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# Crude oil degradation by *Pseudomonas aeruginosa* NCIM 5514: Influence of process parameters

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Petroleum hydrocarbon pollution is a major environmental concern in developing countries as these pollutants cause hazardous effects to the ecosystems and environment. Green technologies using microorganisms for remediation of these pollutants have gained considerable attention. Petroleum hydrocarbon pollutants degrading and biosurfactant producing *Pseudomonas aeruginosa* NCIM 5514 was isolated from crude oil polluted site of Ankleshwar, Gujarat, India. Effect of agitation, temperature, pH, NaCl concentration, petroleum and non-petroleum carbon source and its concentrations, nitrogen sources and inoculum ratio on growth of *P. aeruginosa* NCIM 5514 were studied. Optimum growth of *P. aeruginosa* NCIM 5514 was observed at 1% (w/v) glucose, pH 7.2, incubation at 37°C at 180 rpm with 1% (v/v) inoculum for four days. However, this organism also utilized crude oil and glycerol as sole carbon source. Thus, *P. aeruginosa* used in the presented study here appeared as a mesophilic, halotolerant, aerobic, crude oil utilizer strain. Bioaugmentation studies of this bacterial isolate would help exploring its commercial feasibility in bioremediation of subsurface oil spills.

**Keywords:** Biodegradation, Green technology, Nutrients, Oleophilic, Petroleum hydrocarbons, Pollution, *Pseudomonas*, Salinity

Petroleum hydrocarbon pollutants are classified as priority pollutants[1-3]. Awareness on the harmful effects of pollutants, to the environment as well as human health, has led to notable increase in research on remediation aspects[4-6]. Physical and chemical methods for treatment of these pollutants are not only expensive because of high costs of chemicals and equipments, but also generate excessive amounts of sludge which further pollutes the environment[7-10]. As a better alternative, biological methods are preferred for their simple, cheap and environmentally friendly operations[6,8,11,12]. In biological means, microorganisms play a vital role over other bioremediation agents because of advantages, such as rapid growth with minimum growth requirements[7,10,13].

Various groups of indigenous bacteria, fungi, cyanobacteria and algae are involved in petroleum hydrocarbon utilization/degradation[5,6,14-16]. Among all these groups, bacteria are reported as the most active bioremediation agents[3,10]. Environmental factors and growth conditions like pH, temperature, agitation and oxygen availability, salinity, carbon and nitrogen sources play a vital role in the growth of microorganisms[2,7].Optimization of growth conditions for industrially important microbes prior to field trials have gained considerable momentum worldwide[8,17]. The present work explores effect of environmental as well as nutritional growth conditions for hydrocarbon utilizing and biosurfactant producing *Pseudomonas aeruginosa* NCIM 5514. Growth profile of this organism was performed and results were note in terms of biomass (g/L).

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## Materials and Methods

### Source of *Pseudomonas aeruginosa* NCIM 5514

*P. aeruginosa* NCIM 5514 was isolated by enrichment culture technique from crude oil polluted sub surface soil sample of Ankleshwar Asset, Oil and Natural Gas Corporation (ONGC), Gujarat, India. Crude oil sample was collected from well no. K# X, ONGC, Gujarat. Sampling procedure and physico-chemical characteristics of soil and crude oil have been reported earlier[18,19].

### Culture medium and Reagents

Growth studies were carried out in Bushnell-Hass (BH) medium[12]. The composition of BHM (g/L): 0.2 $MgSO_4$, 0.02 $CaCl_2$, 1.0 $KH_2PO_4$, 1.0 $K_2HPO_4$,

1.0 g $NH_4NO_3$, 0.05 $FeCl_3$, and the pH was adjusted to 7.0-7.2 with 0.01N HCl[20]. The BH and Nutrient agar media were procured from HiMedia (Mumbai, India). All other reagents used were of analytical grade.

**Pre-culture preparation**

Pre-culture was prepared and used as inoculums for examining the effect of various growth parameters on the bacterial isolate.*P. aeruginosa* NCIM 5514 was activated by transferring culture from nutrient agar slants in BH medium, supplemented with 1%, v/v crude oil (K#X) as sole carbon source and incubated for 24 h at 37±1°C, 180 rpm. Pre-culture was prepared in 50 mL BH medium, supplemented with crude oil (K#X, 1%, v/v) by inoculating activated culture broth (1%, v/v, optical density 1.0 at $AU_{600}$) and incubated at 37±1°C, 180 rpm, 24 h[20].

**Growth profile study of *P. aeruginosa* NCIM 5514**

*Growth parameters*

The growth parameters were studied in 250 mL flasks containing 100 mL BH medium. Process parameters studied for crude oil biodegradation included influence of static and shaking (180 rpm) conditions, varying temperatures, pH of the BH medium, NaCl concentrations, petroleum and non-petroleum carbon sources & its concentrations, nitrogen source and inoculum ratio. Succeeding growth condition experiments were performed using optimized preceding growth conditions. These experiments were performed in three sets utilizing resultant optimum parameters and a control devoid of bacterial isolate was prepared for each set of experiments.

Initially, effect of agitation was determined by incubating control and test flasks in static and shaking (180 rpm) conditions at 37±1°C, pH 7.2, 180 rpm for 4 days. Incubations were carried out at temperatures (30, 37, 45 and 50°C), pH of the BH medium (6.0, 7.2, 8.0, 9.0, 10.0 and 11.0) and NaCl concentration [0, 1, 2, 3, 5, 7, 10 and 12% (w/v)]. The effect of petroleum and non-petroleum carbon sources *viz*. crude oil, nonane, decane, dodecane, n-paraffins, kerosene, diesel, xylene, glucose and glycerol (1%, v/v ) or (1%, w/v) & glucose at 1, 2, 3, 4 and 5% concentrations (w/v) was evaluated. For effect of nitrogen sources, ammonium nitrate, potassium nitrate, peptone and tryptone were employed at 1%, w/v with optimum carbon source (1%, w/v), simultaneously one flask was also studied without any additional nitrogen-source. At the end,inoculum ratio (1, 2, 3, 5, 7 and 10%, v/v) was also studied.

*Biomass determination*

Growth was measuredperiodically in terms of biomass using gravimetric method[21]. Ten milliliter (10 mL) sample was withdrawn from flasks and centrifuged at 10000 x g at 4°C for 20 min. The pellet was dried overnight in an oven (70°C) and dry weight of cells was measured gravimetrically.

**Statistical analysis**

Biomass determination for microbial growth was carried as mean±SD to minimize the percentage of error and to achieve significant data. SPSS for Windows, version 16.0 was used for statistical analysis of results.

## Results and Discussion

Petroleum hydrocarbon pollutants degrading and rhamnolipid producing bacteria was isolated from crude oil polluted sub surface soil of ONGC, Ankleshwar asset, Gujarat, India. The isolate was identified as strain of*Pseudomonas* and deposited as *Pseudomonas* sp. NCIM 5514 in the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India[16,19,22,23].The crude oil polluted subsurface soil sample showed 19.57% hydrocarbon utilizing bacterial count in total viable count[18]. *P. aeruginosa* NCIM 5514 showed 99.46% homology with *P. aeruginosa* LMG 1242. The GenBank accession number for 16S rDNA sequence of this isolate is KC713611[16]. *P. aeruginosa* NCIM 5514 is a gram-negative motile bacterium. It produces colonies with diffusible fluorescent yellow-green pigment when grown on Nutrient agar plate. The colonies were medium, irregular, flat, auriculate, smooth and translucent. Photomicrograph of *P. aeruginosa* NCIM 5514 colony on Bushnell-Hass (BH) agar plate supplemented with crude oil is presented earlier[23].

**Growth parameters for *P. aeruginosa* NCIM 5514**

Growth parameter studies of *P. aeruginosa* NCIM 5514 shown in Figs. 1, 2 and 3, revealed that its optimum growth conditions to be 37°C, pH 7.2, glucose (1%, v/v) at 180 rpm with 1%, v/v inoculum for 4 days. Among various carbon substrates tested, glucose (1%, v/v) showed optimum growth (Fig. 2B and C). However, notable growth of *P. aeruginosa* NCIM 5514 was also observed when grown on 1%,

v/v glycerol and 1%, w/v crude oil (Fig. 2B). Halophilic or halotolerant microbial strains have been shown to be useful in bioremediation of oil spills[17]. The isolate could grow at 5%, w/v NaCl concentration (Fig. 2A). As shown in Fig 3A, no profound effect of addition of N-Source in BH medium was found on growth of the bacterial isolate, which indicates amount of $NH_4NO_3$ present in BH medium was sufficient for its growth. *P. aeruginosa* NCIM 5514 is a mesophilic, halotolerant, aerobic crude oil utilizer. Growth optimization studies for hydrocarbon utilizing bacterial consortium (HUBC)has been performed, and 3% v/v crude oil, pH 7.2, incubation at 37ºC at 180 rpm with 2% v/v inoculum were reported as optimum conditions for its growth[17]. Halotolerant HUBC consisting of *Ochrobactrum* sp., *Stenotrophomonas maltophila* and *Pseudomonas aeruginosa* isolates, was able to degrade 83.70%, C8-C35 components of crude oil. Authors suggested its application in paraffin biodegradation and oil spill management[16].

Biodegradation is a primary mechanism for bioremediation which uses oleophilic microbes to remove petroleum hydrocarbon pollutants from the environment[2,3,6,10,12,13,24,25]. Optimizing environmental as well as nutritional parameters for growth of microbes either individual or as consortium, significantly affect microbial degradation rates of total petroleum hydrocarbons[2,8,25,26]. Numerous studies have identified relationships between soil conditions and microbial activity[7,9,13,17]. However to

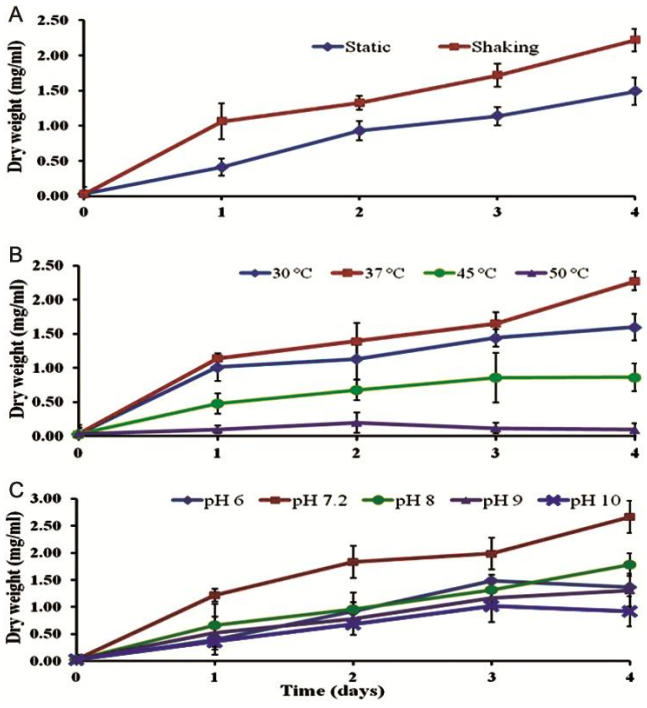


Fig. 1 — Agitation, Temperature and pH optima for *P. aeruginosa* NCIM 5514 [Results are mean of three replicates; error bars represent SD]

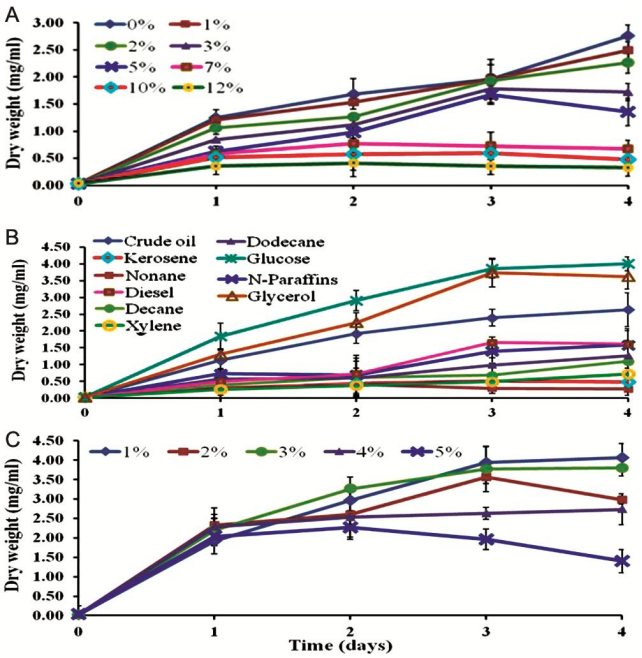


Fig. 2 — Impact of NaCl, C - substrate and its concentration on *P. aeruginosa* NCIM 5514 [Results are mean of three replicates; error bars represent SD]

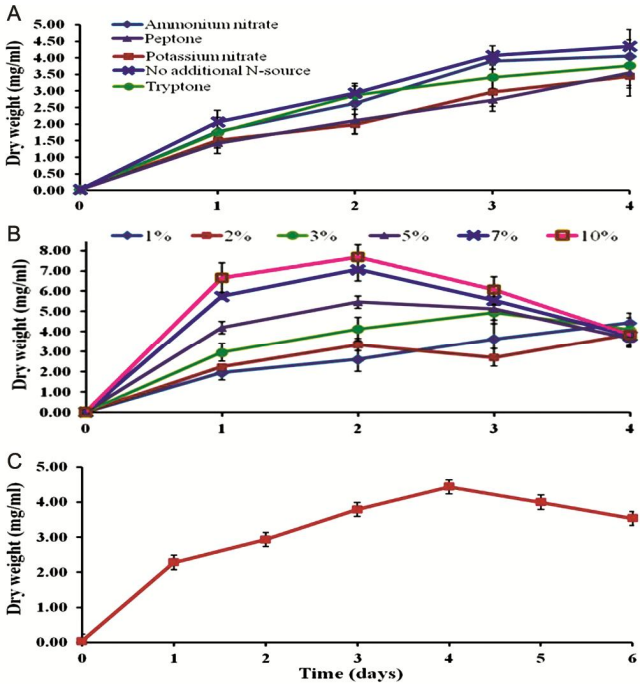


Fig. 3 — Impact of N-source, Inoculum and incubation time on growth of *P. aeruginosa* NCIM 5514 [Results are mean of three replicates; error bars represent SD]

obtain good biodegradation activity enrichment and screening along with optimization ofphysical and chemical conditions *viz.* temperature, pH and type & composition of growth media for potential hydrocarbon degrading microorganisms is very important[2,8,10,16,18]. Researchers have isolated of *Bacillus subtilis* and *P. aeruginosa* from soil contaminated with automobileoil in Tamilnadu, India. Further, they have indicated diesel to be the best carbon source among different carbon sources, such as vegetable oil, kerosene, petrol and diesel studied for their growth[27].

Temperature affects microbial growth rate and thereby, the metabolism as well as physio-chemical state of the contaminants[3,28]. Oxygen acts as substrate in oxygenase catalyzed reactions, also it is an electron acceptor in aerobic metabolism[5,11]. Influence of salinity on microbial growth and bioremediation of petroleum hydrocarbon pollutants has been extensively reviewed[3,7,13]. Type and concentration of carbon and nitrogen source used in culture medium play vital role in microbial growth as these nutrients help in biomass formation[2,8,17,29]. Carbon sources for growth of microorganisms based on their composition can be categorized into two groups, petroleum and non-petroleum[22,24,26]. Rhamnolipid type biosurfactant producing *P. aeruginosa* DS10-129 has been reported from gasoline & diesel oil contaminated sites. For bioremediation of hydrocarbon-contaminated sites and enhanced oil recovery, low-cost renewable carbon source like soybean oil can be used for microbial growth[30]. It is observed that nitrate is the best source of nitrogen for growth and biosurfactant production by microorganisms[31,32].

Polycyclic aromatic hydrocarbon (PAH) degrading *Bacillus* sp. was isolated from the compost of oil sludge and animal manures. This bacterial isolate can be applied in bioremediation of oil sludge. Results of this study also suggest that indigenous bacterial consortium with petroleum oil utilizing capabilities can be used as seed onto crude oil contaminated environment, which could be more ecofriendly approach for bioremediation[6].

*P. aeruginosa* 5514 was successfully employed through *ex-situ* bioaugmentation studies in laboratory simulation experiment for bioremediation of crude oil pollutants[23]and microbial enhanced oil recovery (MEOR)[19]. *P. aeruginosa* NCIM 5514 represents unique properties of carbon spectrum utilization with glucose as best carbon substrate yielding high quantity of rhamnolipid type biosurfactant with desired surface active properties[22]. For MEOR studies, the isolate showed 8.82% oil recovery enhancement as % of residual oil saturation (ROS)[19]. The isolate showed 61.03% biodegradation of C8-C36+ hydrocarbons present in crude oil when analyzed by gas chromatographic methods. These studies highlight potential application of *P. aeruginosa* 5514 in petroleum industry[19,22,23].

## Conclusion

The study explored effect of various environmental and nutritional factors on growth of *P. aeruginosa* NCIM 5514, isolated from petroleum oil well ecosystem of South Gujarat. Maximum growth of *P. aeruginosa* NCIM 5514 was noted at glucose 1%, w/v), pH 7.2, incubation at 37°C at 180 rpm with 1%, v/v inoculum for 4 days. The organism was also able to grow on glycerol and crude oil. The isolate could not grow efficiently when it was grown in Bushnell-Hass (BH) medium supplemented with nitrogen source in addition to carbon source. It showed growth in BH medium supplemented with 5%, w/v NaCl concentration. Over all, the results have substantiated that *P. aeruginosa* NCIM 5514 is a mesophilic, halotolerant, aerobic, crude oil utilizer. There is a need of halophilic or halotolerant microbial strain(s) bioremediation of marine oil spills. Halotolerant nature of this isolate increases its applications in oil spill management.

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