



Role of nutrients in the utilization of polycyclic aromatic hydrocarbons by halotolerant bacterial strain

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Abstract

A halotolerant bacterial strain VA1 isolated from marine environment was studied for its ability to utilize polycyclic aromatic hydrocarbons (PAHs) under saline condition. Anthracene and pyrene were used as representatives for the utilization of PAH by the bacterial strain. Glucose and sodium citrate were used as additional carbon sources to enhance the PAH utilization. The strain VA1 was able to utilize anthracene (73%) and pyrene (66%) without any additional substrate. In the presence of additional carbon sources (glucose/sodium citrate) the utilization of PAH was faster. PAH was utilized faster by VA1 in the presence of glucose than sodium citrate. The strain utilized 87% and 83% of anthracene and pyrene with glucose as carbon source and with sodium citrate the strain utilized 81% and 76% respectively in 4 days. Urea as an alternative source of nitrogen also enhanced the utilization of PAHs (anthracene and pyrene) by the bacterial strain up to 88% and 84% in 4 days. Sodium nitrate as nitrogen source was not able to enhance the PAH utilization rate. Phenotypic and phylogenetic analysis proved that the PAHs utilizing halotolerant strain VA1 belongs to *Ochrobactrum* sp.

Key words: biodegradation; polycyclic aromatic hydrocarbons; halotolerant, *Ochrobactrum* sp.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds composed of two or more fused aromatic rings that are formed by incomplete combustion of organic matter. A variety of PAHs, ranging from naphthalene to high-molecular weight PAHs such as benzo(a)pyrene, are found in the marine environment (Stegeman, 1981). Some PAHs are toxic; others are carcinogenic to marine organisms and may be transferred to humans through seafood consumption (Sikkema et al., 1995).

The phylogenetic and biodegradative diversity of marine and estuarine PAH-degrading microbes has been the subject of several investigations, leading to the isolation of PAH-degrading members of the marine bacterial genera *Cycloclasticus* (Chung and King, 2001; Dyksterhouse et al., 1995; Geiselbrecht et al., 1998), *Neptunomonas* (Hedlund et al., 1999), *Vibrio* (Hedlund and Staley, 2001), *Marinobacter* (Hedlund et al., 2001), *Pseudalteromonas* (Hedlund and Staley, 2006) and *Lutibacterium* (Chung and King, 2001), in addition to those commonly found in terrestrial habitats such as *Pseudomonas*, *Paenibacillus*, *Rhodococcus*, *Tsukamurella*, *Arthrobacter* and

Sphingomonas (Daane et al., 2001). The present study details about the role of nutrients in utilisation of PAHs under saline condition by *Ochrobactrum* sp. strain VA1 isolated from marine environment.

1 Materials and methods

1.1 Source of bacterial strain

The bacterial strain (VA1) was isolated from a bacterial consortium enriched on PAH (anthracene) with marine water samples from seven different sampling sites which included petroleum and coal contaminated sites, salt pans and the sea-port of Chennai, India. The bacterial consortium enriched from different saline environments consisted of three bacterial strains namely VA1, VA2 and VA3 (Arulazhagan and Vasudevan, 2009). The results of PAH (anthracene) clearing zone was used to select the PAH degrading strain for the present study. VA1 degraded 73% of anthracene (3 mg/L) when compared to the strains VA2 (36%) and VA3 (17%) in 4 days at 30 g/L of NaCl concentration (data not shown). Therefore, from the PAHs degrading halotolerant bacterial consortium, VA1 strain was isolated and used in the present study.

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1.2 Enrichment of PAH utilising bacterial strain

The carbon free mineral salts medium (MSM) contained NH_4Cl 2.5 g, KH_2PO_4 5.46 g, Na_2HPO_4 4.76 g, MgSO_4 0.20 g, NaCl 30.0 g, and distilled water 1 L at pH (7.4 \pm 0.2) was used for isolation of PAH degrading bacterial strain. The final pH of the medium was adjusted to 7.4 with 0.1 mol/L NaOH , and the medium was autoclaved (121°C for 15 min) prior to the addition of PAHs. PAHs used in the study, pyrene (purity 99%) purchased from Sigma Aldrich Chem. Co., USA and anthracene (purity 98%) from Merck, India. Stock solutions of each PAH (300 mg/L) were prepared in ethyl acetate.

The bacterial strain was enriched and isolated using mineral salts medium with anthracene as sole carbon source. PAH dissolved in ethyl acetate was added to 250 mL conical flask and after the evaporation of ethyl acetate, the mineral medium (100 mL) was added. The bacterial strain containing 5 mL of 10^4 to 10^5 cfu/mL was added to the mineral medium containing anthracene or pyrene as sole carbon source. The conical flasks were kept in shaker at 150 r/min with 37°C as incubation temperature. After growth was visualized under microscope, 5 mL of enrichment culture was transferred to a fresh medium and incubated under the same conditions. Subsequent identical transfer of culture was performed in the respective PAH containing medium to enrich the bacterial consortium.

1.3 Studies on PAH utilisation

Anthracene was added to the medium at a concentration of 3 mg/L. The bacterial strain isolated from marine environment was grown and the bacterial count was checked everyday. Cell morphology and motility of exponential growing liquid cultures were examined on freshly prepared wet mounts by light microscope (DMLB Type 020-519.509.lb 100t, Leica, Germany). Plate counting was done on nutrient agar medium. The bacterial strain was studied for its growth on anthracene as sole carbon source.

A qualitative assay by the spray-plate method was used to check the utilisation of PAH by the bacterial strain. The clearing zones seen around the colonies indicated the utilization of PAH sprayed on the medium (Kiyohara et al., 1982). For studies on the utilization of PAH, the bacterial strain was inoculated in mineral medium containing respective PAH. The experimental setup consisted of: (I) medium + PAH + bacterial strain; (II) medium + PAH and (III) medium + bacterial strain which served as controls. Glucose and sodium citrate (25 mmol/L) were studied individually as additional carbon substrate along with PAH. The carbon source concentration was selected based on the PAH concentration used in the study. The concentration of carbon source selected also should not affect the PAH degradability by the bacterial strain. The addition of carbon source increases the bacterial cell count and enhanced the degradation of PAHs under saline condition. Urea and sodium nitrate were used as additional nitrogen substrate along with PAH, whereas ammonium chloride present in the medium was removed and the same amount of nitrogen concentration was added in the medium. When additional

nutrients were used along with PAH, medium + additional substrate (carbon) + bacterial strain were used as control. Bacterial consortium was added at concentrations of 10^4 to 10^5 cfu/mL in the medium. The culture prepared in duplicates were incubated at 37°C in shaker at 150 r/min and extracted at every 24 hr time interval for 5 days.

The culture samples were extracted twice with ethyl acetate (V/V) after acidification to pH 2.5 with 1 mol/L HCl . The extracts were filtered through anhydrous sodium sulphate and condensed to 1 mL using rotavapour unit (R124, Buchi, Switzerland) for further analysis in high performance liquid chromatography (HPLC).

1.4 HPLC analysis of PAH utilization

The condensed sample was filtered through 0.2 mm syringe filter and analysed by HPLC, which was performed with Knauer (K501, Knauer, Germany) unit equipped with PAH specific column (Ultrasep ES, B590/02, 250 \times 4 mm, Knauer, Germany) with UV-Vis detector connected to Winchrome software, which was used to process the data. The mobile phase was acetonitrile. Standard solutions of different PAHs were used as reference. The flow rate of the mobile phase was maintained at 1 mL/min. The samples were injected one by one and the utilization rate of PAHs was calculated based on the peak area percent and retention time.

1.5 Phenotypic and phylogenetic analysis

The bacterial strain was analyzed for phenotypic characters using KB003: Hi24 Enterobacteriaceae Identification Kit (Himedia, India).

1.6 Extraction and amplification of bacterial DNA

DNA from the bacterial cell was extracted using Qiagen (QIAamp® DNA stool Mini kit Cat. No. 51504, Germany) DNA isolation kit using the protocol from manufacturer. DNA was eluted in 200 μL of AE buffer and stored at 4°C for further use. The concentrated DNA samples were amplified by polymerase chain reaction (PCR) using Thermal Cycler (Mastercycler® personal, Eppendorf AG, Germany). The primers used were for amplification of general bacterial DNA (Edward et al., 1989). Amplification was performed using 25 pmol of forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-AAGGAGGTGATCCAGCCGCA-3') to the total volume of 50 pmol (Edward et al., 1989). The PCR supermix (invitrogen Cat. No. 10572-014, USA) consisted of tris-HCl (pH 8.4) 22 mmol/L, KCl 55 mmol/L, MgCl_2 1.65 mmol/L, dGTP 220 $\mu\text{mol/L}$, dATP 220 $\mu\text{mol/L}$, dTTP 220 $\mu\text{mol/L}$, dCTP 220 $\mu\text{mol/L}$, and 22 U recombinant *Taq* DNA polymerase/mL. The PCR supermix (40 μL) was mixed with primers (5 μL) and DNA (5 μL) to a total volume of 50 μL in 0.2 mL PCR tubes and loaded into thermal cycler. The PCR reactions occurred through an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 0.45 min, and primer extension at 72°C for 2 min. In the final step, the samples were incubated at 72°C for 10 min. The PCR amplification was verified

by electrophoresis, performed in horizontal submarine apparatus with 1% agarose gel and stained with ethidium bromide. Bromophenol Blue (6×) was used as loading dye. TAE buffer was used as the tank buffer. Electrophoresis was carried out for 2 hr at 50 V. The gel was visualized in an UV illuminator.

1.7 16S rRNA sequential analysis of PAH degrading bacterial consortium

The cyclic sequencing reaction was performed using BigDye terminatorV3.1 cycle sequencing kit (Applied Biosystems, P/N: 4337457, USA). The sequencing reaction mix was prepared by adding 1 µL of BigDyeV3.1, 2 µL of 5× sequencing buffer and 1 µL of 50% DMSO. To 4 µL of sequencing reaction mix 4 pmol of primer (2 µL) and sufficient amount of purified PCR product were added. The constituted reaction was denatured at 95°C for 5 min. Cycling began with denaturing at 95°C for 30 sec, annealing at 52°C for 30 sec and extension for 4 min at 60°C. The cycle was repeated for a total of 30 times in MWG thermocycler. The reaction was then purified on sephadex plate (Edge Biosystems, USA) by centrifugation to remove unbound labeled and unlabeled nucleotides and salts. The purified reaction was loaded on to the 96 capillary ABI 3700 automated DNA analyzer and electrophoresis was carried out for 4 hr. The nucleotide sequences are registered in the computer attached with the ABI 3700 DNA analyzer (ABI 3700, Applied Biosystems, USA).

The nucleotide sequences obtained from the ABI DNA analyzer was studied using BLAST software available in NCBI website (www.ncbi.nlm.nih.gov). After editing the sequence, we analysed with BLAST software to identify the specific type of bacteria corresponding to the nucleotide sequence.

2 Results and discussion

2.1 Isolation of PAH utilising bacterial strain

In this study the PAH utilizing bacterial strain was isolated from a bacterial consortium enriched from the water samples collected from different sites (petroleum or coal contaminated) of seaport of Chennai, India (Arulazhagan and Vasudevan, 2009). PAH utilizing ability of VA1 strain was performed in mineral salt medium with anthracene as sole carbon source. The strain VA1 formed clearing zone in anthracene sprayed mineral salt agar plate indicating the utilization of PAH sprayed on the medium. VA1 strain utilised anthracene and pyrene 73% and 66% as sole carbon source, respectively in 4 days at 30 g/L of NaCl concentration (Figs. 1a and 2a).

2.2 Utilisation of PAHs with additional carbon source

The utilization of PAHs is mainly influenced by chemical and environmental limiting factors (salinity, nutrients, pH, temperature, oxygen) and may not be alone due to the enzymatic capacities of the indigenous hydrocarbon degrading bacterial strains (Atlas, 1981). Due to lack of

nutrients in the saline condition, the consortium was not able to successfully grow and degrade the PAHs. As a result of nutrient deficiency, the bacterial strain was not able to develop complete biosynthetic pathways and have complex nutrient requirements at high saline conditions. The addition of nutrients has demonstrated that critical limiting factors such as salinity and nutrient requirements can be overcome.

In the present study addition of second carbon source enhanced the utilization of PAH. During the PAH utilization glucose and sodium citrate were used separately as additional carbon sources. PAH was utilized faster in the presence of glucose by VA1 than sodium citrate as additional carbon substrate. VA1 utilised anthracene (87%) and pyrene (83%) in 4 days at 30 g/L of NaCl concentration along with glucose as additional carbon source (Figs. 1b and 2b). With sodium citrate as additional carbon source VA1 utilised 81% of anthracene and 76% of pyrene in 4 days at 30 g/L of NaCl concentration (Figs. 1c and 2c). Among the two carbon sources used based on percent of PAH utilized glucose was found to be better in enhancing PAH utilisation. Tam et al. (2002) studied phenanthrene utilization by bacterial consortium isolated from mangrove sediment. The bacterial consortium degraded phenanthrene up to 47% at 20 g/L of salinity, but growth and rate of phenanthrene utilization were influenced by salinity. At higher salinity (35 g/L), the growth and biodegradation of bacterial consortium were inhibited. Tam et al. (2002) also reported that addition of glucose as additional carbon source at 35 g/L NaCl increased the utilization of phenanthrene from 35.6% to 45%. Salinity plays an important role in PAHs utilization.

2.3 Utilisation of PAH with alternate nitrogen source

The utilization of PAH was studied in the presence of ammonium chloride as nitrogen source and with urea and sodium nitrate as an alternate nitrogen sources. With urea as nitrogen source VA1 utilised anthracene (88%) and pyrene (84%) in 4 days at 30 g/L of NaCl concentration (Figs. 1d and 2d). Urea as nitrogen source enhanced the percent utilization of PAHs (Anthracene and pyrene) in 4 day(s). The utilization of PAH in the presence of sodium nitrate as nitrogen source showed no change in PAH utilization. The addition of nitrogen and phosphate was proved to be an effective bioremediation treatment on several shorelines (Swannell et al., 1996, 1999; Venosa et al., 1996). Since increase in salinity and depletion of nutrients inhibit the growth of the bacterial cells. To support the growth of bacterial cells, nutrients such as glucose, yeast extract and acetate were added (Yuan et al., 2000). This shows as new criteria that selection of nitrogen source also plays an important role in the utilization of PAH to support the growth of bacterial strain under saline condition.

2.4 Identification of PAH utilising strain

Phylogenetic analysis based on nucleotide sequences from VA1 strain showed a maximum of 98% identity towards *Ochrobactrum* sp. The phylogenetic tree drawn

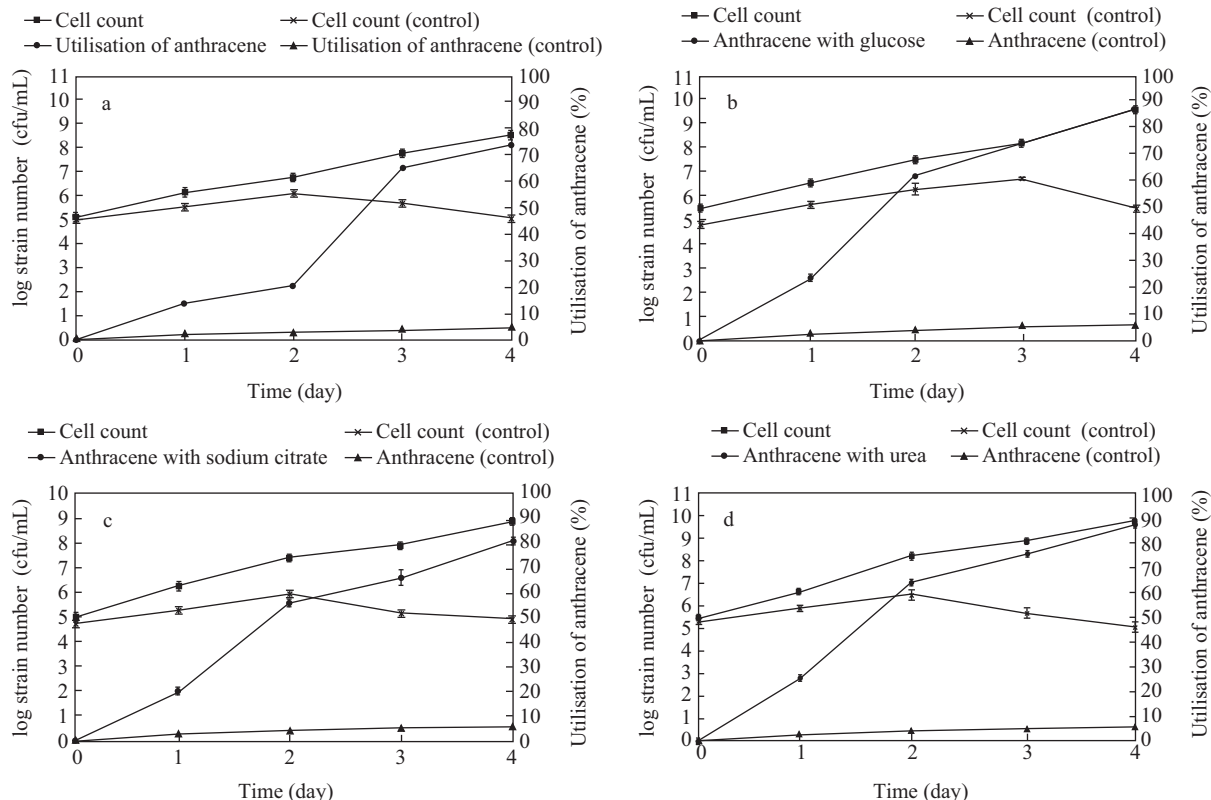


Fig. 1 Utilisation of anthracene by *Ochrobactrum* sp. strain VA1 without additional nutrients (a), and in the presence of glucose (b), sodium citrate (c), and urea (d).

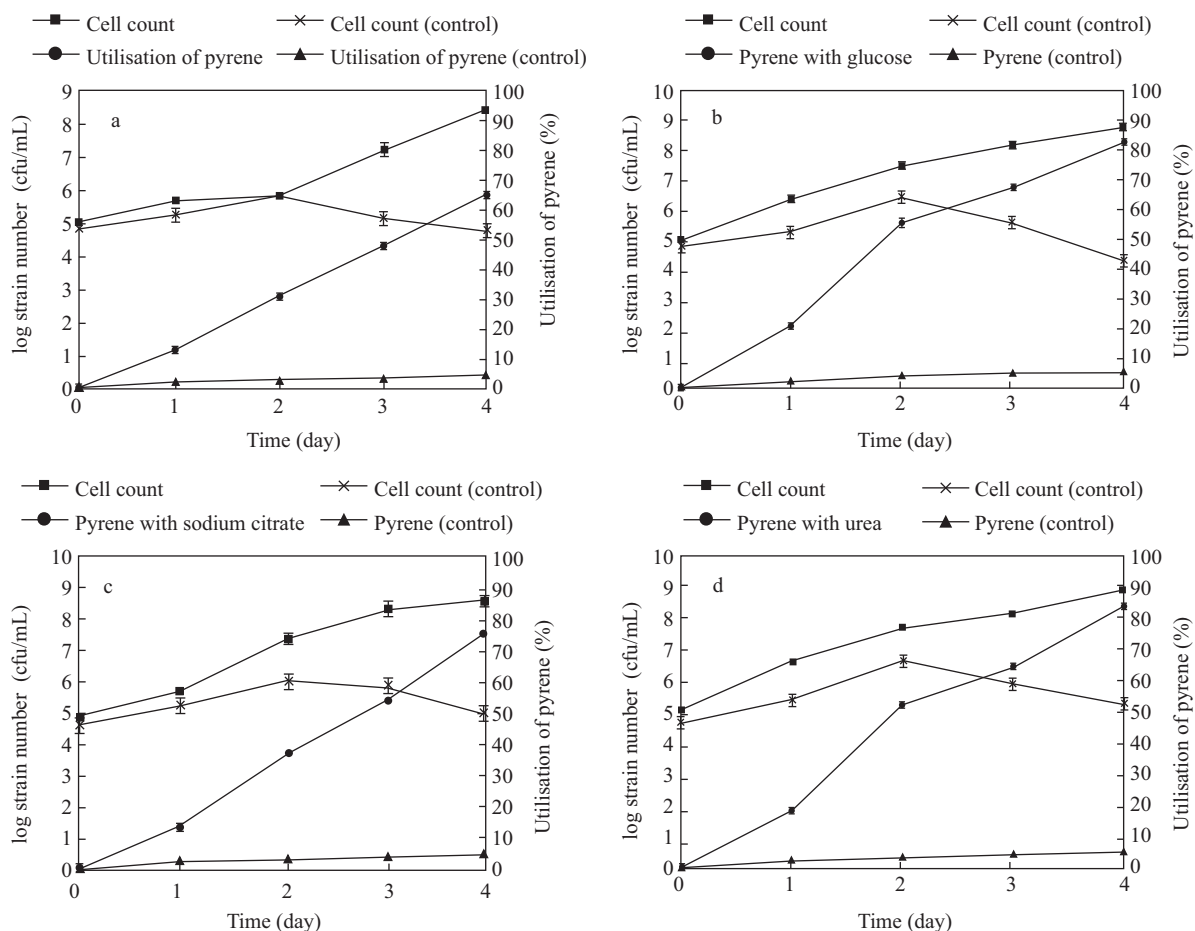


Fig. 2 Utilisation of pyrene by *Ochrobactrum* sp. strain VA1 without additional nutrients (a), and in the presence of glucose (b), sodium citrate (c), and urea (d).

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based on nucleotide sequence and shows the distance relationship between VA2 (*Enterobacter cloacae*) and VA3 (*Stenotrophomonas maltophilia*) strain (Fig. 3). The strain is Gram negative, rod shaped, aerobic and oxidase positive. When the strain was analysed for physiological characters, the strain showed negative result towards *ortho* nitro phenylene β -galactopyranoside, phenylalanine deamination, Methyl Red, voges proskauer and indole reactions. The strain utilized 9 out of 13 carbon sources. Arabinose, adonitol, trehalose and lactose are the carbon sources which were not utilized by the strain (Table 1). Thus from the results of phenotypic and phylogenetic analysis confirmed that the PAH utilizing bacterial strain belongs to *Ochrobactrum* sp.

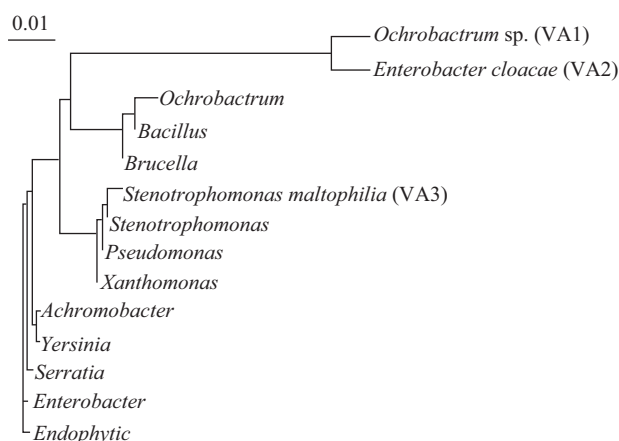


Fig. 3 Phylogenetic tree derived from a Jukes–Cantor evolutionary distances analysis of the 16S rRNA gene sequences of *Ochrobactrum* sp. (VA1), *Enterobacter cloacae* (VA2) and *Stenotrophomonas maltophilia* (VA3) and representative members of *Proteobacteria* group. Scale bar 0.01 substitution per site.

Table 1 Phenotypic analysis of *Ochrobactrum* sp. strain VA1

Name of the biochemical test	VA1	Name of the biochemical test	VA1
Oxidase	+	Esculin	+
<i>ortho</i> -Nitro phenylene β -galactopyranoside	–	Arabinose	–
Lysine decarboxylase	+	Xylose	+
Ornithine	+	Adonitol	–
Urease	+	Rhamnose	+
Phenylalanine deamination	–	Cellobiose	+
Nitrate reduction	–	Melibiose	+
H ₂ S production	+	Saccharose	+
Citrate utilization	+	Raffinose	+
Methyl Red	–	Trehalose	–
Voges proskauer	–	Glucose	+
Indole	–	Lactose	–
Malonate	+		

3 Conclusions

The bacterial strain *Ochrobactrum* sp. VA1 isolated from marine environment was found to be a promising strain for PAH utilization under saline condition. From the above study it was clear that addition of nutrients (carbon-glucose/nitrogen-urea) enhanced the PAH utilization under

saline condition. Thus selection and addition of nutrients play vital role in the utilization of PAH under saline condition.

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