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Salicylic-Acid-Mediated Enhanced Biological Treatment of Wastewater

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Abstract Activated sludge represents a microbial community which is responsible for reduction in pollution load from wastewaters and whose performance depends upon the composition and the expression of degradative capacity. In the present study, the role of salicylic acid (SA) has been evaluated for acclimatization of activated sludge collected from a combined effluent treatment plant followed by analysis of the physiological performance and microbial community of the sludge. The biodegradative capacity of the acclimatized activated sludge was further evaluated for improvement in efficiency of chemical oxygen demand (COD) removal from wastewater samples collected from industries manufacturing bulk drugs and dyes and dye intermediates (wastewater 1) and from dye industry (wastewater 2). An increase in COD removal efficiency from 50% to 58% and from 78% to 82% was observed for wastewater 1 and wastewater 2, respectively. Microbial community analysis data showed selective enrichment and change in composition due to acclimatization by SA, with 50% of the clones showing sequence homology to unidentified and uncultured bacteria. This was demonstrated by analysis of partial 16S rDNA sequence data generated from dominating clones representing the metagenome which also showed the appearance of a unique population of clones after acclimatization, which was distinct from those obtained before acclimatization and clustered away from the dominating population.

Keywords Acclimatization · Activated sludge · Salicylic acid · Microbial diversity · Wastewater treatment

Introduction

The non-performance of waste treatment facilities, especially the combined effluent treatment plants (CETPs) at their optimum levels due to the mixed stream of wastes and the production schedules of different industries, results in a lag period characterized by acclimatization of the

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bacterial biomass to the organic compounds in waste streams followed by detectable biodegradation. Such acclimatization may seldom lead to simultaneous acclimation to structurally related molecules due to induction of enzymes involved in the early steps in the biodegradation pathway, as was first observed by Soulas et al. [1] in the case of soil communities which, when acclimated to 2,4-dichlorophenoxyacetic acid (2,4-D), were simultaneously able to degrade other pesticides.

Isolation of various bacteria from different environmental niches such as CETPs and their role in degradation of a variety of aromatic compounds has been demonstrated in our laboratory [2–8]. Also, there are few reports on the acclimatizing effect of organic compounds on improvement in catabolic capacity of biomass. Buiotron et al. [9] and Khardenavis et al. [10] studied the acclimatizing effect of phenol on sludge biomass and reported a one to twofold increase in degradation capacity. A lag phase of 30 days with acclimatization followed by detectable chloride removal from 2,4-DCP was also observed in an upflow anaerobic reactor [11].

The use of salicylic acid for induction of bacterial cells has been restricted to its medicinal properties [12] with an enhanced resistance of *Escherichia coli* to multiple antibiotics including quinolines, cephalosporins, nalidixic acid, etc. when grown in presence of salicylic acid [13, 14]. With this effect of salicylic acid in view, we acclimatized the sludge biomass to salicylic acid in the presence of wastewater from dye industry followed by studies to degrade different aromatic compounds, viz, phenol, benzoic acid, 2,4-D, and salicylic acid. Acclimatized sludge was also used to treat wastewaters from two CETPs, and change in microbial count and non-culturable diversity was studied over the acclimatization period in order to evaluate the role of degradative genotype which evolved as a result of prior exposure of sludge biomass to the acclimatizing compound.

Materials and Methods

Reactor Setup and Acclimatization of Activated Sludge

The acclimatization studies were carried out in 5-L capacity glass bottle with activated sludge from a CETP. Three liters of this sludge was mixed with 1 L wastewater from a dye industry, and 50 ppm of salicylic acid (SA) was added daily for acclimatization in addition to 5 ppm KH_2PO_4 as a source of phosphate. Aeration was provided by an aquarium pump and spent supernatant was replaced with fresh wastewater at every 48-h interval up to a period of 10 days. The sludge was further aerated up to 17 days with daily addition of salicylic acid and phosphate followed by evaluation of degradation potential of the acclimatized sludge for two different wastewaters: (1) mixed wastewater from dye and bulk drug manufacturing industries (wastewater 1) and (2) dye industry wastewater (wastewater 2). Similar experiment was repeated after a period of 60 days from start-up of reactor, and degradation potential was studied based on COD removal from the wastewaters.

Defining the Eubacterial Diversity

Microbial count (expressed as colony forming units, CFUs) in the sludge during acclimatization was determined at 2, 4, 6, 8, 10, and 17 days on 1× mineral agar medium (MM) [10] containing 1 mmol concentration of either of the following substrates as sole sources of carbon: phenol, benzoic acid, salicylic acid, 2,4-dichlorophenoxyacetic acid, and phthalic anhydride. The composition of the medium is as follows (per liter of distilled

water): $MgSO_4 \cdot 7H_2O$ (10%), 2.0 mL; NH_4Cl (10%), 2.5 mL; $CaCl_2 \cdot 2H_2O$ (10%), 1.0 mL; phosphate buffer (K_2HPO_4+KH_2PO4, 56 mM, pH 7.0), 40.0 mL; Hutner's solution, 20.0 mL; and agar, 20.0 g. The plates were incubated at 30°°C and the number of CFUs was recorded after 48 h.

Change in eubacterial diversity was analyzed by constructing 16S rDNA libraries from the sludge biomass before and after acclimatization with salicylic acid. Polymerase chain reaction (PCR)-compatible total sludge DNA was prepared and 5 μL of the DNA was used as template to amplify 16S rDNA using universal 16S rDNA primers (forward primer 27F 5′-AGAGTTTGATCMTGGCTCAG-3′ and reverse primer 1492R 5′-CGGYTACCTTGT TACGACTT-3′) as reported earlier [15, 16]. The PCR reactions consisted of 30 cycles of denaturation at 94°°C for 1 min, annealing at 55°°C for 1 min, and extension at 72°°C for 1 min, which amplified a 1,466-bp product which was purified using the gel extraction kit from Qiagen (Hilden, Germany) and cloned into pDrive cloning vector (Qiagen PCR cloning plus kit). Recombinant clones were screened by blue-white colony selection followed by plasmid DNA purification from transformants grown overnight in 5 mL Luria–Bertani medium containing 100 μg mL^{-1} ampicillin using Qiagen Q-20 tips. The 16S rDNA insert was amplified by PCR as described above and gel purified, and the 1.5-kb inserts were analyzed by amplified ribosomal DNA restriction analysis using *Alu*I restriction enzyme. Based on the digestion pattern, clones were identified and sequencing was carried out from the 5′ end using the T7 or SP6 primer. Sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/) and phylogenetic tree was constructed using the BOOTSTRAP tree method from Clustal X software developed by Thompson et al. [17].

Degradation Studies

Twenty milliliters 0.1× MM containing different concentrations (0.1, 0.25, 0.5, 1.0, 2.0, and 2.5 mmol) of either phenol, benzoic acid, salicylic acid, or 2,4-D as carbon source in 50-mL conical flasks was used for degradation studies by the 17-day acclimatized sludge. The flasks were kept in an incubator shaker at 30 °C with 150 rpm agitation and samples were withdrawn at 0, 4, 8, 24, and 48 h. Residual concentration of the aromatic compounds was determined by high-performance liquid chromatography (HPLC; Perkin-Elmer, USA) with a 100×4.6-mm Chromolith RP-18e performance column (Merck, Germany) and detected using a UV/visible detector at the following wavelengths: benzoic acid, 255 nm; 2,4-D, 280 nm; phenol, 280 nm; and salicylic acid, 295 nm. HPLC grade acetonitrile and 1 mmol phosphate buffer (pH 7.0) passed through 0.2-μm membrane filter (Millipore) was used as the mobile phase in a ratio of 20:80 at a flow rate of 1.5 mL min^{-1}.

Degradation studies for the two wastewaters (1 and 2) was carried out using 17- and 60-day acclimatized sludge. Of the two wastewaters, 1.5 L was individually taken in 2-L capacity conical flasks to which the 4,000 mg L^{-1} acclimatized sludge was added (expressed as mixed liquor suspended solids, MLSS), while control samples consisted of unacclimatized sludge at a MLSS of 4,000 mg L^{-1}. All the reactors were aerated with an aquarium aerator, and change in COD and biomass (MLSS) were estimated as per the standard methods [18]. The data presented are the average result of three independent experiments.

Results and Discussion

Activated sludge represents a food web in a controlled environment consisting of heterotrophic and autotrophic bacteria, fungi, and protozoa in addition to some rotifers

and metazoans, which obtain energy from carbonaceous organic matter in influent wastewater [19, 20]. Biodegradation by sludge microorganisms requires conditions conducive for adaptation and proliferation of the biomass in the presence of the pollutants in addition to the presence of the necessary catabolic pathways in the microorganisms [21]. Exposure of activated sludge to certain organic compounds can turn on the catabolic capacity for these compounds in addition to similar compounds.

The present study demonstrates the improvement of catabolic capacity of sludge after acclimatization. Degradation analysis was carried after acclimatization of sludge to salicylic acid for 17 days. Since no change in COD removal from wastewater was observed by use of sludge acclimatized to salicylic acid for more than 17 days (data not shown), hence, 17-day period was considered optimum for acclimatization. In order to understand if the effect of acclimatization would last even after stopping the acclimatization to salicylic acid, the sludge was allowed to stand for 60 days without further addition of salicylic acid before assessing its degradative potential and comparing it to the unacclimatized biomass.

Changes in Microbial Count and Diversity During Acclimatization

The change in microbial count in sludge during the acclimatization period is shown in Table 1. Absence of CFUs on phthalic anhydride plates throughout the acclimatization period was attributed to its toxicity. In the case of the remaining substrates, a decline in CFU up to 6 days was followed by a gradual increase in bacterial count in the subsequent sampling. Phenol was found to support the highest growth of bacteria, and though CFU on phenol media plates decreased initially from 433×10^5 to 120×10^5, the microbial count recovered and finally stabilized at 310×10^5 after 17 days of acclimatization. This initial decrease was characteristic of the lag phase in bacteria during which the microbial community adapted to the presence the salicylic acid in the system. Only those bacteria capable of producing inducible enzymes for degradation of salicylate and other structurally related aromatic molecules were acclimatized and survived, thus resulting in an increase in their number following an initial decrease.

The diversity of the different 16S rDNA clones is demonstrated in the form of a dendrogram which shows the relation between clones obtained from sludge samples taken before (B) and after (A) acclimatization with 50 ppm salicylic acid (Fig. 1). The corresponding details of the clone sequences used in tree construction and their homologies

Table 1 CFU formation on substrate agar plates during different stages of acclimatization of sludge (1 mmol concentration of each substrate used).

CFU (±SD)×10^5					
Substrate Time (days)	Benzoic acid	Phenol	Salicylic acid	2,4-D	Pthalic anhydride
0	1,333 (±91)	433 (±33)	421 (±21)	600 (±30)	0
2	850 (±55)	494 (±29)	337 (±17)	125 (±15)	0
4	297 (±27)	120 (±14)	44 (±10)	110 (±5)	0
6	244 (±24)	145 (±18)	26 (±4)	92 (±8)	0
8	94 (±6)	156 (±19)	7 (±3)	47 (±3)	0
10	143 (±7)	170 (±10)	5 (±1)	87 (±8)	0
17	100 (±15)	310 (±25)	4 (±3)	100 (±12)	0

SD standard deviation

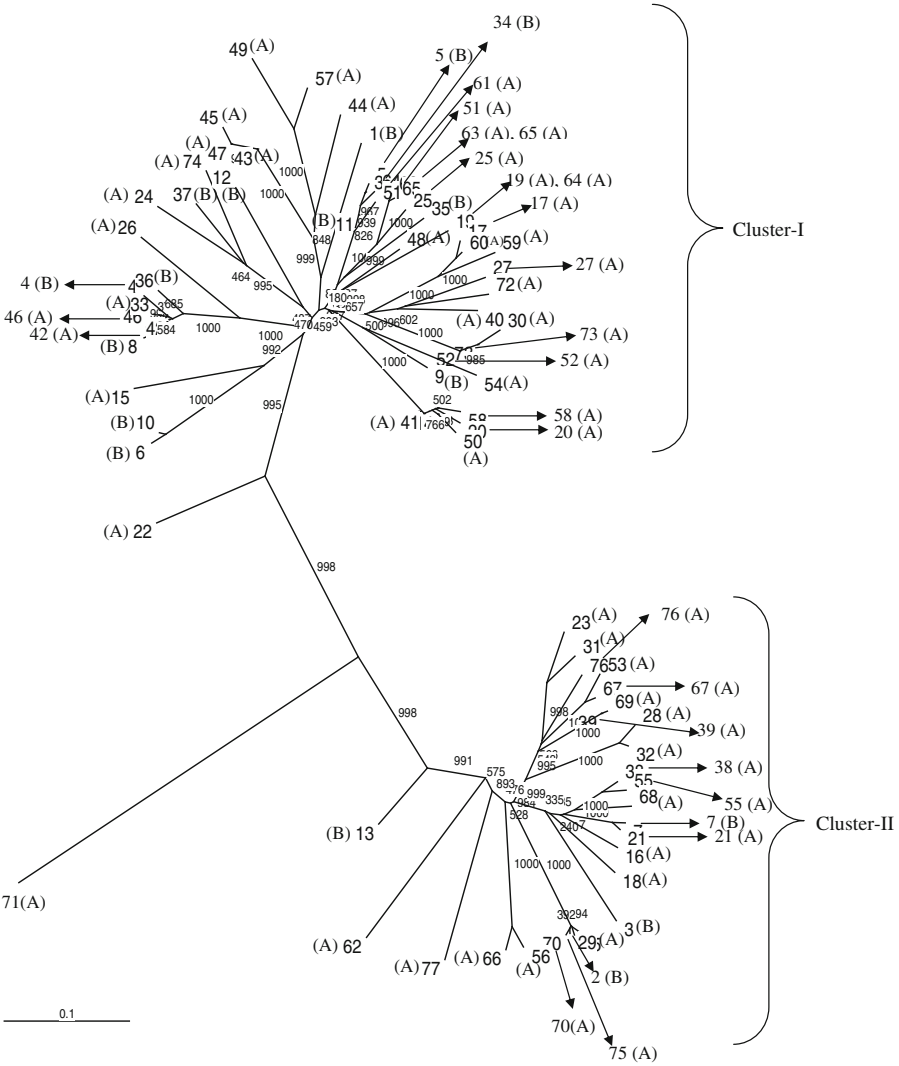

Fig. 1 Phylogenetic tree based on partial 16S rDNA sequences showing relationship between different unculturable bacterial clones observed in activated sludge from CETP before and after acclimatization with salicylic acid. The clones are identified by serial numbers from Table 2. *B* denotes sequences obtained from sludge sample before acclimatization, while *A* denotes sequences obtained from sludge sample after acclimatization to salicylic acid. Bootstrap values are mentioned at branching points. Other sequence details are given in Table 2

to existing sequences in the genome database are described in Table 2. The differentiation between the sequences of B and A samples was best represented using the unrooted tree format which showed two distinct clusters (I and II). It is seen from Fig. 1 that cluster I consisted of clones from both the samples (B and A), while cluster II consisted of unique population of clones which emerged as a result of acclimatization (A), with the exception of four clones which belonged to sample before acclimatization (B).

It is clear from Fig. 1 and Table 2 that clones 1, 9, and 35 (HKT-877, HKT-889, and HKT-918) from cluster I, which were obtained from sample B, were eliminated during

Table 2 Results of the 16S rDNA clone library analyzed by BLAST.

Sr. no.	Clone no.	Accession no. of clone	Acclimatization stage Before/After	Blast result		
				Homology to	Accession no.	% Homology
1	HKT-877	DQ989417	Before	*Clostridium* sp.	X75909	94
				Tissierella praeacuta	X80833	92
2	HKT-882	DQ989418	Before	unidentified bacterium	AF097803	96
				Desulfovibrio inopinatus	AF177276	88
3	HKT-883	DQ989419	Before	*Hyphomicrobium zavarzinii*	Y14305	95
				Hyphomicrobium zavarzinii	Y14305	95
4	HKT-884	DQ989420	Before	*Meiothermus ruber*	L09672	87
				Meiothermus ruber	Z15059	87
5	HKT-885	DQ989421	Before	unidentified bacterium	AF097800	95
				unidentified bacterium	AF097815	93
6	HKT-886	DQ989422	Before	uncultured bacterium SHA-53	AJ249111	96
				uncultured bacterium SJA-61	AJ009469	95
7	HKT-887	DQ989423	Before	*Hyphomicrobium vulgare*	Y14302	95
				Hyphomicrobium zavarzinii	Y14305	94
8	HKT-888	DQ989424	Before	*Meiothermus ruber*	L09672	87
				Meiothermus ruber	Z15059	87
9	HKT-889	DQ989425	Before	*Desulfuromonas acetexigens*	U23140	99
				Desulfuromonas chloroethenica	U49748	94
10	HKT-890	DQ989426	Before	Uncultured bacterium SHA-53	AJ249111	97
				uncultured bacterium SJA-61	AJ009469	96
11	HKT-891	DQ989427	Before	Unidentified bacterium	AF097800	94
				Unidentified bacterium	AF097815	94
12	HKT-892	DQ989428	Before	Uncultured sponge symbiont WS61	AF186448	90
				Rhizosphere soil bacterium RSC-II-7	AJ252671	89
13	HKT-893	DQ989429	Before	*Collinsella aerofaciens*	AJ245919	99
				Collinsella aerofaciens	AB011815	99
14	HKT-894	DQ989434	After	Unidentified bacterium	AF097803	96
				Unidentified bacterium	X84521	93
15	HKT-895	DQ989435	After	Unidentified *Verrucomicrobium* group OPB35	AF027005	98
				Mycoplasma mobile	M24480	91
16	HKT-896	DQ989436	After	*Sphingomonas pituitosa*	AJ243751	96
				Sphingomonas sp.	AB033945	96
17	HKT-897	DQ989437	After	Unidentified bacterium	AB021333	91
				Pseudoxanthomonas sp. M1-3	AB039330	90
18	HKT-898	DQ989438	After	*Rhodobacter veldkampii*	D16421	94
				Rhodobacter sphaeroides	X53855	94
19	HKT-899	DQ989439	After	Uncultured bacterium	AF143826	87
				Mycoplana dimorpha	D12786	88
20	HKT-900	DQ989440	After	Unidentified bacterium	AF097803	96
				Unidentified bacterium	X84521	96
21	HKT-901	DQ989441	After	*Hyphomicrobium vulgare*	Y14302	96
				Hyphomicrobium zavarzinii	Y14305	95
22	HKT-902	DQ989442	After	Unidentified bacterium	X84557	94
				Uncultured eubacterium	AJ232838	94

Table 2 (continued)

Sr. no.	Clone no.	Accession no. of clone	Acclimatization stage Before/After	Blast result		
				Homology to	Accession no.	% Homology
23	HKT-903	DQ989443	After	Uncultured beta proteobacterium	AF204254	96
				Uncultured beta proteobacterium	AF204253	96
24	HKT-904	DQ989444	After	Uncultured eubacterium WR179	AJ233562	91
				Arthrobacter sp. CF-43	AJ243421	89
25	HKT-905	DQ989445	After	Unidentified proteobacterium WR110	X65580	95
				Uncultured eubacterium	AJ233472	96
26	HKT-908	DQ989446	After	*Deinococcus proteolyticus*	Y11331	86
				Desulfuromusa kysingii	X79414	89
27	HKT-909	DQ989447	After	Uncultured freshwater bacterium LD28	Z99999	92
				Uncultured bacterium SY6-54	AF296203	93
28	HKT-910	DQ989448	After	*Deinococcus radiophilus*	Y11333	89
				Deinococcus murrayi	Y13042	88
29	HKT-911	DQ989449	After	Unidentified bacterium	AF097803	96
				Desulfovibrio inopinatus	AF177276	89
30	HKT-912	DQ989450	After	*Pseudomonas stutzeri*	AJ288151	95
				Pseudomonas fragi	D84014	94
31	HKT-913	DQ989451	After	*Thauera selenatis*	Y17591	97
				Uncultured bacterium SJA-186	AJ009507	97
32	HKT-914	DQ989452	After	*Deinococcus radiophilus*	Y11333	90
				Deinococcus geothermalis	AJ000002|	89
33	HKT-915	DQ989453	After	*Meiothermus silvanus*	X84211	89
				Meiothermus silvanus	Y13599	87
34	HKT-917	DQ989430	Before	Unidentified bacterium	AF097800	95
				Unidentified bacterium	AF097815	94
35	HKT-918	DQ989431	Before	*Paracoccus* sp. MBIC4036	AB025192	94
				Paracoccus sp. MBIC4019	AB025190	94
36	HKT-922	DQ989432	Before	*Meiothermus silvanus*	X84211	85
				Meiothermus silvanus	Y13599	85
37	HKT-923	DQ989433	Before	*Microbacterium* sp. *VKM Ac-2016*	AB042081	92
				Rathayibacter festuci	AF159365	92
38	HKT-925	DQ989454	After	*Pseudomonas carboxydohydrogena*	AB021393	96
				Afipia genosp. 4 strain G-3644	U87768	96
39	HKT-926	DQ989455	After	*Frateuria aurantia*	AJ010481	94
				Cimanggu media isolate 88	AF229452	94
40	HKT-927	DQ989456	After	Uncultured bacterium SJA-186	AJ009507	96
				Thauera sp. mz1t	AF110005	95
41	HKT-929	DQ989457	After	Unidentified bacterium	AF097803	96
				Unidentified bacterium	X84521	95
42	HKT-930	DQ989458	After	*Deinococcus proteolyticus*	Y11331	94
				Deinococcus sp. MBIC3950	AB022911	91
43	HKT-931	DQ989459	After	*Polaribacter franzmannii*	U14586	88
				Marine psychrophile IC054	U85883	89

Table 2 (continued)

Sr. no.	Clone no.	Accession no. of clone	Acclimatization stage Before/After	Blast result		
				Homology to	Accession no.	% Homology
44	HKT-934	DQ989461	After	Uncultured eubacterium WCHB1-69	AF050545	92
				Uncultured freshwater bacterium LCK-81	AF107337	89
45	HKT-935	DQ989462	After	Unidentified marine eubacterium	L10944	90
				Unidentified marine eubacterium	L10945	89
46	HKT-936	DQ989463	After	*Meiothermus silvanus*	X84211	84
				Meiothermus silvanus	Y13599	84
47	HKT-937	DQ989464	After	Unidentified marine eubacterium	L10944	91
				Uncultured bacterium MK22	AF087075	87
48	HKT-939	DQ989465	After	Uncultured eubacterium	AJ006014	93
				Sphingomonas stygia	U20775	93
49	HKT-940	DQ989466	After	*Cytophaga*-like bacterium QSSC9-14	AF170754	88
				Cytophaga-like bacterium QSSC9-3	AF170749	89
50	HKT-941	DQ989467	After	Unidentified bacterium	AF097803	96
				Unidentified bacterium	X84521	96
51	HKT-942	DQ989468	After	Alpha proteobacterium A0838	AF235999	95
				Hyphomicrobium sp. *P2*	AF148858	95
52	HKT-944	DQ989469	After	*Pseudomonas* sp. B13	AF039489	97
				Pseudomonas stutzeri	AJ006103	96
53	HKT-945	DQ989470	After	*Pseudomonas* sp.	AJ007004	96
				Pseudomonas alcalophila	AB030583	96
54	HKT-951	DQ989471	After	Desulfococcus multivorans	M34405	96
				Unidentified sulfate-reducing bacterium 2B1	U85478	97
55	HKT-952	DQ989472	After	*Nitrobacter hamburgensis*	L11663	96
				Nitrobacter hamburgensis	L35502	96
56	HKT-953	DQ989473	After	*Leucobacter komagatae*	D45063	96
				Arthrobacter sp. MB6-07 16	U85897	96
57	HKT-954	DQ989474	After	*Cytophaga*-like bacterium QSSC9-14	AF170754	89
				Potato plant root bacterium RC-III-71	AJ252729	89
58	HKT-956	DQ989475	After	Unidentified bacterium	AF097803	96
				Unidentified bacterium	X84521	96
59	HKT-957	DQ989476	After	Uncultured bacterium mle1-45	AF280866	87
				Pseudoxanthomonas sp. M1-3	AB039330	87
60	HKT-958	DQ989477	After	Unidentified bacterium	AB021333	90
				Stenotrophomonas sp.	AJ002814	89
61	HKT-959	DQ989478	After	Unidentified bacterium	AF097803	96
				Unidentified bacterium	X84521	95
62	HKT-960	DQ989479	After	Uncultured eubacterium WCHB1-07	AF050600	90

Table 2 (continued)

Sr. no.	Clone no.	Accession no. of clone	Acclimatization stage Before/After	Blast result		
				Homology to	Accession no.	% Homology
				Uncultured eubacterium WCHB1-26	AF050599	91
63	HKT-961	DQ989480	After	*Bradyrhizobium lupini*	U69637	96
				Nitrobacter winogradskyi	L35506	96
64	HKT-962	DQ989481	After	Unidentified bacterium	AF097800	96
				Filomicrobium fusiforme	Y14313	95
65	HKT-963	DQ989482	After	*Nitrobacter winogradskyi*	L35506	97
				Nitrobacter sp.	L11662	97
66	HKT-964	DQ989483	After	*Oerskovia xanthineolytica*	X79453	93
				Cellulomonas cellulans	AB023355	93
67	HKT-965	DQ989484	After	*Pseudomonas alcalophila*	AB030583	98
				Pseudomonas pseudoalcaligenes	Z76666	98
68	HKT-966	DQ989485	After	*Zoogloea ramigera*	X74915	99
				Zoogloea ramigera	D14255	99
69	HKT-967	DQ989486	After	*Frateuria aurantia*	AJ010481|	94
				Cimanggu media isolate 88	AF229452	93
70	HKT-968	DQ989487	After	Unidentified bacterium	AF097803	96
				Desulfovibrio inopinatus	AF177276	89
71	HKT-969	DQ989488	After	Alpha proteobacterium	AB024595	87
				Uncultured bacterium	AF143826	87
72	HKT-970	DQ989489	After	*Alcaligenes defragrans*	AJ005450	95
				Alcaligenes defragrans	AJ005447	95
73	HKT-971	DQ989490	After	*Pseudomonas stutzeri*	AJ288151	96
				Pseudomonas stutzeri	AF094748	96
74	HKT-972	DQ989491	After	*Microbacterium* sp. SB22	Y07842	96
				Gram-positive bacterium str. 13-2	AB008512	96
75	HKT-973	DQ989492	After	Unidentified bacterium	AF097803	95
				Desulfovibrio inopinatus	AF177276	89
76	HKT-974	DQ989493	After	*Leptothrix* sp. MBIC3364	AB015048	94
				Lautropia sp. oral clone AP009	AY005030	93
77	HKT-975	DQ989494	After	Uncultured bacterium BA2	AF087043	87
				Benzene mineralizing consortium clone	AF029041	85

acclimatization and did not show up in sets of clones obtained from sample A. Similarly, clones 4 and 8 (HKT-884 and HKT-888) from sample B having sequence homology to *Meiothermus ruber* were not seen after acclimatization, though clone 36 (HKT-922) from sample B having homology to *Meiothermus silvanus* was observed after acclimatization in the form of clones 33 and 46 (HKT-915 and HKT-936), and all these clones clustered together in cluster I.

From Table 2, it is seen that of the different clones, nearly 41% of those obtained from sludge before acclimatization showed sequence homology to unidentified uncultured bacteria, while 50% were unidentified or uncultured amongst those obtained after

acclimatization. This indicated that there was an increase in ratio of such bacteria as a result of acclimatization with their possible role in the degradation of different aromatic compounds. Our results are consistent with those obtained by Dojka et al. [22] who used culture-independent methods for analyzing the microbial diversity involved in intrinsic bioremediation of aquifer contaminated with hydrocarbons (mainly jet fuel) and chlorinated solvents and found that approximately 50% of the 812 clones screened by restriction fragment length polymorphisms were unique. In contrast, Popp et al. [23] found the existence of moderate bacterial diversity in clone libraries during bioremediation of soils contaminated with mineral oil hydrocarbons which was dominated by γ-proteobacteria followed by α- and β-proteobacteria with the following dominant genera, e.g., *Pseudomonas*, *Acinetobacter*, *Sphingomonas*, *Acidovorax*, and *Thiobacillus*.

As mentioned above, a unique feature of the tree shown in Fig. 1 was the occurrence of a population of clones which formed a separate cluster from those in cluster I, indicating their distinctness from majority of the population of clones. Of the 26 clones belonging to this cluster (cluster II), 22 belonged to sample A, while only four belonged to sample B. The genetic relatedness of these 26 clones was further clarified from a separate dendrogram which was drawn using sequences of these clones (data not shown). This dendrogram showed that of the four clones from sample B, one (HKT-882) clustered with three clones from sample A (HKT-911, HKT-968, and HKT-973), and all of these clones had sequence homology to the same unidentified bacterium (accession no. AF097803), while clones 3 and 7 (HKT-883 and HKT-887) clustered with clone 21 (HKT-901) and shared sequence homology to *Hyphomicrobium.* The occurrence of these clones from sample B with majority of those of sample A suggested that these bacteria survived the acclimatization to salicylic acid and were also detected in the sets of clones obtained after acclimatization. Cluster II also showed the presence of three clones (HKT-908, HKT-910, and HKT-914) which shared sequence homology with *Dienococcus*, while remaining clones in this cluster were unique to this cluster and did not share sequence homology with those in cluster I. In addition to this, the dendrogram in Fig. 1 also showed a single clone-71 (HKT-969) which did not show any similarity to clones in either of the two clusters and diverged away from the two sets of clones, thereby suggesting that acclimatization enriched a population that was different from the dominating species already present.

Whiteley and Bailey [24] examined bacterial community diversity, distribution, and physiological state with respect to the remediation of phenolic polluted wastewater and highlighted the potential importance of the γ-*Proteobacteria* and the *Cytophaga-Flavobacteria* during this bioremediation process. In our study, the clone library of the biomass before acclimatization demonstrated a very diverse population showing sequences homologous to *Hyphomicrobium*, a soil bacteria reported in methanol-degrading consortium [25], *Desulfuromonas*, a sulfate-reducing bacterium studied mainly for its dehalogenation of di-, tri-, and tetra- chloroethanes [26, 27], and *Collinsella*, a bacterium reported in fecal samples. After acclimatization, besides the unidentified bacteria, certain clone sequences were identified as bacteria showing homology to genera like *Rhodobacte*r [28, 29], *Sphingomonas* [30–32], *Thauera* [33, 34], etc., which are widely reported in the degradation of aromatics.

Degradation of Aromatic Compounds

The effect of acclimatization by salicylic acid on change in microbial population in sludge and its subsequent effect on catabolic potential was confirmed by the degradation studies with different aromatic compounds. Figure 2a–d represents the degradation of benzoate,

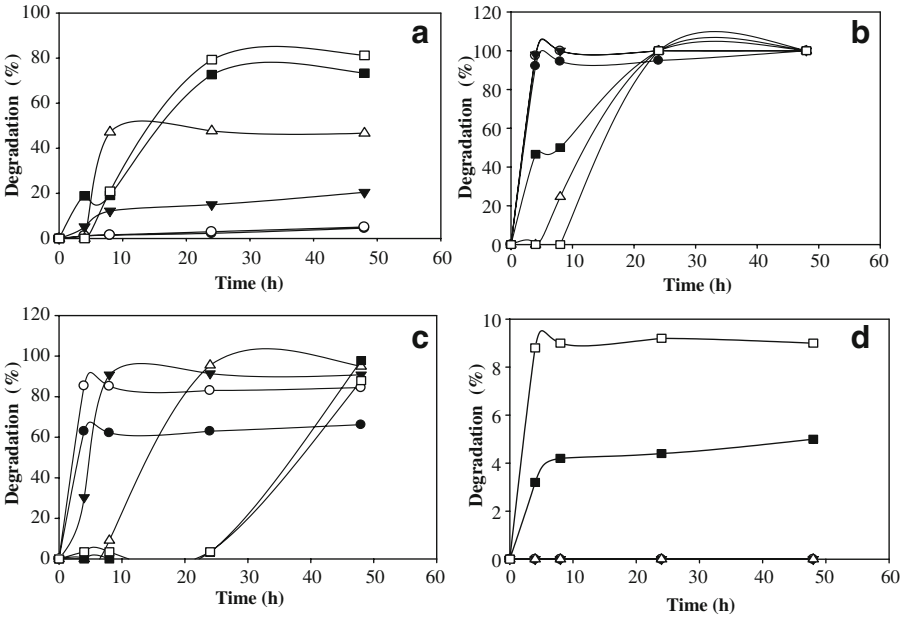

Fig. 2 Degradation of different concentrations of aromatic compounds by acclimatized activated sludge: **a** benzoic acid, **b** phenol, **c** salicylic acid, **d** 2,4-D; 0.1 mmol (*filled circle*), 0.25 mmol (*empty circle*), 0.5 mmol (*inverted triangle*), 1.0 mmol (*triangle*), 2.0 mmol (*filled square*), 2.5 mmol (*empty square*)

phenol, salicylate, and 2,4-D, respectively. Since no CFUs were observed on MM agar plates containing phthalic anhydride, this compound was not included in degradation studies using acclimatized sludge.

In the case of benzoate, no degradation was observed at low concentration even after 48 h, but degradation efficiency improved at higher concentrations (Fig. 2a). Fifty percent benzoate was consumed in 8 h when 1 mmol of the compound was used as sole carbon source. Benzoate (0.5 mmol) remained after 8 h and no further degradation was observed on further incubation. When 2.0 and 2.5 mmol benzoate were used, 75% and 80% degradation was achieved, respectively, in 24 h; however, 0.5 mmol benzoate still remained with no further degradation after 24 h. This indicated that benzoic acid could not serve as carbon source at concentrations lower than 0.5 mmol.

The presence of highest CFUs of phenol-utilizing microbes in sludge at the end of the acclimatization phase was reflected in 100% degradation of phenol at all the concentrations (Fig. 2b), with lower concentrations (0.1, 0.25, and 0.5 mmol) requiring only 4 h for complete removal and up to 24 h being required for complete utilization of higher concentrations of phenol (1.0, 2.0, and 2.5 mmol).

A similar trend is seen in the case of salicylate degradation. At lower concentrations of 0.1, 0.25, and 0.5 mmol, salicylate showed complete degradation in 4 h, with a slight decrease in degradation efficiency at higher concentrations. From Fig. 2c, it is seen that 1 mmol salicylate was completely degraded in 24 h, while 48 h was required for 100% removal of 2 mmol salicylate from the system, and only 88% salicylate removal was achieved at 2.5 mmol initial concentration in 48 h by the acclimatized sludge.

In contrast to the above three compounds, 2,4-D was a comparatively poor substrate and did not show any decrease at 0.5 to 1.5 mmol concentrations. At higher concentrations (2.0

and 2.5 mmol), slight decrease was seen with 1.8% and 6.5% degradation in 8 h and further incubation up to 48 h did not result in any improvement in degradation efficiency (Fig. 2d).

COD Removal from Wastewaters

Figure 3 shows the comparative COD removal from the two types of wastewaters (1 and 2) by sludge acclimatized to salicylic acid over that of unacclimatized sludge. A maximum COD removal of 50% (from wastewater 1) and 78% (from wastewater 2) was seen with unacclimatized sludge in 24 h which improved slightly to 58% and 82% in the case of wastewater 1 and 2, respectively, with 17-day acclimatized sludge (Fig. 3a). This corresponded to a decrease in COD values from 1,100 to 550 mg L^{-1} (unacclimatized sludge) and to 467 mg L^{-1} (17-day acclimatized) in the case of wastewater 1 and reduction from 1,650 to 357 mg L^{-1} (unacclimatized sludge) and 302 mg L^{-1} (17-day acclimatized) with wastewater 2.

In order to confirm the view that the degradative potential acquired by the sludge on account of acclimatization was retained even after the acclimatization was stopped, the above experiment was repeated with the acclimatized sludge which was further incubated for 60 days without the addition of salicylate. Figure 3b shows a decrease in overall treatment efficiency of wastewater 1 over that of 17-day acclimatized sludge (acclimatized sludge 32% and unacclimatized sludge 24% COD removal, respectively). In contrast, there was slight improvement in treatment efficiency of wastewater 2 with COD removal of 85% and 75%, respectively.

Many reports are available on the changes in microbial diversity during bioremediation; however; most of these studies deal with monitoring of in situ changes in diversity, while few studies report the role of changed diversity in bioremediation. Studies by Buitron et al. [9] found the activated sludge to be composed of 11 different strains initially, while only four dominant bacteria remained in sludge after 70 days of acclimatization by phenols with 100% degradation of different phenols in 24 h. Similar decrease in culturable diversity (based on 16S rDNA) and increase in degradative potential of dye industry sludge was reported by Kapley et al. [35] when the sludge was acclimatized to nitroaromatic wastewater. Improvement in biodegradation of petroleum hydrocarbons from 1.7% to 42% in 32 days was also observed by Li et al. [36] with soil bacteria enriched with biological

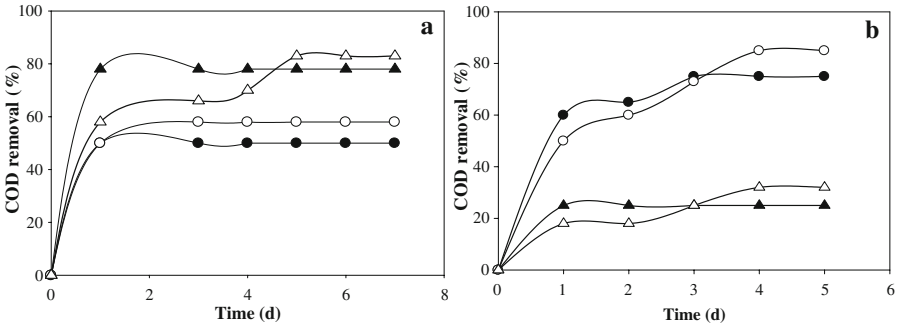

Fig. 3 COD removal from wastewaters by acclimatized activated sludge: **a** 17 days after acclimatization, **b** 60 days after acclimatization. Wastewater 1 + unacclimatized sludge (*filled triangle*), wastewater 1 + acclimatized sludge (*empty triangle*), wastewater 2 + unacclimatized sludge (*filled circle*), wastewater 2 + acclimatized sludge (*empty circle*)

activated carbon. Similar enhancement in catabolic ability of anaerobic sludge for higher concentration of other substituted chlorophenols was reported by Khardenavis et al. [11] on acclimatization of anaerobic sludge to 2,4-DCP for 70 days which also resulted in a twofold increase in anaerobic culturable bacterial count. Recently, phenol-mediated improvement in COD removal by distillery sludge from 19% to 31% for raw distillery wastewater and 85% for ten times diluted wastewater after a short acclimatization period of 13 days was reported by Khardenavis et al. [10].

In the present investigation, though molecular tools were used for tracking the change in unculturable diversity before and after acclimatization and an increase in diversity in addition to increase in degradation potential of sludge biomass was observed, similar studies on acclimatization of sludge by other organic compounds need to be carried out. Also, studies on PCR amplification and probe studies for necessary catabolic enzymes are to be performed in order to further confirm effect of acclimatization in improving the catabolic potential of the sludge biomass.

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References

1. Soulas, G., Codaccioni, P., & Fournier, J. C. (1983). Effect of cross-treatment on the subsequent breakdown of 2,4-D, MCPA and 2,4,5-T in the soil behaviour of the degrading microbial populations. *Chemosphere*, *12*(7–8), 1101–1106. doi:10.1016/0045-6535(83)90263-1.
2. Kapley, A., Tolmare, A., & Purohit, H. J. (2001). Role of oxygen in the utilization of phenol by *Pseudomonas* CF600 in continuous culture. *World Journal of Microbiology & Biotechnology*, *17*, 801–804. doi:10.1023/A:1013526001972.
3. Kutty, R., Purohit, H. J., & Khanna, P. (2000). Isolation and characterization of a *Pseudomonas* sp. strain PH1 utilizing meta-aminophenol. *Canadian Journal of Microbiology*, *46*, 211–217. doi:10.1139/cjm-46-3-211.
4. Moharikar, A., & Purohit, H. J. (2003). Specific ratio and survival of *Pseudomonas* CF600 as co-culture for the phenol degradation in continuous cultivation. *International Biodeterioration & Biodegradation*, *52*, 255–260. doi:10.1016/S0964-8305(03)00114-8.
5. Qureshi, A. A., & Purohit, H. J. (2002). Isolation of consortia for the degradation of *p*-nitrophenol from agricultural soil. *The Annals of Applied Biology*, *140*, 159–162. doi:10.1111/j.1744-7348.2002.tb00168.x.
6. Qureshi, A., Verma, V., Kapley, A., & Purohit, H. J. (2007). Degradation of 4-nitoaniline by *Stenotrophomonas* strain HPC 135. *International Biodeterioration & Biodegradation*, *60*(4), 215–218. doi:10.1016/j.ibiod.2007.03.004.
7. Selvakumaran, S., Kapley, A., Kalia, V. C., & Purohit, H. J. (2007). Phenotypic and phylogenic groups to evaluate the diversity of *Citrobacter* isolates from activated biomass of effluent treatment plants. *Bioresource Technology*, *99*(5), 1189–1195. doi:10.1016/j.biortech.2007.02.021.
8. Thangaraj, K., Kapley, A., & Purohit, H. J. (2007). Characterization of diverse *Acinetobacter* isolates for utilization of multiple aromatic compounds. *Bioresource Technology*, *99*(7), 2488–2494. doi:10.1016/j.biortech.2007.04.053.
9. Buitron, G., Gonzalez, A., & Lopez-Marin, L. M. (1998). Biodegradation of phenolic compounds by an acclimated activated sludge and isolated bacteria. *Water Science and Technology*, *31*(4–5), 371–378. doi:10.1016/S0273-1223(98)00133-4.
10. Khardenavis, A. A., Kapley, A., & Purohit, H. J. (2008). Phenol-mediated improved performance of active biomass for treatment of distillery wastewater. *International Biodeterioration & Biodegradation*, *62*, 38–45. doi:10.1016/j.ibiod.2007.06.016.
11. Khardenavis, A. A., Kapley, A., & Purohit, H. J. (2006). In V. Murugesan, R. Jayabalou, S. Nanjundan & M. Palanichamy (Eds.), Strategies for waste management: Stabilization of upflow anaerobic reactor for dechlorination of substituted chlorophenols. Proceedings of the 8th AANESWM, Anna University, Chennai, Dec. 10–13, pp. 227–234.

12. Price, C. T. D., Lee, I. R., & Gustafson, J. E. (2000). The effects of salicylate on bacteria. *The International Journal of Biochemistry & Cell Biology*, *32*, 1029–1043. doi:10.1016/S1357-2725(00)00042-X.
13. Rosner, J. L. (1985). Nonheritable resistance to chloramphenicol and other antibiotics induced by salicylates and other chemotactic repellents in *Escherichia coli* K-12. *Proceedings of the National Academy of Sciences of the United States of America*, *82*, 8771–8774. doi:10.1073/pnas.82.24.8771.
14. Cohen, S. P., Foulds, J. J., Levy, S. B., & Rosner, L. (1993). Salicylate induction of antibiotic resistance in *Escherichia coli*: Activation of *mar* operon and mar-independent pathway. *Journal of Bacteriology*, *175*, 7856–7862.
15. Purohit, H. J., Kapley, A., Moharikar, A., & Narde, G. (2003). Extraction of activated biological sludge for PCR compatible DNA from effluent treatment systems. *Journal of Microbiological Methods*, *52*, 315–323. doi:10.1016/S0167-7012(02)00185-9.
16. Kapley, A., Baere, T.-De., & Purohit, H. J. (2007). Eubacterial diversity of activated biomass from a common effluent treatment plant. *Research in Microbiology*, *158*(6), 494–500. doi:10.1016/j.resmic.2007.04.004.
17. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, J. D. (1997). The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, *25*, 4876–4882. doi:10.1093/nar/25.24.4876.
18. American Public Health Association. American Water Works Association, Water Environment Federation (1998). *Standard methods for the examination of water and wastewater* (20th ed.). Washington DC, USA: APHA.
19. Fredrickson, A. G., & Stephanopoulus, G. N. (1981). Microbial competition. *Science*, *213*, 972–979. doi:10.1126/science.7268409.
20. Ratsak, C. H., Koouman, S. A. L. M., & Kooi, B. W. (1993). Modelling the growth of an oligochaete on activated sludge. *Water Research*, *27*(5), 739–747. doi:10.1016/0043-1354(93)90136-6.
21. Alexander, M. (1973). Nonbiodegradable and other recalcitrant molecules. *Biotechnology and Bioengineering*, *15*, 611–647. doi:10.1002/bit.260150402.
22. Dojka, M. A., Hugenholtz, P., Haack, S. K., & Pace, N. R. (1998). Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Applied and Environmental Microbiology*, *64*(10), 3869–3877.
23. Popp, N., Schlomann, M., & Mau, M. (2006). Bacterial diversity in the active stage of a bioremediation system for mineral oil hydrocarbon-contaminated soils. *Microbiology*, *152*(11), 3291–3304. doi:10.1099/mic.0.29054-0.
24. Whiteley, A. S., & Bailey, M. J. (2000). Bacterial community structure and physiological state within an industrial phenol bioremediation system. *Applied and Environmental Microbiology*, *66*(6), 2400–2407. doi:10.1128/AEM.66.6.2400-2407.2000.
25. Kuniki, K., Murakami-Nitta, T., Oishi, M., Ishiguro, S., & Kirimura, K. (2004). Isolation of dimethyl sulfone-degrading microorganisms and application to odorless degradation of dimethyl sulfoxide. *Journal of Bioscience and Bioengineering*, *97*(1), 82–84.
26. Lorah, M., & Voytek, M. (2004). Degradation of 1,1,2,2-tetrachloroethane and accumulation of vinyl chloride in wetland sediment microcosms and in situ porewater: Biogeochemical controls and associations with microbial communities. *Journal of Contaminant Hydrology*, *70*(1–2), 117–145. doi:10.1016/j.jconhyd.2003.08.010.
27. Scow, K. M., & Hicks, K. A. (2005). Natural attenuation and enhanced bioremediation of organic contaminants in groundwater. *Current Opinion in Biotechnology*, *16*(3), 246–253. doi:10.1016/j.copbio.2005.03.009.
28. Roldán, M. D., Blasco, R., Caballero, F. J., & Castillo, F. (1997). Degradation of *p*-nitrophenol by the phototrophic bacterium *Rhodobacter capsulatus*. *Archives of Microbiology*, *169*(1), 36–42. doi:10.1007/s002030050538.
29. Song, Z.-Y., Zhou, J.-T., Wang, J., Yan, B., & Du, C.-H. (2003). Decolorization of azo dyes by *Rhodobacter sphaeroides*. *Biotechnology Letters*, *25*(21), 1815–1818. doi:10.1023/A:1026244909758.
30. Corvini, P. F. X., Meesters, R. J. W., Schäffer, A., Schröder, H. F., Vinken, R., & Hollender, J. (2004). Degradation of a nonylphenol single isomer by *Sphingomonas* sp. strain TTNP3 leads to a hydroxylation-induced migration product. *Applied and Environmental Microbiology*, *70*(11), 6897–6900. doi:10.1128/AEM.70.11.6897-6900.2004.
31. Crawford, R. L., & Ederer, M. M. (1999). Phylogeny of *Sphingomonas* species that degrade pentachlorophenol. *Journal of Industrial Microbiology & Biotechnology*, *23*(4–5), 320–325. doi:10.1038/sj.jim.2900729.
32. Wilkes, H., Wittich, R., Timmis, K. N., Fortnagel, P., & Francke, W. (1996). Degradation of chlorinated dibenzofurans and dibenzo-*p*-dioxins by *Sphingomonas* sp. strain RW1. *Applied and Environmental Microbiology*, *62*(2), 367–371.

33. Dilbenedetto, A., Lo Noce, R. M., Narracci, M., & Aresta, M. (2006). Structure–biodegradation correlation of polyphenols for *Thauera aromatica* in anaerobic conditions. *Chemistry and Ecology*, *22* (S1), S133–S143. doi:10.1080/02757540600557975.
34. Shinoda, Y., Sakai, Y., Uenishi, H., Uchihashi, Y., Hiraishi, A., Yukawa, H., et al. (2004). Aerobic and anaerobic toluene degradation by a newly isolated denitrifying bacterium, *Thauera* sp. strain DNT-1. *Applied and Environmental Microbiology*, *70*(3), 1385–1392. doi:10.1128/AEM.70.3.1385-1392.2004.
35. Kapley, A., Prasad, S., & Purohit, H. J. (2007). Changes in microbial diversity in fed-batch reactor operation with wastewater containing nitroaromatic residues. *Bioresource Technology*, *98*(13), 2479–2484. doi:10.1016/j.biortech.2006.09.012.
36. Li, G., Huang, W., Lerner, D. N., & Zhang, X. (2000). Enrichment of degrading microbes and bioremediation of petrochemical contaminants in polluted soil. *Water Research*, *34*(15), 3845–3853. doi:10.1016/S0043-1354(00)00134-2.