Benzene, Toluene and Xylene Tolerant Marine Bacteria from Seawater of Karachi Coastline

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ABSTRACT

Marine bacteria are highly diverse and have been found to possess a unique system of enzymes that enable them to thrive effectively in a complex mixture of organic and inorganic pollutants present in the marine environment. The microorganisms harnessed from the marine environment are eco-friendly and economical, hence they could be effectively integrated with physico-chemical methods for pollution control. During the present investigation, several bacteria isolated from marine environment of Karachi coast were found Benzene, Toluene and Xylene (BTX) tolerant Gram-positive marine bacteria included, primarily *Bacillus* sp., and Gram-negative bacteria of the genus *Pseudomonas* were identified. The 31% of the bacterial isolates showed tolerance to BTX in NB supplemented with 3 % NaCl, comprised of 59 % Gram-positive and 40 % Gram-negative isolates. In contrast, BTX tolerant isolates in NB without 3 % NaCl were 25%, included 57 % Gram-positive and 43 % Gram-negative bacterial isolates. Among Gram-negative isolates, the isolate CMG556 (*Pseudomonas* sp. accession # DQ410039), tolerated 10-15 % v/v of three isomers of xylene (*m*-, *p*-, *o*-), produced hydrophobic mass in the presence of xylene, showed enhanced protease activity, and produced biosurfactant rhamnolipid. CMG556 is a suitable microorganism for further exploration in bioremediation and industrial applications.

1. Introduction

Karachi is one of the most populous and bustling coastal cities situated along the Arabian Sea. It is a business center with over 8000 industries. Coastal cities worldwide typically discharge industrial effluent and municipal waste into their coastal waters. Oil refineries, oil terminals, and shipping activities are significant contributors of organic and inorganic pollution in the coastal marine environment. It exerts detrimental effects on biodiversity, mangrove habitats, coral reefs, and seaweeds in the marine environment (Thiagarajan *et al*., 2025).

Lower molecular weight oil derivatives, such as components of gasoline BTEX (i.e. benzene, toluene, ethylbenzene and isomers of xylene including *m-, p-, o*-xylene) are potential carcinogens and listed as priority pollutant by the Environmental Protection Agency (EPA) in most parts of the world. The shipment of crude petroleum and refined fuel/gasoline across the oceans is a major cause of BTEX pollution in the marine environment. These mono aromatic hydrocarbons are toxic to living cells. The extent of toxicity is based on their partition coefficient in an equimolar octanol-water system, i.e., the log P value; the smaller the value, the greater the toxicity (Inoue & Horikoshi, 1989). Components of BTEX fall within log P values of 1-4, which are extremely toxic to microorganisms.

Microorganisms from diverse environments have been comprehensively studied for their resistance development against organic solvents, including BTEX (Botton and Parsons, 2006). Marine bacteria

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have recently gained significant attention in this context. Therefore, this study was aimed to screen marine bacteria previously isolated from surface seawater of recreation beaches along the Karachi coast line, for tolerance to various concentrations of monoaromatic hydrocarbons (MAH) benzene, toluene and isomers of xylene, to evaluate the toxic effects of these chemicals on number of Gram-positive and Gram-negative marine bacteria.

2. Materials and Methods

2.1 Bacterial isolates and media

Previously isolated 71 marine bacterial strains of Centre for Molecular Genetics, University of Karachi (CMG) from various recreational beaches, seaweeds, harbor Kemari, and offshore seawater samples along the Karachi coastline of the Arabian Sea were selected for this study (Akhtar *et al*., 2013). These strains were grown on Luria-Bertani (LB) medium (Sambrook *et al.,* 1989) and stored at 37 ℃. Strains were checked for purity by Gram-staining. For investigations related to resistance, NB was used. For growth profile, synthetic seawater-agar plates were used; synthetic sea water (SSW), composed of 393.5 mM NaCl, 24.6 mM $MgCl_2.6H_2O$, 7.5 mM $CaCl_2$, 8.9 mM KCl, 0.8 mM KBr, 0.42 mM H_3BO_3, 0.09 mM $SrCl_2$. All chemicals used were of analytical grade from Sigma or Merck.

2.2 Screening of marine bacteria for tolerance to monoaromatic hydrocarbons (MAH)

Tolerance against MAH, such as benzene, toluene and three isomers of xylene; *m*-, *p*-, and *o*-xylene (BTX) was investigated in NB with and without amendment of 3 % NaCl. Tolerance was investigated for growth in the presence of 1 to 5% (v/v) concentrations of BTX. Growth was measured by optical density (OD) using a CaSpec Model-300 spectrophotometer at 600 nm, as described by Copland (1994). The average of three measurements of OD_{600nm} was recorded and isolates which showed average of three measurements optical density (OD_{600nm} > 0.1) were considered as tolerant, while those with optical density OD_{600nm} < 0.1 but > 0.05 were considered as having tolerance potential (+), and isolates with average OD_{600nm} <0.05 were considered sensitive. Percentages of the three classes of isolates were calculated.

2.3 Growth profile of CMG556 (Pseudomonas sp.) in the presence of three isomers of Xylene

Loop full culture from nutrient agar (NA) plates flooded with *p-, m*-, or *o*-xylene was used for the preparation of pre-culture in 250 mL airtight screw cap bottles in a biphasic (polar-aqueous) system. The experiment was set up in three replicates of the test, as well as positive and negative controls. Overnight culture (1 mL) was seeded in 250 mL capacity flasks containing 100 mL of culture medium in the test and positive controls. Test flasks were supplemented with 10 % (v/v) xylene isomers (*m*-, *p*-, and *o*-), while the positive control was without xylene isomers. Negative controls were performed with each of the three xylene isomers, without culture inoculum.

To evaluate the effect of 3 % NaCl on growth profile in presence of 10 % xylene isomers, CMG556 was grown in two set of flasks, one containing NB supplemented with 3 % (w/v) NaCl and the other set containing NB without NaCl.

To determine the degradation potential of CMG556, three sets of SSW-agar plates were prepared. One with carbohydrate (2% gluconate) the positive control, second without carbohydrate supplemented with xylene (200 µL) on the lid of inverted plates served as test plates which contained xylene as sole source of carbon and energy in the form of vapors, and the third set without any carbohydrate and aromatic source i.e. blank plates/negative control. All these plates were spread with 200 µL of fresh overnight culture, sealed with Para film to avoid loss of xylene in the test plates. Positive control was used for growth comparison with the growth on test plates, whereas the negative control was set up to check for autolytic growth of the culture.

All experiments were performed in triplicate. All culture bottles were made airtight with screw caps, inner lined with a Teflon seal, and were agitated at 120 rpm in a shaking incubator at 37 ℃. Samples were collected every 24 h for growth profile, viability, and quantitative protein estimation. The average of the three readings, noted spectrophotometrically at 600nm, was used for graphical representation in Excel version 8.1. Viability of CMG556 in test flasks was checked by streaking on NA plates (Table 1).

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Table 1. Geno- and ecotoxicity (LC50 mg l-1)1) of BTEX

Compound	Carcinogen	LC_{50} Cancer magister	LC_{50} Paleo-monetes pugio	LC_{50} Marone saxitilis
Benzene	+	108	27	5.8
Toluene	-	28	9.5	7.
o-Xylene	-	12	3.7	9.2
m-Xylene	-	6	1.3	1
p-Xylene	-	2	0.86	2
Ethylbenzene	-	13	0.49	4.3
1,2,4-Trimethyl benzene	-	5	5.4	

2.4 Protein assay

Quantitative protein estimations of periodically collected samples were performed using the Bradford (1976) assay, calibrated with a standard curve of bovine serum albumin.

2.5 Protease activity

Marine bacterial isolates were subjected to grow on skim milk agar plates (Nadeem *et al.,* 2006) for enzyme protease production, both in the presence and absence of the organic solvent xylene. The inoculated plated were incubated at 37 ^{o}C. The enzyme protease produces clear zones around bacterial inoculums on skim milk-containing agar plates by cleaving the peptide bonds in the milk proteins. The size of the clear zones was measured in mm by measuring the diameter of the zone around the inoculums after 24 and 48 h.

2.6 Electron microscopy

Samples of cells grown in the presence and absence of xylene, collected on the 3rd and 7th day of incubation, were prepared for scanning and transmission electron microscopy according to Cruden *et al*. 1992. Specimens were analyzed for at least 100 s at a voltage of 15 kV in a JEOL JEM-1200EX transmission electron microscope.

3. Results

3.1 Tolerance to monoaromatic hydrocarbons

Analysis of the data revealed that 25 % of the bacterial strains grown in a biphasic system of variable concentrations of BTEX and NB without NaCl, whereas 31 % of the bacterial strains showed tolerance to variable concentrations of BTEX in a biphasic aqueous-organic system of NB with 3 % NaCl. These tolerant strains were further classified into 71.4 % Gram-positive and 28.6 % Gram-negative strains (Table 4).

Table 2. The carcinogenity and the ecotoxicity towards different marine organisms.

Compound	Mole weight g mole^{-1}	Density g ml^{-1}	Boiling point °C	Water solubility mg l^{-1}	Vapor pressure mmHg	Log K_{ow}
Benzene	78	0.88	80.1	1780	76	2.13
Toluene	92	0.87	110.8	535	22	2.69
o-Xylene	106	0.88	144.4	175	5	2.77
m-Xylene	106	0.86	139	135	6	3.20
p-Xylene	106	0.86	138.4	198	6.5	3.15
Ethyl-benzene	106	0.87	136.2	152	7	3.15

Table-3 Solvent (BTX) tolerance in marine bacteria in presence of 3 % (w/v) NaCl

Compound	Aerobic conditions	Denitrifying conditions	Sulfate-reducing conditions	Iron-reducing conditions	Methanogenic conditions
Benzene	++	-	+	-	+
Toluene	++	++	+	+	+
m-Xylene	++	++	+	+	+
p-Xylene	++	+	+		+
o-Xylene	++	+/-[1)]	-	-	+/-
Ethyl-benzene	++	+/-		-	+/-
1,2,4-Trimethyl-benzene	++				+/-

Among the 25 % of BTX-tolerant isolates, 4.2 %, 8.4 %, 4.2 %, 1.4 %, and 7 % showed tolerance to benzene, toluene, and the three isomers of xylene, i.e., *m*-, *p*-, and *o*- xylene, respectively (Table 5). Likewise, 31 % of the BTX tolerant strains included 8.4 %, 2.8 %, 5.6 %, 4.2 %, 9.8 % respectively, demonstrated tolerance to benzene, toluene, and three isomers *m*-, *p*-, and *o*- of xylene respectively. The strains that showed tolerance to BTX in NB supplemented with 3 % NaCl comprised 59 % Gram-positive and 40 % Gram-negative strains, whereas tolerant strains in NB without 3 % NaCl included 57 % Gram-positive and 43 % Gram-negative strains.

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Table 4. Solvent (BTX) tolerance in isolated marine bacteria in presence of 3 % (w/v) NaCl

Compounds and conc. used	Gram stain	Benzene 0.1% (v/v)	Toluene 0.1% (v/v)	*m*-xylene 1% (v/v)	*p*-xylene 1% (v/v)	*o*-xylene 1% (v/v)
Strain code						
CMG504	*n.i*	-	-	+	-	-
CMG506	*P. stutzeri*	-	-	-	-	+
CMG521	*Xanthomonas maltophilia*	1.317	-	-	-	-
CMG523	*Xanthomonas maltophilia*	-	-	0.470	-	-
CMG524	*P. aeruginosa*	-	-	+	-	0.518
CMG529	*B. polymyxa*	-	-	0.431	-	0.311
CMG530	*B. subtilis*	-	-	-	-	0.582
CMG532	*B. subtilis*	-	-	-	-	0.746
CMG533	*B. subtilis*	1.021	0.184	0.225	-	0.175
CMG535	*Enterobacter cloacae*	1.170	+	-	+	-
CMG536	*B. subtilis*	0.642	0.848	+	-	-
CMG538	*P. pseudomonallei*	-	-	-	-	0.229
CMG539	*P. sp.*	-	+	+	+	+
CMG540	*P. aeruginosa*	+	+	+	+	+
CMG543	*B. pumilus*	+	-	+	-	+
CMG544	*B. pumilus*	+	-	-	+	+
CMG556	*P. sp.**	-	-	1.780	1.675	1.59
CMG557	*B. thruringensis*	+	+	+	-	-
CMG558	*B. thruringensis*	-	-	-	0.164	-
CMG559	*B. licheniformis*	0.321	+	+	-	-
CMG560	*B. polymyxa*	0.37	+	-	-	-
CMG561	*B. polymyxa*	-	-	-	0.284	-

Key: - Growth absent
+ Potential to resist, optical density less than 0.1
Numerals represent optical density measured at 600nm after 48 h
*Identified by 16S rRNA gene sequence.

Table 5. BTX Tolerance of Marine isolates in absence of 3% NaCl in NB

Compound and conc. used	Gram stain	Benzene 0.1% (v/v)	Toluene 0.1% (v/v)	*m*-xylene 1% (v/v)	*p*-xylene 1% (v/v)	*o*-xylene 1% (v/v)
Strain code						
CMG504	*n.i.*	-	-	+	-	-
CMG507	*B.pumilus*	-	-	+	-	-
CMG521	*Xanthomonas maltophilia*	-	1.317	-	-	-
CMG523	*Xanthomonas maltophilia*	-	-	-	+	-
CMG524	*P.aeruginosa*	-	-	0.157	-	0.172
CMG527	*n.i.*	-	+	+	+	+
CMG528	*B.cereus**	-	-	-	-	+
CMG529	*B.polymyxa*	-	-	+	-	-
CMG530	*B.subtilis*	-	-	-	-	0.13
CMG532	*B.subtilis*	-	1.12	-	-	0.252
CMG533	*B.subtilis*	0.118	0.1	0.155	-	0.125
CMG535	*Enterobacter cloacae*	0.5	0.89	-	+	-
CMG536	*B.subtilis*	0.396	0.396	+	-	-
CMG540	*P.aeruginosa*	-	+	-	+	+
CMG548	*n.i*	-	-	+	-	+
CMG549	*B.polymyxa*	-	-	+	-	+
CMG554	*B.subtilis*	+	-	-	-	-
CMG556	*P.sp.**	-	1.2	1.56	1.85	1.79
CMG557	*B.thruingensis*	-	-	-	+	-
CMG559	*B.licheniformis**	+	+	-	+	+
CMG560	*B.polymyxa*		+	+	-	+

Key: - Growth absent
+ Potential to resist, optical density (OD600nm) less than 0.1
Numerals represent optical density (OD600nm) after 48 h
*Identified by 16S rRNA gene sequence.

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Two strains, CMG533 and CMG556, were found to be competitive and were selected for further study. The strain CMG533 showed tolerance to 1 % v/v of benzene, toluene and two isomers of xylene i.e. *m*- and *o*-xylene; whereas CMG556 showed tolerance against 10 % v/v of three isomers of xylene (*m*-, *p*-, *o*-xylene) in aqueous-organic two-phase system of NB with and without 3 % NaCl.

CMG556 showed growth in plates overlaid with three isomers of xylene (Figure 1) within 48 h of incubation, whereas CMG533 showed growth after evaporation of the xylene isomers in more than 7 days. CMG556 showed the deposition of a visible, white, hydrophobic mass at the phase boundary of the aqueous–organic layer. This white, hydrophobic mass was absent in the positive control (without xylene) of CMG556 (Figure 3). CMG533 was unable to produce any hydrophobic mass in the test as well as in the positive control. Streaking during screening experiments revealed viable colonies of CMG556 with yellowish-brown pigmented centers on extended incubation of more than 30 days, while CMG533 turned into a non-viable culture after 7 days of incubation. The yellowish-brown center of the colonies of CMG556 indicated the enzymatic incorporation of oxygen into the MAH, showing the degradation potential of the isolate. Color indicated the oxidative production of hydroxymuconic semialdehyde, which has been known to be produced by enzymatic oxidation of MAH, similar to oxidation of phenol and BTX (Benzene, Toluene & Xylene). Therefore, CMG556 was selected for further characterization.

Further analysis of the data revealed that the tolerant isolates against variable concentrations of BTX belong to Kemari, Green seaweed, Paradise Point, and seawater from the open sea. Whereas sensitive isolates were obtained from Clifton Beach, Cape Monze, and red sea weeds.

3.2 Effect of 3 % NaCl on the tolerance of BTX

Tolerance to BTX in marine bacterial isolates was found to be inhibited in the presence of 3 % NaCl in Gram-positive isolates and increased in Gram-negative isolates. Variability was observed in the percentages of BTX-tolerant Gram-positive and Gram-negative isolates. In NB without 3 % NaCl, (Table 5) 72 % of the isolates were Gram-positive, and 28 % were Gram-negative. However, in NB with 3 % NaCl (Table 4) 57 % isolates were Gram-positive and 43 % were Gram-negative

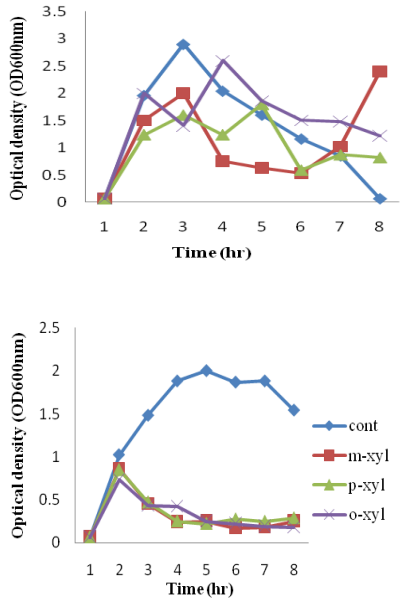

Fig. 1. Growth profile of CMG556 (*Pseudomonas* sp.) in presence of 10% v/v three isomers of xylene in A. unmodified nutrient B. modified nutrient broth with

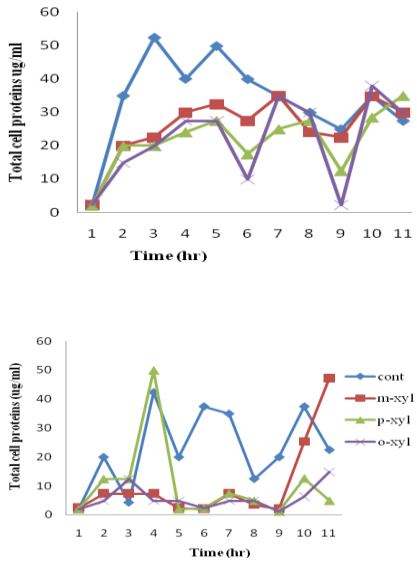

Fig. 2. Estimation of total cell proteins of CMG556 (*Pseudomonas* sp.) in presence of three isomers of xylene in A. unmodified nutrient broth B. modified nutrient broth

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3.3 Total cell protein estimation

Estimation of total cell protein contents of CMG556 revealed decrease in presence of 10 % v/v of three isomers of xylene (*m, p, o*-xylene) with and without 3 % w/v NaCl as compared to respective controls. It indicates that cell death under toxic stress of xylene isomers is further enhanced in the presence of NaCl. The amount of cell proteins in the presence of xylene was inconsistent compared to the control due to the formation of a hydrophobic mass that hindered sampling, resulting in inconsistent protein estimation.

3.4 Protease activity

CMG556 demonstrated protease activity by producing haloes around the inoculums on skim milk agar plates. When inoculums were pre-grown in the presence of xylene or when plates were overlaid with xylene, the halos were increased in size (Figure 2). The protease enzyme acts by cleaving the bonds in the milk proteins, thus leaving clear halos in skim milk plates around the inoculum. It shows increased protease activity of CMG556 in presence of xylene.

3.5 Transmission electron microscopy of CMG556

Transmission electron microscopy (TEM) of CMG556 grown with xylene revealed the toxic effect of xylene isomers on cellular morphology and physiology of the isolates (Figure 4). TEM showed arrested cell division, abnormal growth, and particles of unknown material under the toxic effect of isomers of xylene.

4. Discussion

The marine environment is an extreme habitat inhabited by a highly diverse microbial flora. Adaptation of microorganisms is attributed to their metabolisms and enzyme production, enabling them to thrive in extreme environments. In the present study, potentially active Gram-positive and Gram-negative heterotrophic marine bacteria with tolerance to MAH were found. The occurrence of a considerable proportion of Gram-positive and Gram-negative bacteria tolerant to MAH is attributed to certain anthropogenic and catastrophic activities that release oil and oil-based organic and inorganic compounds into coastal waters (Shoeb *et al.,* 2015). This has increased the selection pressure in favor of bacteria having tolerance against pollutants.

Solvent tolerant bacteria have been isolated from soil or deep sea and identified belong to genera Pseudomonas (Inoue and Horikoshi, 1989; Ramos *et al.,* 1995) and *Rhodococcus* (Paje *et al.,* 1997). So far, several aromatic hydrocarbon-tolerant bacteria have been isolated from the environment, including strains of *P. putida* (Shima *et al.,* 1991) and a mutant of Escherichia coli K-12 (Aono *et al.,* 1991) in the presence of toluene and other alkylbenzenes (Cruden *et al.,* 1992). Further isolates of *A. calcoaceticus*, *P. aeruginosa*, *Pseudomonas* PG-1 have been studied for solvent tolerance (De Smet *et al.,* 1983, Tan & Kong, 2000). Currently, organisms that simultaneously resist and degrade all the different components of BTEX are preferred over those that degrade only one or some of the BTEX compounds (Singh and Celin, 2010). The co-occurrence of tolerance against a variety of toxicants, such as heavy metals and xenobiotics, has been reported, e.g., bacteria resistant to Hg and various xenobiotics (Jaysankar *et al.,* 2006). Tributyltin (TBT) and heavy metals have also been reported to exhibit this co-occurrence (Pain and Cooney, 1998).

Among the various Gram-positive and Gram-negative BTX-tolerant marine isolates in this study, CMG556 belonged to the most vulnerable solvent-tolerant genus of *Pseudomonas* and gained attention for further study. In an earlier study, the number of indigenous marine bacteria, including CMG556, has been reported to exhibit antibiotic and heavy metal resistance, as well as NaCl tolerance (Akhtar *et al.,* 2013). In the present study, CMG556 produced a visible hydrophobic mass at the phase boundary of the aqueous-organic biphasic system and propagated underneath the xylene layer. This observation was in agreement with Al-Tahhan *et al.,* 2000. This hydrophobic mass was found xylene specific. It was found to be absent when CMG556 was grown in the presence of diesel, naphthalene, hexane, and phenol. Accumulated particulate material and bacterial cells without cytoplasmic membranes (protoplasm) were observed in the TEM of xylene-grown CMG556, which agrees with the findings of Kong and Tan (2000) and Holden *et al.* 2002. They have explained

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that these outer cell membrane components, released under the toxic stress of an organic solvent, emulsify the compound and make it available for producing bacteria to act upon for biodegradation. In several studies (Sowbaranika, et al., 2023),, such extra-cellular excreted compounds have been identified as biosurfactant. Biosurfactants are surface-active compounds allow growth on hydrocarbons and provide biocide protection to the producing organism (Bashir *et al.,* 2025). Based on these observations CMG556 was examined for biosurfactant production by documented assays for biosurfactants production (Bashir *et al.,* 2025) and found to produce Rhamnolipid biosurfactant.

Components of BTX are extremely toxic to microorganisms. Most of the documented research showed organic solvent tolerance in Gram-negative bacteria. It is presumed that the outer cytoplasmic membrane of Gram-negative bacteria plays a vital role in protection against the toxicity of organic solvents, whereas Gram-positive bacteria are protected by a single cytoplasmic membrane. This might be the reason why there have been few reports of Gram-positive bacteria tolerant to organic solvents. Those, which are reported include species of *Bacillus*, *Rhodococcus*, and *Enterococcus* (Isken and de Bont, 1998; Peschel *et al*., 2001). Recently, a Gram-positive strain of *Staphylococcus haemolyticus*, tolerant to 100 % toluene, benzene, and *p*-xylene, was isolated from the oil fly larval gut in an overlay plate assay (Nielsen *et al*., 2005). This strain exhibited an antagonistic response, characterized by increased membrane fluidity upon exposure to solvents, through changes in fatty acid composition. This mechanism is commonly found in Gram-negative bacteria which reduces membrane fluidity (Nielsen *et al*., 2005). The mechanism of solvent tolerance in Gram-negative bacteria isolated from soil has been comprehensively studied; however, the mechanism by which marine bacteria account for tolerance to organic solvents remains to be explored. It has been assumed that Gram-negative bacteria isolated from marine environment may have similar mechanisms of tolerance that have been studied in Gram-negative bacteria isolated from soil (Ramos, *et al*., 2002).

An organic solvent-tolerant *Pseudomonas* sp. is important for industrial applications and environmental bioremediation. Such organisms have been reported for applications in solvent bioremediation and biotransformation in non-aqueous media (Isken and deBont, 1998; Pieper and Reineke, 2000; Sardessai and Bhosle, 2004). Some, but not all, organic solvent-tolerant bacteria have been found to be naturally equipped with the ability to produce solvent-stable proteases and lipases (Gupta and Khare, 2006; Rahman *et al.,* 2005, which can help compromise contaminated environments with organic pollutants. Ogino and Ishikawa, 2001, have reported microbes with natural ability to produce protease and lipase in presence of organic solvents. CMG556 (*Pseudomonas* sp.) showed enhanced activity of protease production in presence of three isomers of xylene and 3 % NaCl. The proposed tolerance mechanism of CMG556 involves biosurfactant production and protease activity, although the detailed mechanism is yet to be explored.

This study would be important for introducing a considerable proportion of Gram-positive bacteria, mainly *Bacillus* sp., and Gram-negative bacteria, mainly *Pseudomonas* sp., from the regional sea surface water of recreation beaches. These bacteria were variably tolerant to BTX components. Among the list, CMG556 (*Pseudomonas* sp.) was found to be unique in that it can tolerate and grow in the presence of saturated concentrations (>10 v/v) of three isomers of xylene and is grouped among solvent-tolerant extremophiles. CMG556 possesses the inherent capability to produce biosurfactant and protease enzyme in the presence of xylene. CMG556 is a promising candidate for optimization in industrial applications and environmental bioremediation plans.

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