

Nitrogen Removal by HN-AD Bacteria Immobilized on Modified Absorbent Stone



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<https://doi.org/10.15255/CABEQ.2020.1823>

Original scientific paper

Received: May 16, 2020

Accepted: September 30, 2020

How to simplify the nitrogen removal process, reduce the cost and improve the efficiency has become an urgent problem to be solved. In this research, the isolated HN-AD (heterotrophic nitrification and aerobic denitrification) bacteria were used to remove the nitrogen in wastewater. Modified absorbent stone was used as high-efficiency and low-cost immobilized material. The modification effect was determined by the changes in mechanical strength, Zeta potential, pore structure, micrographs and biomass. The practicability of the modified carrier was further proved by experiments of environmental effect and reuse. The modified carrier had excellent performance. By comparing the degradation effects of immobilized microorganism and free microorganism, it was proved that the immobilized microorganisms have broad application prospects and strong adaptability to environmental factors. Under the optimum conditions (temperature of 30 °C, pH of 7, dissolved oxygen of 3.5 mg L⁻¹), the removal efficiency of ammonia nitrogen reached 100 % in 40 hours, the removal efficiency of total nitrogen reached 60.11 % in 50 hours, and the removal rate of total nitrogen was 2.404 mg-NL⁻¹ h⁻¹ by immobilized microorganisms with the treatment of simulated nitrogen-containing wastewater. This research provides new material for the immobilization of HN-AD bacteria and a new way for nitrogen removal.

Keywords:

immobilized microbial degradation, HN-AD bacteria, modified absorbent stone

Introduction

With the rapid development of economy, the demand for nitrogen-containing raw materials is increasing¹. As a result, a great amount of nitrogen-containing wastewater is discharged. This can cause many serious consequences, e.g., the eutrophication of surface water, destruction of the ecological environment on which aquatic animals and plants depend, and tremendous impact on drinking water safety^{2,3}. At present, it is urgent to develop an efficient and stable technology for nitrogen removal.

Biological treatment for nitrogen removal has attracted extensive attention for its advantages of high efficiency, low energy consumption, and no secondary pollution⁴. Traditional biological treatment generally includes nitrification and denitrification operations. The nitrification and denitrification processes are accomplished by nitrifying bacteria and denitrifying bacteria, respectively⁵. Due to the

different requirements for environment, these two processes cannot be employed simultaneously, but can only be carried out sequentially. So the traditional methods, for instance, anaerobic-anoxic-oxic process and University of Cape Town process, always occupy large areas or are inefficient because of the special or temporal separation⁴. The one-step nitrogen removal technology is in great demand.

The discovery of aerobic denitrificans has inspired the development of novel nitrogen removal technology. Aerobic denitrificans always have the nitrification ability at the same time, so they are also described as HN-AD bacteria⁶. The utilization of HN-AD bacteria can compensate the shortcomings of the common technologies, and achieve low area occupation and high efficiency⁷. At present, a wide variety of aerobic denitrificans have been found, including *Pseudomonas aeruginosa*⁸, *Thiosphaera pantotroph*⁹, *Ochrobactrum* sp¹⁰, *Alcaligenes faecalis*¹¹, *Citrobacter diversus*¹² and so on.

On the other hand, in recent years, the application of microbial immobilization technology in water treatment has attracted increasing attention¹³. This technology is conducive to increasing the con-

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centration of microorganisms (especially those with special functions) in the bioreactor, resisting the adverse effects of environment, improving the treatment efficiency, and promoting the solid-liquid separation after reaction^{14,15}. The application of microbial immobilization technology in nitrogen removal process has also been reported. For example, Ivonne¹⁶ used calcium alginate to encapsulate *Azospirillum brasilense*, and then carried out three-stage advanced treatment on domestic sewage. When the dosage ratio was 10 %, the treatment effect was the best, and the ammonia nitrogen removal efficiency could be more than 50 %¹⁶. Wei used sodium alginate and polyvinyl alcohol to immobilize domesticated activated sludge, and then added it into the reactor with contaminated surface water. The removal rate of ammonia nitrogen reached 90 % through optimization experiments¹⁷. Vanotti used the fixed nitrified sludge to treat nitrogen-containing wastewater, and the removal rate of $\text{NH}_3\text{-N}$ reached $5.67 \text{ mg L}^{-1}\text{d}^{-1}$ ¹⁸. Compared with the traditional denitrification processes, immobilized microorganism technology immobilizes the selected microorganisms on the carrier, thereby making it highly dense, maintaining the biological activity of microorganisms, reducing the loss of microorganisms, enhancing the ability to resist harsh environment, and shortening the denitrification treatment time.

In this research, based on immobilized microorganism technology, the isolated HN-AD bacteria were used to remove the nitrogen in wastewater. Absorbent stone (or porous-stone; travertine) was used as immobilization material. Absorbent stone is abundant in China especially in Shandong province. Compared with traditional materials such as sodium alginate and agar, this immobilization material has a series of advantages, e.g., greater stability, long service life, and low price. The absorbent stone is composed of calcium carbonate and it is commonly used in potted landscape design in China. At present, there are few reports on the environmental application of absorbent stones.

It is well known that stone can be used as filler material of biological filter in wastewater treatment by biofilm process, which shows that stone has certain adhesion ability to microorganisms, but at present the stone used, such as gravel, has compact structure (with small internal pore) and is not conducive to mass transfer and oxygen transfer. The absorbent stone used in this study was of typical porous structure by the appearance, so it may be more suitable as a carrier of microorganisms. At the same time, many studies have explored and analyzed the adsorption function of mineral materials such as volcanic rocks^{19,20} and zeolites²¹, but the adsorption function of mineral materials has not been

used for microbial immobilization. The adsorption function brought by the porous structure of absorbent stone can also increase the microbial immobilization capacity.

In this research, some physical and chemical modification methods were employed to enhance the microbial immobilization performance of absorbent stone. Besides, the treatment conditions were optimized to achieve better nitrogen removal effect. This research provides new material for the immobilization of HN-AD bacteria and a new way for nitrogen removal.

Materials and methods

Materials

All the reagents (analytical grade) were purchased from Beijing Chemical Reagent Factory. The activated sludge used was collected from the oxidation ditch of the sewage treatment plant (Qinhuangdao City, Hebei Province, China). The origin of absorbent stone was Tai'an City, Shandong Province, China.

Culture media and artificial wastewater composition

The enriched medium consisted of tryptone 10 g L^{-1} , yeast extract 5 g L^{-1} and KNO_3 0.1 g L^{-1} . Bromothymol blue (BTB) primary screening medium was composed of KNO_3 1.0 g, $\text{C}_4\text{H}_4\text{Na}_3\text{O}_2$ (succinic acid) 1.0 g, KH_2PO_4 1.0 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05 g, CaCl_2 0.2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0 g, 1 % bromothymol blue 1 mL (per liter). 2 % agar was added when solid medium was required. Denitrification medium (DM) consisted of $\text{C}_4\text{H}_4\text{Na}_2\text{O}_4 \cdot 6\text{H}_2\text{O}$ 13 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g, $\text{NaHPO}_4 \cdot 12\text{H}_2\text{O}$ 7.9 g, KH_2PO_4 1.5 g, KNO_3 3 g, and microelement additive 2 mL (per liter). Nitrification (NI) medium was composed of $\text{C}_4\text{H}_4\text{Na}_3\text{O}_2 \cdot 6\text{H}_2\text{O}$ 11 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 6.7 g, KH_2PO_4 1 g, NH_4Cl 1.5 g and microelement additive 2 mL (per liter). In order to ensure the repeatability of the experimental results, artificial nitrogen-containing wastewater was used for nitrogen removal in this research. The artificial nitrogen-containing wastewater was prepared according to the wastewater concentration of Zhongga Chemical Fertilizer Plant (Qinhuangdao, China). The composition was ammonium chloride 0.46 g L^{-1} , sodium bicarbonate 1 g L^{-1} , sucrose 0.8 g L^{-1} , peptone 0.5 g L^{-1} and potassium dihydrogen phosphate 0.1 g L^{-1} . The ammonia nitrogen concentration of artificial wastewater was 153 mg L^{-1} . The total nitrogen concentration of artificial wastewater was 200 mg L^{-1} . The COD of artificial wastewater was 950 mg L^{-1} .

Isolation of HN-AD bacteria

The samples were obtained from a sewage treatment pond in Qinhuangdao City. The samples were added to sterilized and purified water, and then stirred for 30 min, and placed stably for 10 min. 10 mL supernatant liquor was added into the enriched medium for cultivation (30 °C; 48 h). All the mediums were sterilized (115 °C; 30 min) before use. The enriched samples were then diluted by gradient dilution. The dilution solution of 10^{-4} , 10^{-5} and 10^{-6} were coated on Bromothymol blue (BTB) primary screening medium (solid). After inoculation, they were cultured in an incubator at 30 °C for 2 days. The primary HN-AD bacteria were screened when the growing colonies turned blue or halo appeared around the colonies. The primary screening bacteria were then purified several times. The colonies were picked up with sterilized inoculation ring, and the fresh bacterial suspension was prepared by adding sterile deionized water. The concentration of the bacterial suspension could be seen under OD600 (the OD600 was adjusted to 1.6). The prepared suspension was inoculated into the NI liquid medium for cultivation at 30 °C and 1.5 mg L⁻¹ DO for 4 days. After cultivation, 10 mL suspension solution was taken from each bottle of culture liquid into which nitrite nitrogen chromogenic agent was added, and the non-chromogenic suspension marked, and the cultivation solution checked by quantitative detection. The strains with high ammonia nitrogen removal efficiency and low residual nitrite were selected for conservation. Subsequently, the strains were inoculated into a 250 mL triangular flask with 100 mL DM at 2 %. After 4 days of incubation in a water bath at 30 °C and 1.5 mg L⁻¹ DO, 10 mL suspension solution was taken from each bottle of culture liquid into which nitrite nitrogen chromogenic agent was added, and the non-chromogenic suspension marked, and the cultivation solution checked by quantitative detection. The microorganism with the highest nitrogen removal efficiency was selected as the immobilized bacteria in the follow-up process. The identification of microorganism was carried out by molecular biology method²². The genomic DNA of strain was extracted by precipitation. The 16S rDNA was amplified by the universal primers. The 16S rDNA sequence of the HN-AD bacteria was checked in GenBank.

Preparation of immobilized carriers

The carrier used in this research was absorbent stone (or porous-stone; travertine), also known as calcium carbonate aquatic bryophyte fossil, which is composed of calcium carbonate (calcium oxide content of 55.92 %).

Carrier pretreatment: Firstly, the raw materials were cut into a cylinder of length 3–4 cm and diameter of 1 cm. The materials were then washed repeatedly using distilled water. After washing, the materials were put in a sterilizer (121 °C, 30 min) to remove the microorganisms originally attached to the stone. The carrier was then dried for 24 h at 85 °C in an oven and turned taupe after drying.

Carrier modification: The carriers were heated in a muffle furnace. The temperature was set to 200 °C, 350 °C, 450 °C and the carriers were heated for 150 min, and then cooled. After heat modification, the materials were put into a conical bottle. Lignin cationic surfactant was added with CEC of 50 %, 100 % and 200 %, respectively. After being stirred by a magnetic stirrer for 12 h, the carriers were slightly rinsed 3 times using deionized water, and dried naturally to obtain the modified carrier in the clean bench.

The preparation method of lignin cationic surfactant was as follows. Firstly, 350 g alkali lignin aqueous solution (20 wt%; pH=12) was prepared, and poured into a 500-mL flask. The temperature was raised to 80 °C, and 43.08 g 3-chloro-2-hydroxypropyl trimethylammonium chloride solution was added into the flask with a peristaltic pump at controlled dropping speed of 1 mL min⁻¹. When dropping for 5 min, 22.91 g sodium hydroxide solution (20 wt%) was added. The 3-chloro-2-hydroxypropyl trimethyl ammonium chloride solution then continued to be dropped. After the reaction at 80 °C for 3 h, the reaction solution was diluted 50 times using pure water, and pH adjusted to 7 to precipitate the product. The preparation method of lignin cationic surfactant was provided by the lignin resource research group of South China University of Technology.

Immobilization of microorganisms on carriers: the isolated strain was cultured in enriched medium for 48 h, and the suspension was then centrifuged in a centrifugal tube after sterilization. The supernatant was poured out, and the sediment poured into a conical bottle and diluted using sterile water. The OD₆₀₀ of bacterial suspension was adjusted to 1.6. The prepared carrier was immersed in the prepared suspension for 30 min. As a result of capillarity, the carrier pores were filled up by the bacterial suspension. Finally, the immobilized microbial carriers were prepared.

The sodium alginate immobilized carrier and agar immobilized carrier for contrast experiments were prepared according to a publicly-reported method²³.

Before the wastewater treatment experiment, all the immobilized carriers were washed three times using sterile water.

Nitrogen removal experiment of immobilized microorganism

This research investigated the effects of different environmental factors (temperature: 15 °C, 20 °C, 25 °C, 30 °C, 35 °C; dissolved oxygen: 1.5 mg L⁻¹, 2.5 mg L⁻¹, 3.5 mg L⁻¹, 4.5 mg L⁻¹, 5.5 mg L⁻¹; pH: 5, 6, 7, 8, 9; wastewater concentration: 50 %, 100 %, 150 %, 200 %, 250 %) on the immobilized carrier. The nitrogen removal effect of immobilized HN-AD bacteria and free HN-AD bacteria was compared (the volume of bacterial suspension was the same). The volume of inhalable bacterial suspension of carrier was calculated according to the volume of the inhalable liquid of carrier. The inoculation amount of bacterial suspension was 10 %. These nitrogen removal experiments were carried out in the wastewater treatment unit of the immobilized microbial reactor which we reported previously¹³. At the same time, the recyclable utilization rate of the carrier was also tested. The detection of reusability of the material was carried out under the optimum conditions obtained from previous studies. In each experiment under different conditions, a contrast experiment was set up, with the bacterial solution being replaced by the same volume of sterile water. The residual ammonia nitrogen and TN concentrations in liquids were measured at different retention times.

Analysis methods

Concentrations of ammonia nitrogen (AN), nitrate nitrogen, nitrite nitrogen, and total nitrogen (TN) were measured by Nessler's reagent method, phenol disulfonic acid method, N-(1-naphthyl)-ethylene diamine method, and alkaline potassium persulfate method, respectively²⁴. Dissolved oxygen was determined with a dissolved oxygen meter (HQ30D, HACH, USA). Zeta potential of materials was measured with a Zeta potential analyzer (Nano ZS90, Malvern Company, UK). The mechanical strength of the material was tested with a particle strength tester (JC503-DL4, Beijing Million Electronics Technology Co., Ltd, China). The material specific surface area was determined with a specific surface area tester (F-Sorbx 400, Beijing Jinaipu Technology Company, China). The microstructure of different immobilized carriers was detected using a scanning electron microscope (SUPRA55, Carl Zeiss AG, Germany) for analysis. Infrared detection of materials was measured using a Fourier transform infrared spectrometer (IS10, Thermo Nicolet Corporation, USA). Absorption value was detected using a visible spectrophotometer (DR3900, HACH, USA). The gene automatic sequence was carried out with Gene Sequencer (3730XL, ABI, USA). EDTA titration method was used to determine the

calcium oxide in raw stone (GB/T 3286.1-2012 of China). The cation exchange capacity (CEC) of the material was determined by a publicly-reported method²⁵. The biomass of immobilized materials was measured by the elution, drying, and weighing method²⁶. The inhalable liquid quantity of absorbent stone was tested according to a common method²⁷ as follows: the weight of the carrier was measured firstly, and then the prepared carrier was immersed in deionized water for 30 min (the same as the immobilization time of microorganisms on carriers). Next, the surface of the carrier was cleaned using water-saturated wet filter paper. Finally, the carrier was weighed again to measure the quality of water. The quality was then converted into volume to obtain the carrier's inhalable liquid volume. The removal efficiency of ammonia nitrogen and total nitrogen were calculated as follows:

Removal efficiency of AN (%) = (initial ammonia nitrogen concentration – ammonia nitrogen concentration after treatment)/initial ammonia nitrogen concentration

Removal efficiency of TN (%) = (initial total nitrogen concentration – total nitrogen concentration after treatment)/ initial total nitrogen concentration

Statistical analysis

Three parallel samples of all the above experiments were set. The average value was calculated. At the same time, a one-way ANOVA with Tukey's test was chosen to analyse the significant or insignificant differences between different treatment conditions ($p < 0.05$).

Results and discussion

The isolation of HN-AD bacteria

Although the discovery of HN-AD bacteria has compensated for some shortcomings of previous nitrogen removal processes, many studies have reported that nitrite had accumulated with the use of the HN-AD bacteria^{28,29}. The main reason being that bacteria use oxygen to convert ammonia nitrogen into nitrite nitrogen, and then use oxygen to convert nitrite into nitrate nitrogen. As we know, nitrite is harmful in water. Therefore, it is necessary to isolate HN-AD bacteria without nitrite accumulation in the nitrogen removal system⁶.

In this research, 18 strains were screened by BTB plate, and then the nitrification performance of the 18 strains on NI medium was tested. The strains with ammonia nitrogen removal efficiency above 60 %, and TN degradation rate above 30 % were selected for the color reaction of nitrite. The strains'

accumulated nitrite nitrogen was eliminated, and then the nitrogen removal performance of these strains was also tested in denitrification medium. The strains' accumulated nitrite nitrogen was removed by color reaction again. The strain with the highest removal efficiency was selected as the immobilized bacterium for subsequent research. The results showed that AD-6 was the best HN-AD bacterium, which was *Acinetobacter johnsonii* strain identified by molecular biology. The phylogenetic neighbor-joining tree is shown in Fig. 1. This bacterium had the ability of simultaneous nitrification and denitrification, and no nitrite nitrogen had accumulated. The detected concentration of nitrite nitrogen showed that there was no residual nitrite nitrogen in DM medium after 12 h, and no nitrite nitrogen was detected in NI medium. The total nitrogen removal efficiency of AD-6 in DM medium was 36.8 %.

Preparation and modification of immobilized carrier

In order to improve the nitrogen removal efficiency of bacteria, immobilized carrier was prepared. Absorbent stone was selected as immobilization material, and in order to improve the immobilization effect, the absorbent stone was modified. The modification effect was determined by the changes of mechanical strength, Zeta potential, pore structure, micrographs, and biomass. These parameters are closely related to the performance of the immobilized materials.

Properties of materials before and after modification

Firstly, in order to improve the mechanical properties of the carrier, heat modification was used to treat the material. According to the obtained mechanical strength, 350 °C was the optimal temperature. The mechanical strength of modified carrier was higher than that of the original carrier (average particle strength of the original carrier was 50.4 kg cm⁻²;

average particle strength of modified carrier was 74.5 kg cm⁻²). The heat treatment resulted in the release of water from the stone, which increased the mechanical strength of the stone³⁰. Secondly, due to the electronegativity of the microorganisms used in this research and the electronegativity of the immobilized material, the cationic surfactant was used to modify the material to improve the absorbing capacity of the material to microorganisms. In order to avoid adverse effects on the environment by the commonly-used cationic surfactant, lignin cationic surfactant was used to modify the material. Lignin quaternary amine salts are well-known non-toxic polymers with high positive charge, and they cause no secondary pollution. The results showed that the Zeta potential of the modified material had significantly increased after modification with cationic surfactant. The Zeta potential of the carrier after modification (200 % CEC) at pH=7 reached 8.23, while it was -47.33 before modification. Cationic surfactants have hydrophobic groups of alkyl chain on one end and cationic polar groups on the other. In the process of modification, the cationic end and the negative electricity of mineral surface absorbed each other, and formed a single layer of modification adsorption on the mineral surface. When the amount of modifier was more than one layer, the mineral surface would form bilayer structure. The cationic end of the second layer of modifier was exposed until the bilayer was covered with the whole mineral surface, and the mineral showed positive electricity.

The N₂ adsorption and desorption isotherms of the modified carrier and the original carrier were analyzed and compared. The N₂ adsorption and desorption isotherms of the modified carrier and the original carrier are shown in Fig. 2. It could be seen that the adsorption isotherm had typical characteristics of IUPAV (IV) adsorption isotherm (produced by mesoporous solids, the typical characteristic is that the adsorption curve of isotherm is inconsistent

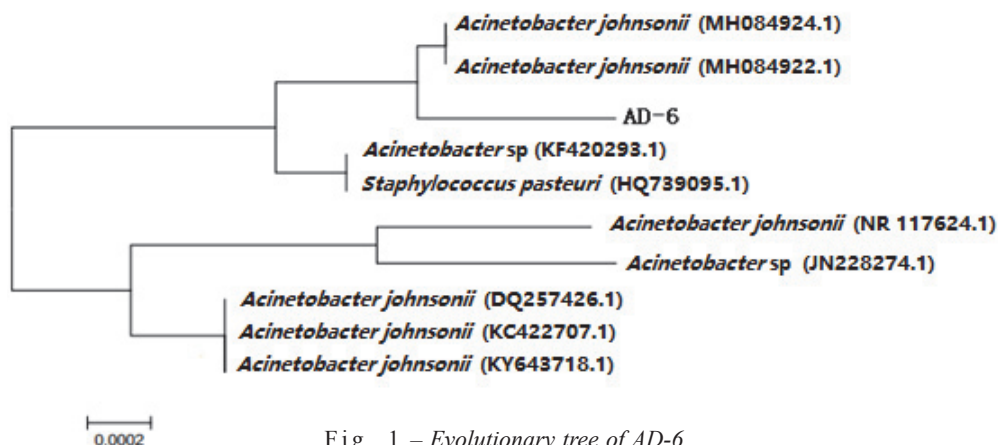


Fig. 1 – Evolutionary tree of AD-6

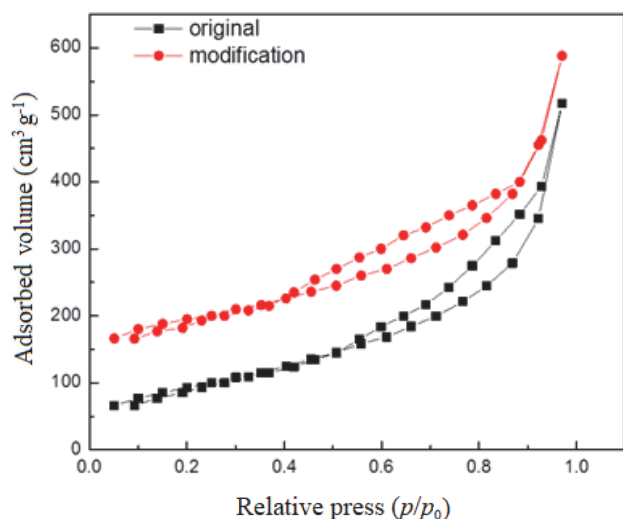


Fig. 2 – N_2 adsorption and desorption isotherms of the modified carrier and the original carrier

with the desorption curve, and a hysteresis loop appears), which indicated that the carrier belonged to the mesoporous material³¹. The pore size distribution was concentrated. The high specific surface area of mesoporous materials determines the application of mesoporous materials in adsorption, catalysis, and separation, etc³². At the same time, the mesoporous structure of the material was not destroyed by modification, and the pore size and specific surface area of the material had improved. The mesoporous structure of the material was conducive to the immobilization of microorganisms.

Microbial immobilization capacity before and after modification

Scanning electron microscopy (SEM) was used to examine the immobilization effect of the modified carrier. Moreover, the biomass of the carriers was measured to determine the change in the microbial attachment ability of the material before and after modification.

Fig. 3 shows the SEM images of different materials. Fig. 3(a) shows the carrier profile before modification, where the carrier profile showed rough texture and close texture. Fig. 3(b) shows the carrier profile after modification, where the lamellar structure increased, and polygonal sheet stacking structure had formed. Fig. 3(c) shows the original carrier with microorganism immobilized. Fig. 3(d) shows the modified carrier with microorganism immobilized. Through the comparison of c and d images, much more microorganisms could be found in the modified material, indicating the improved immobilization performance of the carrier after modification.

At the same time, through biomass measurement, the calculated biomass was 0.036 g g^{-1} and

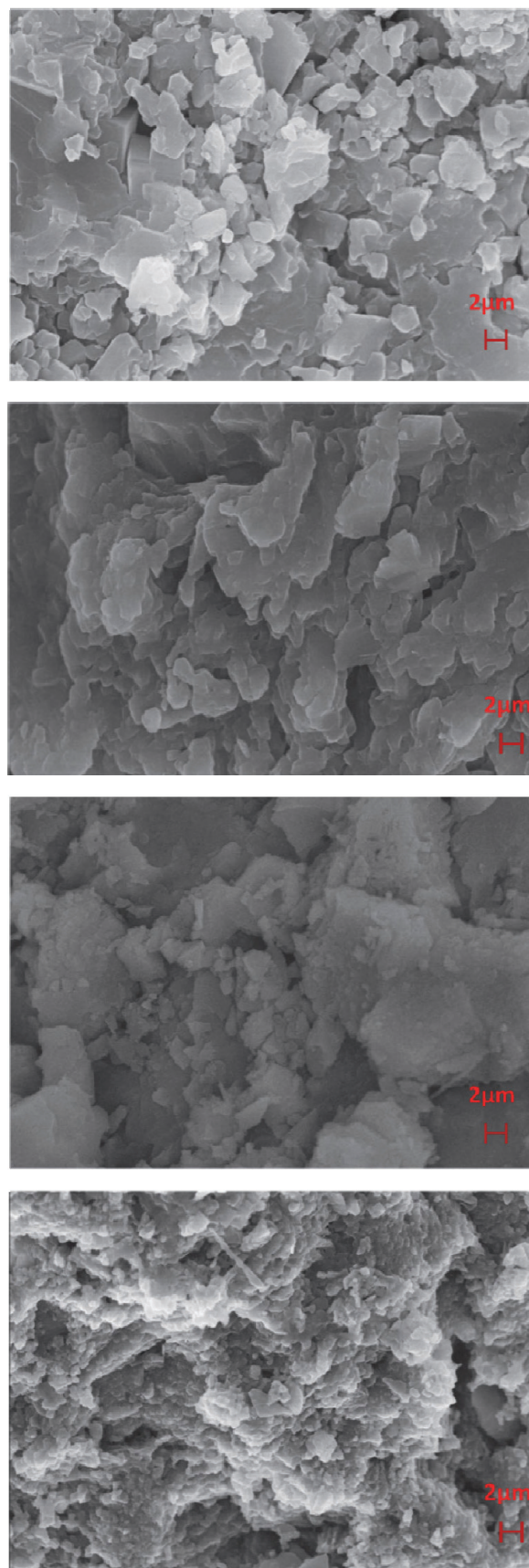


Fig. 3 – SEM analysis of Original carrier without microorganism (a), Modified carrier without microorganism (b), Original carrier with immobilized microorganism (c), and Modified carrier with immobilized microorganism (d)

0.282 g g⁻¹ before and after modification, indicating better immobilization performance of the modified carrier, and the greatly-improved attached biomass. Therefore, in the subsequent experiments, the modified absorbent stone was used as the carrier material to immobilize the strain AD-6.

Nitrogen removal by immobilized strain AD-6

Degradation of nitrogen wastewater by different materials and microorganisms

In order to determine the effectiveness of the immobilized microorganisms, comparative experiments of different immobilized microorganisms (different carriers or different microorganisms) and free microorganisms were designed. At the same time, the nitrogen removal experiments of individual materials (excluding microorganisms) and immobilized materials (with microorganisms) were also carried out to analyze whether the main role of nitrogen removal was microbial metabolism or carrier adsorption. The treatment conditions were as follows: temperature of 30 °C, inoculation amount of 10 % (v/v), pH of 7 and dissolved oxygen of 2.5 mg L⁻¹. The treatment time was 48 h. The experimental results are shown in Table 1.

According to the experimental results, it could be seen that the absorbent stones without the immobilization of microorganisms (including before and after modification) could remove part of the ammonia nitrogen and total nitrogen, indicating that the absorbent stones had a certain adsorption effect resulting from their structure. The materials could be further modified to enhance absorption effect in subsequent research. The application of stone in pollutant adsorption has been extensively investigated. Modified zeolite can be used to adsorb and remove metal ions and organic pollutants, as shown by previous research. Absorbent stones may also have applications in this field. The removal of ammonia nitrogen and total nitrogen by dried or soaked absorbent stones showed little difference, which indicated that the absorbent stones had good mass transfer performance and adequate substance exchange during water treatment process. Secondly, without the immobilization of microorganisms, the removal efficiency of ammonia or total nitrogen was not significantly different before and after modification, but the positive charge on the surface of the material modified by surfactants might affect the adsorption of nitrogen. Thirdly, the removal efficiency of the total nitrogen and ammonia nitrogen of absorbent stones increased significantly after immobilization of microorganisms, which also showed that microorganisms played a major role in the process of nitrogen removal, and the removal efficiency of total nitrogen and ammonia nitrogen were the

highest after heat-surfactant modification. This confirmed a series of conclusions obtained from the determination of the properties of modified materials. Fourthly, the blank and contrast experiments showed that the denitrification effect of absorbent stones was better than that of calcium alginate and agar immobilized carriers, indicating better mass transfer efficiency, better mechanical properties, long service life, and good stability of the new material. The comparison results with activated sludge (free or immobilized) showed that the nitrogen removal efficiency of the isolated HN-AD strain AD-6 was higher than that of activated sludge under current treatment conditions, which verified the superiority of the selected bacteria in nitrogen removal. Finally, by comparing the nitrogen removal effect of free AD-6 bacteria with that of fixed AD-6 bacteria, the immobilized microorganisms treated the ammonia nitrogen completely within 48 h, while the free microorganisms did not completely degrade the ammonia nitrogen. The TN removal efficiency of immobilized microorganisms reached 54.89 %, while that of free microorganisms was 45.65 %. The treatment efficiency of immobilized microorganisms was higher than that of free microorganisms. That was because the carrier could improve the biochemical reaction efficiency of microorganisms. At the same time, with the consumption of dissolved oxygen from outside to inside of the carrier, dissolved oxygen concentration gradient had formed, and the internal micro-oxygen zone was conducive to improving the catalytic performance of enzymes, such as nitrite reductase, thereby improving the denitrification performance³³.

Optimization of nitrogen removal process

Further studies to optimize use of immobilized HN-AD bacteria and analyze the influence factors for nitrogen removal were carried out. At the same time, the adaptability of immobilized microorganisms to environmental factors was also studied. In this study, the effects of treatment time, treatment temperature, dissolved oxygen concentration, pH, wastewater concentration, and material reuse times on the denitrification process were discussed. This is mainly because microorganisms play a major role in the nitrogen removal process. The growth of microorganisms will change in different time, and the microorganisms are sensitive to various parameters of the external environment, such as pH, temperature, and nutrient concentration^{10,12}. Also the number of microorganisms loaded will change with the increase in reuse times¹⁵.

Firstly, this experiment investigated the effect of treatment time on nitrogen removal. The free microorganisms and immobilized microorganisms were compared and analyzed. The treatment condi-

Table 1 – Degradation of nitrogen wastewater by different materials and microorganisms

Individual materials (excluding microorganisms)				Immobilized or free microorganisms			
Materials	Average removal efficiency of TN (%)	Materials	Average removal efficiency of TN (%)	Materials	Average removal efficiency of TN (%)	Materials	Average removal efficiency of TN (%)
Absorbent stone (dry)	6.76±0.52	absorbent stone (aseptic water infiltration)	6.39±0.77	Absorbent stone (immobilized AD-6)	34.44±1.43	Calcium alginate (immobilized AD-6)	50.7±3.80
Heat modified-absorbent stone (dry)	6.82±0.15	Heat modified-absorbent stone (aseptic water infiltration)	6.74±0.44	Heat modified-absorbent stone (immobilized AD-6)	35.81±1.29	Free AD-6	45.65±1.07
Surfactant modified-absorbent stone (dry)	5.17±0.36	Surfactant modified-absorbent stone (aseptic water infiltration)	4.88±0.92	Surfactant modified-absorbent stone (immobilized AD-6)	51.24±0.76	Agar (immobilized AD-6)	46.12±1.23
Heat and surfactant modified-absorbent stone (dry)	5.84±0.17	Heat and surfactant modified-absorbent stone (aseptic water infiltration)	5.90±0.35	Heat and surfactant modified-absorbent stone (immobilized AD-6)	54.89±1.45	Activated sludge	10.43±0.15
Blank test	0			Heat and surfactant modified-absorbent stone (immobilized activated sludge)	15.16±1.22		
Individual materials (excluding microorganisms)				Immobilized or free microorganisms			
Materials	Average removal efficiency of AN (%)	Materials	Average removal efficiency of AN (%)	Materials	Average removal efficiency of AN (%)	Materials	Average removal efficiency of AN (%)
Absorbent stone (dry)	7.43±0.80	absorbent stone (aseptic water infiltration)	7.62±0.14	Absorbent stone (immobilized AD-6)	81.06±4.73	Calcium alginate (immobilized AD-6)	88.7±5.77
Heat modified-absorbent stone (dry)	7.99±1.76	Heat modified-absorbent stone (aseptic water infiltration)	7.77±0.48	Heat modified-absorbent stone (immobilized AD-6)	83.25±1.17	Free AD-6	82.1±3.14
Surfactant modified-absorbent stone (dry)	6.55±1.21	Surfactant modified-absorbent stone (aseptic water infiltration)	5.98±0.86	Surfactant modified-absorbent stone (immobilized AD-6)	90.79±1.05	Agar (immobilized AD-6)	81.43±5.66
Heat and surfactant modified-absorbent stone (dry)	6.99±1.43	Heat and surfactant modified-absorbent stone (aseptic water infiltration)	6.80±0.54	Heat and surfactant modified-absorbent stone (immobilized AD-6)	100 %	Activated sludge	37.51±2.94
Blank test	0			Heat and surfactant modified-absorbent stone (immobilized activated sludge)	41.20±3.28		

The nitrogen removal effect of immobilized microorganisms was compared with that of free microorganisms (the volume of bacterial suspension was the same). The volume of inhalable bacterial suspension of carrier was calculated according to the volume of the inhalable liquid of carrier. The volume of the bacterial suspension in other carriers (sodium alginate immobilized carrier and agar immobilized carrier) and the absorbent stone carrier was also the same. In the blank experiment, no bacteria were added and the same volume of sterile water was added. The volume of activated sludge added in the contrast experiment and the bacterial suspension was the same.

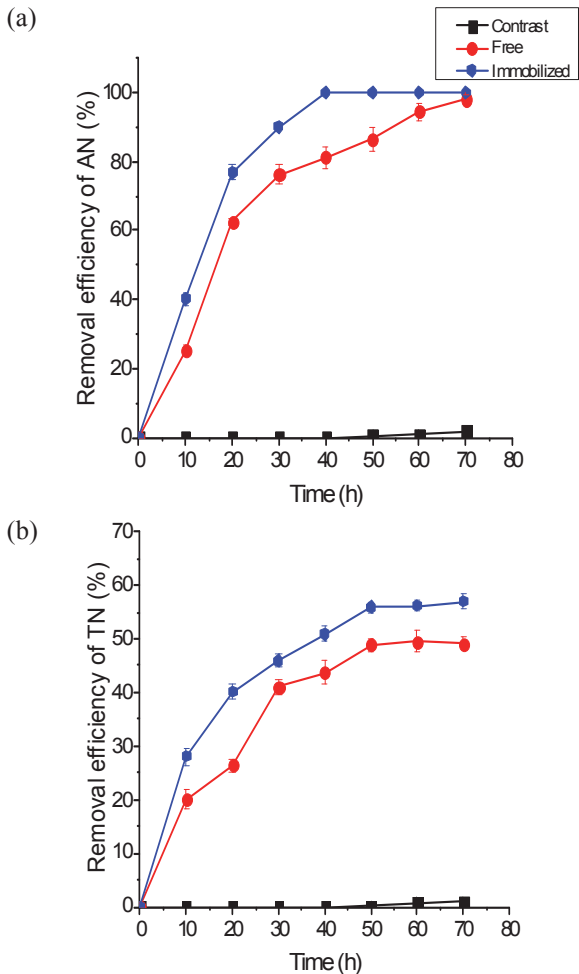


Fig. 4 – Effect of treatment time on AN removal (a) and TN removal (b)

tions were as follows: temperature of 30 °C, inoculation amount of 10 % (v/v), pH of 7 and DO of 2.5 mg L⁻¹. The ammonia nitrogen and total nitrogen concentration was measured at treatment times of 0 h, 10 h, 20 h, 30 h, 40 h, 50 h, 60 h and 70 h, respectively. Then, the removal efficiency was calculated. The results are shown in Fig. 4. In Fig. 4(a), the immobilized microorganisms completely removed ammonia nitrogen in 40 h, while the free microorganisms completely removed ammonia nitrogen in 70 h. The removal rate of ammonia nitrogen by immobilized microorganisms reached 3.825 mg-NL⁻¹ h⁻¹, which was close to that of a novel HN-AD strain reported in a previous research⁶. However, that strain⁶ required the DO concentration of 6.08 mg L⁻¹, so this immobilization microorganism system could reduce the energy consumption of aeration compared to that strain. In Fig. 4(b), the TN removal of immobilized microorganisms was finally stable at 57 %, while that of free microorganisms was stable at about 49 %. And the removal of total nitrogen was stable at 50 h. A one-way ANOVA with Tukey's test was chosen to ana-

lyze the significant or insignificant differences between different treatment conditions. The result suggested that the TN removal rate showed significant differences in free and immobilized states ($p=0.000<0.05$). The total nitrogen removal efficiency of microorganisms had been significantly improved by the immobilization technology.

Generally, the optimal growth temperature of microorganisms is 25–35 °C. High temperature or low temperature will affect the activity of microbial anabolic enzymes. Studies on the effect of temperature on nitrification and denitrification have been reported. The optimal temperature for biological nitrification was 20–30 °C. The nitrification rate decreased when the temperature was below 15 °C, and stopped completely at 5 °C. Denitrification took place in the temperature range of 15–35 °C. When the temperature was below 10 °C or above 30 °C, the denitrification rate decreased significantly³⁴. Fig. 5(a,b) shows the comparison of ammonia nitrogen degradation efficiency between immobilized microorganisms and free microorganisms. Fig. 5(c,d) shows the comparison of TN degradation efficiency between immobilized microorganisms and free microorganisms. At low temperature, the metabolic rate decreased, which affected the biological enzyme activity of strains. But after immobilization, the removal rate was higher than that of free strains. Statistical analysis showed that temperature had a significant effect on the total nitrogen removal of free bacteria ($p<0.05$), but had no significant effect on the immobilized state. After immobilization, the immobilized carrier had a certain protective effect on the strain at low temperature and high temperature. The adaptability of microorganisms to low temperature and high temperature was enhanced, which is beneficial to its application in water treatment process. According to the analysis, the optimal treatment temperature after immobilization was 30 °C.

Oxygen control is very important in the nitrogen removal process. The nitrification process requires the participation of oxygen, but in the denitrification process, if the concentration of dissolved oxygen in the reactor is too high, the molecular oxygen will become the oxygen supply material, which will inhibit the reduction process of nitrate nitrogen. Normally, the DO concentration was mainly from 1.0 mg L⁻¹ to 5.0 mg L⁻¹, and excessive dissolved oxygen concentration might affect the nitrogen removal effect of aerobic denitrifying bacteria³⁵. The effect of DO value is shown in Fig. 6. It could be seen that dissolved oxygen had a greater impact on the nitrogen removal of free microorganisms, especially when dissolved oxygen was higher than 4.5 mg L⁻¹, the nitrogen removal effect decreased. This phenomenon was similar to

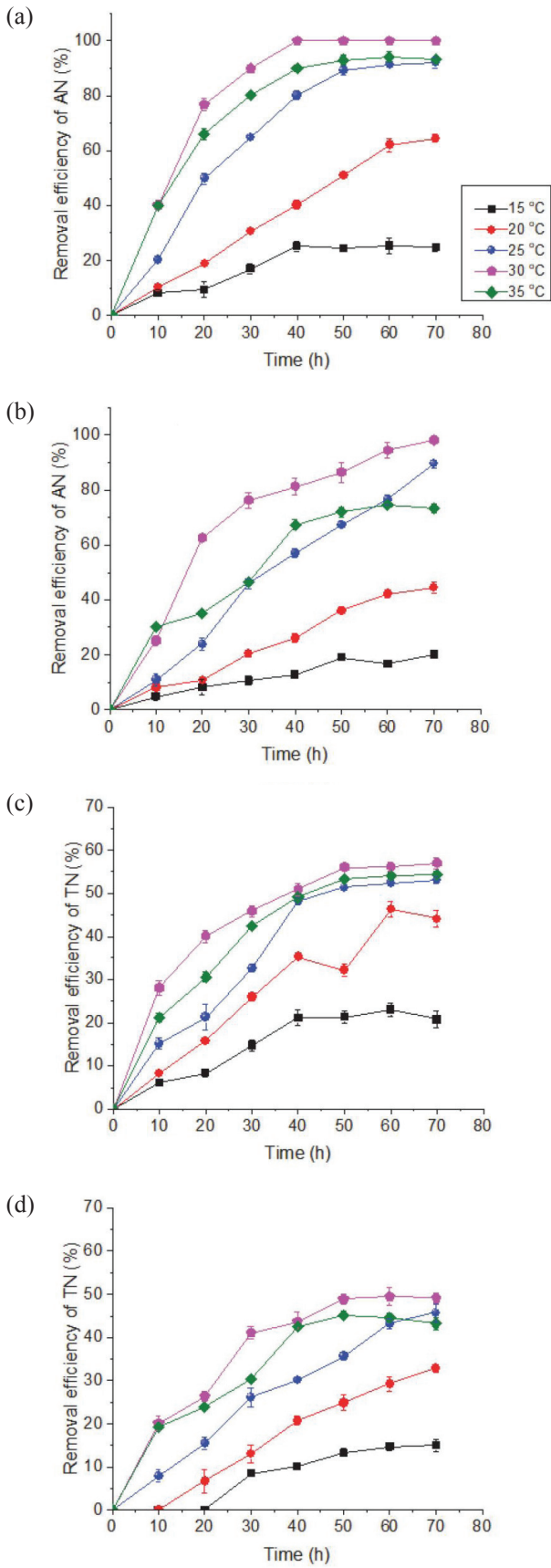


Fig. 5 – Effect of temperature on AN removal by immobilized microorganism (a) and free microorganism (b) and effect of temperature on TN removal by immobilized microorganism (c) and free microorganism (d)

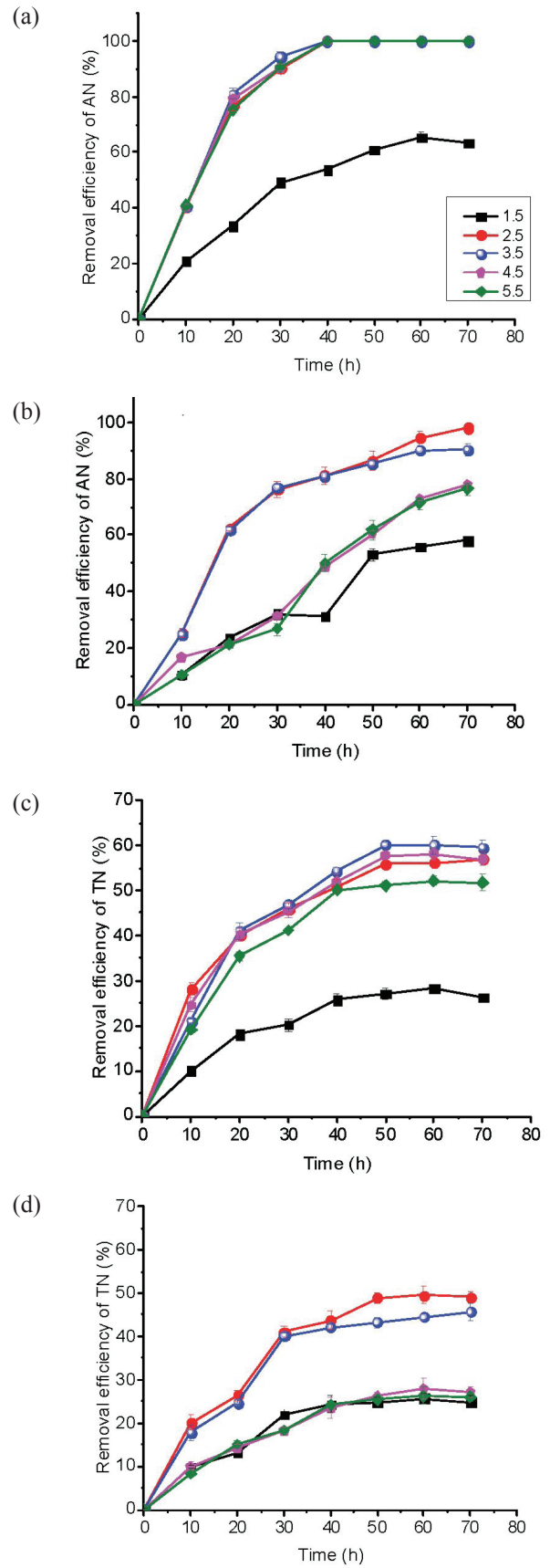


Fig. 6 – Effect of DO on AN removal by immobilized microorganism (a) and free microorganism (b) and effect of DO on TN removal by immobilized microorganism (c) and free microorganism (d)

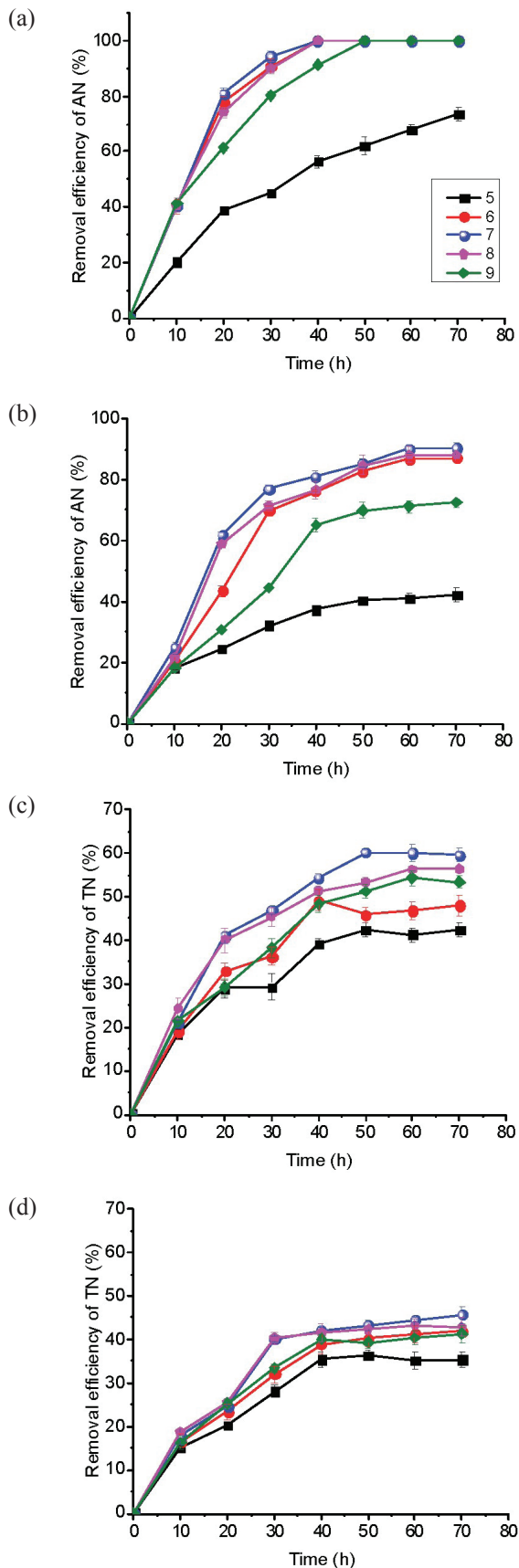


Fig. 7 – Effect of pH on AN removal by immobilized microorganism (a) and free microorganism (b) and effect of pH on TN removal by immobilized microorganism (c) and free microorganism (d)

that reported in a previous research¹². For immobilized microorganisms, the removal efficiency of nitrogen increased first and then decreased with the improving dissolved oxygen concentration, but the overall effect was insignificant. The removal efficiency of total nitrogen reached the maximum when dissolved oxygen solubility was 3.5 mg L^{-1} . The removal efficiency of total nitrogen reached 60.11 % in 50 h, that of total nitrogen of immobilized microorganisms reached $2.404 \text{ mg-NL}^{-1} \text{ h}^{-1}$, and that of free microorganisms was $1.956 \text{ mg-NL}^{-1} \text{ h}^{-1}$ in 50 h, which was much higher than $0.93 \text{ mg L}^{-1} \text{ h}^{-1}$ for *Rhodococcus* sp. CPZ24³⁶. Statistical analysis showed that DO concentration had no significant effect on the nitrogen removal efficiency of free bacteria and immobilized bacteria, which also indicated that the selected aerobic denitrifying bacteria had better tolerance to DO, and DO concentration had weaker impact on immobilized microorganisms than on free microorganisms as a result of the internal environment of the carrier. The micro-oxygen environment could effectively buffer the change in dissolved oxygen and protect microorganisms³⁷.

The pH is also an important factor affecting microbial denitrification process. Slight alkaline conditions are more suitable for ammonia removal³⁸. The pH value in the environment will mainly affect the absorption of nutrients due to the change in the surface charge of cell membrane, thus affecting the nutrient supply³⁹. The activity of microbial enzymes has a certain range of pH adaptation. Over-acidic or over-alkaline environment can reduce the activity or even inactivate microbial enzymes, and reduce the utilization efficiency of microbial nutrients⁴⁰. Fig. 7(a,b) shows the ammonia nitrogen degradation, and Fig. 7(c,d) shows the TN degradation variation. It could be seen from the figures that the removal rates of ammonia nitrogen and TN by free and immobilized microorganisms maintained at a low pH level of 5 and H^+ had a significant effect on nitrification. Under acidic conditions (pH = 5, 6), the removal efficiency of ammonia nitrogen and TN by immobilized microorganisms was higher than that by free microorganisms. Under neutral and weak alkaline conditions (pH = 7, 8), the bacteria showed better activity, while under alkaline conditions (pH = 9), the activity of bacteria was affected and the degradation rate decreased. The optimal pH conditions for nitrogen removal were neutral (pH = 7) or weak alkaline (pH = 7–8). This result was the same as other previously-reported aerobic denitrifying bacteria⁶. The immobilized microorganism had strong adaptability to the wastewater with different pH, and had good removal effect in the pH range of 5–9.

Five concentration gradients were set up to test the tolerance of immobilized microbial carriers to

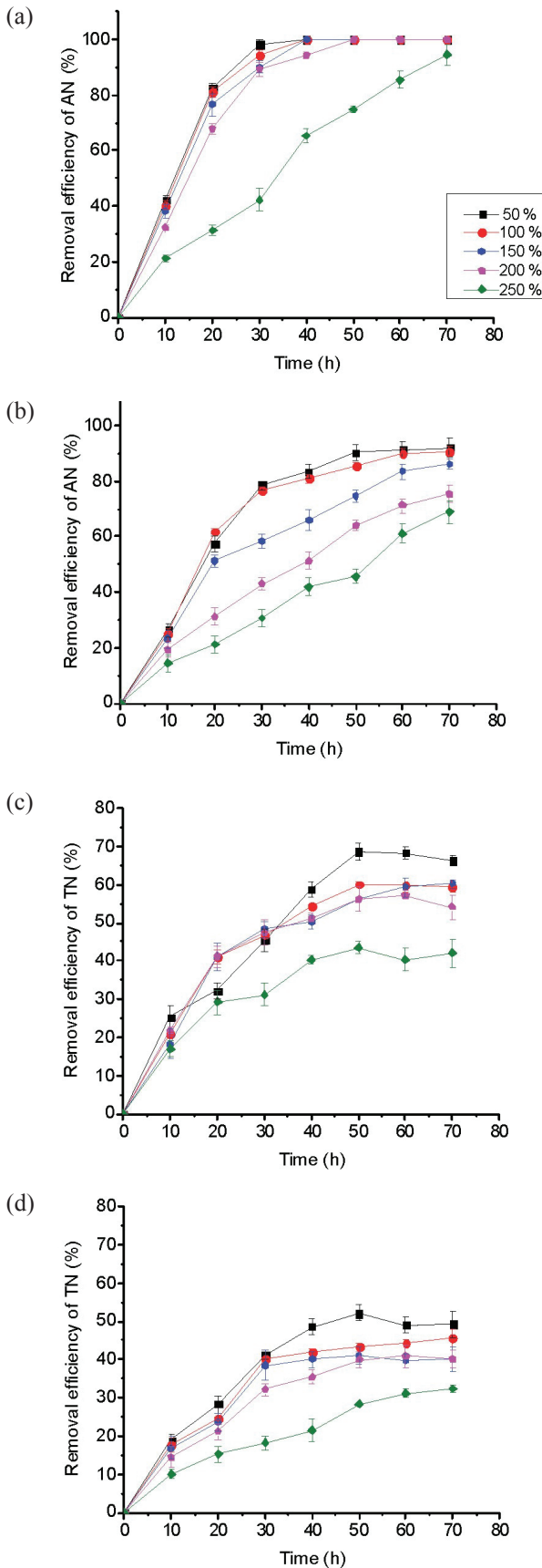


Fig. 8 – Effect of wastewater concentration on AN removal by immobilized microorganism (a) and free microorganism (b) and effect of wastewater concentration on TN removal by immobilized microorganism (c) and free microorganism (d)

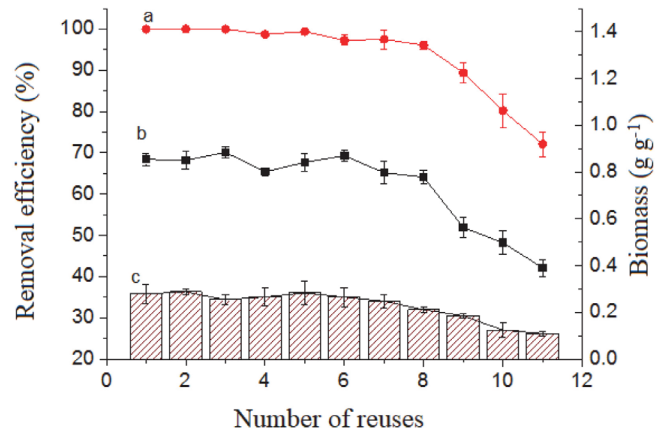


Fig. 9 – Effect of reuse number on AN removal (a), TN removal (b) and Biomass (c)

wastewater concentration. It can be seen from Fig. 8 that the adaptability of immobilized carriers was much better than that of free bacteria in the range of 50 % to 250 % of wastewater concentration. The reason might be that whether in poor or eutrophic state, the carrier could absorb certain nutrients in the water for the survival of bacteria, and enhance the treatment effect. The results showed that with 50 % concentration, the removal efficiency of total nitrogen reached 68.6 % within 50 h. Excessive concentration of wastewater would lead to a greater osmotic pressure on microorganisms, which would affect their normal life activities and finally degrade the pollutants¹³. Compared with activated sludge, when the chloride ion concentration in biochemical system was high, the carbonization and nitrification performance of sludge would be weakened rapidly, resulting in a significant decrease in COD removal rate and nitrite accumulation in nitrification process. The environmental mutation resistance of immobilized microorganisms in this research was strong. When the nitrogen concentration in wastewater was increased by 150 %–200 %, the nitrogen removal efficiency changed little, revealing the wide application prospect of the system.

The recycling times of immobilized materials are important factors for investigating the application of immobilized materials in wastewater treatment. In this research, under the optimal treatment conditions, immobilized microorganisms were used to treat wastewater, and immobilized materials were recycled. The treatment effect is shown in Fig. 9. The degradation rate of ammonia-nitrogen of immobilized microorganisms was almost 100 % in the first eight cycles. At the beginning of the ninth cycle, the removal rate began to decrease slightly. At the end of the tenth and eleventh cycles, the degradation rate of ammonia-nitrogen gradually decreased, and the removal of total nitrogen had a similar trend, which suggested that the bacteria im-

mobilized on the carrier had aged. As the carrier had porous structure, there was enough space for the growth of the bacteria, and the bacteria maintained a high concentration for a long time. With the increase in time, the bacterial membrane immobilized on the carrier would age and fall off, thus affecting the degradation of ammonia nitrogen and total nitrogen. This prediction could be confirmed by measuring the biomass of absorbent stone loaded after each use. When the absorbent stone was reused for the eighth time, the biomass decreased obviously, and then the biomass decreased gradually. During the first eight times of use, the biomass remained stable. The experimental results showed that the immobilized bacteria could be reused at least for 8 cycles, indicating its promising prospects in real applications.

In summary, after immobilization of microorganisms, the nitrogen removal efficiency improved significantly and the environmental tolerance was enhanced. The optimum temperature for wastewater treatment was about 30 °C and the energy consumption was low. The immobilized microorganisms also had good adaptability to different DO concentrations. The DO concentration from 1.5 to 4.5 mg L⁻¹ could achieve good denitrification effect. The immobilized microorganisms had strong adaptability to wastewater with different pH values. This system can treat wastewater with pH from 5 to 9. The immobilized microorganisms in this study had strong environmental mutation resistance. When the concentration of wastewater increased by 150 %–200 %, it had little effect on nitrogen removal efficiency. The modified immobilized material had strong adhesion to microorganisms and stable structure. The experimental results showed that the immobilized microorganisms can be reused for more than eight times in the treatment of nitrogen-containing wastewater, without affecting the treatment effect.

Conclusion

In this study, HN-AD bacteria were isolated, at the same time nitrogen removal experiments were carried out through carrier modification and immobilization. By comparing the degradation effects of immobilized microorganisms and free microorganisms, it has been proved that the immobilized microorganisms have broad application prospects and strong adaptability to environmental factors. Under optimum conditions (temperature of 30 °C, pH of 7, dissolved oxygen of 3.5 mg L⁻¹), the removal efficiency of ammonia nitrogen reached 100 % in 40 hours, the removal efficiency of total nitrogen reached 60.11 % in 50 hours, and the removal rate of TN was 2.404 mg-NL⁻¹ h⁻¹ by immobilized mi-

croorganisms with the treatment of simulated wastewater. When the concentration of wastewater increased by 150 %–200 %, it had little effect on nitrogen removal efficiency suggesting strong application prospects. In the follow-up study, the immobilized microorganisms will be used in the activated sludge system to study its adaptability, and further improve the treatment effect and efficiency of wastewater.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the supports from Natural Science Foundation of Zhejiang Province (LQ20B060004), National Natural Science Foundation of China (No.21606005), the Natural Science Foundation of Hebei Province (No. D2017203317), Key Project of Hebei Education Department (No. ZD2019071), and Zhejiang Shuren University project (2019R017; 2020XZ010). All the authors thank Professor Hongming Lou of South China University of Technology for his help on this topic.

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