The Culture of Salt-Tolerant Strains and its Degradation Performance of High-salt organic Wastewater

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Abstract. High-salt wastewater refers to the wastewater containing more than 1% of total salinity in domestic wastewater and industrial wastewater. The wastewater contains relatively high levels of inorganic ions such as Cl-, SO42-, Na+, and Ca2+, which will inhibit the growth of microorganisms, making it one of the extremely difficult to treat wastewater. In this study, the known saline-alkali soil was used as a sample source, and halophilic strains were screened out using shaking, culture, and domestication methods. The morphological characteristics were observed by light microscopy. Microscopic examination showed that the strain was a milky white colony, round or oval shape, smooth and moist, convex lens surface, moderate area, opaque, bulging in the middle, edge is not spread, the same color on both sides. The effects of salt concentration, culture time, initial organic wastewater concentration, and strain size on the degradation performance of the selected strains were studied. The experimental results show that: when the salt concentration of wastewater is 20%, the initial organic wastewater concentration is 2000mg L-1, the added culture volume is 20 ml, the culture time is 72 hours, the COD removal rate of wastewater can reach 66.4%.

Keywords: High salt wastewater, Salt-tolerant Strains, Degradation performance, COD.

1. Introduction

High-salt wastewater refers to the wastewater containing more than 1% of total salinity in domestic wastewater and industrial wastewater [1]. The wastewater contains relatively high levels of inorganic ions such as Cl-, SO42-, Na+, and Ca2+, also, some short carbon chain organic compounds such as glycerinum [2]. Variety of ions cause high concentration of salt since the complexity of high-salt wastewater, which will restrict the growth of the microbiology. The high-salt wastewater, therefore, becomes one of the crucial pollutants which are relatively difficult to deal with (manage the wastewater by using microbiology is regarded as one of an economical ways). Based on relevant information, the ratio of high-salt wastewater among the total amount of wastewater in China has reached up to 5%, with the annual increasing rate up to 2% [3]. Thus, harmlessly managing the high-salt wastewater plays a
pivotal role within the system of wastewater treatment, also, it is a tough problem to be faced. Therefore, properly management towards high-salt wastewater is not only necessary, but also urgent.

Halophiles refers to the microbiology exist in the high-salt environment. The categories of Halophiles can be split into Extreme Halophiles and Facultative Halophiles according to its capability to adapt the salt. Extreme Halophiles is able to live and breed in the environment whose concentration of NaCl nearly reach the saturation (36%); while the Facultative Halophiles, also known as halotolerant bacteria, is able to grow and breed under the salt or without salt environment. The capability of the tolerance of the salt of such bacteria is 20% for the maximum [9], [10]. Halophiles, usually lives in the high salt environment such as ocean, salt lake, saline-alkali land and base-salt pond, therefore plays a positive role during the process of the high-salt wastewater treatment, since its growth highly relies on the high concentration of salt [11].

This thought was firstly applied by Woolard et al. from Alaska Anchorage University [4]. They proved the feasibility of Halophiles while treating the high-salt wastewater by unfolding the experiment. They firstly extracted the Halophiles from the saltlake by concentration gradient method; then utilized such bacteria to deal with the phenolic wastewater whose concentration is 1% to 15%.

While doing the research about the 35g/L salt wastewater, R.Y.Tokuz et al. found that the concentration of the salt in the wastewater only has tiny influence towards the activated sludge treatment process, since either the SS of outcome water or the rate of oxygen absorption does not have big change [5]. In the study on the application of activated sludge treatment system to treat salt-containing wastewater, M.F.Oda et al. found that the growth of microorganisms was not inhibited in the high-salt environment, on the contrary, it promoted the growth of some halophilic bacteria, increased the concentration of reactive microorganisms, reduced the organic load, and improved the flocculability of the sludge [6]. Kapdan et al. fitted the yield coefficient under different conditions using the stovy-Kincannon model and used halophile H. Lacusroei to degrade the simulated wastewater and measure its residual COD value [7]. Murugavelh et al. degraded high-salt wastewater by using the free cells of Halomonas sp [8]. The results showed that the degradation efficiency was 98.6% and 94.4%, respectively, when the initial concentration of wastewater was 10 and 30mg/L. Even when NaCl concentration increases to 20g L⁻¹, the system can still function normally.

Hilophiles can advance the high-salt wastewater’s restriction function to the bacteria and dramatically improve the degradation of organic compounds. The wastewater with high concentration of salt can be directly degraded by Hilophiles without dilution and pre-option. Moreover, the rate of its extraction is higher compared with the previous halotolerant bacteria. The Hilophiles, therefore, is widely utilized to deal with the biology degradation problem in the high-salt wastewater.

Based on traditional biological methods to handle the high-salt wastewater or obvious inhibition under high-salt waste water, in soil sample collected as the research object, proposed the methods of shock, cultivation and domestication halophilic strains screened out, and by using two different initial screening culture medium (both salt environment) to cultivation of strain, in order to deepen the cognition of halophilic bacteria growth environment. At the same time, the conditions such as simulated salinity of wastewater, culture time of halophile bacteria, initial concentration of simulated wastewater, and volume of inoculated strain were changed, and the optimal treatment environment of the strain was determined in conjunction with the measurement of OD600, removal rate and residual COD, so as to provide method reference and data support for the application of halophile strain in the future.

2. Experiment

2.1. Sample Source and Treatment

There are five kinds of samples, one taken from the back hill of North University of China, marked A, one taken from Longtan Park in Jianaoping District, marked B, and the other three taken from Xiaodian District, Taiyuan City, marked C, D and E respectively.

The prepared soil samples were taken 20g and ground, respectively (to facilitate the suspension during inoculation). Next, 80 ml of distilled water was added to the conical flask, which was placed on
a shaker for shaking at 25 °C, 140 r min⁻¹ for 15 h, so that the bacteria in the soil could be fully dissolved in the distilled water.

2.2. Medium Preparation
Primary screening medium 1 contained NaCl 10 g L⁻¹, glucose 20.0 g L⁻¹, peptone 2.5 g L⁻¹, ammonium chloride (NH₄Cl) 1.0 g L⁻¹, K₂HPO₄ 0.5 g L⁻¹, FeSO₄ 0.01 g L⁻¹, CaCl₂ 0.01 g L⁻¹ and agar 20 g L⁻¹. Distilled water was added to a volume of 1 L. The pH was adjusted to 7.0. The medium was then sterilized with high temperature steam at 121 °C for 20 min.

Primary screening medium 2 was prepared by changing inorganic salts based on the primary screening medium 1 with NaCl 10 g L⁻¹ replaced with MgSO₄ 10 g L⁻¹, and the rest were the same as medium 1.

Re-screening medium was prepared by removing the agar under the condition of initial screening medium to make liquid medium.

2.3. Degradation Characteristics of Salt-Tolerant Strains
The simulated wastewater was prepared. Briefly, 2 g L⁻¹ monosodium glutamate, disodium hydrogen phosphate (Na₂HPO₄) and NH₄Cl (N:P = 2:1) were added to prepare 1 L solution according to the concentration and ratio, which was then dispensed into 5 Erlenmeyer flasks with 100 ml each, and the same amount was taken as raw water to measure the CODCr value.

The influences of different salt concentration, cultivation time, initial organic wastewater concentration and strain volume on the degradation performance of the selected strains were studied.

2.4. Determination Method of CODCr
The potassium dichromate method was used to detect CODCr, and the CODCr was measured according to the GB11914-1989 standard.

2.5. Determination of the Salt-Tolerant Strain Growth Characterization
In this experiment, the turbidimetric method was used to determine the growth curve of the target microorganism. According to direct ratio of the bacterial content to the turbidity of the bacterial suspension, the concentration of the bacterial suspension—the content of the bacteria—could be reflected by the optical density of the measured bacterial suspension. In the experiment, the OD₆₀₀ value was measured and recorded every six hours using an ultraviolet spectrophotometer. By plotting the change curve of the OD₆₀₀ value with time, the growth curve of bacteria under different medium conditions was obtained.

3. Isolation, Purification and Preliminary Identification of Salt-tolerant Strain

3.1. Enrichment, Separation and Purification of Salt-Tolerant Strain
The sample soil was sieved, weighed 10 g and put into a 250 ml Erlenmeyer flask, and then 90 ml of distilled water was added, followed by shaking in a shaker at 120 r min⁻¹ and 35°C for 48 h. On the ultraclean workbench, 1 ml of the upper bacterial suspension was taken using a pipette gun under sterile condition, put into another sterilized test tube containing 9 ml of distilled water, and diluted (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷), followed by shaking and mixing.

In the experiment, the acclimation of microorganisms refers to gradually increase the concentration of inorganic salts during the cultivation process, so that the halophilic bacteria gradually adapt to this breeding environment, so as to acclimate a purer halophilic bacteria community. Namely, after the microorganism enrichment, the colonies on the plate were seeded into the liquid medium (re-screening medium) (the medium with a salt gradient concentration of 1%, 5%, 10%, 15% and 20% was prepared again), respectively for acclimation to make the acclimated colonies contained only halophilic bacteria to achieve the purpose of purification [12].
3.2. Observation of Colonies and Cells of Strains
Gram staining was used for morphological identification of strains. The crystal violet solution was used as the staining agent. Safrane was used as the counterstaining agent, 95% ethanol was used as the decolorizing agent, and Lugo’s iodine solution was used as the dyeing aid. The staining results were observed under a biological microscope.

3.3. Growth Characteristics of Strains
All liquid medium placed in the incubator during the acclimation process were taken out, and a spectrophotometer was used to measure the OD600 of the bacterial solution. The data is shown in Table 1.

| Salt concentration/% | NaCl 1 | NaCl 2 | MgSO4 1 | MgSO4 2 |
|----------------------|--------|--------|---------|---------|
| 1                    | 1.360  | 0.802  | 1.448   | 1.287   |
| 5                    | 1.480  | 0.322  | 1.562   | 1.334   |
| 10                   | 0.199  | 0.203  | 1.546   | 0.304   |
| 15                   | 0.044  | 0.071  | 0.530   | 0.151   |
| 20                   | 0.051  | 0.071  | 0.430   | 0.115   |

Higher value of OD600 indicated more bacterial species in the bacterial solution. Lower value of OD600 suggested less bacterial species in the bacterial solution. When the OD600 value was between 0.6-0.8, it was in logarithmic phase of growth. As shown in Table 1, when the inorganic salt concentration was between 1% and 5%, as the salinity increased, except the bacteria 2 in the primary screening medium 1, other samples showed increased OD600 value by about 8%. When the inorganic salt concentration was greater than 5%, the OD600 value gradually decreased with the most obvious period being 5% to 10%. The reason for this result was that increased salinity killed the bacteria in the bacterial solution, while the halophilic bacteria survived with a small overall number.

3.4. Morphological Characteristics of Salt-Tolerant Strains
It can be seen from Figure 1 that the colony morphological characteristics of the experimental strains were milky white in color, round or oval in shape, smooth and moist, convex lens-shaped surface, moderate area, opaque, middle-raised, uniform edges without spreading, and same color of the front and the back side. Observation showed that the cultivated strain was spherical and the population density was large.

![Figure 1. Morphology of Salt-Tolerant Strains](image)

Bacterial solution cultured for a period of time was smeared, and directly examined with a low magnification and a high magnification. Gram-stained bacteria were observed under a microscope. Gram-positive bacteria (G+) were purple, and Gram-negative bacteria (G-) were not easy to stain and were red. Positive bacteria are recorded as "+" and negative bacteria as ".". It can be seen from Figure 2 that the acclimated product of this experiment was Gram-positive bacteria.
4. Degradation Characteristic of Salt-tolerant Strains

4.1. Effect of Salt Concentration on the Degradation Performance of Salt-Tolerant Strains

Based on the measurement results of the OD<sub>600</sub> of the bacterial liquid in the liquid culture medium, 15% MgSO<sub>4</sub>·7H<sub>2</sub>O was selected for the salinity experiment. MgSO<sub>4</sub>·7H<sub>2</sub>O was added to 5 Erlenmeyer flasks with a salt gradient concentration of 5%, 10%, 15%, 20% and 25% respectively. The selected bacterial solution (10 ml) was seeded into the above 5 bottles of simulated wastewater, respectively, which was then placed in a 25°C incubator for 48 h, and the COD value was measured respectively. Figure 3 shows the degradation performance of salt-tolerant strains on simulated wastewater at different salt concentrations.

![Figure 2. Morphology of Salt-Tolerant Strains under microscope](image)

![Figure 3. Effect of inorganic salt concentrations on residual COD and COD removal rate](image)
It can be seen from Figure 3 that when the inorganic salt concentration was between 5% and 10%, the simulated wastewater COD removal rate was 49.2% and 50.8%, respectively, and when the inorganic salt concentration increased to 15% and 20%, the COD removal rate dropped to 47.6%, which was only 3.2% lower than that in 10% salt concentration. When the inorganic salt concentration continued to increase to 25%, the COD removal rate dropped rapidly to 35%. In other words, when the inorganic salt concentration was less than 20%, the COD removal rate of wastewater could reach more than 47%, indicating that within this salt concentration range, halophilic bacteria could grow normally, and part of carbon in the organism was synthesized by halophilic bacteria into their own organisms, and part of carbon was decomposed into carbon dioxide and water.

In addition, in the experiment, it was observed that the suspended matters in the water in the Erlenmeyer flask with an inorganic salt concentration of 25% after 48 h of reaction were significantly reduced compared with those in other salt concentrations, showing that high inorganic salt concentration had obvious inhibition effect on microorganisms, which hindered the growth of microorganisms and even caused the death of some microorganisms, thereby reducing the processing efficiency.

After a period of acclimation, the selected microorganisms gradually adapted to the high-salt environment and formed a microbial population with salt-tolerant bacteria as the dominant strain. During the cultivation process, it could be preliminary judged that the composition of microorganisms in the sludge and the types of microorganisms have changed significantly. All things considered, the inorganic salt concentration of 20% was selected as the appropriate salt concentration.

### 4.2. Effect of Time on the Degradation Performance of Salt-Tolerant Strains

As above, 2000 mg L⁻¹ of organic wastewater was prepared, added with 10 ml of bacteria, and placed in a constant temperature shaking instrument at 25°C for cultivation. Sample (5ml) was taken at a 12h interval for 6 times. Figure 4 shows the degradation performance of salt-tolerant strains on organic wastewater at different cultivation times.

![Figure 4. Effect of culture time on residual COD and COD removal rate](image-url)

It can be seen from Figure 4 that the removal rate increased with the increase of the incubation time, and the residual value of COD decreased with the increase of the cultivation time. In terms of removal rate, the removal rate in 72h cultivation was 35.96% higher than that in 12h cultivation; in terms of the residual COD value, it was 1.84 times lower at 72 h than that at 12 h. It was believed that due to the increase of the incubation time, the number of autologous reproductions of the strain increased; or the strain adapted to the simulated wastewater environment and was more active, so the removal rate was increasing. Therefore, in the next experiment, the strain culture was carried out for 72 h.
In this experiment, it was found that some of the supernatants in the simulated wastewater were relatively turbid at the beginning. The reason for this phenomenon may due to the increase of the load of salt concentration, and the decomposition of some microbial cells that were not suitable for high-salt environments with cell wall dissolution (the phenomenon of "cytolysis"), thereby causing turbid supernatant.

4.3. Effect of Initial Organic Wastewater Concentration on the Degradation Performance of Salt-Tolerant Strains

Five groups of different organic wastewaters (2000 mg L\(^{-1}\), 1000 mg L\(^{-1}\), 500 mg L\(^{-1}\), 400 mg L\(^{-1}\) and 200 mg L\(^{-1}\)) were prepared, and 10 ml strains were added respectively. The mixture was then placed in a biochemical incubator for 72 h. Figure 5 shows the degradation performance of salt-tolerant strains on organic wastewater at different initial concentrations of simulated wastewater.

![Figure 5. Effect of initial organic wastewater concentration on COD removal rate](image)

It can be seen from Figure 5 that when the initial concentration was between 200-400 mg/l, the removal rate increased, indicating that as the initial concentration increased, the binding sites of organic matter between the halophilic bacteria and the simulated wastewater increased, thereby improving the operating efficiency of halophilic bacteria and increasing the removal rate. When the initial concentration was between 400-1000 mg/l, the removal rate decreased, that was, in this state, the initial concentration increased, but the efficiency of the bacterial species remained in the previous state. When the initial concentration was between 1000-2000 mg L\(^{-1}\), the removal rate increased, indicating that the reaction efficiency of halophilic bacteria at a concentration of 2000 mg L\(^{-1}\) had higher efficiency.

4.4. Effect of Strain Volume on the Degradation Performance of Salt-Tolerant Strains

Organic wastewater (100 ml) was prepared in a 250 ml Erlenmeyer flask, and 5 ml, 10 ml, 15 ml, 20 ml and 25 ml strains were added respectively and incubated for 72 h. Figure 6 shows the degradation performance of selected halophilic bacteria on organic wastewater under different strain volume.
As can be seen from Figure 6, the COD removal rate and the residual COD value gradually performed better as the strain volume increased. As the strain volume gradually increased, the residual COD in the simulated wastewater gradually decreased and the COD removal rate gradually increased. But the removal rate at first rapidly increased and then gradually slowed down. When the strain volume was increased from 5 ml to 10 ml, the removal rate increased by 36.51%, the COD value decreased by 1045.12 mg L⁻¹, and the COD removal rate increased significantly. When the amount of bacteria increased from 20 ml to 25 ml, the maximum removal rate of COD was 66.4%, only an increase of 1.33%. On the one hand, increasing the amount of bacteria increased the microorganisms that degrade organic matter. Under the condition of the same amount of organic matter in the wastewater, the organic matter in the wastewater increased with the increase of the amount of strains. On the other hand, the continuously increased amount of bacteria caused increased competition between them, and thus some microorganisms could not get enough oxygen or nutrients, showing inhibited normal growth of the strain. In addition, in the microscopic observation, it could be found that with the increase of the strain volume of the used bacteria, the density of salt ions around each bacteria was decreasing, which caused this phenomenon. All things considered, the most suitable dosage of the volume of strain was 20 ml.

5. Conclusions
In this paper, the halophilic bacteria were cultured through enrichment, separation, purification and other processes. The effects of salt concentration, cultivation time, initial organic wastewater concentration, and the number of bacterial species on the degradation performance of halophilic bacteria were studied. The survival adaptability of the 1# bacteria is greater than that of the 2# bacteria. The MgSO₄ initial screening medium is more conducive to the growth of strains. The strains obtained from the experiment and domestication are Gram-positive bacteria. When the wastewater salt concentration is 20%, the initial organic wastewater concentration is 2000 mg L⁻¹, and the added bacterial volume is 20 ml, the longer the cultivation time is 72 h, the screened halophilic bacteria have relatively better treatment results for simulated wastewater. Wastewater COD The removal rate can reach 66.4%. It provides data support and theoretical basis for the large-scale application of halophilic microorganism treatment wastewater.

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