 ± 1 , 53 ± 4 , 47 ± 3 , 41 ± 6 , 38 ± 4 , 34 ± 5 and 26 ± 4 for initial DBT concentration (S , mg L⁻¹) of 25, 50, 100, 200, 300, 400 and 500 mg L⁻¹, respectively. Figure 1c represents the variation in maximum biodegradation rate (MBR) (mean value) of FLU and DBT in the log growth phase over the initial concentration range of 25–500 mg L⁻¹. The MBR data can be approximately linearly correlated with initial FLU and DBT concentration, indicating probable first order kinetics of FLU and DBT degradation. The first-order degradation rate constants were determined as 0.026 ± 0.007 day⁻¹ and 0.021 ± 0.007 day⁻¹ for FLU and DBT, respectively. Specific utilization rates (q) of FLU and DBT were determined both for the log and stationary growth phases during the biodegradation of FLU and DBT at various initial concentrations and are provided in SI. In general, an increase in q in the log growth phase was observed with an increase in FLU and DBT concentration, however, unexpectedly low q value was observed at 400 mg L⁻¹ FLU and DBT concentration.

The specific growth rate (μ) was found to lie within a narrow range of 0.32–0.57 day⁻¹ for FLU and 0.24–0.45 day⁻¹ for DBT at their initial concentration ranging from 25 to 500 mg L⁻¹, however, no definite trend was observed (SI). The range of μ values observed during CBZ degradation using the same bacterial strain was 0.96–2 day⁻¹ (Ghosh and Mukherji 2020a), which is much wider compared to that observed for FLU and DBT. The values of μ ranged from 0.72 to 2.04 day⁻¹ for growth of the same strain on pyrene

(25–500 mg L⁻¹) (Ghosh et al. 2014). Thus, the μ values for both CBZ and pyrene degradation are comparable, however, bacterial growth was inhibited on FLU and DBT. In case of FLU and DBT degradation, the log growth phase q values were in the range of 0.01–0.14 mg FLU mg VSS⁻¹ day⁻¹ and 0.01 to 0.18 mg DBT mg VSS⁻¹ day⁻¹, respectively, whereas for CBZ, the values were in the range of 0.002–0.018 mg CBZ mg VSS⁻¹ day⁻¹ range (Ghosh and Mukherji 2020a), which is much lower than for both FLU and DBT. This was due to higher biomass growth obtained in the CBZ degradation studies. It is also possible that much of the degraded FLU and DBT were transformed into metabolites and dead-end products that could not be degraded further as carbon and energy sources. The q values reported for pyrene (25 to 500 mg L⁻¹) degradation by the same strain were within the range 0.01–0.50 mg pyrene mg VSS⁻¹ day⁻¹ (Ghosh et al. 2014), which is higher than the q values on CBZ, FLU and DBT. Table 2 summarizes comparison of bacterial growth and substrate degradation parameters for degradation of CBZ, FLU, DBT and pyrene using the same bacterial strain, *P. aeruginosa* RS1.

FLU (initial concentration 100 mg L⁻¹) degradation rate of 0.8 ± 0.07 mg L⁻¹ h⁻¹ was reported using a bacterial strain, *Pseudomonas putida* ATCC 17514 (Rodrigues et al. 2005). Biodegradation kinetic experiments were conducted on FLU, naphthalene, 1,5-dimethylnaphthalene, and 1-methylfluorene both individually and in mixtures using a bacterial strain, *Shingomonas paucimobilis* EPA505 (Desai et al.

Table 1 Variation of specific growth rate, yield coefficient and best-fit parameter values for logistic and Gompertz models with varying FLU and DBT concentration

Parameters	Initial FLU concentration (mg L ⁻¹)					Initial DBT concentration (mg L ⁻¹)									
	25	50	100	200	300	400	500	25	50	100	200	300	400	500	
Statistics															
Experimental value (log growth phase)															
μ (day ⁻¹)	Mean	0.377	0.567	0.479	0.325	0.387	0.410	0.396	0.306	0.450	0.275	0.244	0.365	0.342	0.353
	SE	0.014	0.086	0.052	0.005	0.013	0.044	0.041	0.023	0.015	0.033	0.016	0.015	0.020	0.015
Linearized logistic model															
μ (day ⁻¹)	Mean	0.468	0.589	0.493	0.419	0.465	0.554	0.459	0.440	0.502	0.373	0.364	0.458	0.394	0.420
	SE	0.006	0.001	0.004	0.003	0.002	0.004	0.002	0.002	0.001	0.004	0.002	0.002	0.002	0.001
Linearized Gompertz model															
t_c (day)	Mean	9.956	7.667	7.112	7.373	9.124	9.242	10.415	6.042	5.783	6.515	7.203	6.758	8.848	6.242
	SE	0.414	0.037	0.113	0.125	0.108	0.076	0.182	0.079	0.037	0.100	0.074	0.054	0.150	0.031
k_g (day ⁻¹)	Mean	0.190	0.228	0.252	0.230	0.194	0.243	0.177	0.240	0.266	0.237	0.216	0.257	0.189	0.260
	SE	0.008	0.002	0.006	0.006	0.003	0.003	0.004	0.005	0.003	0.006	0.004	0.004	0.004	0.003
Y (CFU/mg substrate)	Mean	1.99E+6	9.56E+5	4.44E+5	6.50E+5	7.04E+4	1.67E+5	5.45E+4	5.90E+5	7.05E+5	9.80E+4	6.62E+4	6.91E+4	1.54E+4	5.03E+4

2008). Complete removal of 0.36 mg L⁻¹ FLU was obtained within 4 h of incubation. From the degradation kinetics of FLU conducted at 0.35 mg L⁻¹ initial concentration (below its aqueous solubility), q_{max} and K_s were reported to be 0.96 mg FLU mg protein⁻¹ day⁻¹ and 0.02 mg L⁻¹, respectively. Growth kinetics on benzothiophene (BT) and DBT for an *Acinetobacter* strain yielded μ_{max} , K_s and K_i values of 28.8 day⁻¹, 2.1 mg L⁻¹ and 1.6 mg L⁻¹ for BT and 32.6 day⁻¹, 11.1 mg L⁻¹ and 2.6 mg L⁻¹ for DBT for varying initial concentration of 1.3–33.6 mg L⁻¹ BT and 1.8–46.1 mg L⁻¹ of DBT, respectively (Marzona et al. 1997). It was also reported that the rate of metabolism of DBT was slow and complete removal of DBT could not be achieved even after one week. In the present study, complete removal of DBT was not observed even after 2 weeks of incubation.

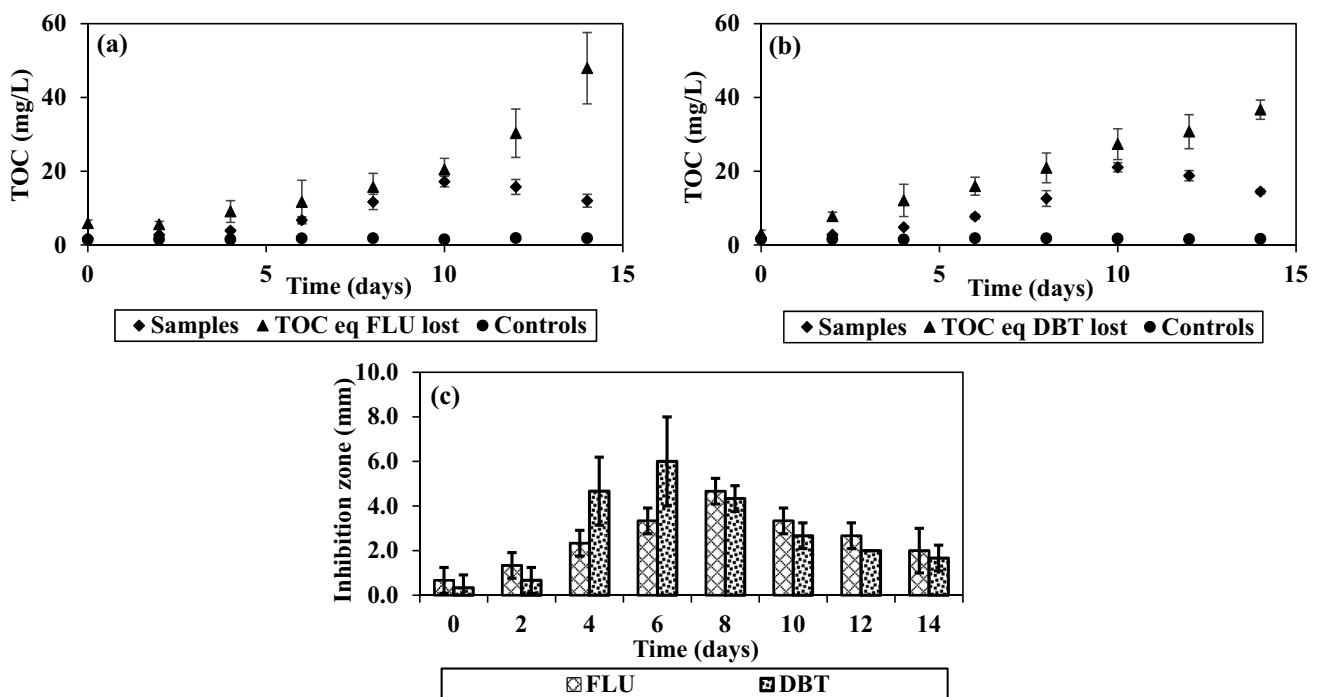
FLU and DBT degradation experiments were conducted at initial concentrations (25–500 mg L⁻¹) much above their aqueous solubility (1.69 mg L⁻¹ and 1.47 mg L⁻¹ at 25 °C for FLU and DBT, respectively). It was concluded that growth of *P. aeruginosa* RS1 over time could be fitted well using the logistic model in both the cases, however, growth was much less on both FLU and DBT, compared to that on CBZ. The yield of cells was also much higher on CBZ (Ghosh and Mukherji 2020a) than on FLU and DBT, which indicates possible accumulation of more dead-end products during degradation of FLU and DBT. Percent degradation of FLU and DBT were significantly reduced when they were applied at a higher initial concentration (200 mg L⁻¹ and more). Specific growth rate (μ) and specific substrate utilization rate (q) obtained from CBZ degradation (25–500 mg L⁻¹) experiment could be fitted well using the Moser model with substrate inhibition and Monod model, respectively (Ghosh and Mukherji 2020a). In case of both FLU and DBT, the value of μ remained relatively invariant over a wide concentration range from 25 to 500 mg L⁻¹ and the average μ values were 0.42 and 0.33 day⁻¹, respectively. However, q showed a clear increasing trend for both FLU and DBT and the average q values were 0.06 mg FLU mg VSS⁻¹ day⁻¹ and 0.07 mg DBT mg VSS⁻¹ day⁻¹, respectively.

Measurement of TOC during degradation of FLU and DBT

It can be seen from Fig. 2a, b that, the maximum TOC was reached on the 10th day of incubation for both FLU and DBT (17 ± 1 mg L⁻¹ for FLU and 21 ± 1 mg L⁻¹ for DBT), indicating the accumulation of metabolites. However, beyond the 10th day, TOC decreased due to the utilization of the metabolites. The presence of TOC even at the end of 14 days indicates the accumulation of some dead-end products. The dissolved concentration of FLU and DBT contributed to the presence of TOC in the controls. This was low throughout the experiment, due to their

Table 2 Comparison of bacterial growth and substrate degradation parameters for CBZ, FLU, DBT and pyrene degradation using *P. aeruginosa* RS1

Strain	Compounds	Concentration range (mg L ⁻¹)	Range of Specific growth rate (μ , day ⁻¹)	Best fit model for specific growth rate	Range of Specific substrate utilization rate (q, mg substrate mg VSS ⁻¹ day ⁻¹)	Best fit model for specific substrate utilization rate	References
<i>Pseudomonas aeruginosa</i> RS1	Carbazole	25–500	0.96–2	Moser	0.002–0.018	Monod	Ghosh and Mukherji (2020a)
	Fluorene	25–500	0.32–0.57	No specific trend seen	0.01–0.14	No specific trend seen	This study
	Dibenzothio- phene	25–500	0.24–0.45	No specific trend seen	0.01–0.18	No specific trend seen	This study
	Pyrene	25–500	0.72–2.04	Monod	0.012–0.504	Linear	Ghosh et al. (2014)

**Fig. 2** TOC profiles of filtered culture broth during **a** FLU and **b** DBT degradation at 100 mg L⁻¹ initial concentration and inhibition zones formed during degradation of **c** 150 mg-TOC L⁻¹ FLU and DBT* by *P. aeruginosa* RS1

low aqueous solubility. The fraction of TOC equivalent of FLU and DBT lost that remained as metabolite at the end of the study were 0.25 ± 0.06 and 0.39 ± 0.03 , respectively, which are both higher compared to that observed during 100 mg L⁻¹ CBZ degradation using the same bacterial strain (Ghosh and Mukherji 2020a). The corresponding values on the 10th day were also higher than for CBZ (17 ± 1 mg L⁻¹ for FLU and 21 ± 1 mg L⁻¹ DBT). Thus,

the high q value obtained over the log growth phase was primarily due to transformation rather than mineralization.

Determination of acute toxicity during degradation of FLU and DBT

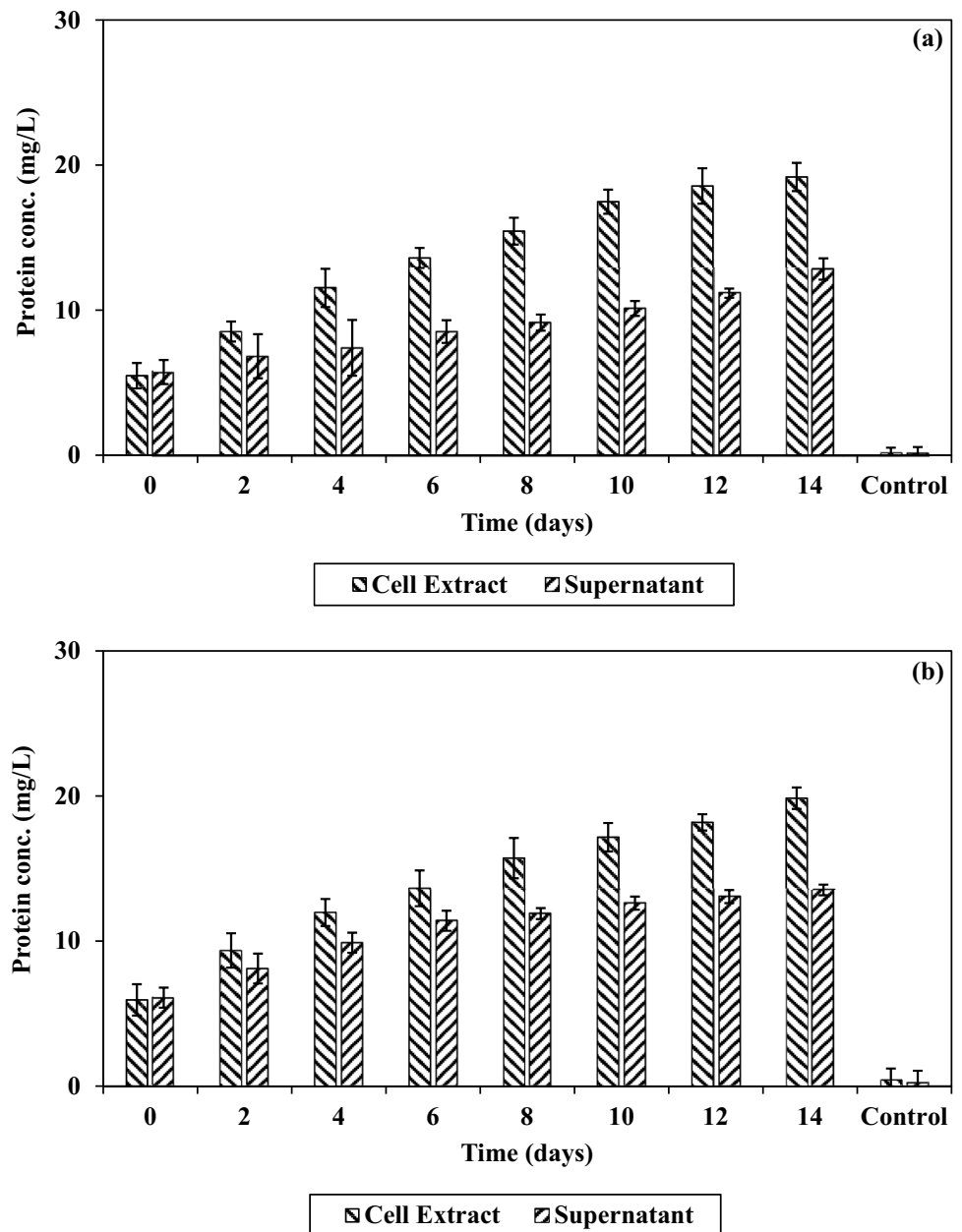
In this study, FLU and DBT were provided as sole substrate and each was added at 150 mg-TOC L⁻¹. Thus,

the initial FLU and DBT concentrations were 159.8 and 191.9 mg L^{-1} , respectively. The profiles of the inhibition zone observed during culture growth is depicted in Fig. 2c. In general, the trends match with the TOC profiles observed during the degradation of the substrates, which indicates that toxicity is caused by the polar metabolites. The maximum diameter of toxic inhibition zone appeared on the 8th day for FLU degradation (5 ± 1 mm) and on the 6th day for DBT degradation (6 ± 2 mm).

Determination of protein concentration

Figure 3a, b represents the protein concentration profiles of culture broths obtained during degradation of 100 mg L^{-1} FLU and 100 mg L^{-1} DBT, respectively. Both the profiles had an increasing trend throughout the incubation period and maximum protein concentration was reached on the 14th day in both the supernatant (13 ± 1 mg L^{-1} for FLU and 14 ± 0.4 mg L^{-1} for DBT) and in the cell extract (19 ± 1 mg L^{-1} for FLU and 20 ± 1 mg L^{-1} for DBT). At any time over the course of FLU and DBT degradation the protein concentration in the cell extract was consistently higher than that in the supernatant. Protein yield of 0.26 mg/mg

Fig. 3 Variation of protein concentration during degradation of **a** FLU and **b** DBT



carbon (equivalent to $\sim 244 \text{ mg L}^{-1}$ protein concentration) was reported in the culture broth collected at the end of log growth phase for a FLU (1000 mg L^{-1}) degrading bacteria, *Pseudomonas vesicularis*, isolated from contaminated soil (Weissenfels et al. 1990). The concentration of protein in the cell broth during DBT (100 mg L^{-1}) degradation by *Sphingomonas* sp. 2mpII was reported to increase from 10 to 27 mg L^{-1} after 12 days of incubation. Subsequent incubation (up to 23 days) reduced protein concentration to 20 mg L^{-1} (Nadalig et al. 2002).

Elucidation of uptake mechanism of FLU and DBT

The surface tension of the culture filtrates had a decreasing trend both during (Fig. 4a) FLU and DBT degradation (each were initially applied at 100 mg L^{-1}) till the 10th day of incubation for FLU and 12th day of incubation for DBT, after which it slightly increased towards the decay phase. Surface tension values varied from 71 ± 2 to $47 \pm 1 \text{ mN m}^{-1}$ for FLU and 72 ± 1 to $52 \pm 1 \text{ mN m}^{-1}$ for DBT over 14 days incubation. During the degradation of both 100 mg L^{-1} FLU and 100 mg L^{-1} DBT, the contact angle values decreased at the end-log phase (Fig. 4b), and again increased in the death phase. The contact angles varied from $43^\circ \pm 3^\circ$ to $63^\circ \pm 1^\circ$ for FLU and $48^\circ \pm 1^\circ$ to $64^\circ \pm 0.2^\circ$ for DBT degradation over 14 days.

An investigation on the uptake mechanism of FLU by *Pseudomonas putida* ATCC 17514 reported that the bacterial cells adhered to the surface of the substrate by modifying their cell surface hydrophobicity through the production of extracellular polymeric substances (Rodrigues et al. 2005). Two different bacterial strains, *Pseudomonas aeruginosa* Y and *Pseudomonas aeruginosa* S have been reported to utilize petroleum hydrocarbons through different mechanisms. The first strain could uptake petroleum hydrocarbons through direct contact mode, while the latter could uptake petroleum hydrocarbons by producing biosurfactant (Song et al. 2006).

Biosurfactant production was reported during degradation of DBT along with several other PAHs and aromatics using a bacterial strain, *Pseudomonas aeruginosa* DHT2 (Kumar et al. 2008). In addition to that, change in bacterial cell surface hydrophobicity (adherence to hexadecane $\sim 50\%$ after 1 week of growth on DBT in basal salt medium) was also observed, which in turn improved the affinity of the cells for DBT. In the present study, growth of the strain was higher on FLU than on DBT. FLU degradation was also significantly higher compared to DBT, which is also evident from the higher value of the first order degradation rate constant in case of FLU. Both the specific growth rate of the strain (μ) and the specific utilization rate (q) of FLU and DBT varied over a narrow range and did not show any specific trend. From the TOC profiles obtained during FLU and DBT degradation experiments, it was hypothesized that much of the degraded FLU and DBT might have been transformed into metabolites and dead-end products that did not degrade further. Accumulation of polar metabolites during FLU/DBT biodegradation might have induced toxic effects on degradation of FLU and DBT. GC \times GC-TOFMS was employed to identify the metabolites of fluorene (FLU) and dibenzothiophene (DBT) (Protocol described in SI). Only one metabolite of FLU, fluorenone was identified in the culture broth obtained during FLU degradation experiments. Fluorenone has been reported to be the first intermediate in one of the FLU degradation pathways (Rodrigues et al. 2005; Sonwani et al. 2019). Medić et al. (2020) reported formation of seven metabolites namely, 9H-Fluoren-9-ol, 9H-Fluoren-9-one (or fluorenone), 1,2-Benzene-dicarboxylic acid, protocatechuic acid, salicylic acid, adipic acid and succinic acid during degradation of FLU by a bacterial strain, *Pseudomonas aeruginosa* san ai using comprehensive two-dimensional gas chromatography–quadrupole mass spectrometry analysis. The authors proposed that the mechanism of FLU degradation involved two different pathways. In one of the pathways, FLU was sequentially converted to 9H-fluoren-9-ol,

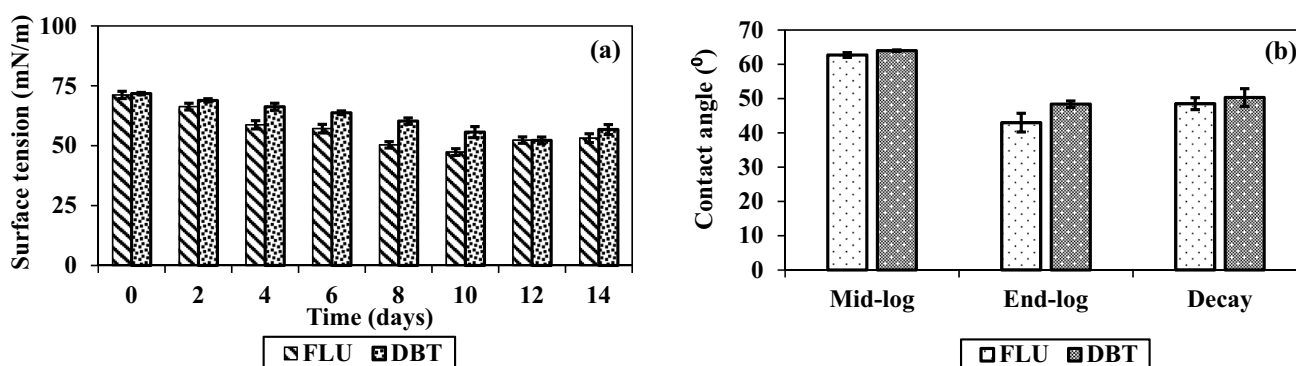


Fig. 4 **a** Surface tension of culture filtrate and **b** water contact angle on bacterial cell surface during degradation of 100 mg L^{-1} FLU and 100 mg L^{-1} DBT

9H-fluoren-9-one (formed by angular deoxygenation), 1,1a-dihydroxy-1-hydro-9-fluorenone, 2'-carboxy-2,3-dihydroxybiphenyl, phthalic acid, protocatechuic acid and adipate. The other pathway indicated sequential conversion of FLU to 3,4-fluorene-diol (formed by lateral deoxygenation), 1-indanone, salicylic acid, catechol and succinate. Adipate and succinate were eventually degraded by the central metabolite pathway. Presence of catechol-1,2-dioxygenase in the culture broths indicated ortho-cleavage of catechol formed during FLU degradation.

No metabolite of DBT could be identified in the current study. Seo (2012) reported dibenzothiophene (DBT) degradation pathway by a bacterial strain, *Mycobacterium aromativorans* strain JS19b1 using GC-MS. Initial deoxygenation occurred at C-1,2 and C-3,4 positions and monooxygenation occurred on the sulphur atom. Sulfur oxidation of DBT resulted in the formation of dibenzothiophene-5,5'-dioxide, initially, which was subsequently transformed to ring-cleavage products, cis-dibenzothiophene dihydrodiols, trans-4-(3-hydroxybenzo[b]thiophen-2-yl)-2-oxobut-3-enoic acid (formed due to meta-cleavage of dibenzothiophene-1,2-diol) and trans-4-(2-hydroxy-benzo[b]thiophen-3-yl)-2-oxobut-3-enoic acid (formed due to meta-cleavage of dibenzothiophene-3,4-diol). The ring-cleavage products were subsequently converted to 2,2'-dithiosalicylic acid via 2-mercaptobenzoic acid.

Protein concentration increased with incubation time and it was consistently higher in the cell extracts compared to that in the supernatant for both FLU and DBT degradation. Surface tension values in culture filtrates during FLU and DBT were not sufficiently low to indicate biosurfactant activity. The observed lowering in surface tension was possibly caused by metabolites. Based on contact angle measurement, it was concluded that hydrophobic cell surfaces of *P. aeruginosa* RS1 possibly played a role in direct interfacial uptake of FLU and DBT. The trends observed in cell surface contact angle during degradation of FLU and DBT indicated that a combination of dissolution and direct interfacial uptake was responsible for their uptake. Further studies on uptake mechanism may explore surface tension variation in dilutions of the culture filtrates to explore if biosurfactant is present at a concentration above the critical micelle concentration (CMC). This study for the first time presents a comprehensive investigation on degradation of both FLU and DBT when applied as sole substrates at varying initial concentration from 25 to 500 mg L⁻¹ by the bacterial strain, *P. aeruginosa* RS1 and also discusses their uptake mechanism. Such studies performed with the *P. aeruginosa* RS1 strain using the culture filtrates after growth on carbazole (CBZ) indicated negligible concentration of biosurfactant with respect to critical micelle concentration (CMC) (unpublished results). The strain was earlier reported to efficiently degrade a multi-ring PAH, pyrene (Ghosh et al. 2014) and a heterocyclic PAH, carbazole (Ghosh

and Mukherji 2020a, 2018) in mineral media and carbazole, fluorene, dibenzothiophene and other PAHs and heterocyclics in refinery wastewater matrix (Ghosh and Mukherji 2020b). Hence, the strain has the capability of degrading a wide range of PAHs and heterocyclics present in both mineral media (synthetic system) and industrial wastewater (refinery wastewater). Some of the other strains of *Pseudomonas* species, such as *Pseudomonas putida* ATCC 17484 (Loh and Yu 2000), *Pseudomonas cepacia* F297 (Grifoll et al. 1995) and *Pseudomonas* sp. LKY-5 (Li et al. 2019) have been reported to efficiently degrade CBZ (4.5 mg L⁻¹ CBZ completely degraded within 18 h), FLU (more than 90% of 831 mg L⁻¹ FLU degraded within 14 days) and DBT (100 mg L⁻¹ DBT completely degraded within 144 h), respectively, under aerobic condition. However, the potential of the strains for degrading the mixtures of CBZ, FLU and DBT or other PAHs and heterocyclics in various environmental matrices (such as, soil, wastewater) were not explored. Hence, the strain, *P. aeruginosa* RS1 is a novel strain, which can be applied to sites contaminated with these PAHs and heterocyclics.

Novelty of the current study can further be explored through conducting LC-MS/MS analysis of the metabolites of FLU and DBT (Ramsauer et al. 2011). This can give more insight on the degradation mechanisms of these substrates employed by the *P. aeruginosa* strain. The molecular structures of the metabolites can also be confirmed through NMR analysis (Aranda et al. 2017). FTIR spectroscopy of residues of metabolite extracts can further reveal the nature of the metabolites formed during their degradation. Wu et al. (2010) reported the formation of carboxyl group containing metabolites during phenanthrene degradation by a bacterial strain, *Flavobacterium* FCN2. Enzymes responsible for FLU and DBT degradation can also be identified through gel electrophoresis followed by HRMS-Orbitrap which can reveal the degradation pathways employed. The α and β subunits of a dioxygenase complex (FlnA1-FlnA2) in *Sphingomonas* sp. strain LB126 was reported to induce angular deoxygenation of FLU (Schuler et al. 2008). DNA extraction followed by qPCR with appropriate primers may be explored to search for presence of specific genes involved in various degradation pathways reported for FLU and DBT (Desta et al. 2019). Gene products of bdsC, bdsA and bdsB genes and various intermediates, such as DBT sulfone, 2'-hydroxybiphenyl-2-sulfinate, and 2-hydroxybiphenyl were identified during DBT degradation by *Bacillus subtilis* WU-S2B and *Mycobacterium phlei* WU-F1 (Kirimura et al. 2004).

Conclusions

The specific growth rate profiles of the bacteria on both FLU and DBT could be better fitted using logistic model. For both FLU and DBT, specific growth rate (μ) values were

within a narrow range and specific substrate utilization rate (q) values showed an increasing trend with increase in initial substrate concentration. During degradation of FLU and DBT, increase in aqueous phase TOC indicated the accumulation of intermediates and disc diffusion studies using *E. coil* revealed the toxic nature of the intermediates. No clear role of biosurfactant on the uptake of FLU and DBT was evident, rather dissolution and direct interfacial uptake possibly facilitated the uptake of FLU and DBT.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s13205-021-02742-7>.

Acknowledgements The authors gratefully acknowledge the Sophisticated Analytical Instrumentation Facility (SAIF), IIT Bombay for providing the HPLC facility. Partial funding for this work was provided by a project funded through IRCC, IIT Bombay (Grant no. 12IRAWD007).

Author contributions PG: experimental analysis, methodology, data processing, investigation, writing-original draft. SM: supervision, review, editing, funding acquisition.

Funding Partial funding for this work was provided by a project funded through IRCC, IIT Bombay (Grant no. 12IRAWD007).

Declarations

Conflict of interest There is no potential conflict of interest of the present study with any person or any organization or agency.

Accession number MTCC 25391 for *Pseudomonas aeruginosa* RS1.

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