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Influence of pH, temperature and nutrient addition on the degradation of atrazine by *Nocardioides* spp. isolated from agricultural soil in Nigeria

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ABSTRACT

Aims: To effectively exploit the atrazine degrading capabilities of *Nocardioides* spp. isolated from agricultural soil samples in Nigeria and ascertain the effect of pH, temperature and nutrient addition on the degradation process.
Methodology and results: Isolates were cultivated on atrazine mineral salts medium at a temperature range of 4 °C - 45 °C and a pH range of 3-10. An optimum atrazine degrading activity was observed in the isolates between temperatures of 25 °C and 37 °C and between pH 5 and 8. Different carbon sources (glycerine, glucose, chitin, cellulose and sodium citrate) and nitrogen sources (urea, biuret, cyanuric acid, potassium nitrate and ammonium chloride) were also added to the medium. The addition of carbon and nitrogen sources did not increase degradation rates although urea and glycerine repressed the degradation ability of the isolates. Statistical analyses of variance at $P < 0.05$ showed no significant differences in the growth and degradation rates by both bacterial isolates under these conditions.
Conclusion, significance and impact study: Atrazine degradation by *Nocardioides* spp. is pH and temperature dependent, and requires no additional sources of carbon and nitrogen. Hence, its use in bioremediation of atrazine contaminated agricultural soil should be explored.

Keywords: atrazine; degradation; pH; temperature; *Nocardioides*

INTRODUCTION

Atrazine [2-chloro-4-(ethylamino)-6(isopropylamino)-1,3,5-triazine], a member of the s-Triazine pesticides, has been widely used all over the world to control a variety of broad leaf and grassy weeds in agriculture, forestry and non-cropped industrial lands (Nousiainen *et al.*, 2014). In spite of its low solubility in water (33 mg/L at 20 °C), atrazine has high mobility in soil, hence can contaminate both groundwater and surface water in regions where it is used (Fan and Song, 2014). It is relatively persistent in the environment, with an average half-life of approximately 60 days in soil. Its residue can remain at sites of application for many years under field conditions, even after discontinued use thus finding its way into drinking water (Perk, 2006; Jablonowski *et al.*, 2011). It has also been classified as a class C carcinogen with adverse effects on different animals and humans (Kolekar *et al.*, 2013). Consequently, regulatory agencies have described atrazine as a target anthropogenic pollutant that requires the urgent development of methods for its removal from contaminated sites (Ghosh and Philip, 2006; Fazlurrahman *et al.*, 2009; Pandey *et al.*, 2009). Different techniques that have been explored for the removal of atrazine from the environment include chemical remediation and bioremediation. However, bioremediation is widely acknowledged as the most suitable method for removal of atrazine (Chirnside *et al.*, 2009; Udiković-Kolić *et al.*, 2012).

Atrazine is a poor energy source due to the highly oxidized carbons in the ring. It is catabolized as a carbon and nitrogen source in limiting environments although, the optimum carbon and nitrogen availability is not known (Dehghani *et al.,* 2013). Theoretically, microorganisms which metabolize nitrogen-containing compounds can be manipulated to selectively metabolize such compounds by inducing nitrogen limitation through the addition of carbon source to increase the C/N ratio (Krutz *et al.*, 2010). Environmental factors including temperature, pH and oxygen content have been observed to influence biodegradation of atrazine at varying degrees (Mahía *et al.*, 2007; Mueller *et al.*, 2010; Nousiainen *et al.*, 2014).

Nocardioides sp. is known to degrade atrazine due to its ability to utilize the pesticide as its sole source of carbon and nitrogen (Topp *et al.*, 2000; Omotayo *et al.,* 2013). Since temperature and pH play significant role in regulating microbial metabolism (Udiković-Kolić *et al.*, 2012), the influence of different temperature and pH

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range as well as sources of carbon and nitrogen on the ability of *Nocardioides* sp. to degrade atrazine must be clearly defined. Therefore, this paper reports the observation on the efficiency of *Nocardioides* spp. in the degradation of atrazine in a liquid culture media when exposed to different regimes of pH and temperatures. The effect of different carbon and nitrogen sources on the biodegradation process was also examined.

MATERIALS AND METHODS

Isolates

Pure isolates of atrazine-degrading bacterial strains used in this study were obtained from an agricultural soil with a history of atrazine application (Omotayo *et al.,* 2013). The strains have been identified and designated as *Nocardioides* sp. strain EAA-3 (GenBank accession number FJ406513) and *Nocardioides* sp. strain EAA-4 (GenBank accession number FJ406514).

Chemicals

Analytical-grade atrazine (98-99.5% purity) was purchased from ChemService Inc. (West Chester, Pa.). The solvents used for the High Performance Liquid Chromatography (HPLC), including the HPLC grade water, acetonitrile and methanol were purchased from Fischer Chemicals, Fischer Scientific (Fair Lawn, NJ, USA). All other reagents were of high purity. Glucose, cellulose, sodium nitrate, chitin, glycerine, urea, biuret, potassium nitrate, ammonium chloride and cyanuric acid were purchased from ChemService Inc. (West Chester, PA, USA).

Atrazine degradation by growing cultures

Bacterial cells were grown in atrazine Mineral Salts Medium (MSM) and then harvested at late exponential phase by centrifugation (Eppendorf 5415D, Hauppauge, NY, USA) at 10,000 rpm for 5 min (Omotayo *et al.,* 2013). Harvested cells were washed twice with sterile MSM. Pelleted cells were re-suspended in atrazine MSM and atrazine degradation was assessed using 10^4 cells/mL of the isolates. (Medium contained 25 mg/L of atrazine). Degradation of atrazine was analyzed in 50 mL of atrazine MSM inoculated with washed cells and incubated at different temperatures on a rotary shaker (Innova 2100, Brunswick Scientific, Enfield, CT, USA) at 150 rpm for 12 days. The pH of the medium was maintained at different values ranging from pH 3-10. Culture samples were drawn at 24 h time intervals for the evaluation of residual atrazine concentration.

Effects of temperature and pH on atrazine degradation

The effect of a range of temperature (4 °C, 10 °C, 25 °C, 37 °C and 45 °C) on atrazine degradation (25 mg/L) by axenic cultures was assessed by incubating the cultures at these temperature values with shaking at 150 rpm for 12 days. The pH of the atrazine MSM was adjusted after preparation and maintained at pH 3, 5, 7, 8 and 10 respectively.

Effects of carbon and nitrogen sources on atrazine degradation

Pure cultures of the *Nocardioides* spp. were tested for their ability to degrade atrazine in the presence of different carbon (0.08 g/L of C equivalent to 2% glucose) and nitrogen (4 mM nitrogen) (García-González *et al.*, 2003) sources. The carbon sources were glucose, sodium citrate, cellulose, glycerin and chitin. The nitrogen sources include urea, cyanuric acid, potassium nitrate, ammonium chloride and biuret. The glucose, glycerine, urea, cyanuric acid and biuret used were separately prepared and filter sterilized using 0.20 µm, Nalgene disposable bottle top filter. All other substrates were autoclaved prior to inoculation.

Determination of microbial growth

Growth of organisms was determined spectrophotometrically (λ_{600nm}) in a Biomate (3) thermospectronic spectrophotometer (Rochester, New York) as described by de Souza *et al.* (1998).

Determination of pH

The pH of the medium was adjusted using a glass electrode pH meter (Beckman). The pH meter was calibrated with pH 7.0 and pH 4.0 buffer standards before use. The pH was determined by inserting the electrode into the liquid medium.

HPLC analytical methods

Atrazine was extracted from the medium with equal volume of acetonitrile and centrifuged at 12,000 rpm for 5 min. The supernatants were pipetted into sterile glass vials for HPLC analysis. This was performed using a Shimadzu LC 10AD VP High Pressure Liquid Chromatography (System Columbia, MD, USA) equipped with an Alltech C-18 Econosphere column (5-µm pore size; length, 15 cm; diameter 4.6 mm; Grace Davison Discovery Sciences, Deerfield, IL, USA). Samples (50 µL) were eluted at 1 mL/min with an isocratic mobile phase of acetonitrile to water (3:2) and quantified with a UV-VIS detector set at 220 nm. Analytical standards of atrazine were prepared in acetonitrile to water solutions (2:3) at concentrations ranging from 1 to 25 mg/L.

Statistical analysis

Determination of mean growth rate constant, mean generation time, analysis of variance (ANOVA), and other statistical tests were performed using the Prism computer software programme version 5.00 (GraphPad Software, San Diego, CA). Significant limits were set at $P < 0.05$.

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RESULTS

Effect of temperature on atrazine degradation

The results of the preliminary analyses of temperatures and pH conditions optimal for atrazine utilization after 12 days of incubation is presented in Figure 1. The degradation of Atrazine was observed only at 25 °C and 37 °C after 12 days of inoculation for both strains of *Nocardioides*. Degradation was negligible at 4 °C, 10 °C and 45 °C within the pH range of 3-10 tested.

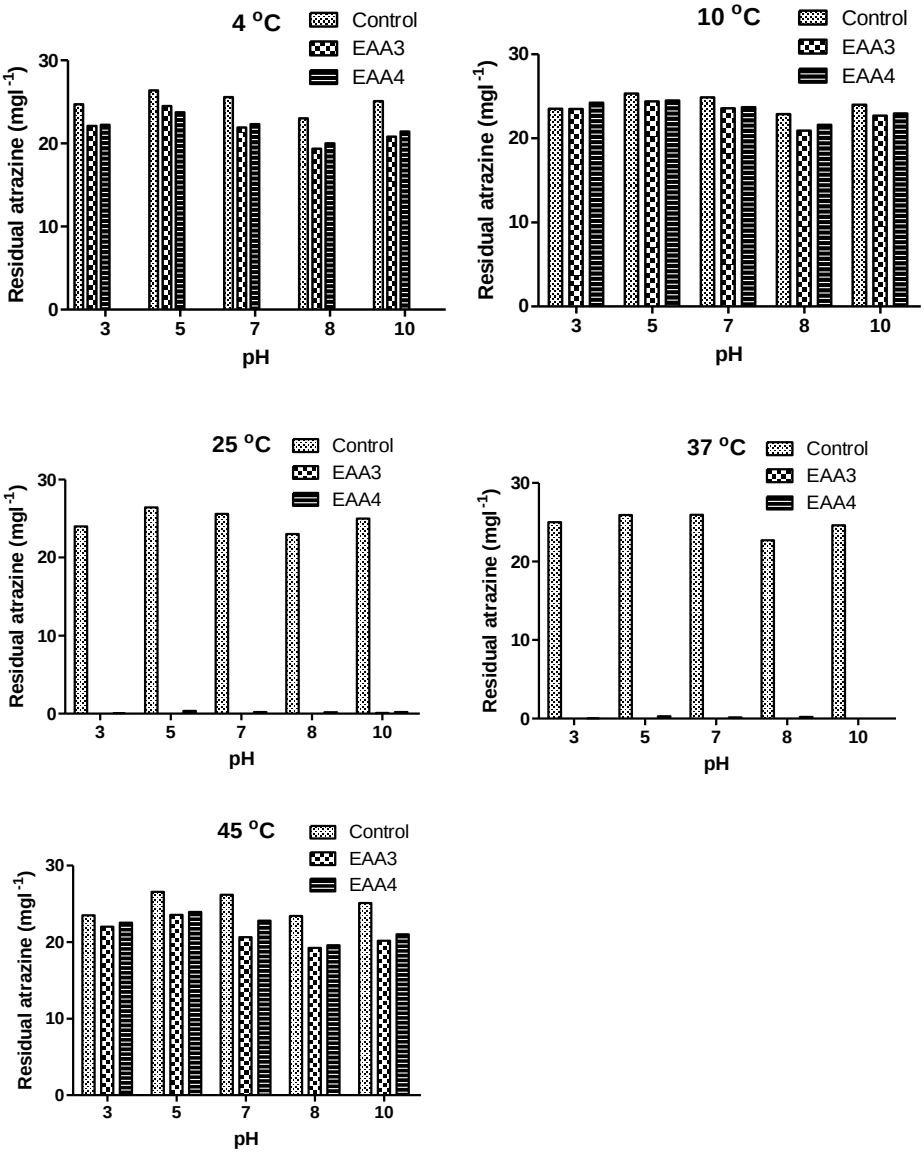

Figure 1: Effect of temperature and pH on atrazine degradation after 12 days of incubation.

Degradation dynamics of atrazine at 25 °C and 37 °C at pH range of 3 to 10

Atrazine degradation was best at pH 5, 7 and 8 (Figure 2). At 60 h of incubation, *Nocardioides* sp. EAA-3 completely degraded atrazine at pH 5, 7 and 8 at both 25 °C and 37 °C; whereas, *Nocardioides* sp. EAA-4 took a longer time (72 h) to completely degrade atrazine at 37 °C for these pH levels. Statistical analyses of variance ($P < 0.05$) showed no significant difference in the degradation rate of atrazine and growth rate constant of both *Nocardioides* spp. strains at 25 °C and 37 °C and at pH 5, 7 and 8 (Table 1). At pH levels 3 and 10, the atrazine degradation rates and growth rates of both strains were considerably low compared to the results obtained at pH 5, 7 and 8.

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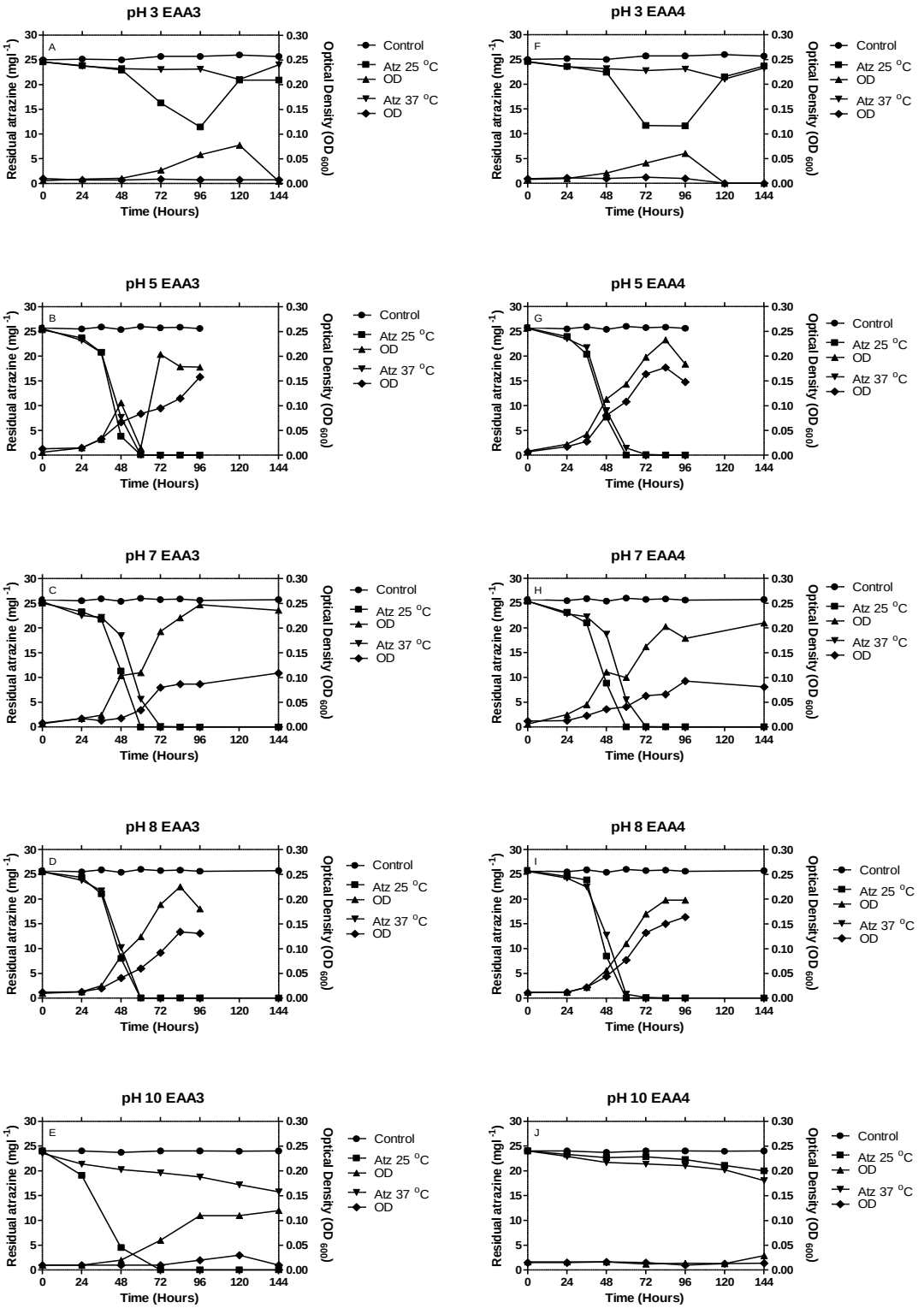

Figure 2: Degradation of atrazine and growth dynamics of *Nocardioides* sp. EAA-3 and *Nocardioides* sp. EAA-4 at 25 °C and 37 °C, pH 3-10. OD, Optical Density; Atz, Atrazine.

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Table 1: Degradation rate and growth rate constant of isolates in atrazine culture medium.

pH	*Nocardioides* sp. EAA3				*Nocardioides* sp. EAA4			
	25 °C		37 °C		25 °C		37 °C	
	Degradation rate (mg/L h)	Growth rate constant (1/h)	Degradation rate (mg/L h)	Growth rate constant (1/h)	Degradation rate (mg/L h)	Growth rate constant (1/h)	Degradation rate (mg/L h)	Growth rate constant (1/h)
3	0.1154	0.0066	0.0215	-0.0012	0.1793	-0.0002	0.0243	-0.0074
5	0.4210	0.0235	0.4260	0.0226	0.4282	0.0190	0.4028	0.0198
7	0.4167	0.0107	0.3282	0.0122	0.4232	0.0101	0.3308	0.0096
8	0.4247	0.0214	0.4750	0.0252	0.4297	0.0247	0.4267	0.0255
10	0.3333	0.0129	0.0547	0.0046	0.0158	0.0034	0.0361	-0.0014

Effect of carbon sources on degradation of atrazine

The breakdown of atrazine by the bacterial isolates was not repressed by the presence of glucose, sodium citrate, cellulose, glycerine and chitin in the culture media (Figure 3). *Nocardioides* sp. EAA-3 showed a higher degradation rate with all carbon sources than *Nocardioides* sp. EAA-4. In cultures with cellulose and chitin as carbon sources, complete degradation was achieved at 96 h while the process took 120 h with glycerine as carbon source although there was a significant level of degradation at 96 h. Statistically, there were no significant difference between the rates of degradation with or without the addition of carbon sources at $P < 0.05$ confidence level; neither was there a significant difference observed with the different carbon sources. Statistical analysis also revealed that the growth of the isolates was not significantly increased by addition of any of these carbon sources. Although, it was observed that the presence of glycerine in the medium repressed bacterial growth completely (Figure 3). The result also showed that the isolates could not utilize the different carbon sources alone as substrate for growth and energy.

Effect of nitrogen sources on atrazine degradation

Inhibition of both atrazine degradation and growth of isolates were observed when urea was used as a nitrogen source (Figure 4). The presence of cyanuric acid, ammonium chloride, potassium nitrate and biuret in the culture media did not show any apparent inhibitory effect on atrazine utilization. Furthermore, there were no statistical differences observed in the catabolism and growth rate between addition of these nitrogen sources to the culture media and the use of atrazine as the sole source of carbon and nitrogen. The result also showed that the isolates could not utilize the different nitrogen sources alone for growth and energy.

DISCUSSION

The influence of media components and physicochemical parameters on degradation of atrazine observed in this study emphasizes the role of abiotic factors in the enhanced degradation of atrazine in the environment. The results showed that the optimal temperature range for atrazine degradation were 25 °C and 37 °C. These temperature values are close to the optimal growth temperature for *Nocardioides* sp., which is 28 °C (Dastager *et al.*, 2008). This explains the negligible degradation and microbial activity observed at 4 °C, 10 °C and 45 °C. Findings from this study confirmed Bernard *et al.* (2005) statement that volatilization and the rate of chemical and microbial degradation are favoured by high temperatures and dissipation of pesticides is significantly faster under tropical conditions (Bernard *et al.,* 2005). Thus, the effect of accelerated degradation in tropical zones might be stronger than in temperate regions (Arbeli and Fuentes, 2007).

It was observed that atrazine degradation was higher at pH 5, 7 and 8. This rate is consistent with findings from similar studies (Korpraditskul *et al.*, 1993; Pussemier *et al.*, 1997; Nelieu *et al.*, 2000). Several studies have emphasized that an increase in soil pH results in an increase in soil microbial biomass and enzymatic activities, which in turn helps the microbial community to adapt and develop gene-enzyme systems for the enhanced degradation of pesticides (Bardgett and Leemans, 1995; Singh *et al.*, 2003). There have been reports of a nonspecific relationship between high pH and rapid biodegradation of triazine herbicides (Houot *et al.*, 2000). Although Korpraditskul *et al.* (1993) reported that at pH 5, atrazine degradation was due to chemical degradation as a result of hydrolysis of chlorine. This study observed degradation of atrazine by the bacterial isolates at this pH value. This agrees with reports by Mueller *et al.* (2010) who documented rapid atrazine dissipation with half-lives of less than 4 days in soil of pH 5.5 *ex-situ* and less than 8 days *in-situ*.

The addition of different carbon sources to atrazine degrading cultures showed no significant difference in the rate of degradation of atrazine. Hence, the presence of these carbon sources in the soil may not promote or limit the persistence of atrazine in the soil and surface water. Other researchers however reported otherwise. Ngigi *et al.* (2013), for instance, reported that the addition of sucrose as a carbon source reduced the rate of degradation of atrazine. Also, Dehghani *et al.* (2013) reported that the addition of sodium citrate increased rate of degradation of atrazine by a mixed consortium because

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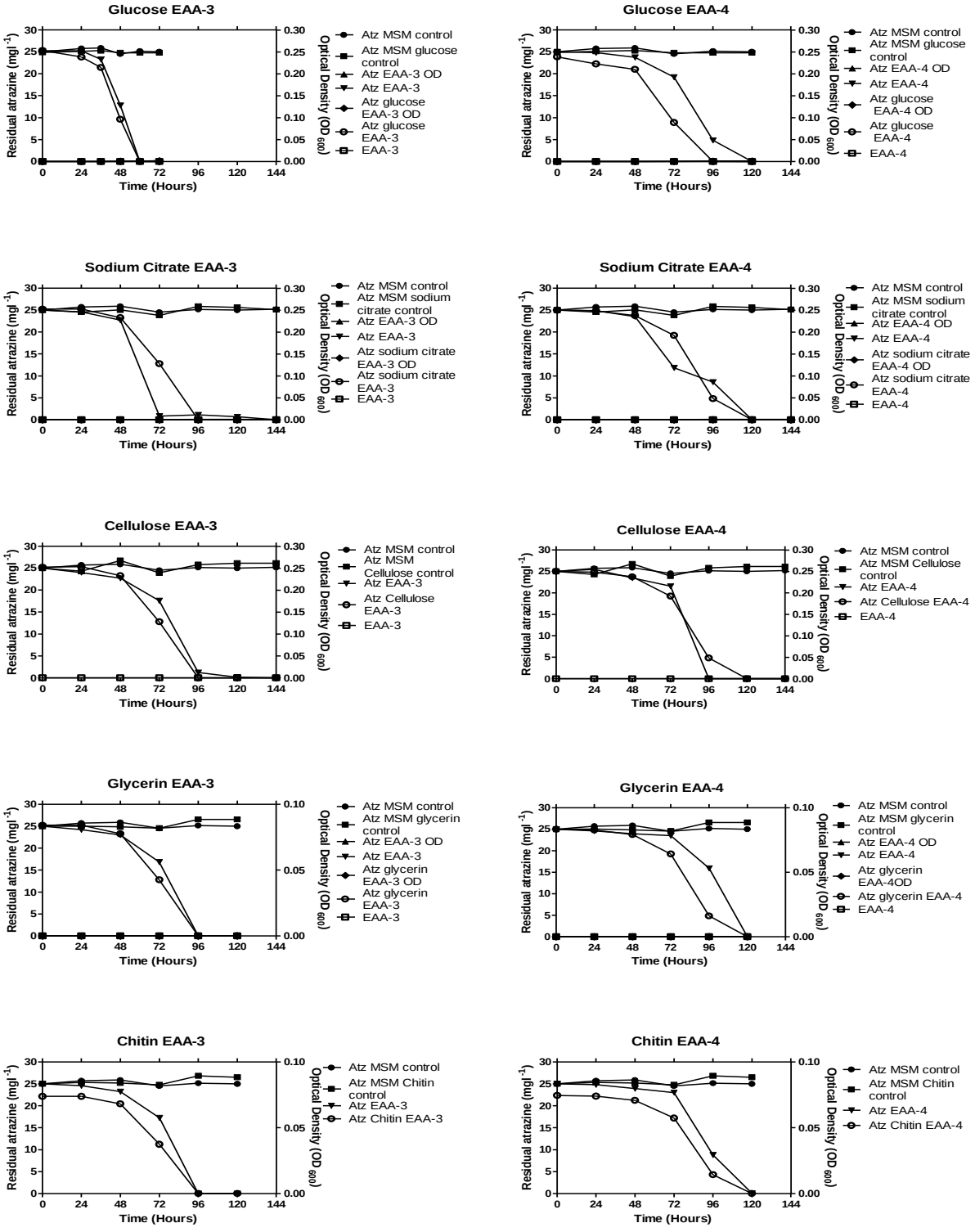

Figure 3: Effect of addition of carbon substrates on degradation of atrazine by *Nocardioides* sp. EAA-3 and EAA-4 at 25 °C, pH 7. Atz, Atrazine; MSM, Mineral Salts Medium; OD, Optical Density.

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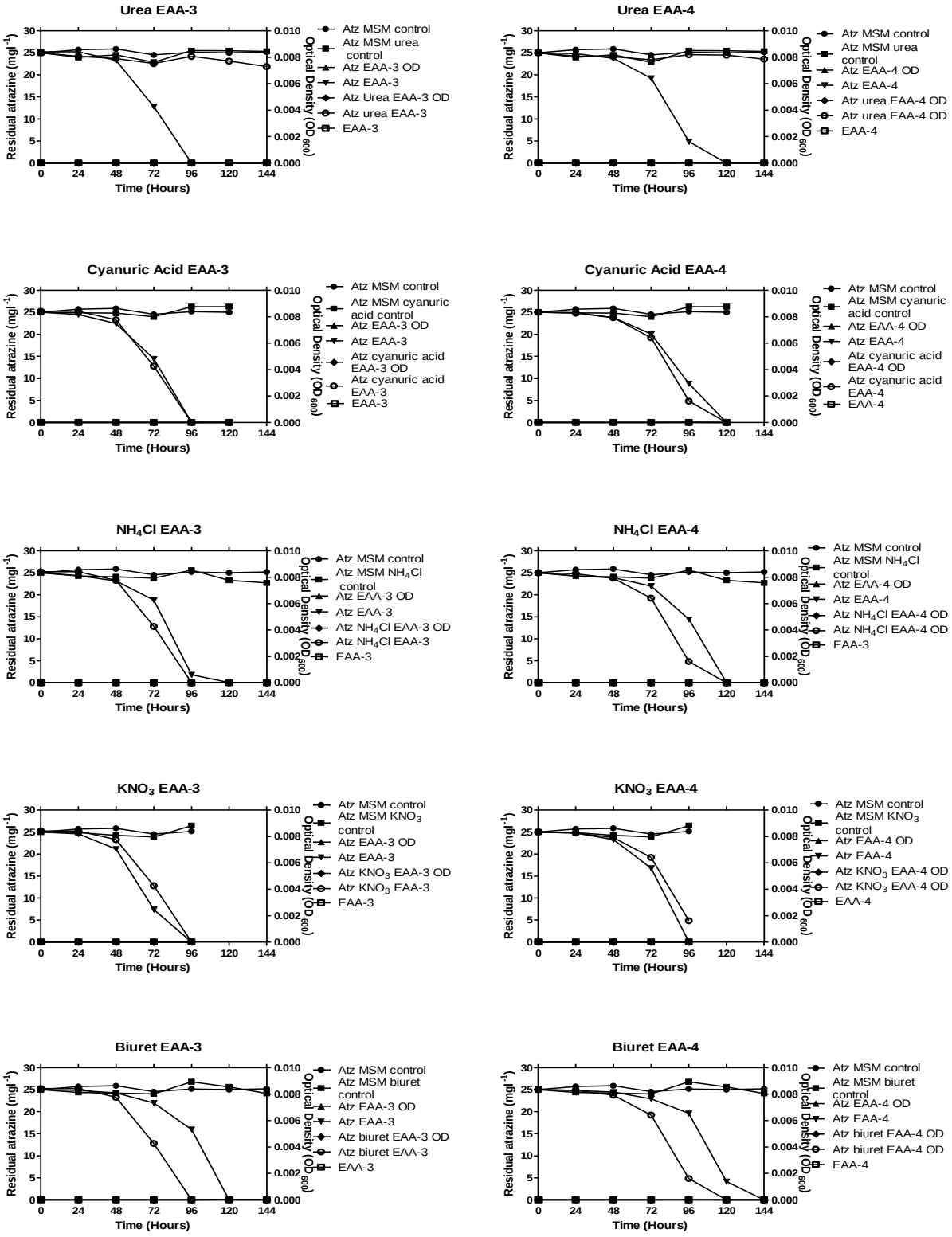

Figure 4: Effect of addition of nitrogen substrates on degradation of atrazine by *Nocardioides* sp. EAA-3 and EAA-4 at 25 °C, pH 7. Atz, Atrazine; MSM, Mineral Salts Medium; OD, Optical Density.

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it initiated the co-metabolism of atrazine.

The result obtained for the repression of atrazine degradation in the presence of urea is similar to the findings of Barra Caracciolo *et al.* (2005). According to García-González *et al.* (2003), atrazine does not stimulate its own catabolism when a repressing nitrogen source is present. Therefore, the addition of urea to the soil might increase weed control and hence the persistence of the herbicide in soil and surface water. Unlike urea, the addition of other nitrogen sources (ammonium chloride, potassium nitrate, cyanuric acid and biuret) had no repressive effect on atrazine degradation. Similar results for buriet and cyanuric acid were demonstrated by García-González *et al.* (2003). Struthers *et al.* (1998) showed that the addition of nitrates (KNO_3 or NH_4NO_3) in amounts far above those required for cell growth to Minimal Salts Medium resulted in growth of cells and complete loss of atrazine from the medium. Although, the bacterial isolates in this study mineralised atrazine in the presence of ammonium, several studies have reported otherwise. For instance, Mandelbaum *et al.* (1993) and García-González *et al.* (2003) reported that the presence of ammonium in the growth medium did not stimulate atrazine degradation, suggesting that ammonium-mediated repression operates regardless of the presence of the herbicide in the culture medium. The addition of nitrogen sources in the form of fertiliser is a typical agricultural practice. The ability of *Nocardioides* sp. to degrade atrazine both in the presence and absence of additional carbon and nitrogen sources demonstrates its suitability for bioremediation of atrazine contaminated agricultural soils and the cost effectiveness of the process since additional nutrients may not be required.

CONCLUSION

Nocardioides spp. are able to utilise atrazine as the sole source of carbon and nitrogen in a process which is both temperature and pH dependent. The evidences presented in this study demonstrate the ability of *Nocardioides* spp. to effectively degrade atrazine in conditions similar to those in agricultural soils (presence of additional carbon and nitrogen sources). They also suggest the suitability and potential use of the strains in the bioremediation of atrazine contaminated agricultural soils.

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