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Changes in *Pseudomonas sp.* CY growth in the presence of atrazine

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Abstract

Microbial degradation, compared with many other degradation processes, is the most important pathway for the depletion of triazine herbicides in soil. The aim of this study was to determine the growth potential of *Pseudomonas* sp. CY in the presence of atrazine and additional carbon (sodium citrate) and nitrogen (ammonium-nitrate) sources. The experiment was performed with five treatments: i) 100 mg/L atrazine (control); ii) One hundred mg/L atrazine + sodium citrate (0.3 %, w/v); iii) One hundred mg/L atrazine + sodium citrate (0.3 %, w/v) + ammonium nitrate (0.6 %, w/v); iv) Atrazine (300 mg/L) + sodium citrate (0.3 %, w/v) and v) Atrazine (500 mg/L) + sodium citrate (0.3 %, w/v). The bacterial count was determined after incubation (7 days at 30°C) using the agar plate method, while atrazine degradation was determined by measuring the optical density at 221 nm. *Pseudomonas* sp. CY can partially utilize atrazine as the sole source of carbon and energy. The highest values of the bacterial count were determined at the highest initial atrazine concentrations; however, bacterial growth was not detected in these treatments. A significant impact of citrate on bacterial growth and atrazine degradation was observed, while the addition of nitrate decreased the atrazine degradation rate. This study confirmed that *Pseudomonas* sp. CY can be used as a prominent candidate for the remediation of atrazine-affected environments.

Key words: ammonium-nitrate, atrazine, bacterial growth, *Pseudomonas* sp., sodium-citrate

Introduction

The use of pesticides is strongly recommended in contemporary agricultural production. One of the pesticides that has been frequently applied during the last decades of the 20th century is atrazine ($C_8H_{14}CIN_5$). However, their widespread use has led to soil contamination (Duan et al., 2016). Atrazine is one of the most frequently detected pesticides In various agricultural soils. Furthermore, atrazine has phytotoxic (Wang et al., 2015), carcinogenic, and teratogenic (Sun et al., 2020) effects. Atrazine was also detected in surface and subsurface waters, although its use was prohibited by the EU in 2003, and Amadori et al. (2016) reported the widespread use of atrazine worldwide. Considering the half-life of atrazine, which varies from one month to approximately a year (Diana et

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al., 2000), it is important to find an environmentally friendly and low-cost approach for the depletion of atrazine in the environment.

The ability of several bacterial genera to degrade atrazine has been well described (Bazhanov et al., 2016; Zhu et al., 2019). Nevertheless, in most cases, the low efficiency of microbial strains to degrade atrazine has been observed (Solomon et al., 2013). In addition, owing to the lack of microbe-based products for atrazine removal from the environment (Zhu et al., 2019), it is necessary to find more efficient microbial strains capable of cleaning atrazine-affected ecosystems. In this study, a pure culture of *Pseudomonas* sp. strain CY was used to estimate growth estimation in the presence of atrazine. This strain showed the ability to grow on benzene (Lalević et al., 2006).

Apart from degradation *via* direct metabolism, microbial strains are capable of the co-metabolic degradation of atrazine (Cupul et al., 2014). *In vitro* experiments have shown that the addition of other energy and nutrient source(s) may stimulate microbial activity (Ngigi et al., 2013). Although additional C sources stimulate atrazine mineralization (Dehghani et al., 2013), inhibition of this process after the addition of external N sources has been observed in other studies (Clausen et al., 2002). In these studies, various external C and N sources were used: acetate, pectin (Rhine et al., 2003), and sucrose (Wang et al., 2011) as C sources, whereas NH_4NO_3, cyanuric acid (Rhine et al., 2003), nitrate, urea (Bichat et al., 1999) and organic amendments (Forouzangohar et al., 2005) were used as N sources. Hence, it is evident that additional nutrient sources may play a crucial role in the microbial mineralization of atrazine (Struthers et al., 1998).

The objective of this study was to determine the effect of secondary nutrient sources (sodium citrate and ammonium nitrate) on the growth of *Pseudomonas* sp. CY in the presence of atrazine to estimate the efficiency of atrazine biodegradation under laboratory conditions.

Material and methods

Gram-negative *Pseudomonas* sp. CY, previously isolated from kerosene, was maintained in the Laboratory of Environmental Microbiology, Faculty of Agriculture, University of Belgrade, Serbia.

The liquid mineral salt medium (Mandelbaum et al., 1993) was inoculated with a suspension of pure cultures of *Pseudomonas* sp. CY (0.5 %, v/v). The initial population density of *Pseudomonas* sp. CY were determined on nutrient agar (Torlak, Serbia). Atrazine (diluted in methanol) and external C and N sources were added as nutrient sources for bacterial growth. The experiment was performed within five treatments: i. 100 mg × l^{-1} of atrazine (100 A); ii. One hundred mg × l^{-1} atrazine + 0.3 % (w/v) sodium citrate (100 AS); iii. One hundred mg × l^{-1} atrazine + 0.3 % (w/v) sodium citrate + 0.6 % (w/v) ammonium-nitrate (100 ASA); iv. 300 mg × l^{-1} atrazine + 0.3 % (w/v) sodium citrate (300 AS) and 500 mg × l^{-1} atrazine + 0.3 % (w/v) sodium citrate (500 AS). Incubation was performed in an

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orbital shaker (Biosan ES-20, Latvia) for seven days at 30°C. All experiments were performed in triplicates.

Samples for the determination of bacterial count were taken after 24, 48, 72, and 96 h and at the end of the incubation period. Bacterial counts were determined using nutrient agar (Torlak, Serbia) and expressed as colony-forming units (CFU) per mL. The atrazine degradation was estimated using a spectrophotometer (T70 UV/VIS Spectrometer, PG Instruments Ltd., UK) at 221 nm (Moreira et al., 2017). Optical density (OD_{221}) readings were used to determine the atrazine concentration in the liquid cultures at the beginning of the experiment, and after 24, 48, 72, 96, 120 and 168 h of incubation.

The results were statistically analyzed using the software package SPSS 20. To determine the statistically significant differences in the obtained values, one-way analysis of variance (ANOVA) was performed, followed by post-hoc Tukey's test ($p = 0.05$) for the values between different treatments at the same time interval, as well as between the same treatments on different time intervals.

Results and discussion

The results showed that the bacterial count, optical density of liquid cultures, and atrazine degradation were affected by the concentrations of atrazine, external nutrient sources, and incubation time. In this study, the initial population density of *Pseudomonas* sp. CY was 3.8×10^7 CFU x ml^{-1}.

As can be seen from Table 1, in all samples a decrease in the bacterial count compared with the initial population count was observed. During incubation, the presence of atrazine in all treatments hindered bacterial growth during the first 48 h of incubation. Zhang et al. (2012) revealed that pollutants might be accumulated in the microbial cells and cause metabolic disorders. Furthermore, they reported that atrazine inhibits bacterial growth. In the control, the lowest bacterial count was observed compared to other treatments, which suggests that the addition of external nutrient sources accelerated bacterial growth, particularly in the 100 AS and 100 ASA treatments. The addition of external nutrients and energy sources does not inhibit the microbial performance (Kannika et al., 2010). Moreover, the complex structure of atrazine contributes to its low bacterial growth (Sharma et al., 2019).

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Table 1. Bacterial count (x 10^6 CFU/mL) in the liquid medium supplemented with atrazine and external nutrient sources

Treatment	Time (hours)					
	24	48	72	96	120	168
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD
100 A (control)	8.03^{aA} ± 0.67	2.92^{bA} ± 0.65	3.65^{cA} ± 0.52	6.22^{dA} ± 1.39	2.31^{eA} ± 0.10	6.51^{fA} ± 1.97
100 AS	6.37^{aB} ± 1.14	3.07^{bB} ± 0.42	4.28^{cB} ± 0.30	5.15^{dB} ± 0.70	12.38^{eB} ± 0.10	5.42^{fB} ± 0.98
100 ASA	6.29^{aB} ± 0.56	4.36^{bC} ± 0.12	5.28^{cC} ± 0.18	5.98^{dC} ± 0.89	9.18^{eC} ± 0.15	7.77^{fC} ± 2.19
300 AS	10.99^{aC} ± 1.22	10.03^{bD} ± 1.48	9.30^{cD} ± 0.10	9.94^{dD} ± 0.20	9.80^{eD} ± 2.62	9.40^{fD} ± 1.99
500 AS	13.11^{aD} ± 1.11	13.11^{aE} ± 1.02	11.97^{bE} ± 0.96	9.39^{cE} ± 3.58	11.51^{dE} ± 0.12	12.49^{eE} ± 0.16

a, b, c - Values of the same treatment at different time intervals marked with different letters, are significantly different ($p<0.05$).

A, B, C - Values of different treatments at the same time interval, marked with different letters, are significantly different ($P<0.05$).

Although the highest *Pseudomonas* sp. CY counts were detected at 300 AS and 500 AS, a negligible bacterial growth was observed after 72 and 96 h, respectively. According to these results, higher atrazine concentrations remarkably decreased bacterial growth, which has been previously confirmed (Zhu et al., 2019). On the other hand, treatments with citrate (100 AS), and citrate + nitrate (100 ASA) showed more pronounced bacterial growth compared to the control; in both treatments, *Pseudomonas* sp. CY stationary phase was reached between 120 and 168 h of incubation. The addition of nitrate (100 ASA) resulted in a significantly higher cell number compared to 100 AS.

Ramadan et al. (1990) pointed out that some microbes are more likely to use nutrients and energy sources than pollutants. Sharma et al. (2019) found that several N sources are a better substrates for bacterial growth than atrazine. However, remarkable shifts in *Pseudomonas* sp. counts were observed during incubation. These changes may be linked to atrazine degradation (Yale et al., 2017). In most samples, the highest OD_{221} was recorded at the start of the incubation period (Table 2), which indicated that *Pseudomonas* sp. strain CY was able to use atrazine. Nevertheless, an irregular degradation trend was observed in all treatments, as previously reported (Alattas et al., 2023).

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Table 2. Optical density (OD_{221}) of the liquid cultures in the presence of atrazine and external nutrient sources

Treatment	Time (hours)						
	0	24	48	72	96	120	168
	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD	$\bar{x}$ ± SD
100 A (control)	1.41^{aA} ± 0.08	1.38^{aA} ± 0.16	0.38^{bA} ± 0.06	0.51^{cA} ± 0.13	1.02^{dA} ± 0.34	0.35^{bA} ± 0.19	1.08^{eA} ± 0.50
100 AS	1.33^{aB} ± 0.10	1.05^{bB} ± 0.27	0.40^{cA} ± 0.10	0.64^{dB} ± 0.07	0.81^{eB} ± 0.17	2.13^{fB} ± 0.13	0.87^{gB} ± 0.24
100 ASA	1.34^{aB} ± 0.06	1.03^{bB} ± 0.13	0.65^{cB} ± 0.02	0.83^{dC} ± 0.04	0.97^{eC} ± 0.22	0.34^{fA} ± 0.25	1.33^{aC} ± 0.53
300 AS	2.33^{aC} ± 0.08	1.96^{bC} ± 0.29	1.28^{cC} ± 0.50	1.30^{cD} ± 0.59	1.75^{dD} ± 0.05	2.44^{eC} ± 0.03	1.65^{fD} ± 0.48
500 AS	2.46^{aD} ± 0.05	2.37^{bD} ± 0.27	2.32^{cD} ± 0.28	2.15^{dE} ± 0.23	1.64^{eE} ± 0.86	2.47^{aC} ± 0.04	2.25^{fE} ± 0.04

a, b, c - Values of the same treatment at different time intervals marked with different letters, are significantly different ($p<0.05$).

A, B, C - Values of different treatments at the same time interval, marked with different letters, are significantly different ($P<0.05$).

Significantly higher ($p<0.05$) OD_{221} values were observed in the 300 AS and 500 AS treatments than in the control and other treatments. At 300 AS and 500 AS, OD_{221} declined by 72 h, and 96 h of incubation was followed by a rapid increase up to 120 h. According to the obtained OD values and comparison with other treatments, *Pseudomonas* sp. CY was unable to degrade atrazine at 300 and 500 AS. The prolonged lag phase and adaptation period detected in these treatments may be crucial for low bacterial growth and inefficient atrazine degradation (Zhao et al., 2018). In the other treatments, including the control, the CY strain showed a faster acclimation period. However, complete degradation of atrazine was not observed in these treatments. In our study, an incubation period of 168 h was used to determine the atrazine degradation. Mandelbaum et al. (1993) revealed that atrazine mineralization is a relatively slow process, whereas Silva et al. (2004) suggested that atrazine degradation should be estimated after an adaptation period of 28 days. In addition, Khatoon and Rai (2020) pointed out the importance of some environmental factors during atrazine biodegradation by microbes, which possess enzymes and/or genes responsible for the formation of degradation products (Liang et al., 2022).

The results presented in Table 2 show that in the control and 100 ASA, after 120 h of incubation, the lowest OD_{221} values were observed, while in 100 AS the lowest values were detected after 48 h of incubation. Significant differences in OD_{221} values among the control, 100 AS, and 100 ASA groups were observed during incubation. Although these data suggest that the addition of citrate plays a significant role in atrazine biodegradation (Mandelbaum et al. 1993), our results showed that, in most samples, the OD_{221} values were significantly lower in the control. This observation was previously

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reported (Kannika et al., 2010), pointing out that the addition of inorganic N decreased the atrazine degradation process, which is similar to our data. The increase in OD_{221} values at the end of the incubation period and the irregular curve of atrazine biodegradation could be attributed to the degradation of metabolites, which may serve as an additional nutrient sources and accelerators of atrazine degradation (Piutti et al., 2002).

Our results show the absence of a correlation between bacterial growth and atrazine degradation. Zhang et al. (2019) reported that *Klebsiella variicola* FH-1 reached log phase after 12-16 h of incubation, but the atrazine degradation rate was very low. Furthermore, they suggested that atrazine is not an optimal nutrient source for bacterial growth, but its degradation rate depends on the presence of external N-source(s). Taking into account that the selection of external C and N sources (Deghani et al., 2013) and environmental factors (Qu et al., 2020) plays a crucial role in atrazine biodegradation, optimization of environmental factors, such as temperature and pH, and addition of various C and N sources, coupled with detection of degradation products, will be involved in future research of atrazine biodegradation by *Pseudomonas* sp. CY.

Conclusion

The present study demonstrated that atrazine can be degraded by *Pseudomonas* sp. CY. Significant shifts in the bacterial count and optical density were observed during incubation. Shifts in cell number were mainly caused by external C and N sources rather by the initial atrazine concentrations. The addition of citrate increased bacterial growth and atrazine degradation, suggesting that *Pseudomonas* sp. CY has promising potential for applications in atrazine-affected environments. Further research will be focused on the optimization of critical parameters in *in vitro* conditions and on the determination of the role of various C and N sources during *Pseudomonas* sp. CY-assisted atrazine degradation.

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Promene u rastu *Pseudomonas sp.* CY u prisustvu atrazina

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SAŽETAK

Mikrobijalna degradacija je, u poređenju sa drugim degradacionim procesima, najvažniji način razgradnje triazinskih herbicida u zemljištu. Cilj ovog istraživanja je bio da se determiniše potencijal rasta *Pseudomonas* sp. CY u prisustvu atrazina i dopunskih izvora ugljenika (natrijum citrat) i azota (amonijum nitrat). Eksperiment je obavljen u pet tretmana: 1. 100 mg/l atrazina (kontrola); 2. 100 mg/l atrazina + natrijum citrat (0,3 %, w/v); 3. 100 mg/l atrazina + natrijum citrat (0,3 %, w/v) + amonijum nitrat (0,6 %, w/v); 4. 300 mg/l atrazina + natrijum citrat (0,3 %, w/v) i 5. 500 mg/l atrazina + natrijum citrat (0,3 %, w/v). Broj bakterija je određen nakon inkubacije (7 dana na 30ºC) metodom agarnih ploča, dok je koncentracija atrazina određena merenjem optičke gustine na talasnoj dužini od 221 nm. *Pseudomonas* sp. CY parcijalno može da koristi atrazin kao jedinstveni izvor ugljenika i energije. Najveća brojnost bakterija je ustanovljena pri najvećim početnim koncentracijama atrazina; međutim, rast bakterija u ovim tretmanima nije detektovan. Konstatovan je značajan uticaj citrata na rast bakterija i degradaciju atrazina, dok je dodavanje nitrata rezultiralo smanjenjem stepena degradacije atrazina. Ova istraživanja potvrđuju da se *Pseudomonas* sp. CY može koristiti kao značajan činilac remedijacije životne sredine kontaminirane atrazinom.

Ključne reći: amonijum-nitrat, atrazin, rast bakterija, *Pseudomonas* sp., natrijum-citrat

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