ORIGINAL ARTICLE

Isolation of Atrazine Degrading Bacteria in Semi-Salinity Medium

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Abstract

Background: Atrazine is a widely used herbicide. The increasing salinity of many water resources has had a negative effect on atrazine biodegradation. The aim of this study was to isolate atrazine degrading bacteria in semi-salinity media.

Methods: Nine selected bacterial species were cultivated on the mineral salt broth culture medium containing atrazine (50, 100, 500 mg/L), NaCl concentration (10 g/L), and 2% (wt/vol) agar. The bacteria with higher growths in the atrazine medium (500 mg/L) were selected. Then, those with higher growths were transferred to the medium with atrazine concentration of 1000 mg/L. The atrazine biodegradation rates by *Ochrobactrum oryzae* and consortium bacteria (all of the nine bacteria species) were compared by cultivating separately on the mineral salt broth containing atrazine concentration of 30 mg/l, and NaCl concentration of 10 g/L in the incubation time of 10 day and HPLC analysis.

Results: The results indicated that *Ochrobactrum oryzae* had the highest growth compared to the other investigated bacteria (*Acinetobacter radioresistens, Paenibacillus lautus*, and *Bacillus sp)* in the mineral salt broth culture medium containing atrazine concentrations (1000 mg/L), NaCl (10 g/L), and 2% (wt/vol) agar. In the *Ochrobactrum oryzae* and bacterial consortium comparison, atrazine biodegradation rate in the culture medium containing NaCl, by *Ochrobactrum oryzae,* was higher than bacterial consortium and atrazine biodegradation rate in the culture medium with no NaCl addition, by *Ochrobactrum oryzae,* was lower than bacterial consortium.

Conclusion: Based on the results, *Ochrobactrum oryzae* was significantly capable of atrazine biodegradation in the semi-salinity aqueous environment.

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Introduction

Atrazine, 6-chloro-N2-ethyl-N4-isopropyl-1, 3, 5 atrazine, 4-diamine, is a herbicide that has been widely used in corn and sorghum production and horticultural and forests; it is also used to control many broad-leaf and some grassy weeds. This herbicide has long-term reproductive and endocrine-disrupting influences and is a probable human carcinogen.[1,2] Its extensive use has caused environmental concern due to repeated detection of atrazine in water resources at concentrations that exceed the maximum contaminant level of 3 µg/L.[3] Atrazine is the second most repeatedly detected pesticide in the countries affiliated to EPA in the investigated drinking water.[4] It has been usually reported that the observed

concentration of atrazine in the groundwater is higher than the maximum contaminant level of 3 µg/L in the United States and 0.1 µg/L in Europe.[5]

Although this herbicide was banned in Europe since 2003, it is still utilized as a main herbicide in Egypt.[6] Water resources contamination and soil pollution by atrazine

have become an international critical issue in today's world.[1,7] Atrazine is moderately resistant in the environment and its half-life is one to twelve months.[8] Due to low biodegradability, persistence and partial water solubility of this herbicide, it has a high potential to contaminate water resources and is an environmental contaminant.[9,10]

Several environmental parameters such as temperature, pH, hardness, and salinity can affect the level of toxicity of xenobiotic to microorganisms. Salinity is a major factor in microorganism dispensation and preservation. Salinity tolerance by atrazine biodegradation bacteria is a main issue in the atrazine biodegradation as well.[11] Through increase in salinity, the cellular osmotic pressure enhances, and protein structure of cells is destroyed.[12] Although several biological and chemical processes are used to remove atrazine, biological process has the main role in atrazine biodegradation in the soil.[13,14] The herbicides are utilized as the energy source by metabolic activity. Since most of the herbicides are new and exotic for microorganism, microbial adaptation is used for treating the polluted soil.[14] Bioremediation is considered as a harmless, efficient, and economical biotechnological pathway for the removal of herbicides.[6] Several studies reported the biodegradation of atrazine by various bacteria such as *Pseudomonas, Acinetobacter, Agrobacteriium, Rhodococcus* and *Arthrobacter*.[15-19] Detection of atrazine degrading microorganisms is of great importance for reduction or eradication of the adverse effects of this compound to human health and environment.[6] *Ochrobactrum* has been repeatedly used for various contaminant removals.[6,12,20,21] Reyad and colleagues showed that *Ochrobactrum oryzae* can be used effectively to biodegrade high atrazine concentrations.[6] Zhang and colleagues investigated the metabolic ability of bacterial consortium which contained four isolated bacterial species for atrazine biodegradation.[22] Wang and colleagues used *Arthrobacter DAT*$_1$ for the treatment of high atrazine polluted soil.[23] Kolic and colleagues reported the high capability of *Ochrobactrum* for reducing atrazine in the culture media.[24]

In the study of Toe and colleagues, salinity had the exponential inhibitory effect on the atrazine biodegradation.[25] Results of another study also showed that *Ochrobactrum oryzae,* compared to other investigated bacteria, had a higher growth capability in a rich salinity medium.[12]

Widespread use of atrazine and its low biodegradability are the factors that increase the environmental contamination by this compound. Since ground water salinity has been increased due to climatic change, one of the main factors that intensify the low biodegradability of atrazine is salinity. It is highly probable that the concentration of atrazine in many ground water resources has an increasing trend. The present study aimed to assess nine selected bacterial species for atrazine biodegradation and isolation of bacterial species with highest atrazine degradation capability and determine the percentage of atrazine removal.

Materials and Methods

Chemical and Analytical Method

All the chemicals were purchased from Merck (Germany). Atrazine standard was provided by Sigma-Aldrich, USA. High performance liquid chromatography (HPLC) (Waters YL9100HPLC SYSTEM, USA) system with C18 columns (CP-SIL 5 CB column model, 250*4.6 mm, 5 µm) was used to determine the amount of atrazine and experimented prior to the samples injection. For atrazine detection in the samples, Detector (UV absorbance) at 224 nanometer wave length was also used. The mobile phase contained methanol and water (50/50 V/V) with a flow rate of 1 mL/min. The retention time for atrazine was 8.679 minutes. The detection limit for the sample was 0.001 mg/L.

Preparation of Enrichment Culture Medium

For bacterial growth, we used mineral salts broth (MSB) containing atrazine. The enrichment culture medium included 1.6 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, and 0.02 g $CaCl_2$, 1 mL of salt solution, 1 mL of vitamin solution, 1 mL $FeSO_4.6H_2O$, and 30 mg atrazine in one liter sterile deionized water. Moreover, we also added the vitamin solution containing 100 mg/L thiamine and 40 mg/L biotin and $FeSO_4.6H_2O$ solution containing 5 mg/L of the salt solution including 2 g/L boric acid, 1.8 g/L $MnSO_4.H_2O$, 0.2 g/L $ZnSO_4$, 0.1 g/L $CuSO_4$, and 0.25 g/L Na_2MoO_4. Vitamin and $FeSO_4.6H_2O$ solutions were maintained at 4°C. After autoclaving, cycloheximide (25 mg/L) was added to prevent fungal growth. The medium culture pH was set to 7- 7.5. The culture mediums were placed in a dark place on a shaker (100 rpm) at room temperature for inhibiting the growth of algal. To inhibit bacterial growth, sodium azide was added at a concentration of 1 g/L to the controls.[8]

Isolation of Atrazine Degrading Bacteria in Semi-Salinity Medium

Nine selected bacteria including *Ochrobactrum*

oryzae, Sphingomonas yanoikuyae, Bacillus sp, Serratia marcescens, Pseudomonas aeruginosa (types I and II), Acinetobacter radioresistens, Bacillus subtilis, and Paenibacillus lautus were separated from the agricultural corn field soils.[26] The enrichment culture mediums were sub-cultured in a one-week period, and sub-cultured by transferring 10 mL of old culture to 90 mL of freshly prepared culture containing atrazine and salt for 6 months to adapt the bacteria with the herbicide and NaCl.

Agar at the concentration of 2% (wt/vol) was added to the liquid culture medium at the higher atrazine concentration (50,100,500, and 1000 mg/L) and NaCl (10 g/L). Atrazine was added from a stock solution (1000 mg/L in methanol). The solid culture medium was used to differentiate the bacteria with the higher growth capability. For providing visual examination of clearing the zones surrounding atrazine-degrading bacteria, a cloudy solid culture medium containing atrazine was utilized.

After selecting the bacteria with higher growth in the medium containing atrazine at the concentration of 50, 100, 500 mg/L, they were transferred to the culture medium with higher atrazine concentration (1000 mg/L). The isolated bacteria species were kept in sterile water at -4 °C temperature.[8]

Then, the isolated bacteria with the higher growth and the consortium of nine selected bacteria species were cultured separately in the mineral salt broth containing atrazine concentration of 30 mg/L and NaCl concentration of 10 g/L. The samples were incubated at room temperature and placed in dark for 10 days. After 10 days of inoculation, the remaining atrazine was measured by HPLC.

Type of the Study

This study was conducted at the bench scale at the laboratory.

Statistical Analysis

The atrazine removal data presented here are the mean values of three replications. Standard errors were calculated for all the values using MS Excel 2007. In this study, one factor at a time approach was used for optimizing the removal conditions.

The Study Date and Place

This experimental study was conducted in the second half of 2015 and all experiments were carried out at the laboratory.

Results

Nine selected bacteria species (*Ochrobactrum oryzae, Sphingomonas yanoikuyae, Bacillus sp, Serratia marcescens, Pseudomonas aeruginosa (types I and II), Acinetobacter radioresistens, Bacillus subtilis, and Paenibacillus lautus*) were studied in the solid mineral salt medium containing 2% (wt/vol) agar, atrazine concentration of 50 mg/L, NaCl concentration of 10 g/L after days1, 3, 5, 7, 10 of incubation periods. The highest growth rate on the first day was related to *Ochrobactrum oryzae*, *Bacillus sp, Paenibacillus lautus, Acinetobacter radioresistens, Sphingomonas yanoikuyae, Serratia marcescens, Pseudomonas aeruginosa, (types I and II), and Bacillus subtilis*, respectively. Among these bacteria, *Ochrobactrum oryzae, Bacillus sp, Paenibacillus lautus, Acinetobacter radioresistens, and Pseudomonas aeruginosa (types II)* had a faster growth. The highest growth rates after 10 days were related to *Ochrobactrum oryzae, Acinetobacter radioresistens, and Bacillus sp*. In addition, the lowest growth was related to *Serratia marcescens* (Figure 1).

Next, the growth rate of the selected bacteria

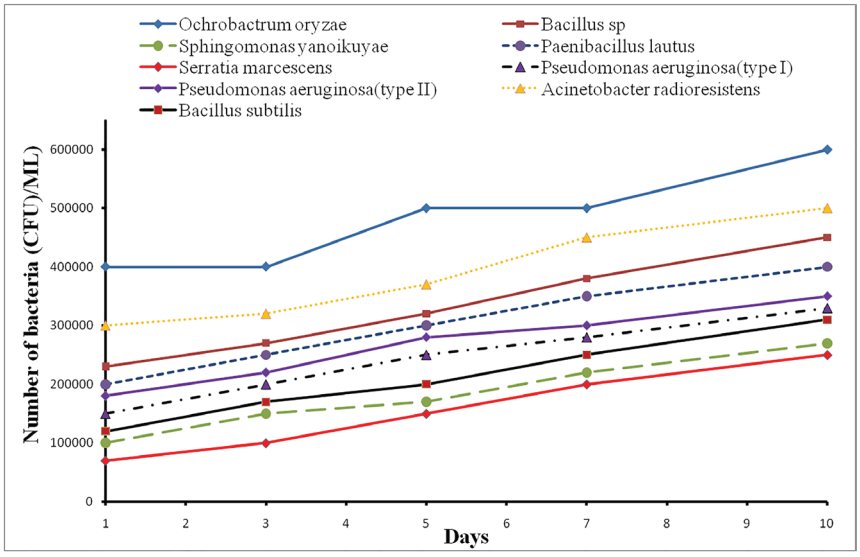

Figure 1: Comparison of the growth rate of bacterial species (mineral salt medium culture containing atrazine 50 mg/L and NaCl 10 g/L) during the 10 days of incubation.

species was studied in the same medium except at higher atrazine concentration (100 mg/L). The highest growth rates on the first day were related to *Ochrobactrum oryzae, Bacillus sp, and Acinetobacter radioresistens, Paenibacillus lautus* respectively. Also, *Sphingomonas yanoikuyae, Serratia marcescens, Pseudomonas aeruginosa, (types I and II),* and *Bacillus subtilis* had no growth. Among these bacteria during 10 days of incubation period, *Ochrobactrum oryzae, Bacillus sp, Acinetobacter radioresistens, Paenibacillus lautus* had a faster growth and *Sphingomonas yanoikuyae, Serratia marcescens, Pseudomonas aeruginosa, (types I and II), and Bacillus subtilis* showed a slower growth. The highest growth rates on the tenth day were related to *Ochrobactrum oryzae, Bacillus sp, Acinetobacter radioresistens, and Paenibacillus lautus*. The growths of *Sphingomonas yanoikuyae, Serratia marcescens, Pseudomonas aeruginosa, (types I and II), and Bacillus subtilis* were almost similar (Figure 2).

Subsequently, the growth rates were examined at 500 mg/L. The highest growth rates on the first day were related to *Ochrobactrum oryzae, Acinetobacter radioresistens, Bacillus sp and Paenibacillus lautus*. Moreover, *Sphingomonas yanoikuyae*, *Serratia marcescens*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (types I and II) had no growth at all. *Pseudomonas aeruginosa* (types I), *Sphingomonas yanoikuyae*, *Serratia marcescens*, and *Bacillus subtilis* had no growth on the third day; also, *Sphingomonas yanoikuyae*, *Serratia marcescens* and *Bacillus subtilis* had no growth on the fifth day. The highest growth rates on the tenth day were related to *Ochrobactrum oryzae, Acinetobacter radioresistens, Bacillus sp, and Paenibacillus lautus*. Furthermore, the growths of *Sphingomonas yanoikuyae, Serratia marcescens, Pseudomonas aeruginosa* (types I), and *Bacillus subtilis* were nearly the same (Figure 3).

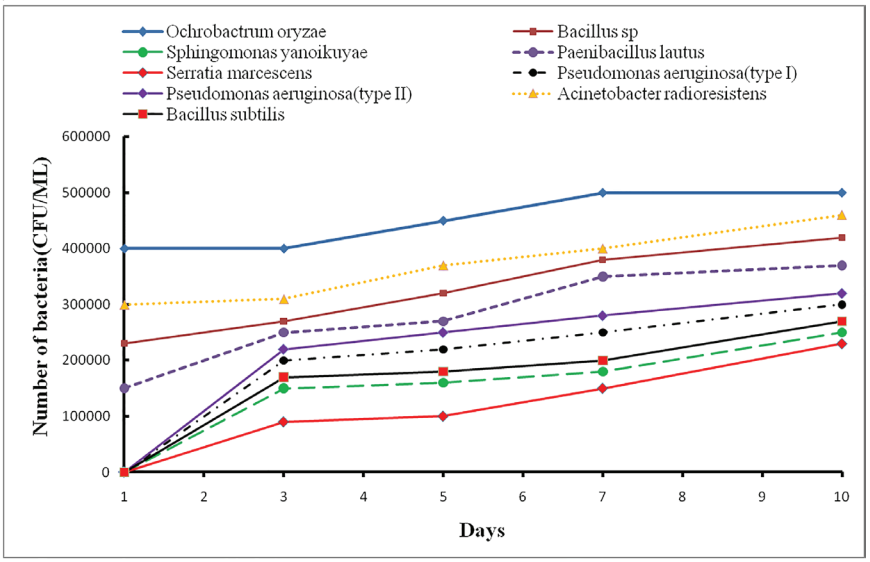

Figure 2: Comparison of the growth rate of bacterial species (mineral salt medium culture containing atrazine 100 mg/L and NaCl 10 g/L) during the 10 days of incubation.

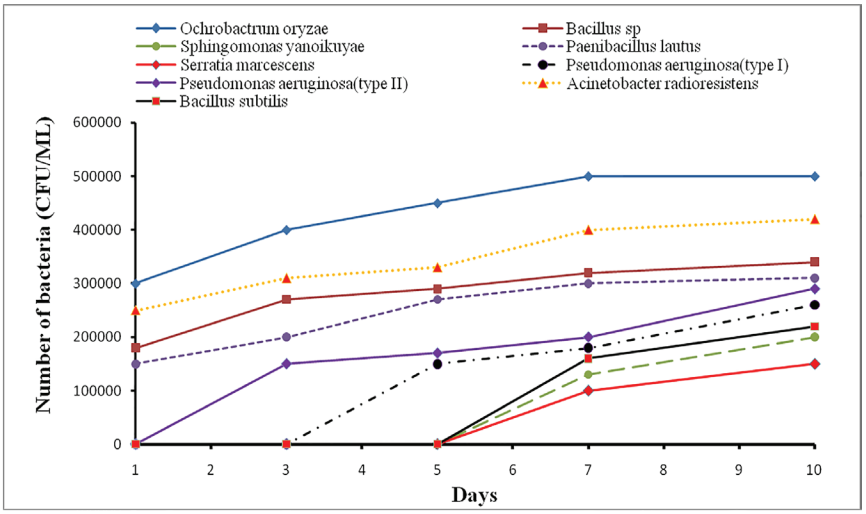

Figure 3: Comparison of the growth rate of bacteria species (mineral salt medium culture containing atrazine 500 mg/L and NaCl 10 g/L) during the 10 days of incubation.

Isolation of Atrazine Degrading Bacteria in Semi-Salinity Medium with the Highest Growth Rate

Bacteria species of *Ochrobactrum oryzae, Acinetobacter radioresistens, Bacillus sp, Paenibacillus lautus*, which had the highest growth rate on the solid mineral salt medium containing atrazine concentration of 500 mg/L, and NaCl concentration of 10 g/L, were transferred to the culture medium with 1000 mg/L atrazine concentration. *Ochrobactrum oryzae* was selected with the highest growth rate and clearing zone around the colony on the solid media. The highest growth ability on the first day was related to *Ochrobactrum oryzae*, and bacterial species of *Acinetobacter radioresistens*, *Bacillus sp* had a slight growth. In addition, *Paenibacillus lautus* had no growth on the first day. The highest growth rates on the 10-days of incubation were in the following order: *Ochrobactrum oryzae, Acinetobacter radioresistens,Bacillus sp and Paenibacillus lautus* (Figure 4). Therefore, *Ochrobactrum oryzae* had the maximum growth capability on the solid culture medium compared to the others (Figure 4).

Next, there was an attempt to determine the biodegradation rate of atrazine using the consortium of selected bacterial species and comparing with *Ochrobactrum oryzae*. Atrazine biodegradation efficiency by the nine selected bacterial consortium was compared with *Ochrobactrum oryzae* in the MSB media containing atrazine concentration of 30 mg/L and time incubation of 10 days (Figure 5). Atrazine biodegradation rates in the culture medium containing NaCl (10 g/L) by *Ochrobactrum oryzae* and bacterial consortium were 30.33% and 21.46%, respectively. Also, the atrazine biodegradation rates in the culture medium with no NaCl addition by *Ochrobactrum oryzae* and bacterial consortium were 31.46% and 36.7%, respectively (Figure 5).

Discussion

According to the results obtained by nine bacterial species, it was observed that the studied bacteria had a higher growth rate in the low initial atrazine concentrations compared to the high one (Figures 1-3). Dehghani and colleagues also observed that atrazine biodegradation rate decreased by increasing the initial atrazine concentration.[1] On the contrary, another study found that atrazine biodegradation rates were increased by increasing the initial atrazine

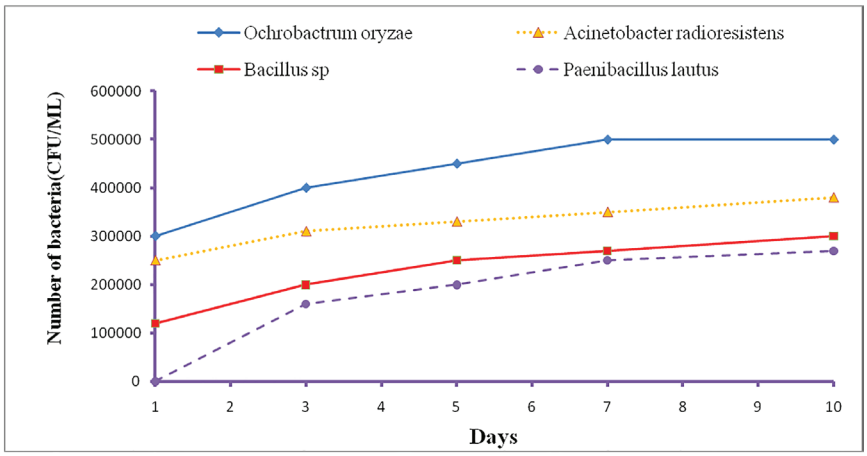

Figure 4: Comparison of the growth rate of bacterial species (mineral salt medium culture containing atrazine 1000 mg/L and NaCl 10 g/L) during the 10 days of incubation.

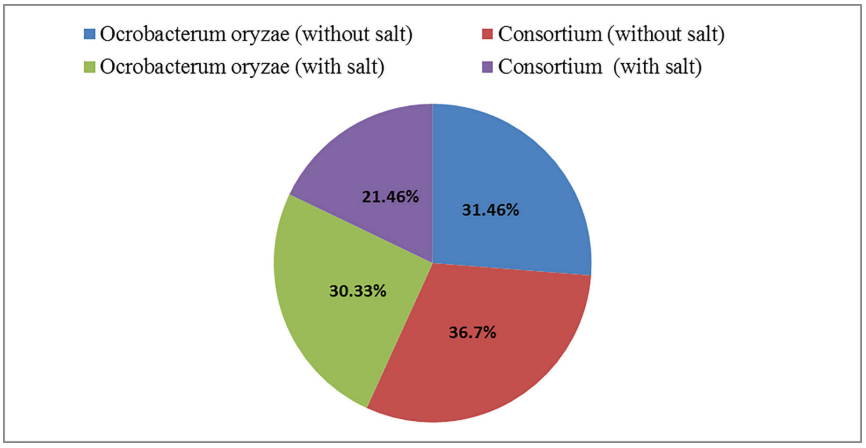

Figure 5: Atrazine biodegradation rate by *Ochrobactrum oryzae* compared with nine selected bacterial consortium in the aqueous media (with NaCl and with no NaCl).

concentration using acclimated *Pseudomonas aeruginosa* and *Pseudomonas flourescens*.[27] Dehghani and colleagues demonstrated that alachlor biodegradation rate reduced significantly by increasing the initial concentration. They have reached this conclusion because of the complicated interaction between bacterial activity and nutrient availability.[28]

Based on the results, growth capability of all the bacteria species has been increased over the incubation time periods. For example on the first day, bacterial species of *Ochrobactrum oryzae, Bacillus sp, Acinetobacter radioresistens,* and *Paenibacillus* had a high growth capability compared to others, but bacterial species of *Sphingomonas yanoikuyae, Serratia marcescens, Pseudomonas aeruginosa (types I and II),* and *Bacillus subtilis* did not show any growth. During the 10 days of incubation, the growth capability of *Ochrobactrum oryzae, Bacillus sp, Acinetobacter radioresistens,* and *Paenibacillus lautus* in the medium containing atrazine and salt increased significantly and the others had a slower growth (Figure 3). Reyad and colleagues studied the effect of atrazine concentration (100 – 500 ppm) as a sole carbon and nitrogen source on the growth of *Ochrobactrum oryzae*. They found that the maximum optical density (0.61) was recorded at 400 ppm of atrazine after 12 days of incubation, and the growth capability of *Ochrobactrum oryzae* at all concentrations was increased over the incubation time periods.[6]

According to the results, bacterial species of *Ochrobactrum oryzae, Bacillus sp, Acinetobacter radioresistens,* and *Paenibacillus lautus* had a higher growth capability in the atrazine concentrations of 50, 100, 500 mg/L (Figures 1-3) than the rest of the studied bacteria. Many investigations also reported that these bacteria had a higher ability for atrazine biodegradation.[6,29-31] Samaei and colleagues expressed the higher growth capability of *Ochrobactrum oryzae* and *Acinetobacter radioresistens* in the presence of high NaCl concentration (4%).[13] On the contrary, other investigations demonstrated a very high efficiency of atrazine biodegradation by *Pseudomonas aeruginosa* and *Bacillus subtilis*.[27,32] Smith and colleagues reported the inability of *sphingomonas yanokuyae* to use atrazine as a nitrogen source.[33]

Based on the results, atrazine biodegradation rates by *Ochrobactrum oryzae* and bacterial consortium in the presence of salt were 30.33% and 21.46%, respectively. Also, atrazine biodegradation rates by *Ochrobactrum oryzae* and bacterial consortium in the culture medium with no NaCl addition were 31.46% and 36.7, respectively (Figure 5). *Ochrobactrum* had a high potential for removing many pollutions.[6,20,21,34] In addition, *Ochrobactrum oryzae* had the ability for growth in salty media and it was halo-tolerant.[12]

According to the data obtained in the present study, atrazine biodegradation efficiency by *Ochrobactrum oryzae* and bacterial consortium in the MSB culture medium with no NaCl addition was higher than the medium containing NaCl. This indicates that salinity had the inhibitory effect on the bacterial growth capability and atrazine biodegradation efficiency. Also, in other investigations, the inhibitory effect of salinity on the biodegradation ability of the bacteria was presented.[35,36] In the study of Ugar and colleagues, COD removal decreased from 96% to 32% by increasing the salinity from 0 to 6%.[37] Studies by Nitisoravut and Klomjek also reported that the effect of salinity on BOD removal appeared to approach the exponential phase in the constructed wetland. They also showed that salinity inhibited the metabolism of microorganisms in the wetland environment, which may be critical for the proper functioning and maintenance of the system.[38]

The difference between Atrazine biodegradation rate by *Ochrobactrum oryzae* in the mineral salt broth culture medium with no NaCl addition and NaCl addition was only 1.13%. However, the difference between Atrazine biodegradation rate by bacterial consortium in the mineral salt broth culture medium with no NaCl addition and NaCl addition was more than 15%. Therefore, it can be concluded that NaCl addition to mineral salt broth had a lower inhibitory effect on the atrazine biodegradation capability *by Ochrobactrum oryzae* than bacterial consortium. The major limitation of this study was the cost analysis, including high cost of HPLC analysis and laboratory materials.

Conclusion

Atrazine is a selective herbicide which has adverse effects on the human health and environment. Since the salinity in many water resources are increasing, there are many concerns about the negative effects on the atrazine biodegradation. Based on the results, the selected bacterial species had a higher growth capability in low atrazine concentrations compared to the high concentrations. Moreover, *Ochrobactrum oryzae* had a higher growth ability in all of the atrazine concentrations compared to the other investigated bacteria. Atrazine biodegradation rate by *Ochrobactrum oryzae* in the mineral salt broth containing NaCl was more than consortium bacteria, which showed that *Ochrobactrum oryzae* is halo-tolerant. Moreover, atrazine biodegradation rate by *Ochrobactrum oryzae* in the mineral salt broth with no NaCl addition was lower than the consortium bacteria. This may be due to the presence of different bacteria in the consortium. It can be concluded that *Ochrobactrum oryzae* can be used for the remediation of atrazine in the semi-salinity medium.

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Conflict of Interest: None declared.

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