Environment, Resource and Ecology Journal (2023)
Clausius Scientific Press, Canada

DOI: 10.23977/erej.2023.070302
ISSN 2616-3756 Vol. 7 Num. 3

# *Screening and Characterization of Nitrogen Removal Functional Bacteria in Landfill Leachate*

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***Keywords:*** Ammonia nitrogen degradation rate, microbe, landfill leachate, separation and purification, evolutionary tree

***Abstract:*** In this paper, the microorganisms collected from the leachate of the landfill drainage outlet of the landfill were separated and purified for many times, and the sterilized and diluted leachate was added and physicochemical treatment as a blank control, and the ammonia nitrogen degradation rate was used as the evaluation index to measure the effect of purified and domesticated strains in treating leachate. After cultivation and screening, the final ammonia nitrogen degradation rates of different strains 3, 4, 7, 9, 10, 12 and 13 were 57.14%, 38.96%, 85.26%, 92.72%, 66.53%, 95.62% and 74.84%, the degradation rate of No. 12 strain was as high as 95.62%, and the lowest degradation rate was 38.96% for No. 4 strain. The research showed that the degradation effects of different strains were different, and strains with good degradation of ammonia nitrogen were screened out. In summary, the microorganisms isolated from the landfill leachate can degrade the ammonia nitrogen present in themselves, thereby reducing the pollution of its water quality, and indirectly or directly affecting the effect of environmental indicators.

## 1. Introduction

### 1.1. Context and Meaning

#### 1.1.1. Analysis of the Current Situation of Garbage Treatment and Landfill Leachate in China

With the development of China's economy, the amount of garbage continues to grow. According to data from China's environmental protection department, in 2021, the annual output of urban waste in China will be as high as 150 million tons, and it is expected to be 4 in 2030 100 million tons, 5. In 2050, 300 million tons, the total amount of domestic waste and unit output are increasing at a rate of more than 10% per year [1]. In contrast, the world's annual output of garbage is 490 million tons, and the average annual growth rate of the world's garbage is 8.42%, and China's garbage disposal demand is increasing. In waste treatment, the leachate produced by garbage treatment accounts for about 25% of the amount of waste. Obviously, as the total amount of waste produced grows, so will the landfill leachate. Urban and rural waste generation also continues to grow.

Under the background of rural revitalization, rural garbage management has become an important

part of rural work. Rural garbage is mainly food waste and agricultural production waste, which is characterized by the generation of garbage, but also produces sewage, which is the root cause of rural garbage pollution treatment. Therefore, in terms of garbage management, the national government should increase investment to improve garbage treatment infrastructure such as garbage transfer, landfill, and garbage incineration. However, in terms of landfill leachate treatment, a complete landfill leachate disposal system has not been established, which is a weak link in domestic waste control. Paying attention to the research of the theory of landfill leachate control can promote the improvement of the comprehensive level of garbage treatment in China, and the treatment of domestic waste should be promoted and applied as much as possible to 3R technology.

Landfill leachate is mainly a highly polluting secondary polluted wastewater generated from sanitary landfills or waste incineration power plants, with high concentrations of organic matter, sulfate ions, heavy metal ions and ammonia nitrogen [2]. Among them, ammonia nitrogen is one of the sources of endogenous pollution. At present, China's municipal solid waste produces about 29 million tons of fresh leachate every year, and the concentration of pollutants contained in 1 ton of leachate is equivalent to about 100 tons of municipal wastewater. The concentration of ammonia nitrogen in landfill leachate is generally high, and under China's guarantee requirements. Its concentration indicators are lower and sensitive areas are more stringent. If the ammonia nitrogen discharge exceeds the standard, it is easy to make the water body eutrophication, which will cause serious harm to the atmosphere, water body, soil, etc; Therefore, there is an urgent need to research and develop advanced landfill leachate treatment technologies.

### 1.1.2. Landfill Leachate Treatment Process

Before the landfill leachate treatment process entered China, China's waste treatment focused on the treatment of solid waste, such as crushing, landfilling, and recycling. Gradually, the landfill leachate has attracted widespread attention from the society, and the introduction and development of its technology have continued to mature. In recent years, the technical methods of domestic landfill leachate mainly include physical and chemical method, land method, biological method and the comprehensive use of series and parallel connection of this method. According to different landfill leachate water quality and the requirements for the degree of treatment, the landfill leachate treatment system is a combination of process units. The pretreatment process is generally precipitated first, and then ammonia blown off by adding alkali, and acid is added into the oxidation pond, using physical and chemical methods; On-site treatment mainly exercises a variety of mature anaerobic and aerobic treatment processes, using biological treatment methods; The post-treatment process usually adopts physical and chemical methods such as coagulation precipitation, filtration, adsorption, and reverse osmosis [3].

### 1.1.3 Problems with Landfill Leachate Treatment in China

Landfill leachate is a landfill secondary pollution wastewater, because it contains a large number of organic matter, heavy metal ions, ammonia nitrogen and other characteristics and now the national environmental protection department standards are increasingly strict, so that its treatment process is complex, high energy consumption, time-consuming and labor-intensive, and requires equipment maintenance costs, depreciation rates, high costs and other characteristics. The result is now a large amount of landfill leachate treatment is a key issue.

At present, the research on landfill leachate in China is not extensive enough, generally for "young" landfill leachate (landfill period $< 5$ years), because it is easy to be degraded by microorganisms, anaerobic + aerobic treatment methods are adopted; For "old" landfill leachate (landfill period of 5-10 years), because it is difficult to be biodegraded, C/N is very low, biochemical capacity is poor,

etc., and no cost-effective process technology has been developed, so it requires high costs. The biological treatment leachate method is an important clean technology to be promoted, and the process flow is divided into aerobic, anaerobic and anaerobic and aerobic biological treatment Combined. Aerobic biological treatment can effectively remove ammonia nitrogen, thereby BOD and COD in wastewater. Anaerobic treatment has obvious effects in high concentrations of organic wastewater [4].Therefore, one technology cannot be relied on to maximize the treatment of domestic waste leachate, but the combination of the two technologies needs to be used to play 1+1>2 effect to meet new specifications and standards, thus extending the technology to a wider range.

In view of China's current landfill leachate process technology and current laws and regulations, more practical process applications are needed to optimize and improve. Therefore, it is necessary to build a sound landfill leachate treatment process evaluation standard system, reduce the treatment cost of landfill leachate by optimizing the process steps, and monitor the quality of waste water control at all times during the treatment process to avoid secondary pollution.

### 1.1.4. Research Status of Microbial Degradation of Ammonia Nitrogen in Landfill Leachate

The temperature, moderation, nutrients and other conditions of the landfill are suitable for the growth and reproduction of microorganisms, and the microbial species in the leachate better reflect the richness and diversity of microorganisms in the garbage. According to relevant studies, Yue Bo et al. investigated the abundance of methane bacteria in garbage and leachate, and concluded that the number of methanosaur in landfill and leachate in aerobic was about 48 and 59 times that of anaerobic respectively, indicating that the environment is more conducive to microbial reproduction and growth [5].

The microbial species in landfill garbage are basically the same as those contained in landfill leachate, mainly including 7 types of bacteria such as methanogenic bacteria, nitrosifying bacteria, nitrifying bacteria, denitrifying bacteria and sulfate-reducing bacteria, and some pathogenic bacteria and pathogenic microorganisms [6], such as Huang Linan's article mentions the structure and diversity of methanogenic bacterial communities in leachate; However, with long-term aerobic exposure of leachate or anaerobic landfill, the mortality of microorganisms in the leachate will increase.

In the process of aerobic degradation of garbage, decomposition will release a large amount of water and heat, resulting in an increase in temperature, exceeding the optimal temperature of some microorganisms, which will affect the growth and survival of microorganisms; Too high or too low pH of landfill leachate can also lead to microbial inactivation, and if multiple conditions are restricted at the same time, it will greatly accelerate the inactivation of microorganisms [3].

The microorganisms in the landfill leachate not only degrade solid waste to produce high-concentration leachate, but also have other microbiota participating in biological treatment. The results show that the diversity of bacterial populations in landfills fluctuates with the increase of landfill depth, and the density and quantity are also "uneven" - uniformity", and the hierarchy of dominant and non-dominant species changes greatly [5].

In recent years, in the study of the treatment effect of microbial dominant bacteria, Liu Jianhong et al. used photosynthetic bacterial mixed cultures to conduct static tests on leachate, and the removal rates of CODcr and BOD reached 84.4% and 79.7%, respectively Sulfide and $NH_3$-N removal rates were 97.3% and 69.2%;

## 1.2. Research Content

In this paper, the landfill leachate in landfill was taken as the research object, the microbial communities were determined according to the domestic waste leachate of different time spans, and

the accelerated decomposition of domestic waste leachate by microbial communities was discussed. The dominant microbial species were cultivated under the existing experimental equipment conditions, so as to domesticate colonies with efficient degradation of ammonia nitrogen. It provides a solid foundation for future research on whether the microorganism can realize the transformation and application of resources to domestic waste leachate.

In this paper, biological treatment technology is mainly selected. Its advantage lies in the fact that biological treatment technology has important advantages such as high speed, rapid consumption, high efficiency, low cost, mild reaction conditions, and secondary pollution protection in the treatment of environmental pollutants [7].Use existing resources for integration, so as to achieve reduction, recycling, and harmlessness. In the research process, the existing energy and materials are used as much as possible to solve the environmental pollution of domestic waste leachate, combined with the broad market prospects envisaged by technological development, which is highly valued by the government, enterprises and scientific research talents, and reflects the long-term strategy of sustainable development from the ideological point of view. Apply the effective degradation of microorganisms to convert them into effective control of environmental pollution.

The research content is as follows:

(1) Identify community species of microorganisms with different time spans;

(2) Cultivation of identified microbial communities;

(3) Selection and domestication after the microbial cultivation process;

(4) Study on the effect of microorganisms on nitrogen removal treatment of leachate;

(5) explore the favorable direction of the intrinsic mechanism of microorganisms;

## 2. Materials and Methods

### 2.1. Experimental Materials

(1) Microbial source: Obtain landfill leachate from different sampling points in the landfill and store it in a refrigerator at 4 ℃ for use.

(2) Experimental reagents: landfill leachate stock solution, experimental strains, various media materials [8] (Gao's No. 1 culture materials, beef paste protein medium materials, enrichment media and other reagents), Gram stain, cedar oil, Toluene, Knott's reagent, spectrophotometric drugs, distilled water and other loss materials.

(3) Experimental instruments: conventional glass instruments, samplers, autoclaves, induction cookers, petri dishes, sterilization basic materials, UV spectrophotometers, refrigerators, artificial climate chambers, ultra-clean workbenches, bacterial incubators, shakers, high-speed centrifuges, microscopes, pH test strips, pipettes, pipettes, inoculation tools, etc.

### 2.2. Experimental Methods

#### 2.2.1. Medium Preparation

(1) Gao medium: $MgSO_4 \cdot 7H_2O$ 0.5g, soluble starch 20g, agar 15.25g, NaCl 0.5g, $FeSO_4 \cdot 7H_2O$ 0.01g, $KNO_3$ 1g, $K_2HPO_4 \cdot 3H_2O$ 0.5g, water 1000ml, pH value 7.4~7.6, store after sterilization.

(2) Beef paste protein medium: peptone 10g, beef paste 3g, agar 20g, NaCl 5g, water 1000ml, pH value 7.4~7.6, stored after sterilization.

#### 2.2.2. Microbial Inoculation

The experimental species in this paper were taken from the sampling point of the landfill leachate discharge outlet in the sanitary landfill of domestic waste, and the leachate stock solution was taken

from three different sampling points, and obtained by culture. Two bottles of 1000 ml stock solution were taken at each sampling point, and the obtained samples were preserved [9] immediately brought back to the laboratory, and 1 ml of the required stock solution was taken for dilution factor ($10^{-3}$, $10^{-5}$, $10^{-7}$), and apply on 20~25 ml of different media according to the concentration gradient.

#### 2.2.3. Microbial Selection and Purification

The inoculated culture base was placed in a constant temperature incubator at 25~30 ℃ and cultured upside down, and the colonies were to grow out of the plates, and the appearance and morphology of the colonies were observed every 6~8 hours, and the colonies were recorded in time Morphological characteristics [10], after the strain is roughly formed, placed in the ultra-clean workbench to select a single plant for purification, using the inoculation ring to pick out all different forms of single plant colonies, so as to obtain uncontaminated strains, using the five-step line drawing method to draw lines Purify 2~3 times until a single form of purebred strain is obtained and purified The single colonies are numbered [11].

#### 2.2.4. Identification of Strains

##### 2.2.4.1. Morphological Identification

The dominant hyphae after the above screening were repeatedly inoculated on sterilized medium plates with a sterilized inoculation ring, cultured under suitable growth conditions, waited for purified uncontaminated single colonies to grow on the dish, and morphological observation and identification of the strain [12] and staining to determine that the strain was $G^+/G^-$.

(1) Preparation: take the active growth period strains according to the conventional method of smearing (should not be too thick), dry and fixed.

(2) Primary dyeing: add grass-sensitive iron crystal violet dyeing solution dropwise to cover the coated part, dye for 1~2min and then pour off the dye, ash with water until the outflow water is colorless.

(3) Mordant: first wash away the residual water traces with iodine solution, and then cover it with iodine solution for 1min, pour off the iodine solution, and wash with water until the outflow water is colorless

(4) Decolorization: suck off the residual water on the slope sheet with absorbent paper, tilt the slide, add 95% ethanol decolorization (generally 20~30s) with a dropper on a white-~ackground, and immediately wash off the thanol with water when the effluent is colorless.

(5) Counterstaining: suck off the residual water on the slide with absorbent paper, dye with saffin counterstaining solution for 2min, wash with water, and absorb the residual water to dry or dry with a hair dryer and cold air.

(6) Microscopic examination: oil mirror observation [13].

##### 2.2.4.2. Molecular Biology Identification

Purified dominant cultures were sent to a genetic sequencing company for 16SrDAN sequencing. The 16S rDNA sequencing results of the dominant strain were entered into the website and BLAST compared with the known sequences in the database, so as to find out the sequence information of the top three similarities for comparison and perform homology analysis [14]; The sequences of each strain were then imported into MEGA 7.0 software to construct phylogenetic trees and step trees, and finally molecular biology identification was carried out [15].

### 2.2.5. Preparation of Bacterial Liquid

The dominant strain on the plate was quantitatively removed with a sterilized tip and inoculated into enriched liquid medium ($NH4Cl$ 0.5g, $CH_3COONa$ 3.5 g, $MgSO4·7H_2O$ 0.05g, $K_2HPO4$ ·3H2O 0.2g, NaCl 0.12g, MnSO4 ·$H_2O$ 0.01g, $FeSO_4$ ·7$H_2O$ 0.01g), distilled water 1000mL, pH 7.0-7.4, at 30 ℃, 150r/ Under min conditions, the OD600 was measured by shaking the shaker every 3h, and the growth of each dominant strain was plotted to obtain the concentration of bacteria in the logarithmic phase.

### 2.2.6. Ammonia Nitrogen Degradation Experiment in Landfill Leachate

(1) Draw the spectrophotometric calibration curve of ammonia nitrogen Natsch's reagent [16]: in eight 50ml colorimetric tubes, add 0.00, 0.50, 1.00, 2.00, 4.00, 6.00, 8.00 and 10.00ml ammonia nitrogen standard solution, and the corresponding ammonia nitrogen content is 0.0, 5.0, 10.0, 20.0, 40.0, 60.0, respectively. 80.0 and 100 μg, add water to the reticle. Add 1.0 ml of potassium sodium tartrate solution, shake well, and then add 1.5ml or 1.0ml of Knotler reagent, shake well. After 10 min of placement, at a wavelength of 420 nm, use a 20mm cuvette with water as a reference to measure the absorbance. The calibration curve was drawn with the blank-corrected absorbance as the ordinate and its corresponding ammonia nitrogen content (μg) as the abscissa [17].

(2) Sample determination:

Dominant culture leachate culture solution: take 50ml of sample and determine absorbance according to the same method steps as the standard curve.

Blank test: Replace the water sample with distilled water and pretreat and determine it according to the same method steps as described above [18].

(3) Results calculation: ammonia nitrogen mass concentration calculation method formula [19]:

$$\rho N = \frac{As - Ab - a}{b \times v} \tag{1}$$

In the formula,

$\rho_N$—the mass concentration of ammonia nitrogen in the water sample (in N), mg/L;

As — absorbance of the water sample;

Ab—absorbance of the blank test;

a—the intercept of the calibration curve;

b—the slope of the calibration curve;

V—sample volume, ml.

## 3. Results and Analysis

### 3.1. Identification Results of Dominant Strains

Table 1: The Comparison of morphological characteristics of dominant strains.

| Colony characteristics | Shape | Color | Humidity | Transparency | Degree of binding |
|---|---|---|---|---|---|
| Strain 3 | round bulge | yellow | moister | opacity | not combined |
| Strain 4 | Smooth round | yellow | dry | opacity | not combined |
| Strain 7 | round | rosiness | moister | opacity | not combined |
| Strain 9 | smooth round | white | moister | translucent | not combined |
| Strain 10 | round bulge | ivory | moist | translucent | not combined |
| Strain 12 | irregular grains | yellow | moist | opacity | not combined |
| Strain 13 | folded | yellow | drier | opacity | combined |

The comparison of morphological characteristics of dominant strains is showed Table 1.

### 3.2. Strain-Phylogenetic Tree Construction

(1) Dominant strain 3 evolutionary tree: The 16S rDNA sequence of dominant strain 3 was compared with the sequence in the database, and the gene homology was searched by BLAST to construct its phylogenetic tree, as shown in the figure 1 is shown. The resulting strain 3 was 99.33%, similar to Pseudomonas cold-tolerant, so strain 3 belonged to the genus Pseudomonas.

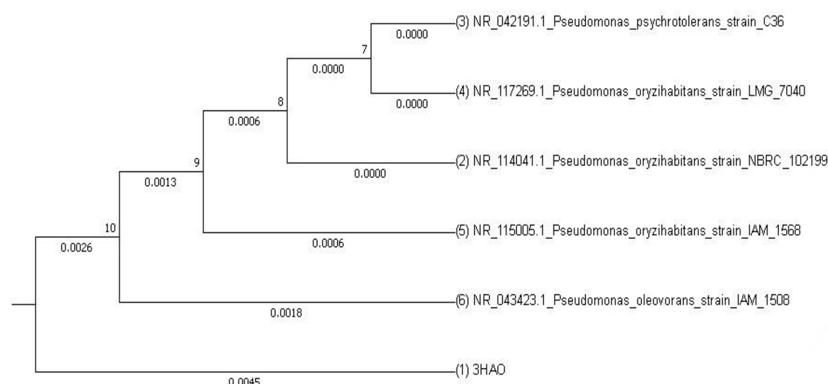



Figure 1: Phylogenetic tree of strain 3

(2) Dominant strain 4 evolutionary tree: The 16S rDNA sequence of the screened dominant strain 4 was compared with the sequence in the database, and the gene homology was searched by BLAST to construct its phylogenetic tree, as shown in Figure 2. The resulting strain 4 is 99.67% similar to Sphingosine monosphingosine, so strain 4 belongs to the genus Sphingosine.

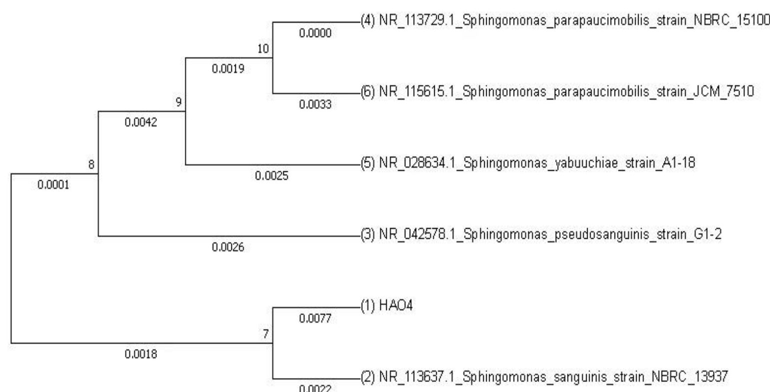



Figure 2: Phylogenetic tree of strain 4

(3) Dominant strain 7 evolutionary tree: The 16S rDNA sequence of the screened dominant strain 7 was compared with the sequence in the database, and the gene homology was searched by BLAST to construct its phylogenetic tree, as shown in Figure 3. The resulting strain 7 is 99.34% similar to sphingosine monobacteria, so strain 7 belongs to the genus Sphingosine monocella.

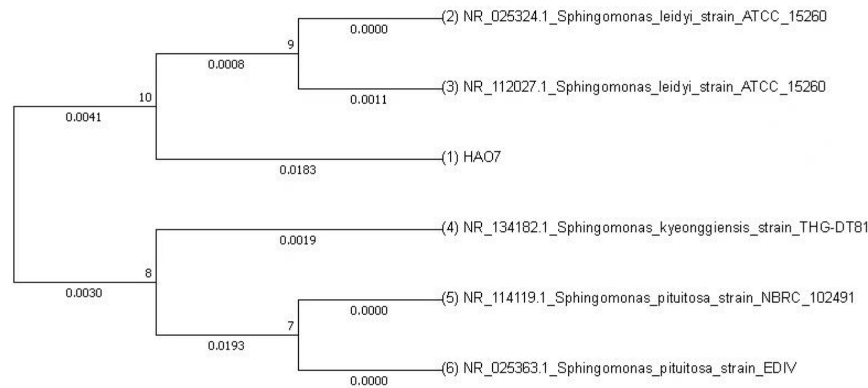



Figure 3: Phylogenetic tree of strain 7

(4) Dominant strain 9 evolutionary tree: The 16S rDNA sequence of the screened dominant strain 9 is compared with the sequence in the database, and gene homology is searched for with BLAST and constructed. Its phylogenetic tree, as shown in Figure 4. The resulting strain 9 is similar to Pseudomonas knotorii 96.77%, so strain 7 belongs to the genus Pseudomonas knotnoti.

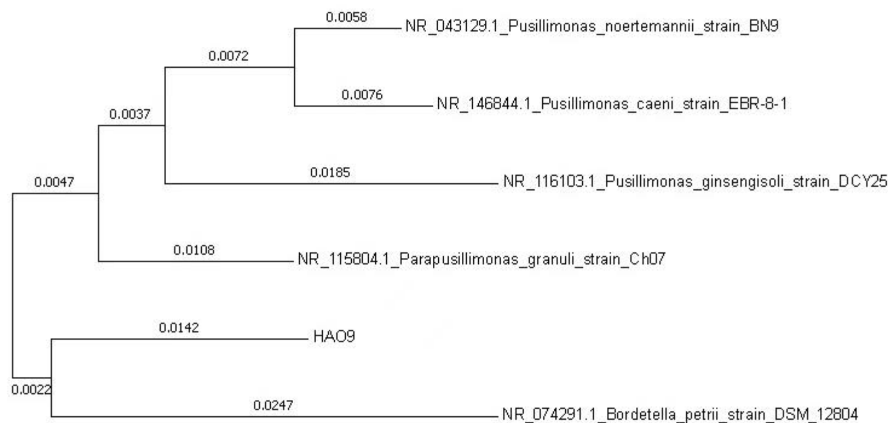



Figure 4: Pylogenetic tree of strain 9

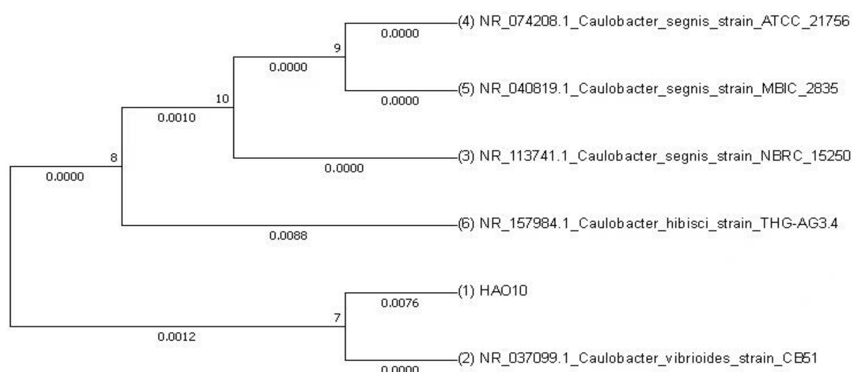



Figure 5: Phylogenetic tree of strain 10

(5) Dominant strain 10 evolutionary tree: The 16S rDNA sequence of the screened dominant strain 10 is compared with the sequence in the database BLAST searches for gene homology and constructs its phylogenetic tree, as shown in Figure 5. The resulting strain was similar to Vibrio 96.57%, so

strain 10 belongs to the genus Stem fungi.

(6) Dominant strain 12 phylogenetic tree: The 16S rDNA sequence of the screened dominant strain 12 is compared with the sequence in the database BLAST searches for gene homology and constructs its phylogenetic tree, as shown in Figure 6. The resulting strain 12 was similar to Pseudomonas rice 99.77%, so strain 12 genera in the genus Pseudomonas fultifolia.

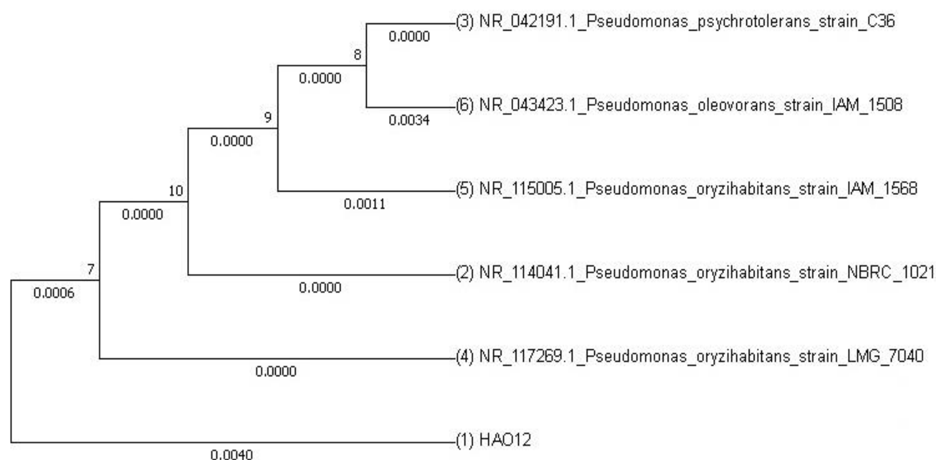



Figure 6: Phylogenetic tree of strain 12

(7) Dominant strain 1 3 evolutionary tree: The 16S rDNA sequence of the screened dominant strain 13 is compared with the sequence in the database BLAST searches for gene homology and constructs its phylogenetic tree, as shown in Figure 7. The resulting strain 13 was similar to Pseudomonas 99.66%, so strain 13 belonged to the genus Pseudomonas.

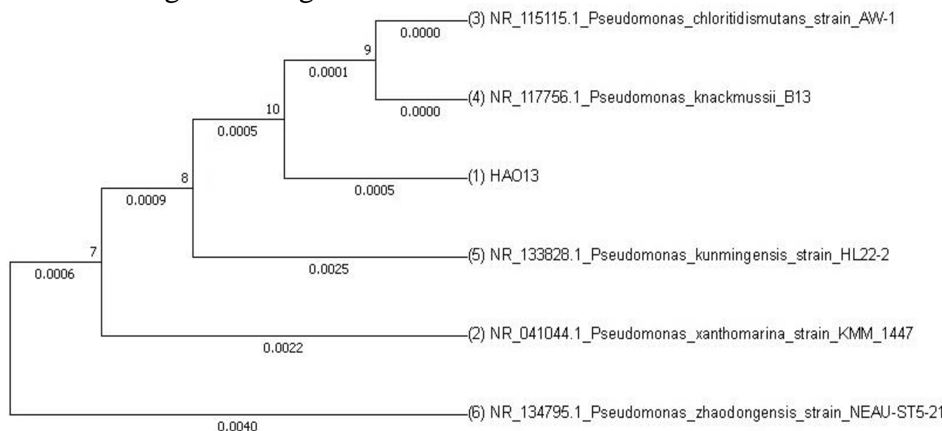



Figure 7: Phylogenetic tree of strain 13

(8) As shown in Figure 8, the step size test merge tree of the strain is the construction of the evolutionary tree of the dominant strain and its similar strains, and the similar strains of the screened dominant strains can be seen from the figure, as well as the homology between each other. The three dominant strains 3, 1 2 and 1 3 belong to the same branch, and the more similar dominant strains 3 and 1 2 belong to the same branch. The No. 9 strain had strong homology with the dominant strains 3, 1 2 and 13. The other branch is the dominant strain 4, 7, 10. The homology of the dominant strains 4 and 7 was higher than that of the dominant strains 10.

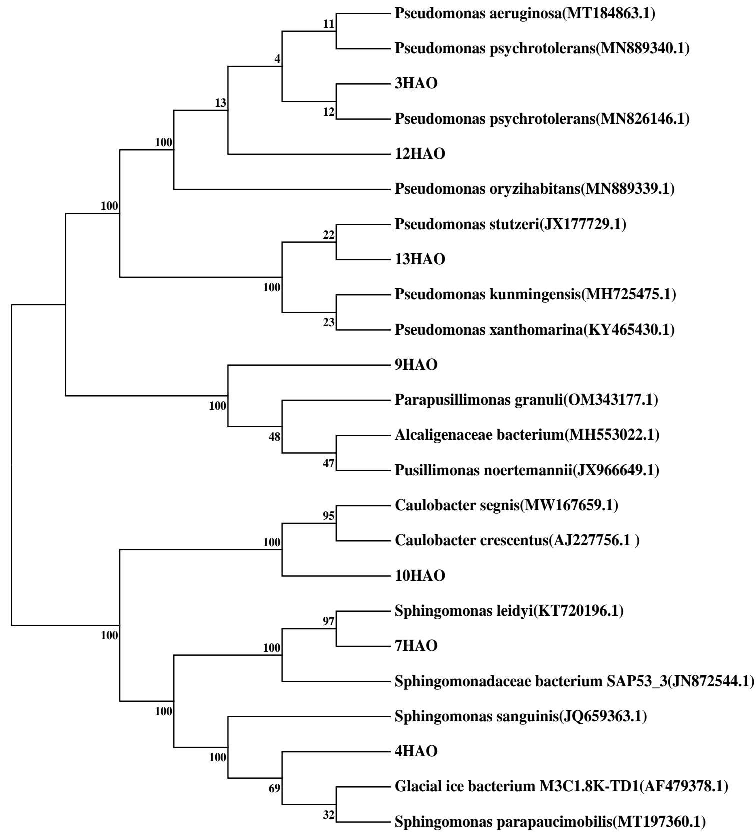
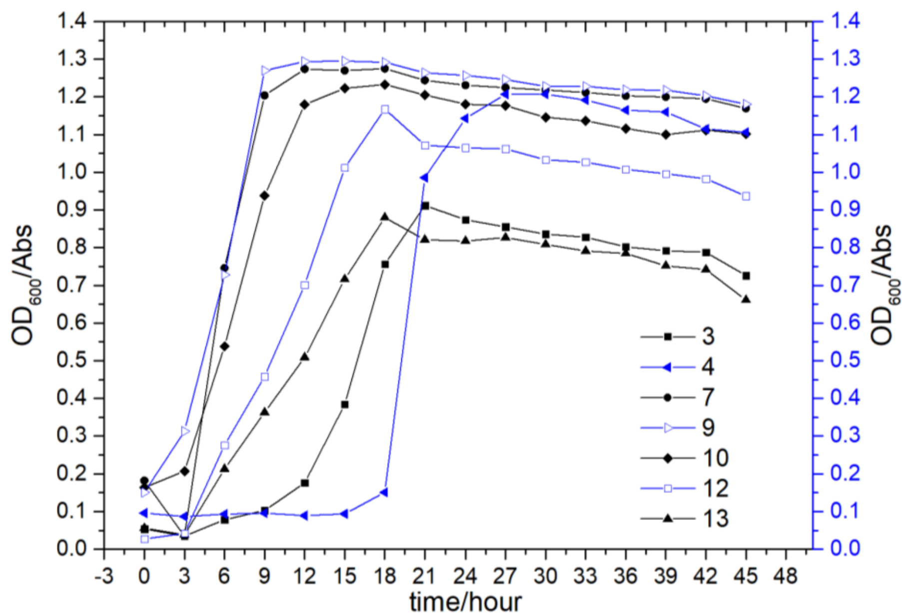



Figure 8: Bootstrap consensus tree of strain

## 3.3. Determination of Growth $OD_{600}$ of Potential Strains




Note: Figures 9 in which 3, 4, 7, 9, 1 0, 1 2, and 13 represent the dominant strains, respectively. (Same as below)

Figure 9: Growth curve of dominant strains

### 3.4. Degradation Rate of Dominant Strain $NH_4^+$-N (Standard Curve Regression Equation $y=0.0033x+0.0248$, $R^2=0.9996$)

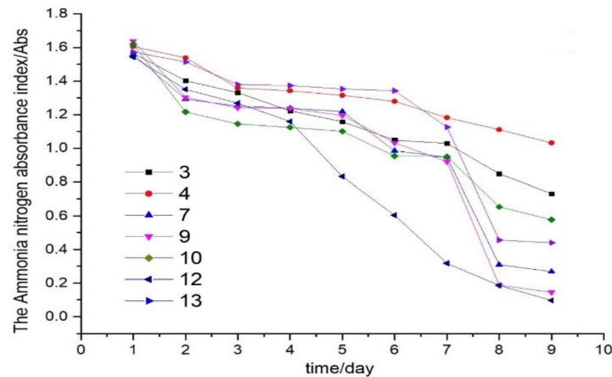



Figure 10: The Ammonia nitrogen absorbance index graph

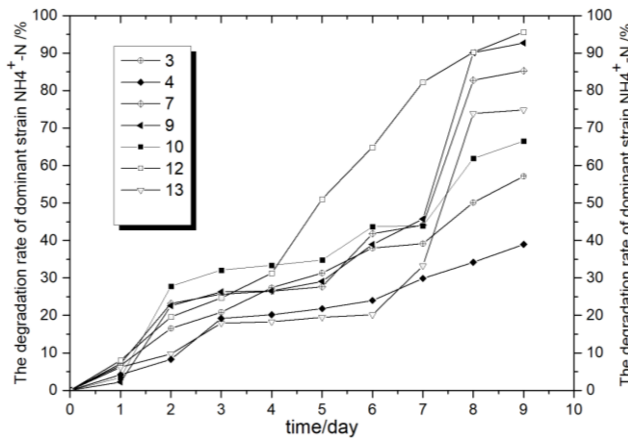



Figure 11: The degradation rate of dominant strain $NH_4^+$-N

## 4. Conclusions

(1) The growth curves of dominant strains 3, 4, 7, 9, 10, 12, 13 in the medium are shown in Figure 9. It can be seen from Figure 9 that 0~3h is the adaptation period of 7, 9, 10, 12, 13 strains, 3h After the strains began to enter the logarithmic growth phase, the number of strains 7 and 9 reached the maximum between 12～18h, and the number of strains reached the maximum when strains 10 reached 18h, and strains 12, 13 The strain reaches maximum at 18 h; Among them, 0~12h is the adaptation period of strain 3, and the strain begins to enter the logarithmic growth phase after 12h, and the number of strains reaches the maximum at 21h. 0~18h is the adaptation period of strain 4, and the logarithmic growth phase begins after 18h, and the number of strains reaches the maximum between 27～30h. In summary, in the subsequent ammonia nitrogen removal rate experiment, in order to ensure that all 7 strains have good activity and the largest number of strains, the largest bacterial solution at the maximum time of each bacterial solution $OD_{600}$ should be selected.

(2) As shown in Figure 10, the process of absorbance reduction directly represents the effect of degradation through the effect of the dominant strain on the diluted landfill leachate, and then the degradation rate of each strain is calculated through a series of unit shrinkage, formula application, and result calculation.The degradation rates of ammonia nitrogen of the seven dominant strains are shown in Figure 11. It can be seen from the figure that within 10 days, the ambient temperature is

30 ℃, the speed is 200r/min, and the inoculation amount is 10%, and the time of inoculation is the maximum $OD_{600}$ value of each strain. The ammonia nitrogen degradation rates of each bacterium were (12)95.62%> (9)92.72%> (7)85.26% > (13)74.84%（10）66.53%＞（3）57.14%＞（4）38.96%. Ammonia nitrogen degradation in the diluted landfill leachate up to 85% or more is strains 12, 9, 7; Degradation rates higher than 6 0% have strains 7, 13, 10; The degradation rate is less than 60%, and there are strains 5, 6. Therefore, in summary, the isolated strains selected in this paper have obvious degradation effects on the degradation index of ammonia nitrogen in the diluted landfill leachate, and the isolated strains can also be further studied, and the maximum degradation rate range of the strains can be found in the subsequent experimental parameter exploration. Therefore, it is applied to all kinds of sewage, sludge or other pollution, and also plays a certain auxiliary role in subsequent research and development.

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