Biodegradation of atrazine by Cryptococcus laurentii isolated from contaminated agricultural soil

Evy Alice Abigail M., Lakshmi V and Nilanjana Das*

Environmental Biotechnology Division
School of Bio Sciences and Technology, VIT University, Vellore-632014, Tamil Nadu, India

ABSTRACT

Atrazine is one of the most environmentally prevalent s-triazine – ring herbicides. The widespread use of atrazine and its toxicity necessitates search for remediation technology. As atrazine is still used in India as a major herbicide, exploration of atrazine degrading microorganisms are of immense importance. Considering lack of reports on yeast as degrader of atrazine, studies were carried out on atrazine degradation using Cryptococcus laurentii, a yeast species isolated from contaminated agricultural soil. The influence of various factors viz. as additional carbon source, additional nitrogen source, pH, temperature, shaking speed, inoculum volume and initial atrazine concentration on growth and atrazine degradation were studied. Cells grown in media with atrazine as sole carbon and nitrogen source showed more degradation compared to media containing additional carbon and nitrogen sources. The optimum growth conditions for atrazine degradation by C. laurentii were found to be at pH 7, temperature 30 °C, shaking speed 120 rpm, inoculum size 3 % (w/v) with initial atrazine concentration 150 ppm. 100 % degradation of atrazine was achieved by C. laurentii within 9 days. GC-MS analysis detected the formation of four metabolites viz. hydroxyatrazine, deethylatrazine, deisopropylatrazine and deethyldeisopropylatrazine during the process of degradation of atrazine. This is the first report on atrazine degradation using yeast species.

KEYWORDS: Atrazine, Biodegradation, Yeast.

INTRODUCTION

Atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-1, 3, 5-triazine) is a selective, systemic triazine herbicide, widely used for the control of annual broadleaf and grassy weeds in corn, maize and low brush blueberries. Although banned for use in many European countries, atrazine is still a commonly used herbicide in many countries like China, India and USA [1, 2]. The world market for atrazine is worth over $400 million at the user level. Due to its moderate water solubility and low volatility, atrazine remains highly mobile in soil. Researches have illustrated the toxic effects of atrazine in algae, aquatic plants, aquatic insects, fishes and mammals [3]. Presence of atrazine at ppb levels has shown to disrupt sexual development in amphibians and thus may pose serious ecological risks [4]. Atrazine is considered as a potential environmental contaminant [5, 6], and considered as one of the worst ground water pollutant.

The United States Environmental Protection Agency (USEPA) in 2006 started reviewing the possible association of atrazine with carcinogenic effects in humans. As this pollutant may be a hazard to human health, much attention has been paid for its removal from environment [7].

Several methods are available for atrazine removal from contaminated soil, water and wastewater. Among these, the most commonly used techniques are chemical treatment, incineration, adsorption, phytoremediation and
biodegradation. Most commonly employed chemical methods for the remediation of atrazine bearing wastewaters are photolysis, hydrolysis, dehalogenation and oxygenation. Conventional methods of atrazine removal have some disadvantages. These methods are either very costly producing other toxic substances or are not feasible [8]. Removal of atrazine from drinking water by adsorption using granular activated carbon or powdered activated carbon has been recognized as the best available technology. But the feasibility of a specific adsorbent-adsorbate system must be examined in the laboratory and there are several reports on success and failure of adsorption process on atrazine. Hence from health, environment and economic point of view, it is necessary to find out the other mode of atrazine removal. Microbial methods of atrazine degradation are better alternative to this problem. There are several reports on atrazine degradation using bacteria [3, 9-17] and fungi [3, 18-21]. Reports are scanty regarding the potentiality of yeast as degrader of atrazine. Therefore, the aim of the present investigation was to study the biodegradation of atrazine using the yeast species Cryptococcus laurentii isolated from contaminated agricultural soil samples.

MATERIALS AND METHODS

Chemicals
Atrazine (99% pure) was purchased from Sigma Aldrich, Bangalore, India. All other chemicals purchased are of analytical grade from Himedia limited, Mumbai, India and Sigma Aldrich, Bangalore, India.

Growth Media and Culture conditions
The mineral salt medium used in this study contained (g/L): K2HPO4, 0.4; KH2PO4, 0.2; NaCl, 0.1; MgSO4.7H2O, 0.5; MnCl2, 0.01; Fe (SO4)3, 0.01; Na2MoO4, 0.01; pH 6; temperature 30 °C and atrazine 50 ppm was used for enrichment. For solid medium, 2% (w/v) agar was added to the same atrazine containing liquid mineral salt medium. Stock solution of atrazine was prepared with a concentration 500 ppm in methanol that was sonicated to reduce the particle size of the atrazine crystals and was diluted to the required concentrations for the degradation studies.

Enrichment and Identification of Atrazine degrading yeast
Surface soil was collected from atrazine contaminated sugarcane fields, Vellore, India, for isolating atrazine degrading yeast. The collected agricultural soil was enriched in the above mentioned mineral salt medium containing atrazine for 7 days. After enrichment, mixed culture was streaked further to obtain pure atrazine degrading yeast cultures. Isolated pure culture of yeast species were then stored in solid mineral salt medium containing atrazine (50 ppm) for further degradation studies. Isolated yeast was identified using VITEK 2 compact yeast card reader with the software 05.01 from Council for Food Research and Development (CFRD), Kerala, India.

Effect of Growth Parameters on Atrazine Degradation
To investigate the effect of growth parameters on atrazine degradation, yeast isolate (2 % (w/v)) was grown in atrazine (50 ppm ) containing liquid medium of pH 6.0 maintained at 30 °C in 100 ml Erlenmeyer flask for 5 days on an orbital shaker at 120 rpm. Yeast cells in log phase of the growth were harvested and used as inoculum.

To study the effect of additional carbon sources, dextrose, fructose, lactose, maltose and sucrose were added at 1 gm/l concentration to atrazine containing media. The effect of additional nitrogen sources on degradation of atrazine was also studied by adding ammonium nitrate, ammonium sulfate, magnesium nitrate, sodium nitrate and urea at 1 gm/l concentration to atrazine containing media.

The effects of different growth parameters viz. pH (5-9), temperature (20- 40 °C), shaking speed (80 -140 rpm) and inoculum volume (1-5%) on atrazine degradation were studied in the medium containing atrazine as sole carbon and nitrogen source. The atrazine degrading efficiency of the isolated yeast species was tested by varying atrazine concentration in the range of 50-250 ppm. During the experiment, samples were collected periodically at different intervals of time for estimation of biomass and atrazine degradation. All the experiments were done in triplicates to reduce experimental errors.

Analytical Methods
A UV- visible spectrophotometer was used to monitor the cell density by measuring the turbidity at 600 nm. The cell dry weight was measured by drying the cell pellet at 80 °C in hot air oven. Using Gas chromatography (GC), atrazine concentration and metabolites formation were studied. At each sampling time, 1 ml of culture supernatant was extracted with 2 ml of ethyl acetate and further concentrated by air flow to 0.1 ml before analysis [20]. GC analysis was performed with a gas chromatograph (JEOL GCMATE II) equipped with an autosampler, flame ionization and thermal control detector and polysilphenylene-siloxane GC column (DB-5 column, 30 m X 0.25 mm i.d., 0.25 μm film thickness). The operating conditions were as follows: injector temperature 280 °C and transfer...
line temperature 300 °C, injection volume was 1.0 mL with splitless injection. The initial temperature was 90 °C, held for 0.5 min, then was increased to 160 °C at 15 °C/min and to 280 °C, was held for 5 min. The flow rate was 1.5 mL/min with vacuum compensation at constant flow with helium as carrier gas. Mass spectroscopy (MS) coupled with electron impact ionization at 70 eV was used to identify the metabolites formed during atrazine degradation. A solvent delay was set at 8 min. The EI-MS experiments were performed with unit mass resolution and with a scan time of 1.55 s [22]. Identification of atrazine and its metabolites in the samples was based on the detection of their base peak ions as well as comparison of their retention times with those of authentic standards.

RESULTS AND DISCUSSION

Isolation and Identification of Atrazine degrading yeast

The yeast species capable of degrading atrazine was isolated using enrichment technique. The isolated yeast species was identified as Cryptococcus laurentii using VITEK 2 compact yeast card reader from Council for Food Research and Development (CFRD), Kerala.

Effect of growth parameters on atrazine degradation

The effects of various parameters such as additional carbon and nitrogen sources, pH, temperature, shaking speed, inoculum size on yeast biomass growth and atrazine degradation were studied with initial atrazine concentration of 50 ppm. To study the effect of initial atrazine concentration, atrazine was added at varying concentrations ranging from 50-250 ppm to the mineral salt media.

Atrazine degradation in media containing atrazine as sole carbon and nitrogen source showed the maximum degradation of 80% (Fig. 1). Cells grown in media with additional carbon sources showed comparatively less degradation and less biomass yield. Similar results were observed in case of Agrobacterium radiobacter J14a, where the additional carbon sources such as sucrose did not significantly increase the atrazine degradation rate [23].

Additional nitrogen sources also showed a negative effect on atrazine degradation as well as biomass yield of the yeast species (Fig. 2). Similar results were reported in atrazine biodegradation by indigenous soil populations where the presence of preferential nitrogen sources is reported detrimental to atrazine degradation [24]. In bacterial strains such as Pseudomonas, Klebsiella etc., nitrogen amendments were found to decrease the atrazine degradation rates [25]. In fungi, atrazine mineralization was suppressed by nitrogen supplements, suggesting that additional nitrogen could alter the microbial process and carbon uptake and thus decreased atrazine degradation rate [26].

Figure 1: Effect of additional carbon sources on atrazine degradation by C. laurentii.
The effects of temperature on growth and atrazine degradation were examined (Fig. 4). The degradation efficiency was found to be maximum (90%) at 30 °C. A decline in the degradation efficiency was observed for temperatures below and above 30 °C.

The effect of shaking speed on the yeast growth and atrazine degradation efficiency was determined by varying the shaking speed at 80, 100, 120, 140 and 160 rpm. At 120 rpm maximum growth and atrazine degradation of 88% was achieved within 5 days (Fig 5). Significant decrease in the biomass production and atrazine degradation was observed at other shaking speeds.
The effect of inoculum size on caffeine degradation is shown in figure 6. Atrazine degradation was increased with increase in inoculum size. Highest atrazine degradation of 95 % was achieved with an inoculum size of 3 % (w/v). At inoculum size of 4 and 5 % (w/v), the biomass as well the degradation efficiency decreased to 67 and 35 % respectively. Thus, an inoculum size of 3 % (w/v) was found to be optimum for atrazine degradation.

To study the effect of initial atrazine concentration on degradation efficiency, the yeast species was grown in medium containing atrazine of concentration varying from 50-250 ppm. Complete degradation of atrazine was observed up to a concentration of 150 ppm (Fig 7). Above this concentration, atrazine degradation and biomass yield were relatively less.
Analysis of degradation products

The metabolites produced during atrazine degradation by yeast species were identified using GC-MS. The GC-MS results of atrazine showed retention time of 13.72 min (Fig 8) with a molecular weight of 214.59. At 3rd and 6th day, four metabolites of atrazine were observed (Fig 8a, b), with retention time of 12.78, 13.52, 15.01 and 15.56 min which were identified as hydroxyatrazine with molecular weight 197.23, deethylatrazine with molecular weight 187.63, deisopropylatrazine with molecular weight 173.63, and deethyldeisopropylatrazine with molecular weight 145.55 respectively. Gas chromatogram (Fig 8c) did not show the existence of any compound which confirmed complete atrazine degradation within 9 days. Results of the present study indicated that the yeast species *C. laurentii* degraded atrazine to form polar dechlorinated metabolite hydroxyatrazine and N-dealkylated metabolites viz. deethylatrazine, deisopropylatrazine and deethyldeisopropylatrazine. Similar mechanisms of atrazine degradation in microorganisms were previously reported [28]. In some bacterial strains the biodegradation of atrazine initiate through N-dealkylation of the lateral ethyl and isopropyl chains to deethylatrazine and deisopropylatrazine [21]. In *Nocardia* sp., the dechlorination of atrazine resulted in the formation of hydroxyatrazine which was further degraded to N-ethyllumelide. In fungi such as *Pleurotus pulmonarius*, atrazine was degraded to produce mainly the N-dealkylated metabolites deethylatrazine, deisopropylatrazine and deethyl-deisopropylatrazine [20]. In case of *Aspergillus* sp., *Rhizopus* sp., *Fusarium* sp., *Penicillium* sp. and *Trichoderma* sp., the degradation of atrazine
proceeds through N-dealkylation leading to two main degradation products, deethylatrazine and deisopropylatrazine [21].

Figure 8: Gas Chromatogram of (a) Atrazine and its degradation products (b) at 3rd day (c) at 6th day and (d) at 9th day showing complete degradation.
CONCLUSION

This study has shown that the yeast species Cryptococcus laurentii isolated from contaminated agricultural soil can serve as an efficient degrader of atrazine. Degradation efficiency was found to be greatly influenced by the factors viz. pH, temperature, shaking speed and initial atrazine concentration. Complete degradation of atrazine was noted when it was used as sole carbon or nitrogen source in the growth medium. Additional carbon and nitrogen sources showed negative effects on atrazine biodegradation. The present knowledge concerning the degradation of atrazine using yeast species may be relevant for the development of effective bioremediation strategies for contaminated agricultural soil.

REFERENCES


Available online at www.scholarsresearchlibrary.com