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GROWTH KINETIC STUDIES AND ANTIBIOTIC RESISTANCE PROFILE OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM COASTAL ENVIRONMENT, KARACHI, PAKISTAN

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ABSTRACT

The present study deals with the isolation and growth kinetics of *Pseudomonas aeruginosa* .The plastic debris was collected from the coast of Karachi, Pakistan and was used as a source of isolating microorganism having ability to degrade plastic. *Pseudomonas aeruginosa* has a wide catabolic potential, commonly known as a species of bioremediation and a versatile toxic organic compound degrader. During this research plastic was degraded using the mineral salt medium. Before entering into the growth inhibition phase, bacterial strains show a relationship (what type of relationship?) between plastic concentration and bacterial growth. As the plastic concentration increased the generation time (g) was increased and growth specific rate (μ) decreases. The hydrophobicity of isolated bacteria was evaluated by Salt Aggregation Test (SAT).The results depict that the stain were hydrophobic and were able to grow on hydrocarbon i.e n-heptane and Xylene. Scaning Electron microscope (SEM) analysis revealed that the strains were exhibiting adherence on plastic.

Keywords Growth kinetics , Plastic , hydrophobicity, SAT

INTRODUCTION

For the remediation of pollution also called biodegradation or bioremediation has been extensively applied as the answer of environmental problem as they are natural , more efficient and environmentally acceptable. The major threat to the environment associated with plastic is its slow rate of degradation or almost non-biodegradable under normal conditions. Plastic are enlisted as recalcitrant compound, their potential degrading microbes has been a matter of curiosity since 1961 after work of Fuhs (1961), reported microorganism can consume paraffin as sole source of carbon (Kale *et al*., 2015).

Pseudomonas is characterized as a gram-negative, versatile and adaptable microorganism can grow easily in the entire environment, it has minimal nutrient requirement, and it will grow in the absence of O_2, if NO_3 is available as a respiratory electron acceptor. It is also shows a high degree of resistivity against antibiotics and saline. Isolation of bacteria from the site of contamination has an advantage that culture is adaptable in specific environment. Hussein *et al* is his research work isolated *P. aeruginosa* from industrial discharge; contaminated with metal as a result the strains were tolerant against Chromium, Copper, Nickel and Cadmium contamination (Awasthi *et al.,* 2015). *P. aeruginosa* responsible to degrade number of pollutants including crude oil (Zhang *et al.,* 2005, Emtiazi *et al.,* 2005), phenol, Benzoic acid (Razika *et al.,* 2010), Naphthalene, Pyrine and Phenanthrene (Sharma and Pathak 2014) n-alkanes (Chayabutra 2000) , diesel fuel (Erikson *et al*., 2001) , oil sludge (Mishra *et al*., 2001), bitumen or polycyclic aromatic compounds (Wolf and Bachofen 1991). Enzymes play key role for degradation of polymers. The versatility of *P. aeruginosa* against different pollutants is prolific producer of a number of extracellular enzymes.

In the view of plastic waste treatment chemical, biological and many other degradation techniques has been extensively study previously (Aamere *et al*., 2008).Polythene ready from the low-cost is petrochemical stocks extracted from oil or gas through efficient catalytic polymerization of ethylene monomers .Several microorganism such as bacteria and fungi are involved in the degradation of synthetic plastic. During the mechanism of degradation the polymer first converted into monomers then they are further mineralized. Polyethylene's has been reported to degrade in the existence of Streptococcus, Staphylococcus, Pseudomonas and Micrococcus (Kathiresan 2003; Yoon *et al.* 2012), *Flavobacterium, Agrobacterium, Xanthomonas* and *Bacillus* spp. (Gupta *et al*., 2010). According to the results of Rajandas *et al.* (2012), 50 and 61% of degradation achieved by *Pseudomonas aeruginosa* and *Microbacterium paraoxydans* inoculated with polythene plastic. *Pseudomonas aeruginosa* have also been reported to cause in situ colonization and substratum deterioration of plasticized PVC (Booth and Robb, 2007). Usha *et al.* (2011), recorded up to 62.71×10^4 and 56.52×10^4, the fungal count ranged from 44.32×10^2 and 35.62×10^2 and

actinomycetes count ranges from $72.54x10^4$ and $64.75x10^4$ on the surface of plastic film, and identified the microbial species associated with plastic degradation were *Pseudomonas* sp., *Bacillus* sp., *Staphylococcus* sp., *Aspergillus nidulans*, *Aspergillus flavus* and *Streptomyces* sp.

The present study aims to demonstrate the biodegradation potential of metabolic versatile *Pseudomonas aeruginosa* isolated from costal environment of Karachi. Their growth kinetics were performed under different concentration of plastic in minimal media.

MATERIALS AND METHODS

Bacterial strain

Pseudomonas aeruginosa was isolated from contaminated soil with plastic at the Clifton beach, Karachi. The strain was characterized by morphological and biochemical tests like Gram staining, , catalase, motility pigment production, oxidase, growth on cetrimide agar, ability to grow at 42°C.The culture was maintained on nutrient broth tubes at 4^{o} C with successive subcultures acquired every one month.

Real-time 16S rDNA PCR analysis

Colonies were struck out for pure culture and analysed using real-time 16S rDNA PCR analysis. DNA purification and extraction was done via QIA (Qiagen) amp (amplification) DNA Mini-kit. Qiagen proteinase K (20 µl) (20 mg/mL) was added for every 200 µL of serum sample. Then 200 µL of buffer Al was added, vortexed for 15 seconds, then incubated at $56C^0$ for 10 minutes. After incubation 200 µL of 100% ethanol was added, centrifuged for 20 sec and transferred the filter into Qiagen Amplification spin column and centrifuged at 6000g or 8000RPM for 1 minute. Then transferred concentrate into the new collection tube, added 500 µL buffer aw1 centrifuged at 8000rpm for 1 minute and replaced the collection tube .Added buffer aw-2 500 µL, and centrifuged at max speed for 3 minutes. Discarded and replaced filtrate tube. Added 200 µL elusion buffer AE. Kept at room temperature for 1 minute then centrifuged at 8000 RPM for 1 minute. The resulting Elute was loaded onto the Qiagen amplification DNA mini kit Column. Amplification reaction volume was 25µL including 12.5 µL master mix, 5 µL elute, 1.25 µL forward primer,1.25 µL reverse primer and 5 µL RNAs free water. Primer sequences were as follows: forward Primers, 5'AAC TGG AGG AAG GTG GGG AT3'; reverse primer, 5'AGG AGG TGA TCC AAC CGC A-3.

Minimal salt medium (MSM)

For study the growth kinetics of *Pseudomonas aeruginosa* all the enrichment was done in minimal salt media contained 15(g/L*)* KH_2PO_4, 1.0 (g/L) NH_4NO_3, 2.5 g NaCl , 5 (g/L) NH_4Cl and 33.9 (g /L) $Na_2HPO_4.7H_2O$. Media was also supplemented with 2 mL of 1M $MgSO_4$ and 0.1 mL of 1M $CaCl_2$. The pH of media maintained at 7.2.

Preparation of innoculum and growth kinetic studies

Inoculum was prepared by taken loopful of bacterial culture innoculated into 6 ml of nutrient broth and incubated at 37 ºC for 24 hours under shaking condition (160 rmp).0.2 ml (Vi Macfarland`s Index) of 24 hours grown culture media tranfered into 50 mL of minimal salt medium fortified with different concentration of plastic (1g, 3.5g and 5g) .One flask used as control in which culture is seeded without plastic. All the flask were incubated at shaking condition for 40 hours at 37 ºC, sampling were withdraw for check the OD_{600} at every 5 hours interval by using UV-spectrophotometer (UV-1100Pc,Macy).Generation time (g) and specific growth were calculated for the growth curve obtained by plotting OD_{600} against time.

Growth Kinetic studies with Hydrocarbon

Overnight culture of *Pseudomonas aeruginosa* were centrifuged and washed thrice with Phosphate urea magnesium (PUM) buffer, contain 17 g of K_2HPO_4 , 1.8 g Urea, 7.5 g KH_2PO_4, and 0.2 g of $MgSO_4$ in distilled water. The washed cells were resuspended in PUM buffer until its reached to $3 X 10^8$ cfu/mL (Macforland Index). 0.2 mL of culture transferred into the flask containing 49 mL mineral media. The carbon source was used as 2% of xylene and n-heptane, respectively. Control without carbon source performed. All the flask were incubated at shaking condition for 18 hours at 37 ºC and tested by Miles and misra technique. All the experiments were carried out in triplicate.

Salt Aggregation Test (SAT)

Selected culture of *Pseudomonas aeruginosa* were aggregated by combining 10 µL volumes of the bacteria suspension with equal volumes of a series of ammonium sulphate of varying molarities (0.2 - 4.0 M, pH 6.8) in a microplate wells. The reaction mixture causing maximum agglutination was considered positive whereas absence of

agglutination was considered as negative. Classification of results was expressed as: < 1.0 M = strongly hydrophobic, 1.0 – 2.0 M = Hydrophobic, > 2.0 M = Hydrophilic (Nwanyanwu *et al*., 2012).

Antibiotic resistivity test

Susceptibility testing was done using disk diffusion test method with isolated strain of *Pseudomonas aeruginosa*. The strains were tested against Ceftriaxone (CRO; 30 µg), Tobramycin (NN; 10 µg), Ampicillin (AMP: 10 µg), Amoxyillin (AMC; 30 µg), Gentamycin (GM; 10 µg), Amikacin (Ak; 30 µg), Vancomycin (VA; 30 µg), Cefixime (CFM; 5 µg) Piperacillin (PP; 100 µg), norflaxin (NOR; 10 µg) and Ciproflaxacim (CIP; 5µg). All the Experiment set were performed in triples.

Multiple antibiotic resistance (MAR) index were calculated by using formula of MAR= a /b, where "a"donates as number of antibiotics to which the test isolate resistance and " b" donates ad total number of antibiotic tested for susceptibity evaluation (Krumperman, 1983).

Scanning Electron Microscopy (SEM) of *Pseudomonas aeruginosa*

For visualization of bacteria, the specimen was prepared by taking 5 ml of nutrient broth in corning tube with culture and incubated at 37 C for 25 hours. After one day of incubation 20% of 2 mL glucose was added in incubated tubes. The entire sample was discarded from corning tube after 24 hours and 5 mL of 99% methanol were added and left the tubes for15 minutes before discarded. After this whole procedure, 0.1 % crystal violet was added and left for 20 minutes, then washed with sterilized water. Samples were cut into miniature pieces, air dried and coated with 300°A gold and analysis under high resolution electron microscope (Jsm-6380 A, Japan).

RESULTS AND DISCUSSION

In order to study the growth kinetics of *P. aeruginosa* on plastic we performed numbers of experiments. Plastic degrading microbes can easily isolate from a number of sites such as dumping area soil, marine water or mangroves soil (Rutkowska *et al.,* 2002). We isolate the cells of strain from a coastal environment of Karachi embraced with huge amount of plastic pollution. *P. aeruginosa* is a highly versatile and adaptable organism that is able to grow, survive and persist under a broad range of environmental conditions and can be found in most of the natural environments, including terrestrial, marine and freshwater habitats (Green *et al*., 1974; Goldberg, 2000; Spiers *et al*., 2000).

Strain IES-1 was isolated from the enrichment culture of coastal area soil, contaminated with plastic debris. Isolate were rod-shaped, gram-negative bacterium, growth best at the pH ranged from 4.0 to 10.0, with optimal growth at pH 7.0. After growing on Nutrient agar followed by centrimide agar at 37°C for 24 h, the cells formed green, round, moist and glossy colonies, approximately 1.0 mm in diameter. The general characteristics of *P. aeruginosa for* identification are described in table 1. Further confirmation was done by real time 16s rDNA PCR and tested against degradation by screening their growth kinetic at different concentration of plastic (0.01 ,0.03,0.05 gm).Results of optical density and log of O.D were measured and represented in (Fig. 5). Generation time and specific growth rate are recorded in (Table 1).When compared with control test (grow without plastic), the growth pattern was different and required extra adaptation time for growth. At higher concentrations of *plastic, bacteria* accumulated more biomass (as reflected by OD_{600}), and also required more time to reach the stationary phase.

The response of *Pseudomona*s *aeruginosa* growth was determined by using different concentration of plastic. The results of growth kinetics in nutrient broth and in minimal media presents direct relationship between plastic concentration and bacterial growth. As the plastic concentration increased the generation time (g) was increased. The specific growth rate which expressed as the rate of growth of organisms, increased (Table 1, Fig. 7).

The specific growth rate at different substrate concentration were calculated by measuring the slopes of increasing optical densities at 600 nm. From the results it is obvious that the specific growth rate increase an increase in the substrate concentration. The growth rate (µ) of an organism is not constant, but it may vary from environmental conditions such as concentration of substrate and temperature. As in our work increased substrate (plastic) concentration in minimal media the decline in graph is noticed (Fig. 6).This decline indicates the inhibition in growth, which can be caused by damaged or disruption of the cell membrane at high concentration of plastic. According to Narayan (2006) the degradation of plastic molecule is mostly contribute as a result of the production of the extracellular enzyme. Microorganism utilized the compound as a carbon and energy source. The subsequent breakdown fragments must be absolutely used by the microorganisms, otherwise there is the prospective for environment and health significances. However, biodegradability decreases with increase in molecular weight, while monomers, dimers and repeating units degrade easily (Okmoto *et al.,* 2003)

Table 1. Biochemical test for the identification of *Pseudomonas aeruginosa (IEs-1).*

Test perform	Result
Gram reaction	Positive
Motility	Motile
Growth Temperature	37 °C
Citrate	Positive
Catalase	Positive
Gelatine hydrolysis	Positive
H_2S production	Negative
Lactose fermenter	Negative
Glucose	Negative
Sucrose	Negative
Manitol	Negative
Cetramide agar (CAB)	Dark green flourscent colonies

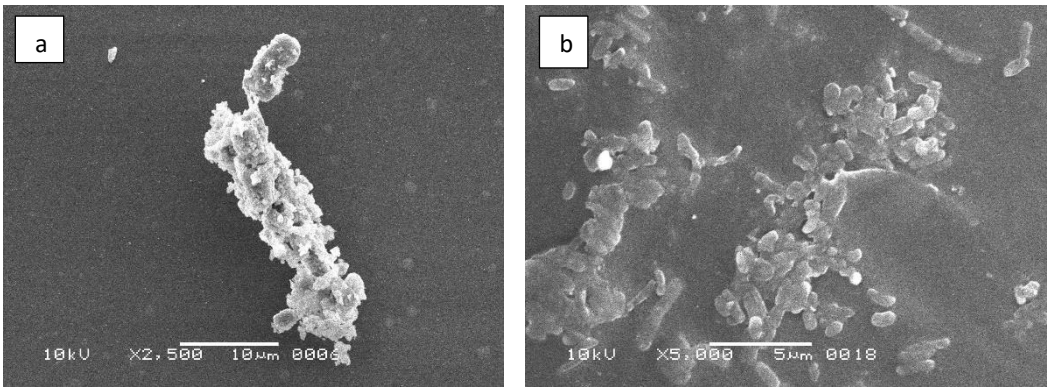

Fig. 1. Cellular morphology of (A) *Pseudomonas aeruginosa* (IES-1) at 2,500X (B) on Surface of plastic 5000X

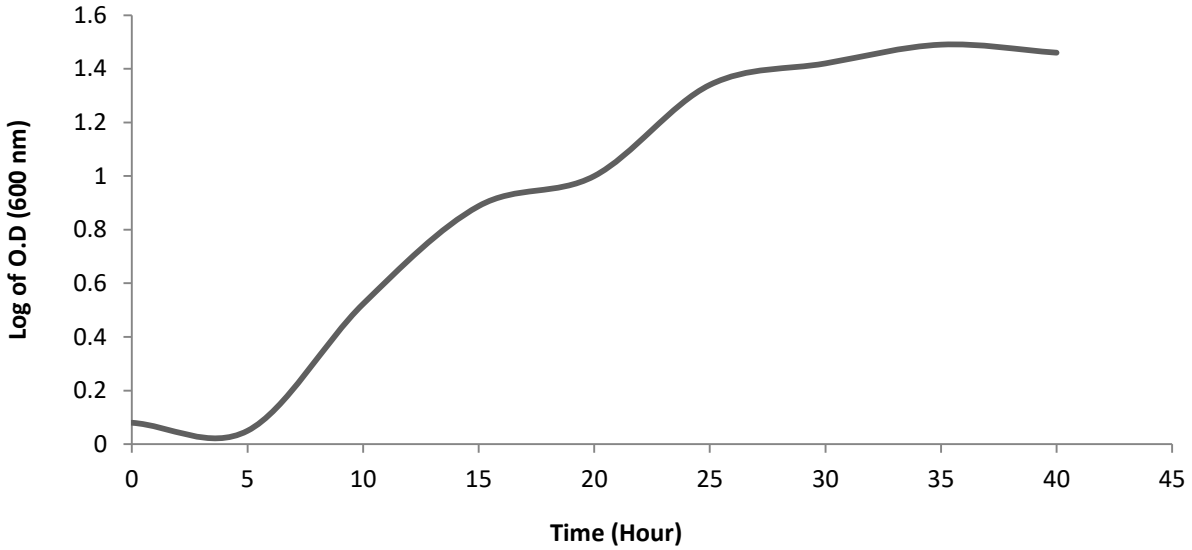

Fig. 2. Growth curve of *Pseudomonas aeruginosa* in nutrient broth supplemented by Plastic.

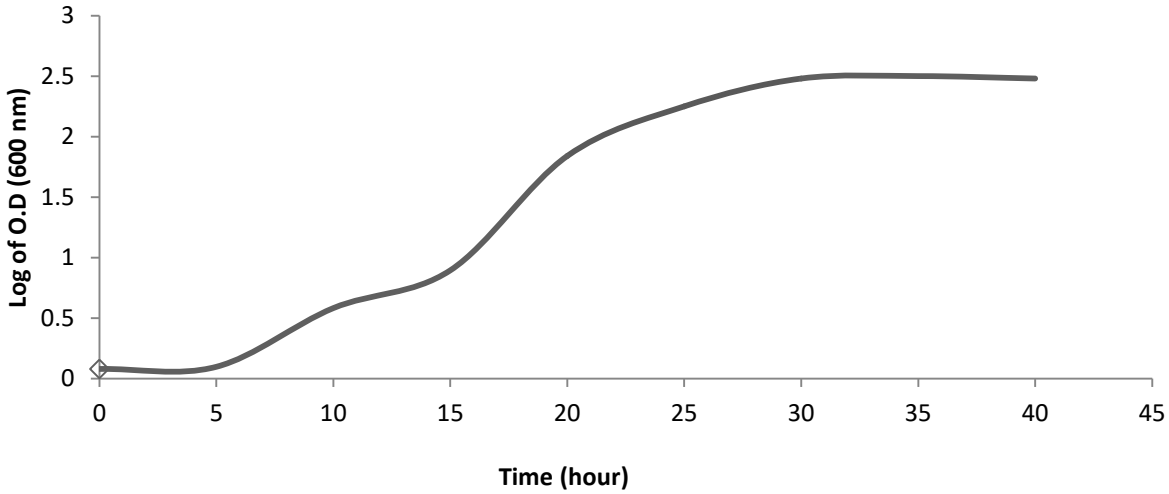

Fig. 3. Growth curve of *Pseudomonas aeruginosa* in nutrient broth.

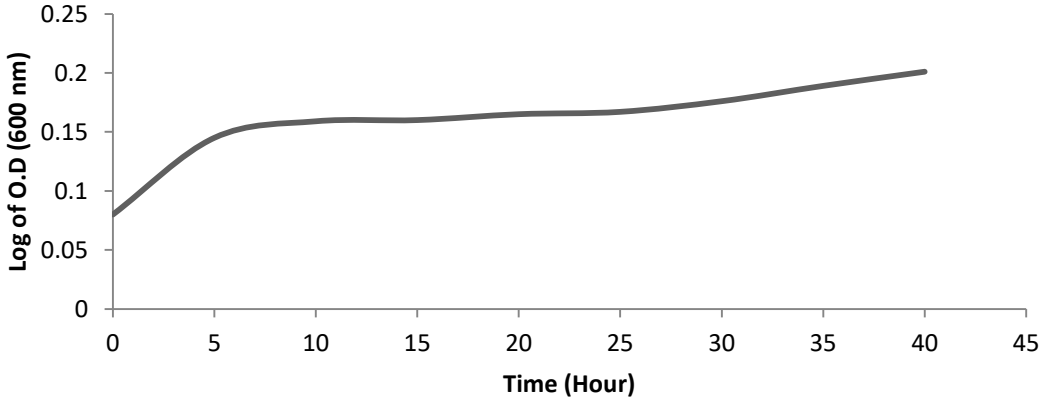

Fig. 4. Growth curve of *Pseudomonas aeruginosa* in Minimal media.

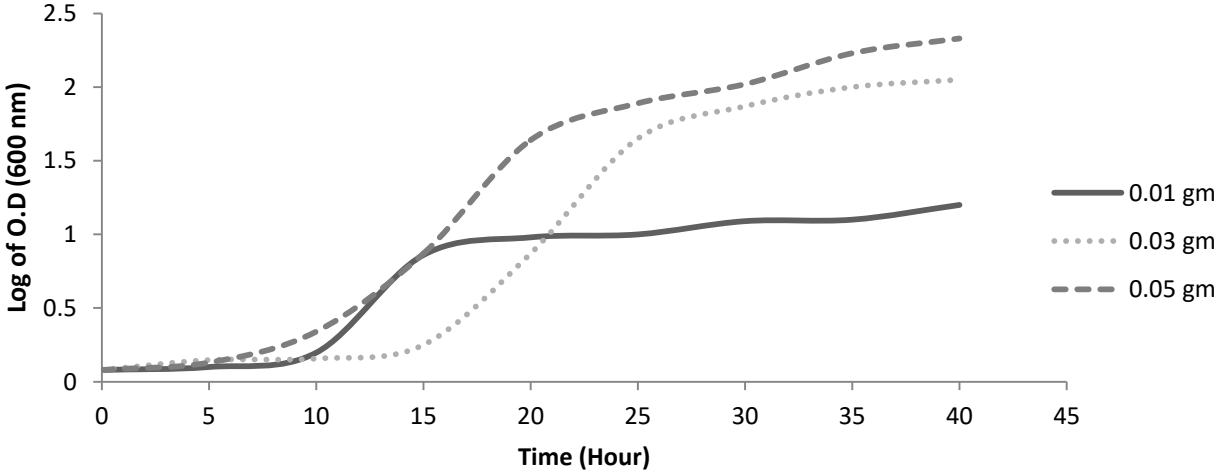

Fig. 5. Growth kinetics of plastic degradation by *Pseudomonas aeruginosa* (IES-1) in MM medium supplemented with (a) 0.01 gm (b) 0.03 gm (c) 0.05 gm of plastic.

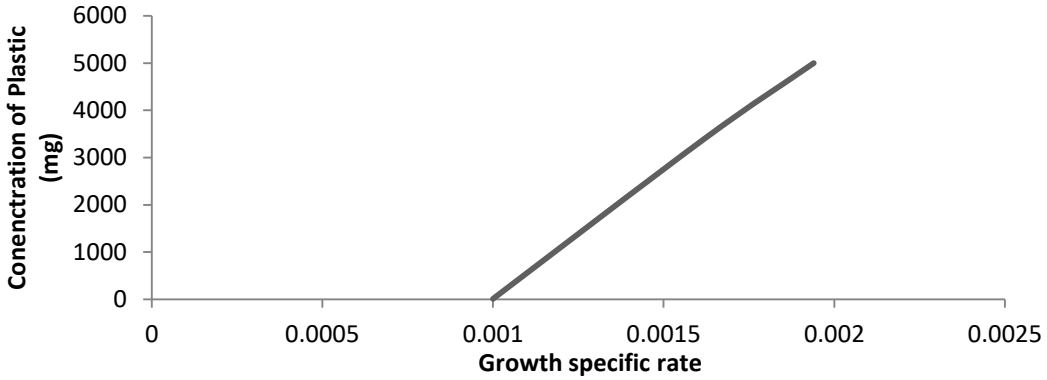

Fig. 6. *Psedomonas aeruginosa* growth curve in proportion to mass of plastic.

Table 2. Generation time and Growth specific rate of *Pseudomonas aeruginosa* (IES-1) at different concentration of plastic.

Concentration of plastic (mg)	**Generation time (min)**	**Growth rate (min^{-1})**
10	500	0.0010 min^{-1}
3400	515	0.00162
5000	617	0.00194
Control	1000	0.002
Culture with plastic in N.b	588	0.0017
Culture in N.b	470	0.0021

N.B represents Nutrient broth

Table 3 . Generation time and Growth specific rate of *Pseudomonas aeruginosa* (IES-1) with Xylene and n-heptane.

Name of hydrocarbon	**Generation time (min)**	**Growth rate (min^{-1})**
n-Heptane	92	0.010min^{-1}
xylene	118	0.008 min^{-1}
Control	218	0.0045 min^{-1}

Table 4. Salt aggregation test with *Pseudomonas aeruginosa* give visible cramp at various reactions of mixture

S.No	**Concentration of $(NH_4)_2SO_4$**	**Result**
1	0.2 M	Negative
2	0.8 M	Negative
3	1 M	Negative
4	1.5 M	Negative
5	2 M	Negative
6	2.5 M	Positive
7	3 M	Positive
8	3.5 M	Positive

Table 5. Multiple antibiotic resistance (MAR) of *Pseudomonas aeruginosa.*

	No. of antibiotics		
Strain	**Resistance**	**Tested**	**MAR Index**
Pseudomonas aeruginosa (IES-1)	*9*	*11*	*0.8*

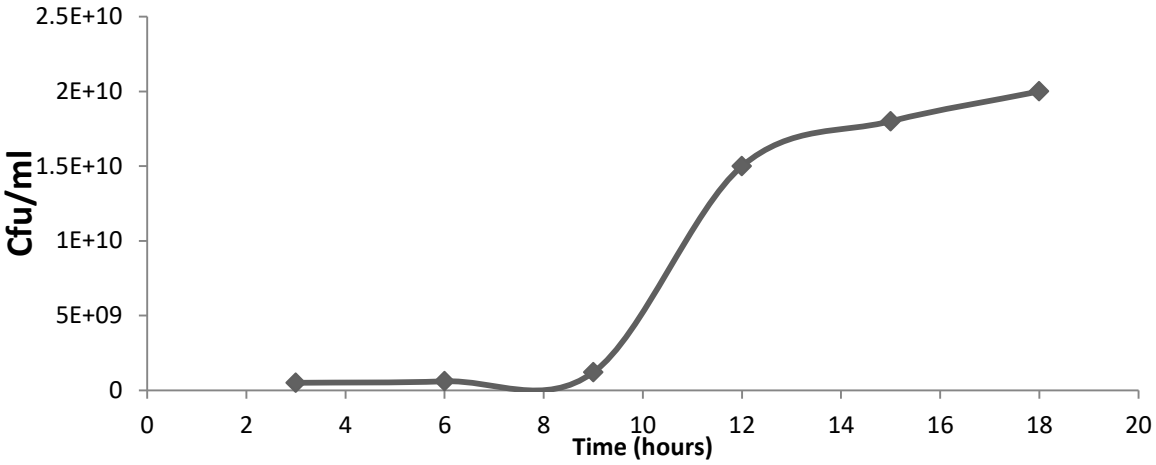

Fig. 7. Logarithmic phase of *Pseudomonas aeruginosa* (IES-1) with 5 ml of xylene.

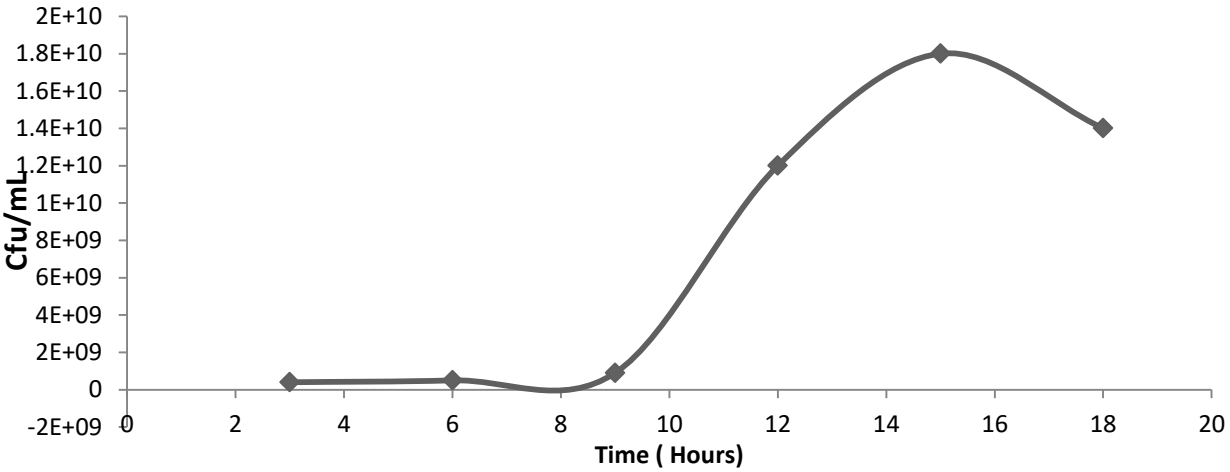

Fig. 8. Logarithmic phase of *Pseudomonas aeruginosa* (IES-1) with 5 ml of n-heptane.

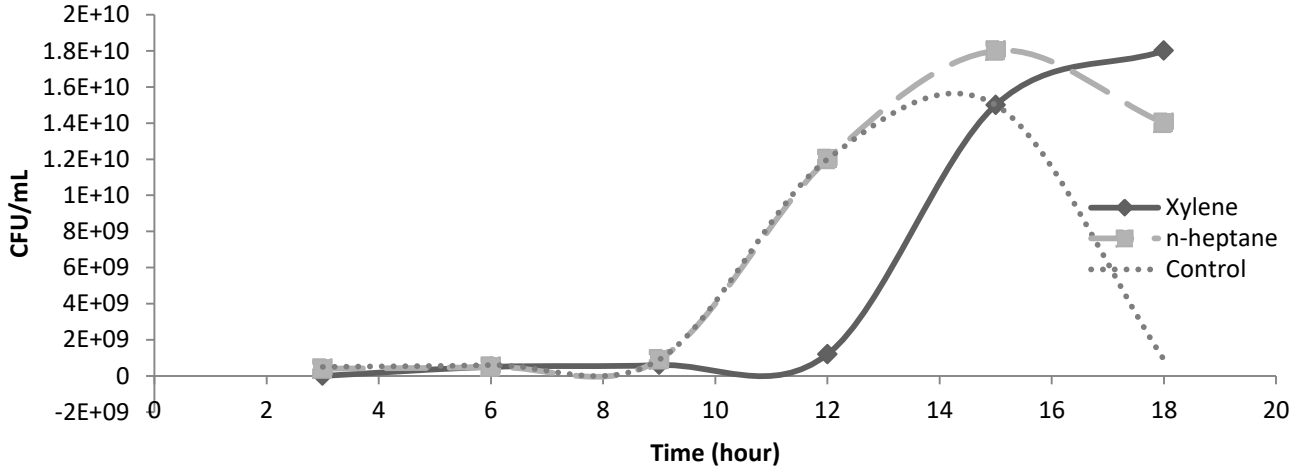

Fig. 9. Comparitive Logarithmic phase of *Pseudomonas aeruginosa* (IES-1) with (a) Xylene (b) n-heptane and (c) Control.

For testing the further degradability of isolated bacteria, the strain was trying to grow on liquid growth culture with the subsequent amount of n-hexane and octane. The strain effectively mineralized long chain of alkane as a sole source of carbon within 18h of study. The alkanes (n-hexane and octane) utilization was studied by monitoring its cell growth in minimal media. Cell multiplying start immediately after incubation and culture reached to the exponential phase with 10h. Of both the alkanes, based on generation time n-hexane was probably the best available carbon source in this set of experiment (Table 3). The stains of *P. aeruginosa* can grow in the presence of *n*-tetrodecane, *n*-docosane, *n*-triacontane, and *n*-tetrocontane (Liu *et al*., 2014). His study showed the complete mineralization of *n*-hexadecane within 36h.

Jeon and Mal (2016) reported in his research work that the strain of *Pseudomonas aeruginosa* detected in soil or water has two alkane monooxygenases (AlkB1and AlkB2), one rubredoxin reductase (RubB) and two rubredoxins (RubA1 and RubA2), all these enzymes contribute to the degradation of alkanes.

Inspired with the numerous advantages of this bacteria, the strains of *P. aeruginosa* has increased resistance to many antibiotics due to several adaptations mechanisms, such as multiple enzyme secretion, membrane impermeability and biofilm formation (Henwood *et al*., 2001; Landman *et al*., 2007). Multiple antibiotic resistance (MAR) is a good and useful assessment tool for risk associated with antibiotic. In case of *Pseudomonas aeruginosa* the MAR index is 0.8. The MAR value higher than 0.2 specifies the high risk of contamination by often overused of antibiotics (Riaz *et al*., 2011). The resistivity of bacteria against multiple antibiotics is mostly associated with presence of resistance genes in plasmids, encoding a single antibiotic resistance phenotype.On the bases of current finding, the MAR index of *Pseudomonas aeruginosa* is 0.8,confirming that the strain is adaptable for high antibiotic use and selective pressure in the environment.

Microbial adhesion is important for the bioremediation. Cell surface hydrophobicity was assessed by Salt Aggregation Test (SAT).The isolated strain of *Pseudomonas aeruginosa* showed maximum aggregation from 2.5 to 4 M ammonium sulphate. This indicates that strain of *Pseudomonas aeruginosa* is hydrophobic.The values of SAT are believed to depend on microbial culture's age, as well as the diversity of cells outer membrane composition and cell charge (Kadam *et al*., 2009; Nwanyanwu and Abu, 2013). As the outer membrane has fimbriae which play a principal role in cell-surface hydrophobicity. Other than this pili and flagella also serve as hydrophobins and preeminent hydrophobic character on organisms in which they occur (Deziel *et al*., 2001,Christian *et al*., 2007)

The culture morphology of isolated *Pseudomonas aeruginosa* was studied from the scanning electron microscope (SEM) (Fig. 1). Micrographs reveals that the strain are rod in structure with approximately 2-4 µmm in size. From scan micrographs it was observed that the cells growth in minimal media with plastic has formed clustered and scattered arrangement as compared with the cells grows in simple media. This behavior of isolated strain in the presence of plastic was attributed as a defense mechanism.

CONCLUSION

This study covers the major concern of selection of *Psedomonas aeruginosa* for the remediation of plastic pollution. The obtained results indicated that the isolated strain degraded plastic by stimulated the growth of culture, however, as the concentration of plastic increased a marked reduction in optical density noted, this indicates the suppression of bacteria enzyme with increase the dose or it may lead to the bacteria stress condition.

The overall finding of research study suggested that *Psedomonas aeruginosa* a versatile microbes,having multiple resistant gene can grow different concentration of plastic as they also degraded and tolerate hydrocarbon (n-hexane and xylene). Growth kinetics parameters determined the removal of plastic with bacteria growth as they utilized as a sole source of carbon. If an appropriate and optimum operation condition maintained this bacteria are efficiently useful for the treatment of plastic contamination.

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