Biodegradation of atrazine by three strains: identification, enzymes activities, and biodegradation mechanism

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ABSTRACT

Biodegradation could remove atrazine from contaminated soil and water. In this work, three isolated bacterial strains D2, D6, and D17 were identified as Solibacillus, Bacillus, and Arthrobacter, respectively. They can rapidly degrade atrazine and effectively alleviate the phytotoxic effects of atrazine, with degradation efficiency following D2 > D6 > D17. Besides having the highest amount of exoenzyme and endoenzyme, the highest exoenzyme ratio in strain D2 also contributed to the highest atrazine degradation, as exoenzyme facilitated the direct reaction with atrazine in a short time and alleviated the stress of pollutant on strains, while more endoenzyme for D6 and D17 indicated degradation acted only after across the membrane, which needs more time to degradation. Furthermore, trzH in strain D2 can degrade atrazine to hydroxylatrazine. The biodegradation product of atrazine by strain D2 was cyanuric acid via dichlorination, hydroxylation, hydrodealkylation, methylation, dealkylation, elimination, and hydrolysis.

Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-S-triazine) is a widely used triazine pesticide. Due to its persistence and low adsorption on soil [1], atrazine is frequently detected in soil, groundwater, and surface water by rain and irrigation [2,3]. Atrazine inhibits plants’ growth rate [4], photosynthetic efficiency, and metabolic pathways [5], and impacts the reproductive and developmental systems of living organisms [6]. For example, severe neurodevelopmental and neurological damage may occur when male zebrafish are exposed to atrazine during development [7]. Li et al. [8] reported that chronic exposure of humans to trace amounts of atrazine in polluted environments may disrupt the dopaminergic system and lead to cause midbrain iron deposition. Therefore, it is important to find an efficient way to remediation of atrazine from contaminated soil or water environment.

At present, besides physical adsorption [9], photocatalysis [10], and advanced oxidation [11], bioremediation [12–14] based on biostimulation and bioaugmentation can achieve economical and efficient degradation of atrazine. Previous studies selected some bacterial and fungal organisms, which can use atrazine as their sole nitrogen source for growth, and convert atrazine into cyanoacetic acid with the help of hydrolase, such as Arthrobacter sp. C2 [15], Klebsiella sp. FH-1 [16], Paenarthrobacter sp. W11 [17], Arthrobacter sp. DNS10 [18], and Pseudomonas sp. ZXY-1 [19]. However, different strains have different degradation ability for atrazine. After incubation for 5 d, the atrazine was completely degraded from 100 mg·L⁻¹ by Arthrobacter sp. C2, and its degradation rate was highest at pH of 9, but significantly inhibited when pH<6 [15]. An atrazine degradation rate by Pseudomonas sp. ZXY-1 was 12.73 mg L⁻¹ h⁻¹ under the condition of 34°C and pH of 9.0 [19]. Most of the isolated atrazine degradation strains have a high removal rate under alkaline conditions, but the soil pH in the Yangtze River delta region and southwest of China was neutral or slightly acidic, furthermore, local strains are more suitable for remediation of contaminated soil, so it is needed to select some strains from Yangtze River delta region with high efficiency in removing atrazine under neutral and slightly acidic conditions.

Previous studies have found four pathways of atrazine degradation (Figure 1), which are dechlorination, dealkylation, hydroxylation, and ring cleavage. The degradation pathway of each bacterium was closely related to its degradation genes. For instance, atzA, B, C, and aztDEF genes contained in Pseudomonas sp. strain AKN5 in the dechlorination pathway were responsible for complete atrazine mineralization [20]. The Inga striata, Caesalpinia ferrea [21], and bacillus velezensis MHNK1 [22] could degradation of cyanuric...
acid to urea, then further decomposed into carbon dioxide and ammonia under the help of atzA, B, C, and atzD, E, F. However, many environmental factors affect the biodegradation of atrazine, including pH, temperature [23], soil nutrients, organic matter, and humus content [24]. Moreover, Zhang et al. [25] found that different microbes use different degradation pathways for organics, highlighting the need to further study the pathways by which new strains degrade atrazine.

Key enzymes play an important role in the degradability of bacteria microorganisms [26]. Enzymes released by microorganisms can promote pollutant degradation through catalysis [27]. Many recent studies have demonstrated that enzymatic catalysis is an environmentally friendly solution to degrade and detoxify pesticides in soil and aqueous media [28]. The extracellular and intracellular enzymes of microorganisms have different catalysis effects on pollutants [29]. However, few studies have systematically studied one specific atrazine degrading strain, to determine the contribution of degrading enzymes and to explore the degradation mechanism of the strain. This highlights the need to search for suitable enzymes, including hydrolases for degrading atrazine residues, to identify efficient ‘green’ solutions to biodegrade polluting chemicals.

In this study, three atrazine-degrading strains named D2, D6, and D17 were isolated from long-history atrazine soils in the Yangtze River delta region, China. The main objectives of this study were to 1) Identification of the three strains by morphology observation, physiological characterization, and 16S rRNA sequence homology analysis; 2) Effects of pH, temperature, and atrazine initial concentration on the biodegradation efficiency of the three strains, and the detoxification of three strains on plants; 3) the degradation mechanisms by examining enzyme activities, analysis of degradation product and degradation genes. In summary, this study will provide new strains

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Figure 1. Atrazine-degradation pathways and genes.
for atrazine degradation, which will expand the atrazine biodegradation gene pool.

2. Materials and method

2.1 Chemicals

Atrazine (>97%) was obtained from the Shanghai McLean Biochemical Technology Co., Ltd. China. Other chemical products and chromatography-grade organic solvents, such as methanol, were purchased from Asia Pacific, Ltd., Suzhou, China.

2.2 Isolation and identification of atrazine-degrading strains

The topsoils (0–10 cm) were collected from five maize fields with a long history application of atrazine in Jiangsu Province. These samples were used to screen for atrazine-degrading strains. Soil samples (5 g) were weighed into a conical flask containing 50 mL of a sterile mineral salt’s medium (MSM, MgSO4 · 7H2O: 0.1 g; K2HPO4: 1 g; KH2PO4: 1 g; FeSO4 · 7H2O: 0.025 g; CaCl2: 0.025, H2O 1000 mL, pH 7.0) and 50 mg L−1 atrazine. The mixture was shaken at 30°C and 150 rpm in a dark culture. The incubation was repeated three times with fresh medium at 30°C and 150 rpm for 7 d each. Subsequently, at the end of incubation, the culture medium was gradient diluted with sterile normal saline to 10−3, 10−4, 10−5, 10−6, and 10−7 concentrations. After 5 days of incubation in a constant temperature incubator, the resulting colonies were isolated and purified by morphology. The pure colonies of each isolate were fully mixed with the sterile salt medium containing 10 mg L−1 atrazine to assess the degradation ability. Finally, three strains capable of degrading atrazine, named D2, D6, and D17, were isolated. These samples were repeatedly drawn to ensure that single strains could be obtained, and then preserved using an inclined surface temperature and glycerin.

The isolated strains were identified based on morphological characteristics, 16S rRNA gene sequence analysis, and biochemical properties. The pure strains were inoculated in solid LB medium to observe the colony morphology. The surface morphology of strains D2, D6, and D17 was also observed by scanning electron microscopy (SEM, Quanta FEG 250). The isolated strains were determined using molecular characterization (16S rRNA sequencing), with the DNA extracted and stored at −4°C. The primer set utilized in the polymerase chain reaction (PCR) included 27 F (5’AGAAGTGTGACCTTGTAGCG-3’) and 1492 R (5’ TACGCGTACCTTGAACCTT-3’). The PCR procedures were as follows: initial denaturation for 3 min at 95°C, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 56°C for 20 s, extension at 72°C for 50 s, and a final step of repair extension at 72°C for 5 min.

The sequencing of PCR products were completed by Shanghai Tianlin Biotechnology Co., Ltd., Wuxi, China. BLAST alignment analysis of each measured sequence was conducted to identify the strain, and MEGA7.0 was used to construct the phylogenetic tree (using the neighbor-joining method) for identifying bacteria and genetic distances (between bacteria).

2.3 The biodegradation of atrazine at different pH, temperature, and initial concentration

The three strains were individually inoculated into a 50 mL LB liquid medium and then cultured at 30°C for 12 h at 150 rpm. Each culture medium was centrifuged in a 3500-rpm centrifuge for 10 min and washed three times with sterile water. The upper liquid was then discarded.

To explore the biodegradation of atrazine under different environmental factors, the bacterial suspensions of D2, D6, and D17 were inoculated with a 2% inoculation amount (The initial concentration of three strains was OD600 = 1) into 50 mL of MSM at four different initial atrazine concentrations (10 mg L−1, 20 mg L−1, 50 mg L−1, and 100 mg L−1), three different pH values (5.0, 7.0, 9.0), and four different temperatures (10°C, 20°C, 30°C, and 40°C), respectively. Each treatment was conducted in triplicate in an inorganic salt medium, with atrazine serving as the only carbon and nitrogen source. Samples were collected every 12 hours. To explore the degradation of atrazine and growth of strains, additional samples were collected to detect the concentration of atrazine using the high-performance liquid chromatography (HPLC, LC-20AT, Shimadzu, Japan) and the OD600 values of strains using the ultraviolet spectrophotometer every 2 hours. After each sample was filtered through a 0.22 μm membrane, the atrazine concentration in the remaining solution was measured.

2.4 Detoxification of isolated strains on the phytotoxic effects of atrazine

Because atrazine is commonly used in corn fields, and wheat is a rotation plant of corn, wheat (Jimai 22) was used to evaluate the detoxification ability of isolated strains (D2, D6, and D17) in an atrazine-contaminated solution. Uniformly-sized wheat seeds were selected and soaked in a 20% H2O2 solution for 20 min (sterilization and disinfection) and then soaked in distilled water for 3 h. Finally, the seeds were laid in a petri dish with wet filter paper and screened at 28 ± 2°C for germination. The hydroponic experiment was conducted using a biochemical incubator, with three plants individually secured in a sterilized transparent cup with 120 mL of sterile Hoagland solution spiked with 5 mg L−1 atrazine. Then, 400 μL (around 1.7 × 109 cells·mL−1) of the bacterial inoculum was introduced into the solution. The roots were then rinsed three times with sterile ddH2O and
were secured into vials with cotton pads, ensuring that the root was submerged in the solution. The system was then incubated at room temperature (28 ± 2°C) under 14/10 h light-dark cycles.

To determine the influence of the strains or atrazine on wheat growth, five treatments were performed: without strain and atrazine, containing atrazine, containing atrazine and strain D2, containing atrazine and strain D6, and containing atrazine and strain D17, and named as CK, WAT, WAT-D2, WAT-D6, and WAT-D17, respectively. Each treatment was replicated three times. After 8-day-and-night incubation, plant height, root length, fresh weight, dry weight, and root activity for each treatment were measured.

2.5 Extraction of bacterial crude enzyme extract

The single colonies were inoculated in an LB liquid medium for 12 h at 30°C and 150 rpm. Then, the bacterial liquid was centrifuged at 4°C and 10,000 rpm for 20 min. The extracellular enzyme was collected from the supernatant through a 0.22 μm membrane. The cells were washed 3 times with PBS phosphate buffer at pH 7.4 and then added to 20 ml of 20% glycerol in phosphate buffer. Afterward, the cells were ultrasonically crushed in an ice water bath for 20 min. The harvested cells were ultrasonicated 20 times for 10s at 15s intervals in 400 watts at 4°C. After ultrasonication, the supernatant was centrifuged at 4°C and 10,000 rpm for 30 min and then filtered through 0.22 μm membrane to obtain the intracellular enzymes. The intracellular enzymes or extracellular enzymes were added to atrazine solution at an initial concentration of 10 mg·L⁻¹, and after 2 h of incubation, the remaining atrazine concentration in the solution were measured by high-performance liquid chromatography (HPLC). The enzyme activity was calculated according to the atrazine concentration. Every treatment was performed in triplicate. The inactivated enzymes were the control. One unit of enzyme activity (U) was defined as the total amount of enzyme required to degrade 1 μmol of atrazine per minute at 30°C.

In this experiment, the protein concentration was determined using Coomassie Bright Blue. When the Coomassie G-250 dye was combined with the protein, the maximum absorption peak (λmax) changed to 595 nm, at the same time, the absorbance was measured to determine the amount of binding protein.

The specific activity was calculated as the ratio of enzyme activity and protein content in solution, expressed in Equation (1).

\[
\text{Specific activity (U · mg}^{-1} \text{)} = \frac{\text{One unit of enzyme activity (U)}}{\text{Protein mass (mg)}}
\]

2.6 Identification of atrazine-degrading genes

The degrading genes of strain D2 were amplified by PCR. The pure colony suspension of isolated D2 was incubated on an LB medium at 30°C and 150rpm for 12 h. The DNA was extracted from 1 ml of suspension of isolated D2. PCR was performed using available primers (trzN-F: 5′-ATGATCCTGATCCGCGGACTGA, trzN-R: 5′-CTACAAGTCTTGTGAATGAGTG-3′, atzA-F: 5′-TTGCGGTGCAGGTGTCTGATG-3′, atzA-R: 5′-TGACGAACGGCGTCAATTC-3′) to determine the presence of two atrazine degrading genes (atzA and trzN) in strain D2. The total genomic DNA in D2 was used as the PCR template. PCR reactions were conducted using the following thermocycler program: preheating 5 min at 95°C, 35 cycles of denaturation 1 min at 94°C, annealing for 1 min at the optimal temperature, and extension 2 min at 72°C, and then an additional extension of 10 min cycle at 72°C. PCR products were examined using 1% agarose gel electrophoresis.

2.7 Detection of atrazine and its metabolites

Atrazine was detected by HPLC (LC-20AT, Shimadzu, Japan) with a Shim-Pack VP-ODS liquid chromatography column (150 mm×4.6 mm, pore size 5 μm). The mobile phase V (methanol): V (water) = 60:40 at a flow rate of 1.0 ml·min⁻¹, a column temperature of 30°C, and the injection volume was 10 μL. Compounds in the samples were detected using a PDA detector at 220 nm and the retention time of atrazine was 7.0 min.

To investigate the degradation mechanism of strain D2, the degradation products of atrazine in the solution of strain D2 degradation were measured. The strain D2 was added into MSM medium with atrazine initial concentration of 100 mg·L⁻¹, and then cultured at 30°C and 150rpm. After incubation for 12 h, Extracted samples were cyclic for 60s, centrifuged at 17,000 rpm for 15 min, and filtered with 0.22 μm filter membrane. Extracted samples (described above) were used for HPLC-MS studies. A high-performance liquid chromatograph with Ultimate 3000 (Thermo Fisher Scientific, USA) and a resolution mass spectrometer 5600 QTOF (AB Sciex, Framingham, USA) were used to determine the atrazine metabolites. The gradient elution was performed on an analytical column (ACQUITY UPLC HSS T3 1.8 μm 2.1 × 100 mm column) at 40°C with water (containing 2 mM ammonium acetate) and acetonitrile. The MS conditions were as follows: scan range, 50–1200 m/z, collision energy, 30 eV. The pressure of atomization (GS1) was 60 psi, the auxiliary pressure was 60 psi, and the pressure of the Curtain Gas was 35 psi. The source temperature was 650°C, and the spray voltage was +5000 V or −4000 V.
2.8 Data analysis

The atrazine degradation rate was determined by Equation (2). All presented data were expressed as the mean ± standard error (SE) around the mean.

where \( Q \) is the atrazine degradation rate, \( \% \); \( C_0 \) is the initial atrazine concentration, mg·L\(^{-1}\); and \( C_1 \) is the concentration of the residual atrazine in the solution, mg·L\(^{-1}\).

\[
Q = \frac{C_0 - C_1}{C_0} \times 100\%
\]  

(2)

All data represent at least three independent experiments. Microsoft Excel 2019 (Microsoft, Washington, USA) was used to process the experimental data. The figures were visualized using Origin software (Origin 2018, USA).

3. Results and discussion

3.1 Isolation and identification of the atrazine-degrading strains

Eighteen degrading strains were isolated from five maize (corn) fields in Jiangsu Province that had a long history of atrazine application. They were then placed in an inorganic salt medium to validate their effect on atrazine degradation. The 18 strains were added to atrazine solution with an initial concentration of 10 mg·L\(^{-1}\) respectively. After incubation for 1 hour, the concentration of residual atrazine was determined by HPLC, to verify the degradation effect of the strains on atrazine. Ultimately, three strains that effectively degraded atrazine were selected and named D2, D6, and D17, respectively, which used atrazine as the sole source of carbon and nitrogen.

Figure 2 shows the preliminary morphological observations for strains D2, D6, and D17 (A\(_1\), A\(_2\), and A\(_3\)). The three strains are gram-positive bacteria (Figure 2 B\(_1\), B\(_2\), and B\(_3\)). The morphology of D2 was light yellow, with neat edges and a smooth surface, while the D6 and D17 were round and milky white with smooth surfaces (Figure 2 C\(_1\), C\(_2\), and C\(_3\)). The sizes of strains are 1.17–1.58 \( \mu m \) for D2, 2.65–2.89 \( \mu m \) for D6, and 0.86–1.05 \( \mu m \) for D17 (Table 1), respectively. The indole test, MR test, V-P test, and citrate utilization test were negative for the three strains. Both strains D6 and D17 can assimilate starch.

![Figure 2](image-url)
The 16S rRNA gene fragment obtained by PCR amplification was sequenced. The genes of strains D2, D6, and D17 were 1421 bp, 1418 bp, and 1403 bp in length, respectively. They were uploaded to the NCBI database (GenBank accession numbers OP605344, OP605383, and OP605387), and were used to conduct the BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search in the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) database. The nucleotide sequences were analyzed using BLAST in the NCBI database. The target sequences, the homologous sequences, and the exogenous sequence were analyzed using MEGA 7.0 software, and the phylogenetic trees based on 16S rRNA were constructed using the neighbor-joining method (Figure 3). The results of the genetic distance showed that strain D2 was closely related to Solibacillus, showing over 99% homology in the 16S rRNA (Figure 3a). Strain D6 was identified as Bacillus, with a nucleotide sequence similarity as high as 99.93% (Figure 3b). Strain D17 was identified as Arthrobacter, with a high nucleotide sequence similarity of 99.93% (Figure 3c).

### 3.2 Biodegradation of isolated strains

The influence of initial atrazine concentration on its degradation by the different strains was used to evaluate the strains’ growth abilities [30]. As Figure 4 shows, compared with the initial concentration, the residual atrazine concentration of CK (control, only contains atrazine) after 48 h incubation was negligible change when the initial atrazine concentration increased from 10 to 100 mg·L\(^{-1}\), indicating that atrazine was a stable pollutant. However, a sharp decline of atrazine concentration was observed in the three species of strain treatments after 48 h incubation, especially for the lower initial concentrations. Due to the low tolerance of the strain to atrazine, atrazine degradation of many strains decreases or even disappeared with the increasing initial concentration. The strain with the highest tolerance to atrazine was strain D2, followed by D6 and D17. After 48 h incubation, the degradation rates of atrazine at levels of 10, 20, 50, and 100 mg·L\(^{-1}\) were 0.833, 1.667, 4.167, and 4.167 mg·L\(^{-1}\)·h\(^{-1}\) by D2, 0.208, 0.417, 0.198, and 0.398 mg·L\(^{-1}\)·h\(^{-1}\) by D6, and 0.208, 0.163, 0.198, and 0.398 mg·L\(^{-1}\)·h\(^{-1}\) by D17, respectively. Strain D2 had the strongest biodegradation ability and shortest adaptation period. Compared with D2, the lower degradation for D6 and D17 was attributed to lower degradation rates and longer bacterial adaptation periods. The higher atrazine residual concentration for strain D6 compared to D17 at 12 h and 24 h indicated that strain D6 had a longer adaptation period than D17. Previous research found that the atrazine degradation rate at 50 mg·L\(^{-1}\) in a liquid medium was 100% by Citricoccus sp. strain TT3 after 66 h [31], and 81.5% by Klebsiella sp. FH1 after 264 h [32]. The degradation of 100 mg·L\(^{-1}\) in a liquid medium was 100% by Arthrobacter sp. C2 after 72 h [15]; and strain ATLJ-5 and ATLJ-11 reached a degradation rate of approximately 99% after 7 days [30]. Strain D2 completely degraded atrazine at initial concentrations of 10, 20, and 50 mg·L\(^{-1}\) within 12 h and 100 mg·L\(^{-1}\) within 24 h, indicating that strain D2 was more efficient for atrazine degradation than the above-mentioned strains. The average concentration of atrazine in the natural environment was 33.3 ng·L\(^{-1}\) [31], so strains D2, D6, and D17 can adapt to most natural environments and achieve the degradation of atrazine.

Most strains were temperature sensitive. Figure 5 (a, b, and c) shows the influence of temperature on the biodegradation of atrazine by the isolated strains D2, D6, and D17. The strains D2, D6, and D17 completely degraded atrazine at 20°C and 30°C. However, the degradation of atrazine by D2 was only 22.24% and 56.23%, and that by strains D6 and D17 was less than 20% at temperatures of 10°C and 40°C, respectively. These results suggested that the degradation of atrazine at lower and higher temperatures decreased because of the inhibition of microbial activity and the optimal performance at 20–30°C. Similar results were found in atrazine-degrading strains Arthrobacter sp. C2 [15] and Citricoccus sp. strain TT3 [30].

Figure 5 (d, e and f) shows the degradation of atrazine by the three strains at pH 5.0, 7.0, and 9.0. After 24 h incubation, the removal rates of atrazine by

| Biochemical characteristics | D2          | D6          | D17         |
|----------------------------|-------------|-------------|-------------|
| Size                       | 1.17–1.58 μm| 2.65–2.89 μm| 0.86–1.05 μm|
| Gram stain                 | +           | +           | +           |
| Cell shape                 | Fusiform    | Rods        | Short rods  |
| Margin                     | Smooth      | Smooth      | Smooth      |
| Color                      | Faint yellow| Milky white | Milky white |
| Configuration              | Circular, Convex| Circular, Convex| Circular, Convex |
| Amylohydrolysis            | +           | –           | –           |
| Indole test                | –           | –           | –           |
| MR test                    | –           | –           | –           |
| V-P test                   | –           | –           | –           |
| Citrate utilization        | –           | –           | –           |
| Nitrate reduction          | +           | –           | –           |
| Catalase                   | +           | –           | –           |

Symbol ‘+’ indicates positive and ‘–’ indicates negative.

Table 1. Morphological and biochemical characteristics of strain D2, D6 and D17.
D2 at pH 5.0, 7.0, and 9.0 were 91.5%, 100%, and 100%, respectively. This indicated that D2 could effectively degrade atrazine in slightly acidic, neutral, and alkaline environment. D6 and D17 were more pH sensitive than D2. The atrazine removal rates by D6 at pH 5.0, 7.0, and 9.0 were 26.39%, 31.79%, and 10.80%, and were 18.27%, 20.79%, and 15.08% by D17 at 24 h. The atrazine removal rates at pH 5.0, 7.0, and 9.0 were 100%, 100%, and 15.21% by D6, and 73.25%, 72.59%, and 11.79% by D17 at 36 h incubation, respectively.

The biodegradation of atrazine by strains D6 and D17 decreased at pH 9.0. This phenomenon significantly differed from some reported strains, such as *Arthrobacter* sp. C2 [15] and *Pseudomonas* sp. ZXY-1 [19], whose biodegradation of atrazine was highest at a pH of 9.0 and was inhibited at pH < 6. However, strains with higher biodegradation efficiency in neutral and slightly acidic environments are more suitable for the

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**Figure 3.** Phylogenetic tree of the strain D2(a), D6 (b) and D17 (c) based on 16S rRNA gene sequence using neighbor-joining method.
remediation of atrazine-contaminated soil in the Yangtze River Delta and southwest in China, because the soil pH in these regions is neutral and slightly acidic. The results showed that the most suitable culture environment for selected strains was 20–30℃ and a pH of 5.0 – 7.0.

Among the three isolated strains, strain D2 showed its higher atrazine removal rate under a wide range of temperature and pH conditions, which demonstrated its strong atrazine removal effect and adaptability in actual site remediation. Compared with the reported degrading bacteria (Table 2), strain D2 has stronger degradation efficiency on atrazine than published strains.

### 3.3 Degradation and growth of strains

The residual concentration of atrazine and strain biomass were measured to analyze the correlation between the atrazine removal and strains growth (Figure 6). Based on the tolerance and biodegradation of the three strains (Figure 4), the initial atrazine concentrations were 100 mg·L⁻¹ for strain D2, and 10 mg·L⁻¹ for D6 and D17, respectively. The OD₆₀₀ concentration is considered as the biomass of the strain and is commonly used to analyze the growth of strains. It is usually measured using a UV-Vis spectrophotometer at a wavelength of 600 nm. The growth curves of three isolated strains in LB medium fitted with the modified Gompertz model(Fig. S1) and the fitted parameters are shown in Table S1. The growth of strains D2, D6, and D17 was well fitted by the modified Gompertz model with high R² (0.991–0.998). As shown in Figure 6, the biomass of strains D2, D6, and D17 increased with the increase of incubation time and reached the peak after incubation for 20, 26, and 30 h, respectively, indicating that the three strains entered a stable phase. The max OD₆₀₀ concentration of strain D2 was 0.108, which was 1.51 times higher than that of strain D6 and 2.32 times higher than that of strain D17. Furthermore, D2 had the shortest stagnation period for strain growth. At the same time, the concentration of atrazine decreased with the increase in incubation time. The results also showed that the atrazine removal rates by D2, D6, and D17 were 100%, 69.1%, and 40.7% at 24 h incubation, respectively, and 100%, 100%, and 100% at 36 h incubation. A significant negative correlation was founded between the residual atrazine concentration and the strain biomass (OD₆₀₀), indicating that the biodegradation of the strain contributed to atrazine removal. The most biodegradation by strain D2 was attributed to its largest biomass and shortest stagnation period. As such, it quickly adapted to the solution environment and grew, with atrazine as its single source of carbon and nitrogen.

### 3.4 Detoxification of isolated strains on the phytotoxic effects of atrazine

Table 3 shows the growth of the wheat in CK, WAT, WAT-D2, WAT-D6, and WAT-D17 after 8-days of cultivation. Compared with the CK treatment, the

Figure 4. The residual concentration with incubation time by three strains at initial atrazine concentrations of 10 (a), 20 (b), 50 (c) and 100 (d) mg·L⁻¹.
Figure 5. Degradation of atrazine by strain D2(a), D6(b) and D17(c) at different incubation temperatures (10, 20, 30, and 40°C) and the degradation of atrazine by strain D2 (d), D6(e) and D17(f) at different pH (5.0, 7.0, and 9.0), respectively.

Table 2. Atrazine-degrading bacteria and fungi.

| Name of strain                  | Initial Atrazine concentration(mg/L) | Degradation rate (%) | Degradation time | Corresponding references |
|---------------------------------|--------------------------------------|----------------------|-----------------|--------------------------|
| Arthrobacter sp. C2             | 100                                  | 100                  | 3d              | [15]                     |
| Paenarthrobacter sp. W11        | 100                                  | 97.1                 | 60 h            | [17]                     |
| Arthrobacter sp. DNS10          | 100                                  | 38.57                | 48 h            | [18]                     |
| Pseudomonas sp. ZXY-1           | 100                                  | 100                  | 12 h            | [47]                     |
| Klebsiella sp. FH-1             | 50                                   | 81.5                 | 11d             | [16]                     |
| Arthrobacter sp. ZXY-2          | 50                                   | 100                  | 6h              | [19]                     |
| Strain ATLJ-5 and ATLJ-11       | 50                                   | 99                   | 7d              | [30]                     |
| Citricoccus sp. strain T3       | 50                                   | 100                  | 66 h            | [31]                     |
| Pseudomonas sp.                 | 20                                   | 100                  | 24 h            | [48]                     |
| Achromobacter sp.               | 20                                   | 39                   | 48 h            | [48]                     |
| Pleuratus ostreatus INCQS 40310 | 10                                   | 82                   | 22d             | [40]                     |
| Pencillium sp. yz11-22N2        | 8                                    | 91.2                 | 5d              | [49]                     |
| D2                              | 100                                  | 100                  | 20 h            | /                         |
| D6                              | 100                                  | 100                  | 28 h            | /                         |
| D17                             | 10                                   | 100                  | 32 h            | /                         |

leaf height, root length, fresh weight, dry weight, and root activity of wheat in the WAT treatment were reduced by 28.41%, 55.75%, 51.65%, 62.10%, and 11.54%, respectively, which due to the toxic effect of atrazine on wheat growth or the induction of its apoptosis [33]. Atrazine controls the weeds, or other sensitive plant parts, by disturbing the photosynthesis of the plant seeding [34].
Supplementing the samples with the three strains reduced the toxic effect of atrazine on wheat growth, and significantly improved the physiological parameters of wheat. As a result, the leaf height, fresh weight, dry weight, and root activity of wheat for WAT-D2, WAT-D6, and WAT-D17 treatment increased by 1.41% – 12.22%, 4.46% – 46.79%, 9.80% – 17.15% and 8.93% – 18.68%, respectively, compared with the WAT. The wheat grown in WAT-D2 had the largest biomass. With the introduction of the strains at a 2% level v/v, atrazine was rapidly degraded within 2 d. This indicated that strains alleviated the phytotoxicity of atrazine to the wheat, and promoted the development of wheat roots and leaves. Jiang et al. [36] reported that strain PAS18 alleviated the disturbance of 20 mg·kg\(^{-1}\) atrazine on plant growth and physiology, with both the shoot length and root length being larger than with the atrazine exposure treatment. Therefore, utilizing degrading microorganisms may be an efficient, ecologically safe method for removing toxic influences on sensitive crops.

### 3.5 Atrazine degradation by crude enzyme extracts

Recent studies [26–28,37,38] found that the key to the microbial metabolism of xenobiotic compounds was the active proteins or enzymes of applied microorganisms. Figure 7(a) shows the activities of extracellular and intracellular enzymes of three strains.

The activated extracellular and intracellular enzyme activities of D2 were 1.437 and 1.306 U·mg\(^{-1}\), respectively, which were 3.44 and 1.48 times higher than the corresponding activities of D6, respectively, and 4.79 and 1.34 times higher than the corresponding activities of D17, respectively. The exoenzyme activity of strain D2 was higher than the endoenzyme activity, on the contrary, the exoenzyme activity of strains D6 and D17 was 52.62% and 69.14% lower than its endoenzyme, respectively. A sharp decline of enzyme activity could be found for devitalized exoenzyme and devitalized endoenzyme, due to the high temperature killing the strains.

To determine the effect of the crude enzyme on atrazine removal, activated and devitalized exoenzymes and endoenzymes were compared for the degradation of atrazine at 30°C for 2 h (Figure 7b).

| Treatment | Leaf height / cm | Root length / cm | Leaf fresh weight/g | Root fresh weight/g | Leaf dry weight/g | Root dry weight/g |
|-----------|-----------------|-----------------|--------------------|--------------------|------------------|------------------|
| CK        | 16.4 ± 1.07     | 16.3 ± 0.12     | 1.28 ± 0.06        | 0.452 ± 0.01       | 0.283 ± 0.006    | 0.0890 ± 0.001   |
| WAT       | 11.7 ± 1.11     | 7.20 ± 0.56     | 0.525 ± 0.02       | 0.310 ± 0.01       | 0.117 ± 0.008    | 0.0240 ± 0.001   |
| WAT-D2    | 16.6 ± 0.35     | 15.1 ± 0.36     | 1.41 ± 0.02        | 1.13 ± 0.01        | 0.359 ± 0.004    | 0.0900 ± 0.002   |
| WAT-D6    | 16.7 ± 0.55     | 16.6 ± 1.09     | 1.32 ± 0.01        | 0.621 ± 0.01       | 0.334 ± 0.007    | 0.0920 ± 0.001   |
| WAT-D17   | 18.4 ± 0.35     | 12.5 ± 0.61     | 1.34 ± 0.01        | 0.465 ± 0.02       | 0.349 ± 0.005    | 0.0870 ± 0.003   |

CK, WAT, WAT-D2, WAT-D6 and WAT-D17 represent the sample without strain and atrazine, containing atrazine, containing atrazine and strain D2, containing atrazine and strain D6, and containing atrazine and strain D17, respectively.
The total atrazine degradation by crude enzyme extracts (contains both exoenzyme and endoenzymes) was 84.3% for strain D2, 44.3% for D6, and 27.0% for D17, respectively. The greatest degradation of atrazine by D2 was attributed to the highest extracellular and intracellular enzyme activities (Figure 7 a and b), which was consistent with a study by Santillan et al. [39], who posited that the pollutant degradation of the crude enzyme solution was positively correlated with enzyme activity. Zhao et al. [19] suggested that there were several degrading genes in the crude enzyme solution, which contributed to the biodegradation of pollutants. Compared with the activated exoenzyme and endoenzyme treatments, a significant decrease in the atrazine degradation by the inactivated extracellular and intracellular enzymes could be found (Figure 7b), proving that the microbial degradation of atrazine might be through an enzymatic reaction and that both the intracellular and extracellular proteins played an important role in atrazine degradation. Previous studies also found that both intracellular and extracellular enzymes were involved in the degradation of atrazine [40] and that the atrazine degradation by enzyme extracts of strain ACB contributed approximately 71% of degradation in 6 h [38]. However, in this study, the contribution of exoenzyme and endoenzyme to atrazine degradation was different for strains D2, D6, and D17. For strain D2, the contribution of exoenzyme to atrazine degradation was higher than that of endoenzyme, but for strain D6 and D17, the contribution of endoenzyme was greater than that of exoenzyme.

The contaminant-crude enzyme extracts system is a free-cell system, which is conducive to the direct reaction of pollutants with the target and improves the efficiency of pollutant degradation. However, in a cell-based living system, the cellular membrane limits contaminant bioavailability [41], contaminants are first carried over the membranes to be metabolized [42], so atrazine degradation by isolated three strains probably included three processes: extracellular degradation, cell adsorption, transport, and intracellular degradation. Similar degradation mechanisms were seen in a separate study [43]. Because of the lipophilic nature of atrazine, the cell membrane of the strain adsorbed atrazine and transferred it into the cell via material transport. Then, endoenzymes were involved in the degradation of atrazine. So, in addition to having the highest amount of exoenzyme and endoenzyme, the higher ratio of extracellular enzyme activity of strain D2 also contributed to the highest atrazine degradation by strain D2, as it facilitated the direct reaction of atrazine in a short time and alleviated the stress of pollutant on strains. In contrast, in addition to having a lower amount of exoenzyme and endoenzyme, the lower atrazine degradation by D6 and D17 was attributed to lower ratio of extracellular enzyme activity but higher ratio of intracellular enzyme activity. It indicated that the endoenzyme only acted after atrazine was transferred into the cell, which needed more degradation time. The lower biomass and longer stagnation period of strains D6 and D17 also contributed to lower atrazine degradation (Figure 6).

3.6 Biodegradation pathway of atrazine based on HPLC-MS and PCR

Strain D2 was selected to further analyze the degradation products and pathway of atrazine because of its highest atrazine-degradation behavior (Figure 5). Atrazine metabolites were extracted and characterized using HPLC-MS analysis. Table 4 shows the molecular and structural formulas of metabolites, which were compared with the measured and theoretical m/z values and fragmentation data [44]. Fig. S2 shows the mass spectrum of atrazine metabolites. After 12 h incubation, seven metabolites detected by HPLC-MS were propazine, desethylatrazine, atraon, simazine, propazine-2-hydroxy, atrazine-2-hydroxy, and simazine-2-hydroxy, which retention time at 25.125, 24.300, 22.867, 21.375, 21.192, 19.036, and 16.361 min, respectively and corresponding m/z values of 230.1167,
Table 4. Intermediates of atrazine degradation by strain D2 identified by HPLC-MS.

| Name | RT (min) | Compounds | Formula | Molecular weight | Mass-to-charge ratio (m/z) |
|------|----------|-----------|---------|------------------|---------------------------|
| MEA  | 25.125   | Propazine  | C₇H₈N₂O₃ | 229.71           | 230.1167                  |
| ATZ  | 23.159   | Atrazine   | C₇H₈N₂O₃ | 215.72           | 216.1014                  |
| Atraton | 22.867   | Atraton    | C₇H₈N₂O₃ | 211.26           | 212.15059                 |
| DMA  | 21.375   | Simazine   | C₇H₈N₂O₃ | 201.66           | 202.0854                  |
| HMEA | 21.192   | Propazine-2-hydroxy | C₇H₈N₂O₃ | 211.26           | 212.1506                  |
| HA   | 19.036   | Atrazine-2-hydroxy | C₇H₈N₂O₃ | 197.24           | 198.1349                  |
| DEA  | 24.300   | Desethylatrazine | C₇H₈N₂O₃ | 187.63           | 188.06975                 |
| HDMA | 16.361   | Simazine-2-hydroxy | C₇H₈N₂O₃ | 183.21           | 184.11929                 |

Figure 8. Agarose gel electrophoresis (1%) of PCR products (a) and PCR amplification (b) of Atrazine degrading genes in strain D2.
Figure 9. The probably degradation pathway of atrazine by strain D2 based on UPLC-MS analysis.

188.06975, 212.15059, 202.0854, 212.1506, 198.1349, and 184.11929, respectively.

PCR analysis was carried out to identify the atrazine degrading genes in strain D2 (Figure 8). After amplification, trzN and atzA encodes encode the dechlorination hydrolase. Although it is noted that gene atzA might be present with a weak band of ~500bp by agarose gel electrophoresis, we failed to isolate this product. The trzN gene had obvious bands, indicating the presence of trzN gene in strain D2. PCR amplification of trzN gene in strain D2 was shown in Figure 8(b). The trzN is the atrazine chlorohydrolase gene, which initiates the biodegradation of atrazine by displacing chlorine from the atrazine ring [31,45]. The atrazine chlorohydrolase gene trzN can degrade atrazine to hydroxyatrazine.

Based on the results of HPLC-MS analysis, four possible pathways of atrazine degradation by strain D2 were identified (Figure 9).

1. The hydroxyl group was first substituted for chlorine to produce dechlorinated hydroxylated atrazine (HA, 2-hydroxy-4-ethylamino-6-isopropylamino-1,3,5-triazine), and then the methyl group of HA was substituted for H to produce atratone. The degradation gene of trzN in D2 detected by PCR (Figure 8) also proved strain D2 can degrade atrazine to hydroxyatrazine.
2. Atrazine was demethylated to produce simazine (DMA, 6-chloro-N,N-diethyl-1,3,5-triazine-2,4-diamine), and the chlorine was replaced by a hydroxyl group and converted into Simazine-2-hydroxy (HDMA, 2-Hydroxysimazine; 4,6-Bis (ethylamino)-1,3,5-triazin-2(1H)-one), and then to ammeline (DEDIA) by dechlorination.
3. Atrazine was initially converted by N-dealkylation to desethylatrazine (DEA, 1,3,5-Triazine-2,4-diamine,6-chloro-N2-(1-methylethyl)), then to deethyldeisopropylated atrazine (DACT, 6-chloro-1,3,5-triazine-2,4-diamine), and was then finally hydrolyzed into cyanuric acid.
4. The methylation of atrazine (MEA, 6-chloro-N,N'-diisopropyl-1,3,5-triazine-2,4-diamine) converted atrazine into propazine-2-hydroxy (HMEA, 2,6-bis(propan-2-ylamino)-1-H-1,3,5-triazin-4-one) via dechlorination, followed by demethylation of HMEA to Simazine-2-hydroxy (HDMA).

Compared with the reported strains, such as Arthrobacter sp. C2 [15], Citricoccus sp. strain TT3 [31], and Klebsiella variicola Strain FH-1 [32], the strain D2 in this paper had more degradation products and more degradation pathways for atrazine. For example, four metabolites were detected in the degradation solution of Arthrobacter sp. C2 and two degradation pathways were proposed [15]. The main atrazine degradation pathway by [46] Citricoccus sp. strain TT3 [31] was the conversion of atrazine to hydroxyatrazine, followed by hydrase-catalyzed conversion of hydroxyatrazine to N-isopropylam ide, and finally, N-isopropylamide is hydrolyzed to cyanuric acid, which is the first of the four degradation pathways for isolated D2 in this paper. However, in this paper, seven metabolites were detected in the degradation solution of strain D2 and four degradation pathways were proposed, indicating that strain D2 can degrade atrazine more rapidly and efficiently.

4. Conclusions

Strains D2, D6, and D17 identified as Solibacillus, Bacillus, and Arthrobacter, respectively were able to rapidly degrade atrazine in solution and effectively alleviate the phytotoxic effects of atrazine. The greatest biodegradation by Strain D2 was attributed...
to the largest biomass and shortest stagnation period in wide temperature and pH ranges. Both the total mass of crude enzyme extracts (containing both exoenzyme and endoenzyme) and types of enzymes played an important role in atrazine biodegradation. In addition to having the highest amount of exoenzyme and endoenzyme, the highest exoenzyme ratio in strain D2 also contributed to the highest atrazine degradation, as exoenzyme facilitated the direct reaction with atrazine in a short time and alleviated the stress of pollutant on strains, while endoenzyme was the main active substance for atrazine degradation by D6 and D17, so the concomitant could decrease the biomass of strains, transferred pollutant into the cell, and intracellular degradation, which resulted in the lower degradation. The biodegradation mechanism of strain D2 was the degradation of atrazine to propazine, desethylatrazine, atraton, simazine, propazine-2-hydroxy, atrazine-2-hydroxyl, and simazine-2-hydroxy, by dechlorinated hydroxylation, hydrodealkylation, methylation, dealkylation, elimination, and hydrolyzation. The degradation gene of trzn in D2 detected by PCR also proved strain D2 can degrade atrazine to hydroxyatrazine. Compared with the reported strains, strain D2 had more degradation products and more degradation pathways for atrazine than some reported strains, which contributed strain D2 can degrade atrazine more rapidly and efficiently. These results suggest that the isolate strains D2, D6, and D17 could be candidates for the detoxification of atrazine-contaminated soil and water. In particular, strain D2 has strong atrazine degradation efficiency and strong environmental adaptability.

**Author’s contributions**

LND, BZ and QS performed the experiment; LND, and QS had literature search; LND, YL and SYW contributed significantly to data analysis; YL, LND,QS and SL performed the manuscript preparation; YL and QS performed the manuscript editing and manuscript review. All authors read and approved the final manuscript.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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