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Phenol tolerance of bacteria- a case of spontaneous or adaptive mutation?

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Abstract

Origin of mutation is a long lasting dilemma that often haunts researchers at their study. The same query comes up at our bacterial phenol degradation study. Phenol that is one of the most toxic, carcinogenic chemicals can be tolerated by some bacteria those can not only tolerate but also use phenol as substrate by enzymatic degradation. However, like other toxic materials, phenol also exhibits substrate inhibition at higher concentration. Primarily to find out the substrate inhibition, we isolated and identified six phenol degrading bacteria (GSI-1 to 6) (*Streptococcus urinalis*, *Pseudomonas* sp., *Bacillus* sp., *Staphylococcus epidermidis*, *Rhodococcus* sp. and *Micrococcusluteus*) from polluted waters of West Bengal, India, and studied their tolerance level at different phenol concentrations (200 mg l^{-1} to 1500 mg l^{-1}); from the study 1200 mg l^{-1} phenol concentration was defined as inhibitory concentration. The growth rate study up to the inhibitory phenol concentration (1200 mg l^{-1}) showed higher growth rate of the isolates at lower phenol concentrations (200 - 600 mg l^{-1}) defining substrate inhibition. However, to our surprise in the further study of phenol degradation at that inhibitory concentration, all of these previously acclimatized bacteria were able to nearly completely degrade the 1200 mg l^{-1} phenol concentration- presumably due to mutation. But question arises as we obtained this same trend for phylogenitically distantly located bacterial strains- that the origin of this mutation is spontaneous or adaptive attributed by the phenol to the organisms to tolerate the inhibitory phenol concentrations.

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Introduction

Phenol is one of the most common toxic organic compounds produced naturally as well as artificially and causes environmental pollution (Chung *et al.*, 1998; Wei *et al.*, 2008). Even at low concentration phenol is stable with its toxic, mutagenic property (Autenrieth *et al.*, 1991), and also cause undesirable chemical changes to soil and water(Przybulewska*et al.*, 2005). Thus, it was regarded as a priority contaminant by the U.S. Environmental Protection Agency (Keith and Telliand 1979; Sung *et al.*, 2000; Singh *et al.*, 2009). Phenol is present in waste water from manufacturing industries such as refineries (6-500 mg l^{-1}), cooking operation (28-3900 mg l^{-1}), coal processing (9-6,800 mg l^{-1}) etc. (Busca *et al.*, 2008). Biological treatment by utilizing different types of microbes has proved to be the most effective and economical approaches for the removal of organic water pollutants like phenol. Phenol and phenolic compound are toxic, even as low as 0.05% concentrations, to most microorganisms (Kahru*et al.*, 2002). They can inhibit the growth of the microbial strains, and hence is used as an antimicrobial agent. However, there are microbes those are capable of assimilating phenol as a sole source of carbon and energy (Przybulewska*et al.*, 2005). Thus the biotechnological method of degrading these toxic organic pollutants is based on the capability of those microorganisms to utilize phenolic compounds.

It is previously reported that the phenol-degrading microorganisms display substrate inhibition at higher phenol concentrations (Banerjee and Ghoshal, 2010). At the same time phenol degrading bacteria can use phenol as their carbon source. Thus there are two functions of phenol that are mutually contradicting. Thus studies should be performed to know whether phenol is at all a substrate inhibitor for phenol degrading bacteria – the knowledge which may be important for bio-remedial application of pollutant degrading bacteria. In the current study we investigate the existence of substrate inhibition property of phenol for phenol degrading bacteria by an easier way.We tally the growth rate of bacteria with their capacity to tolerate the inhibitory phenol concentration. As a part of the study, we isolated four bacteria from two regions of West Bengal, India containing petroleum and leather waste *viz.* Haldia, and Calcutta Leather Complex at Bantala in east Kolkata, India, and reported their phylogenetic positions based on the 16S rDNA sequence analysis and biochemical testing. The isolated bacteria were further investigated to find out their growth rate at different phenol concentration as well as their degradation nature at those concentrations, with the comparison of the two types of data.

Materials and methods

Study sites

Phenol degrading bacteria were collected from different polluted sites, viz. Haldia Petro Refinery Waste Canal and Bantala Leather Complex Waste Canal, West Bengal, India during November 2014 to December 2014. Haldia is a small industrial city of West Bengal, India with petrochemical industries, located at125 km south-west of Kolkata and 50 km from the Bay of Bengal at the confluence of three rivers Hooghly, Haldi and Rupnarayan. The rivers receive effluent from the industries, thus the West Bengal Pollution Control Board detected presence of phenol in water, however, only up to 1.6 mg l^{-1} - that implies a regular treatment by the industry. However, the treatments are often chemical treatments those again bring some additional chemicals to the water flow. On the other hand, Calcutta Leather Complex is a 4.5 square kilometre stretched industrial complex at Bantala in east Kolkata, India, that very often use tannin, a poly phenolic compound for lather processing. An effective effluent treatment system for proper disposal of sludge is still lacking in this complex. The effluent often mixes with the neighbouring water canals which are connected with local rivers those are intern connected with Bay of Bengal, polluting the water courses.

Four samples were collected from each sampling site during morning hours (8:00 hr-10:00 hr), thoroughly mixed for heterogeneity reduction. Top layers (0–14 cm) of bottom sediment were collected with the aid of an Ekman dredge (capture area: 52 x 26 cm^2); water

samples were taken from 0.5 m depth. One litre of water and about 1 kg sediment samples were collected from each sampling spot in sterile containers and immediately stored in ice-box and brought to laboratory as soon as possible.

Isolation of bacteria by enrichment method

Ten grams of each sediment sample was homogenized in sterile 0.85% saline solution. One hundred microlitres of the 10% (w/v) suspension, water sample, and their dilutions (10-fold dilutions in 0.85% saline) were added in 100 ml minimal medium (MM) ; (MM: KH_2PO_4 2.75 g l^{-1}; K_2HPO_4 2.25 g l^{-1}; $(NH_4)_2SO_4$ 1 g l^{-1}; NaCl 0.10 g l^{-1}; $CaCl_2$ 0.01 g l^{-1}; $MgCl_2 .6H_2O$ 0.20 g l^{-1}; $FeCl_3 .6H_2O$ 0.02 g l^{-1}; pH 7.5). Phenol at the rate 100 mg l^{-1} was used as sole source of carbon in the medium. The enrichment broths were incubated in 250 ml flask at 30°C on rotary shaking incubator at 120 rpm for a week. A volume of 1 ml of the enriched media was plated onto MM supplemented with 15% agar and 200 mg l^{-1} phenol, and again incubated at 30°C. Colonies with different morphologies were re-streaked on Reasoner's 2A (R2A) agar for pure culture isolation supplemented with 200 mg l^{-1} of phenol.

Initial screening of bacterial strains by quantitative phenol degradation study To screen the isolates for higher phenol degrading ability, one loop-full of overnight culture was inoculated in test tubes containing 5 ml of MM medium supplemented with phenol at varying concentration ranging from 400 to 1500 mg l^{-1} and incubated at 30°C under shaking at 120 rpm. At an interval of 6 hours, aliquots were pipetted out, centrifuged at 10,000×g for 10 min, and 4-amino antipyrine method was adapted to measure the unused amount of phenol in MM media by the bacterial isolates (Yang and Humphrey, 1975). In brief, samples were added with 0.05 N sodium bicarbonate and 0.6% (w/v) amino antipyrine solution, followed by 2.4% potassium ferric cyanide solution. After an incubation of 15 minutes the colour were measured at 510 nm using UV-visible spectrophotometer (Hitachi U-2900). Autoclaved uninoculated medium served as control.

Identification of isolates

Six phenol degrading bacterial isolates with prominent phenol degradation property were characterized on the basis of morphological such as Gram staining and biochemical characteristics such as cytochrome oxidase, catalase, oxygen requirements, and acid production from sugars and sugar alcohols like glucose, arabinose, cellobiose, fructose, galactose, inulin, mannitol, mannose, sorbitol, sucrose and trehalose following standard protocols (Maitra*et al.*, 2015). Strains were further identified by PCR amplification and sequencing of 16S rDNA. Here bacterial genomic DNA was isolated by phenol chloroform DNA extraction technique from a freshly growing culture on TSA, and the gene encoding the 16S rRNA was amplified by the polymerase chain reaction (PCR). The PCR reaction mix was set up as per Maitra*et al.*(2015) using 0.2 mM bacterial universal primers 8F (5´-AGAGTTTGATCCTGGCTCAG-3´) and U1492R (5´-GGTTACC TTGTTACGACTT-3´), dNTPs at concentration 10 mM each, 1.5 mM of $MgCl_2$, 10× PCR buffer and 0.5 U of proof-read Taq DNA polymerase (Sigma), following cycle condition of 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec, extension at 72°C for 2 min and a final extension at 72°C for 5 min. The PCR products were purified from agarose gel with the NucleoSpin Gel and PCR Clean up kit (Macherey-Nagel) and sequenced following Sanger sequencing method. The sequence chromatograms were trimmed by Sequence Scanner v1.0 software (Applied Biosystems, Inc., USA), checked for chimera with Bellerophon (http://compbio.anu.edu.au/bellerophon/bellerophon.pl), forward and reverse sequences were aligned with Codon Code Aligner to make contig (CodonCode Corporation, US), contig sequences were blasted in GenBank, Ribosomal Database Project (RDP), Greengenes, and compared with the type strain sequences and the closest match of phylogenetic affiliation was used to assign them to the specific taxonomic groups.

Determination of growth rate of bacteria

250 ml conical flask containing 200 ml of MM media with phenol as sole carbon source was used for this purpose. The test bacteria (GSI-1 to 6) were grown overnight in tryptic soy broth (TSB), and followed by growth were spread on to TSA (Tryptic Soya Agar). From these plates, bacteria were washed in normal saline (0.85%) by repeated centrifugation, and inoculated at a concentration of 9.18 x 10^9 - 1.04 x 10^{10} CFU (set by dissolving bacteria at 0.85% saline to achieve transmittance 10 NTU. The flasks were incubated in a shaker (120 rpm) at 37°C. The initial concentration of phenol was maintained in the range of 200–1200 mg l^{-1}. Samples were collected at an interval of 12 hours for cell concentration measurement. The specific growth rate (μ; h^{-1}) of cells in the batch culture was determined at the exponential phase of the growth curve using the logarithm of bacterial population determined from absorbance unit at 600 nm (OD).

Degradation study at inhibitory phenol concentration

The experiment was set up as the previously described growth rate study experiment. 250 ml conical flask containing initial phenol concentration 1200 mgl^{-1} as sole carbon source in 200 ml of MM media was inoculated with test bacteria. The acclimatized test bacteria those were previously used in growth rate experiment were further grown on TSA and TSB to obtain the desirable concentration before inoculation. At an interval of 6 hours, aliquots were pipetted out, and divided into two factions. One fraction was centrifuged at 10,000 × g for 10 min, and 4-amino antipyrine method was adapted to measure the remaining phenol in the media. The other fraction was studied spectrophotometrically for bacterial growth at 600 nm. Experiments were carried out in cotton plugged flask so that there is continuous exchange of atmospheric oxygen in the system, and autoclaved uninoculated medium served as control.

Results

Isolation of phenol degrading bacteria

A total of 23 strains having different colony morphology on MM agar media supplemented with 200 mg l^{-1} of phenol as sole source of carbon were isolated from sediment and water sources.

Since phenol is soluble in water, no halo zone indicating degradation of phenol was present. Instead all colonies were picked up just by viewing the differences in morphology (Fig. 1).

Table 1. Phenol degradation capacity of the selected bacterial isolates.

Strain	Phenol concentrations							
	200 mg l^{-1}	400 mg l^{-1}	600 mg l^{-1}	800 mg l^{-1}	1000 mg l^{-1}	1200 mg l^{-1}	1400 mg l^{-1}	1500 mg l^{-1}
GSI-1	+++	+++	+++	+++	+	+	-	-
GSI-2	+++	+++	+++	+	+	Very low	-	-
GSI-3	+++	+++	+++	++	+	+	-	-
GSI-4	+++	+++	+++	+	+	+ (?)	-	-
GSI-5	+++	+++	+++	+	+	Very low	-	-
GSI-6	+++	+++	+++	++	+	+	-	-

+, growth; -, no growth; + (?), doubt about growth.

Initial screening of bacterial strains by quantitative phenol degradation study

Initial degradation study revealed an extended ability of the isolated bacteria to tolerate phenol concentration as high as 600 mg l^{-1}. However, only six isolates exhibited ability to tolerate as high as 800 mg l^{-1} initial phenol concentration. For all these six isolates, a profuse growth was obtained at lower concentrations (200-600 mg l^{-1}), and beyond 800 mg l^{-1} thegrowth was lesser, and there was very little growth at 1200 mg l^{-1} initial phenol concentration. Above 1200 mg l^{-1} (i.e. at 1400 and 1500 mg l^{-1} initial phenol concentration) no visible growth was found for all these six isolates, and thus we took the 1200

mg l^{-1} phenol concentration as the threshold inhibitory phenol concentration for all the six strains (Table 1).

Identification of isolates after quantitative phenol degradation study

Through the initial screening in MM containing different concentration of phenol, six potent strains (having the potency of degrading 800 mg l^{-1} phenol or more concentrations) (Table 1) were selected for identification and further study, and were renamed for garbage sediment isolates or GSI-1 to 6.

Table 2. Biochemical and physiological characteristics of the selected phenol degrading strains.

	GSI-1	GSI-2	GSI-3	GSI-4	GSI-5	GSI-6
Gram's Reaction	+ve	+ve	+ve	+ve	+ve	+ve
Cell Shape	Coccid	Rod	Rod	Coccid	Short rod	Cocci in clusters
Catalase	-	+	+	+	+	+
Oxidase	-	+	-	-	-	+
O_2 requirement	FAn	A	A	FAn	A	A
Glucose	+	+	-	+	+	+
Mannose	-	-	+	+	-	+
Galactose	-	-	+	+	-	+
Sucrose	+	-	+	+	+	-
Fructose	+	+	+	+	+	-
Mannitol	-	-	+	-	+	-
Sorbitol	-	-	-	-	+	+
Dulcitol	-		-	-	-	-
Inulin	-		+	-		-
Cellobiose	-		-	-	-	-
Trehalose	+	-	+	-	+	-
Arabinose	-	-	+	-	-	-

A, aerobe; FAn, facultative anaerobe; +, positive; -, negative.

Table 3. Identification of PMB isolates by 16s rDNA sequencing.

Isolate	Number of nucleotide compared	Identification	Closest match and its accession code	Identity	Accession number
GSI-1	1407	*Streptococcusurinalis*	*Streptococcus* sp. 2285-97T (AJ131965)	100%	KU707916
GSI-2	721	*Pseudomonas* sp.	*Pseudomonas nitroreducens* IAM 1439T (AM088473)	91%	KT364748
GSI-3	810	*Bacillus* sp.	*Bacillus megaterium* ATCC 14581T (GU252112)	99%	KU189229
GSI-4	718	*Staphylococcusepidermidis*	*Staphylococcus epidermidis* NBRC 100911T(AB681292)	100%	KU871116
GSI-5	1434	*Rhodococcus* sp.	*Rhodococcusimtechensis* RKJ300T (AY525785)	99%	KU687353
GSI-6	1418	*Micrococcusluteus*	*Micrococcus luteus* DSM 20030T (AJ536198)	100%	KU707915

Isolates GSI-1 to 6 were identified using biochemical and molecular biological techniques. Since our isolation approach was aerobic, most of the strains were found to be aerobes, as evidenced by oxygen requirement test (Table 2). Based on biochemical (Table 2) and physiological characters as well as SSU sequences, the bacteria have been identified as members of genera *Streptococcus*, *Pseudomonas*, *Bacillus*, *Staphylococcus*, *Rhodococcus*, and *Micrococcus* (Table 3). Phylogenetically, the isolates were distributed among *Gammaproteobacteria*, *Actinobacteria*and*Firmicutes* (Fig. 2).

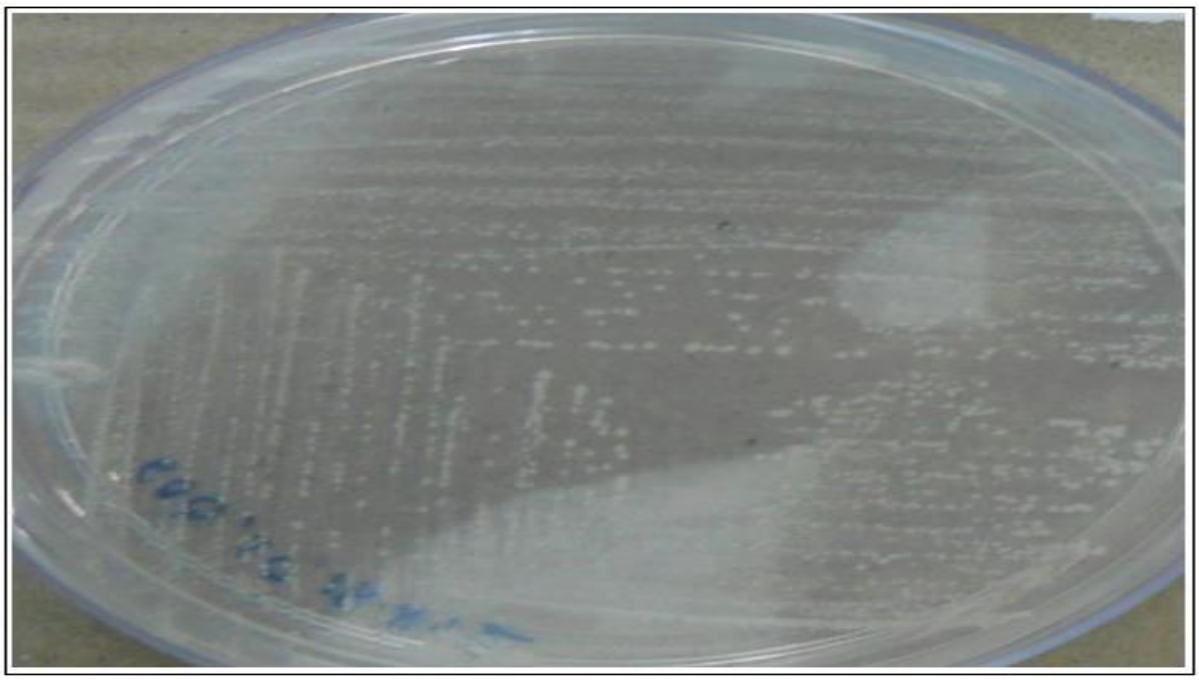

Fig. 1. Phenol degrading bacteria on MM media, showing no visible change of media for phenol degradation.

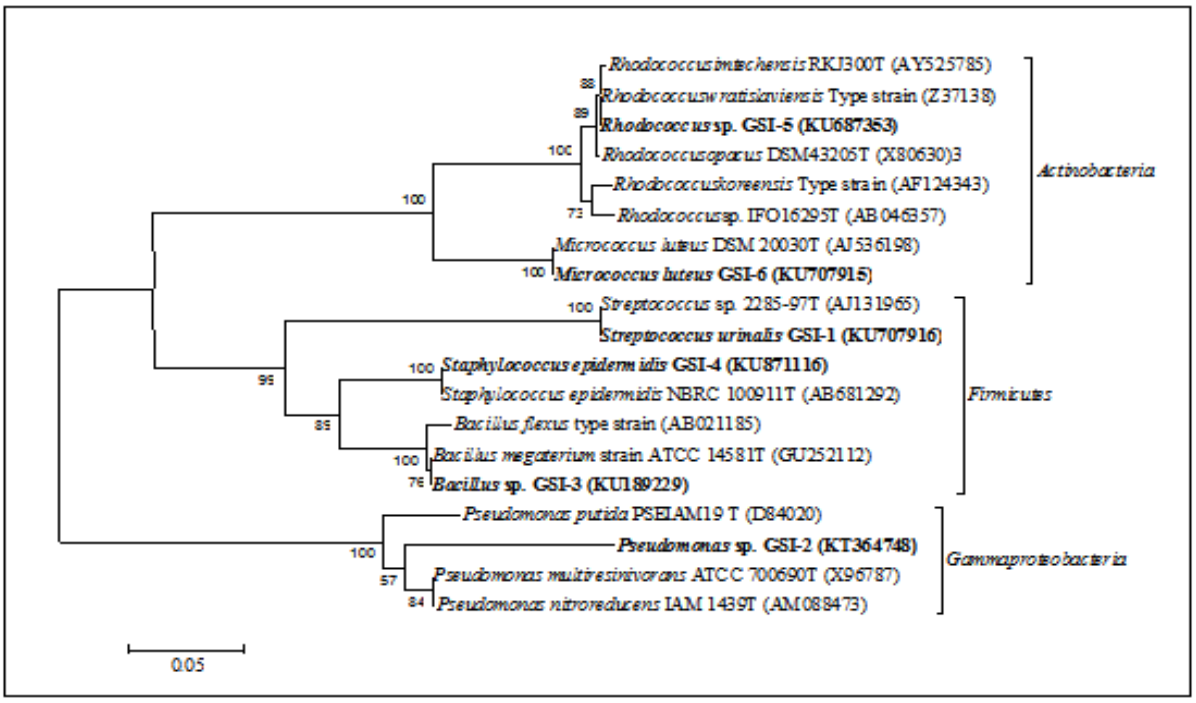

Fig. 2. Phylogenetic tree showing the relationship among phenol degrading bacteria antheir closest type strains. The GenBank accession numbers of the type strains and studied strains are shown in parenthesis following species names. Distance matrix was calculated by Kimura's 2-parameter model and Bootstrap analysis was calculated with 1000 replicates bootstrap. The scale bar indicates 0.05 substitutions per nucleotide position.

Determination of growth rate of bacteria

The analysis of growth rate of bacteria pointed out higher growth rate at lower phenol concentration (highest growth rate: GSI-1 at 200 mg l^{-1}; GSI-2 at 600 mg l^{-1}; GSI-3 at 200 mg l^{-1}; GSI-4 at 600 mg l^{-1}; GSI-5 at 400 mg l^{-1} and GSI-6 at 400 mg l^{-1}) as a general feature for our studied strains (Fig. 3). However, for two strains GSI-1 and GSI-3 an increase in the growth rate was observed at concentration 1200 mg l^{-1} initial phenol concentration.

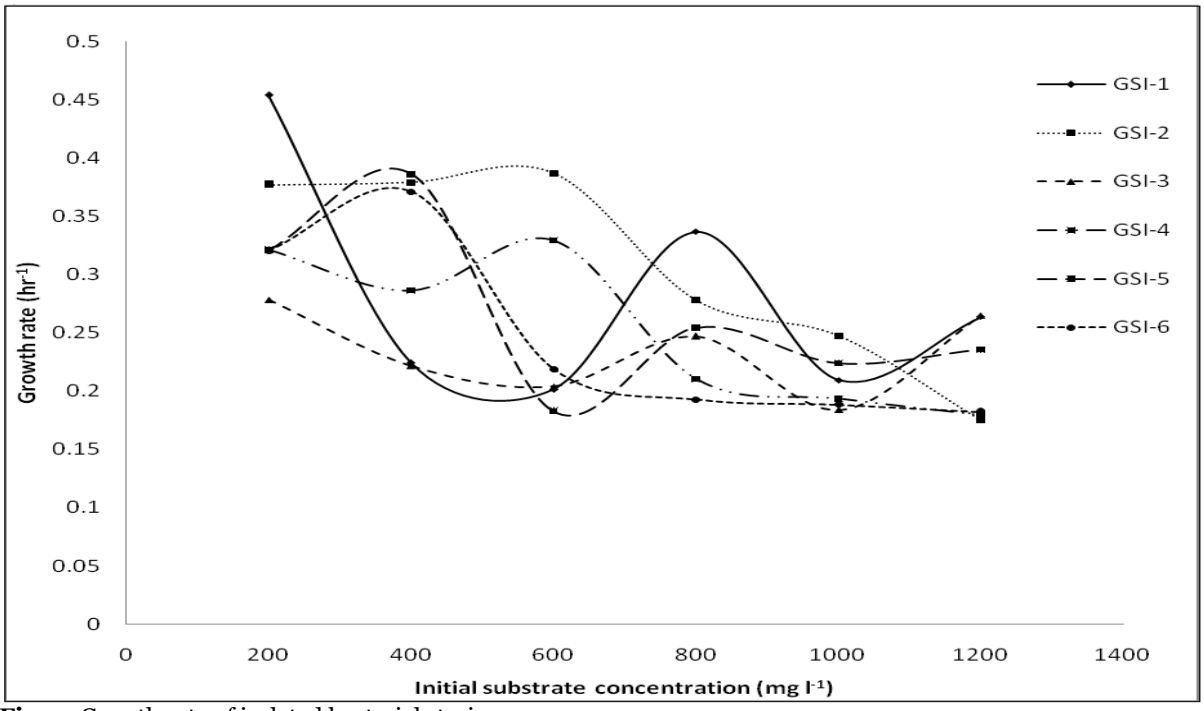

Fig. 3. Growth rate of isolated bacterial strains.

Degradation study at inhibitory phenol concentration

Further degradation study to correlate degradation study with growth rate study showed a lower lag phase (up to about 50 hours) than the previous degradation study (lag phase data for previous study was obtained by visual checking of growth), followed by exponential log phase growth pattern.

There was a stationary phase for all the isolates. For all the isolates nearly complete phenol degradation within 120 hours (Fig. 4).

Discussion

Phenol inhibits growth of the bacteria, more so when its concentration is high enough; obviously there are certain phenol degrading bacteria those can survive the toxic effect of phenol, but for them alsophenol may act as growth inhibitor at a significantly higher concentration- a phenomenon known as substrate inhibition (Molin and Nilsson, 1985; Yang and Lee, 2007).

Biodegradation of phenol at higher concentration by bacterial species is thus hampered by this substrate inhibition problem (Lob and Tar, 2000).

Substrate inhibition study

In our case, the initial growth rate study indeed indicated that all our studied strains showed substrate saturation phenomenon; the growth was lower as phenol concentration increases beyond 800 mg l^{-1}, very low was at 1200 mg l^{-1},while no growth at 1400 mg l^{-1} and 1500 mg l^{-1}initial phenol concentration. Thus, we presumed the phenol concentration 1200 mg l^{-1} as the threshold inhibitory phenol concentration. This substrate inhibition may have two probable reasons.

The enzyme systems involves in phenol degradation pathway (like phenol hydroxylase) may show a feedback inhibition process. Else, the phenol itself showed the inhibition to growth of bacteria by its toxic property.

Growth rate study

As we studied the growth rate for the isolates, two isolates GSI-1 and GSI-3 showed a higher growth rate at 1200 mg l^{-1} initial phenol concentration- which did not tally with the previous degradation study result.

For the rest of the strains, growth rate was slow at higher concentration of phenol.

Study of phenol degradation at inhibitory phenol concentration

Next we studied the efficacy of our isolates to survive and degrade the inhibitory phenol concentration i.e 1200 mg l^{-1} which was obtained from the first degradation experiment. It was surprising that all the isolates showed a moderate growth pattern at that concentration. Even initial lag phase was smaller than the previous degradation study, and even after completion of log phase when bacteria enter stationary phase, then too we got phenol degradation by bacteria.

Another striking feature of this second degradation study was that we observed almost complete degradation of phenol at the end of the experiment.

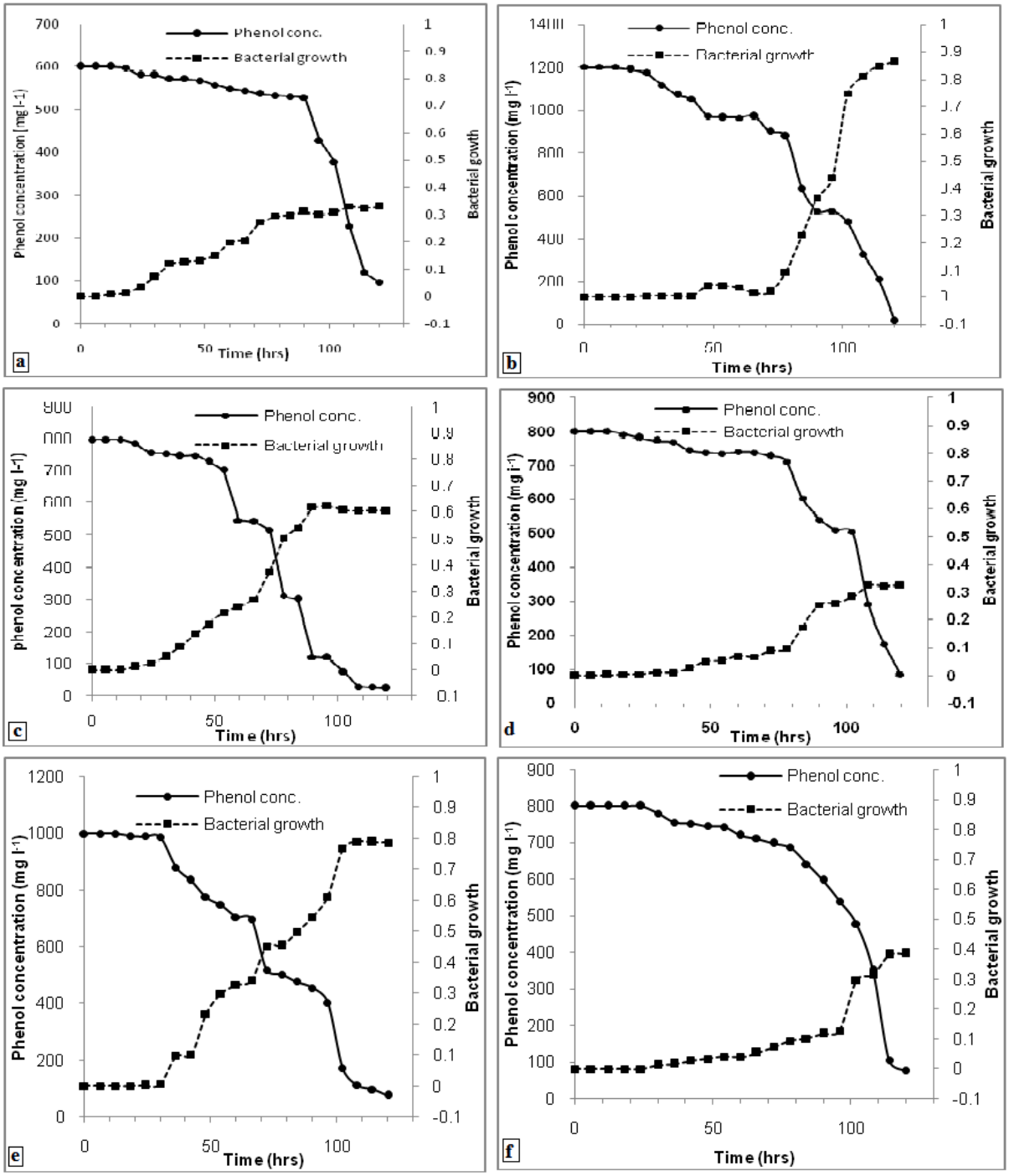

Fig. 4. Phenol degradation study by the isolated strains. a.: GSI-1; b.: GSI-2; c.: GSI-3; d.: GSI-4; e.: GSI-5; f.: GSI-6.

If phenol were indeed a toxic growth inhibitor then such situation should not had arrived at all. The chance of feedback inhibition of enzyme system also got nullified, since the degradation of phenol never stopped throughout our experiment, even at stationary phase- proving phenol was used there as nutrient. Now question arises that what changes occur in the test bacteria during acclimatization that help them to combat with a phenol concentration that was previously toxic.

One possible answer could be a mutation. Now, if we consider the mutation as spontaneous one, contrary question comes that why we obtained the same mutation trend for all of our studied bacteria belonging to distant families like *Gammaproteobacteria*, *Actinobacteria* and *Firmicutes*. And at this situation, the long dilemma of origin of mutation really arises, is it really spontaneous or an adaptive one (Cairns *et al.*, 1988) that was induced by the higher phenol concentration over the enzyme system, or any other part of bacteria to help them not to succumb to that higher phenol concentration, instead use them as nutrient.

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Conflict of interest statement

The authors hereby declare no conflict of interest.

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