ISSN 1001-0742
CN 11-2629/X

# JES

# JOURNAL OF ENVIRONMENTAL SCIENCES

June 1, 2014 Volume 26 Number 6
www.jesc.ac.cn

## Special issue: Sustainable water management for green infrastructure

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Science Press

Sponsored by
Research Center for Eco-Environmental Sciences
Chinese Academy of Sciences

ISSN 1001–0742 **Journal of Environmental Sciences** Vol. 26 No. 6 2014

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## Special Issue: Sustainable water management for green infrastructure



Serial parameter: CN 11-2629/X*1989*m*162*en*P*23*2014-6

Journal of Environmental Sciences 26 (2014) 1369–1374

Available online at www.sciencedirect.com

Journal of Environmental Sciences

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# Characterization of a salt-tolerant bacterium *Bacillus* sp. from a membrane bioreactor for saline wastewater treatment

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ARTICLE INFO

Article history:

*Keywords:*
salt-torlerant bacterium
saline wastewater
bacteria
*Bacillus*
16S rRNA
DOI: 10.1016/S1001-0742(13)60613-0

ABSTRACT

High salt concentrations can cause plasmolysis and loss of activity of cells, but the salt-torlerant bacterium can endure the high salt concentrations in wastewater. In this research 7 salt-torlerant bacteria, which could survive in dry powder products and could degrade organic contaminants in saline wastewater, were isolated from a membrane bioreactor. The strain NY6 which showed the fastest growth rate, best property for organic matter degradation and could survive in dry powder more than 3 months was selected and characterized. It was classified as *Bacillus aerius* based on the analysis of the morphological and physiological properties as well as the 16S rRNA sequence and Neigh bor-joining tree. The strain NY6 could survive in the salinity up to 6% and the optimal growth salinity is 2%; it belongs to a slightly halophilic bacterium. The capability of its dry powder products for COD removal was 800 mg COD/(g·day) in synthesized saline wastewater with salinity of 2%. According to salt-tolerant mechanism research, when the salinity was below 2%, the stain NY6 absorbed $K^+$ and $Na^+$ to maintain osmotic equilibrium, and when the salinity was above 2%, the NY6 kept its life by producing a large amount of spores.

## Introduction

Saline wastewaters are generated by some industries and domestic activities. Especially, the city, which is located on coastal areas, can produce much more saline wastewater. In these cities the seawater is used for cooling and toilet-flushing, even in some industries (Artiga et al., 2008). Moreover, certain industrial sectors, such as agro-food, petroleum and leather industries, also generate highly saline wastewater (Lefebvre and Moletta, 2006). With increasing amount of saline wastewater, the interest in the saline effluent treatment processes, both for salt and organic matter removal, has been increasing rapidly. Several kinds of measurements such as physico-chemical means and biological processes can be applied to treat saline effluents. But physico-chemical techniques are more energy-consuming, and their start-up and running costs are high. Therefore, the saline wastewater is often treated by biological processes employing large amounts of microorganisms. But the activities of microorganisms, e.g. bacteria are usually affected by high salt concentration, which can lead to the low COD removal efficiency and bulking of the activated sludge. Because high salinity (> 1%) can cause plasmolysis and make the cells lose activity (Jiang et al., 2013), which is a challenge for the traditional biological treatment.

In order to solve this problem, the salt-tolerant microorganisms are employed in the wastewater treatment. The salt-tolerant microorganisms are metabolically different and can adapt to extreme salinity; these microorganisms are good candidates for the bioremediation of hypersaline environments and treatment of saline effluents. According to the extent of their halotolerant characteristics, the salt-tollerant microorganisms are categorized as slightly,

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moderately and extremely halophiles (Ventosa et al., 1998; Ventosa, 2006; Deorsola, 2013). Numbers of salt-torlerant bacteria being used in treating saline wastewater have been reported. Kargi and Dincer (2000) found that the use of halophilic microorganisms in an activated sludge process resulted in a better treatment performances at salt contents above 2%. Cuadros-Orellana et al. (2006) found the *Halobacteriaceae* had the ability to degrade *p*-hydroxybenzoic acid under hypersaline conditions.

In recent years, a large amount of bio-products of microorganisms for special use have been produced in the market. These microorganisms can usually survive in the dry powder for a long time and maintain at a relative high concentration of activated cells. While few studies have focused on the special salt-tolerant bacteria, which can survive in dry powders and have strong ability to degrade organic matter in saline wastewater. The objective of this study is to characterize and identify some salt-tolerant bacteria and can degrade organic contaminants from a membrane bioreactor for saline wastewater treatment on a ship. Their phenotypic characteristics, phylogenetic affiliation, and salt-tolerance mechanism were also studied.

## 1 Materials and methods

### 1.1 Chemicals and media

Chemicals used in this study were of analytic grade. The salt medium contained: $NH_4Cl$ (10 g/L); $KH_2PO_4$ (2.2 g/L); sucrose (25 g/L); peptone (10 g/L); agar powder (20 g/L); beef extract (6 g/L); NaCl (20–120 g/L); pH (5.0–9.0). The salinity wastewater contained: sucrose (0.3 g/L); $NH_4Cl$ (0.1 g/L); $KH_2PO_4$ (0.022 g/L); NaCl (20–120 g/L); pH (5.5–8.0). The solution pH was adjusted with $NaHCO_3$ and HCl.

### 1.2 Quality analysis of wastewater and activated sludge

Chemical oxygen demand (COD) was analyzed with $K_2CrO_4$ as a oxidizer. High dose of $HgSO_4$ (at the mole ratio of $HgSO_4$:$Cl^-$ = 10:1) was added to the samples to eradicate the chloride interference.

Activated sludge was used as a starting material for the isolation of pure cultures. The activated sludge was collected from the aeration tank of a membrane bioreactor operating for saline wastewater treatment for more than 12 mon on a ship. The dry sample of activated sludge was obtained by drying at 40°C in a oven until the weight of the sample reach a steady value.

### 1.3 Domestication and isolation of bacteria

Enrichment cultures were obtained using salt medium with the addition of the dry powder. After 48 hr culturing at shaking speed of 130 r/min and 37°C, high concentration of bacteria could be found. Inoculate (1 mL) from the $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$ dilutions of microcosm sediments were transferred with pipette onto the salt medium agar surfaces of the Petri dishes and incubated for 24 hr at 37°C. After 24 hr, different morphological colonies with a good growth on a surface salt medium agar plate were isolated and the streaked plate method was used to purify colonies. These bacterial colonies were aseptically removed from the mixed-culture plates and grown in salt medium agar.

### 1.4 Characteristics of the isolated bacterial strains

The strains were characterized by morphological and biochemical tests as described previously (Gerhardt et al., 2007; Mata et al., 2002). To determine the optimum temperature, pH, salinity and carbon:nitrogen:phosphor (C:N:P) weight ratio for the growth of the strains, the cultures were incubated in the temperature range from 20 to 50°C, pH range from 6 to 8, salinity range from 0 to 6% and C:N:P ratios of 100:10:1, 100:5:1, 100:2:1 and 100:1:1. pH value was adjusted by $KH_2PO_4$ and NaOH. After shaking at 130 r/min and 37°C for 24 hr, bacterial growth was measured as optical density at 600 nm using a spectro-photometer. The survival rate of dry powder was determined according to double dilution method.

### 1.5 Spores dying and calculation

Firstly, bacteria are placed on a slide andheat fixed. The slide is then suspended over a water bath with some sort of porus paper over it, so that the slide issteamed. Then Malachite Green is applied to the slide, which can penetrate the tough walls of the spores, staining them green. After 5 min, the slide is removed from the steam, and the paper towel is removed. After cooling, the slide is rinsed with water for 30 sec. The slide is then stained with diluted Safranin for 2 min, which stains most other bacterial bodies red or pink. The slide is then rinsed again, and blotted dry withbibulous paper. After the staining, the spores present blue-green color, while the bacterial bodies show pink color.

The spores and bacterial bodies can be counted on the slides by the microscope from the color differences. The spore rate can be calculated by the number of spores divided by the sum of the number of spores and bacterial bodies.

### 1.6 Strain identification

The strain's 16S ribosomal RNA (16S rRNA) sequence was identified. Genomic DNA of the isolate was extracted with a DNA extraction kit according to the manufacturer's recommended procedure. The extracted DNA was stored at –20°C until further processed.

The 16S rRNA was amplified with conserved primer (forward prime 5-AGAGTTTGATYMTGGCTCAG-3′ reversed primer, 5′TACGGHTACCTTACGACT-3′) as reported earlier (Selvakumaran et al., 2008). The reaction mixture was prepared to a total volume of 100 μL con-

taining 10 μL buffer, forward primer 3 μL, reversed primer 3 μL, dNTP 8 μL, Taq polymerase 0.5 μL, template 1 μL, dd$H_2O$ 74.5 μL. The reaction mixture was incubated in a thermal cycler at 94°C for 4 min for initial denaturation, followed by 30 cycles of 1.5 min at 94°C, 1 min at 55°C and 1.5 min at 72°C, and a final extension at 72°C for 10 min. PCR products were purified using PCR production purification kit, and were sent to Harbin SettlebioTechnologies Co. Ltd. (China) for sequencing. The sequence of 16S rRNA was submitted to the National Center for Biotechnology Information. The sequence was compared with 16S rRNA gene sequences available in the GenBank database. Distances were calculated and clustering was performed with the neighbor-joining method (Saitou and Nei, 1987).

To ascertain the phylogenetic affiliation of the novel strains, the almost-complete 16S rRNA gene sequences of the isolate was aligned with related species of the genus *Bacillus* using CLUSTAL W (Thompson et al., 1994).

### 1.7 COD removal experiments

Cells were cultured in 300 mL Erlenmeyer flasks containing 150 mL of wastewater after sterilized with 2% NaCl. Then the cells were isolated from the flasks with centrifugation method and made them into dry powder at 40°C.

The medium was inoculated with the dry powder and incubated aerobically at 37°C on a rotary shaker (130 r/min) for 1 day. The COD of influent and effluent were measured by standard methods, and the ability of the dry powder sample for COD removal was calculated according to the amount of the addition of sample and the amount of COD removal during 1 day.

### 1.8 Salt-tolerance mechanism

The stain NY6 was cultured in 300 mL Erlenmeyer flasks containing 150 mL of wastewater with different salinities of 0.5%, 2%, 4%, 6%, and incubated aerobically at 37°C on a rotary shaker (130 r/min) for 3 day. Then a part of bacterial sludge was collected by centrifuge (4000 r/min, 5 min), and was washed in buffer for 3 times. The bacterial sludge finally re-suspended in sterile deionized water. Plate the bacterial suspension before and after a heat treatment (60°C for 15 min), and calculate the spore rate of NY6 at different salinities. The other part of bacterial sludge was made by dry powder. The dry powder was digested with high concentrated $HNO_3$ (98%) for 4 hr. Then the suspension was collected with centrifuge (12000 r/min, 30 min) , and $K^+$, $Na^+$, $Ca^{2+}$, $Mg^{2+}$ in the suspension were measured by inductively coupled plasma method.

## 2 Results and discussion

### 2.1 Selection of the bacteria with strongest ability for salt tolerant

As a result, seven salt-torlerant bacteria (NY1–NY7) were obtained. All of them showed certain adaptability to survive in different salinity from 0 to 6% (**Fig. 1a**). NY6 has stronger salt-torlerant ability compared to others. It can survive in the salinity up to 6% with the optimal salinity 2%. It should belongs to the slightly halophilic bacteria (Le et al., 2009; Asad et al., 2007). In this work, the activited sludge was sampled from a membrane bioreactor for saline wastewater treatment on a ship. The salinity of the wastewater is in the range from 1% to 3%. It can be understandable that the strains can adapt to the salinity of 2%. It can also be seen that NY6 presents the fastest velocity of growth among the 7 strains (**Fig. 1b**). Its lag phase was very short, indicating that bacteria could adapt themselves to growth conditions quickly. The exponential phase was 48 hr. After 48 hr the strain was being stationary phase. In addition, under optimum conditions (pH 7, time 24 hr, salinity 2%), the degradation efficiency of COD for NY1–NY7 were 20.2%, 15.9%, 14.6%, 52.5%, 53.8%,

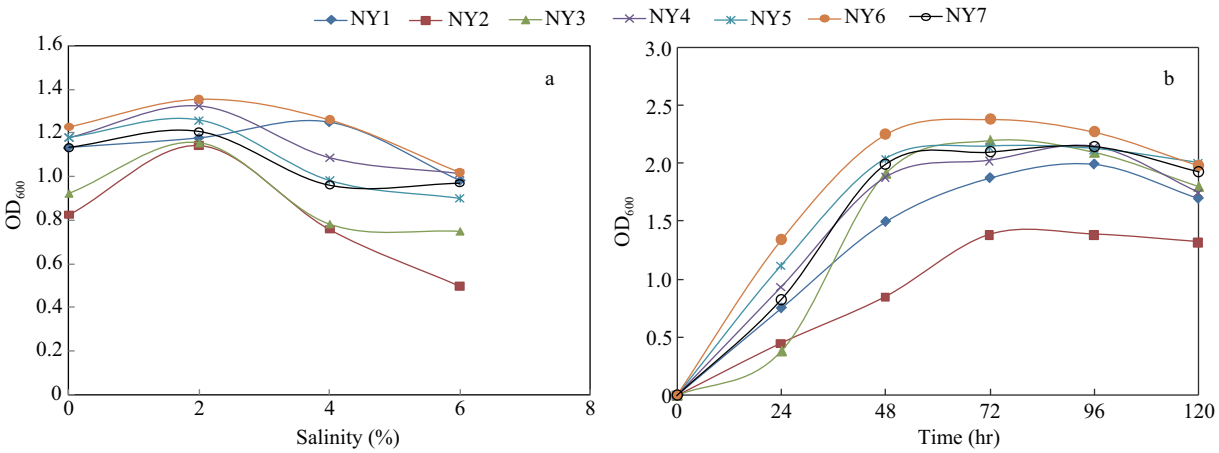


**Fig. 1** Effect of salinity on the growth of seven stains (a) and the growth curve of strains with salinity 2% (b) at pH 7 for 24 hr.

56.0% and 33.5%, respectively. NY6 shows the highest value. The above results show that NY6 is a good candidate bacterium with stronger ability for salt-tolerant and COD degradation.

### 2.2 Biochemical characterization of the salt tolerant strain NY6

NY6 shows positive results for gram staining, spore staining, gelatinase, catalase reaction, gelaune liquefaction (**Table 1**). In contrast, it shows negative results for the urease test and arginine dihydrolase test, and its colony appears ivory white, irregular on the edge, fold and raised on the surface (**Fig. 2**). According to phenotypic characterization of the strain and in comparison to other study (Tuo et al., 2012) the strain was tentatively identified as a kind of bacillus bacteria. These results can premilinary explain the ability of salt-tolerant ability of the NY6. It is known that bacillus bacteria are a kind of bacteria which have the abilities for the moisture retention, organic matter degradation and odder removal. It is also reported that bacillus bacteria can keep activity in dry powders with spores producing (Agarwal et al., 2010). In this research, it is noticed that even in the dry powder after 3 months, NY6 can still keep living. Many researches have reported the application of special bacillus bacteria in wastewater treatment and medical therapy (Zhang et al., 2013). One of the character of bacllus is that they can survive in many aggressive environment and can be made into multi-bioproducts. It can be predicted that the salt-tolerant bacillus can show potential application value for saline wastewater treatment. Therefore, NY6 was furhter indentified with 16S rRNA and its environmental factors and salt-tolerant mechanism will be discussed in the following sections.

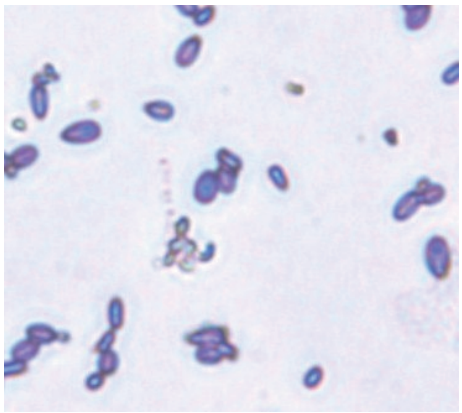

**Fig. 2** Spores dying of the strain NY6. The bacteria cell present pink color, while the spores show blue-green color.

**Table 1** Identification and biochemical tests of NY6

| Characteristic | NY6 | Characteristic | NY6 |
|---|---|---|---|
| Gram staining | + | Sucrose | + |
| Spore staining | + | Starch | + |
| Gelatinase | + | Arginine dihydrolase | – |
| Catalase reaction | + | $CH_3COONa$ | + |
| Urease | – | Polyethylene glycol | + |
| Citrate utilization | + | D-Sorbitol | + |
| Xylitol | + | 6% NaCl | + |

### 2.3 Strain identification with 16S rRNA sequence

The 16S rRNA gene was amplified from genomic DNA, purified and sequenced as described earlier (Shivaji et al., 2000), and the 16S rRNA fragment for NY6 was sequenced and submitted to the National Center for Biotechnology Information for BLAST analysis. Alignment of the strain indicated that the partial 16S rRNA sequence of NY6 is 99% identical to *Bacillus* sp. strains, and all the closest strains belonged to Bacillus. Thus, we designated NY6 as *Bacillus* sp. The phylogenetic position of the strain NY6 was constructed from evolutionary distance values by the neighbor joining method as shown in **Fig. 3**. Phylogenetic tree indicated that strain had the closest relation ship with *Bacillus aerius*. Shivaji et al. (2006) found *Bacillus aerius* in air samples, which was collecting at altitudes of 24 km, and it could tolerates up to 11.6% NaCl and resistant to UV radiation.

### 2.4 Effect of environmental factors on the growth of strain NY6

The effects of pH, temperture, C:N:P ratio and type of carbon source on the NY6 growth were investigated. As shown in **Fig. 4**, the biomass concentration of strain NY6 increased with increasing pH, reached the highest at pH 6, and then decreased with further increase in pH. From 20°C to 37°C, the number of bacteria increases to the maximum, and then began to decrease. Therefore, it can be concluded that the optimal temperature for strain NY6 is 37°C. The optimal ratio of C:N:P for strain NY6 is 100:5:1. The strain NY6 could grow with carbon sources including glucose, sucrose and $CH_3COONa$, and glucose is the optimal carbon source. The surviving spores and COD removal ability were further tested under optimal conditions (pH 6.0, temperature 37°C, carbon source glucose, C:P:N 100:5:1 and salinity 2%). The surviving spores of dry powder was $1.972 \times 10^8$ cfu/g. The COD removal

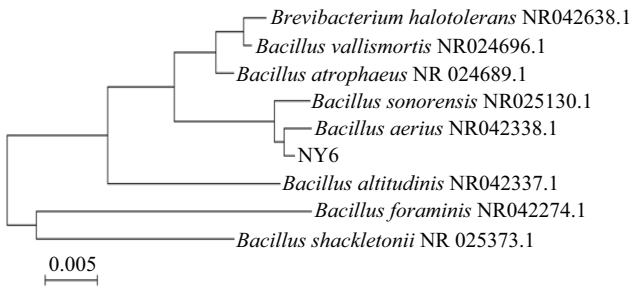


**Fig. 3** Neighbor-joining tree showing the position of strain HSA6 to a selected number of members of bacteria.

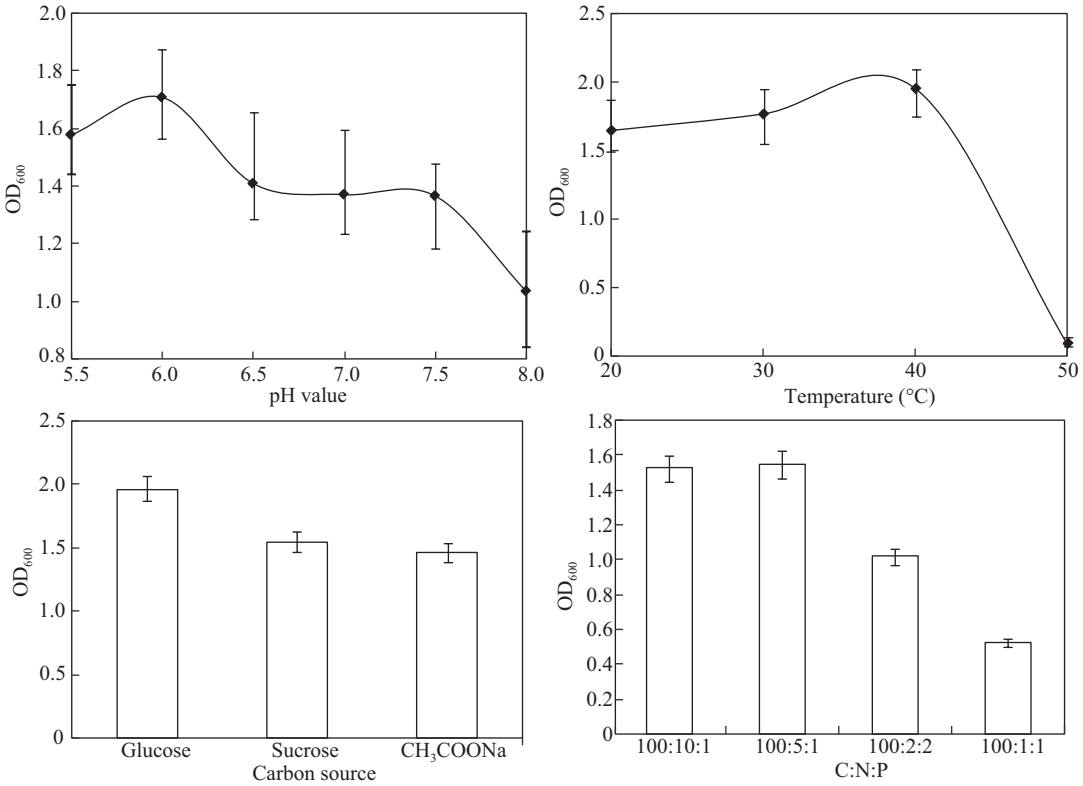


**Fig. 4** Effect of pH, temperature, C:N:P ratio and carbon sources on the growth of strain NY6. Experimental condition: pH 7, 24 hr, salinity 2%.

ability of dry NY6 powder was 800 mg COD/(g·day).

### 2.5 Salt-tolerance mechanism

The spore rate of stain NY6 in different salinities 0.5%, 2%, 4%, 6% is shown in **Fig. 5**. The spores in salinity 0.5% have similar number to that in salinity 2%, and then with salinity increasing, the number of spores began to increase. It seems when the salinity is over 2%, the bacillus cells will produce large amount of spores to keep survival. But when the salinity is below 2%, it is shown that the there is little difference of the spores producing.

The $Ca^{2+}$ and $Mg^{2+}$ concentration in the bacillus body decreases little by little as salinity rises from 0.5% to 6% (**Fig. 5**). While for $K^+$ concentration increases to the maximum with increasing salinity from 0.5% to 2%, and

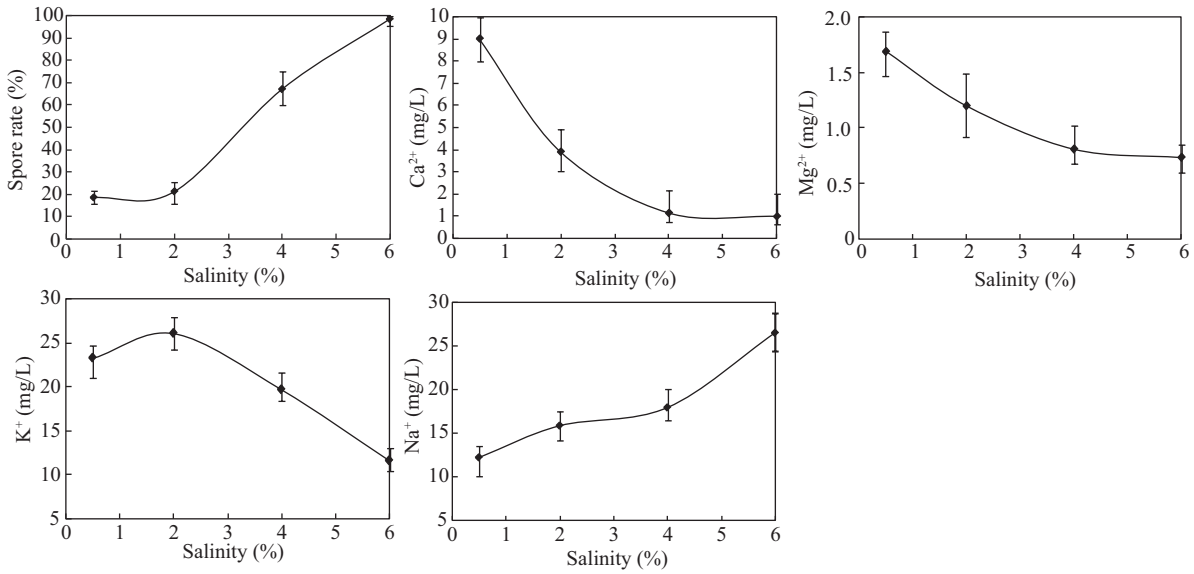


**Fig. 5** Effect of salinity on spore rate and the ion concentration of strain NY6 at pH 7 for 24 hr.

then began to decrease, And $Na^+$ concentration increased with increasing salinity from 0.5% to 6%.

It can be concluded that the stain NY6 achieves osmotic equilibrium by absorbing $K^+$ and $Na^+$ when the salinity is lower than 2%. On the contrary, the $Ca^{2+}$ and $Mg^{2+}$ were released at salinity of 0.5% to 2% to keep the equilibrium of the electrical charge of the bacterial bodies. When the salinity was over 2%, only $Na^+$ concentration increased, because of the interference of external environment, and the stain NY6 produced a large amount of spores. Under those conditions, the NY6 produced spores to keep its survival.

## 3 Conclusions

The salt-tolerant strain NY6 shows the good ability for organic matter degradation and salt-tolerance. It is classified to *Bacillus aerius* and belongs to a slightly halophilic bacterium. The capability of its dry powder sample for COD removal is 800 mg COD/(g·day) in synthesized saline wastewater with 2% salinity. When the salinity was below 2%, the stain NY6 absorbed $K^+$ and $Na^+$ to maintain osmotic equilibrium, and when the salinity was above 2%, the NY6 kept its life by producing a large amount of spores. This research suggests that NY6 may be a new candidate which can be made into dry powdered bio-products for engineering application for saline water treatment.

### Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 51108112), the Natural Science Foundation of Heilongjiang Province (No. E201252), the Maixin Pathology Foundation (No. m1101), the Fundamental Research Funding of Harbin Engineering University (No. HEUFT06029), and the Open Project of State Key Laboratory of Urban Water Resource Environment, Harbin Institute of Technology (No. ESK201004).

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**Journal of Environmental Sciences (Established in 1989)**

**Vol. 26 No. 6 2014**

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ISSN 1001-0742