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Atrazine biodegradation efficiency, metabolite detection, and *trzD* gene expression by enrichment bacterial cultures from agricultural soil

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Abstract: Atrazine is a selective herbicide used in agricultural fields to control the emergence of broadleaf and grassy weeds. The persistence of this herbicide is influenced by the metabolic action of habituated native microorganisms. This study provides information on the occurrence of atrazine mineralizing bacterial strains with faster metabolizing ability. The enrichment cultures were tested for the biodegradation of atrazine by high-performance liquid chromatography (HPLC) and mass spectrometry. Nine cultures JS01.Deg01 to JS09.Deg01 were identified as the degrader of atrazine in the enrichment culture. The three isolates JS04.Deg01, JS07.Deg01, and JS08.Deg01 were identified as efficient atrazine metabolizers. Isolates JS04.Deg01 and JS07.Deg01 produced hydroxyatrazine (HA) *N*-isopropylammelide and cyanuric acid by dealkylation reaction. The isolate JS08.Deg01 generated deethylatrazine (DEA), deisopropylatrazine (DIA), and cyanuric acid by *N*-dealkylation in the upper degradation pathway and later it incorporated cyanuric acid in their biomass by the lower degradation pathway. The optimum pH for degrading atrazine by JS08.Deg01 was 7.0 and 16S rDNA phylogenetic typing identified it as *Enterobacter cloacae* strain JS08.Deg01. The highest atrazine mineralization was observed in case of isolate JS08.Deg01, where an ample amount of *trzD* mRNA was quantified at 72 h of incubation with atrazine. Atrazine bioremediating isolate *E. cloacae* strain JS08.Deg01 could be the better environmental remediator of agricultural soils and the crop fields contaminated with atrazine could be the source of the efficient biodegrading microbial strains for the environmental cleanup process.

Key words: Atrazine, Biodegradation, *Enterobacter cloacae*, Deethylatrazine, Cyanuric acid, *trzD* gene
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1 Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropyl amino-1,3,5-triazine) is a member of chlorinated *s*-triazine group of herbicides, which is moderately mobile and highly persistent in soil (Cohen *et al.*, 1984). It is used as a selective herbicide to control emergence of broadleaf and grassy weeds in corn, pineapple, sorghum, cotton, and other crops and as a non-selective herbicide on non-cropped industrial lands and fallow lands (Seiler *et al.*, 1992; Ghosh and Philip, 2006).

The contamination of soil, groundwater and surface waters by herbicides poses major environmental problems. Annually, 340 t of atrazine of the technical grade was consumed and it was the second most highly consumed herbicide in India (Kadian *et al.*, 2008). At present, the most common approach is containment, which is a costly process and involves the removal of highly contaminated soil to landfill sites. Microbial bioremediation is an effective and cheapest process that can be used for degrading the toxic compounds present in contaminated environments (Ganesh-Kumar *et al.*, 2010; Kalimuthu *et al.*, 2011). Hence the bioremediation methods for in situ treatments are needed as alternative and supplementary approaches

for cost-effective and residue-free clean-up. Persistence of atrazine in soil and subsurface environments plays a major role in biodegradation of atrazine by the microorganisms. However, atrazine can be degraded mainly by biological processes *N*-dealkylation, dechlorination, and ring cleavage (Struthers *et al*., 1998).

Atrazine can be degraded by *Arthrobacter* sp. (Wang and Xie, 2012), *Chelatobacter heintzii* (Rousseaux *et al*., 2001), *Rhodococcus* sp., *Acinetobacter* sp., *Streptomyces* sp., *Pseudomonas aeruginosa*, *Clavibacter michiganense* (Popov *et al.*, 2005), *Enterobacter cloacae* (Shapir *et al.*, 2006), *Bacillus megaterium*, *Alcaligenes faecalis*, *Klebsiella ornithinolytica*, and *Agrobacterium tumefaciens* (Siripattanakul *et al.*, 2009). Under aerobic conditions, *Rhodococcus* strain TE1 can metabolize the atrazine into deethylatrazine (DEA) and deisopropylatrazine (DIA) (Behki *et al.*, 1993). *Pseudomonas* strain ADP metabolizes atrazine into cyanuric acid via three enzymatic steps, encoded by the genes *atzABC* (de Souza *et al.*, 1998) and cyanuric acid acts as a nitrogen source for many bacteria.

Atrazine mineralization pathway (Fig. 1) generally begins by hydrolytic dechlorination catalyzed by atrazine chlorohydrolase (*atzA* or *trzN* gene product) to produce hydroxyatrazine (HA). HA is converted into two different aminohydrolases encoded by *atzB* and *atzC* in the upper degradation pathway. Later, cyanuric acid is metabolized into carbondioxide by the hydrolytic reactions encoded by *trzD/atzD*, *trzE/atzE*, and *trzF/atzF* in the lower degradation pathway (Shapir *et al.*, 2002; Udiković-Kolić *et al*., 2010).

Persistence of agrochemicals in the arable soil is influenced by the metabolic action of habituated native microorganisms and understanding of those microorganisms involved in removal of pollutants from the contaminated regions is a prime natural resource in maintaining the environmental health. This work provides information on the occurrence of several atrazine-degrading bacterial strains with faster metabolizing ability. In this study, the atrazine-degrading bacteria were isolated in the agricultural field by an enrichment culture technique and the degradation ability was tested by analyzing the breakdown metabolites of atrazine. The efficient degrader was characterized in terms of 16S ribosomal RNA (rRNA) gene typing and atrazine catabolic gene expression. The efficient atrazine metabolizer was also found to harbor the gene cluster with *trzD* in catabolizing cyanuric acid in the upper degradation pathway.

2 Materials and methods

2.1 Soil sample collection

The soil samples were collected from an agricultural field cultivated with cotton and sorghum in Madurai, India. The sampling sites had been sprayed with atrazine as a pre-emergence herbicide for more than a decade to control broad leaf and grass weeds. The sampling sites had a red loamy soil type, pH 7.2 with organic matter of 1.45%. The top 10 cm of soil was collected randomly from 10 different sites, pooled together, air dried, graded through a 3-mm sized mesh, stored at 4 °C, and used for enrichment and isolation of atrazine-degrading microorganisms.

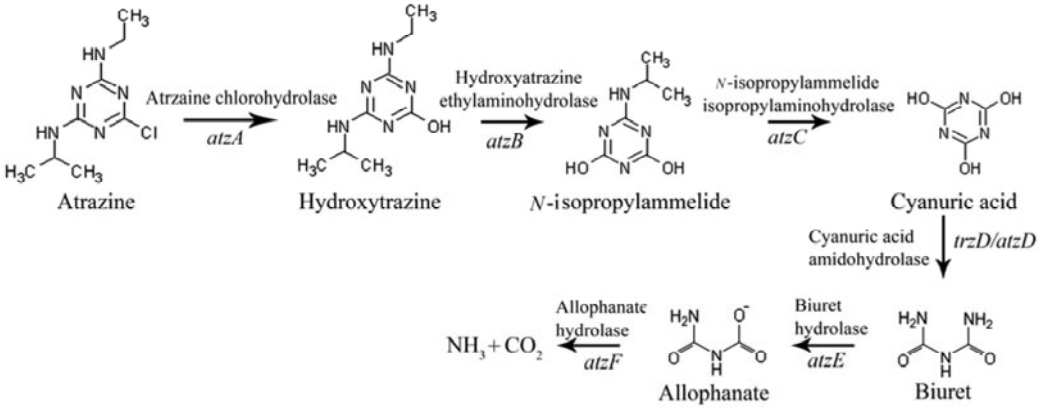

Fig. 1 Atrazine biodegration pathway illustrating the genes catalyzing the hydrolytic reactions

2.2 Enrichment and isolation of atrazine-degrading microorganisms

Atrazine (99% purity) was purchased from Sigma-Aldrich, India, for enrichment and degradation analyses. The composition and physical condition of the minimal medium were adopted according to Seto *et al.* (1995) with slight modification: 1.7 g KH_2PO_4, 9.8 g Na_2HPO_4, 1.0 g $MgSO_4{\cdot}7H_2O$, 0.95 mg $FeSO_4{\cdot}7H_2O$, 10.75 mg MgO, 2.0 mg $CaCl_2$, 1.44 mg $ZnSO_4$, 0.25 mg $CuSO_4{\cdot}5H_2O$, 0.25 mg $CoSO_4{\cdot}7H_2O$, 0.06 mg H_3BO_3, 51.3 μl concentrated HCl, 10 μmol/L $MnCl_2$, and 1 L deionized water (the medium with a final pH of 5). Enrichment of the atrazine-degrading microorganism was performed in 100 ml mineral medium supplemented with 2.5 mg atrazine (Topp *et al.*, 2000) as the sole carbon and nitrogen source from the 5 g soil sample and incubated aerobically by agitating at 200 r/min at 30 °C for 7 d.

After a week, 5.0-ml culture was transferred to a new flask, holding 100 ml fresh mineral medium with 100 μg/ml of atrazine and the enrichment process was repeated for two weeks by increasing the concentration of atrazine by 100 μg/ml every week. Enrichment cultures which degraded atrazine were monitored every week by streaking onto the atrazine mineral salt agar medium. The colonies which developed clear zones were pure cultured, and stored in 15% glycerol at −20 °C. Their morphological and physiological characteristics were determined by conventional biochemical methods (Cappuccino and Sherman, 2004).

2.3 Atrazine degradation analysis by high-performance liquid chromatography (HPLC) and mass spectrometry

Bacterial cell cultures incubated in a mineral medium with 0.1 mmol/L atrazine were centrifuged at 10000 r/min for 10 min, supernatant was filtered thrice through Whatmann No. 2 filter paper, and the final filtrate was filtered through a 0.22-μm filter. HPLC analysis was performed by a Shimadzu LC-6AD liquid chromatograph equipped with Shim-pack CLC ODS (4.6 mm×15 mm) reverse-phase C18 column, system controller-SCL-6B, and data processor-CR-5A, and the metabolites were detected by a UV-Vis (195–700 nm)-SPD-6AV detector. The samples were analyzed by an isocratic elution in the HPLC system with the mobile phase of 75% 0.05 mol/L KH_2PO_4 (pH 2.4) and 25% HPLC-grade acetonitrile with a flow rate of 1 ml/min and detection wavelength at 213 nm.

Atrazine and the degradation metabolites (2-HA, deisopropyl-2-HA, and cyanuric acid) were identified with the authentic standards and quantified by peak area integration of the HPLC chromatogram. Degradation metabolites of atrazine herbicide in the cell-free supernatants were determined by mass spectrometry with an ion trap mass spectrometer (LC-MS, Bruker, Germany) with electrospray ionization mode with nitrogen as a nebulizer gas at 1.12×10^5 Pa. The capillary voltage was set at 36000 V, and two skimmers of ion trap parameters set at 29.0 and 16.0 V respectively, and scanned from *m*/*z* 20 to 400 (Piutti *et al.*, 2003).

2.4 Genomic DNA isolation and 16S rRNA gene typing

A single colony of atrazine-degrading bacteria was grown in Luria-Bertani broth overnight at 37 °C and the genomic DNA was isolated by the alkaline lysis method according to Sambrook and Russel (2001). 16S rRNA gene specific universal primers 27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-GGTTACCTTGTTACGACTT-3′ were synthesized from Sigma-Genosys, India, and 16S rRNA gene was amplified using a reaction mixture containing 5 μl of 10× Taq-Buffer, 1.2 μl of 10 mmol/L dNTPs, 0.05 U of Taq DNA polymerase enzyme (Sigma, USA), 4 μl of each primer (10 pmol/L), and 2 μl of 10 ng template DNA. The amplification reaction was performed in a thermal cycler (MyCycler, Bio-Rad, USA) according to Satheeja Santhi and Jebakumar (2011). The polymerase chain reaction (PCR) product was resolved by electrophoresis in a 1% (w/v) agarose gel in 0.5× Tris-acetate-EDTA (TAE; pH 8.0) to confirm the expected size of the product. PCR-amplified 16S rRNA gene was purified and sequenced using Applied Biosystems 3730XL DNA Analyzer. Phylogenetic analysis was performed by the Ribosomal Database Project (RDP)-II database (Cole *et al.*, 2003), ClustalW by neighbour joining method, and the phylogenetic tree was analysed using PHYLIP 3.68 (Felsenstein, 2008) using bootstrapping over 1000 replicates.

2.5 Atrazine catabolic gene *trzD* transcript analysis

Total RNA was isolated from 1.5 ml bacterial culture JS08.Deg01 exposed to atrazine at 72 h using the diethyl polycarbonate (DEPC) (Summers, 1970). The complementary DNA (cDNA) complementary to

transcripts was prepared with SuperScript® II Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's instructions using oligo$(dT)_{25}$ as template-primer. The real-time PCR amplification was performed using the ABI PRISM 7900HT (Applied Biosystems) with 2.0 μl cDNA product. The amplification of targeted *atzD* gene (558 bp) was performed by the primers AtzD392F, 5′-ACGCTC AGATAACGGAGA-3′ and AtzD949R, 5′-TGTCGG AGTCACTTAGCA-3′. Likewise, to detect the *trzD* gene (663 bp), the primer pairs TrzD274F, 5′-CAC TGCACCATCTTCACC-3′ and TrzD936R, 5′-GTT ACGAACCTCACCGTC-3′ (Martinez *et al.*, 2001; Fruchey *et al.*, 2003) were used. The size of the PCR products was confirmed by electrophoresis on 1.5% agarose gel.

2.6 Nucleotide sequence accession number

The 16S rRNA gene sequence of the atrazine-degrading isolate *E. cloacae* strain JS08.Deg01 was deposited in the GenBank nucleotide database of the National Center for Biotechnology Information (NCBI) under the accession number of FJ810807.

3 Results

3.1 Enrichment, morphological, and physiological characteristics of atrazine-degrading bacteria

Nine bacterial isolates JS01.Deg01 to JS09.Deg01 which were degrading the atrazine, were isolated from atrazine-enriched mineral medium after the fourth week of enrichment, and they possessed a good growth rate and formed a clear zone on a mineral medium supplemented with atrazine agar plates. The bacterial cells that degrade the herbicide appeared as smooth glistening, opaque, and raised circular colonies on the surface of the atrazine-enriched agar minimal medium. Under a light and phase contrast microscope, the bacterial isolates were identified as oval to rod shaped cells and the Gram staining showed that they belonged to the Gram-negative type except JS04.Deg01 and JS07.Deg01. The biochemical tests were mainly performed to identify the physiological variation of microorganisms based on their biochemical properties. The results of biochemical tests (Table 1) depicted the physiological diversities of the isolates JS04.Deg01, JS07.Deg01, and JS08.Deg01.

Table 1 Biochemical characteristics of atrazine-mineralizing bacterial isolates

Biochemical assay	JS04.Deg01	JS07.Deg01	JS08.Deg01
Gelatin hydrolysis	−	−	−
Citrate utilization	−	+	+
Starch hydrolysis	+	−	+
Lipid hydrolysis	−	+	+
Triple-sugar iron	−	+	−
Urea hydrolysis	+	−	+
Methyl red	−	+	+
Vogus-Proskauer	−	−	−
Phenylalanine test	−	−	−
Catalase test	+	+	+
Nitrate reductase	−	+	+
H_2S test	−	+	−
Gram staining	Gram+	Gram+	Gram−

−: negative; +: positive

The growth pattern of atrazine-degrading bacteria was analyzed at pH 4.0, 5.0, 6.0, 7.0, and 8.0 in atrazine-containing mineral medium at 37 °C and 200 r/min. The bacterial isolates grew well in the pH range of 6.0 to 8.0. However, at pH 7.0, the efficiency of growth was high for the three isolates namely JS04.Deg01, JS07.Deg01, and JS08.Deg01. At lower pH (pH 4.0 and 5.0), the growth of bacterial isolates and atrazine degradation appeared to be slow (Fig. 2). Comber (1999) also reported the slow degradation rate of atrazine at pH 4.0.

3.2 Analysis of degradation products of atrazine

Occurrence of a significant level of residues of atrazine in groundwater appears to be caused by the environmental persistence of atrazine (Agertved *et al.*, 1992; Widmer and Spalding, 1995; Kolpin *et al.*, 1998). Microorganisms can break down the atrazine into the final form as cyanuric acid and CO_2. This study has shown that potentially stable atrazine can be degraded over time by the mineralization activity of bacterial isolates (Fig. 3). The quantification of atrazine degradation was performed by spiking the standard atrazine into an HPLC unit under identical conditions and it is concluded that all the isolated bacteria showed the degradation of atrazine and 50% atrazine degradation was achieved at 74, 62, and 47 h by JS04.Deg01, JS07.Deg01, and JS08.Deg01 isolates, respectively. JS04.Deg01, JS07.Deg01, and JS08. Deg01 isolates showed higher atrazine degradation efficiencies of 92%, 94%, and 97%, respectively, in 6 d of incubation.

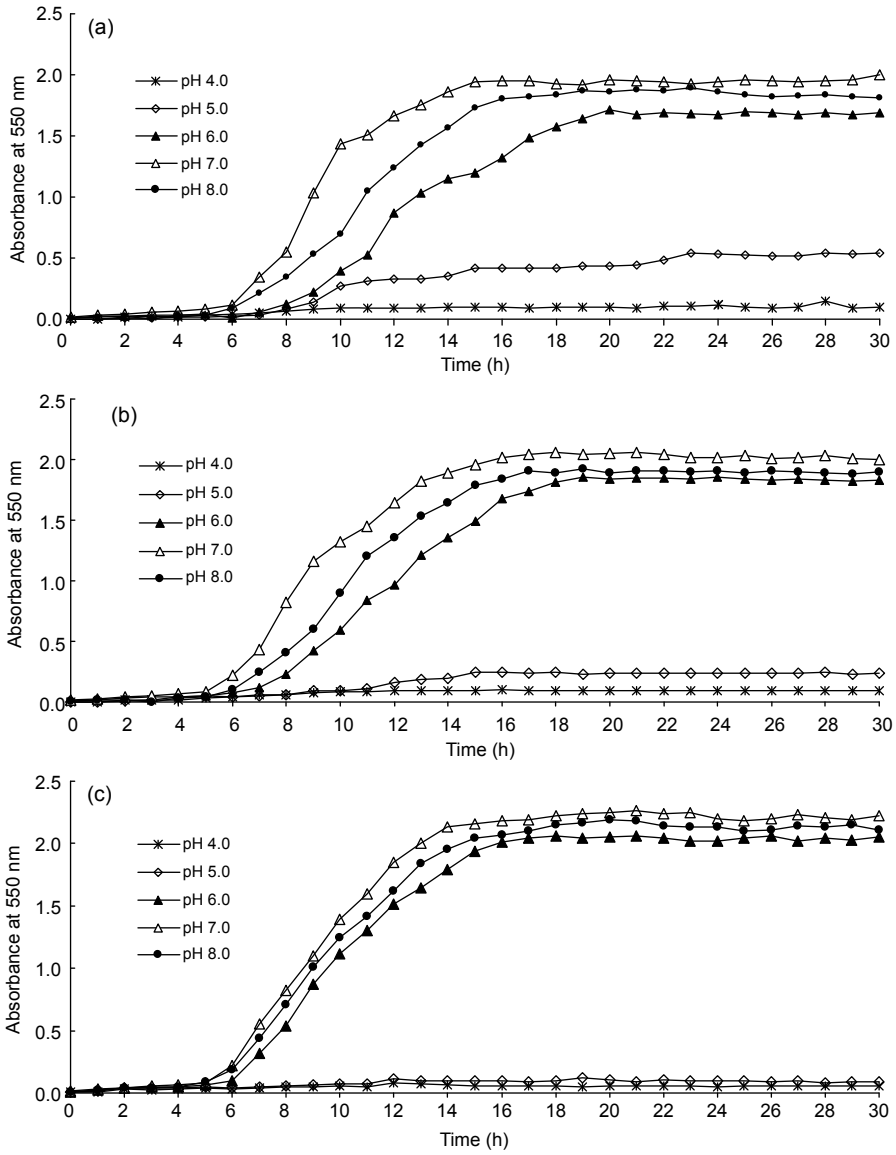

Fig. 2 Growth pattern of the bacterial isolates JS04.Deg01 (a), JS07.Deg01 (b), and JS08.Deg01 (c) at different pH ranges in the atrazine-containing mineral medium

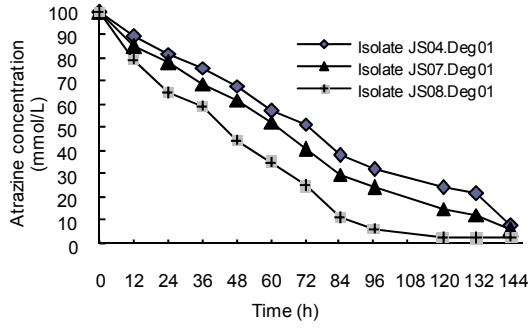

Fig. 3 Dissipation of atrazine in the spent medium by the metabolization of bacterial isolates JS04.Deg01, JS07.Deg01, and JS08.Deg01

In the case of JS08.Deg01 isolate, the rapid degradation of atrazine was attained and the quantity of cyanuric acid production was higher when compared to the other isolates. After 3-d incubation, more than 90% of atrazine in the spent medium was utilized by the JS08.Deg01 microorganism. The atrazine-degrading isolates were compared with each other with respect to the generation of cyanuric acid, the product of the upper degradation pathway of atrazine metabolism beyond which cyanuric acid is converged into carbondioxide and ammonia by hydrolytic ring cleavage in the lower degradation pathway.

3.3 Mass spectrometric analysis of metabolites

Atrazine was rapidly degraded by JS08.Deg01 and it produced the metabolites. The mass spectrometry analysis revealed that the metabolites produced by the isolate during atrazine degradation were DEA, DIA, and cyanuric acid. Formations of DEA and DIA in the incubation mixture revealed that the atrazine was degraded by *N*-dealkylation reaction, which was in accordance with Hanioka *et al.* (1999). The product of atrazine biodegradation is the decarboxylation reaction product. Cyanuric acid was also detected in the spent medium of isolate JS08.Deg01 from 65 to 95 h, and cyanuric acid was not detected after 95 h (Fig. 4). The substantial decrease in the amount of cyanuric acid in the medium suggests that it could be metabolized into CO_2 by the microbial cells.

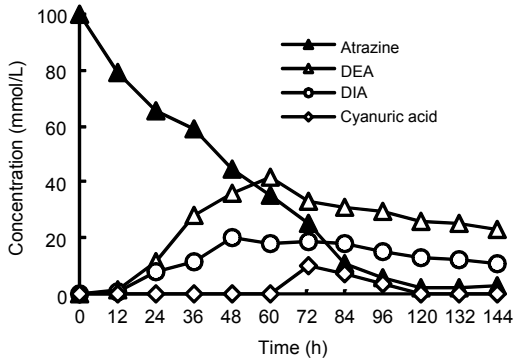

Fig. 4 Removal of atrazine and formations of its degradation metabolites, deethylatrazine (DEA), deisopropylatrazine (DIA), and cyanuric acid in the spent atrazine mineral medium of bacterial isolate JS08.Deg01

The isolates JS04.Deg01 and JS07.Deg01 showed the production of metabolites by the dechlorination reaction of atrazine. They produced HA and *N*-isopropylammelide in the culture supernatant from 80 h onwards and their levels were accounted for as 20% and cyanuric acid in the spent medium was 45% when compared to the level of the parent compound, atrazine (Fig. 5). Earlier reports also suggest that the atrazine-degrading bacterial strain *Nocardioides* sp. SP12 could produce cyanuric acid (Piutti *et al.*, 2003) and *Pseudomonas* sp. could produce HA, *N*-isopropylammelide, cyanuric acid, ammonia, and CO_2 in atrzine biodegradation (Yanze-Kontchou and Gschwind, 1994; Wackett *et al.*, 2002). The JS08.Deg01 isolate produced DEA, DIA, and cyanuric acid as degradation byproducts. Similar to this result, the *Rhodococcus* strain B-30 released the *N*-dealkylated metabolites of atrazine, such as DEA and DIA (Behki and Khan, 1994).

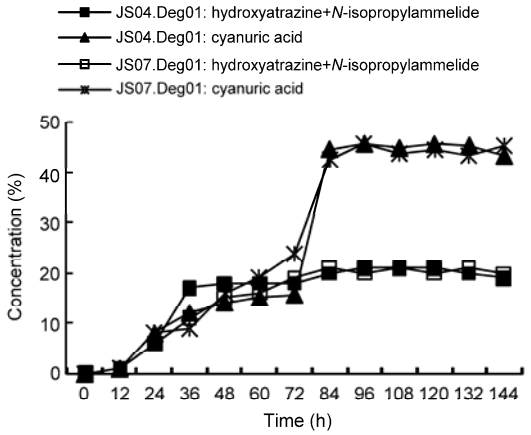

Fig. 5 Degradation metabolites hydroxyatrazine, *N*-isopropylammelide, and cyanuric acid produced by the isolates JS04.Deg01 and JS07.Deg01

3.4 Phylogenetic analysis of atrazine-degrading microorganism

When compared to the other eight strains, JS08.Deg01 isolate exhibited the rapid degradation of atrazine and produced a higher quantity of cyanuric acid. It also showed a more than 90% reduction in the atrazine level on the 3rd day of incubation. Due to the faster degradation rate, the JS08.Deg01 isolate was selected for the amplification of the 16S rRNA gene by PCR. The amplified gene product was subjected to sequencing and phylogentic analysis. Results from 16S rDNA sequence analysis revealed that there was a significant genetic diversity of the efficient atrazine-degrading isolate JS08.Deg01 when compared to the 16S rDNA sequence of the bacterial strains deposited in the nucleotide databases. Phylogenetic analyses by BLAST, ClustalW, and PHYLIP revealed that the isolate JS08.Deg01 exhibited sequence similarity to the genus *Enterobacter*, the members of the phylum Proteobacteria, class γ-proteobacteria, order Enterobacteriales and family Enterobacteriaceae. JS08.Deg01 exhibited 96.8% (1381/1426) and 96.9% (1382/1426) sequence similarities to the 16S rRNA gene sequences of the closely related type strains, *E. cloacae* ATCC13047T^T (AJ251469) and *E. cloacae* LMG 2683^T (Z96079), respectively (Fig. 6).

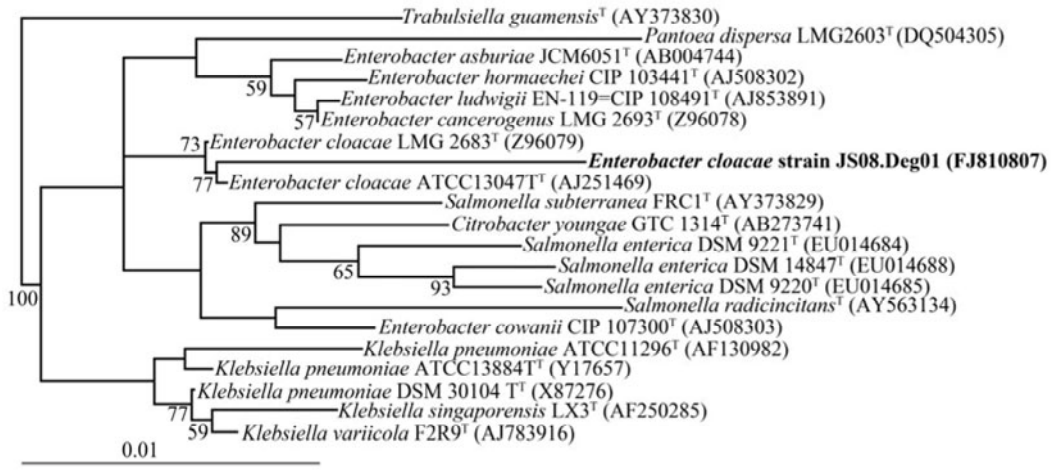

Fig. 6 Phylogenetic tree constructed by neighbor-joining method illustrating the clustering of partial 16S rRNA gene sequences of atrazine-degrading bacteria *Enterobacter cloacae* strain JS08.Deg01 derived from agricultural field soil

The tree was constructed with *Trabulsiella guamensis* as an out group and the tree topology was analyzed by parsimony analysis (DNAPARS) with bootstrapping over 1000 replicates using PHYLIP package. Scale bar 0.01 represents the nucleotide substitution level at 0.01%

3.5 Expression analysis of atrazine catabolic gene

The two major enzyme homologs encoded by *atzD* and *trzD* genes were generally reported in atrazine metabolizing bacterial populations (Fruchey *et al.*, 2003). Hence, the specific PCR primers to detect the *atzD* and *trzD* genes in the *E. cloacae* strain JS08.Deg01 were used. The real-time quantitative PCR (RT-qPCR) analysis of atrazine catabolic genes (*atzD* and *trzD*) showed that the atrazine-degrading gene, *trzD*, appears to be constitutively expressed upon exposure of JS08.Deg01 to atrazine and the expression of the *trzD* gene was highly conserved and widely dispersed in atrazine-degrading microbial isolate JS08.Deg01. Since the atrazine degradation was the highest in the JS08.Deg01 strain, the level of *trzD* mRNA expression appeared to be highest after atrazine exposure. However, the expression of the *atzD* gene was not observed in the bacterial isolate JS08.Deg01 after exposure to atrazine.

The bacterial 16S rRNA gene is often used as an internal control in relative quantification gene expression studies in RT-qPCR in prokaryotes. Hence the 16S rRNA was used as normalization control in *atzD* or *trzD* gene expression analysis. The RT-qPCR analyses of *atzD* and *trzD* gene transcripts showed that the *trzD* gene was expressed to the level of 542.66±26 relative mRNA number (gene was expressed as mRNA copy number per 10^6 16S rRNA gene) and the level of *trzD* mRNA of JS08.Deg01 isolate correlated well with the atrazine mineralization activity (Fig. 7).

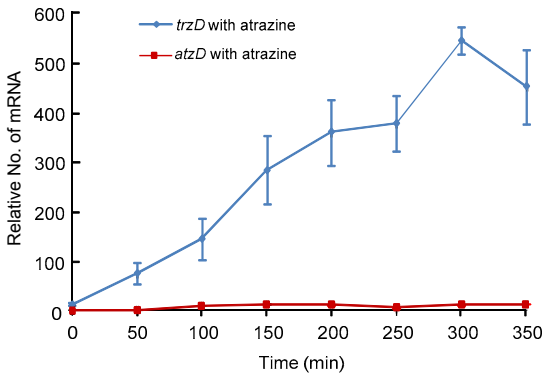

Fig. 7 RT-qPCR analysis depicting the relative numbers of *atzD* and *trzD* mRNA expressed by *Enterobacter cloacae* JS08.Deg01 when incubated with atrazine

Data are expressed as mean±standard error, n=3

4 Discussion

Prolonged exposure of microbial communities to the xenobiotic compounds facilitated the biodegradation and thereby reduced toxicity in the contaminated sites (Zhang *et al.*, 2011; Ganesh-Kumar *et al.*, 2013; Suzuki *et al.*, 2013) and applications of sustainable

and economically feasible various remediation technologies maintain the quality of soils and waters (Lone *et al.*, 2008). The microbial degradation plays the primary role in the reduction of toxic chemicals from contaminated sites. The bacterial enrichment cultures exhibited rapid growth in the atrazine-containing mineral medium, and a simultaneous reduction in the level of atrazine in the spent medium suggested that they could be considered as the potential degraders of the herbicide, atrazine. Atrazine was primarily degraded at the stationary growth phase of *Rhodococcus* TE1 (Behki *et al.*, 1993) and the bacterial strains isolated from this study were also involved in the degradation of atrazine after the log phase of the growth cycle. The optimal pH range for atrazine degradation was reported as 6.0–9.0 by *Arthrobacter* sp. strain HB-5 (Wang *et al.*, 2011) and 5.0–10.0 by *Arthrobacter* sp. strain DAT1 (Wang and Xie, 2012). Similar to these reports, the broad optimum pH range, exhibited by bacterial isolates JS04.Deg01, JS07.Deg01, and JS08.Deg01 with respect to growth in atrazine-supplemented medium, implies their ability to survive and metabolize atrazine in contaminated environments (Wang *et al.*, 2011).

The atrazine metabolizing bacterial isolates derived from enrichment cultures varied with respect to the degradation product of atrazine catabolism. Six of the isolates mineralized atrazine into HA and they did not produce cyanuric acid. The rest of the three bacterial isolates (JS04.Deg01, JS07.Deg01, and JS08.Deg01) degraded atrazine to cyanuric acid in the spent medium. Moreover, these isolates exhibited high degradation rates of atrazine and were highly efficient in reducing the persistent nature of the herbicide. In general, enzymatic actions of amidohydrolases release cyanuric acid by the ring cleavage mechanism of the atrazine (Eaton and Karns, 1991; Karns and Eaton, 1997; Topp *et al.*, 2000). Hence, these enzymes and respective genes are presumably present in the isolates JS04.Deg01, JS07.Deg01, and JS08.Deg01, which degrade triazine ring carbon and utilize triazine ring as a nitrogen source for their growth.

The phylogenetically unknown strain JS08.Deg01 exhibited >3% sequence divergence to the closest matching type strains: *E. cloacae* ATCC13047T 3.2% and *E. cloacae* LMG 2683 3.1%. Based on the level of sequence divergence as described by Stackebrandt and Goebel (1994), the atrazine-degrading isolate JS08.Deg01 was described as a new strain of *E. cloacae.* Since the 16S rRNA gene sequence diversity is significantly high (Fig. 6) and denotes the novel strain of *E. cloacae*, the members of the genus *Enterobacter* represent the major bacterial community in agricultural fields (McInroy and Kloepper, 1995; Seghers *et al.*, 2004). These bacteria are able to degrade the atrazine (Cook, 1987) and *E. cloacae* strain 99 has cyanuric acid hydrolase which can convert cyanuric acid to ammonia (Shapir *et al.*, 2006). Phylogenetic analysis also showed that the strain JS08.Deg01 and its closely related type strains were involved in the decontamination of environmental pollutants such as triazine-degrading *Agrobacterium* (Struthers *et al.*, 1998) *Klebsiella* (Hapeman *et al.*, 1995), *Pseudomonas* (Mandelbaum *et al.*, 1995), and *Rhodococcus* species (van Zwieten and Kennedy, 1995). Hence the identification of JS08.Deg01 as *E. cloacae* represents a new strain of the bacterial group to metabolize the atrazine herbicide.

Many studies suggested that the phylogenetically distinct bacteria metabolizing cyanuric acid was attributed to the enzymes encoded by *atzD* and *trzD* genes (Fruchey *et al.*, 2003; Cheng *et al.*, 2005). These genes are widespread and more prevalent in such bacterial strains; however, the strains lacking the *atz* genes contained *trzD*. In other words, these bacteria were known to harbor either *atzD* or *trzD*, but not both genes. The *atzD* and *trzD* genes are known to produce the cyanuric acid hydrolase that encodes the production of biuret (Karns, 1999; Fruchey *et al.*, 2003; Cheng *et al.*, 2005). When compared to the rest of the atrazine-mineralizing strains reported in the present study, the isolate JS08.Deg01 showed the production of cyanuric acid upon exposure to atrazine from 60 to 120 h, beyond which the presence of cyanuric acid was not detected in the spent medium (Fig. 2). To determine whether the bacterial isolate JS08.Deg01 harbors an atrazine metabolizing cluster containing either *atzD* or *trzD*, the gene transcripts of *atzD* or *trzD* were analyzed by RT-PCR. The results of this study inferred that atrazine mineralization was demonstrated mainly by the *trzD* catabolic genes in *E. cloacae* JS08.Deg01 isolate. This concept was also evidenced by the previous studies (Karns, 1999; Cheng *et al.*, 2005) in which they reported that the

E. cloacae bacterial isolates lacking the *atzD* genes generally contained *trzD* genes. The *trzD* gene is responsible for encoding an enzyme homolog, cyanuric acid hydrolase, which hydrolyzes cyanuric acid into a product known as biuret.

Microbial growth and atrazine dissipation in the enrichment cultures and spent medium revealed that the bacterial isolates inhabiting the soil have an ability to metabolize the atrazine for their growth and development. Based on the performance of atrazine biodegradation, isolates JS04.Deg01, JS07.Deg01, and JS08.Deg01 are able to remove the atrazine efficiently. However, the JS08.Deg01 strain is the best degrader of atrazine. Further studies on the ability to decontaminate the atrazine by *E. cloacae* JS08.Deg01 strain at a field site by constructing microcosm experiments will provide an insight into the amelioration of an herbicidal-contaminated environment for the welfare of living organisms.

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Compliance with ethics guidelines

Robinson David Jebakumar SOLOMON, Amit KUMAR, and Velayudhan SATHEEJA SANTHI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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