Benzene utilization as growth substrate by a newly isolated Aerococcus sp. strain BPD-6 indigenous to petroleum hydrocarbon contaminated oily sludge

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Abstract— Monoaromatic hydrocarbons such as benzene have been found in sludge created by oil production facilities and industries. Benzene is considered as a carcinogenic substrate and biodegradation, primarily under aerobic conditions, is an important environmental process for water and soil associated benzene remediation. *Aerococcus* sp. strain BPD-6 isolated from petroleum hydrocarbon contaminated oily sludge was characterized in terms of its growth in presence of benzene (50–1000 mg L^{-1}) as the sole carbon and energy source in mineral salts medium. The growth kinetics of the strain was analysed and the Haldane model was found to be a good fit for the experimental data with kinetic constants maximum specific growth rate (μ_{max}) = 0.02157 h^{-1}, half saturation constant K_s = 19.56 mg L^{-1} and substrate inhibition constant K_i = 1584 mg L^{-1}. The biodegradation rate kinetic parameter was obtained for the first order kinetic model and thus the results indicate that this Gram-positive strain can potentially be utilized in bioremediation of benzene containing oily sludge.

Index Terms— *Aerococcus* sp., Benzene, Bioremediation, Gram-positive, HPLC, Oily sludge.

1 INTRODUCTION

Environmental contamination by volatile organic compounds (VOCs) like benzene, toluene, ethyl benzene and xylenes originating from petrochemical and allied industries is of great public health concern [1]. Benzene is widely used during plastic, detergents and pesticide production [2]. It has been considered as a priority pollutant worldwide only because of their large migration abilities, solubility in water, toxicity, and volatility [3]. According to the US Environmental Protection Agency (1996) and the Agency for Toxic Substances and Disease Registry (2005) the maximum permissible level of benzene in water is 0.5 ppm.[4] Human exposure to benzene is a global environmental problem. It is known as highly toxic and carcinogenic pollutant.[5] After inhalation or absorption, benzene targets organs viz. liver, kidney, lung, heart and brain etc. Benzene causes haematotoxicity through its phenolic metabolites that act in concert to produce DNA strand breaks and chromosomal damage along with it also causes apneas, lung cancer or leukemia and other adverse effects [1], [5], [7]. Therefore improper waste disposal or accidental spills of benzene poses acute threat to human health as well as to natural ecosystems [6]. The Clean Air Act Amendments of 1990 proposed by the US Environmental Protection Agency places special emphasis on the handling, usage and treatment of monoaromatic compounds like benzene. Hence there is a pressing need for development of efficient and cost effective technologies for the treatment of such pollutant [1], [5].

Bioremediation of such organic solvents has been considered as an adjunct/alternative to the traditional physical and chemical treatment methods that are costly, energy intensive and produce hazardous by-products [8]. Biodegradation employing growing cells is an excellent alternative as viable cells provide rich metabolic flux in the form of enzymes capable of degrading organic compounds [9], [10]. Furthermore, benzene compounds are easily degraded aerobically via oxygenase-catalysed reactions, while such compounds tend to persist under anaerobic conditions. In order to properly design and model biodegradation processes it is necessary to determine the degradation kinetics of benzene compound by bacterial isolate. Degradation of benzene component by pure bacterial strains has been well studied, such as benzene biodegradation by *Pseudomonas putida* F1 occurs through oxidation of aromatic ring via a dioxygenase mediated reaction that results in the production of catechol [11]. Likewise biological treatment methods using bioscrubbing, biofilters or two phase partitioning bioreactors have been implemented for the biodegradation of benzene [12].

Many researchers have undertaken fundamental investigations for the degradation of benzene by microorganisms. Benzene degradation by Gram negative bacteria including *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Pseudomonas putida* has been extensively studied [13], [14], [15]. However report on the utilization of benzene by Gram positive bacteria is

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scarce and particularly by *Aerococcus* sp. has not been reported so far. It was earlier considered that the outer membrane present in Gram-negative bacteria shields the organisms from the attack of the organic lipophilic compounds. Fahy et al. (2008) demonstrated that Gram positive bacteria belonging to the genera *Bacillus, Rhodococcus*, *Staphylococcus* and *Arthrobacter* have good tolerance to various concentrations of benzene and were better contenders than the Gram negative ones as well as grew well in groundwater microcosm imitating the natural circumstances [16]. Diverse kinetic substrate utilization and inhibition models like Monod and Haldane equations have been used to describe the growth dynamics of microorganisms on hydrocarbons [17]. A variety of organic substrate concentrations ranges and environmental conditions influence the kinetic parameters such as half saturation constant K_s and substrate inhibition constant K_i are employed to describe the biodegradation process of the microorganisms. These kinetic parameters depend on the type of cell and its culture environments [18]. Thus the knowledge of the growth kinetics and degradative potential of the indigenous microorganisms is vital in order to design appropriate biological reactors and to ensure the long term stability of the scaled up process for the treatment of contaminated sites.

Previously, we have isolated and identified a Gram positive bacteria *Aerococcus* sp. strain BPD-6 from petroleum hydrocarbon containing oily sludge [19]. The strain was characterized in terms of its benzene utilization potential as a sole source of carbon and energy during its growth. The best physicochemical conditions suitable for the growth of this strain and consequently the degradation kinetics of the monoaromatic compound benzene were also examined.

2 EXPERIMENTAL SECTIONS

2.1 Chemicals

Benzene, methanol, acetonitrile and n-hexane were purchased from Merck (India), while media components were procured from HiMedia (India). All the solvents used in this study were HPLC grade and other chemicals were analytical grade with 99-100% purity.

2.2 Microorganism and growth conditions

The strain BPD-6 was isolated from a petroleum hydrocarbon containing sludge collected from a storage reservoir of Bharat Petroleum Corporation Limited, Durgapur, India [19]. Luria-Broth (LB) and mineral salt medium (MSM) of culture media were used for the growth of this strain. The composition of MSM used was as follows (g L^{-1} of deionized water): K_2HPO_4, 0.348; KH_2PO_4, 0.272; NaCl, 4.68; NH_4Cl, 1.07; KCl, 1.49; Na_2SO4, 0.43; $MgCl_2.2H_2O$, 0.2; $CaCl_2.2H_2O$, 0.03.Additionally 2.5 ml trace element from stock solution composed of (g L^{-1} of deionized water): EDTA, 0.05; $MgSO_4.7H_2O$, 3; $MnSO_4.H_2O$, 0.5; NaCl, 1; $FeSO_4.H_2O$, 0.1; Anhy.$CaCl_2$, 0.1; Al $(SO_4)_2$, 0.01; H_3BO_3, 0.01; $Na_2MoO_4.2H_2O$, 0.01; Na_2 SeO_3, 0.001. The pH of the medium was adjusted to 7.2 and the medium was sterilized by autoclaving (121°C and 15lb/seq. inch for 15 min) prior to the addition of the trace element solution (filter sterilized) and the organic substrate of benzene.

2.3 Morphological, biochemical and physiological characterization of the isolate

The bacterial strain BPD-6 was isolated and identified by color and colony morphology. Gram staining and other biochemical tests like indole production, methyl red (MR), Voges-Proskauer (V-P), citrate utilization test, casein hydrolysis, nitrate reduction, oxidase activity, catalase and urease activity were tested. Acid and gas production from glucose, sucrose, fructose, maltose, galactose, manitol, lactose, inositol, arabinose, mannose and salicin were also performed using standard protocol. Anaerobic growth was observed on anaerobic agar plates in anaerobic jar with gas pack and anaerobic indicator (HiMedia, India).

2.4 Batch growth and degradation study

All batch benzene degradation experiments using strain BPD-6 were carried out by acclimatization of the strain to the increasing concentration of benzene 10–50 mg L^{-1} as the sole source of carbon. The experiments were conducted in 100 mL serum bottles containing 25 mL of MSM supplemented with 50 mg L^{-1}, 100 mg L^{-1}, 200 mg L^{-1} and 400 mg L^{-1} of benzene as sole source of carbon and energy. The culture serum bottles were closed with butyl rubber caps and sealed with aluminium crimps to prevent volatization of benzene and incubated in an orbital shaker maintained in dark at 37°C and 150 rpm. Before, sealing 2% of the acclimatized culture was added to each serum bottles directly under aseptic conditions. All the experiment was carried out in duplicate.

Un-inoculated bottles were kept under the same conditions to determine the abiotic losses during the experiment. Samples were collected in polytetrafluoroethylene (PTFE) tubes at various time intervals from the starting of the experiments, using air tight disposable syringe and subsequently centrifugation was carried out at 10,000 rpm for 10 min at 4°C in a Centrifuge (Eppendorf, Germany) to separate cell mass and the supernatant for extraction of residual benzene concentrations. The results obtained from the same set of duplicate experiments were averaged and are plotted.

Different physicochemical factors such as pH, temperature and salinity were assayed in presence of benzene as a substrate during growth of the strain BPD-6 and examined the optimum pH, temperature and salinity. The pH was maintained in the medium ranging from (2–12) using 1M HCl or 1M NaOH and the NaCl salt concentration and temperature were maintained 1.0–6.0 g L^{-1} (w/v) and 20–60°C, respectively. In all the cases, 2% v/v of acclimatized inoculum was taken and inoculated in 25 mL MSM containing 100 mg L^{-1} of benzene. All the experimented serum bottles were incubated in dark at 37°C under shaking conditions (150 rpm) till the

growth reached the late log phase. Samples were collected at regular intervals of 4 hours up to 36 hours of time and the bacterial cell concentration were determined by UV–visible spectrophotometer (U–2800, Hitachi) by measuring the absorbance or optical density (OD) of the cell suspensions at 600 nm.

2.5 Software used

Regression analysis was performed with the data analysis tool pack of Microsoft Excel®. The model equations were solved using nonlinear regression method using GraphPad Prism® 6.0 based on Windows 7.

2.6 Analytical methods

Biodegradation of benzene compound by bacteria was determined by quantifying the amount of aqueous residual benzene in the culture medium at different time intervals using high performance liquid chromatography (HPLC). Benzene was extracted with a mixture of equal volume of n-hexane and culture medium (1:1, v/v) and the extracted samples were filtered through 0.45µm pore size filter and 20µl sample was analysed in reversed phase C18 column equipped with a 4.6 × 250mm, 5µm particle size, with UV-Visible detector at an excitation wavelength of 208 nm. The mobile phase was a mixture of acetonitrile and de-ionized water at a ratio of 75:25 with a constant flow rate of 1 ml min^{-1}. The samples were injected individually and the residual benzene concentration was calculated from the standard. Biomass concentration in the samples was monitored by measuring its absorbance at 600 nm wavelength using a UV–Visible Spectrophotometer, Model 23.

2.7 Scanning electron microscopy (SEM)

The strain BPD-6 cells were growth in MS medium in the presence of glucose (0.5%) as well as in benzene (50 mg L^{-1}) and the log phase cells were separated fixed with 2.5% (v/v) glutaraldehyde in phosphate buffer for 12-18 hours, rinsed in buffer and then dehydrated by sequential immersion in increasing concentration of ethanol (30, 50, 70, 90 and 100% alcohol) and centrifuged for 5 min each. Subsequently the specimens were coated with gold to be finally observed with a Hitachi S-530 scanning electron microscope at an acceleration voltage of 25 kV.

3 RESULTS AND DISCUSSION

3.1 Morphological, biochemical and physiological characterization of the isolate

The strain BPD-6 was isolated from petrochemical oily sludge was round-shaped and formed entire, convex, smooth and light yellow color colonies. As shown in Table 1, this strain was gram positive, Voges-Proskauer test positive bacterium and also able to hydrolysis casein as a substrate. The strain BPD-6 was negative for catalase, oxidase, indole production, methyl red test and the strain was unable to reduce nitrate. No growth was observed under anaerobic conditions. The strain produced gas or acid from glucose, sucrose, fructose, maltose, galactose, manitol, lactose, mannose and salicin but no gas or acid was produced from arabinose and inositol. By analyses and comparing 16S rRNA gene sequence, this strain was identified previously as *Aerococcus* sp. The Genbank accession number of *Aerococcus* sp. strain BPD-6 is JN377811.1 [19].

Table 1. MORPHOLOGICAL, BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF *AEROCOCCUS* SP. STRAIN BPD-6 ISOLATED FROM PETROLEUM OILY SLUDGE.

Characteristic properties	Growth of strain BPD-6
Color of colonies	Light yellow
Form	Round
Margin	Entire
Elevation	Convex
Gram staining	Gram positive, round shaped
Indole production	-
Methyl red	-
Voges-Proskauer	+
Citrate utilization	-
Nitrate reduction	-
Oxidase test	-
Catalase test	-
Urease	-
Casein hydrolysis	+
Anaerobic growth	-
Carbohydrate utilization	
Glucose	+
Sucrose	+
Fructose	+
Maltose	+
Galactose	+
Manitol	+
Lactose	+
Inositol	-
Arabinose	-
Mannose	+
Salicin	+

3.2 Effect of initial benzene concentration on the growth of the isolate

The ability of the isolate to utilize benzene was indicated by an increase in turbidity of the medium measured at 600 nm. Fig. 1 shows the change of biomass concentration in the culture at different time intervals at different initial benzene concentrations ranging from 50–1000 mg L^{-1}.

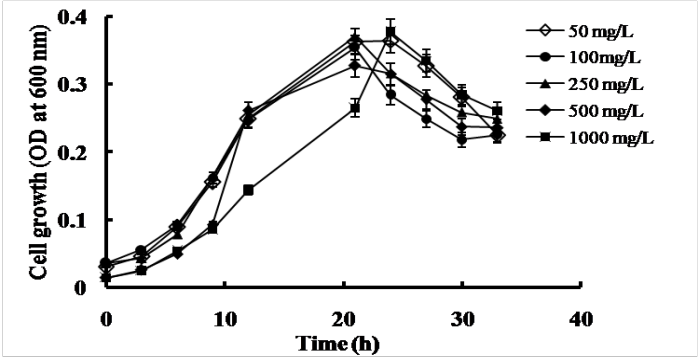

Fig. 1. Growth profile of the *Aerococcus* sp. strain BPD-6 at different benzene concentrations (50–1000 mg L^{-1}) as sole carbon and energy sources and growth was observed at 600 nm.

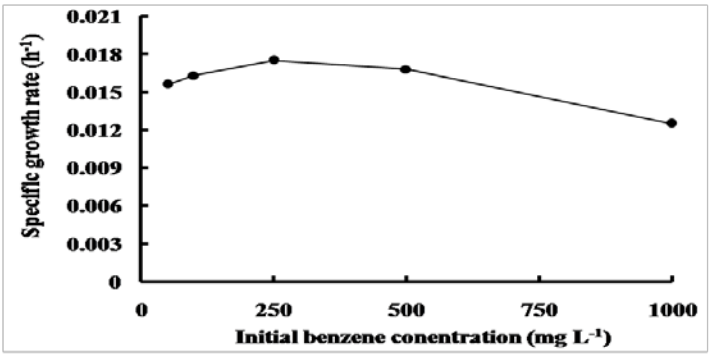

Fig. 2. Specific growth rate versus initial benzene concentration using free cells of *Aerococcus* sp. Strain BPD-6.

Biomass concentration increased with increase in time until all benzene was utilized by the microorganism. It was observed that a lag phase of around 6 hours in the growth profile was evident at all concentrations. The lag phase observed in this is due to the utilization of benzene by the culture and could be ascribed to the highly toxic nature of the compound. The experimental specific growth rate (μ) data were plotted against initial concentration of benzene in order to show the variation in the experimental specific growth rate against initial substrate concentration (Fig. 2). Higher initial concentration of the substrate enhances the reaction rate and is responsible to render the driving force to subdue the mass transfer resistance of benzene between the two phases of aqueous and gaseous phases [20]. Thus in this plot a typical trend has been observed, specific growth rate increased with the increase in initial benzene concentration level, and then it started decreasing with the increase of benzene concentration which is in good agreement with the results and showed that though higher concentrations of benzene usually gave higher bacterial growth, this ceased when a threshold concentration was reached [13], [15]. The curve showed the maximum specific growth rate μ_{max} was reached at a benzene concentration of 250 mg L^{-1} and then the specific growth rate gradually decreased. It was evident that beyond 250 mg L^{-1} the inhibition became evident. Thus the parameter specific growth rate μ has been found to be strongly related to initial benzene concentration S.

3.3 Kinetic modelling of the isolate during growth

Since the degradation of a substrate by an organism proceeds with cell mass growth, the kinetic parameters were evaluated on the basis of various growth models such as Monod, Haldane, Yano, Aiba and Tessier at the optimum growth temperature of 37°C and pH 7.0. The value of μ is determined at the exponential phase of the growth curve.

$$\mu X = \frac{dX}{dt}$$

Where μ in the above expression represents the specific growth rate of the biomass (X) which is a strong function of the substrate concentration. Benzene can be utilized as growth promoting substrate at lower concentrations while it could inhibit growth of the microorganism especially at higher concentrations for acclimatized populations and is represented by the extended and modified forms of the Monod's non-inhibitory kinetics equation which include the additional factor of inhibition constant (K_i) [21]. Various single substrate growth inhibitory kinetic models (Haldane, Aiba, Teissier and Yano and Koga) mentioned in Table 2 represent the growth kinetics data of an inhibitory compound. Here in the present study, four growth kinetic models are considered for all substrate concentration values, and are fitted to the experimental data. Where μ is the specific growth rate (h^{-1}), S is the substrate concentration (mg L^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), K_s is the half saturation coefficient or the substrate affinity constant (mg L^{-1}) (the affinity of a bacterium for a substrate), K_i is the substrate inhibition constant (mg L^{-1}). The values of kinetic parameters and correlation coefficients (R^2) of four substrate inhibition models have been shown in Table 2. Fig. 3 displays the results of all four kinetic models and the results of experimental run. At high substrate concentration, the predictions of models such as Haldane, Aiba, Teissier and Yano differ and so at high substrate concentration there should be distinction among the models. The Monod model does not fit the experimental data at all. The calculations for standard deviation of predicted values of μ from experimental values show that Yano and Koga and Aiba model with R^2 values (0.9683 and 0.9028) respectively followed by Haldane model with R^2 value (0.8680) are more suitable model for full sub-

strate concentration range of (50–1000) mg L^{-1} taken. Based on this study specific growth rate of *Aerococcus* sp. BPD-6 was assumed to be inhibited by the production of metabolites or products and higher initial substrate concentrations. All the models except Monod, adopted in this study have generally been used to describe substrate inhibition on growth of the isolate.

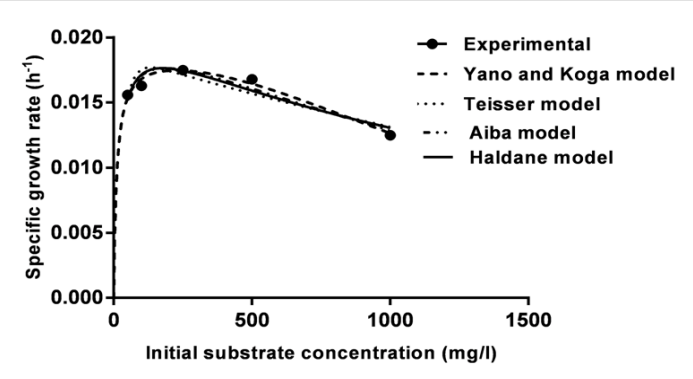

Fig. 3. Comparison of growth kinetic models. The software Prism 5.0 is employed to process the experimental data.

Therefore, it is more probable that these models fitted the experimental data obtained in the study quite well. However, some models showed slight differences in the values of biokinetic constants, such as μ_{max}, K_s and K_i, probably due to their differences in origin of development. For example, Haldane model is based only on the effect of substrate on growth of a culture [22]. Aiba model is based on that substrate inhibition could be due to formation of intermediates and products or altered activity of enzymes [23].

TABLE 2. ESTIMATED VALUES OF BIOMASS GROWTH KINETIC MODEL PARAMETERS FOR BENZENE DEGRADATION.

Models	Equations	μ_{max}(h^{-1})	K_s(mg L^{-1})	K_i(mg L^{-1})	R^2	RMSE values
Haldane	$\mu=\frac{\mu_{max}S}{K_s+S+\frac{S^2}{K_i}}$	0.02157	19.56	1584	0.8680	0.0009967
Yano and Koga	$\mu=\frac{\mu_{max}S}{K_s+S+\frac{S^2}{K_i^2}}$	0.0175	11.08	1456	0.9683	0.0004883
Aiba	$\mu=\frac{\mu_{max}S\exp(-\frac{S}{K_i})}{K_s+S}$	0.02115	18.04	2091	0.9028	0.0008554
Teissier	$\mu=\mu_{max}[\exp(-\frac{S}{K_i})-\exp(-\frac{S}{K_s})]$	0.01874	28.44	2818	0.7707	0.0013140

It is evident that the maximum specific growth rate (μ_{max}) of the bacterium *Aerococcus* sp. strain BPD-6 is 0.02157 h^{-1}, which lies below the range of literature results 0.096–0.62 h^{-1} (Table 3). This value of μ_{max} indicates that substrate is degraded by microorganism at a reasonable rate K_s is defined as the substrate concentration at which μ is equal to half μ_{max} [24]. A low apparent K_s value expresses that the microorganism can efficiently remove the substrate at maximum rate until a low concentration value is reached [25]. The value of K_s obtained from Haldane model is 19.56 mg L^{-1} and falls slightly on the lower side range but is within the limits of literature ranges (1.65–71.18 mg L^{-1}).

TABLE 3. COMPARISON OF BENZENE HALDANE KINETIC PARAMETERS FROM PUBLISHED STUDIES.

Microorganisms	μ_{max} ($hour^{-1}$)	Ks(mg L^{-1})	Ki(mg L^{-1})	References
Aerococcus sp. strain BPD-6	0.02157	19.56	1584.0	This study
Pseudomonas putida MTCC 1194	0.1631	71.18	340.15	[13]
Ralstonia sp. strain YABE411	0.096	3.589	1235.0	[38]
Pseudomonas aeruginosa	0.3	30.0	130.0	[14]
Pseudomonas fluorescens	0.0973	64.3	170.2	[15]
Pseudomonas putida F1	0.62	1.65	180.0	[39]

Thus the substrate is oxidized at maximum rate until a low concentration value is reached and the strain can grow in such low concentration of substrate. The inhibition constant K_i is 1584 mg L^{-1} and on the higher range when compared to the values reported in literatures (130.0–1235.0 mg L^{-1}). A large K_i value indicates that the culture is less sensitive to substrate inhibition, so high value of K_i shows that the inhibition effect can be observed at high benzene concentration [26]. When the organic contaminant surpasses the K_i value, the rate of degradation decreases leading to accumulation of substrate thus affecting the efficacy of the treatment process. Thus these values of kinetic parameters showed a high efficiency of microbial culture *Aerococcus* sp. strain BPD-6 to grow in presence of benzene, and so a complete degradation of the compound in the process.

3.4 Kinetic modelling during benzene biodegradation

Fig. 4 shows the time profile of benzene biodegradation for concentration ranging from (50–1000) mg L^{-1} using the acclimatized culture of *Aerococcus* sp. strain BPD-6. The benzene concentration was found to decrease with time showing the consumption of benzene by the microbe as they utilized it as a carbon source for their growth [27]. It was observed that the isolate degraded benzene in about 66 hours for 50, 100 and 250 mg L^{-1} initial concentrations but no noticeable complete depletion of benzene was observed beyond the time span of 66 hours for the concentrations of 500 and 1000 mg L^{-1}. This is may be probably due to the accumulation of toxic metabolites and products which inhibit the degradation of the substrate at higher concentration. It is important to elucidate the enzymatic mechanisms underlying the pollutant removal as they determine both the process efficiency and long term stability, particularly of benzene which proceeds via toxic intermediates, which could be even more toxic than their parent compound [28]. The pollutants and their metabolites might potentially reduce process performance as they exhibit a high toxicity towards microbial communities.

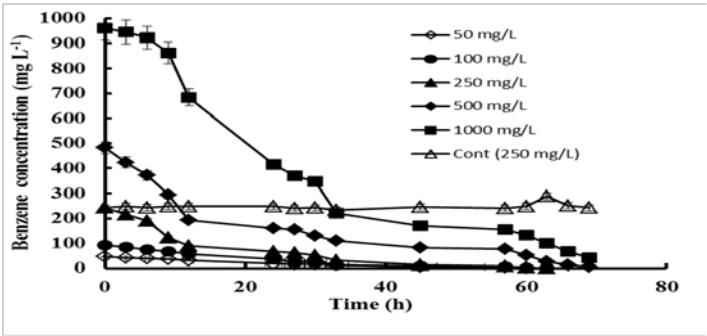

Fig. 4. Potential of *Aerococcus* sp. Strain BPD-6 to utilize benzene at different initial benzene concentrations (50–1000 mg L^{-1}).

Thus in order to have an insight into the mechanism of benzene degradation, the rate of substrate degradation was calculated. The rate of disappearance of substrate is dependent on the substrate concentration. Initial substrate concentration S_0 which changes with time and half saturation constant K_s can be described by

First-order reaction: $S = S_0 e^{-kt}$, where $S_0 << K_s$

The rate constant can be calculated from the slope value of the plot i.e.

$$-\ln \frac{S}{S_0} = kt$$

Where S_0 is the initial concentration of benzene (mg L^{-1}), S is the concentration of benzene at time t (mg L^{-1}), t is time (h), and k is the observed first-order reaction rate constant for the biodegradation of benzene (h^{-1}). For first-order kinetics, it is assumed that the microbial concentration remains constant over the entire experiment. First order kinetics was calculated using acclimatized culture over a concentration ranges from 50–1000 mg L^{-1}. The first-order kinetics is said to be valid if a linear relationship is achieved upon plotting the logarithm of residual substrate concentration versus time [29]. The slopes of the linearly fitted data were 0.0562, 0.0633, 0.0734, 0.0492 and 0.0412 (Table 4) for benzene concentration of 50, 100, 250, 500 and 1000 mg L^{-1}, respectively. The rate constants first increased and then gradually decreased while the benzene concentration was increased. That may be probably the result of substrate inhibition at high benzene concentration [30]. Nakhla et al. (2003) have also reported that the biodegradation of monoaromatic compounds follows the first-order kinetic model [31].

TABLE 4. PARAMETERS OF FIRST ORDER KINETIC MODELS AT DIFFERENT INITIAL BENZENE CONCENTRATIONS

Sl. No.	Initial benzene concentration (mg L^{-1})	First Order kinetics (k)	R^2 values
1	50	0.0562	0.9194
2	100	0.0633	0.9377
3	250	0.0734	0.9233
4	500	0.0492	0.8449
5	1000	0.0412	0.9560

3.5 Calculation of decay constant

After the complete consumption of substrate, the microorganism showed decline in its growth. This phase is denoted by some cell population becoming food for the rest of the cell population. Thus the following expression denotes the cell endogenous metabolism

$$\frac{dX}{dt} = -k_d X$$

Where k_d denotes endogenous-decay coefficient (h^{-1}) of the biomass (X)

Presuming k_d is independent of initial substrate concentration, benzene substrate concentration of 1000 mg L^{-1} was chosen for the calculation of decay coefficient. The value of k_d is determined at the decline phase of the growth curve. The negative slope gives endogenous rate coefficient. The values of the decay rate coefficients obtained is 0.0082 h^{-1} with R^2 value 0.9712. The growth rate will be reduced with the value of decay coefficient and thus wash-out condition may occur at lower dilution rates. So in a scaled up continuous reactor, organisms with higher decay rate coefficient should be operated at lesser dilution rate [32].

3.6 Scanning electron microscopy (SEM)

The Scanning Electron Microscopy was used to study and understand the morphological changes on LB solid plate in the presence of benzene at a concentration of 100 mg L^{-1} using cells from exponentially growing cultures. There was no marked distortion but little shrinkage of the cells in the presence of benzene as observed under the microscope (Fig. 5a and 5b). This indicates that *Aerococcus* sp. strain BPD-6 is quite resistant to the compound benzene.

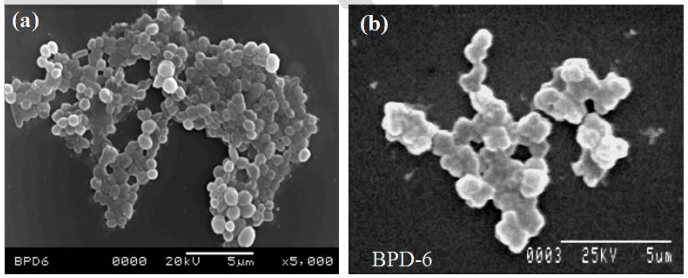

Fig. 5. SEM micrograph of *Aerococcus* sp. strain BPD-6 (a) Control cell grown in presence of glucose. (b) cells grown in presence of benzene as carbon source.

3.7 Physicochemical factors affecting the growth in presence of benzene

Availability of macronutrients, enzyme activity and stability are highly pH and temperature dependent. High acidic environment and high basic environment increases the toxicity due to production of metabolites [33]. Moreover cell biomass gets inhibited at low pH and at high pH due to complete loss of activity for most of the enzymes. Further increased or low temperatures would bring about decrease in enzymatic reaction rates in the microorganism and affect the bioavailability of benzene. Besides elevation in temperature causes decrease in oxygen solubility resulting in inhibition of growth of aerobic microorganisms [34]. Also high salinity effects cell num-

bers and distribution, resulting in reduced microbial metabolic rates [35]. Thus comparisons of the growth by *Aerococcus* sp. strain BPD-6 incubated at various pH, temperatures and salinity were made as degradation of the contaminant relies on the growth of the microorganism.

3.7.1 Effect of initial pH on the growth of the strain

To investigate the effect of pH, on the growth of *Aerococcus* sp. strain BPD-6 was studied experimentally at different initial pH values varying from (2.0–12.0) under identical environmental conditions like temperature at 37°C and the initial concentration of benzene at 100 mg L^{-1}. Most of the organisms cannot endure pH levels above 9.5 or below 4.0. However, the optimum pH for different microorganisms lies in different ranges [36]. Consequently, the effect of pH on growth of *Aerococcus* sp. strain BPD-6 is in agreement with most microorganisms, which favour growth at pH levels ranging from 6.0–8.0 [37]. Subsequently, growth profile study at varying pH was carried out (Fig. 6a). It could be seen that this bacterium grew efficiently at almost neutral pH. Further growth however significantly declined when the pH was less than 5.0. Maximum growth was obtained at pH value 9.0, but the important thing which was observed in the present study was that the isolated strain could grow at the pH values 8.0, 11.0 and 12.0 as indicated by high growth at these pH values which is in context with the properties of *Aerococcus* sp. which are alkaline tolerant in nature and indicating that *Aerococcus* sp. can be applied to different climatic condition on wide range of pH. In the present work, the pH of medium is taken to be 7.0 for further degradation studies.

3.7.2 Effect of temperature on the growth of the isolate

The isolate was tested for growth on 100 mg L^{-1} benzene and at optimum pH of 7.0 on a broad range of temperatures and subsequently six temperatures (20, 30, 37, 40, 50 and 60°C) as shown in (Fig. 6b) were chosen for the determination of acclimatization time in order to avoid long lag phases during biodegradation measurements. Growth of the strain increased at the temperatures 30°C and 37°C and slightly at 40°C while it decreased acutely at incubation of 20°C and 50°C and inhibited totally at 60°C. Results thus indicated that at 37°C, the incubated strain exhibited shorter lag time relative to when incubated at 30°C. For this isolate the optimum growth temperature of 37°C indicates that the isolated strain is mesophilic in nature.

3.7.3 Effect of salinity on the growth of the isolate

The effect of salinity on the growth of the strain was observed in the batch set up containing 6 different NaCl concentrations (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 g L^{-1}. The maximum growth obtained at various salt concentrations in the MS media is shown in (Fig. 6c). It was observed that the isolate grows exceptionally well upto concentration of 6.0 g L^{-1} and also maximum growth is observed at 4.0 g L^{-1}. Thus the optimum growth of the isolate is in the salt concentration range of 4.0–6.0 g L^{-1}.

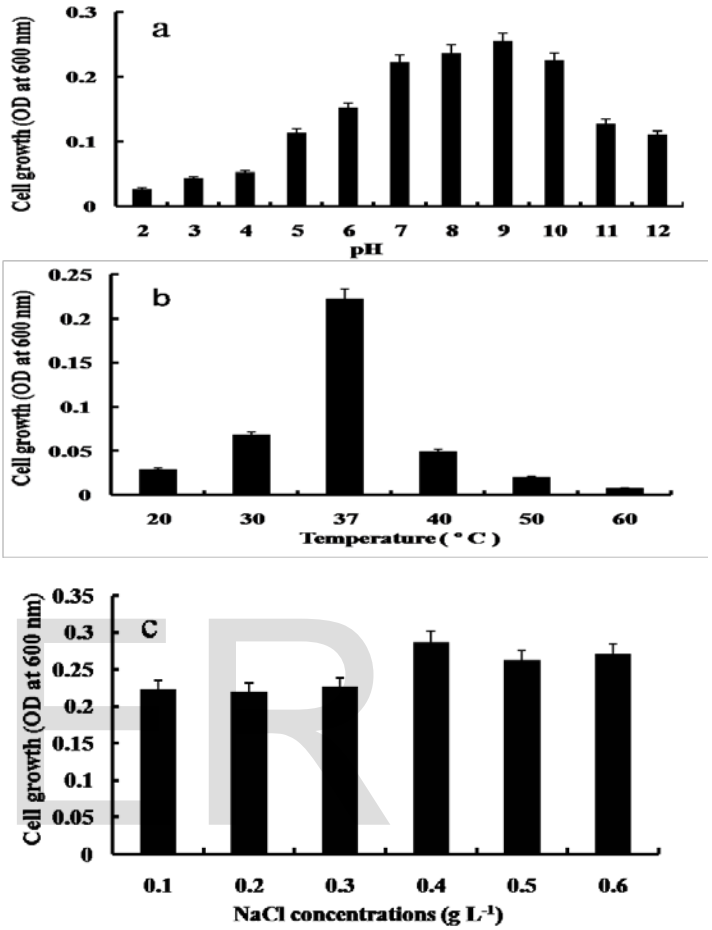

Fig. 6. Growth of *Aerococcus* sp. Strain BPD-6 in MSM at 100 mg L^{-1} of benzene as sole source of carbon, (a) Growth at different pH range (2–12), (b) Growth at different temperature (20–60°C) and (c) Growth at different NaCl concentration (1–6 g L^{-1}).

4 Conclusions

The present study found out the following salient observations for benzene degradation and consequently growth of the indigenous Gram positive bacteria *Aerococcus* sp. strain BPD-6, isolated from petroleum oily sludge. To our best knowledge, this is the first report of benzene utilization by *Aerococcus* sp. The lag phase of this bacterium was found to be around 10 hours indicating that the strain is highly active and has the ability to degrade the contaminant in less time. Growth kinetic parameters for this strain according to the Haldane model were μ_{max} = 0.02157 h^{-1}.The maximum specific growth rate indicated that strain degraded the contaminant in less time. Ks = 19.56 mg L^{-1} and Ki= 1584 mg L^{-1}. High K_i value reflects the ability of the strain to grow at relatively high benzene concentration. First order kinetic models were tested and the kinetic model fitted well for the biodegradation of benzene with de-

cay coefficient found to be 0.0082 h^{-1}. Thus this study revealed the potential of an indigenous microbial culture *Aerococcus* sp. in treating oily sludge containing highly recalcitrant compound such as benzene. Further studies are warranted to elucidate the mechanism of benzene degradation in terms of enzymes involved which would facilitate in development of enhanced remediation strategy for monoaromatic compounds from petroleum oil sludge.

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