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Atrazine degradation in liquid culture and soil by a novel yeast *Pichia kudriavzevii* strain Atz-EN-01 and its potential application for bioremediation

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ABSTRACT

A novel yeast strain, Atz-EN-01 isolated from contaminated-agricultural soil was found to be highly effective in degrading atrazine in liquid culture and soil. The molecular characterization based upon partial 18S rDNA and ITS regions identified the strain Atz-EN-01 as *Pichia kudriavzevii*. The yeast could degrade atrazine completely within 7 days with a rate constant of 0.31 per day following the first order kinetic model. The time in which initial atrazine concentration (500 mg/L) was reduced by 50% (half-life) was 2.2 days under optimal conditions (pH 7.0, temperature 30˚ C, inoculum size 3% (v/v) and shaking speed 120 rpm). The analysis of the metabolites using GC-MS identified the formation of 3 intermediates *viz.* hydroxyatrazine, N-isopropylammelide and cyanuric acid. The enzyme atrazine chlorohydrolase exhibited maximum activity during degradation. Based upon the intermediates identified by GC-MS and FT-IR analysis, the sequential process of atrazine degradation was proposed. In soil bioremediation experiment, inoculation of soil with Atz-EN-01 promoted effective degradation than did the control. To the best of our knowledge, this is the first report on *Pichia kudriavzevii* strain Atz-EN-01 which can serve as a potential agent for in-situ bioremediation of atrazine contaminated environment.

INTRODUCTION

The accumulation and persistence of toxic pesticides in water and soil represents a major problem today. The s-triazine herbicides are the most commonly used pesticides in agriculture and forestry throughout the world. Atrazine, 2-chloro-4-ethylamino-6-isopropylamino -1, 3, 5-triazine, a member of s-triazine group of herbicides is widely used for the control of grassy and broad leaf weeds in corn, sorghum and sugarcane crops (Shahitha, 2012). Atrazine strongly inhibits photosynthesis by blocking the electron transport in photosystem II leading to chlorophyll destruction (Christopher *et al.,* 2010). In the environment, the persistence of atrazine varies according to the soil types and its half life usually ranges from 21 days to 1 year (Zhang *et al.*, 2012a). As it might represent a long-time threat, its ubiquity in water and soil has been considered to be a potential environmental concern. Several researchers reported atrazine as an endocrine disruptor and also as inducer of liver cancer (Siripattanakul *et al.,* 2009). With regard to the situations, studies focused on efficient remediation strategies for atrazine polluted environments and reduction of its contamination to environmentally safe level is an urgent need.

Bioremediation has attracted increasing attention as a safe, effective, and cheap biotechnological approach for pesticide elimination (Chen *et al.,* 2011). Until now, most of the research has focused on the isolation of atrazine degrading bacteria (Li et al 2008a; Siripattanakul *et al.,* 2009; Arbeli *et al.,* 2010; El sebai *et al.,* 2012) and fungi (Mougin *et al.,* 1994; Masaphy *et al.,* 1996; Vasilchenko *et al.,* 2004; Gonçalves *et al.,* 2012; Doruk *et al.,* 2012).

However, little information is available on the role of yeast in the atrazine degradation process. In our previous study, we reported atrazine degradation for the first time by a yeast isolate *Cryptococcus laurentii* (Abigail *et al.,* 2012). In the present study, we isolated a highly effective atrazine degrading yeast *Pichia kudriavzevii* strain Atz-EN-01.

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It was found that this strain could use atrazine as its sole carbon and energy source for growth. With the goal of elucidating a possible application of *Pichia kudriavzevii* strain Atz-EN-01 in remediation of atrazine contaminated environments, we studied Atz-EN-01 mediated degradation of atrazine in liquid culture and soil samples and identified the metabolites of atrazine degradation. This article highlights an important use of a pure culture of isolated yeast strain for the cleanup of atrazine contaminated environment.

MATERIALS AND METHODS

Chemicals

Atrazine (99 % purity) was purchased from Sigma Aldrich Chemical Co. All the organic solvents used for residue analysis were of analytical grade and purchased from SRL India Pvt. Ltd. All the culture media components used were procured from Himedia. Rest of the chemicals used throughout the study were obtained from standard manufacturers.

Enrichment and isolation of yeast strain

The soil used for the isolation of degrading yeast was collected from sugarcane field in Vellore district, Tamil nadu (India) which had been exposed to atrazine herbicide for several years. Table 1 shows the physical and chemical properties of the soil used for enrichment.

The degrading yeast was isolated using an enrichment culture technique. The mineral salt medium (MSM) contained (g/L distilled water): K_2HPO_4 -0.4, KH_2PO_4 -0.2, NaCl -0.1, $MgSO_4.7H_2O$ -0.5, $MnCl_4$ -0.01, Fe $(SO_4)_3$ -0.01, and Na_2MoO_4 -0.01. The final pH was adjusted to 6.0. The collected soil sample was added to 250 ml Erlenmeyer flask with MSM (5 g per 100 ml medium) supplemented with 10 mg/L atrazine as the sole source of carbon and nitrogen. Yeast was grown for 7 days at 28±2˚ C with shaking.

The enrichment cultivation was performed under the same conditions and repeated three times with different concentrations (10, 25 and 50 mg/L) of atrazine in the media. The yeast present in the enrichment culture was isolated onto Yeast Extract Peptone Dextrose (YEPD) agar plates. A pure culture was made by serial subculture and the yeast was named as Atz-EN-01.

Table. 1: Physical and chemical properties of the soil used for yeast isolation.

Properties	
pH	6.58
Total organic carbon (%)	2.65
Total nitrogen concentration (ppm)	14
Available phosphorus (ppm)	40.6
Sand (%)	60
Slit (%)	11
Clay (%)	28
Structure	Sandy Clay loam soil

Taxonomic identification of the strain Atz-EN-01

The partial 18S rDNA and ITS sequences of Atz-EN-01 strain were amplified using PCR with the following primers: UL18F: 5'-TGTACACACCGCCCGTC - 3' as forward and UL28R: 5'- ATCGCCAGTTCTGCTTAC-3' as the reverse. The amplified sequences were eluted and purified using clean GeNei™ kit (Bangalore) and sequenced using the primer, UL620R: 5'-TGGTCCGTGTTTCAAGA-3'. The sequencing results were subjected to bioinformatics analysis for identification of the isolate.

Assembled and trimmed sequences for 18S rDNA and ITS regions sequences were submitted to GenBank under accession number KC886644. Nucleotide sequence similarities were determined using BLAST (NCBI database). The sequences were aligned using multiple sequence alignment software CLUSTALW (1.81). Phylogenetic tree was then constructed by neighbour-joining method using TREEVIEW software (1.6.6) based on partial 18S rRNA and ITS gene sequences of 12 strains phylogenetically close to the isolated strain (Kumar *et al.*, 2004b). The robustness of the tree topology was assessed by bootstrap analysis with 1000 resembling replicates.

Optimal conditions for atrazine degradation by Atz-EN-01

The yeast strain Atz-EN-01 was inoculated into MSM containing 50 mg/L atrazine and incubated at 30 °C on a rotary shaker (120 rpm) for 3 days. 24 h grown culture of Atz-EN-01 (OD_{600}=0.1) was inoculated for optimization studies.

The residual atrazine concentration was determined at 1 day interval. To optimize degradation conditions in liquid medium, the effects of pH (5.0-9.0), temperature (25-40 °C), shaking speed (80-140 rpm), inoculum size (1-5 % (v/v) and initial atrazine concentration (50-700 mg/L) on atrazine degradation were investigated. Duplicates were maintained for each experiment and uninoculated flasks were maintained as abiotic control.

Kinetics of atrazine biodegradation by Atz-EN-01 strain

The experimental data on the degradation kinetics of atrazine by strain Atz-EN-01 was fitted with first order kinetic equation to derive the half life period and degradation rate constant for various concentrations (50-700 mg/L). The first order kinetic equation is commonly expressed as follows:

$$C_t = C_0 e^{-kt}$$

where, C_0 is the initial concentration of atrazine, C_t is the concentration of atrazine at time t, k is degradation constant and t is reaction time. It can be written in the linearized form as:

$$\ln C_t = \ln C_0 - kt$$

The biodegradation half-life ($T_{1/2}$) of atrazine by Atz-EN-01 can be calculated from the formula:

$$T_{1/2} = \ln 2/k$$

Metabolites characterization by GC-MS and FT-IR analysis

Residual atrazine was extracted from cell free supernatants (9000 X g for 20 min, 4 ºC) with equal volume of ethyl acetate and was concentrated to 0.1 mL. Aliquots of 2-5 µL were injected directly for gas chromatography-mass spectroscopy

analysis (GC-MS) into JOEL GC MATE II equipped with capillary column (DB-5; 30m X 0.25 mm X 0.25 µm) and electron capture detector (ECD), with splitless injector operated at 70 eV. Helium was used as a carrier gas at a flow rate of 1.5 mL per min. The temperatures of the transfer line and the ion trap were 280 and 300˚C, respectively. The concentration of atrazine in the samples was calculated by comparing the peaks formed with that of the peaks obtained in the abiotic control. For the identification of degradation products, mass spectra of the products were compared with the mass spectra of the standards.

The infrared spectrum of the degraded products extracted from the culture medium was recorded on an IR affinity-1 FTIR spectrophotometer (Shimadzu) in the 4000-400 cm^{-1} spectral region at a resolution of 4 cm^{-1} after mixing with 0.23 mm by KBr pellet. A possible degradation pathway was deduced according to the instrument analysis performed.

Enzyme assays

The activities of the enzymes viz. atrazine chlorohydrolase, hydroxyatrazine ethylaminohydrolase and N-isopropylammelide isopropylaminohydrolase, which may be involved in the degradation of atrazine, were also assayed in the present study following the standard procedures. The cells of strain Atz-EN-01 were grown in MSM media containing atrazine (50 mg/L) for 24 hrs. Then the medium was centrifuged and the collected cell pellet was suspended in phosphate buffer (pH 7.0), sonicated and finally centrifuged to serve as crude extract for enzyme assays. Atrazine chlorohydrolase activity was monitored by measuring the decrease in reaction absorbance of atrazine at 225 nm (Bouquard *et al.,* 1997).

Hydroxyatrazine ethylaminohydrolase activity was measured spectrophotometrically at 242 nm by monitoring the decrease in absorbance of hydroxyatrazine. The reaction mixture contained hydroxyatrazine and crude enzyme extract (Seffernick *et al.,* 2007). Finally, N-isopropylammelide isopropylaminohydrolase activity was determined for enzymatic conversion of N-isopropylammelide to cyanuric acid by measuring the N-isopropylammelide absorbance at 240 nm (Shapir *et al.,* 2002). One unit of enzyme activity was defined as the conversion of 1 µmol of reaction substrate to product per min.

Biodegradation of atrazine in soil by Atz-EN-01

Soil samples were collected from the nursery in VIT University, Vellore, Tamil Nadu, India which had never been treated with atrazine. The top soil (0-10 cm) samples were dried at room temperature and sieved to 5 mm. One set of soil sample was sterilized for three consecutive days in order to confirm the absence of spore bearing microbes.

Both fresh and sterile soils were used for the biodegradation experiments. Atrazine dissolved in methanol was added to soil samples to give concentrations of 25, 50 and 100 mg/kg soil. After mixing and solvent evaporation, the soil was inoculated with yeast strain to give a final concentration of 1.8 x 10^8 cells per gram of soil and then incubated at 30 °C. Uninoculated soil sample served as controls. All the soil samples were incubated in the dark and the concentration of atrazine in the soils was determined at regular intervals. Soil moisture content was maintained at 25% water holding capacity with regular addition of distilled water as needed.

To extract atrazine, 5 g of soil samples were extracted twice with 50 ml of methanol. The mixture was centrifuged after incubating for 1 h on a rotary shaker. The supernatant was concentrated by rotary evaporation and re-dissolved in methanol for GC-MS analysis.

RESULTS AND DISCUSSION

Isolation and identification of strain Atz-EN-01

In the present study, an atrazine degrading yeast, designated as Atz-EN-01, was isolated from contaminated agricultural soil through enrichment culture technique. Strain Atz-EN-01 could utilize atrazine as the sole source of carbon and energy for growth in MSM. When grown on YEPD agar, the yeast colonies were found to be creamy white, small circular, non mucoid, with raised filiform margin (Figure 1A, B).

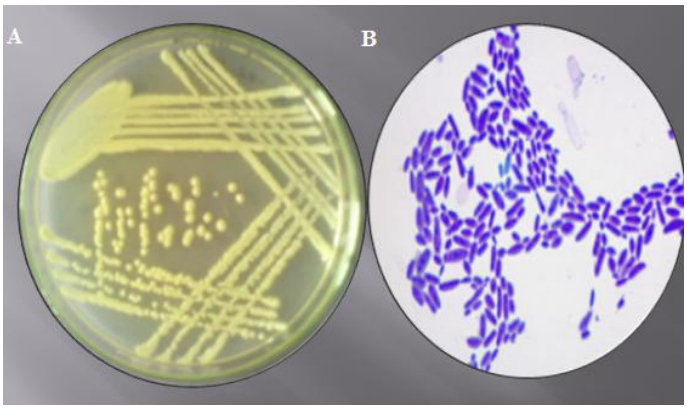

Fig. 1: Morphology of *Pichia kudriavzevii* strain Atz-EN-01 A. On YEPD agar plates after 48 h of incubation B. Under X100 magnification of microscope.

Morphological and 18S rRNA gene analysis were used to identify the strain Atz-EN-01. The 18S, ITS regions and D1/D2 rRNA (542bp) gene from Atz-EN-01 were amplified, and the sequenced fragment was submitted in the GenBank database (Accession number KC886644). Partial 18s rRNA gene sequence analysis of Atz-EN-01 strain showed 99% homology with *Pichia kudriavzevii* strain NRRL Y-5396 (EF 550222.1).

The phylogeny of Atz-EN-01 was identified by constructing a phylogenetic tree using TREEVIEW software (1.6.6). The phylogentic tree (Figure 2) showed that Atz-EN-01 exhibited highest identity with *Pichia kudriavzevii* NRRL Y-5396. Therefore, the isolated atrazine degrading yeast was designated as *Pichia kudriavzevii* Atz-EN-01. Our results revealed that the isolated yeast *Pichia kudriavzevii* Atz-EN-01 showed potentiality of atrazine degradation in liquid culture and soil. Furthermore, this is the first report of the isolation and identification of a yeast strain that was capable of degrading high concentrations (500 mg/L) of atrazine.

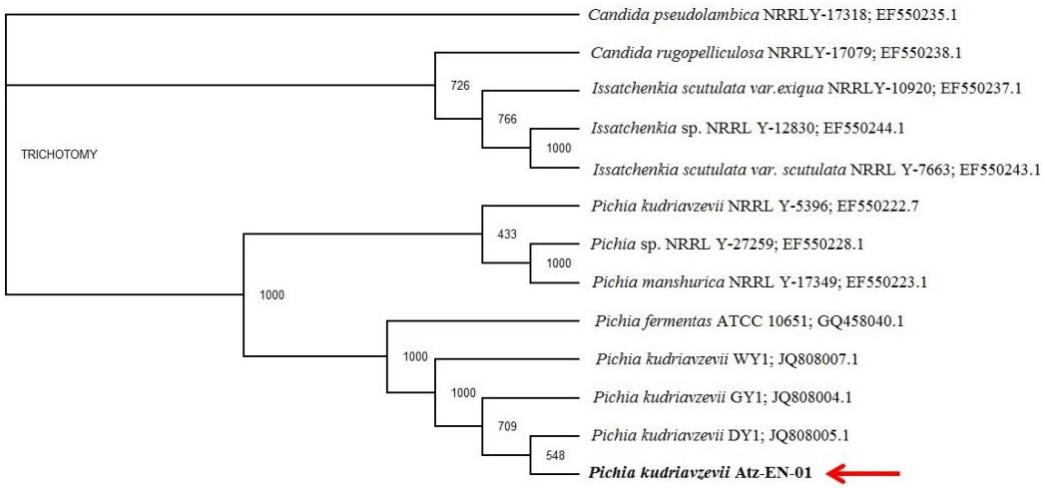

Fig. 2: Phylogenetic tree based on 18S rRNA gene sequences of strain Atz-EN-01 and related species by the neighbour-joining approach. The Genbank accession number for each microorganism used in the analysis is shown after the species name.

Optimal conditions for degrading atrazine by strain Atz-EN-01

The environmental fate of atrazine is largely dependent on various factors *viz* pH, temperature and atrazine concentration (Kodama *et al.*, 2001). For effective degradation study, the initial concentration of atrazine was maintained at 50 mg/L in the optimization tests where pH, temperature, shaking speed and inoculum volume was investigated. The effect of various parameters on growth and atrazine degradation by *Pichia kudriavzevii* Atz-EN-01 is depicted in Figure 3. The effect of pH on atrazine degradation at the different pH (5.0, 6.0, 7.0, 8.0 and 9.0) tested is shown in Figure 3A. The degradation of atrazine was found to be proportional to the cell dry weight. Strain Atz-EN-01 showed better degradation at an initial pH of 7 which was considered as optimal pH. Similar findings were reported in bacterial degradation of atrazine (Dai *et al.*, 2007) and fungal degradation of simazine (Kodama *et al.*, 2001). In case of temperature, maximum biomass and best course of degradation was observed at incubation temperature of 30 °C (Figure 3B). Synchronous observations at 30°C were reported earlier in fungal strain *Phanerochaete chyrososporium* ME446 (Doruk *et al.*, 2012) and in bacterial strain L-6 (Li *et al.*, 2012b). To demonstrate the effect of agitation on degradation of atrazine, experimental flasks were incubated at different shaking speeds ranging from 80-120 rpm. The degradation was more prominent at 120 rpm within 3 days (Figure 3 C) and hence was considered optimum. In case of inoculum size, larger the inoculum size greater the efficiency of degradation of toxic compounds (Guillen-Jimeneza *et al.*, 2012). Illustration in Figure 3D, depicts the yeast strain, Atz-EN-01 at different dosage added into the MSM containing atrazine. The atrazine degradation rate was found to be proportional to the inoculum size. Inoculum size of 3% (v/v) showed rapid and complete degradation of atrazine in 2 days. When the yeast inoculum size was increased from 4 to 5% (v/v), no significant difference was noted in the rate of degradation. Attainment of equilibrium by the microbial load might be the probable reason for this observation. So, inoculum size of 3% (v/v) was considered most appropriate for atrazine degradation by Atz-EN-01 strain. Similar results were reported by other researchers in case of other pollutant (Kumar *et al.*, 2006).

The concentration of target pollutant affects the activity of degrading microbes significantly. The effect of various concentrations of atrazine on the yeast growth and degradation is shown in Figure 3E. The rate of atrazine degradation decreased with increase in initial atrazine concentration. Strain Atz-EN-01 showed tolerance of atrazine up to 700 mg/l. At concentrations between 100-500 mg/L, complete degradation of atrazine occurred and beyond 500 mg/l, the degradation rate significantly reduced. This limited growth and atrazine degradation at higher concentrations could be attributed to the toxicity at higher concentrations of atrazine. Thus, the optimal concentration was found to be 500 mg/L for strain Atz-EN-01 where complete degradation occurred within 7 days. In a recent study, fungi *Phanerochaete chyrososporium* ME446, degraded 74% of atrazine (50 mg/L) at the end of 14 days (Doruk *et al.*, 2012). Gonclaves et al. (2012) reported atrazine removal of 73.5, 73.42 and 68.42% at an initial concentration of 1.5 g/L in 15 days by three filamentous fungi isolated from swine wastewater. Atrazine degradation by the white rot fungus *Phanerochaete chrysosporium* demonstrated 48% decrease of the initial concentration of 2 µM atrazine in first 4 days of incubation (Mougin *et al.*, 1994).

Previously we reported a yeast strain *Cryptococcus laurentii* which could degrade atrazine (150 mg/L) within 9 days (Abigail *et al.*, 2012). In the present study, the yeast strain Atz-EN-01 supersedes the previous reports presented. Strain Atz-EN-01 could degrade 500 mg/L atrazine within 7 days which portraits degradation at much higher concentrations compared to other degrading fungus reported so far. Therefore, the ability of *P. kudriavzevii* Atz-EN-01 to efficiently degrade atrazine at reasonably high concentrations is a very positive attribute from the point of view of its expected use in bioremediation of atrazine contaminated environment.

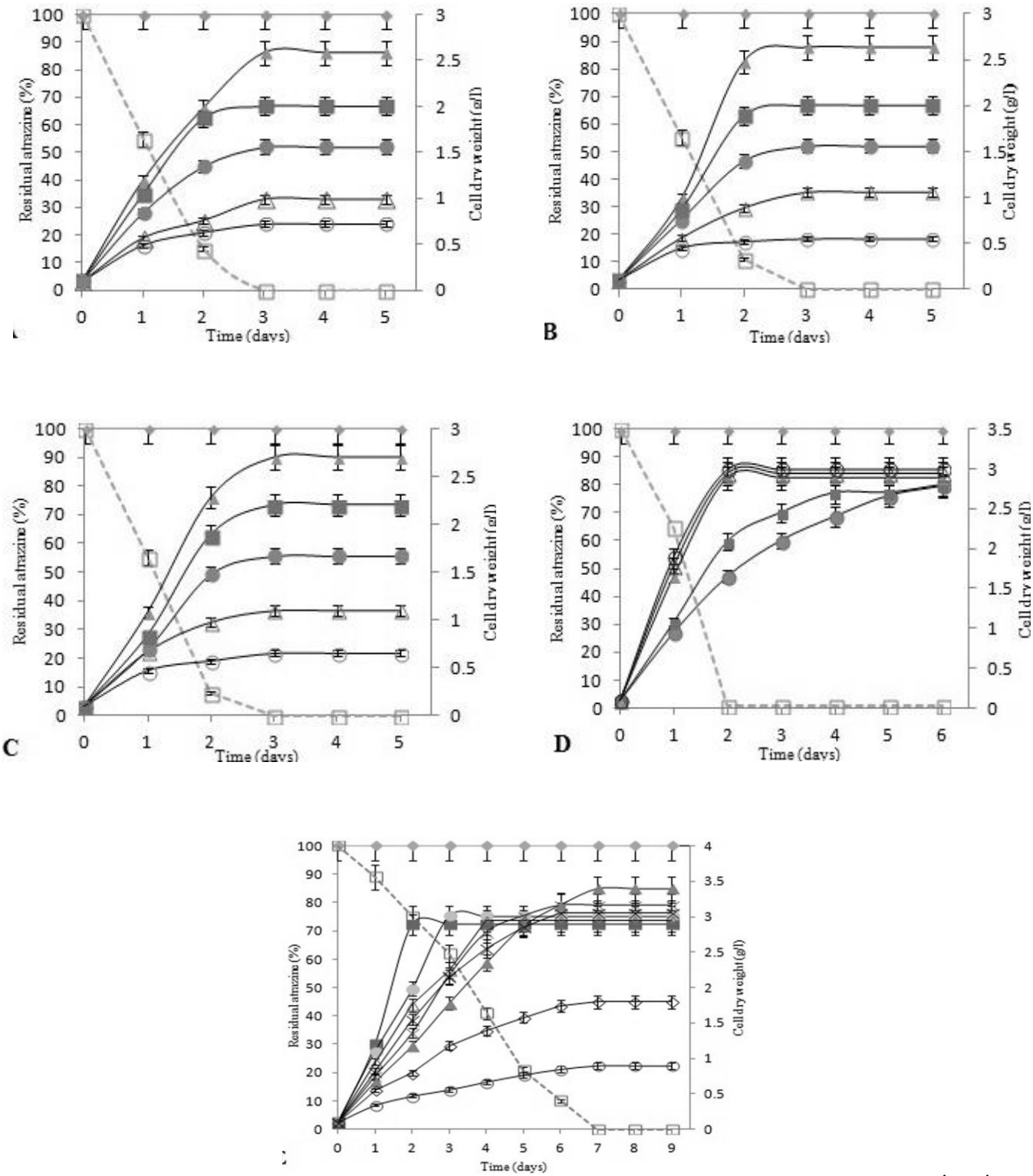

Fig. 3: The effect of culture conditions on growth and degradation of *P. kudriavzevii* strain Atz-EN-01 strain were evaluated at A pH-5(O), 6 (Δ), 7(▲), 8(■), 9(●),residual atrazine concentration at optimum pH 7 (□); B temperature-20 (Δ), 25 (●), 30 (▲), 35 (■), 40(O), residual atrazine concentration at optimum temperature 30 (□); C shaking speed- 80 rpm (Δ), 100 rpm (●), 120 rpm (▲), 140 rpm (■), 160 rpm (O), residual atrazine concentration at optimum shaking speed 120 rpm (□);D inoculum size- 1 % v/v (●),2% v/v (■), 3% v/v(▲), 4% v/v (Δ), 5% v/v(O), residual atrazine concentration at optimum shaking speed 120 rpm (□); E initial atrazine concentration- 50 mg/l (■), 100 mg/l (●), 200 mg/l (Δ), 300 mg/l (X), 400 mg/l ( ), 500 mg/l (▲), 600 mg/l (◇), 700 mg/l (O), residual atrazine concentration at optimum atrazine concentration 500 mg/l (□) ,were evaluated. Abiotic control (◆) was set for each experiment. Error bars represent the standard deviation of the mean.

Kinetics of degradation by strain Atz-EN-01

Degradation kinetics of atrazine was fitted to first order kinetic equation in order to study the effect of atrazine concentration on degradation.

The degradation rate constant and half life periods for various concentrations of atrazine were calculated from the slope of the plot ln C_t Vs t (Figure 4). As shown in Table 2, the rate of atrazine degradation was maximum with the minimum initial concentration of 50 mg/L. As the atrazine concentration increased, there was a constant reduction in the degradation rate till the optimum concentration of 500 mg/L was reached. After which a drastic reduction in the degradation rate and simultaneous increase in the half life was noted. At an optimum concentration of 500 mg/L atrazine, the degradation rate and half life were 2.22 per day and 0.311 days.

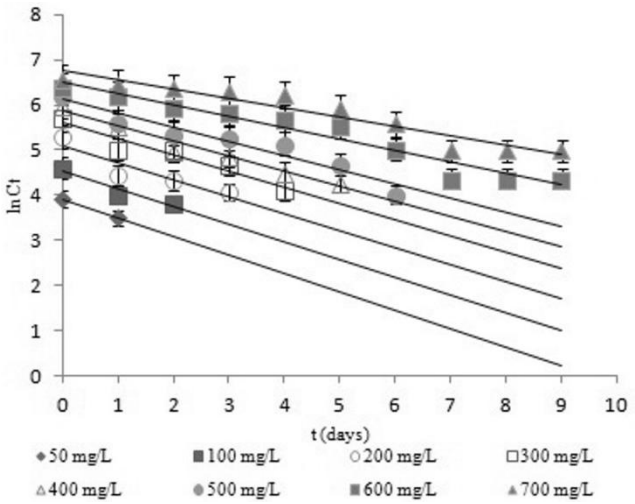

Fig. 4: Atrazine degradation by strain Atz-EN-01 was evaluated with first order kinetics, plotted with ln C_t versus time (days) for various initial atrazine concentrations (50–700 mg/l). Each point represents the mean of two replicates, and error bars are standard deviations.

Degradation products

The degradation products formed by strain Atz-EN-01 while degrading atrazine were extracted at different intervals and identified by GC-MS. Figure 5 presents the results of GC-MS analysis of atrazine degradation. There was an apparent peak, P at a retention time (RT) of 13.72 min corresponding to atrazine standard (m/z 215.0).

The GC-MS of the degradation products revealed the formation of metabolites designated as M1 (RT 12.87, m/z (M+1) of 198.23), M2 (RT 6.16, m/z (M+1) of 171.19) and M3 (RT 2.87, m/z (M+1) of 129.86).

The degradation products M1, M2 and M3 were identified as hydroxyatrazine, N-isopropylammelide and cyanuric acid by comparing the MS profiles of their authentic standards. The characteristic fragment ion peaks of M1, M2 and M3 area shown in Figure 6 A,B and C.

Atrazine as well the degraded metabolites were observed to decrease throughout the experiment and disappeared completely after 7 days of incubation. The formation of metabolites was further confirmed by FT-IR spectroscopy and the results were presented in Table 3. The above results were validated and the pathway of atrazine degradation by strain Atz-EN-01 is shown in Figure 7.

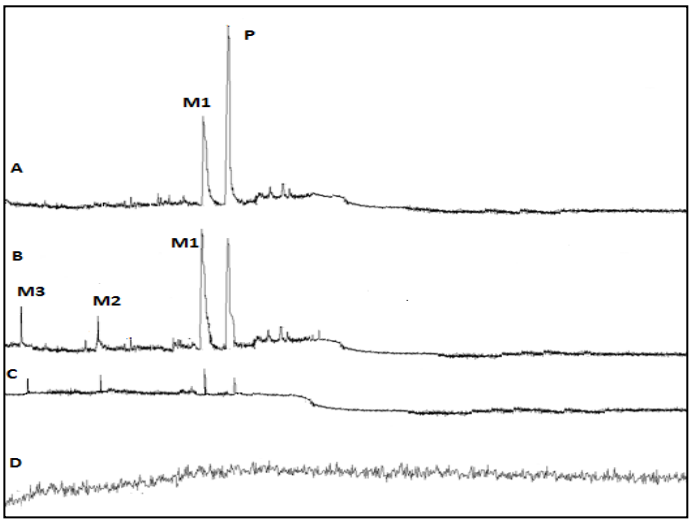

Fig. 5: Gas chromatographic-mass spectroscopic (GC-MS) analysis of intermediates during atrazine degradation by yeast *Pichia kudriavzevii* strain Atz-EN-01 incubated at 30 ˚C with 500 mg/L for 7 days. GC Chromatogram of extracted culture sample at various intervals A) 2 days, B) 4 days, C) 6 days D) 7 days of incubation. Complete mineralization was seen after 7 days with no evident peaks in GC chromatogram.

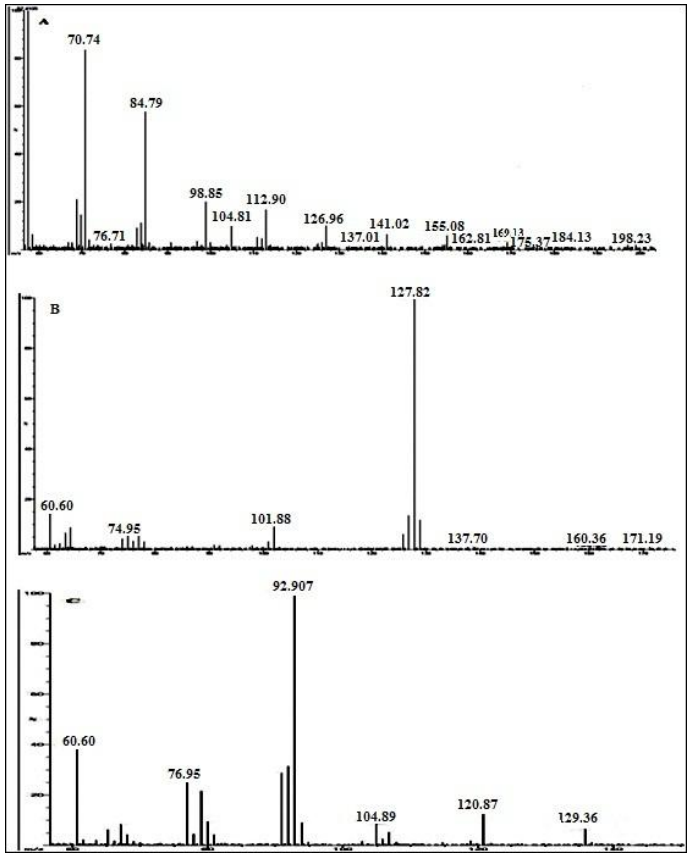

Fig. 6: Mass spectrum of atrazine metabolites produced during atrazine degradation by strain Atz-EN-01. A. hydroxyatrazine; B. N-isopropylammelide; C. Cyanuric acid.

Table. 2: Kinetic parameters of degradation of different concentration of atrazine by *P. kudriavzevii* strain Atz-EN-01.

Initial atrazine concentration (mg/l)	Kinetic parameters		
	K (per day)	R^2	$T_{1/2}$ (days)
50	0.412	1	1.682
100	0.3926	0.918	1.765
200	0.3797	0.849	1.825
300	0.3584	0.928	1.934
400	0.3345	0.966	2.072
500	0.3118	0.928	2.223
600	0.2543	0.939	2.725
700	0.2042	0.911	3.394

Table. 3: FTIR spectral peaks for atrazine and degraded products.

	Origin	Group frequency (cm^{-1})	Assignment	Suggested Compound
Before degradation	C-Cl	734.21	C-Cl stretch	Atrazine
	C=C	1620.21	C=C stretch In the aromatic ring	
	C-C	1066.64, 1138.00, 1116.78	C-C skeletal vibration of the aromatic ring	
	C-H	862.18	Aromatic C-H out of plane bend	
After degradation	O-H	3236.55	Polymeric O-H stretch	Cyanuric acid/Hydroxyatrazine
		3471.87	Dimeric O-H stretch	Isopropylammelide/Cyanuric acid
	C-H	3028.24	C-H stretch	Ring backbone in the degraded products.
		850.61	1, 4, Disubstitution (para)	Cyanuric acid/Isopropylammelide
	C-C	1080.14,1049.28	C-C skeletal vibration	Ring backbone in the degraded products.
	C=C-C	1633.71, 1618.28	Aromatic ring stretch C=C-C	
	C=O	1400.32	Carboxylate	Cyanuric acid

Table. 4: Specific activities of enzymes in the cell-free extracts of *P. kudriavzevii* strain Atz-EN-01 grown on atrazine.

Enzyme	Substrate	Specific activities (µmol/min/mg of protein)
Atrazine chlorohydrolase	Atrazine	0.162
Hydroxyatrazine ethylaminohydrolase	Hydroxyatrazine	0.132
N-isopropylammelide isopropylaminohydrolase	N-isopropylammelide	0.121

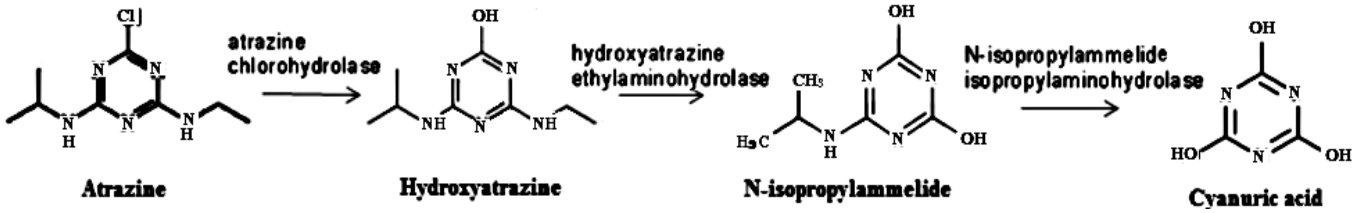

Fig. 7: Proposed degradation pathway of atrazine by *Pichia kudriavzevii* Atz-EN-01.

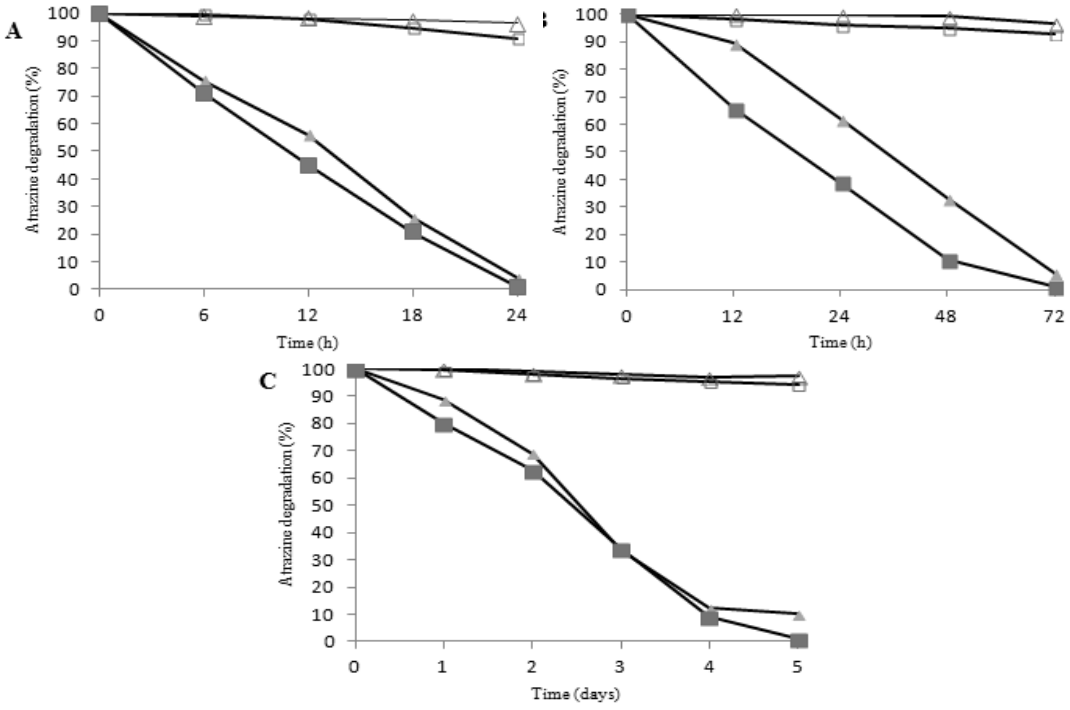

Fig. 8: Atrazine degradation in inoculated and uninoculated soil microcosms. A. 25 mg/L B. 50 mg/L C. 100 mg/L. (△) Sterilized soil; (□) Fresh soil; (▲) sterilized soil + Atz-EN-01; (■) fresh soil Atz-EN-01. Error bars represent the standard deviation of the mean.

In the present study, yeast strain Atz-EN-01 was found to degrade atrazine into three metabolites *viz*. hydroxyatrazine, N-isopropylammelide and cyanuric acid. At the end of biodegradation by strain Atz-EN-01, no persistent and accumulative products were detected, possibly that the metabolites might have been immediately degraded by the strain. In earlier studies, it was reported that microbial degradation of atrazine resulted in the formation of metabolites *viz*. hydroxyatrazine, deethylatrazine, deisopropylatrazine, N-isoproylammelide and cyanuric acid (Strong *et al.*, 2002; Wackett *et al.*, 2002; Wang *et al.*, 2011).

The strain Atz-EN-01 degraded atrazine by initial dechlorination to yield hydroxyatrazine which was further hydrolyzed to form N-isopropylammelide and cyanuric acid. There are reports on dechlorination and further hydrolysis of atrazine by other workers (Kumar *et al.*, 2006; Wang *et al.*, 2011; Strong *et al.*, 2002). In this context, the microbial degradation of atrazine to hydroxyatrazine has important environmental implications because hydroxyatrazine was reported as non-herbicidal with negative human health impacts (Wackett *et al.*, 2002). Similar breakdown pathway for atrazine had been reported in other species *viz* *Pseudomonas* sp. ADP (Wackett *et al*., 2002), *Nocardiodes* (Devers *et al.*, 2007) and *Arthrobacter* sp. (El Sebai *et al.*, 2012; Zhang *et al.*, 2011b; Sajjaphan *et al.*, 2004). On contrast, in fungi, *Pleurotus pulmonarius* (Masaphy *et al.*, 1996) and *Phanerochaete chrysosporium* (Mougin *et al.*, 1994) a different mode of atrazine degradation was identified through the formation of toxic metabolite deethylatrazine and deisopropyl atrazine.

Enzyme assays

The cell free extracts of *Pichia kudriavzevii* strain Atz-EN-01 grown on atrazine for 24 h showed the activities of enzymes *viz*. atrazine chlorohydrolase, hydroxyatrazine ethylaminohydrolase and N-isopropylammelide isopropylaminohydrolase. The specific activities of the enzymes are presented in Table 4. The results suggested the prominence of dechlorination in atrazine degradation. The activity of hydroxyatrazine ethylaminohydrolase exceeded that of the activity of N-isopropylammelide isopropylaminohydrolase. The enzyme assays confirmed the involvement of these three enzymes during atrazine degradation by *P. kudriavzevii* strain Atz-EN-01.

Biodegradation of atrazine in soil

The degradation of atrazine in soil samples by Atz-EN-01 strain, with different treatments, including sterilized soil, sterilized soil + atrazine, fresh soil and fresh soil + atrazine are presented in Figure 8. Within a day, Atz-EN-01 strain could completely degrade 25 mg/kg of atrazine added to the fresh soil whereas the control fresh soil showed 10.3% reduction in the initial atrazine concentration (Figure 8A). This reduction in the fresh soil may be probably due to adsorption of atrazine onto the soil particles. When atrazine was added at concentrations of 50 and 100 mg/kg to fresh soil inoculated with strain Atz-EN-01, complete degradation was observed within 3 and 5 days (Figure 8B, C) respectively whereas their respective controls showed degradation of 8.7 and 6.8% of added atrazine. In another set of treatment, Atz-EN-01 strain added to sterile soil containing 25, 50 and 100 mg/kg of atrazine showed degradation percentages of 96.2%, 94.5% and 90.7% respectively, while those of the sterilized soil (control) were 3.72%, 6.91% and 9.78% at the time intervals mentioned for the other treatment.

The soil bioremediation studies demonstrated rapid and complete atrazine degradation by Atz-EN-01strain under co-culture conditions. Low concentrations of the herbicide were degraded faster than the higher concentration. At all atrazine concentrations, fresh soil showed better degradation compared to sterilized soil. This suggested that indigenous microbes in fresh soil might have played some role in enhancing atrazine degradation by Atz-EN-01 strain. This explains the synergistic activity between the indigenous microbes and Atz-EN-01 strain. The small decrease in atrazine concentration in both sterilized and fresh soil controls may be attributed to the adsorption of atrazine on to the soil particles as well as the organic matter present in the soil. It is thus proved that the presence of degrading microbes in the soil results in the enhanced dissipation of atrazine when compared to the uninoculated control soils. Therefore, we suggest that strain Atz-EN-01 can be a good candidate for establishing bioremediation strategies for the decontamination of atrazine contaminated soils.

CONCLUSION

In conclusion, obtained results here showed that the new yeast *Pichia kudriavzevii* strain Atz-EN-01 isolated from contaminated agricultural soil was proficient for atrazine biodegradation in various environments. The involvement of three key enzymes in the degradation of atrazine was elucidated and the atrazine degradation pathway in the strain Atz-EN-01 was proposed. Hence, the new soil yeast, *Pichia kudriavzevii* Atz-EN-01 could be implied for developing a bioremediation strategy of atrazine contaminated soil and aqueous environment.

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