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Presence of Catechol Metabolizing Enzymes in *Virgibacillus Salarius*

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Abstract – Hydrocarbon contamination of marine ecosystems has been a major environmental concern. Hydrocarbon metabolizing capacity of four halotolerant bacteria (*Bacillus atrophaeus, Halomonas shengliensis, Halomonas koreensis,* and *Virgibacillus salarius*) isolated from saline soil of Khambhat, India was investigated. Presence of catechol metabolizing enzymes (catechol 2,3 dioxygenase, chlorocatechol 1,2 dioxygenase, and protocatechuate 3,4 dioxygenase) was checked in *V. salarius*, as only this among all the test organisms could grow on the hydrocarbon substrates used, and compared with *Pseudomonas oleovorans*. Effect of salinity of the growth medium on activity of catechol metabolizing enzymes was also studied. Catechol 2,3 dioxygenase activity in both the organisms was more susceptible to increase in salinity of the growth medium than chlorocatechol 1,2-dioxygenase activity. To the best of our awareness, this is the first report of catechol metabolism in *V. salarius*. *V. salarius* was found to be capable of weak biofilm formation. As *V. salarius* is capable of growing at high salt concentration, alkaline pH, hydrocarbon degradation, and also of growth in presence of various metal ions, it can be an attractive candidate for bioremediation of marine oil spills. Organisms like *V. salarius* can also serve as a model for multiple stress tolerance in prokaryotes.

Keywords – Catechol, Catechol 2,3 Dioxygenase, Chlorocatechol 1,2 Dioxygenase; Salinity; Halotolerant; Marine Oil Spill; Bioremediation

1. Introduction

Hydrocarbon (HC) contamination has been a major environmental concern since long. Most of the components of petroleum are amenable to biodegradation, albeit at slow rates. Petroleum can enter the biosphere by natural seepage and due to oil spills (Atlas & Bartha, 1998). Leaks and accidental spills occur frequently during transportation, production, exploration, refining, and storage of petroleum and its derivatives. The amount of natural crude oil seepage has been estimated to be 600,000 metric tons per year with an uncertainty of 200,000 metric tons per year (Kvenvolden & Cooper, 2003). Release of crude oil and other hydrocarbons into marine waters has been a major threat to marine biodiversity. Dissolved aromatic components of petroleum even at a low ppb concentration can disrupt the chemoreception of some marine organisms. Another disturbing possibility is that some condensed polynuclear components of petroleum that are carcinogenic and relatively resistant to biodegradation may move up marine food chains and taint fishes or shellfishes, which further can be used as food or feed, and thus can harm higher life forms through biomagnification.

Hydrocarbon metabolizing capacity has been demonstrated in many microorganisms such as *Pseudomonas putida* (Gibson et al., 1970), *Pseudomonas oleovorans* (Zhou et al. 2011), *Pseudomonas picketti* (Kukor & Olsen, 1991), *Burkholderia xenovorans* (Goris et al., 2004), *Arthrobacter* sp. (Kallimanis et al., 2007), *Janibacter* sp. (Nguyen et al., 2011), *Mycobacterium* sp. (Mundle et al., 2012), *Nocardia hydrocarbanooxydans* (Kalme et al., 2008), etc. However all the microorganisms possessing hydrocarbon metabolizing machinery are not capable of surviving low temperature, high salinity, and alkaline pH characteristic of marine ecosystems. Low temperatures of the marine environment provide no appreciable opportunity for a large part of microbial community to remain in active physiological state (Atlas & Bartha, 1998). For this reason halotolerant/halophilic microorganisms possessing hydrocarbon metabolizing enzymes and capable of growth at low temperature can be of special interest for bioremediation of marine environments polluted with petroleum hydrocarbons. Quite a few reports on hydrocarbon metabolizing halophilic microbes are available (Dalvi et al., 2012; Liu et al., 2012). There have been reports on certain halophilic actinomycetes known for HC degradation (Solanki & Kothari, 2011).

This study was designed to investigate hydrocarbon metabolizing capacity of four halotolerant bacteria (*Bacillus atrophaeus, Halomonas shengliensis, Halomonas koreensis,* and *Virgibacillus salarius*) previously isolated by us from saline soil of Khambhat, Gujarat, India (Solanki & Kothari, 2012). Presence of catechol metabolizing enzymes was checked in *V. salarius*, as only this among all the test organisms could grow

on the hydrocarbon substrates used. Effect of salinity of the growth medium on activity of catechol metabolizing enzymes was also studied. Catechol is the key reaction intermediate in the microbial metabolism of aromatic hydrocarbons including phenol, benzoic acid, toluate, naphthalene, salicylate (Dagley et al., 1960), and substituted catechols are intermediates in the catabolism of methylated and chlorinated derivatives of these compounds. Various pathways involving different enzymes are employed by different hydrocarbon degraders for catechol metabolism. Present study also involved a comparison between *V. salarius* and *P. oleovorans* with respect to activity of three different catechol metabolizing enzymes viz. catechol 2,3 dioxygenase, chlorocatechol 1,2 dioxygenase, and protocatechuate 3,4 dioxygenase. Role of cholorocatechol 1,2-dioxygenase in hydrocarbon metabolism in enviormental samples is known (Alfreider et. al. 2003). Hydrocarbon metabolizing enzymes like alkane-monooxygenase from *P. oleovorans* has previously been reported (Staijen et. al. 2000). This organism was suggested to be an efficient tetrahydrofuran (THF) degrader, and to posses two distinguished benzene pathway (Zhou et. al. 2011).

2. Materials and Methods

2.1. Test organisms

Following four halotolerant organisms isolated by us from saline soil of Khambhat, India (Solanki & Kothari, 2012) were investigated for their hydrocarbon metabolizing capacity, and compared with *Pseudomonas oleovorans* (MTCC 617) procured from Microbial Type Culture Collection (MTCC), Chandigarh:

1. *Bacillus atrophaeus*
2. *Halomonas shengliensis* (GenBank accession: JX081416)
3. *Halomonas koreensis* (GenBank accession: JX081417)
4. *Virgibacillus salarius* (GenBank accession: JX081418)

All the cultures were maintained on halophilic nutrient agar (Sodium chloride 60 g/l, Casein peptone 10 g/l, Yeast extract 5 g/l, Sucrose 5 g/l, Agar agar 15g/l, pH 8).

2.2. Qualitative screening for growth on hydrocarbons

All the four test organisms were streaked on minimal media (K_2HPO_4 5 g/l, NH_4Cl 2 g/l, $MgSO_4$ 1 g/l, NaCl 60 g/l, Agar agar 15 g/l, pH 8) supplemented with 1-5%v/v benzene, toluene, ethylbenzene, or xylene (BTEX) to check whether test bacteria can use these hydrocarbons as sole source of carbon. Additionally minimal media plates supplemented with petrol (1-3%v/v) were also streaked with *V. salarius*. Plates with identical medium composition with above mentioned hydrocarbon concentration, and supplemented with 0.1% yeast extract (HiMedia, Mumbai), were also simultaneously streaked to check whether presence of organic substance like yeast extract can enhance growth of test bacteria on hydrocarbon containing media. Incubation was continued upto one week at 35 ℃. Plates were observed everyday for checking growth of the test bacteria.

After confirming ability of *V. salarius* to utilize benzene, toluene, and ethylbenzene as sole carbon source, this organism was challenged with various combinations of these three compounds, with each compound added at 1%v/v. Incubation was continued upto one week at 35 ℃. Plates were observed everyday for checking growth of the test bacteria.

2.3. Enzyme assays

V. salarius or P. oleovorans was allowed to grow on minimal media (supplemented with 2%v/v petrol) plates (containing three different salt concentarions viz. 6%, 10%, and 15% NaCl) for 72 h at 35℃. Growth from this plate was used to prepare suspension of test organism in 5 mL of 0.1 M potassium phosphate buffer (pH 7). Turbidity of this suspension was adjusted to 0.5 at 625 nm (Thermo scientific spectronic 20D+). This standardized suspension was then subjected to cell lysis using the method described in Harley & Prescott (2002) with some modification. Briefly, 5 mL of cell suspension was mixed with 0.05 mL of lysozyme (HiMedia, Mumbai) solution (2 mg/mL). This mixture was gently agitated and kept at room temperature for 1 h. Then the tubes containing this reaction mixture were put in chilled water for 30 min, followed by addition of chloroform (Merck) into it, and then incubated for 10 min at room temperature, following which the content was centrifuged (7500 rpm for 10 min), and the supernatant was used as cell lysate. The lysate was subjected to heat treatment at 60 ℃ for 10 min prior to use. This was done to allow the ring fission product to accumulate by inactivating the enzymes involved in their further metabolism (Spain & Nishino, 1987).

Enzyme activity in the cell lysate was determined for different enzymes as described below (Kukor et al, 2007):

2.3.1. Catechol 2,3-dioxygenase (C23O)

The activity of C23O was determined spectrophotometrically from the rate of product accumulation. Briefly, 3.72 ml of 50 mM potassium phosphate buffer (pH 7.5), 0.18 ml of 10 mM catechol (Merck, Mumbai; made fresh for each use), and 0.1 ml of cell lysate was mixed in a test tube (final volume: 4 mL). The rate of increase in absorbance at 375 nm was monitored for 1 h.

2.3.2. Chlorocatechol 1,2-dioxygenase (Catechol dioxygenase II)

The activity of chlorocatechol 1,2-dioxygenase was determined spectrophotometrically from the rate of accumulation of the product chloro-*cis,cis*-muconic acid at 260 nm for 1 h. The assay mixture contained, in a final volume of 3 ml, 1 ml of 0.1 mM catechol, 0.5 ml of 1 mM EDTA, 1 ml of 33 mM Tris-HCl buffer (pH 8), and 0.5 ml of cell lysate.

2.3.3. Protocatechuate 3,4-dioxygenase

This enzyme was assayed by monitoring the decrease in A_{290} due to the degradation of the substrate protocatechuate (MP Biomedicals). The assay mixture contained, in a final volume of 3 ml, 0.3 ml of 50 mM tris-acetate (pH 7.5), 0.12 ml of 10 mM protocatechuate, and 0.1 ml of cell lysate.

2.4. Biofilm formation

Standardized inoculum was added into the wells of a 96-well surface treated polystyrene microtiter plate (HiMedia TPP96). Uninoculated sterile medium was put in wells corresponding to sterility control. Biofilm formation was tested in three different media: (a) minimal media, (b) halophilic nutrient broth (HNB), and (c) brain heart infusion (BHI; HiMedia) broth (pH 7.3) supplemented with 2% sucrose. Total volume of the content filled in the wells was kept 300 μl. Incubation was carried out at 35˚C for 48 h under static condition. Following incubation, degree of biofilm formation was assessed through crystal violet assay (Rukayadi & Hwang, 2006, Hirshfield et al., 2009). Briefly, biofilm-coated wells of microtitre plates were washed twice with 200 μL of 50 mmol L^{-1} phosphate buffer saline (PBS; pH 7.0) in order to remove all non-adherent (planktonic) bacteria, and air-dried for 45 min. Then, each of the washed wells was stained with 110 μL of 0.4% aqueous crystal violet solution for 45 min. Afterwards, each well was washed twice with 300 μL of sterile distilled water and immediately de-stained with 200 μL of 95% ethanol. After 45 min of de-staining, 100 μL of de-staining solution was transferred to a new well and the amount of the crystal violet stain in the de-staining solution was measured with microplate reader (BIO-RAD 680) at 655 nm. Magnitude of adherence was determined as described by Mathur et al. (2006).

2.5. Statistical analysis

All the experiments were performed in triplicate, and measurements are reported as mean ± standard deviation (SD). Statistical significance of the data was evaluated by applying *t*-test using Microsoft Excel®. *P* values less than 0.05 were considered to be statistically significant.

3. Results and Discussion

3.1. Results of qualitative screening

After streaking all the four test organisms on minimal media containing BTEX compounds, only *V. salarius* was found to be capable of growth on different concentrations of benzene, toluene, or ethylbenzene (Table 1). It was unable to grow on xylene. This pattern of growth on toluene, benzene, and ethylbenzene but not on xylene is normally observed in organisms with the ring-oxidising dioxygenase pathway (Kukor et al., 2007). In absence of yeast extract *V. salarius* was able to grow in presence of upto 5 %v/v benzene, toluene, and ethylbenzene. However it required longer incubation for growth on ethylbenzene. Presence of yeast extract in the medium was not found to have any stimulatory effect on growth of *V. salarius* on BTEX compounds. For example, it was able to grow on benzene (5 %v/v) and ethylbenzene (3 %v/v) faster in absence of yeast extract than in its presence. Similar inability of yeast extract to induce hydrocarbon degradation was reported by Dibble & Bartha (1979). This is in contrast to positive influence of pure or complex organic compounds on hydrocarbon degradation reported in literature. Ganesh & Lin (2009) reported diesel degradation rates to be influenced positively by glucose in gram-positive isolates. Positive influence of other carbon sources on hydrocarbon degradation may be attributed to the possible cometabolic hydrocarbon transformation. Such cometabolic transformation may or may not take place under a given set of biotic and abiotic experimental factors.

Table 1. Qualitative screening of *V. salarius* for growth on BTEX compounds

Test compound	Benzene (v/v)					Toluene (v/v)					Ethylbenzene (v/v)				
Conc.	1%	2%	3%	4%	5%	1%	2%	3%	4%	5%	1%	2%	3%	4%	5%
Incubation time (h)	With yeast extract														
48	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-
72	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-
96	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Without yeast extract														
48	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
72	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
96	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+): growth; (-): no growth

Once *V. salarius* was identified to be capable of utilizing benzene, toluene, or ethylbenzene as sole carbon source, it was challenged with combination of these compounds (each at 1 %v/v), and was found capable of growth on media containing all these three compounds at a time (Table 2).

Table 2. Growth of *V. salarius* in response to simultaneous challenge with two or more hydrocarbon compounds

Incubation Time (h)	B+T	T+E	B+E	B+T+E
48	+	+	+	-
72	+	+	+	+
96	+	+	+	+

(+): growth; (-): no growth;
B: Benzene, T: Toluene; E: Ethylbenzene

3.2. Detection of catechol metabolizing enzymes

Following identification of *V. salarius* as capable of hydrocarbon degradation, assays for detecting activity of three different catechol metabolizing enzymes were made in the cell lysates of *V. salarius*, and *P. oleovorans* grown on media containing 2 %v/v petrol as the sole source of carbon, at three different salt concentrations. Additionally these assays were also made in the cell lysate obtained by lysis of mixed suspension containing cells from both the organisms in equal proportion.

Both *V. salarius* and *P. oleovorans* were found to possess C23O activity, when grown at 6% as well as 10% salt concentration (Fig 1-2). *P. oleovorans* was not capable of growth at 15% salt concentration. C23O activity was detected by measuring product accumulation at 375 nm over a period of 1 h. When grown at 6 % salt concentration C23O from *P. oleovorans* showed 20.87% higher activity than *V. salarius,* while the latter showed activity 10.86% higher than that of mixture of both the organisms (Table 3). When grown at 10% salt concentration, *V. salarius* registered a 75.18% higher C23O activity than that of *P. oleovorans*, while the mixture had C23O activity similar to *V. salarius*. Thus C23O from *V. salarius* was less susceptible to increase in salinity of the growth medium than that from *P. oleovorans.* The former experienced a 42.17% and 87.61% decrease at 10% and 15% (Fig 3) salt concentration as compared to the activity at 6% salt concentration, which is the optimum salt concentration for *V. salarius* growth (Solanki & Kothari, 2012). The latter experienced a heavy decrease of 88.12% with increase in salinity from 6% to 10% (Table 4).

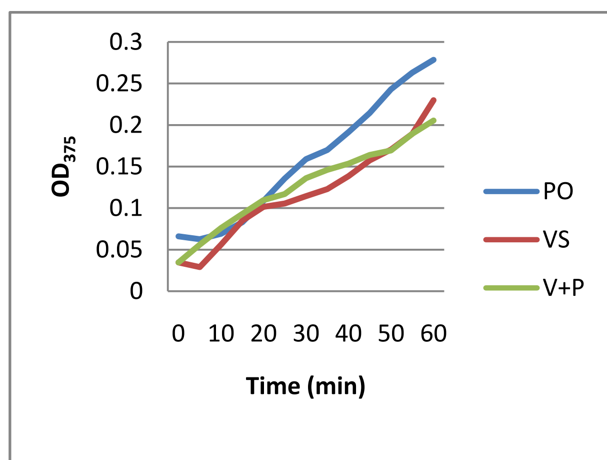

Fig. 1. Comparison of C23O activity at 6% salt concentration.

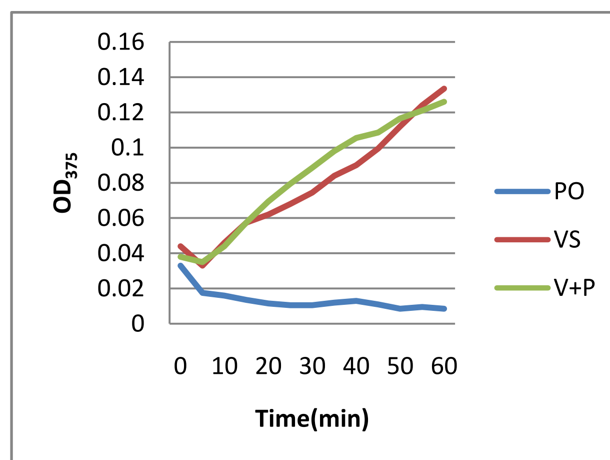

Fig. 2. Comparison of C23O activity at 10% salt concentration.

(PO*:* *P. oleovorans*; VS: *V. salarius*; V+P: mixture of *P. oleovorans* and *V. salarius.*)

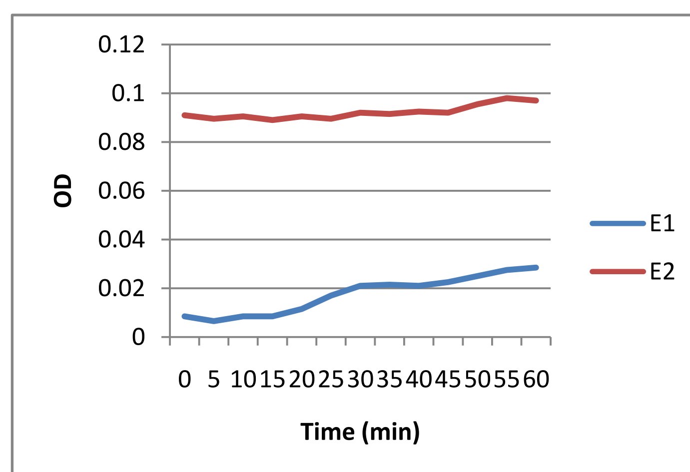

Fig. 3. C23O and chlorocatecho 1,2 dioxygenase activity in *V. salarius* at 15% salt concentration.

E1: catechol 2,3-dioxygenase, E2: chlorocatechol 1,2-dioxygenase.

Table 3. Comparison of C23O and catechol dioxygenase II activity based on maximum absorbance at 375 nm and 260 nm respectively

Enzyme	Salt conc in growth medium (%)	*V. salarius*	*P. oleovorans*	Percent difference	*V. salarius*	Mixed suspension	Percent difference
		OD_{375} (Mean±SD)			OD_{260} (Mean±SD)		
C23O	6	0.230±0.001	0.278±0.002	-20.87**	0.230±0.001	0.205±0.002	10.86**
	10	0.133±0.020	0.033±0.004	75.18*	0.133±0.020	0.126±0.001	5.26
Catechol dioxygenase II	6	0.521±0.028	0.417±0.006	19.96*	0.521±0.028	0.372±0.008	28.60**
	10	0.403±0.005	0.403±0.018	00	0.403±0.005	0.257±0.003	36.23**

**$p<0.01$, *$p<0.05$; % difference shown is for *V. salarius* with respect to either *P. oleovorans* or the mixture

Table 4. Effect of salinity on catechol metabolizing enzymes

Enzyme	Organism	Salt concentration (%) 6	Salt concentration (%) 10	Percent difference	Salt concentration (%) 6	Salt concentration (%) 15	Percent difference
		OD_{375} (Mean±SD)			OD_{260} (Mean±SD)		
C23O	*V. Salarius*	0.230±0.001	0.133±0.020	42.17*	0.230±0.001	0.028±0.000	87.61**
	P. oleovorans	0.278±0.002	0.033±0.004	88.12**	-	-	-
	Mixture	0.205±0.002	0.126±0.001	38.69**	-	-	-
Catechol dioxygenase II	*V. Salarius*	0.521±0.028	0.403±0.005	22.66*	0.521±0.028	0.097±0.001	81.41**
	P. oleovorans	0.417±0.006	0.403±0.018	03.40	-	-	-
	Mixed suspension	0.372±0.008	0.257±0.003	30.98**	-	-	-

**$p<0.01$; *$p<0.05$; '-': *P. oleovorans* could not grow at 15% salt concentration.

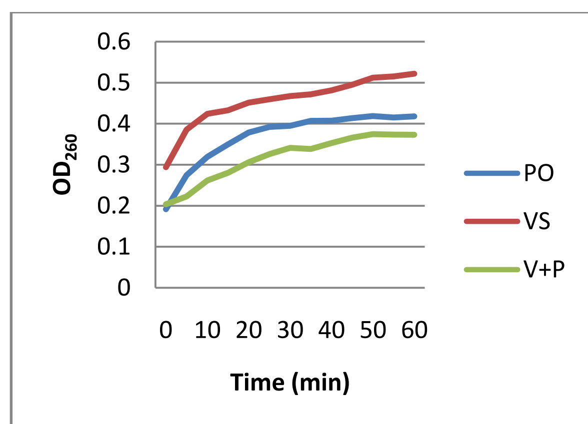

Fig. 4. Comparison of catechol dioxygenase II activity at 6% salt concentration

Chlorocatechol 1,2-dioxygenase activity was detected in both *V. salarius* and *P. oleovorans* grown at 6% and 10% salt concentration (Fig 4-5). *P. oleovorans* grown at 6% salt concentration had a 20% lesser activity than that of *V. salarius*, however chlorocatechol 1,2-dioxygenase activity was identical in both these organisms when grown on 10% salt concentration (Table 3). Activity of this enzyme in *P. oleovorans* was not affected by increase in salinity of the growth medium from 6% to 10%. At both the salt concentrations the mixture showed activity lesser than either of the organisms in pure culture.

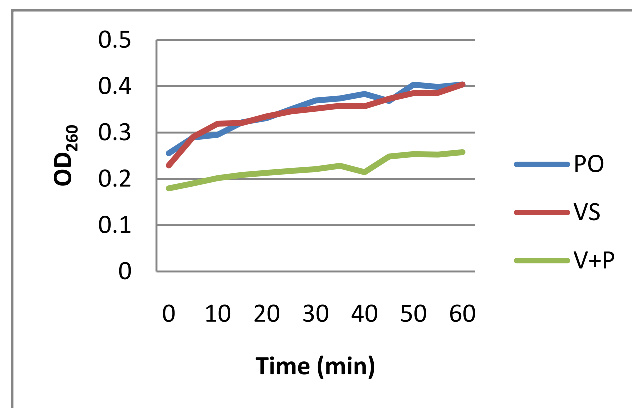

Fig. 5. Comparison of catechol dioxygenase II activity at 10% salt concentration

C23O activity in both the organisms was more susceptible to increase in salinity of the growth medium than chlorocatechol 1,2-dioxygenase activity. Salt concentration has varied effect on HC degradation depending on the type of environment and the type of organisms involved. Higher salt concentrations usually tend to inhibit degradation (Martins & Peixoto, 2012). The ability of variation in salinity to affect the rate of HC degradation appears to be dependent on the natural variation in salinity regime of the sample source (Kerr & Capone, 1988). In this study *V. salarius* was found to possess catechol 2,3-dioxygenase, and chlorocatechol 1,2-dioxygenase activity, when grown at salt concentration ranging from 6-15% (60-150 g/L). Sea water salinity is usually around 35 g/l (Le Borgne et. al., 2008), therefore potential organisms to be employed for marine oil spill bioremediation should be capable of survival and hydrocarbon degradation at salt concentration typical of marine environments.

Hydrocarbon degradation capacity of otherwise efficient organisms can be impaired with increase in salinity, if they are not able to adapt to saline or hypersaline conditions (Margesin & Schinner, 2001). Minai-Tehrani et al.(2006) reported 41% crude oil degradation in soil samples with no added NaCl, while only 12% was observed in samples from the same soil subject to 50 g/l NaCl after 120 days. As a consequence of a "salting out" effect, hydrocarbons are less bioavailable in hypersaline environments than in non-saline ones. Solubility of hydrophobic compounds in water is reduced in presence of soluble salts (Martins & Peixoto, 2012).

3.3. Biofilm formation by V. salarius

After confirming presence of catechol metabolizing enzymes in *V. salarius*, and its comparison with those of *P. oleovorans*, biofilm formation capacity of these two organisms was investigated (compared) in three different media. Results of crystal violet assay (Table 5) indicated that *P. oleovorans* formed better ($p < 0.01$) biofilm than *V. salarius* in BHI, while the latter formed a better ($p < 0.01$) biofilm in minimal medium. Both the organisms showed nearly equal biofilm formation in halophilic nutrient broth, with *P. oleovorans* being slightly better ($p < 0.01$). These results suggest that *V. salarius* can grow in biofilm form with minimal nutrient supply, even if no exogenous supply of organic nutrients is provided. This particular feature of biofilm formation with no fastidious nutrient requirements is attractive with respect to bioremediation of marine oil spills, because using such organisms can reduce the cost behind nutrient augmentation, which often is done during bioremediation operations (Zhu et al., 2004). As scarcity of mineral nutrients is considered as one of the principal forces limiting the biodegradation of polluting petroleum in the sea (Atlas & Bartha, 1998), organisms capable of growth with minimal nutrient requirements can be of special interest. Some organisms apply adhesion in biofilm formation as behavioural strategy to acquire carbon and energy from hydrophobic organic compounds (HOCs) contained in marine aggregates. HOCs are weakly soluble in water, resulting in low bioavailability. *Marinobacter hydrocarbanoclasticus* SP17 was reported for biofilm formation at HOC-water interface resulting in increased bioavailability of HOCs (Grimaud et al., 2012). Physiological and proteomic studies have revealed biofilm formation to be an efficient strategy for colonization of hydrophobic interfaces (Ballihaut, et al., 2004; Vaysse , et al., 2009; Vaysse , et al., 2011). Biofilm-mediated enhanced crude oil degradation in *Pseudomonas* species was reported by Dasgupta et. al. (2013). They reported biofilms formed in presence of crude oil to accumulate higher biomass with greater thickness compared to the biofilm produced in presence of glucose as sole carbon source.

Table 5. Results of assay for detection of biofilm formation

Organism	Media	OD_{655} (Mean ± SD)	Adherence (Degree of Biofilm formation)
P. oleovorans	Minimal broth	0.017 ± 0.000	Weak
	BHI	0.161 ± 0.005	Moderate
	HNB	0.043 ± 0.000	Weak
V. salarius	Minimal broth	0.085 ± 0.001	Weak
	BHI	0.096 ± 0.000	Weak
	HNB	0.037 ± 0.000	Weak

This study has identified *V. salarius* to posses C23O and catechol dioxygenase II activity, comparable to that of *P. oleovorans* at low salinity. Reports on catechol 2,3-dioxygenase from *Pseudomonas* species are available in literature (Murakami et. al., 1998; Cao et. al., 2008). When grown at higher salt concentration, *V. salarius* had better catechol metabolizing capacity. Dihydroxylated intermediates such as catechol and methylcatechols are generated during metabolism of hydrocarbon compounds such as toluene. These intermediates are further converted into those which can enter the TCA cycle. Metabolism of dihydroxylated compounds involves cleavage of the aromatic ring either between (*ortho*-cleavage) or adjacent to (*meta*-cleavage) the hydroxyl groups. In the latter case, aromatic ring cleavage is mediated by an extradiol catechol dioxygenase, commonly designated (methyl) catechol 2,3-dioxygenase (C23O) or metapyrocatechase, which is believed to have a key role in the degradation of aromatic molecules in the environment (Kita et al., 1999). Intradiol cleavage by catechol dioxygenases results in formation of *cis,cis*-muconic acid, which can be detected by measuring absorbance at 260 nm. Extradiol ring cleavage catalyzed by C23O results in formation of 2-hydroxy muconate semialdehyde, whose accumulation was detected in the C23O assay employed in present study at 375 nm. Thus C23O from *V. salarius* can be said to be carrying out extradiol ring cleavage. C23O was found to be the key enzyme responsible for dearomatization of napthelene by *Pseudomonas putida* (Ibrahim, 2003).

Chlorocatechol 1,2-dioxygenase (also called catechol dio-

xygenase II) is reported to possess a broad substrate specificity and relatively low k_{cat} values with catechol and chlorocatechols (Kukor et al.,2007). The broad substrate tolerance of this enzyme is particularly noted with gram-negative organisms such as *Pseudomonas* (Alfreider et. al., 2003). Chlorocatechol 1,2-dioxygenase activity in gram-positive bacterium *Rhodococcus erythropolis* was reported by Maltseva et. al. (1994). Its activity results in formation of chloro-*cis,cis*-muconic acid, whose accumulation was detected by measuring absorbance at 260 nm, in the enzyme assay employed in this study. Further metabolism of it can proceed by a modified β-ketoadipate pathway.

Protocatechuate 3,4-dioxygenase activity was found neither in *V. salarius* nor in *P. oleovorans*. Besides catechol and methylcatechol, protocatechuate is a common ring cleavage substrate found in aromatic catabolic pathways. Protocatechuate 3,4-dioxygenase catalyses intradiol cleavage of protocatechuate to yield β-carboxy-cis,cis-muconic acid.

Among the four halotolerant strains used in this study only *V. salarius* was found to be capable of hydrocarbon degradation. Though *Halomonas shengliensis* has been described as a crude oil utilizing bacterium (Wang et. al., 2007), the *H. shengliensis* strain employed in this study was not found to degrade the hydrocarbon compounds tested. To the best of our awareness, this is the first report of catechol metabolism in *V. salarius*. Identification of novel hydrocarbon degrading organisms and detecting specific enzyme activities in them is important with respect to microbial bioremediation. It also can pave way for realizing the possibility of transgenic exploitation of bacterial degradative genes in higher organisms (Liao et al., 2006). As *V. salarius* is capable of growing at high salt concentration (0.5-16%, with 6% being optimum), alkaline pH (pH range 6-10), hydrocarbon degradation, and also of growth in presence of various metal ions (Solanki & Kothari, 2012), it can be a more attractive candidate for bioremediation over those many organisms capable of hydrocarbon degradation but not able to survive high salinity, alkaline pH, and presence of metal ions. Organisms like *V. salarius* can also serve as a model for multiple stress tolerance in bacteria.

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Note

Meera Panchal and Namrata Srivastava contributed equally to this work.

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