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Biostimulation and bioaugmentation for the enhanced atrazine degradation in semi-saline medium

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ABSTRACT

Although microorganisms are excellent degraders of herbicide combinations in aqueous solutions, some amendments may be needed to stimulate a more rapid degradation of the herbicide in a restricted period. This paper demonstrates the use of biostimulation and bioaugmentation as robust remediation strategies for the rapid cleanup of atrazine-polluted semi-saline medium. A bacterial strain, *Ochrobactrum oryzae* (*O. oryzae*), capable of utilizing atrazine for growth was isolated from semi-e medium. Factors, such as stimulant type, concentration of atrazine, time of inoculation, and pH of medium, were analyzed to determine their effects on the atrazine removal in semi-saline medium. Maximum atrazine removal (46.6%) using *O. oryzae* was obtained in a semi-saline medium containing 4 g/L of sodium citrate, 1.5 mg/L of K_2HPO_4, and 0 mg/L of potassium nitrate at pH = 7 after 10 d of inoculation. The atrazine removal (%) varied significantly depending on the stimulant type, concentration of atrazine, inoculation time, and pH of medium. The results show biostimulation and bioaugmentation may be useful biodegradation tools for atrazine-polluted, semi-saline medium.

Keywords: Biostimulation; Bioaugmentation; Atrazine; *O. oryzae*; Stimulant; Semi-saline

1. Introduction

Atrazine is a selective herbicide that has been applied to control the growth of grassy and broadleaf weeds [1]. Because of low vapor pressure, long half-life in soil, and high mobility, atrazine has contaminated various ecosystems [2]. Atrazine is persistent in various environments and so, it remains in the soil and aqueous media for an extended period [3]. Due to its relative mobility and leaching property in the soil, atrazine has a potential to reach to and contaminate water resources [2,4–6]. Due to its high permeability into the soil, concentrations above 3 μg/L were reported in many groundwaters [7]. Atrazine contamination in water resources, through agricultural run-off, could lead to serious environmental effects due to its persistence, toxicity, and low biodegradability [8,9]. Also, atrazine is a probable human carcinogen [1] and an endocrine disruptor with adverse effects on nervous, immune, and cardiovascular systems [10].

Biodegradation is one of the most critical processes through which organic wastes are degraded by living organisms [11,12]. Pesticides' biodegradation is already a widespread method and can be performed in various media including soil, sediments, surface water, and groundwater. Through this process, pesticides are degraded by extracellular enzymes of bacteria, but this method does not cause complete contaminants removal [13].

Atrazine is more degraded from the soil by biological processes than chemical processes [14]. Many different methods have been used to remove this herbicide and its metabolites [5,14,15]. Reyad et al. [16] showed that *Ochrobactrum oryzae* (*O. oryzae*) can be used for treating agricultural

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wastewaters, contaminated with high concentrations of atrazine. Kolić et al. [17] isolated bacteria with high capability of atrazine degrading including *Arthrobacter* sp., *Ochrobactrum* and *Pseudomonas* sp., *Agrobacterium J14*, *Rhodococcus erythropolis*, *Pseudaminobacter*, and *Nocardioides*, which can use atrazine as a source of carbon and nitrogen; they can completely mineralize the ring of the compound. According to Strong et al. [18], the addition of carbon source prevents atrazine biodegradation, but it stimulated the increase of inorganic phosphate. Instead, Chung et al. [19] concluded that the addition of carbon source enhances the rate of atrazine biodegradation. On the other hand, Ostrofsky et al. [20] exhibited that cyanuric acid can enhance atrazine's mineralization through the stimulation of microbial population and activity. Willems et al. [21] showed that with the help of the presence of simple carbon sources, 96% of atrazine could be mineralized in 7 d. Many studies demonstrated that high concentrations of nitrate prevent atrazine mineralization [22–24]. Bichat et al. [25] found that degradation of atrazine by *Pseudomonas ADP* was not much affected by the external source of nitrogen. However, Bach et al. [26] reported that the addition of inorganic nutrients, such as phosphorus and nitrogen, decreased the biodegradation rate.

During the last decade, atrazine has been widely used to control grassy and broadleaf weeds in Iran. Since atrazine is resistant to environmental media, groundwater contamination with this herbicide is very likely. In recent years, Iran has experienced increases in the salinity of water resources due to low precipitation and a hotter, drier climate [27–29]. Due to the high toxicity of atrazine and its adverse effects on human health, concerns about its biodegradability in semi-saline media have increased. The present study aims to evaluate the effect of stimulant type, concentration of atrazine, time of inoculation, and pH of medium on atrazine removal using *O. oryzae* in a semi-saline medium.

2. Material and methods

2.1. Reagents and analytical method

All chemicals were of reagent grade and purchased from Merck (Germany). Atrazine was provided by Sigma-Aldrich, USA. A high-performance liquid chromatography (HPLC; Waters YL9100HPLC SYSTEM, USA) system with C_{18} columns (CP-SIL 5 CB column model, 250 × 4.6 mm, 5 μm) was calibrated to detect residual atrazine in the samples. The HPLC mobile phase included methanol and water (50/50 V/V) with a flow rate of 1 mL/min. For the detection of residual atrazine in the samples, a UV absorbance detector was used at a wavelength of 224 nm. The retention time for atrazine in the chromatogram was 8.679 min. The detection limit for the atrazine sample in the HPLC system was 0.001 mg/L. In this research, erlenmeyer flasks with a volume of 50 mL were used as bioreactors.

2.2. Isolation of bacteria

In a previous study conducted by the authors of this research [30], *O. oryzae,* which is a bacterial species that shows high atrazine utilization in semi-saline medium, was isolated. Nine selected bacterial species (*O. oryzae*, *Sphingomonas yanoikuyae*, *Bacillus* spp., *Serratia marcescen*, *Pseudomonas aeruginosa*, *Acinetobacter radioresistens types I and II*, *Bacillus subtilis*, and *Paenibacillus lautus*) were cultivated on mineral salt broth (MSB) culture medium containing atrazine (50, 100, or 500 mg/L), NaCl (10 g/L), and 2% (W/V) agar. Bacterial species that showed higher growth rates in the atrazine medium (500 mg/L) were selected and transferred to a medium containing 1,000 mg/L atrazine. The results indicated that *O. oryzae* had the highest growth rate compared with the other investigated bacteria (*A. radioresistens, P. lautus*, and *Bacillus* spp.) in the MSB culture medium containing atrazine (1,000 mg/L), NaCl (10 g/L), and 2% (W/V) agar.

2.3. Preparation of the culture medium

O. oryzae was cultured in MSB culture medium containing 30 mg of atrazine. The enriched culture medium contained 1.6 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.2 g of $MgSO_4{\cdot}7H_2O$, 1 g of NaCl, 0.02 g of $CaCl_2$, 1 mL of salt solution, 1 mL of vitamin solution, and 1 mL of $FeSO_4{\cdot}6H_2O$. It was adjusted with 30 mg atrazine solution in 1 L sterile deionized water. The vitamin solution contained 100 mg/L of thiamine and 40 mg/L of biotin. The $FeSO_4{\cdot}6H_2O$ solution contained 5 mg/L of $FeSO_4{\cdot}6H_2O$, and the salt solution contained 2 g/L of boric acid, 1.8 g/L of $MnSO_4{\cdot}H_2O$, 0.2 g/L of $ZnSO_4$, 0.1 g/L of $CuSO_4$, and 0.25 g/L of Na_2MoO_4. The vitamin and $FeSO_4{\cdot}6H_2O$ solutions were kept at 4°C. After the prepared culture medium was autoclaved, 25 mg/L of cycloheximide was added to the medium to prevent fungal growth. A solution of H_2SO_4 and NaOH was used to adjust the pH of the culture medium to 7–7.5. To avoid algal growth and photolysis reactions, culture media were incubated under aerobic conditions on a reciprocal shaker (Model E5650 Digital Benchtop Reciprocal Shaker, Eberbach, Germany) in the dark at room temperature (100 rpm). Sodium azide (1 g/L) was also added to control flasks to inhibit microbial growth [31].

2.4. Effects of the carbon, nitrogen, and phosphorus sources

To investigate the effects of different sources on atrazine biodegradation by *O. oryzae*, MSB culture medium containing 30 mg/L of atrazine and 10 g/L of NaCl was prepared in several bioreactors. Then, carbon sources (glucose, sodium citrate, and sucrose) were added to the bioreactors at concentrations of 2 and 4 g/L. Thereafter, *O. oryzae* was cultured in the bioreactors. To induce growth of the bacterial species, the bioreactors were placed in the dark at room temperature for different durations (0–50 d). After ensuring growth of the bacterial species, the residual atrazine was measured by the HPLC system. Finally, the optimal carbon source was selected based on the sample that had the lowest amount of residual atrazine (4 g/L sodium citrate).

In the next step, MSB culture medium containing 30 mg/L of atrazine, 10 g/L of NaCl, and the optimal carbon source (4 g/L sodium citrate) was prepared in several bioreactors. KNO_3 at concentrations ranging from 20 to 90 mg/L were added to the bioreactors, since 20, 40, and 90 mg/L KNO_3 contain 12.26, 24.53, and 55.19 mg/L NO_3^-, respectively. Thereafter, *O. oryzae* was cultured in the bioreactors. To induce growth of the bacterial species, the bioreactors were kept in the dark at room temperature for different durations (0–50 d). After ensuring growth of the bacterial species, the residual atrazine was measured by the HPLC system. Finally, the optimal

nitrogen source was selected based on the sample that had the lowest amount of residual atrazine (no nitrogen source).

Eventually, MSB culture medium containing 30 mg/L of atrazine, 10 g/L of NaCl, the optimal carbon source (4 g/L sodium citrate), and the optimal nitrogen source (no nitrogen source) was prepared in the bioreactors. Then, K_2HPO_4 at concentrations of 1–2 mg/L were added to the bioreactors as K_2HPO_4 concentrations of 1, 1.5, and 2 mg/L contain 0.54, 0.81, and 1.08 mg/L PO_4^{3-}, respectively. Thereafter, *O. oryzae* was cultured in the bioreactors. For inducing growth of the bacterial species, the bioreactors were kept in the dark at room temperature for different durations (0–50 d). After ensuring growth of the bacterial species, the residual atrazine was measured by the HPLC system. Finally, the optimal phosphorus source was selected based on the sample that had the lowest amount of residual atrazine (1.5 mg/L K_2HPO_4).

In order to ensure the homogeneous distribution of bacterial species throughout the bioreactor space, the optical density (OD) of the bacterium was adjusted at a wavelength of 600 nm (OD = 1). All the experiments were performed in three replicates for each concentration. Control (sample without bacterial inoculation) and blank (sample with no sources) samples were also analyzed [32]. After determining the optimal sources, we determined the time required by the bacterial species to reach a growth plateau.

2.5. The effect of pH

The effect of pH on atrazine biodegradation was evaluated at pH 5.5–9. First, MSB medium containing an optimal carbon source (4 g/L sodium citrate), an optimal phosphorus source (1.5 mg/L K_2HPO_4), and an optimal nitrogen source (that is, is no nitrogen source) was prepared in the different bioreactors, and the pH was adjusted by adding 0.1 N HCl and 0.1 N NaOH. Then, for culturing *O. oryzae* bacteria in the bioreactors, the bioreactors were situated in a dark place at room temperature for 10 d. To ensure a homogeneous distribution of bacterial isolates throughout the bioreactor space, the OD of the bacteria was adjusted at a wavelength of 600 nm (OD = 1). All the experiments were performed in triplicate for each pH [32]. A control sample without bacterial inoculation was also analyzed. The residual atrazine was measured after 10 d of inoculation using an HPLC system.

2.6. Statistical analysis

All data are expressed as the mean ± standard error (n = 3) and were evaluated by Kruskal–Wallis test; $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. The effects of carbon sources

Fig. 1 shows atrazine removal (%) by *O. oryzae* with different carbon sources (glucose, sucrose, and sodium citrate) at different concentrations (2 and 4 g/L) after 10 d of inoculation in a semi-saline medium. The atrazine removal was in the range of 11.6–31.1%. The highest percentage of atrazine removal (31.1%) was obtained in the medium with 4 g/L of sodium citrate, followed by the medium with 4 g/L of sucrose (23.2%). The least atrazine removal (11.6%) occurred with the addition of glucose at a concentration of 2 g/L. The

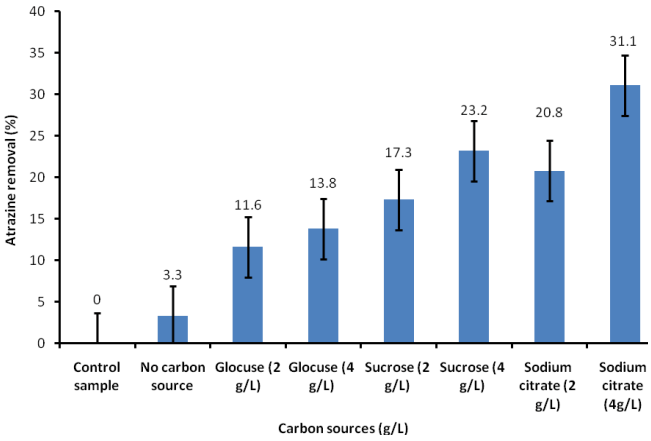

Fig. 1. Atrazine removal (%) by *O. oryzae* with different carbon sources (glucose, sucrose, and sodium citrate) at different concentrations (2 and 4 g/L) after 10 d of inoculation in a semi-saline medium.
*Each value consists of mean ± standard error (n = 3).

maximum atrazine removal in the current study (31.1%) was lower than that found by Lin et al. [33] (87.72%), this might be due to the high salinity that prevented *O. oryzae* growth [2,34]. Derakhshan et al. [35] resulted that increasing salinity up to 40 g/L NaCl in influent flow could inhibit atrazine biodegradation process strongly in the moving bed biofilm reactor (MBBR). We can conclude that the level of atrazine removal varied significantly depending on the carbon source type and concentration; it increased with increasing carbon source concentration. According to Table 1, statistical analysis showed that there was a significant difference between adding different carbon sources and atrazine removal (%; $p < 0.05$).

Our results also indicated that atrazine removal was very low (3.3%) when no carbon source was added to the culture medium. Different studies have demonstrated that the addition of a proper carbon source can increase atrazine biodegradation [7,17,32]. Dehghani et al. [32] showed that sodium citrate and sucrose play important roles in atrazine biodegradation. In addition, using two carbon sources together, such as sodium citrate and sucrose, could support a higher consortium growth rate compared with the use of sodium citrate alone [32]. Many bacteria cannot use the carbon in an atrazine ring to produce energy for growth, as only alkyl groups provide the carbon necessary for bacterial growth [36]. Therefore, providing an additional carbon source could increase the rate of biodegradation through co-metabolism.

3.2. The effect of a nitrogen source

Fig. 2 shows atrazine removal (%) by *O. oryzae* with a nitrogen source (potassium nitrate) at different concentrations) 20–90 mg/L) and an optimal carbon source (4 g/L sodium citrate) after 10 d of inoculation in a semi-saline medium. The results showed that after increasing the potassium nitrate concentration from 0 to 90 mg/L, the atrazine removal decreased from 33.3% to 25.6%. The atrazine removal in culture media with 0, 20, 40 and 90 mg/L of potassium nitrate was 33.3%, 28.6%, 28.3%, and 25.6%, respectively. Thus, the

Table 1
Comparison of mean atrazine removal (%) with different carbon sources

	N	Mean	Standard deviation	Chi-square	df	Asymptotic significance
Control sample	3	0.0000	0.00000	22.839	7	0.002
No carbon source	3	3.3333	0.00000			
Glucose (2 g/L)	3	11.6666	0.57735			
Glucose (4 g/L)	3	13.8888	0.57735			
Sucrose (2 g/L)	3	17.3333	0.00000			
Sucrose (4 g/L)	3	23.2222	0.57735			
Sodium citrate (2 g/L)	3	20.8888	0.57735			
Sodium citrate (4 g/L)	3	31.1111	1.00000			

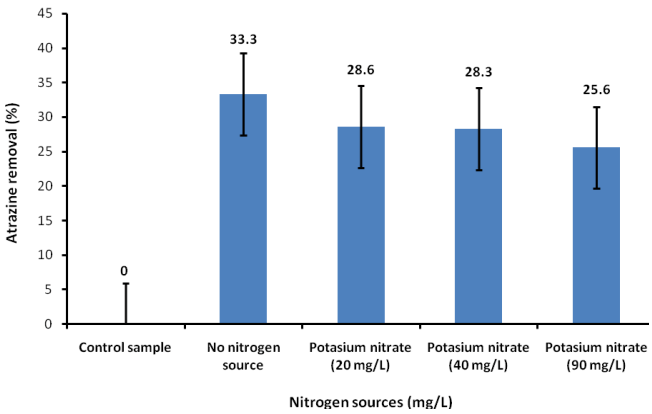

Fig. 2. Atrazine removal (%) by *O. oryzae* with a nitrogen source (potassium nitrate) at different concentrations) 20, 40, and 90 mg/L) and an optimal carbon source (4 g/L sodium citrate) after 10 d of inoculation in a semi-saline medium.
*Each value consists of mean ± standard error (n = 3).

addition of a nitrogen source resulted in a decrease of the atrazine biodegradation by *O. oryzae*. Moreover, according to Table 2, statistical analysis showed that there was a significant difference between adding different nitrogen sources and atrazine removal (%; $p < 0.05$). Many other studies have also reported that atrazine biodegradation dropped significantly in the presence of nitrogen [14,32,37,38]. Derakhshan et al. [39] showed that atrazine biodegradation rapidly declines in anaerobic MBBR from 74% ± 0.05 in the presence of nitrate to 9.12% only 3 d after the nitrate was eliding from the influent. In this study, it can be concluded that *O. oryzae* has no tendency to utilize atrazine given a sufficient nitrogen source. The disruption of the enzymatic system participating in triazine-ring degradation after the addition of nitrogen could be another reason for the decrease in atrazine biodegradation [40]. Also, high nitrogen concentrations repress the activity of catechol, 2,3-oxygenase and protocatechuate, which are essential enzymes in triazine-ring degradation [37]. Elevated levels of nitrogen have been shown to inhibit the degradation of atrazine and dichlorophenoxyacetic acid (2,4-D) by some bacteria and fungi [41].

3.3. The effect of phosphorus sources

Fig. 3 shows atrazine removal (%) by *O. oryzae* with a phosphorus source (K_2HPO_4) at different concentrations (1–2 mg/L), an optimal carbon source (4 g/L sodium citrate), and an optimal nitrogen source (that is, no nitrogen source) after 10 d of inoculation in a semi-saline medium. Our results showed that the atrazine removal at concentrations of 1, 1.5, and 2 mg/L of K_2HPO_4 was 24.4%, 38.2%, and 24%, respectively. These results indicate that increasing the K_2HPO_4 up to a concentration of 1.5 mg/L led to a significant increase in atrazine removal, but after that atrazine removal decreased with an increasing concentration of K_2HPO_4. According to Table 3, statistical analysis showed that there was a significant difference between adding different phosphorous sources and atrazine removal (%; $p < 0.05$). Many other investigations have also shown that supplying mineral phosphorus is an essential step in the biodegradation process and can increase the rate of microbial metabolism [26,42–44]. Also, phosphorus plays a significant role in improving biomagnification and biostimulation [42,45]. Atrazine utilization by

Table 2
Comparison of mean atrazine removal (%) with different nitrogen sources

	N	Mean	Standard deviation	Chi-square	df	Asymptotic significance
Control sample	3	0.0000	0.00000	13.251	4	0.010
No nitrogen source	3	33.3333	1.52753			
Potassium nitrate (20 mg/L)	3	28.6666	0.00000			
Potassium nitrate (40 mg/L)	3	28.3333	1.00000			
Potassium nitrate (90 mg/L)	3	25.6666	0.00000			

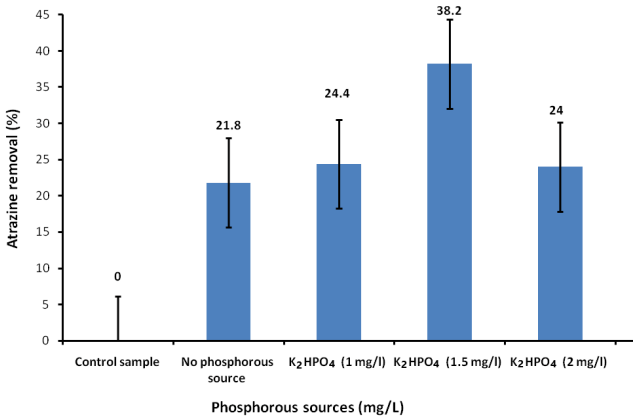

Fig. 3. Atrazine removal (%) by *O. oryzae* with a phosphorus source (K_2HPO_4) at different concentrations (1, 1.5, and 2 mg/L), an optimal carbon source (4 g/L sodium citrate), and an optimal nitrogen source (that is, no nitrogen source) after 10 d of inoculation in a semi-saline medium.
*Each value consists of mean ± standard error (n = 3).

bacteria increases in the presence of sufficient phosphorus, but excessive concentrations of this nutrient can have an adverse effect on biodegradation [46].

3.4. The effect of time

Atrazine removal (%) using *O. oryzae* with an optimal carbon source (4 g/L sodium citrate) and optimal nitrogen source (no nitrogen source) in semi-saline medium during different time intervals is shown in Fig. 4. Whereas, on days 0, 10, 15, 20, 25, 30, 40, and 50, there was 30, 20.6, 19, 16.4, 14.8, 13.5, 13, and 12.9 mg/L concentration of atrazine, respectively. As well as, on days 0, 10, 15, 20, 25, 30, 40, and 50, there was 0%, 31.2%, 36.6%, 45.3%, 50.6%, 54.8%, 56.6%, and 57% removal of atrazine, respectively. Our results showed that atrazine removal (%) increased when the time was increased from 0 to 50 d. Moreover, atrazine removal (%) reached an almost steady level after 30 d. The results revealed that almost half of the atrazine (14.8 mg/L) was degraded after 25 d of incubation, and a minimal amount of atrazine (0.1 mg/L) was biodegraded at 40 and 50 d.

Atrazine removal (%) using *O. oryzae* with an optimal carbon source (4 g/L sodium citrate), optimal nitrogen source (no nitrogen source), and optimal phosphorus source (1.5 mg/L K_2HPO_4) in a semi-saline medium during different time intervals is presented in Fig. 5. As it is evident from Fig. 5, on

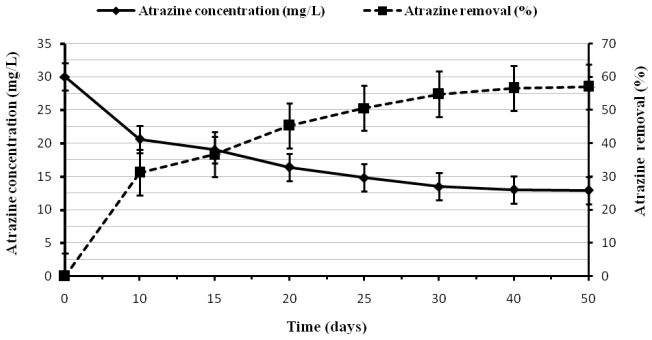

Fig. 4. Atrazine removal (%) using *O. oryzae* with an optimal carbon source (4 g/L sodium citrate) and optimal nitrogen source (no nitrogen source) in a semi-saline medium during different time intervals.
*Each value consists of mean ± standard error (n = 3).

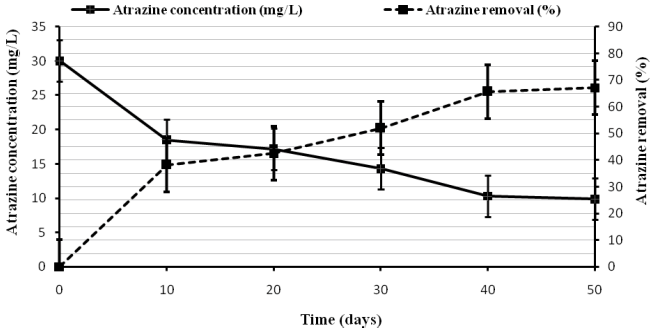

Fig. 5. Atrazine removal (%) using *O. oryzae* with an optimal carbon source (4 g/L sodium citrate), optimal nitrogen source (no nitrogen source), and optimal phosphorus source (1.5 mg/L K_2HPO_4) in a semi-saline medium during different time intervals.
*Each value consists of mean ± standard error (n = 3).

days 0, 10, 20, 30, 40, and 50, there was 30, 18.5, 17.2, 14.3, 10.3, and 9.87 mg/L concentration of atrazine, respectively. Also, on days 0, 10, 20, 30, 40, and 50, there was 0%, 38.2%, 42.5%, 52%, 65.6%, and 67.1% removal of atrazine, respectively. Our results showed that atrazine removal (%) increased as the time increased from 0 to 50 d. Moreover, atrazine removal (%) reached an almost steady level after 40 d.

The plot of the atrazine concentration (Ln C/C_0) during the incubation period (50 d) after the addition of *O. oryzae* is presented in Fig. 6. The results showed that atrazine removal (%) using *O. oryzae* under optimal conditions were followed by a pseudo-first-order reaction.

Table 3
Comparison of mean atrazine removal (%) with different phosphorous sources

	N	Mean	Standard deviation	Chi-square	df	Asymptotic significance
Control sample	3	0.0000	0.00000	13.188	4	0.010
No phosphorous source	3	21.8888	0.57735			
K_2HPO_4 (1 mg/L)	3	24.4444	1.00000			
K_2HPO_4 (1.5 mg/L)	3	38.2222	0.00000			
K_2HPO_4 (2 mg/L)	3	24.0000	0.57735			

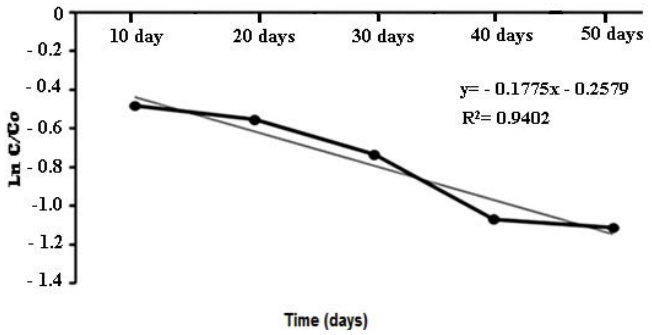

Fig. 6. The plot of the atrazine concentration (Ln C/C_0) during the incubation period after the addition of the *O. oryzae*.

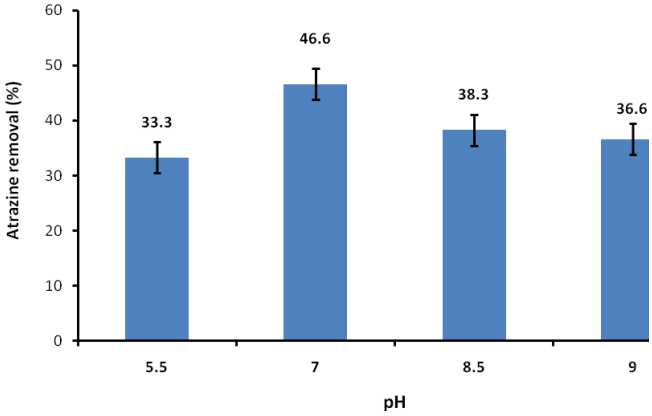

Fig. 7. Atrazine removal (%) using *O. oryzae* with an optimal carbon source (4 g/L sodium citrate), optimal nitrogen source (no nitrogen source), and optimal phosphorus source (1.5 mg/L K_2HPO_4) at different pH levels (5.5, 7, 8.5, and 9) in a semi-saline medium after 10 d of inoculation.
*Each value consists of mean ± standard error (n = 3).

3.5. The effect of pH

Atrazine removal (%) using *O. oryzae* with an optimal carbon source (4 g/L sodium citrate), optimal nitrogen source (no nitrogen source), and optimal phosphorus source (1.5 mg/L K_2HPO_4) at different pH levels (5.5–9) in a semi-saline medium after 10 d of inoculation is illustrated in Fig. 7. Atrazine removal (%) at pH levels of 5.5, 7, 8.5, and 9 was 33.3%, 46.6%, 38.3%, and 36.6%, respectively. The results showed that maximum and minimum atrazine removal (%) obtained when the semi-saline medium's pH was 7 and 5.5, respectively. Atrazine removal (%) varied significantly depending on pH. Dehghani et al. [32] also found that a pH level of 7 was optimal for the biodegradation of atrazine using a bacterial consortium.

4. Conclusions

The results demonstrated that *O. oryzae* in the optimal condition, 4 g/L sodium citrate (31.1%), no nitrogen source (33.3%), 1.5 mg/L K_2HPO_4 (38.2%), and pH level of 7 (46.6%) effectively removed atrazine from the semi-saline medium after 10 d. The incubation period was an important factor in atrazine biodegradation using *O. oryzae*. Atrazine removal (%) with an optimal carbon source was 57% after 50 d. In addition, atrazine removal (%) with optimal carbon and phosphorus sources was 67.1% after 50 d. Kinetic experiments verified that the biodegradation process was followed by a pseudo-first-order reaction. Providing additional carbon and phosphorus sources may increase atrazine biodegradation through co-metabolism. However, nitrogen did not appear to effect *O. oryzae* efficacy. *O. oryzae* may be a good atrazine biodegradation tool for semi-saline media; however, further studies analyzing its potential application in semi-saline media are warranted.

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