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ORIGINAL PAPER

# Isolation of a *Pseudomonas aeruginosa* strain from soil that can degrade polyurethane diol

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**Abstract** Polyurethane diol (PUR-diol), a synthetic polymer, is widely used as a modifier for water-soluble resins and emulsions in wood appliances and auto coatings. Non-biodegradability of polyurethanes (PUR) and PUR-based materials poses a threat to environment that has led scientists to isolate microbes capable of degrading PUR. However, the bio-degradation of PUR-diol has not yet been reported. In this study, we report isolation of a soil bacterium that can survive using PUR-diol as sole carbon source. PUR-diol degradation by the organism was confirmed by thin layer chromatographic analysis of the conditioned medium obtained after the growth wherein a significant reduction of PUR-diol was observed compared to non-inoculated medium. To quantify the PUR-diol degradation, a sensitive assay based on High Performance Thin Layer Chromatography has been developed that showed 32% degradation of PUR-diol by the organism in 10 days. Degradation kinetics showed the maximal depletion of PUR-diol during logarithmic growth of the organism indicating a direct relation between the growth and PUR-diol degradation. Mutagenic study and GC-MS analysis revealed that esterase activity is involved in this degradation event. The ribotyping and metabolic fingerprinting analysis showed that this organism is a strain of *Pseudomonous aeruginosa* (*P. aeruginosa*). It has also been observed that this strain is able to degrade Impranil DLN$^{TM}$, a variety of commercially available PUR. Therefore this study identifies a new bacterium from soil that has the potential to reduce PUR-related waste burden and adds a new facet to diverse functional activities of *P. aeruginosa*.

**Keywords** Polyurethane diol · Bioremediation · Pseudomonas

## Introduction

Materials made of plastics and synthetic polymers are extensively used in various walks of life. Plastics are widely used because of their easy availability, proven strength, light weight and above all, durability. However, the major problem associated with the plastics is their non-degradability, which poses a significant threat to the environment. Moreover, as landfill sites for safe disposal of plastic become limited, indiscriminate disposal of plastics are causing water and land pollution problems. Plastics can enter into drainage

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lines and choke them resulting in floods in local areas. Plastics are also a major marine pollutant throughout the world and thus affect marine ecosystem. Additionally indiscriminate disposal of plastic in hill areas and its deposition in soil over time causes landslides. Such environmental issues have raised concern regarding the usage of plastics. However, as plastics have become an integral part of modern life style, it is extremely difficult to avoid using these materials. Therefore, the current situation demands new tools and technologies for the remediation of plastic-related environmental pollution. Microbes are known for surviving in niches that are rich in recalcitrant material such as synthetic polymers. This observation suggests that microbes surviving in such environments may have potential to utilize synthetic polymers that are apparently not biodegradable and thus such microbes could provide valuable tools to remediate plastic wastes in a cost effective manner. Consistent with this idea, there are reports of microbial degradation of synthetic polymers such as polyurethanes (PUR) (Barratt et al. 2003; Kim and Rhee 2003; Cosgrove et al. 2007; Nakajima-Kambe et al. 1999, 1995).

PUR, derived from the condensation of isocyanates and polyalcohols, are an important and versatile class of thermoplastic non biodegradable polymer. They are used in a wide variety of products in domestic, medical, automotive and industrial fields. In the previous studies, both bacteria and fungi have been shown to degrade PUR. However, majority of the reports describe fungus-mediated degradation. With the increasing application of plastics, several synthetic polymers and raw material for plastic manufacturing industry are being developed that are chemically diverse. Polyurethane diol (PUR-diol) is one of such type of polyurethanes that is used as a modifier for water-soluble resins and emulsions in wood, appliances and auto coatings. However, no biodegradation studies have been carried out till date on PUR-diol and this prompted us to make an effort for the isolation of microbes able to degrade PUR-diol.

In this study we report isolation of a bacterium from soil that can efficiently degrade PUR-diol. We have also developed a sensitive method utilizing High Performance Thin Layer Chromatography (HPTLC) to quantify the degradation ability of this organism. In addition, we have made attempts to gain an insight into the mechanism involved in the degradation process.

## Materials and methods

### Growth medium

The selection and culture of the isolated organism was carried out in a medium containing PUR-diol (Sigma–Aldrich) (average mol.wt. ~320) as sole carbon source. The medium comprises a basal medium {0. 2% $KH_2P0_4$; 0.7% $K_2HP0_4$; 0.1% $(NH_4)_2S0_4$; 0.01% $MgS0_4.7H_20$; 0.0001% $ZnS0_4.7H_20$; 0.00001% $CuS0_4.7H_20$; 0.001% $FeSO_4.7H_20$; 0.0002% $MnS0_4.H_20$; the final pH was 7.2} supplemented with either 1.0% PUR-diol or 0.3% Impranil DLN$^{TM}$ as required. Cells were also grown in Luria broth {NaCl (10 $gl^{-1}$), Tryptone (10 $gl^{-1}$), yeast extract (5 $gl^{-1}$)} as required. 2.0% agar was added for making solid medium.

### Collection of the soil samples and isolation of bacteria

Soil samples were collected from different solid waste dumping sites that are rich in plastic disposal at Kolkata (formerly known as Calcutta), India. Samples were collected 5 cm below the land surface in sterile containers. One gram of each soil sample was dispersed in 9 ml of 0.85% NaCl in sterile test tubes. Thereafter, a series of dilution from $10^{-2}$ to $10^{-10}$ were prepared in 0.85% NaCl. A 0.2 ml aliquot of the appropriate dilution was spread aseptically onto agar plates containing PUR-diol as sole carbon source. Plates were incubated at 37°C for 48–72 h.

### Rhodamine plate assay

This assay was performed as described previously (Howard et al. 2001). PUR-diol-rhodamine B agar plates were prepared by adding rhodamine B (0.001% wt/vol) to the selection medium containing 1.0% PUR-diol as sole carbon source. Wells of 1.0 cm diameter were made and loaded either with the cell lysate or with an overnight saturated culture of the organism. The plates were incubated at 37°C for 48 h. After the incubation plates were visualized under UV light.

### Thin Layer Chromatography (TLC)

TLC aluminium sheet coated with silica gel 60 $F_{254}$ (Merck) was used for this assay. Aliquots (500 µl) of

either, the inoculated or the non-inoculated media, incubated at 37°C for 5 days in shaking conditions, were extracted with equal volumes of ethyl acetate. 10 µl of each of these extracts was spotted on the TLC plate and chromatographic separation was carried out using a solvent system composed of 6:2:1 ethyl acetate, n-hexane and methanol. After the run, 5% ethanolic sulphuric acid was sprayed over the plate, and heated until spots appeared.

### High Performance Thin Layer Chromatography (HPTLC)

Hundred and fifty milliliter of medium containing PUR-diol as sole carbon source was inoculated with the organism and incubated at 37°C. Thereafter, 1 ml aliquot of the culture medium was taken out at different time points of growth (as indicated in Fig. 2) and centrifuged. Cell free conditioned media was subjected to ethyl acetate extraction and then applied for HPTLC analysis. A Camag (Switzerland) HPTLC system with a Linomat V sample applicator, a Camag twin trough plate development chamber, Camag TLC scanner 3 and Wincats integration software were used in the present work. Aluminum backed HPTLC plates of 0.2 mm layers of silica gel 60 $F_{254}$ (Merck) was used in this assay. Ten microliter of organic extract of each of the sample solutions were applied on the plate. The chromatographic run was made in twin-trough chamber saturated with a solvent system composed of ethyl acetate and hexane in 3:1 ratio. After the run, solvent was evaporated from the plate and dipped into freshly prepared solution of 5% ethanolic sulphuric acid. Thereafter, the plate was heated in an oven for 3 min at 150°C. After color development, plates were scanned in absorbance/transmittance mode at 450 nm with 40 mm/s scan speed.

### Esterase plate assay

For this assay, 20.0 g of a tributyrin agar base (Fluka) containing peptone and yeast extract was added to 1 l water. Thereafter, it was autoclaved. To it, sterile glyceryl tributyrate (Sigma-Aldrich) and rhodamine B was added sequentially to a final concentration of 1.0 and 0.001% respectively. Thereafter, it was poured into petridishes. Saturated cultures of the isolated organism and *E. coli* were loaded in wells made on this agar plate and incubated at 37°C for 48 h. After the incubation the plate was visualized under UV.

### UV mutagenesis

One ml saturated culture from the isolated microorganism was centrifuged, washed and resuspended in 0.85% NaCl. Thereafter, the cell suspension was exposed to 15 Watt UV-bulb, (Philips India Ltd., India) at a distance of 30 cm for a predetermined period of 5 min such that it did not exceed 50% killing of the organism. Thereafter, cells were plated onto Luria agar plates and incubated overnight at 37°C. Approximately 1000 colonies from this plate were streaked on Luria agar as well as plate containing PUR-diol as sole carbon source. After 48 h incubation, candidates that showed impaired growth only on PUR-diol containing plate were selected for further study.

### p-nitro phenyl acetate (pNPA) assay

Cells were grown at 37°C to mid log phase and harvested. Cell lysates were made in lysis buffer containing 50 mM Tris–Cl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 50 mM PMSF and 1.0% sodium deoxycholate. Protein content of the lysate was estimated by Folin–Lowry method as previously described (Lowry et al. 1951). Thereafter, esterase activity was measured by incubating cell lysates containing 50 µg of total protein with 50 mM of pNPA (Hi-Media) at 37°C for 1 h. After the incubation, reaction was stopped by the addition of 0.1 N NaOH. Absorbance of esterase breakdown product of pNPA, was measured spectrophotometrically at a wavelength of 405 nm.

### Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The breakdown product of PUR-diol was identified by GC-MS as described previously (Nakajima-Kambe et al. 1997). Cells were grown for 7 days in medium containing PUR-diol as the sole carbon source. Thereafter, 5 ml culture was centrifuged. The cell-free supernatant was acidified to pH 1.0 with 1 N HCl and extracted three times with ethyl acetate. Two microliter of the organic extract was then injected into a DB-17 capillary column (0.25 mm × 30 m) attached to a gas chromatography/mass spectrometry

apparatus (model JMS-700, Thermo Scientific, USA). The injection temperature was 230°C. The oven temperature program was as follows: 80°C initial, 230°C final, and rate of increase 10°C/min. Helium was used as the carrier gas.

### Isolation of genomic DNA

Bacterial genomic DNA was prepared by CTAB/NaCl method essentially as described previously (Ausubel et al. 1994). Briefly, 1.5 ml of the saturated culture was centrifuged for 5 min. The cell pellet was resuspended in 567 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8). Thereafter, 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase-K were added. The solutions were mixed thoroughly and incubated at 37°C for 1 h. To this, 100 µl of 5 M NaCl and 80 µl of CTAB/NaCl solution was added and incubated at 65°C for 10 min to remove cell wall debris, denatured proteins and polysaccharides. Thereafter, chromosomal DNA was extracted with an equal volume of a mixture of chloroform:isoamyl alcohol (24:1). The aqueous phase was again extracted with phenol:chloroform:isoamyl alcohol mixture (25:24:1). The chromosomal DNA was precipitated by adding 0.6 volume of 2-propanol and centrifuged at 12000*g* for 10 min. The DNA pellet was then washed with 70% ethanol, dried and dissolved in 50 µl TE buffer.

### Ribotyping and construction of phylogenetic tree

Partial amplification of the 16S rRNA gene was done by performing PCR with bacteria specific universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492 R (5′-TACGGTTACCTTGTTACGACTT-3′) and genomic DNA isolated from the organism as template. The amplified PCR product was gel purified and sequenced in automated genetic analyzer (ABI 3100, USA) using primers 27F (5′-AGAGTTTGATC CTGGCTCAG-3′) and 1492 R (5′-TACGGTTACCTT GTTACGACTT-3′). Sequencing reaction was performed using Big Dye Terminator Cycle sequencing Kit V3.1 (Applied Biosystems, USA) following manufacturer's protocol. The partial 16S rDNA sequence of the isolated organism was compared by BLAST search analysis (http://www.blast.ncbi.nlm.nih.gov) with those available in the public databases. Thereafter, identification to the species level was determined by 16S rDNA sequence similarity of >95% with that of the prototype strain sequence in the GenBank (http://www.ncbi.nlm.nih.gov/genbank).

To construct phylogenetic tree sequence alignment of the 16S rDNA fragment and comparison was performed using the multiple sequence alignment program CLUSTAL X (v 1.83) with default parameters and the data converted to PHYLIP format. Thereafter, bootstrap analysis was performed as described previously (Felsenstein et al. 1981, 1985), on several random samples taken from the multiple alignment analysis. Minor modifications in the alignment were made using the BIOEDIT sequence editor. Thereafter, unrooted phylogenetic tree was constructed using neighbor-joining (NJ) method. TREEVIEW (Win32) program was used to display the phylogenetic relationship.

### Metabolic fingerprint analysis by BiOLOG

Metabolic fingerprint analysis of the organism was performed by examining the carbon source utilization pattern using GN2 microplate system in BiOLOG instrument (Biolog, Inc., USA) as per manufacturer's protocol. Briefly, the organism was streaked onto Biolog universal media (BUG) plate and incubated for overnight at 37°C. Next day, single colonies were picked and inoculated in a special gelling inoculating fluid (GN-IF) at the recommended cell density. Thereafter, 150 µl of the cell suspension was added onto each well of GN2 micro plate containing tetrazolium dye and different carbon source. During the incubation because of respiration the dye gets reduced resulting in purple colour. Intensity of the purple colour will depend on the carbon source utilization ability of the organism in each well. The color intensity of each well was measured and then keyed into Biolog Microsoft computer, which automatically cross references the pattern to an extensive library of species. Based on the adequate match, the identification of the organism was made.

## Results

### Isolation of a soil bacterium that can utilize PUR-diol as sole carbon source

To isolate microbe(s) that can survive using PUR-diol as sole carbon source, soil samples were collected

from sites rich in plastic waste and dissolved in normal saline (0.85% NaCl). Different dilutions of soil samples were spread on agar plates containing PUR-diol as sole carbon source. Plates were incubated for 48–72 h at 37°C. After the incubation period several colonies grew on selection plates. Among these, bacterial candidates were selected based on colony morphology and microscopic examination. For further confirmation these candidates were streaked again onto plates containing PUR-diol as sole carbon source. The candidate that exhibited best growth was chosen for further studies. Figure 1a (right panel) shows that the isolated organism can grow well in solid medium containing PUR-diol as sole carbon source. In contrast, *E. coli* exhibits no or very little growth on this plate. The most likely explanation for this observation is that the isolated organism can degrade PUR-diol and is able to utilize the breakdown products as carbon source for its survival. However, it could also be possible that the isolated organism is able to fix carbon from air or alternatively it is able to degrade agar used in solid medium and utilize the breakdown products of agar. To rule out these possibilities, we have examined the growth of the isolated organism in both liquid and solid medium having identical compositions except these media lacks PUR-diol. Our results showed no growth of the isolated organism in selection medium lacking PUR-diol (data not shown). Failure to grow in medium lacking PUR-diol excludes the possibility that the isolated organism utilizes either aerial carbon dioxide or agar present in the solid medium for its survival on selection plate.

To examine the ability of this organism to degrade another variety of commercially available, widely used polyester PUR we tested its ability to grow in agar plates containing Impranil DLN$^{TM}$ as sole carbon source. Our results showed considerable growth of this organism on plate containing Impranil DLN$^{TM}$ as sole carbon source. A clear zone surrounding the growth of the organisms was appeared (Fig. 1a, middle panel) that can only be attributed to the utilization of Impranil DLN$^{TM}$ by the isolated organism.

To further confirm, that the isolated organism is able to degrade PUR-diol, we performed thin layer chromatography (TLC) and rhodamine plate assay. In the TLC assay, we investigated the amount of PUR-diol present in media, which were either inoculated or not inoculated with the isolated organism after 5 days of incubation at 37°C. The result showed a significant reduction in spot intensity of PUR-diol in the medium inoculated and incubated with the organism compared to the non-inoculated medium of similar volume (Fig. 1b, Supplementary Fig. 1). Co-migration of the spots in lanes 1 and 2 with the spot of the pure PUR-diol (lane 3) confirms that the spots in lane 1 and 2 are PUR-diol. This result clearly demonstrated the utilization of PUR-diol by the isolated organism. In rhodamine plate assay, we exploited the fluorescent property of the rhodamine. Upon irradiation with UV, it emits pink fluorescence when conjugated with the PUR-diol in an agar medium containing both. If an organism breaks down PUR-diol during its growth then there will be a reduction in the level of PUR-diol-rhodamine complex in the vicinity of the growth and a concomitant reduction in pink fluorescence. For this assay, either the saturated culture was spotted or the cell lysate prepared from saturated culture was applied to wells on PUR-diol plates containing rhodamine B and incubated for 48 h at 37°C. After the incubation, plates were visualized under UV. Results showed clear zone surrounding either the growth of the organism (Fig. 1c, left panel) or the well where the lysate was applied (Fig. 1c, right panel) indicating the breakdown of PUR-diol. Based on these results it can be concluded that the isolated organism is able to utilize PUR-diol as sole carbon source and thus can survive on selection plate.

## Isolated organism degrades PUR-diol efficiently

To quantify the efficacy of PUR-diol degradation, we have developed an HPTLC-based sensitive assay to measure the amount of PUR-diol present in the conditioned medium at different time points during the growth of this organism. In this assay we first established $R_f$ value of PUR-diol under the experimental condition. Thereafter, components of the conditioned medium were separated under the same experimental condition. Based on the $R_f$ value the peaks corresponding to PUR-diol were identified and marked with * in Fig. 2a. The area under each peak that represents the amount of PUR-diol present in the medium were determined and plotted. Figure 2b shows the relative amount of PUR-diol present in the conditioned medium at indicated time points. Our results showed that after 10 days of incubation the

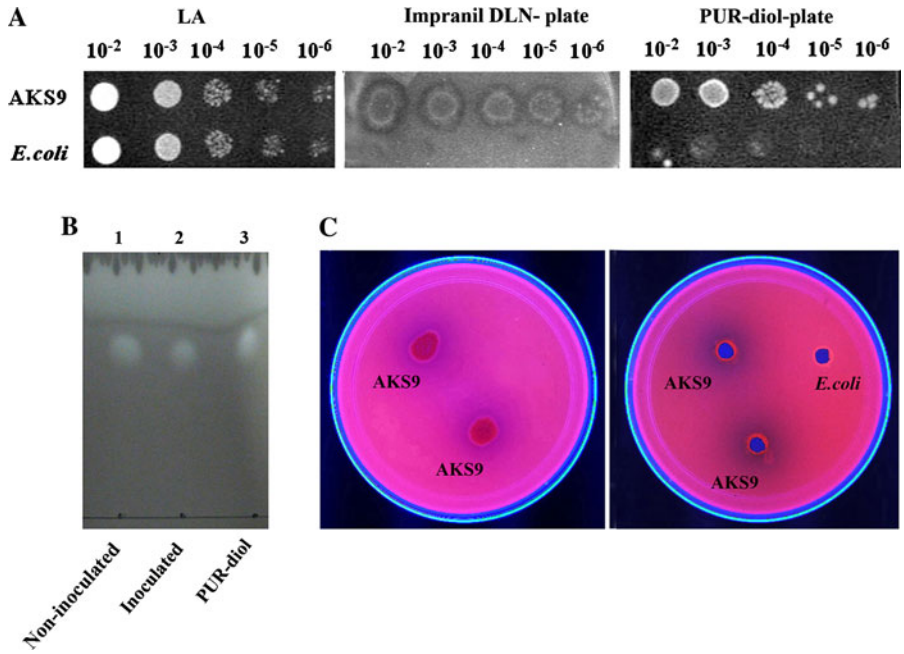


**Fig. 1** Isolated organism degrades PUR-diol. **a** Isolated organism can survive on plate containing PUR-diol as sole carbon source. Equal number of cells from a saturated culture was spotted in different dilutions on LA plate (*left panel*), plate containing Impranil DLN™ as sole carbon source (*middle panel*) and on plate containing PUR-diol as sole carbon source (*right panel*). **b** TLC assay for PUR-diol in the inoculated and non-inoculated media. The inoculated and the non-inoculated media obtained after the incubation for 5 days at 37°C and pure PUR-diol were extracted with ethyl acetate. Ten microliter from each of these organic extracts were spotted on silica gel plate and run using a solvent system composed of 6:2:1 ethyl acetate, n-hexane and methanol. Spots were visualized by staining with 5% ethanolic sulphuric acid followed by heating. This data is representative of five independent experiments with identical results. **c** Rhodamine plate assay. Either 5 µl of the saturated culture or 20 µl of cell lysates obtained from the same culture of the isolated organism were respectively spotted (*left panel*) or loaded into the wells (*right panel*) on rhodamine B containing PUR-diol plates. Thereafter, the plates were incubated for 48 h at 37°C. Lysate from *E. coli* culture was used as negative control. AKS9 indicates the isolated organism

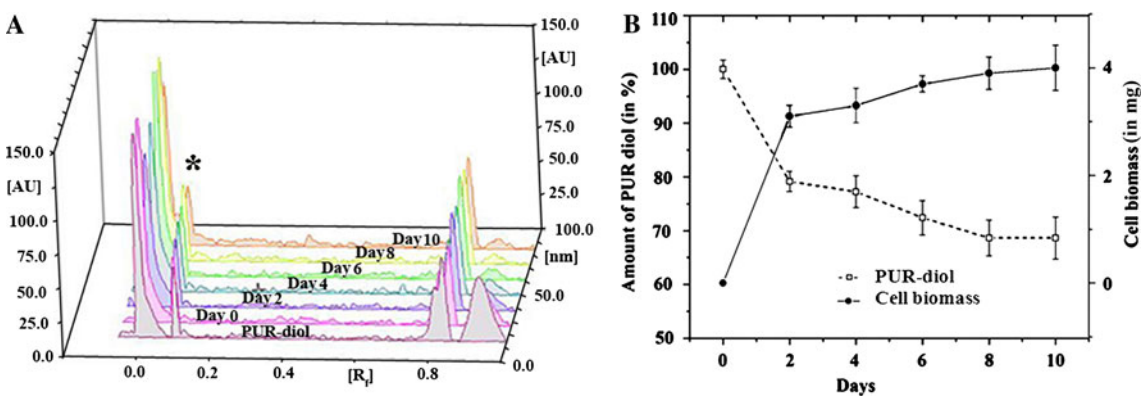


**Fig. 2** PUR-diol was significantly reduced in conditioned medium. **a** Band profile obtained after HPTLC analysis of the pure PUR-diol and conditioned media at days 0, 2, 4, 6, 8, 10. Peak corresponding to PUR-diol is marked with asterisk (*). **b** Densitometric analysis of the PUR-diol bands and cell biomass at the indicated time points. This data is representative of three independent experiments with identical results. *Error bar* indicates standard deviation (±SD)

amount of PUR-diol in the medium was reduced by 32%. However, the maximum depletion was observed in first 48 h (Fig. 2b) and during this period 22% of PUR was found to be degraded. Thus the result showed a considerable breakdown of PUR-diol by the isolated organism.

## Esterase activity is involved in PUR-diol breakdown by the isolated organism

Previous studies indicate that an esterase activity is involved in PUR degradation (Akutsu et al. 1998). Therefore, the PUR-diol degradation performed by our isolated microorganism may also require an esterase activity. To check whether the isolated organism shows any esterase function, plate assay with tributyrin, a substrate for esterase, was performed (Kouker and Jaeger 1987). In this assay, 5 µl of the overnight culture was spotted on a plate containing tributyrin and rhodamine B, incubated at 37°C for 48 h and visualized under UV light. A clear zone surrounding cell growth was observed (Fig. 3a), indicating the substrate utilization by an esterase. This result shows that the isolated organism has

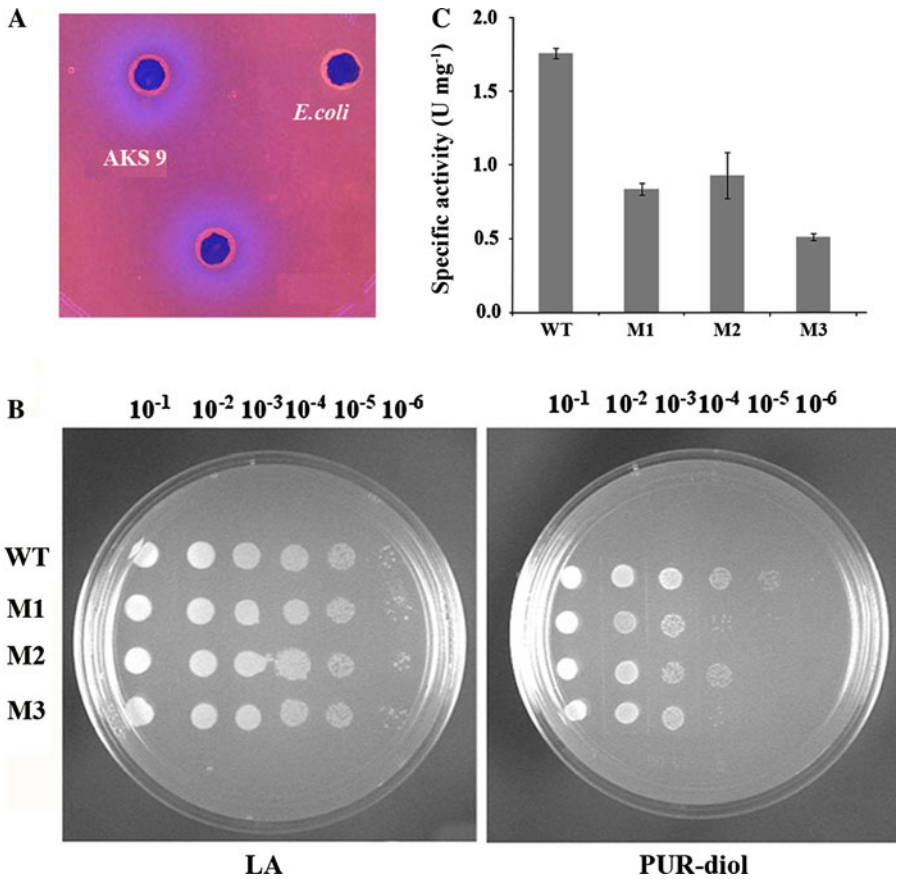


**Fig. 3** Esterase activity is involved in PUR-diol breakdown event. **a** Isolated organism exhibits esterase activity. Ten microliter of the saturated culture of the organism was applied in holes punched in agar plate containing tributyrin (a substrate for esterase) and rhodamine B and incubated at 37°C for 48 h. After the incubation plate was visualized under UV. AKS9 indicates the isolated organism. *E. coli* was used as a negative control. **b** Mutants exhibit growth defect only on plate containing PUR-diol as sole carbon source. Wild type and mutant strains obtained after UV-mutagenesis were grown in PUR-diol-broth up to saturation and different dilutions (containing equal number of cells) were spotted on LA plate (*left panel*) and on plate containing PUR-diol as sole carbon source (*right panel*) and incubated at 37°C. **c** Mutants exhibit reduced esterase activity. Mutants and wild type strains were grown to mid log phase in PUR-diol broth and harvested. Lysates were made and pNPA assay was performed utilizing equal amount of protein. Specific activity for each was determined and plotted. U denotes activity of the enzyme. *Error bar* indicates standard deviation (±SD)

considerable esterase activity that could act on ester bonds present in PUR-diol and cause PUR-diol degradation.

To verify the possible involvement of esterase(s) we have isolated loss of function mutants of the isolated organism by UV mutagenesis (see "Materials and Methods"). Three candidates were selected based on considerable growth impairment on selection plates containing PUR-diol as the sole carbon source compared to wild type cells (Fig. 3b, right panel). However these candidates exhibited growth similar to wild type when they were spotted on Luria agar plate (Fig. 3b, left panel). This result demonstrates that isolated mutants exhibit growth abnormality only with respect to utilization of PUR-diol as sole C-source. We also examined the PUR-diol degrading ability of these mutants by TLC. Consistent with the impaired growth, mutants showed reduced ability to degrade PUR-diol in medium compared to the wild type (Supplementary Fig. 2). Thereafter, we examined the esterase activity of these mutants by pNPA assay as described in "Materials and Methods" to test whether this loss of function is associated with the altered esterase function of the isolated organism. The result of pNPA assay showed significant reduction in esterase activity in the lysates obtained from mutants than esterase activity present in cell lysate from the wild type strain (Fig. 3c). This result suggests a correlation between esterase activity of the organism and its PUR-diol utilization ability, which can only be possible if esterase activity is involved in PUR-diol degradation.

If esterase activity is involved in the breakdown of PUR-diol, similar to PUR-breakdown PUR-diol breakdown event may generate a polyol. To examine the presence of any polyol in the conditioned medium, we performed GC-MS analysis using DB17 column as described in "Materials and Methods". For this experiment the organic extract of the conditioned medium obtained after the growth at 37°C for 7 days and non-inoculated medium incubated at the same condition were subjected to GC-MS. GC-MS analysis showed the presence of a peak (marked with *) only in the conditioned medium (compare Spectrum II and Spectrum III of Fig. 4a). The corresponding mass spectra of this peak showed an *m/z* value of 45.0 (Fig. 4b, Spectrum II) which is found to be similar to the *m/z* value of diethylene glycol, a known polyol, obtained from existing database. To confirm the peak is indeed diethylene glycol, we determined the retention time of pure diethylene glycol under the same experimental condition and indeed it was found to be similar to that observed for the peak obtained from conditioned medium (Fig. 4a, Spectrum I, marked with *). The corresponding mass spectra of this control diethylene glycol also showed a peak with *m/z* value of 45.0 (Fig. 4b, Spectrum I). Since this peak was identified as diethylene glycol, a known polyol, and absent in the non-inoculated medium, we consider diethylene glycol to be a PUR-diol breakdown product resulting from microbial activity. The polyol product can only be obtained by hydrolytic cleavage of ester bond present in PUR-diol and thus the PUR-diol degradation by the isolated organism can be attributed partly, if not fully, to its esterase activity.

## PUR-diol utilizing bacteria was identified to be a strain of *P. aeruginosa*

To identify the isolated organism, 16S r DNA sequencing was performed. Segment of 16S r-RNA gene was amplified by PCR using specific primers and the amplified product was sequenced. BLAST search (http://www.blast.ncbi.nlm.nih.gov) analysis was carried out for the obtained 16S rDNA in the GenBank (http://www.ncbi.nlm.nih.gov/genbank) database. Results showed a sequence identity of 99.0% with *P. aeruginosa.* The 16S rDNA sequence was submitted to GenBank (Accession no: GQ203623).

As the BLAST analysis revealed the alignment of the 16S rDNA sequence with several species of *Pseudomonas* genus, we used the most similar sequences to construct a phylogenetic tree for our organism. The phylogenetic relationship was inferred using the maximum-likelihood approach. It was evident from the phylogenetic tree (Fig. 5) that the isolated organism lies in gamma proteobactor order of gram negative bacteria *Pseudomonas* and showed a close relationship with its nearest neighbours. The isolated organism was assigned as *P. aeruginosa* AKS9.

To further characterize the isolated organism, several biochemical tests including gram staining were performed. The results showed that the isolated organism is a rod shaped bacteria with gram-negative character (data not shown). In addition, the organism was found to be oxidase, catalase and lipase positive but negative in indole and hydrogen sulphide production and urease test (Table 1). All these results are

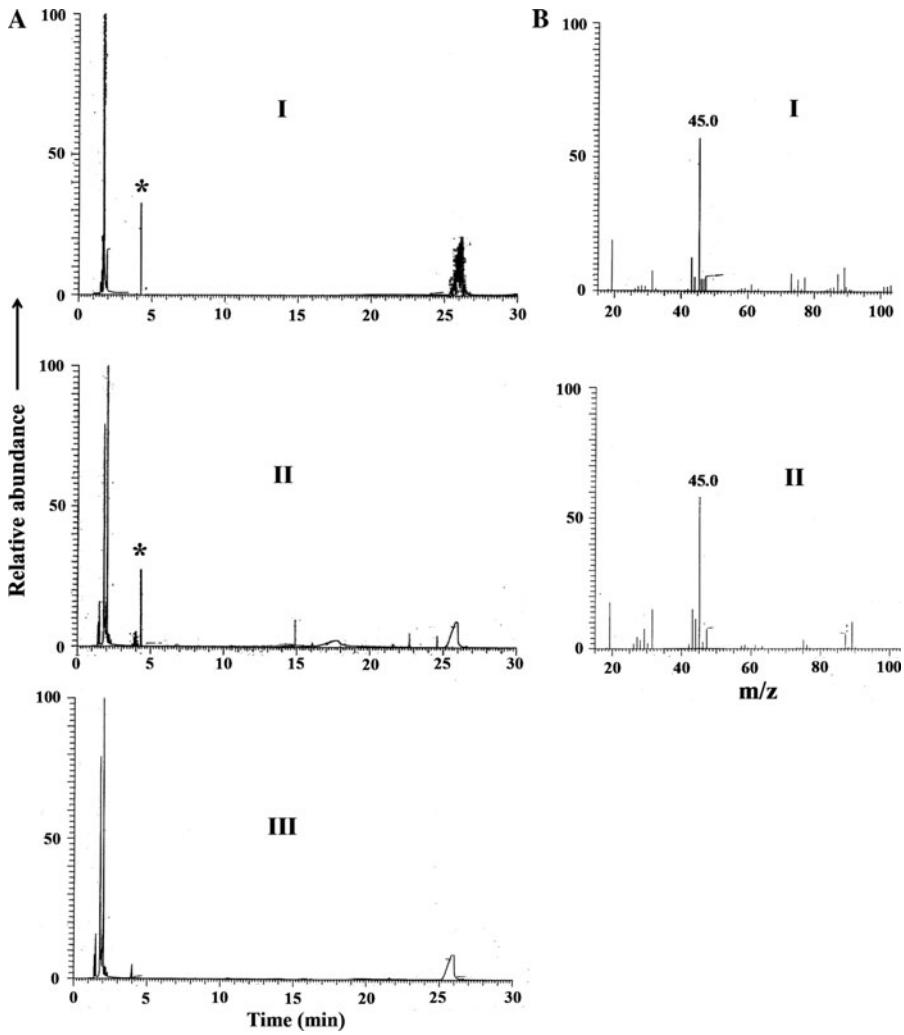


**Fig. 4** PUR-diol breakdown by the organism generates diethylene glycol in the medium. **a** Gas chromatograms of pure diethylene glycol (Spectrum I), organic extract from conditioned medium (Spectrum II), and from non-inoculated medium (Spectrum III). Peaks corresponding to diethylene glycol are marked with *. **b** Mass spectrum for the peaks obtained in GC analysis. Peaks for diethylene glycol are marked with its *m/z* value (45.0)

consistent with the different phenotypic characteristic properties of *P. aeruginosa.* We have also examined the metabolic fingerprinting for the organism by analyzing its carbon source utilization spectrum using GN2 microplate system in BiOLOG as described in "Materials and Methods". Consistent with the ribotyping result, C-source utilization spectrum was found to match with that of *P. aeruginosa* from database. Thus, all these results indicate that the isolated organism is a strain of *P. aeruginosa*.

## Discussion

To address the problem of bioremediation of synthetic polymers, we have explored the ability of microorganisms surviving in an environment rich in apparently non-biodegradable waste. Among different synthetic polymers that are marketed, the biodegradability of a polymer called PUR-diol, which has wide range of application in the plastic industry, was investigated. There are reports of microbial degradation of PUR and majority of these studies report fungus mediated degradation (Howard 2002). Therefore, in this study we aimed at isolating organism of bacterial origin that can degrade PUR-diol. Based on the survival ability on selection plate containing PUR-diol as only carbon source, we obtained several organisms capable of utilizing PUR-diol. However, as mentioned before we proceeded with an isolate of bacterial origin that exhibited best growth on the selection plate. The TLC analysis and rhodamine-based plate assay clearly demonstrate the ability of this isolate to degrade PUR-diol.

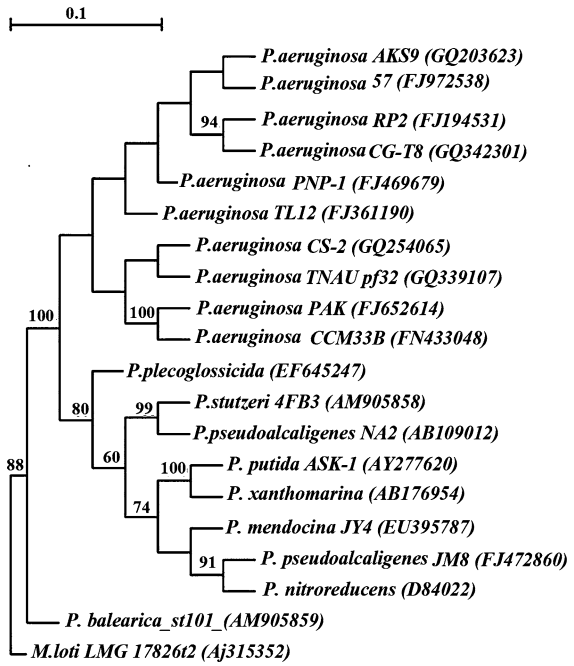


**Fig. 5** Phylogenetic tree for the isolated organism. Unrooted tree based on 16S rDNA sequence showing the phylogenetic relationships of *P. aeruginosa AKS9* with its nearest neighbours. Bootstrap values (expressed as percentages of 1,000 replications) are given at the branching points. Bar indicates 0.1% sequence divergence. *Mycobacterium loti* LMG 17826t2 (AJ 315352) was used as outgroup

**Table 1** Biochemical characteristics of the isolated organism

| Biochemical test | Results |
|---|---|
| Oxidase test | + |
| Catalase test | + |
| Indole production test | − |
| Citrate utilization test | + |
| Hydrogen sulphide production test | − |
| Motility test | − |
| Oxygen tolerance test | Aerobic |
| Lipase production | + |
| Nitrate reduction test | + |
| Urease test | − |

16S rDNA sequence analysis and biochemical tests revealed the isolated organism to be a strain of *P. aeruginosa*. There are reports of polyurethane degradation by several species of *Pseudomonas*. In a previous report laboratory strains of *P. aeruginosa* and *P. putida* have been shown to degrade PUR-paints in military aircrafts (El-Sayed et al. 1996). There are also reports of polyester PUR degradation by others *Pseudomonas* species like *P. fluorescens* (Howard and Blake 1999) and *P. chlororaphis* and (Ruiz et al. 1999). In a different study, Kay et al. (1991) showed the ability of a *P. aeruginosa* strain to degrade PUR only in presence of Corynebacteria. These two isolates together can degrade PUR upon incubation for a period of 12 weeks. In the same study, seven other bacteria were also shown to degrade PUR only when the media was supplemented with yeast extract. In contrast, the *P. aeruginosa* strain that we have isolated from soil by itself markedly degrades PUR-diol within a very short period of time. It can even exhibit this ability to degrade PUR-diol in a medium that is completely devoid of yeast extract (data not shown). The efficiency of this organism to degrade PUR-diol was also highlighted in this study by looking into the kinetics of PUR-diol depletion with time. None of the previous reports were directed towards addressing this important issue. Most of the earlier studies were directed only towards identifying organisms capable of degrading various forms of PUR. These studies tested PUR degradability by either changes in physical properties like tensile strength or by looking into the formation of some clear zone in the plate assay. Few reports also involved the measurement of weight of the soil buried polyurethane or polyurethane cubes. With the existing information it is very difficult to have an idea about the efficacy of PUR-degradation by these previously identified organisms. In this context, for the first time we exploited HPTLC to follow the degradation process of a polymer quantitatively. The HPTLC-based assay clearly demonstrates that in 10 days of time the isolated organism can degrade 32% of total PUR-diol present in the growth medium. It also shows that the majority of PUR-degradation by this organism took place during the first 48 h of growth. Interestingly, this time period corresponds to the logarithmic growth phase of this organism in this special medium (Fig. 2b), indicating a linear relation between PUR-breakdown and the growth of the organism. This result strongly supports the view that the isolated organism degrades PUR-diol and utilizes it for growth and survival. The direct correlation between PUR-degradation and growth of this organism also validates the sensitivity of this newly adopted method. Therefore, this HPTLC-based assay can be used to follow the kinetics of microbial

degradation of several other synthetic polymers that are extractable in organic solvent and thus can be an effective tool in comparing the efficiency of polymer degradation by different isolates.

Several soluble and membrane bound lipases, esterases, proteases and polyurethenase are reported to be involved in the breakdown of different types of PUR (Howard and Blake 1999; Ruiz et al. 1999; Akutsu et al. 1998; Vega et al. 1999). In this study, by isolating mutants we have shown that esterase activity is involved in the breakdown event of PUR-diol by this organism. These mutants exhibit reduced esterase activity and are defective in their ability to utilize PUR-diol as sole carbon source. However, we failed to obtain any mutant exhibiting complete loss of PUR-diol utilization property. This result indicates that the degradation of PUR-diol by this organism may involve more than one esterase or different enzyme activities in addition to esterase.

In an effort to gain further insight into the degradation process of PUR-diol, we have performed GC-MS analysis of the conditioned medium for the detection of some break down products. Despite the wide application of PUR-diol in polymer industry, no chemical structure of this compound is available in the literature. Conventionally polyol is linked to isocyanate by an ester bond in PUR. Given that esterase activity is involved in the PUR-diol breakdown, the generation of a polyol compound like diethylene glycol is likely after the breakdown of PUR-diol. Congruent with the expectation, GC-MS results showed the presence of diethylene glycol only in the conditioned medium. This result not only bolstered the involvement of esterase activity in PUR-diol breakdown but also confirms the presence of diethylene glycol as the polyol segment of the PUR-diol used in this study.

In this study for the first time we report the isolation, identification and elucidation of PUR-diol degrading property of a soil bacterium *P. aeruginosa* AKS9. This ability to degrade PUR-diol adds a new facet to the diverse functional activities of *P. aeruginosa*. Our study revealed that the ability of this isolated organism to degrade PUR-diol is mediated partly, if not fully, by its esterase activity. We also provided a new assay system based on HPTLC to quantify polymer degradation. In our case this assay revealed a marked reduction of PUR-diol only within 2 days. Isolation of an organism from soil rich in plastic disposal where polymers are an abundant carbon source holds the promise to provide with a potent source of polymer degrading enzyme(s).

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**Conflict of interest** Authors declare no conflict of interest.

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