# Screening of Highly-efficient Triethylamine-degrading Bacteria

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**Abstract** Triethylamine is used in the production process in many fields, and it will enter the environment and cause pollution in the process of use. As a pollutant, triethylamine will cause great harm to aquatic organisms and affect the development of aquaculture. It is very important to degrade triethylamine. Traditional degradation methods have the disadvantages of low efficiency and high cost. It is safer, more effective and cheaper to degrade triethylamine by microbial decomposition. In this study, a strain (*Microbacterium actinomyceterae* CC-VM-Y (T)) which can degrade triethylamine efficiently was successfully screened, providing an effective and economical method for degrading triethylamine and protecting aquaculture environment.

**Key words** Triethylamine; Strain; Screen

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Triethylamine is a kind of aliphatic amine. In the process of production and use, a lot of triethylamine enters the environment, causing pollution. It is a toxic substance to aquatic organisms such as fish, causing degeneration and necrosis of organs such as fish gill and liver easily, and it has a slight neurotoxic effect. Under the influence of triethylamine, the activities of S-GPT and cholinesterase in fish blood increase, hepatic glycogen becomes disordered and deposited, and alkaline phosphatase content increases. When triethylamine concentration is greater than 11 mg/L, the growth of goldfish is obviously affected[1]. Triethylamine will cause serious damage to aquaculture, and meanwhile, the accumulated toxicity will also cause harm to people who eat these aquatic organisms. Therefore, it is very important to degrade triethylamine.

Traditional pollutant treatment methods all have some defects, and can't completely remove pollutants. It is a safer, more effective and cheaper way to treat the increasing environmental pollutants by microbial decomposition. In this study, triethylamine-degrading bacteria in sewage were screened to obtain strains with strong triethylamine-degrading ability, hoping to help sewage treatment plants to purify sewage better and protect aquaculture from sewage.

## Materials and methods

### Experimental materials

Samples were taken from a sewage treatment plant in Tangshan City.

### Media

The used triethylamine inorganic-salt medium (g/L) was composed of NaCl 1.0, $K_2HPO_4$ 1.5, $KH_2PO_4$ 0.5, pH 7.0, and triethylamine hydrochloride was added as carbon source and nitrogen source at a desired concentration.

Triethylamine hydrochloride was prepared into a solution with triethylamine content of 10 000 mg/L using deionized water. The solution was filtered with an organic filter membrane (0.22 μm) and then added in proportion.

Trace element formula: Manganese sulfate monohydrate 1.69 g/L, cobalt chloride hexahydrate 0.24 g/L, boric acid 1.16 g/L, sodium molybdate dihydrate 0.024 g/L, ferrous sulfate heptahydrate 2.78 g/L, zinc sulfate heptahydrate 1.15 g/L, and copper sulfate pentahydrate 0.38 g/L.

Selecting culture medium: When culturing triethylamine-degrading bacteria, the addition concentration was 0.5 – 1.5 g/L to the triethylamine inorganic salt culture medium.

LB medium (g/L): Peptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH 7.0.

Finally, the concentration of triethylamine in the culture medium was determined to be 100 mg/L. There were reasons for choosing this concentration, the first of which was that according to the literature relevant to triethylamine, the samples were collected from activated sludge in industrial sewage treatment plants, which is consistent with the samples in this study, and the concentration of triethylamine in enrichment media in the literature was 100 mg/L. Second, after measuring the concentration of triethylamine in the collected samples, the concentration was about 50 mg/L, and when the concentration was selected as 100 mg/L, the strain would not stop growing due to too-high or too-low concentration.

### Enrichment and isolation of triethylamine-degrading bacteria

First, 5 g of activated sludge and 10 ml of wastewater were taken from a wastewater treatment tank of the sewage treatment plant and added into l00 ml of inorganic salt culture medium containing 100 mg/L triethylamine, respectively, and the samples were cultured at 28 ℃ and 180 r/min for 5 d. Next, 5 and 10 ml were, respectively, transferred into triethylamine inorganic salt

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culture medium with the same concentration, and the transfer operation was performed continuously for 3 times. The effective enrichment liquid (turbid culture solution) was diluted and coated on plates of inorganic salt culture medium containing 100 mg/L triethylamine, and cultured at a constant temperature of 28 ℃. Single colonies with different morphologies were selected and purified by streak inoculation, and then stored on a slant culture medium.

**Quantitative determination of triethylamine on isolated and purified strains**

The strains were inoculated into the screening liquid medium containing 100 mg/L triethylamine content, and cultured with shaking at 28 ℃ for 3 d. The triethylamine content in each fermentation broth was determined by bromophenol blue spectrophotometry according to national standard GB/T 14377-1993. The degradation rate was calculated for statistics. Through the observation in the process of screening bacteria and the determination of degradation rate, dominant degrading strains were determined.

Degradation rate = [(Control − Strain absorbance)/Control] ×100%

**Determination of triethylamine in water by bromophenol blue spectrophotometry**

**Determination range of triethylamine** The determination range of triethylamine was 0.5 – 3.5 mg/L. When the triethylamine content in water samples was greater than 3.5 mg/L, it could be determined by the method after dilution.

The determination by spectrophotometry was performed at 410 nm[13].

**Reagents** Chloroform; sodium carbonate; nitric acid solution: 10% (V/V); triethylamine: greater than 99%; bromophenol blue.

A 0.5 mol/L sodium carbonate solution was prepared. First, 53 g of sodium carbonate was weighed and dissolved in 500 ml of water. Next, the obtained solution was transferred into a 1 000 ml volumetric flask, diluted with water to constant volume, and shaken to mix well.

A bromophenol blue developer was prepared according to 0.1 g/100 ml. First, 0.1 g of bromophenol blue was weighed, and dissolved in 100 ml of chloroform, and the obtained solution was shaken to mix well. Next, insoluble substances in the solution were removed using a quantitative medium-speed filter paper, and the solution was stored in a brown bottle. It was stable at room temperature for at least one month.

A 1 500 mg/L triethylamine stock solution was prepared. First, 50 ml of water was added into a 100 ml volumetric flask, 0.15 g (0.2 ml, accurate to 0.000 1 g) of triethylamine was weighed with an injection syringe by the decrement method, and carefully poured into the volumetric flask, which was then gently shaken to fully dissolve triethylamine. The solution was diluted with water to constant volume and mixed evenly. The concentration of the prepared triethylamine stock solution was calculated, and it was refrigerated in a refrigerator. It could be stable for at least one month. It should be noted that the needle tip must be sealed with a rubber block during weighing to prevent leakage of triethylamine.

A 100 μg/ml triethylamine intermediate solution was prepared by diluting an appropriate amount of triethylamine stock solution with water.

After the preparation of the triethylamine intermediate solution, 5.00 ml was pipetted and diluted with water to 100 ml and shaken to mix well.

**Steps** First, 200 μl was pipetted from the blank control of triethylamine liquid culture medium and samples, respectively, and the obtained liquids were diluted to 10 ml with water. Next, 1 ml of sodium carbonate solution was added, and the solutions were shaken to mix well and then added with 10 ml of chloroform with a burette. The solutions were shaken for 1 min (about 150 times) while paying attention to releasing the gases. After standing and layering, clean degreased cotton was used to wipe away any trace moisture that might be present at the neck of the funnel. The initial filtrate was discarded, and the extract was then added into a clean container. Subsequently, 5 ml was accurately transferred into a 10 ml stoppered colorimetric tube with an undivided pipette, added with 0.5 ml of bromophenol blue indicator, and shaken to mix well. After standing at room temperature for 15 min, with chloroform as the reference solution, and the absorbance of each tube was measured at the wavelength of 410 nm using 3 cm cuvettes.

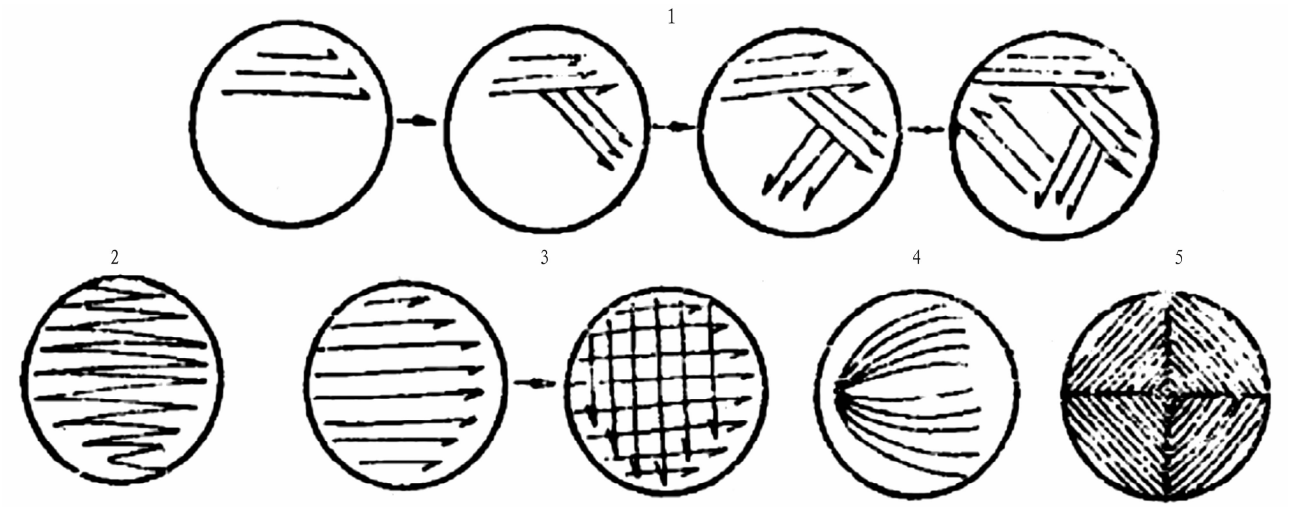
**Determination** Samples were determined within 24 h.

Each water sample containing suspended solids was filtered using a 0.45 μm filter membrane, and water samples were drawn from it.

A water sample (the triethylamine content was less than 30 μg, and the volume was not more than 10 ml) adjusted to a neutral state was drawn and added into a 60 ml separatory funnel. Next, water was added to 10 ml, and colorimetry was performed according to the same steps of making the standard curve. Next, the absorbance was determined, and the average absorbance of the blank solution was deducted to find corresponding triethylamine content from the standard curve[2].

**Secondary isolation and purification of dominant triethylamine-degrading bacteria**

The streak plate method was adopted to isolate triethylamine-degrading bacteria from test tubes in aseptic environment. A sterile inoculation ring was used to take a little culture and streak it on a plate. There are many streaking methods, and the common methods which can get single colonies easily are oblique line method, curve method, grid method, radiation method, four-grid method and so on (Fig. 1). When an inoculation ring moves backward on the surface of the culture medium, the bacterial liquid on the inoculation ring is gradually diluted, and finally single cells are scattered on the drawn line, and each cell grows into a colony after culture[3].




1. Oblique line method; 2. Curve method; 3. Grid method; 4. Radiation method; 5. Four-grid method.

**Fig. 1 Streak plate method**

**Parallel control experiment on dominant degrading strains**

A control experiment was carried out on triethylamine-degrading bacteria with a degradation rate over 50%. Three parallel controls were made for each strain, using ordinary inorganic medium and glucose-added medium, respectively, in order to detect whether the degradation efficiency of triethylamine-degrading bacteria is stable and efficient, and whether the presence of carbon and nitrogen sources other than triethylamine in the medium will affect the degradation efficiency of triethylamine-degrading bacteria. The following experimental steps were the same as the bromophenol blue spectrophotometry for the determination of triethylamine in water in "Determination of triethylamine in water by bromophenol blue spectrophotometry".

**Sequencing of 16S rDNA sequence for triethylamine-degrading strains**

In this experiment, the universal primers 27f[4] (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492r[5] (5'-GGT TAC CTT GTT ACG ACT T-3') were used to amplify the 16S rDNA of triethylamine-degrading bacteria with stable and high degradation efficiency.

**PCR system** The PCR system was 50 μl in volume, containing 10 × PCR buffer (TianGen) 5 μl, dNTP (2.5mM) (TianGen) 4 μl, 27f (10 μM) 2 μl, 1492r (10 μM) 2μl, rTaq (5U/μl) (TianGen) 0.4 μl, Templet DNA 10 – 100 ng, and $DdH_2O$ 36.6 μl.

**PCR program** The reaction procedure of PCR was started with pre-denaturation at 94 ℃ for 4 min, followed by 30 cycles of denaturing at 94 ℃ for 45 s, annealing at 54 ℃ for 45 s and extending at 72 ℃ for 45 s, and finally completed with extending for 10 min at 72 ℃. The PCR products were detected by 1.0% agarose gel electrophoresis.

## Results and Analysis

**Enrichment and isolation results of triethylamine-degrading bacteria**

After the enrichment and isolation of triethylamine-degrading bacteria in our experimental materials, we obtained 81 strains after bacteria selection and purification. The strain number was named SHN according to the activated sludge.

**Standard curve of triethylamine**

The 100 mg/L triethylamine medium was diluted to the concentrations of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.5 mg/L, respectively. The triethylamine standard curve obtained by quantitative determination was shown in Fig. 2.

**Re-screening results of triethylamine-degrading bacteria**

Three strains, SHN 6-1, SHN 6-2 and SHN 6-3, were found to have a degradation rate of more than 60% through re-screening.

Degradation rate = [(Control – Strain absorbance)/Control] × 100%

Because there were many strains to be tested, 81 strains were divided into four batches for testing, and the testing time of each batch was close to one week. The results of all quantitative determination are attached below.

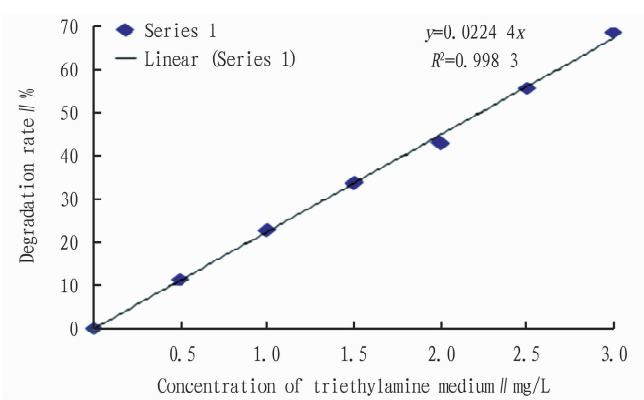



**Fig. 2 Standard curve of triethylamine**

The degradation rates of triethylamine in the first batch were recorded for 3 d. Among all the strains, SHN6-2 showed a degradation rate of more than 50%, and the degradation rates of remaining strains were all below 10%. The degradation rate of SHN6-2

reached 54.487% on the first day, 64.026% on the second day and 71.157% on the third day. The degradation rate increased with the continuous growth of the strain.

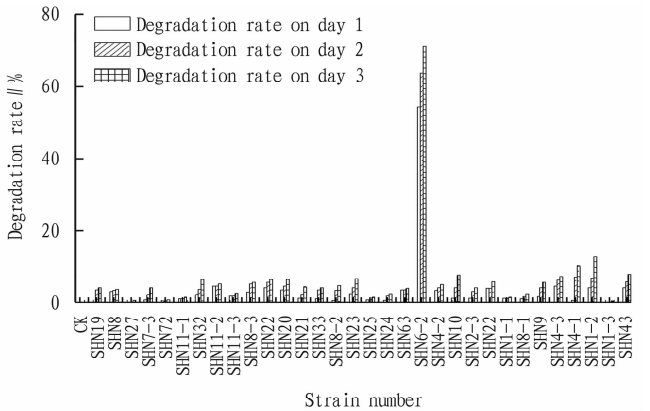



**Fig. 3 Triethylamine degradation results of the first batch of strains**

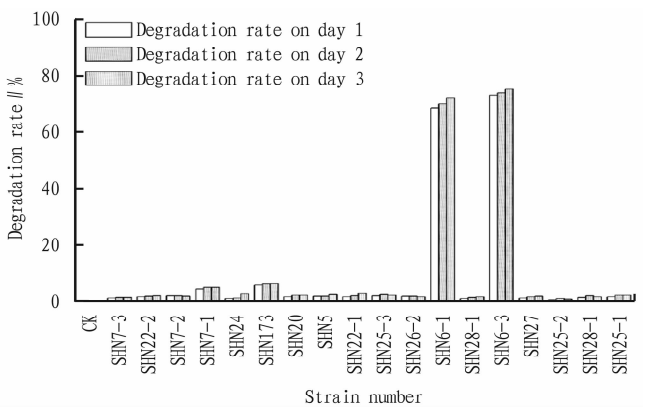



**Fig. 4 Triethylamine degradation results of the second batch of strains**

The second batch of strains was also determined for 3 d. Among them, only two strains, namely SHN6-1 and SHN6-3, exhibited degradation rates of over 50%, both of which were as high as 68%. The degradation rates of other strains were below 10%. The degradation rates of these two strains increased gradually in the three days, and there was little difference.

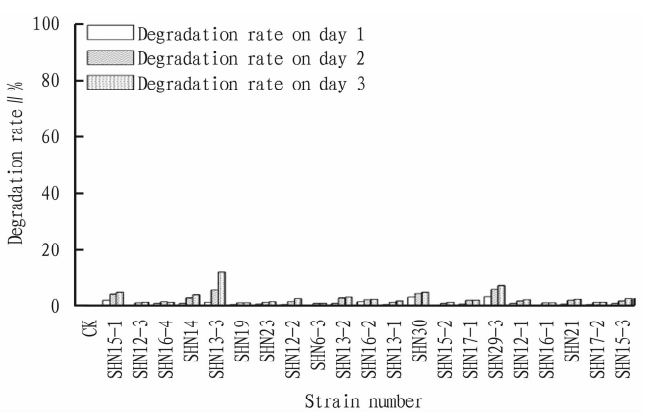



**Fig. 5 Triethylamine degradation results of the third batch of strains**

The third batch of strains was determined for 3 d. Among them, no strain showed a degradation rate over 50%. Only one strain of SHN13-3 exhibited a degradation rate of 12.076% on the third day, and the values of remaining strains were all below 10%.

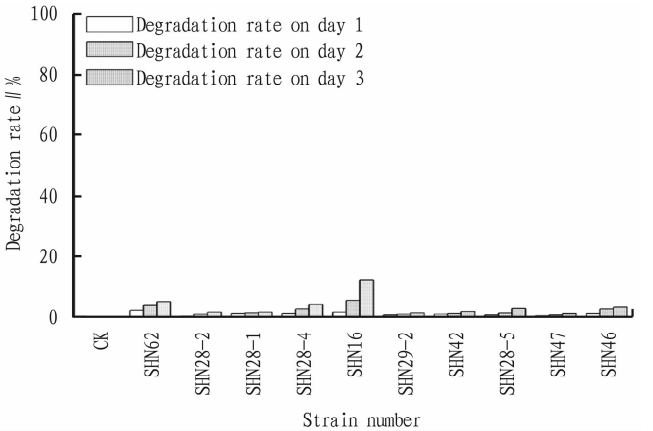



**Fig. 6 Triethylamine degradation results of the fourth batch of strains**

The three-day determination of the fourth batch of strains showed that the number of strains with a degradation rate over 50% was 0. The degradation rates of all strains were below 10%.

To sum up, through the comprehensive analysis of these four batches of experimental data, we found that three triethylamine-degrading strains were dominant, namely, SHN6-1, SHN6-2 and SHN6-3. Therefore, we purified and isolated these three strains again, and a comparative experiment was done to determine that these three strains are efficient and stable triethylamine-degrading bacteria.

**Table 1 Control experiment on triethylamine-degrading bacteria with high degradation rates**

| Strain | Inorganic culture medium | Degradation rate // % | Glucose-added medium | Degradation rate // % |
|---|---|---|---|---|
| 0 | 0.478 | | 0.476 | |
| SHN6-1-1 | 0.156 | 67.364 | 0.152 | 68.067 |
| SHN6-1-2 | 0.185 | 61.297 | 0.146 | 69.328 |
| SHN6-1-3 | 0.126 | 73.640 | 0.102 | 78.571 |
| SHN6-2-1 | 0.138 | 71.130 | 0.096 | 79.832 |
| SHN6-2-2 | 0.126 | 73.640 | 0.078 | 83.613 |
| SHN6-2-3 | 0.196 | 58.996 | 0.089 | 81.303 |
| SHN6-3-1 | 0.122 | 74.477 | 0.161 | 66.176 |
| SHN6-3-2 | 0.126 | 73.640 | 0.172 | 63.866 |
| SHN6-3-3 | 0.122 | 74.477 | 0.167 | 64.916 |

The control experiment was carried out on triethylamine-degrading bacteria with a degradation rate over 50%, *i.e.*, SHN6-1, SHN6-2 and SHN6-3. Three parallel controls were made on each strain. The strains were cultured using two kinds of culture media, one of which was inorganic culture medium, and the other was glucose-added culture medium. The purpose was to examine whether glucose as a carbon source affects the degradation efficiency of the strain to triethylamine. The results showed that there was little difference in the degradation rate between the inorganic culture

medium and glucose-added culture medium, and the reason why the data of the latter was slightly larger than that of the former might be that the strains in glucose-added culture solution grew better, so the degradation rate was better.

Efficient and stable triethylamine-degrading strains SHN6-1, SHN6-2 and SHN6-3 were identified, and these three strains had also been isolated and purified. Then, the 16S rDNA fragments of these three strains were amplified by PCR and sequenced. Through PCR sequencing, it was determined that these three strains of bacteria were all of the same species, named *Microbacterium arthrospirae* CC-VM-Y (T).

## Conclusions and Discussion

In this study, sludge and water samples were taken from a sewage treatment plant in Tangshan City. Triethylamine-degrading bacteria were primarily screened and re-screened. Through the enrichment and isolation of triethylamine-degrading bacteria, the degradation rate of triethylamine in water was determined by bromophenol blue spectrophotometry, and three strains with high degradation rates were selected, namely, SHN6-1, SHN6-2, and SHN6-3. The three strains were amplified by PCR and sequenced. Finally, they were found to be the same strain, named *M. arthroporaea* CC-VM-Y (T).

Triethylamine is a typical organic small molecular substance which is degradation-resistant in wastewater, and there is little research on its degrading microorganisms. This study aimed to screen new strains that can degrade triethylamine efficiently, so as to help sewage treatment plants purify water better and reduce the effects of harmful substances in sewage to the surrounding environment and the development of aquaculture. Microbial resources are huge and charming. We should make use of modern advanced science and technology to make microbial resources better serve aquaculture and all mankind. Further exploration and discovery are needed for other uses of microbial resources by future generations.

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