Study on the capability of a bacterial strain for aniline degradation under some specific circumstances

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**Abstract.** The purpose of the study is to explore the characteristics of a laboratory cultured bacterial strain *Delftia* sp. named AD1 for aniline degradation. Several degradation conditions were studied to provide information for biological treatment of aniline-containing wastewater. The bacterial strain AD1 is salt-tolerant, and the most suitable salt concentration for the growth of AD1 and the biodegradation of aniline is 5% (NaCl w/v), but when the salinity surpasses 5%, the growth of AD1 and the biodegradation of aniline could be restricted. The copper ion (Cu^{2+}) had the most significant restriction impact on the growth of AD1, but up to 99.9% of aniline (initial concentration: 600mg/L) can be degraded under the effect of heavy metal ion. Both the single bacterial strain AD1 and mixed bacterial strains (AD1 and nitrifying bacteria) can efficiently degrade aniline, and the degradation rate of aniline for both groups reached 99.9%. However, the mixed bacterial strains did not show palpable advantage of the speed of aniline degradation over single bacterial strain AD1. Though nitrifying bacteria in the mixed bacterial strains was expected to degrade the ammonia nitrogen produced in aniline biodegradation process, there was no conspicuous difference in the concentration of ammonia nitrogen and total nitrogen between the samples of single bacterial strain AD1 and mixed bacterial strains.

1. **Introduction**

Aniline is a kind of highly toxic cyclic aromatic compound which could impair people’s liver, nervous system and respiratory system [1]. It is commonly used as the raw material or intermediate in printing and dyeing industry, pharmaceutical industry and coking industry [2]. Owing to the prosperous development of industry in China and many other developing countries, it is urgent to find an efficient and stable method for aniline degradation.

The biodegradation of aniline was proved to be feasible and only result in limited level of secondary contamination [2,3]. The biodegradation of aniline by bacterial consortia was reported to have the characteristics to endure the environment which was acidic and of elevated salinity, hence this method do not need pre-treatment [4]. Besides, *Delftia* sp. was reported to have strong ability for the degradation of aniline [5], so the research group chose the isolated bacterial strain *Delftia* sp. named AD1 for aniline biodegradation and mainly studied on the capacity of biodegradation under some specific circumstances. The experiment is basically divided into two parts:

1. By setting a series of salinity gradients in the growth medium, the research group compared the biodegradation rate to find out a relatively suitable salt concentration for the growth of AD1 and the biodegradation of aniline. Additionally, the research group compared the influence of several kinds of heavy metal ions on the growth of AD1 and the biodegradation rate through adding heavy metal compounds into the growth medium.
(2) Ammonia nitrogen is one of the intermediates in the process of the biodegradation of aniline. Considering that ammonia nitrogen may lead to secondary contamination, the research group mixed the AD1 with nitrifying bacteria in order to degrade the ammonia nitrogen released in the process of biodegradation of aniline [6]. By setting two separate mix ratios, the research group compared the biodegradation rate and other statistics to explore whether the mixed bacterial strains which consist of AD1 and nitrifying bacteria can contribute to reduce the concentration of ammonia nitrogen.

2. Materials and methods

2.1. Materials
The bacterial strain AD1 was isolated from the activated sludge of sewage treatment in Wuhan and stored in the refrigerator. The chemicals used for the experiment were analytical grade.

2.2. Experimental procedure

2.2.1. The growth of bacterial strain
(1) Preparation of inorganic salt culture medium
Culture medium formula for AD1: add the chemicals in table 1 into 1L of distilled water.

| Chemical                  | Quality (g) |
|---------------------------|-------------|
| NaH₂PO₄·2H₂O              | 0.26        |
| Na₃HPO₄·12H₂O             | 1.0079      |
| (NH₄)₂SO₄                 | 2           |
| MgSO₄·7H₂O                | 0.2046      |
| KCL                       | 0.2         |
| Fe(NO₃)₃·9H₂O             | 0.0134      |

The culture medium for mixed bacterial stains which consist of AD1 and nitrifying bacteria is the same with the formula in Table 1.

(2) Preparation of the plate culture medium
Add 150mL of inorganic salt culture medium into two 250 mL conical flasks separately, ensure two conical flasks contains different kinds of culture medium (formula 1 and 2). Then add 2.7g of agar powder, shake up. Tighten the lid of flasks, wrap the lid with medical gauze, then wrap the pipette tips with papers and put the conical flasks, petri dishes, pipette tips into the autoclave. After autoclaving, place the items into the ultra-clean workbench and sterilize them under ultraviolet light for 20 minutes. After that, ventilate the ultra-clean workbench for 15 minutes. Apart from the items before, alcohol lamp, lighter, inoculation loops, pipettes, aniline were also sterilized. Add 88.2μL of aniline into the AD1 culture medium, shake up. Add the two kinds of inorganic salt medium into petri dishes, ensure the height of medium is about 2/3 of the height of the petri dish. After the medium is cooled down and solidified, inoculate the bacteria into the petri dish. Stick the label to the bottom of the petri dish and
invert it. Then put the petri dishes into an incubator for cultivation (28°C), observe the growth of bacterial stain in the next few days.

(3) Preparation of bacterial solution
The steps of autoclaving and sterilization in this process were similar to the preparation of the plate culture medium. The difference is that the permeable sealing films and the rubber bands were also sterilized in the ultra-clean workbench. Add 88.2μL of aniline into the culture medium (formula 1), inoculate the bacteria strain grown in the plate medium into the culture medium, shake up. Seal the flasks with permeable sealing film, write the information on the label, and place the flasks in a shaking table. Observe the turbidity of the culture medium in the flasks in the next few days. Measure the OD600 after two or three days. To ensure the bacteria is in logarithmic growth phase, the bacterial solution with the OD600 about 0.52 is suitable for the subsequent experiments.

2.2.2. Experiment on the biodegradation capability of the mixed bacterial strains
(1) Measure the OD600 of the cultured bacterial solution. After the OD600 is larger than 0.52, dilute the bacterial solution with inorganic salt culture medium (formula 1).
(2) Prepare four of 250mL conical flasks, add 150mL of inorganic salt culture medium (formula 1) into the conical flasks. After autoclaving and ultraviolet sterilization, ventilate the ultra-clean workbench. Inoculate AD1 and nitrifying bacteria into the inorganic salt culture medium (formula 1), the mix ratio of AD1 and nitrifying bacteria (volume ratio) was set to 1:1. The inoculation amount was set to 5%. More specifically, add 7.5 mL of AD1 solution and 7.5 mL of nitrifying bacteria solution into 150 mL of culture medium (formula 1). A total of four samples were tested, two flasks of culture medium with the mix ratio of 1:1, one with only 7.5 mL of AD1, and the other one without bacterial solution. Put the inoculated medium into the shaking table, and periodically measure the measurement indexes of the samples, including OD600, concentration of aniline, ammonia nitrogen, nitrate nitrogen and total nitrogen. Change the mix ratio of AD1 and nitrifying bacteria to 1:3, repeat the procedure with another four samples.

2.2.3. Experiment on the effects of salinity
The research group chose NaCl, and set 0%, 0.5%, 1%, 1.5%, 2%, 5%, 10%, 20% eight salinity gradients to test the salt tolerance of the bacterial strain AD1 as well as the biodegradation rate of aniline. Set two samples for each salinity gradient, prepare sixteen conical flask (100 mL), add 50 mL of culture medium (formula 1) into the conical flasks. The bacterial strain was inoculated into the culture medium (formula 1) with aniline (concentration 600 mg/L) as the sole carbon source and nitrogen source, the inoculation amount was 5%. The shaking table was set to 20°C and 180 r/min, and samples were taken out every 12 hours to measure the concentration of the aniline remained. After autoclaving, ultraviolet sterilization, ventilation and inoculation, put the flasks into a constant temperature incubator, measure the samples after 12h, 24h, 36h, 48h, 60h, 72h. The measurement indexes were OD600, concentration of aniline and ammonia nitrogen.

2.2.4. Experiment on the effects of heavy metal
The research group chose four kinds of heavy metal compounds, and set two concentration gradients for each kind of heavy metal compound to test the effects of heavy metal ion on the growth of AD1 as well as the biodegradation rate of aniline. The research group chose four kinds of heavy metal compounds, and set two samples for each concentration of heavy metal compound, sixteen samples in total. Prepare sixteen conical flasks (100 mL). Based on the results of preliminary experiment in subsection 2.2.3, 2g of NaCl was added into each of 50mL conical flask (16 conical flasks in total), and different kinds of heavy metal compound were also added to the corresponding conical flask. The addition: 0.00125g/0.00250g of CuSO4·5H2O, 0.00144g/0.00288g of ZnSO4·7H2O, 0.00099g/0.00198g of MnCl2·4H2O, 0.00097g/0.00194g of K2CrO4. Then 50mL of culture medium (aniline as sole carbon source and nitrogen source) was added into each conical flask.
After autoclaving, ultraviolet sterilization, ventilation and inoculation, put the flasks into a constant temperature incubator, measure the samples after 12h, 24h, 48h, 72h. The measurement indexes were OD_{600}, concentration of aniline and ammonia nitrogen.

Table 3. Chemicals and quality for each sample

| Heavy metal compound | Concentration (g/L) | Quality (g) | Sample quantity |
|----------------------|--------------------|-------------|-----------------|
| CuSO₄·5H₂O           | 0.025              | 0.00125     | 2               |
|                      | 0.05               | 0.0025      | 2               |
|                      | 0.0288             | 0.00144     | 2               |
| ZnSO₄·7H₂O           | 0.05751            | 0.0028755   | 2               |
|                      | 0.0198             | 0.00099     | 2               |
| MnCl₂·4H₂O           | 0.0396             | 0.00198     | 2               |
|                      | 0.0194             | 0.00097     | 2               |
| K₂CrO₄               | 0.0388             | 0.00194     | 2               |

3. Results and discussion

3.1. The characteristics of biodegradation of aniline by mixed bacterial strains

It can be seen from the four Figures in Figure 1 (the second group has sodium succinate hexahedron for extra carbon source, concentration in the culture medium: 3.076g/L) that there was no significant discrepancy between OD_{600} statistics, which means growth of the single bacterial strain and mixed bacterial strains (AD1 and nitrifying bacteria mix ratio 1:1) were similar, the mix of bacterial strain did not reveal palpable advantage in the growth of bacteria. The maximum OD_{600} of the single strain AD1 and the mixed strains reached the maximum of 1.381 and 1.199 respectively. The OD_{600} of the second group at 72h was much higher than that of the first group with the same ratio, indicating that AD1 and nitrifying bacteria may restrict each other's growth due to the competition for nutrients between two kinds of bacterial strain, by adding sodium succinate hexahedron as extra carbon source, the competition can be alleviated, hence the mixed bacterial in the second group can grow better.
Figure 1. Biodegradation of aniline by mixed bacterial strains

It can be seen from Figure 6(b) and Figure 6(d) that both single bacterial strain AD1 and mixed bacterial strains can efficiently degrade aniline, and the degradation rate of aniline both reached 99.9%, which indicates that the nitrifying bacteria did not contribute to the degradation of aniline. A certain amount of ammonia nitrogen was accumulated in the process of aniline biodegradation by AD1. In the introduction, it was expected that the nitrifying bacteria in the mixed bacterial strains can degrade the ammonia nitrogen produced in the biodegradation process. However, according to Figure 6(b) and Figure 6(d), there was no significant difference in ammonia nitrogen concentration between the group of single bacterial strain AD1 and mixed bacterial strains after 72 hours. This may owe to the competition between two bacterial strains. Besides, high concentration of aniline can inhibit the growth of nitrifying bacteria.

Compare Figure 6(b) with Figure 6(d), when the ratio of AD1 and nitrifying bacteria is 1:1, it took nearly 60 hours to degrade 99.9% of aniline; while the ratio of AD1 and nitrifying bacteria is 1:3, it took merely 36 hours to degrade 99.9% of aniline. In addition, there was no significant difference in aniline biodegradation ability between single bacterial strain AD1 and mixed bacterial strains. Besides, the concentration of residual ammonia nitrogen in single bacterial strain group was lower than that of mixed strains after 72h. Generally speaking, the mixed strain’s capability of degrading the ammonia nitrogen and total nitrogen produced in the process of aniline biodegradation did not show obvious advantage upon single strain. However, the addition of sodium succinate hexahedron may contribute to strain ammonia nitrogen produced in the process of aniline biodegradation.

3.2. The influence of salinity upon the growth of AD1 and aniline biodegradation

According to the procedure in subsubsection 2.2.3, the statistics are shown in Figure 2:

Figure 2. The influence of salinity upon aniline biodegradation
It can be seen from Figure 2 that as the salinity gradient increases, the inhibition on aniline biodegradation became more obvious. As for AD1, the optimal salinity range for aniline biodegradation is 0.5%~2%. After 60 hours, the degradation rate of sample 0.5% and sample 2% both reached 100%. When the salinity reached 5%, the biodegradation rate of aniline in 0~60h was much lower than the biodegradation rate of sample 2%, indicating that the biodegradation of aniline began to be inhibited. It is mainly because the bacteria need to absorb nutrients and also need to adapt to the influence of NaCl in the early stage of growth. But the aniline of sample 5% was fully degraded after 60h. After the salinity exceeds 5%, the bacterial strain did not grow normally. For example, the aniline was not degraded in sample 10% and sample 20%. The possible reason is that the salinity in the culture medium can affect the nutrient absorption process of the bacterial cells. When the salinity exceeds 5%, the osmotic pressure of the environment increases, which makes it more difficult for bacteria to absorb nutrients, hence the growth and metabolism of bacteria is inhibited. When the salinity is too high (about 10% or higher), it will cause internal dehydration or even the death of the bacteria.

3.3. The effect of heavy metal ions on the growth of AD1 and aniline biodegradation

According to the procedure in subsubsection 2.2.4, the statistics are shown in Figure 3:

**Figure 3.** The influence of heavy metal ions on the growth of AD1 and aniline biodegradation

For the samples which contained CuSO₄, it can be seen from Figure 3 that the adaptation period of AD1 for copper ion (Cu²⁺) was relatively longer (48 hours). From 48h to 72h AD1 was in logarithmic growth phase, and OD₆₀₀ reached the maximum of 0.749 and 0.644 at 72 h. In the end, 99.8% of aniline was degraded, and the minimum concentration of aniline was 1.140 mg/L.

It also can be seen from the Figure 3 that the adaptation periods of AD1 for zinc ion (Zn²⁺), manganese ion (Mn²⁺) and chromium ion (Cr⁷⁺) were relatively shorter (12hours).

For the samples which contained K₂CrO₄, the AD1 was in logarithmic growth phase from 12h to 48h, and OD₆₀₀ reached the maximum of 0.648 and 0.542 at 48h. In the end, 97.0% and 97.9% of aniline was degraded, and the minimum concentration of aniline was 1.134mg/L and 3.402mg/L respectively.

For the samples which contained MnCl₂, the AD1 was in logarithmic growth phase from 12h to 48h, and OD₆₀₀ reached the maximum of 0.616 and 0.648 at 48h. In the end, 96.6% and 97.0% of aniline was degraded, and the minimum concentration of aniline was 0mg/L and 1.134mg/L respectively.

For the samples which contained ZnSO₄, the AD1 was in logarithmic growth phase from 12h to 24h, and OD₆₀₀ reached the maximum of 0.686 and 0.716 at 24h. In the end, 85.8% and 90.6% of aniline was degraded, and the minimum concentration of aniline were both 3.402mg/L. The biodegradation rate was lower in these four samples compared with other twelve samples probably owe to the insufficient of carbon source. The OD₆₀₀ began to decrease after reaching the maximum, which may also prove that the bacterial strain was led to autolysis. The growth curve of AD1 gradually stabilized after 48h.
4. Conclusions
The research group basically studied on the capability of aniline degradation of the bacterial strain *Delftia* sp. named as AD1 under some specific circumstances. The experiment included three parts.

1. The growth of the single bacterial strain (AD1) and mixed bacterial strains (AD1 and nitrifying bacteria) did not reveal palpable discrepancy. The *Delftia* sp. bacterial strain AD1 and nitrifying bacteria may restrict each other's growth due to the competition for nutrients, and this situation can be alleviated by adding sodium succinate hexahedron as extra carbon source. Both the single bacterial strain AD1 and mixed bacterial strains can efficiently degrade aniline, and the degradation rate of aniline for both groups reached 99.9%. However, the mixed bacterial strains did not show significant advantage in the speed of the degradation of aniline.

Though nitrifying bacteria in the mixed bacterial strains were expected to degrade the ammonia nitrogen produced in the aniline biodegradation process, there was no significant difference in the concentration of ammonia nitrogen and total nitrogen between the single bacterial strain AD1 and mixed bacterial strains. This may owe to the competition between two kinds of bacterial strain or the inhibition of aniline on the growth of nitrifying bacteria. When the ratio of AD1 and nitrifying bacteria is 1:1, it took nearly 60 hours to degrade 99.9% of aniline; while the ratio of AD1 and nitrifying bacteria is 1:3, it took merely 36 hours to degrade 99.9% of aniline.

2. AD1 is salt-tolerant, and the most suitable salinity for the growth of AD1 and the biodegradation of aniline is 5%, but when the salinity is higher than 5%, the bacteria will have difficulty in absorbing nutrients due to the high external osmotic pressure, even cause the death of the bacterial strain.

3. The heavy metal ions did not have a strong effect on the growth of AD1 and the biodegradation of aniline. The copper ion (Cu²⁺) had the most significant impact on the growth of AD1, it took relatively longer time for AD1 to adapt to the copper ion in the culture medium (48 hours).

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