

Biodegradation kinetics of acenaphthene by *Sphingobacterium* sp. strain RTSB isolated from a petroleum-contaminated soil

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Acenaphthene metabolism was previously reported by a Gram-negative *Sphingobacterium* sp. strain RTSB. The degradation pathway was found to proceed involving the key intermediate 1-naphthoic acid and processed via *trans*-3-carboxy-2-hydroxybenzylidene-pyruvic acid to salicylic acid and catechol. The present article demonstrates the biodegradation kinetics of acenaphthene along with the fate of some of the major metabolites, 1-naphthoic acid, 1-acenaphthenol, 1-acenaphthenone, acenaphthenequinone, salicylic acid, and catechol; where, the maximum accumulated amount of 1-naphthoic acid measured was 302.1 mg l⁻¹ during the mid-log stage of growth. The degradation of acenaphthene was observed to follow a first order kinetic model with a rate constant 0.528 d⁻¹ and half-life of 1.31 days.

Keywords: Acenaphthene, biodegradation, kinetic study, 1-naphthoic acid, *Sphingobacterium* sp.

POLYCYCLIC aromatic hydrocarbons (PAHs) include a cluster of widely present contaminants due to their potential carcinogenic, mutagenic, and/or toxic properties¹⁻³. PAHs are considered persistent organic pollutants because of their high stability and hydrophobic nature, and because of these, cleaning up of sites polluted with PAHs by conventional techniques are somewhat difficult. Consequently, bioremediation studies of PAHs have acquired substantial interest nowadays and numerous microorganisms capable of degrading diverse PAH molecules have been reported⁴⁻⁸.

According to the U.S. Environmental Protection Agency, acenaphthene is considered as one of the 16 priority PAH pollutants⁹ and released in the ambience primarily by anthropogenic and pyrolytic processes in addition to emissions and effluents from different industries^{10,11}. In recent years, extensive use of acenaphthene in several industries¹¹ resulted in their high level discharge and accumulation in the environment, which caused an alarming concern among the environmental scientists. In the past few decades, acenaphthene bioremediation studies involving

various bacterial sp. had gained great interest and were reported^{10,12-24}. There are diverse reports of acenaphthene assimilation by a solitary isolate being able to degrade acenaphthene as the individual carbon source^{15,18,19,22-24} along with co-metabolism studies^{10,12,24,25}; and depending on the bacterial sp. under examination, numerous metabolic pathways exist. In an earlier study, a comprehensive report of acenaphthene biodegradation was made by *Sphingobacterium* sp. strain RTSB via 1-naphthoic acid, which was then degraded via *trans*-3-carboxy-2-hydroxybenzylidene-pyruvic acid to salicylic acid and catechol¹⁹.

Even though aerobic biodegradation is a vital technique for the atmospheric reduction of PAHs, potential estimation of PAH biodegradation rates is more significant to predict environmental destiny as well as to manipulate bioremediation efforts. Information related to biodegradation rate is very much desired for metabolic regulation and engineering of PAH assimilation pathways as well as for the enhancement of degradation competence. Although there are several reports of biodegradation studies of acenaphthene by different microorganisms, neither of them revealed any detailed kinetic study of the degradation pathway. In this study, both kinetic and metabolic aspects of acenaphthene biodegradation and the outcome of metabolic intermediate 1-naphthoic acid together with other metabolites, viz. 1-acenaphthenol, 1-acenaphthenone, acenaphthenequinone, salicylic acid and catechol were explored in the acenaphthene degradation pathway by *Sphingobacterium* sp. strain RTSB. Therefore, the present study may be useful in refining the competence of acenaphthene degradation along with other PAHs and to remediate the environment contaminated with PAHs.

Materials and methods

Isolation of the bacterial strain

Isolation of the *Sphingobacterium* sp. strain RTSB, a novel acenaphthene degrading bacterium, was carried out by enrichment culture technique¹⁹ from the soil contaminated with petroleum with acenaphthene (1 g l⁻¹), as the solitary carbon and energy resource.

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Media and culture conditions

Growth of the bacterial cells was carried out in a liquid mineral salt medium (MSM, pH 7.2) containing 3.34 g K_2HPO_4 , 2.0 g NH_4Cl , 0.87 g NaH_2PO_4 , 200 mg $MgSO_4 \cdot 7H_2O$, 123 mg nitrilotriacetic acid, 12 mg $FeSO_4 \cdot 7H_2O$, 3 mg $ZnSO_4 \cdot 7H_2O$, 3 mg $MnSO_4 \cdot H_2O$ and 1 mg $CoCl_2 \cdot 6H_2O$ per litre.

Degradation experiments

Acenaphthene degradation experiments were conducted at 28°C on a rotating shaker (180 rpm) in a 100 ml conical flask having 25 ml of MSM (pH 7.2) supplemented with acenaphthene (1 g l^{-1}) as the solitary source of carbon, and the resultant metabolites of the acenaphthene degradation pathway were isolated and recognized by combined chromatographic and spectrometric techniques¹⁹. To optimize the conditions of acenaphthene assimilation by the strain RTSB, replicate bacterial cultures in MSM with 1 g l^{-1} of acenaphthene were incubated at different incubation temperatures and pH values in the range of 22°C to 42°C, and 5.0 to 9.0 respectively. The degradation process was monitored with different preliminary acenaphthene concentrations ($0.05\text{--}5.0 \text{ g l}^{-1}$) and incubated for diverse periods at optimized temperature and pH. To estimate the remaining acenaphthene in the culture medium at each sampling point, a spent broth was centrifuged at 8000 g for 12 min and the pH of supernatant liquids was adjusted to 1.5–2.0 by concentrated HCl. The reaction mixtures were then extracted thrice with ethyl acetate, dried, and evaporated under reduced pressure at 40°C; and the residual acenaphthene in the medium was quantified through HPLC analysis.

For the measurement of 1-naphthoic acid degradation, the substrate was supplemented in the range of $0.05\text{--}1.0 \text{ g l}^{-1}$; whereas in the other experimentation, 0.1 g l^{-1} of 1-naphthoic acid was added in each installment (total 0.6 g l^{-1} of 1-naphthoic acid) at 24 h intervals. Acenaphthene and 1-naphthoic acid degradation experiments were conducted with 1% inoculums (OD_{600} adjusted to 0.5) and 2% inoculums (OD_{600} adjusted to 0.25) respectively, when incubated with acenaphthene (1 g l^{-1}) for 72 h and 1-naphthoic acid (0.5 g l^{-1}) for 48 h. Based on the amount of cellular protein, cell growth was estimated according to the process of Lowry *et al.*²⁶. Unless mentioned otherwise, all experiments were carried out in triplicate sets.

Analysis and quantification of the metabolites

Following incubation, at different time intervals, centrifugation of both the spent broth and resting cell cultures were performed at 8000 g for 12 min and extracted with ethyl acetate thrice after adjusting the pH of the supernatants to 1.5–2.0 by concentrated HCl. Later, the extracts

obtained were combined, dried out, and evaporated under reduced pressure at 40°C and the resultant residues were used for further analyses. Characterization and quantification of acenaphthene, 1-naphthoic acid, and the resulting metabolites were carried out using HPLC system (Waters Corp., USA) equipped with a $5 \mu\text{m}$, $4.6 \text{ mm} \times 250 \text{ mm}$ analytical Inertsil ODS-3 column (MetaChem Technologies, Germany), fitted-out through a protector column filled with an identical immobile phase and connected to a Waters 515 solvent supply arrangement. Elution of the metabolites was performed employing a planned gradient of a methanol-water solvent system at a flow speed of 1.0 ml per min and detected at 254 nm by a 486 adjustable absorbance UV detector (Waters). The movable phase was a linear gradient (45 min) from 45% (v/v) to 95% (v/v) of aqueous methanol, withholding at 90% for 10 min, and subsequently 95% to 45% (v/v) of the same solvent system within 5 min. By comparing the retention times with that of the authentic compounds analysed in identical conditions, the eluted metabolites were identified. The quantity of unaltered acenaphthene along with residual and/or accumulated 1-naphthoic acid and other metabolites were also measured employing HPLC analyses using version 2.15.01 of the Millennium Session Manager Software package¹⁹. Without any refinement, analytical grade acenaphthene, 1-naphthoic acid along with other chemicals and reagents were used to carry out all the experiments.

Results and discussion

An acenaphthene-degrading bacterial strain RTSB was isolated from a petroleum-polluted soil and was identified as *Sphingobacterium* sp. Isolation and categorization of diverse metabolites recommended the conversion of acenaphthene through 1-naphthoic acid via 1-acenaphthenol, 1-acenaphthenone, and acenaphthenequinone in the upper stage of assimilation; followed by the transformation of 1-naphthoic acid to salicylic acid and catechol through *trans*-3-carboxy-2-hydroxybenzylidenepyruvic acid, ultimately entering into the TCA cycle intermediates¹⁹. It is the first comprehensive report of acenaphthene assimilation with a *Sphingobacterium* sp. strain, competent of utilizing acenaphthene as the only carbon source; where, within 120 h of incubation, over 90% acenaphthene was found to be degraded.

Acenaphthene degradation experiments were performed at different incubation temperatures and pH values in the array of 22°C to 42°C (Figure 1 a) and 5.0 to 9.0 (Figure 1 b) respectively, to optimize the condition of acenaphthene assimilation by the strain RTSB (with optimum 1 g l^{-1} acenaphthene concentration). The optimal circumstances for the growth of *Sphingobacterium* sp. strain RTSB with acenaphthene (1 g l^{-1}) were set on as 28°C and pH 7.2 in a shake culture situation at 180 rpm.

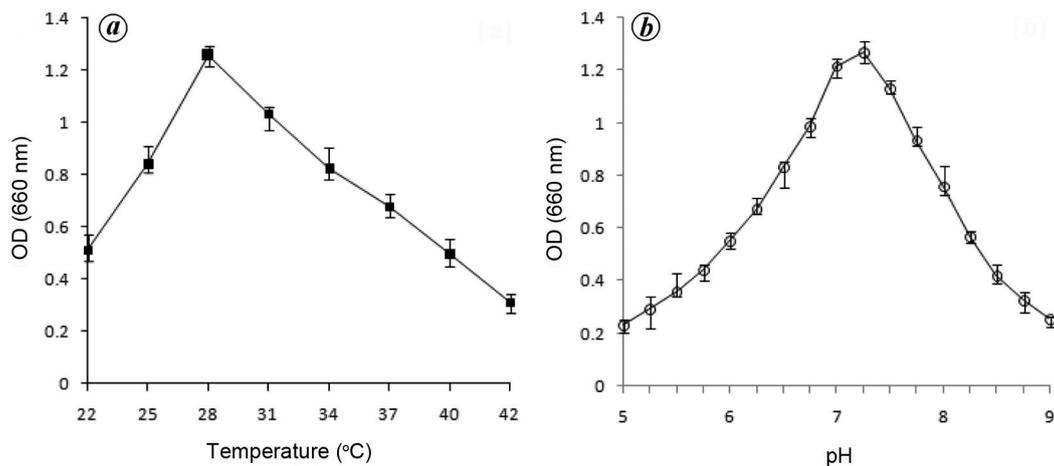


Figure 1. Growth of acenaphthene by *Sphingobacterium* sp. strain RTSB in MSM (a) at different incubation temperatures (■) and (b) different pH values (○) with initial acenaphthene concentration of 1 g l^{-1} . The errors of three independent samples are indicated by bars.

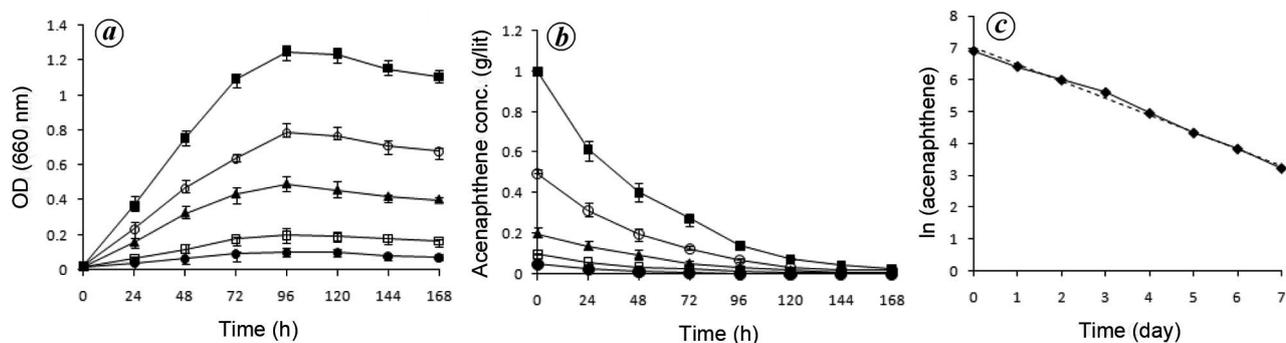


Figure 2. (a) Growth and (b) degradation of acenaphthene by *Sphingobacterium* sp. strain RTSB in MSM at different initial acenaphthene concentrations of 0.05 g l^{-1} (●), 0.1 g l^{-1} (□), 0.2 g l^{-1} (▲), 0.5 g l^{-1} (○), and 1 g l^{-1} (■), (c) Plot of the natural logarithm of acenaphthene concentration against degradation time by *Sphingobacterium* sp. strain RTSB. The errors of three independent samples are indicated by bars.

Growth summary of *Sphingobacterium* sp. strain RTSB in the course of acenaphthene assimilation at diverse preliminary concentrations is represented in Figure 2a. The doubling time found was 32.18, 31.26, 29.96, 28.32 and 26.27 h respectively, during bacterial growth with 0.05, 0.1, 0.2, 0.5 and 1 g l^{-1} of initial acenaphthene concentrations in MSM medium. The precise growth rate observed was 0.0327, 0.0341, 0.0362, 0.0391 and 0.0423 h^{-1} respectively, under similar set of initial acenaphthene concentrations. Figure 2b describes the condition of acenaphthene at the time of incubation by *Sphingobacterium* sp. strain RTSB at various preliminary concentrations of the substrate. At the time of cultivation, bacterial degradation of acenaphthene encountered a linear decline phase associated with the log-phase growth of *Sphingobacterium* sp. strain RTSB, when a linear correlation was observed among acenaphthene depletion rates and the primary acenaphthene concentration. The plot of the normal logarithm of acenaphthene concentration against time shown in Figure 2c is linear ($R^2 = 0.9947$) under ex-

perimental setting and is appropriate to the Monod first-order kinetic equation²⁷ as $\ln C = -Kt + A$, where C , t , K and A represent the acenaphthene concentration, time, first-order rate constant and a constant respectively. The rate constant K and the constant A determined from the experimental outcome were 0.528 d^{-1} and 7.536 respectively. Consequently, the first-order equation might be demonstrated as $\ln C = -0.528t + 7.536$ and the half-life ($t^{1/2} = 0.693/K$) was 1.31 d. Therefore, in this case, acenaphthene assimilation by *Sphingobacterium* sp. strain RTSB is predicted to follow first-order reaction kinetics.

A steady increase in acenaphthene degradation rate was primarily noticed with an augment in initial acenaphthene concentration up to 1 g l^{-1} . A rapid fall of acenaphthene concentration was observed in the beginning phase of incubation followed by stagnation. Assimilation of acenaphthene by bacterial cells along with the adsorption of substrate to the cell wall could be the possible reason behind this type of speedy primary decline of the concentration^{28–30}. However, the relative assimilation rate of

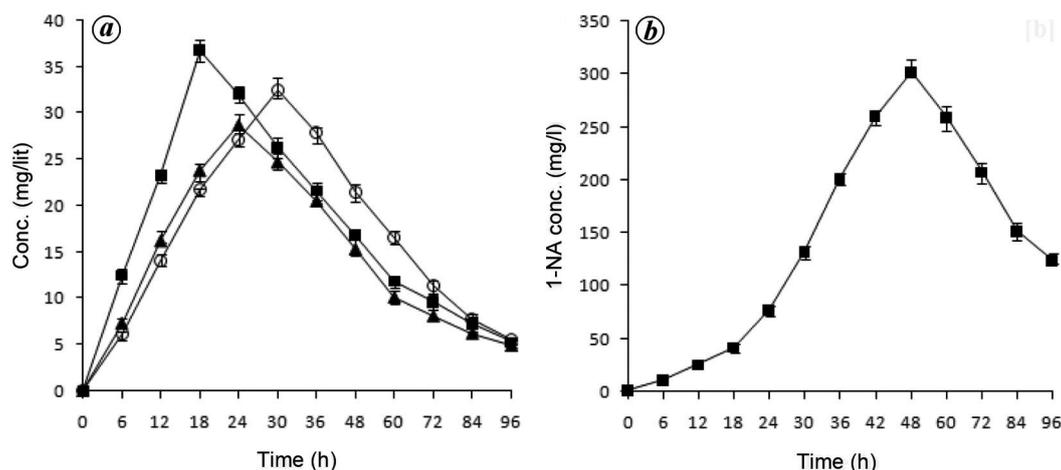


Figure 3. Accumulation and utilization of (a) 1-acenaphthenol (■), 1-acenaphthenone (▲) and acenaphthenequinone (○) and (b) 1-naphthoic acid (■) during incubation of acenaphthene up to 96 h by *Spingobacterium* sp. strain RTSB in MSM with initial acenaphthene concentration of 1 g l^{-1} . The errors of three independent samples are indicated by bars.

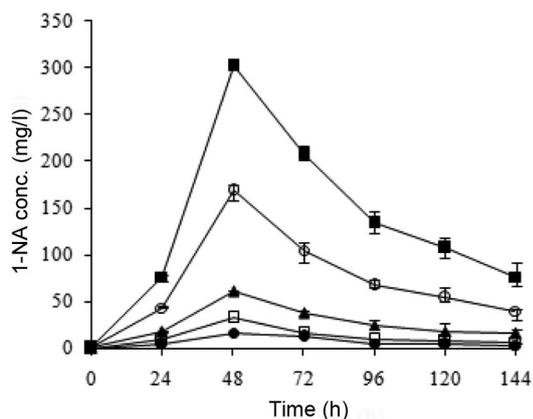


Figure 4. Accumulation along with the utilization of 1-naphthoic acid during incubation of acenaphthene up to 144 h by *Spingobacterium* sp. strain RTSB in MSM at diverse preliminary acenaphthene concentrations. The symbols are identical as in Figure 2.

acenaphthene (per gram) falls steadily ahead of 1 g l^{-1} of concentration; and a severe decrease in its degradation rate was observed over 5 g l^{-1} of its primary concentration. The declining rate of biodegradation of acenaphthene ahead of 1 g l^{-1} of concentration maybe because of the accumulation of increased levels of 1-naphthoic acid and other toxic metabolites generated at the time of the degradation process. Formation and utilization of various metabolites in the earlier stages of acenaphthene degradation, viz. 1-acenaphthenol, 1-acenaphthenone and acenaphthenequinone, involved in the conversion of acenaphthene to 1-naphthoic acid were monitored kinetically using 1 g l^{-1} of initial acenaphthene concentration (Figure 3a). It was observed that the maximum amount of 1-acenaphthenol, 1-acenaphthenone and acenaphthenequinone accumulated was 36.8 (18 h), 28.7 (24 h), and 32.5 (30 h) mg l^{-1} respectively and simultaneously the amount

of 1-naphthoic acid accumulated was 41.9 (18 h), 76.9 (24 h), and 132.5 (30 h) mg l^{-1} respectively (Figure 3b).

Figure 4 denotes 1-naphthoic acid accumulation and utilization trends by the strain RTSB in acenaphthene degradation pathway. It was observed that the maximum accumulation of 1-naphthoic acid occurred in the mid-log stage of growth regardless of primary acenaphthene concentration, and the highest amount of accumulated 1-naphthoic acid quantified was 302.1 mg l^{-1} (with 1 g l^{-1} of initial acenaphthene concentration). It was also observed that, with the rise in initial acenaphthene concentration, the amount of accumulated 1-naphthoic acid also increased (457.7 and 638.3 mg l^{-1} with 2 and 4 g l^{-1} of initial acenaphthene concentrations respectively). The accumulation of 1-naphthoic acid in the mid-log phase was perhaps ascribed by the fact that the genetic basics accountable for the assimilation of acenaphthene to 1-naphthoic acid and metabolism of 1-naphthoic acid via the metabolite *trans*-3-carboxy-2-hydroxybenzylidene-pyruvic acid¹⁹, be in two diverse degradative operons, and the dissimilar metabolic directive of these two operons result in the accumulation of the said metabolite. Since acenaphthene is solely processed via 1-naphthoic acid, it can be perhaps concluded that the action and/or expression of the group of enzymes involved earlier, that transform acenaphthene to 1-naphthoic acid is higher than that of the set of enzymes associated in the lower degradation pathway¹⁹. However, ahead of the mid-log stage, a steady reduction of 1-naphthoic acid concentration was observed possibly due to low availability of its precursor acenaphthene in the culture medium as well as due to the existence of elevated concentration of enzymes concerned in the ring-cleavage of 1-naphthoic acid.

It was reported earlier that *Spingobacterium* sp. strain RTSB grown on acenaphthene showed a positive

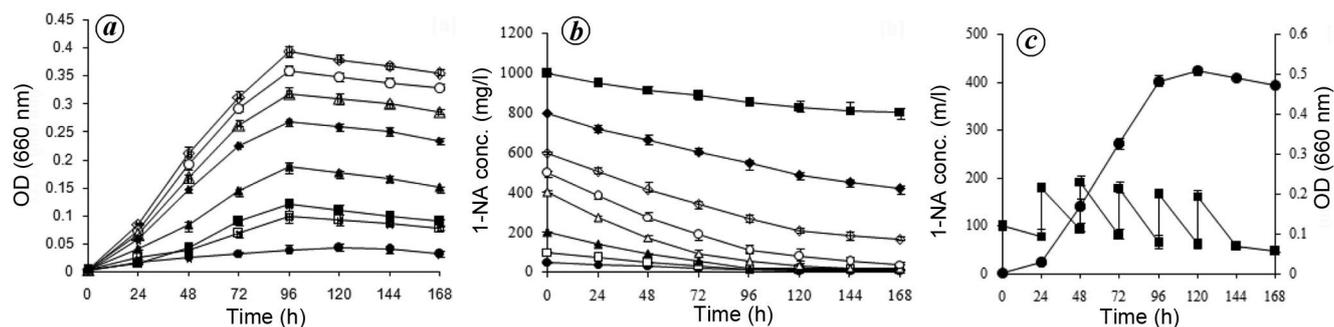


Figure 5. (a) Growth and (b) degradation of 1-naphthoic acid acenaphthene by *Sphingobacterium* sp. strain RTSB in MSM at various initial 1-naphthoic concentrations of 0.05 g l^{-1} (●), 0.1 g l^{-1} (□), 0.2 g l^{-1} (▲), 0.4 g l^{-1} (△), 0.5 g l^{-1} (○), 0.6 g l^{-1} (◇), 0.8 g l^{-1} (◆) and 1 g l^{-1} (■), (c) Growth (●) and degradation (■) of 1-naphthoic (0.6 g l^{-1}) supplemented in installments, 0.1 g l^{-1} at 24 h intervals.

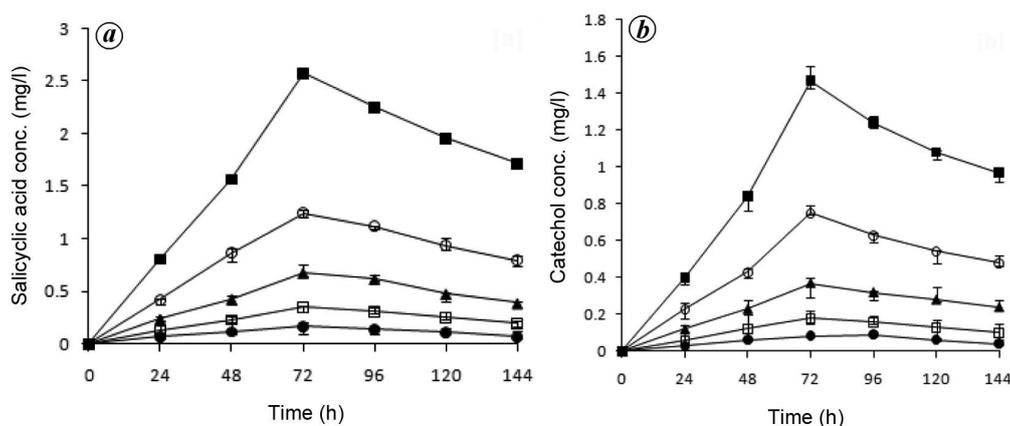


Figure 6. Accumulation and utilization of salicylic acid (a) and catechol (b) during incubation of acenaphthene by *Sphingobacterium* sp. strain RTSB in MSM at different preliminary acenaphthene concentrations. The symbols are identical as in Figure 2.

response in oxygen uptake studies, as well as it could utilize 1-naphthoic acid as the growth substrate¹⁹. Figure 5a illustrates the growth curve of RTSB at different initial concentrations of 1-naphthoic acid (0.05 , 0.1 , 0.2 , 0.4 , 0.5 , 0.6 , 0.8 and 1 g l^{-1}) as the growth substrate, while the biodegradative outcome of 1-naphthoic acid at the time of incubation (taking the same set of substrate concentration) is represented in Figure 5b. It was seen that with an initial increase of 1-naphthoic acid concentration up to 0.6 g l^{-1} , the growth increases. However, a steady decrease in growth was documented with an additional raise in the concentration. A possible toxicity of the substrate at an elevated preliminary concentration to the organism could be the reason behind this type of decline in growth. This fact was also supported by the observation that the fall in 1-naphthoic acid concentration was quite steady at low primary concentration, but at an elevated concentration, the lowering was less steady. This observation was confirmed by adding 0.6 g l^{-1} of 1-naphthoic acid in installments (with 0.1 g l^{-1} in six installments) at 24 h intervals (Figure 5c), when 30.2% more growth yield was observed compared to that when the same amount (0.6 g l^{-1}) was added at once at zero hour. In addition to 1-naphthoic acid, fate of the two

additional metabolites downstream of the acenaphthene degradation pathway, namely salicylic acid and catechol, was also investigated (Figure 6). Salicylate hydroxylase mediated conversion of salicylic acid to catechol was observed in this case, which subsequently metabolized to 2-hydroxyruconaldehyde acid by the activity of catechol-2,3-dioxygenase, finally forming TCA cycle intermediates¹⁹. The accumulated amount of salicylic acid and catechol observed was 2.58 and 1.47 mg l^{-1} respectively after 72 h of incubation with 1 g l^{-1} of acenaphthene. Very low amounts of accumulation of these two metabolites were due to the high level activities of 3-formylsalicylate oxidase and catechol-2,3-dioxygenase¹⁹.

Conclusion

The kinetic and metabolic studies on PAH assimilation are fundamental and significant for better understanding of PAH biodegradation pathways, and to assemble and engineer bioprocessing plans to get better degradation competence. In the present article, a comprehensive description is offered on the kinetic and metabolic aspects of acenaphthene assimilation pathway by *Sphingobacterium* sp. strain RTSB, which follows the first-order

reaction kinetics. The experimental results indicated that apart from the alteration in temperature, pH value, and substrate concentration, acenaphthene biodegradation by the strain RTSB is also influenced by the metabolic regulation and on the gathering of growth inhibitory metabolites, for instance, 1-naphthoic acid. Therefore, the kinetic and metabolic aspects of acenaphthene assimilation by the *Sphingobacterium* sp. strain RTSB are interesting, which may offer a superior option for microbial choice in the bioremediation of PAHs and/or other toxic organic pollutants.

Conflict of interest: The author declares no conflict of interest.

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ACKNOWLEDGEMENTS. I acknowledge Dr Tapan K. Dutta, Bose Institute, Kolkata for providing instrumental and laboratory facilities. This study was supported by the University Grants Commission of India.

Received 24 October 2019; revised accepted 6 November 2020

doi: 10.18520/cs/v120/i5/926-931