

# Degradation of N-methylpyrrolidone in High Salinity Wastewater by a Halotolerant Microorganism

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## Abstract

N-methylpyrrolidone (NMP), a nitrogen-containing heterocyclic compound, is widely used in lithium batteries and the refining of lubricants. However, it is also very harmful to human health and the environment. Although NMP is biodegradable, industrial high salinity wastewater can stop microorganisms from growing. To effectively degrade NMP in high salinity wastewater, a halotolerant strain CCZU-X was isolated from sea shrimps. The strain was identified as *Staphylococcus lentus* through morphology observation and 16S rDNA sequencing. The effects of processing conditions such as salt concentration, pH, and temperature on degradation of high salinity NMP-containing wastewater were investigated using single-factor experiments. Quantitative analysis of degradation efficiency of NMP was conducted by high-performance liquid chromatography. The optimal conditions for CCZU-X to degrade NMP in high salinity wastewater were determined to be at pH 7.0 and 35 °C, and the maximum salt tolerance was 25%. Under optimal conditions (pH 7.0, 35 °C, 1% salt, 2000 mg/L NMP), the NMP degradation efficiency of CCZU-X reached 93%. This strain can effectively degrade NMP in high salinity NMP-containing wastewater, thus can be potentially used in industrial applications.

**Keywords:** Halotolerant bacteria, Degradation, N-methylpyrrolidone

## 1 Introduction

N-methylpyrrolidone (NMP) is a colorless and transparent aprotic liquid with a slight ammonia odor. NMP is miscible with water and common solvents like ethanol, acetaldehyde, ketones, and aromatic hydrocarbons (Bhandari et al., 2019). Because of its unique physiochemical properties, NMP has been used as an organic solvent in various fields, such as electrode auxiliary materials for batteries (Liu et al., 2019), solvents for pharmaceutical production, and cleaning agents for semiconductor production facilities and circuit boards (Hilton-Proctor et al., 2019). However, NMP harms health and the environment. NMP

has a lethal concentration (LC<sub>50</sub>) of more than 5100 mg/m<sup>3</sup> for acute inhalation for rats and mice. The range of acute oral lethal dose (LD<sub>50</sub>) ranges from 3605 to 7725 mg/KG body weight (Ansell & Fowler, 1988), and the range of acute percutaneous LD<sub>50</sub> is between 5000-7000 mg/KG body weight (Poet & Crbader, 2010). In addition, NMP is toxic to reproduction. The Globally Harmonized Classification and Labeling System of Chemicals issued by the United Nations lists the acute toxicity of NMPs as Category 4 (Fine & Mullin, 2017). Therefore, it is necessary to treat the residual NMP in industrial production waste liquids. Akesson's research showed that NMP is very stable thus not easily degradable by chemical methods (Akesson & Paulsson, 1997), but according to Casi, it is prone to biodegradation under aerobic conditions (Shu et al., 2014). Thus, biodegradation methods can be really effective at degrading NMP in wastewater.

Existing NMP wastewater treatment often use physical and chemical methods (Loh et al., 2018). These methods are complicated and costly, and requires a series of specific equipment (Chen & Xu, 2010). And little research covered NMP treatment in high salinity wastewater. In this study, a highly efficient halotolerant strain CCZU-X was isolated from sea shrimps and was identified by observation and 16S rDNA sequencing. Its salt tolerance and degradation characteristics were studied. Test were done in high salinity wastewater to simulate the conditions in practical applications.

## **2 Materials and Methods**

### *2.1 Materials*

The strain was found on the surface and inside of spoiled sea shrimps (very high salinity).

### *2.2 Main Equipments*

The main instruments used in this study included a THZ-072HZ constant temperature shaker (Shanghai Gaming Biotechnology Co., Ltd.), an SW-CJ ultra-clean bench (Suzhou Antai Air Technology Co., Ltd., Sujing Group), a PHS-3C (08) laboratory pH meter (Shanghai Yindian Scientific Instrument Co., Ltd), and an API bacteria identification instrument (Merrier Diagnostic Products (Shanghai) Co., Ltd).

### *2.3 Media*

Luria-Bertani (LB) liquid media were prepared from peptone (1%), yeast powder (0.5%), and sodium chloride (added as needed in the gradient of 1%, 5%, 10%, 15%, 20%, 25%).

LB solid media were composed of peptone (1%), yeast powder (0.5%), sodium chloride (1%), and agar (1.5-2%).

Simulated wastewater consisted of glucose (10 g/L), sodium chloride (20 g/L), calcium chloride (40 g/L), and NMP (2 g/L).

### *2.4 Screening of the Halotolerant Strain*

The edible sea shrimps was cut into small pieces and weighed (5 g). The samples were poured into 50 mL of the sterilized LB medium with 1% salt and incubated in a constant

temperature shaker at 37 °C and 180 rpm for 12 hours. 1 mL of the bacteria culture was transferred to a 5% salt medium with a pipette. This procedure was repeated until the culture was transferred to a 25% salt medium. Then a certain amount of the culture solution was coated on the solid LB medium with different dilution ratio and incubated at 37 °C for 24 hours. Single colonies with rounded edges and uniform color were inoculated into LB liquid media and incubated for 24 hours. The culture solution was coated on the solid LB medium and single colonies were inoculated into LB liquid media again. This procedure was repeated three times.

### *2.5 16S rDNA Sequence Analysis*

The 16S rDNA sequence was determined by Biotech Bioengineering (Shanghai) Co., Ltd. The measured sequences were compared with the 16S rDNA sequences in GeneBank for homology analysis.

### *2.6 Determination of Growth Conditions of the Strain*

#### *2.6.1 Determination of Salt Tolerance of the Strain*

The dominant strains were inoculated into standard LB media and cultivated overnight. The culture was inoculated (2%) into LB media with salt concentration 1%, 5%, 10%, 15%, 20%, and 25% respectively, and cultivated under 37 °C and 180 rpm. Samples were taken every 2 hours and OD<sub>600</sub> was determined with a spectrophotometer. The results are the average of 3 parallel experiments.

#### *2.6.2 Effect of Initial NMP Concentration on the Growth of the Strain*

The dominant strains were inoculated into standard LB media and cultivated overnight. The culture was inoculated (2%) into LB media with NMP concentration 0, 500, 1000, 1500, and 2000 mg/L respectively, and cultivated under 37 °C and 180 rpm. Samples were taken 24 hours later and OD<sub>600</sub> was determined with a spectrophotometer. The results are the average of 3 parallel experiments.

### *2.7 Degradation Characteristics of the Strain*

To study the degradation characteristics of the strain, three factors that may affect the NMP degradation ability of the strain were selected to optimize: initial salt concentration, degradation temperature, and initial pH.

The purified strain was inoculated into the LB liquid media and collected in centrifuge pellets after 12 hours of cultivating. To study the effect of salt concentrations on the NMP degradation efficiency of the strains, the precipitated strain was introduced into simulated wastewater with 1%, 5%, 10%, 15%, 20%, and 25% salt. The samples were incubated in a shaker at 180 rpm and 37 °C. As of the effect of temperature on the NMP degradation efficiency, the precipitated strain was introduced into the simulated wastewater, and cultured in a shaker at 25, 30, 35, 40 and 45 °C and 180 rpm. To investigate the effect of initial pH on the NMP degradation efficiency, the precipitated strain was introduced into simulated wastewater with pH 5, 6, 7, 8, and 9. The samples were incubated in a shaker at 180 rpm.

From all experimental groups, samples were collected every 12 hours for determination of NMP content. The results were the average of 3 parallel experiments.

### 2.8 Analytical Method

Determination of NMP concentration: High-performance liquid chromatography (HPLC) was used to prepare standard curves using standard solutions at different concentrations, and the corresponding NMP concentrations were calculated according to the standard curves.

$$\text{NMP degradation rate} = \frac{\text{Concentration}_{\text{before}} - \text{Concentration}_{\text{after}}}{\text{Concentration}_{\text{before}}} \cdot 100\%$$

Chromatographic conditions were as follows: C18 column, 4.6  $\mu\text{m} \times 250$  mm, 5  $\mu\text{m}$ ; mobile phase: methanol: water = (30:70) and flow rate: 1.0 mL/min; column temperature: 30 °C; detection wavelength: 210 nm; injection volume: 20  $\mu\text{L}$ .

## 3. Results

### 3.1 Isolation and Purification of the Strain

After isolation and purification, a strain of halotolerant bacteria that can tolerate high salt concentration (25%) was obtained from the shrimps and named CCZU-X. After the strain was cultured on LB medium for 24 hours at 37 °C, it formed milky white colored single colonies. These small round colonies are moist and have neat edges. The cells are Gram-positive and have a spherical shape.

### 3.2 16S rDNA Sequence Phylogenetic Tree

The 16S rDNA sequence analysis of CCZU-X was performed, and the measured gene sequence was compared with the 16S rDNA sequence reported in Genbank for homology. A phylogenetic tree was constructed using MEGA5.1. Results showed that the 16S rDNA sequence of CCZU-X was similar to *Staphylococcus lentus* (Figure 1). Considering its colony morphology, this strain was initially named as *S. lentus* CCZU-X, and its phylogenetic tree is shown in Figure 1.

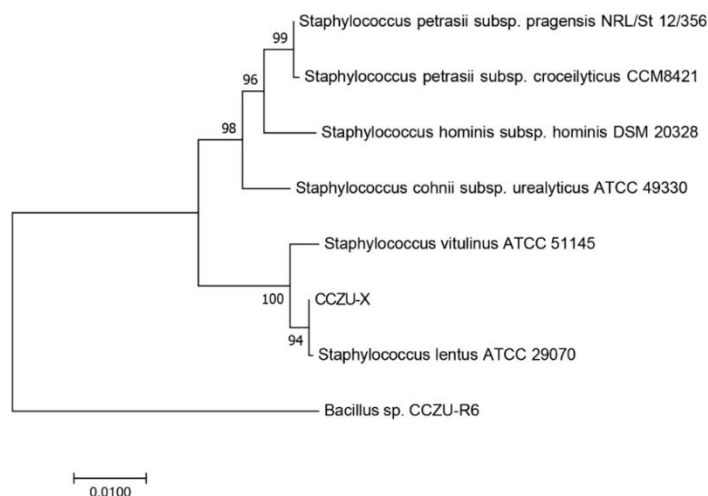


Figure 1. Phylogenetic tree of CCZU-X

### 3.3 Salt Tolerance of the Strain

The growth of the strain in the LB medium with different salt concentrations is shown in Figure 2. The growth rate and size of the colony decreased with the increase of salt concentration in the media, indicating the strain is halotolerant instead of halophilic. When salt concentration reached 25%, the growth of the strain was slow, as shown by insignificant OD<sub>600</sub> values; but when it was coated on plates, single colonies with good growth status appeared. When the salt concentration was between 15% and 20%, the growth rate and size of the colony were reduced compared to the case of low salt concentration, but the growth was not stagnated, and the strain can still adapt to the saline environment. Compared to general halotolerant strains, CCZU-X performed well at salt tolerance, with a maximum salt tolerance of 25%.

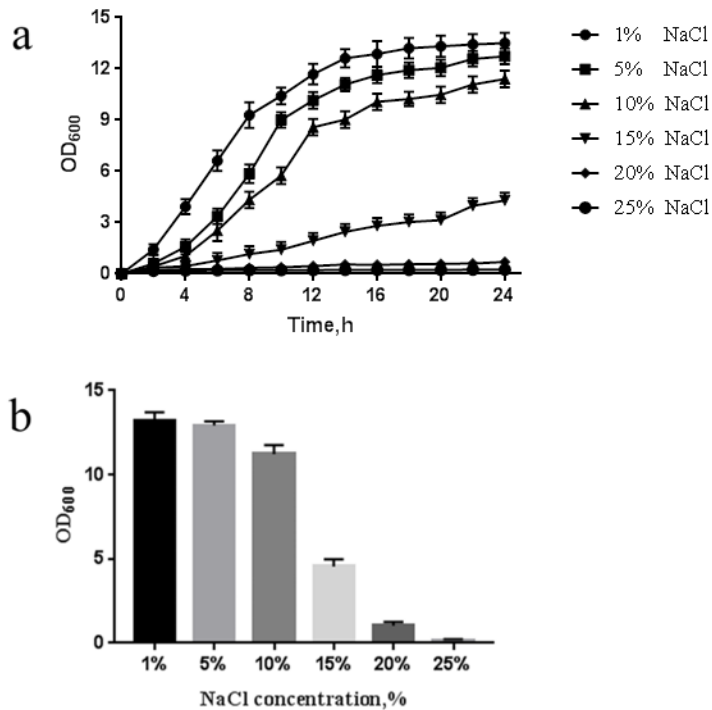


Figure 2. CCZU-X growth in LB medium with different salt concentrations:(a) Density of CCZU-X under different salt concentrations within 24 hours. (b) Final density of CCZU-X under different salt concentrations after 24 hours

### 3.4 Effect of Initial NMP Concentration on the Growth of the Strain

NMP has negative effects on the growth of microorganisms, hence, the effect of initial NMP concentration on the strain growth should be studied. The growth of the strain under different NMP concentrations are shown in Figure 3. With increasing NMP concentration in the medium, the strain growth decreased, but it managed to develop under an NMP concentration of 2000 mg/L. This indicates that CCZU-X is a viable option for biodegradation of NMP in common industrial wastewater.

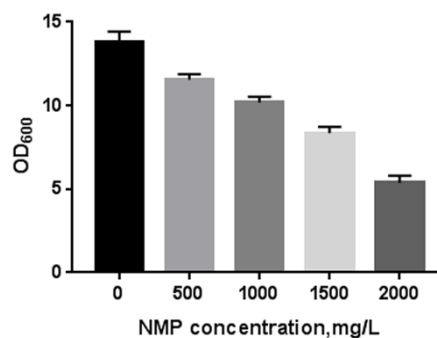


Figure 3. 72hours density of CCZU-X under different initial NMP concentrations

### 3.5 NMP Degradation Characteristics of the Strain

#### 3.5.1 Effect of Initial Salt Concentrations on NMP Degradation

Initial salt concentration affects the growth and thus the NMP degradation efficiency of the strain. NMP degradation of the strain under different initial salt concentrations is shown in Figure 4. As salt concentration increase, NMP degradation was reduced. After 72 hours, NMP degradation of CCZU-X reached 92.1% under 1% salt, and 3.8% under 25% salt. In common NMP-containing wastewater, salt concentration is between 2% and 5%. At 5% salt, CCZU-X can degrade 75.1% of the NMP in 3 days. This implies that CCZU-X can effectively degrade NMP under common high salinity conditions.

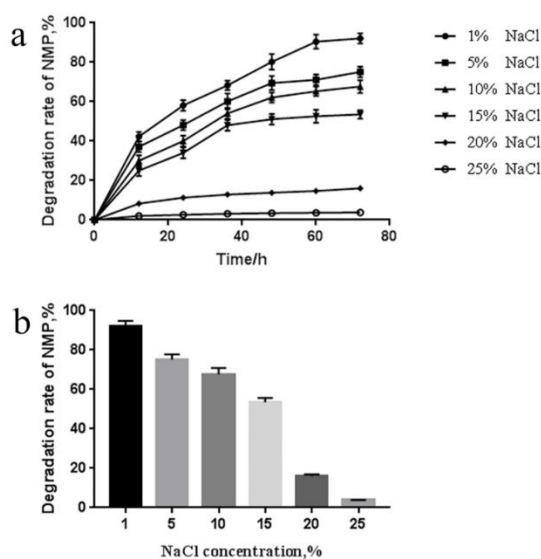


Figure 4. NMP degradation under different initial salt concentrations:(a) Percentage of NMP degraded at different salt concentrations within 72 hours. (b) Final percentage of NMP degraded under different salt concentrations after 72 hours

#### 3.5.2 Effect of Temperature on NMP Degradation

Temperature affects the growth and degradation characteristics of microorganisms. Enzyme activity maximizes at certain temperature, and only at the appropriate temperature should the microorganism be utilized(Chookietwattana et al., 2012). NMP degradation of the stain under different temperatures is shown in Figure 5. NMP degradation maximized at 93.0% under 35°C.

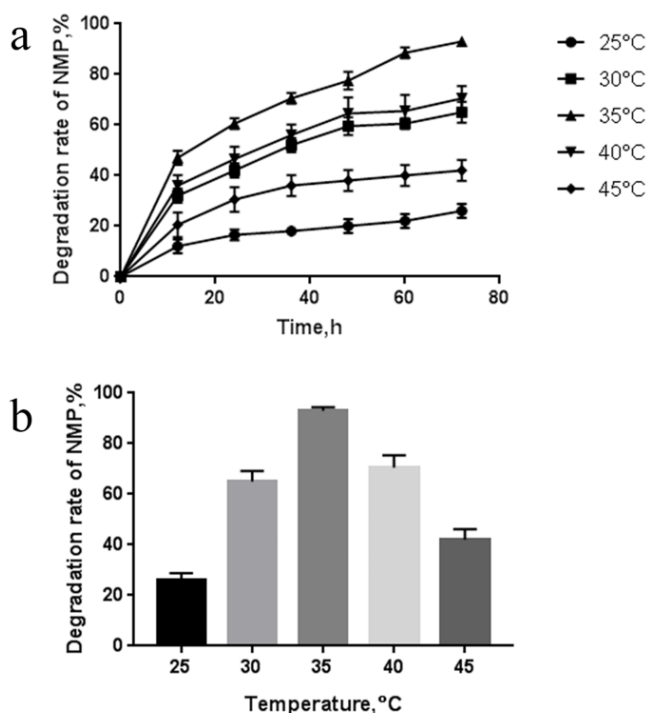


Figure 5. NMP degradation of CCZU-X at different temperatures:(a) Percentage of NMP degraded at different temperatures within 72 hours. (b) Final percentage of NMP degraded at different temperatures after 72 hours

### 3.5.3 Effect of Initial pH on NMP Degradation

Initial pH affects the growth and metabolism of the strain. The pH directly affects the charge of cell membranes of microorganisms and changes the ionization of organic matter in the medium (Chookietwattana et al., 2012). Figure 6 shows the NMP degradation of CCZU-X at 35 °C, 180 rpm and different initial pH within 72 hours in a simulated wastewater containing 2000 mg/L NMP. The optimal pH for NMP degradation is 7.0, at which the average NMP degradation rate reached 90.5% (Figure 6). This result is consistent with the effect of pH on the growth of the strain and indicates that neutral condition is optimal for utilization of strain CCZU-X.



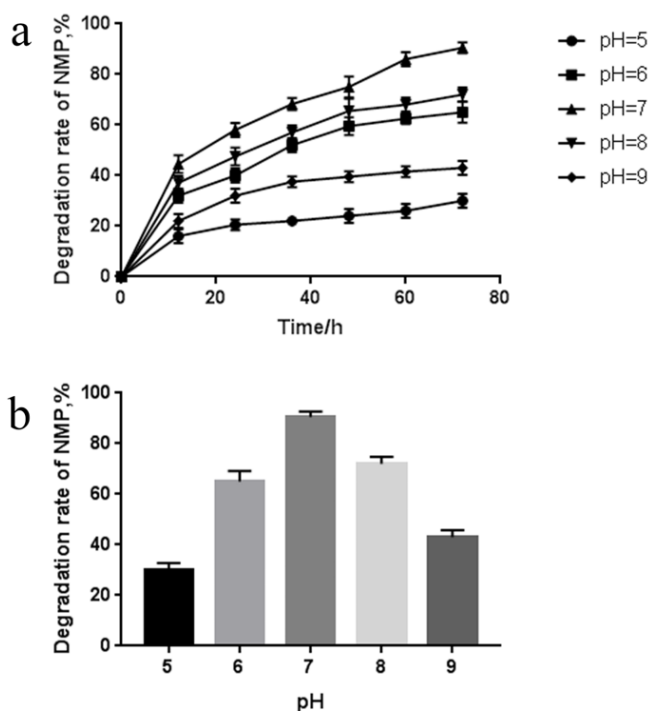


Figure 6. NMP Degradation of CCZU-X at different pH:(a) Percentage of NMP degraded at different pH within 72 hours. (b) Final percentage of NMP degraded at different pH after 72 hours

#### 4. Discussion

NMP is a nitrogen-containing heterocyclic compound widely used in the chemical industry, but it is also a major pollutant. It is very important to remove NMP from industrial wastewater. In previous reports, microorganisms have been used to degrade nitrogen-containing heterocyclic pollutants(Kaiser et al., 1996), however, in high salinity conditions, few known microorganisms can effectively degrade NMP due to high osmotic pressure. In this study, a strain that can tolerate a salt concentration of 25% and degrade 93% (under 1% salt) of the 2000 mg/L NMP present in simulated wastewater was found. Based its morphology and 16S rDNA gene sequence, it was named as *S. lentus* CCZU-X. The optimal conditions for NMP degradation in wastewater by CCZU-X were identified to be at pH 7.0 and 35 °C.

CCZU-X can tolerate high salt concentration and effectively degrade NMP. It is a good candidate for studying the mechanism of NMP degradation and can be potentially used in the treatment of NMP in industrial wastewater.

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## Conflict of Interest

The authors declare no conflict of interest.

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