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Nitrification characteristics of nitrobacteria immobilized in waterborne polyurethane in wastewater of corn-based ethanol fuel production

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Abstract

A technology to achieve stable and high ammonia nitrogen removal rates for corn distillery wastewater (ethanol fuel production) treatment has been designed. The characteristics of nitrifying bacteria entrapped in a waterborne polyurethane (WPU) gel carrier were evaluated after acclimation. In the acclimation period, nitrification rates of WPU-immobilized nitrobacteria were monitored and polymerase chain reaction (PCR) was also carried out to investigate the change in ammonium-oxidizing bacteria. The results showed that the pellet nitrification rates increased from 21 to 228 mg-N/(L-pellet·hr) and the quantity of the ammonia oxidation bacteria increased substantially during the acclimation. A continuous ammonia removal experiment with the anaerobic pond effluent of a distillery wastewater system was conducted with immobilized nitrifying bacteria for 30 days using an 80 L airlift reactor with pellets at a fill ratio of 15% (*V*/*V*). Under the conditions of 75 mg/L influent ammonia, hydraulic retention time (HRT) of 3.7–5.6 hr, and dissolved oxygen (DO) of 4 mg/L, the effluent ammonia concentration was lower than 10 mg/L and the ammonia removal efficiency was 90%. While the highest ammonia removal rate, 162 mg-N/(L-pellet·hr), was observed when the HRT was 1.3 hr.

Key words: immobilized nitrobacteria; corn distillery wastewater; ethanol fuel production; nitrification rate; waterborne polyurethane gel

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Introduction

Distillery wastewater is the main byproduct originating in distilleries, and its volume is approximately 10 times that of the ethanol produced (Krzywonos et al., 2009). Utilization of the distillery wastewater has become a serious issue. The distillery wastewater is relatively non-toxic, but it usually contains a high concentration of complex organic matter and suspended solids. The treatment methods for distillery wastewater can be classified into two major groups. One includes the processes of recycling and utilizing valuable solids from corn distillery wastewater, and the other comprises both aerobic and anaerobic processes, by which the distillery wastewater can be biodegraded (Li et al., 2006; Rajeshwari et al., 2000). It was reported that an anaerobic-aerobic process had superior performance in the degradation of high concentrations of organic matter. However, the traditional aerobic process is ineffective for wastewater containing high concentrations of ammonia.

Therefore, immobilized bacteria are being considered for application in nitrification processes. Compared with conventional processes, the use of immobilized bacteria has the following advantages: the compact physical structure of the carrier pellets, high biomass retention, and premium tolerance with respect to variations in load and pollution levels (Sumino et al., 1992). At the same time, a screen is used to separate the entrapped bacteria from the wastewater to prevent the washout of bacteria (Asano et al., 1992; Dong et al., 2011). Thus, gel entrapment is able to prolong the biomass retention times even under short hydraulic retention times (HRT). However, little effort has been made to evaluate the nitrification activity of gel carriers of microorganisms for distillery wastewater.

In this research, nitrification by immobilized bacteria pellets was studied to design a novel advanced treatment technology for the anaerobic pond effluent of a distillery wastewater system. Pellets of waterborne polyurethane (WPU) immobilized nitrifying bacteria were prepared and acclimated. The growth and distribution of immobilized nitrobacteria were observed by scanning electron microscope (SEM) and analyzed by polymerase chain reaction (PCR) during the acclimation process. Characteristics of the nitrification processes with WPU-immobilized nitrify-

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ing bacteria pellets were investigated for the development of a corn-based ethanol fuel production wastewater treatment system.

1 Materials and methods

1.1 Reagents

Polyether polyol, isophoronediisocyanate (IPDI), and 2-hydroxylethyl methacrylate (HEMA) were purchased from Aldrich (USA). Reagents such as NH_4Cl, $NaHCO_3$, Na_2HPO_4, and H_3BO_4 were purchased from Sinopharm Chemical Reagent Co. (China). All chemicals used were of analytically pure grade.

1.2 Synthesis of waterborne polyurethane (WPU)

Polyether polyol, IPDI, and HEMA (with a molar ratio of 1:2:2) were mixed with catalyst (Dibutyltin dilaurate, DBTL) in a 500 mL flask and allowed to react in vacuum at 60–70° for 5 hr. The WPU prepolymer was dripped into a vessel containing distilled water at the stage of reaction completion, which took another 12 hr. The final solution was stirred vigorously for 1 hr so that a stable WPU emulsion was obtained.

1.3 Immobilization of nitrobacteria in WPU

In this research, the nitrifying bacteria (seed sludge) were obtained from the aeration tank of the Minhang Municipal Wastewater Treatment Plant, Shanghai, China. After 2 months of acclimation, suspensions of nitrifying bacteria were concentrated to the density of 20 g/L by centrifugation and the concentrate was entrapped in the WPU gel carrier. The entrapped concentrate was then mixed with a WPU prepolymer emulsion and a promoter (*N,N,N',N'*-tetramethylenediamine). To initiate polymerization, potassium persulfate, an initiator, was added to the beaker. The mixture was allowed to stand for 5 min at a room temperature of 25°C. Consequently, WPU-immobilized nitrifying bacteria in the form of an elastic gel was obtained. The resulting polymerized gel carrier was cut into 3 mm cubes and then washed thoroughly with distilled water.

1.4 Analytical methods

Dissolved oxygen (DO) and pH were measured daily. The temperature of the reactor was monitored using an online probe. Ammonium nitrogen (NH_4^+-N), nitrate-nitrogen (NO_3^--N), nitrite-nitrogen (NO_2^--N), and mixed liquor suspended solids (MLSS) were determined according to standard methods (APHA, 1999).

1.5 Experimental setup

Biological nitrification was carried out in an up-flow inner circulation aerated reactor, as shown in Fig. 1. The reactor (with 80 L operating volume) made of acrylic glass was fed with ammonia wastewater. The filling ratio of immobilized pellets was 15% (*V*/*V*). Air was supplied through a sintered glass ball fixed at the column bottom. The pellets rose up to the top by aeration and dropped down to the bottom of the reactor by gravity. Thus, inner circulation was formed, which ensured complete mixing and contact between wastewater and immobilized bacteria. At the top of the reactor, pellets and wastewater were separated by a diagonal filter mesh. The wastewater was pumped by variable speed peristaltic pumps. The HRT was controlled by adjusting the feed flow rate.

1.6 Acclimation of immobilized nitrobacteria in WPU to the synthetic wastewater

An activity recovery experiment of the nitrifying bacteria immobilized in WPU was conducted for over 90 days in the reactor. The compositions of the synthetic ammonia wastewater are shown in Table 1. The influent ammonia concentration was increased with operating time to evaluate the performance of the immobilized bacteria from stages I to V, and analysis was carried out every day. At the end of each acclimation stage, several pellets were taken from the reactor and refrigerated at 4°C until bacterial DNA extraction and PCR amplification experiments were carried out. Biomass subsamples were freeze-thawed three times and DNA was extracted

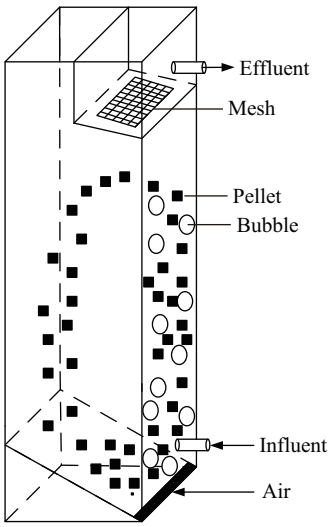

Fig. 1 Schematic diagram of the up-flow inner circulation aerated reactor.

Table 1 Compositions of the synthetic ammonia wastewater*

Acclimation stages	Ammonia (mg/L)	NH_4Cl (mg/L)	Na_2HPO_4 (mg/L)	NaCl (mg/L)	KCl (mg/L)	$CaCl_2$ (mg/L)	$MgSO_4$ (mg/L)	$NaHCO_3$ (mg/L)
I	45	172.2	52.2	23.1	10.7	8.13	18.5	527.4
II	90	344.4	104.4	46.2	21.4	16.3	37.0	1054.8
III	150	573.6	174.1	77.2	36.0	27.1	61.6	1754.8
IV	200	764.9	232.1	102.9	48.0	36.1	82.2	2339.8
V	360	1376.2	417.6	184.4	86.4	64.8	147.4	4211.7

* 150 L influent tank.

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(Ausubel et al., 1990). To evaluate the activity and quantity of the ammonia-oxidizing bacteria, the DNA fragments encoding the *amoA* gene were amplified using the forward primer A189 (GGNGACTGGGACTTCTGG) and reverse A682 (GAASGCNGAGAAGAASGC) (Zhou et al., 2007). The whole volume was made up to 20 μL, and PCR was conducted in a reaction solution containing (μL) template DNA 2.0, 10×PCR buffer 2.0, $MgCl_2$ 1.2, dNTPs 0.5, PCR primers 2.0, Taq DNA polymerase 0.5 and sterile water 11.8. After being mixed on a vibrating mixer, PCR amplification was carried out using a DNA thermal cycler at 96°C for 4 min, 95°C for 45 sec, 58°C for 45 sec, and 72°C for 90 sec for 29 cycles (Holmes et al., 1995). A positive control was carried out to ensure the accuracy of results. The preparation of amplified samples for gel electrophoresis was carried out with gel concentration 1.5% and electrophoresis voltage 100 V, and the electrophoresis imaging was performed under ultraviolet light.

1.7 Conversion of synthetic wastewater to distillery wastewater system

Distillery wastewater was substituted for the synthetic wastewater as the influent after the acclimation period. Most of the organic matter and some of the nitrogen in the distillery wastewater were removed by anaerobic treatment. The nitrification process of the anaerobic pond effluent adopted the WPU-immobilized nitrifying bacteria pellets in place of the traditional biological treatment. The characteristics of the effluent from the anaerobic pond containing the distillery wastewater were as follows: NH_4^+-N 30.1–156.2 mg/L, NO_2^--N 0.04–5.53 mg/L, NO_3^--N 0.20–6.88 mg/L, COD 47–374 mg/L and pH 6.94–7.99. A continuous ammonia removal experiment with the immobilized nitrifying bacteria was carried out in the aerobic reactor with pellets with fill ratio 15% by volume. The process continued for 45 days and was divided into two stages. During the first stage, the ammonia processing capacity of the WPU-immobilized nitrifying bacteria pellets with distillery wastewater was tested for 30 days. The ammonia nitrogen removal load under different HRT was investigated for 15 days in the second stage.

2 Results and discussion

2.1 Mechanisms of WPU immobilization nitrobacteria hydrogel formation

Due to their flexibility and non-biodegradability, synthetic gels are promising for the application of entrapping nitrifying cells for wastewater treatment systems (Leenen et al., 2001). Among all of the synthetic polymers used for microorganism immobilization, polyurethane is stands out due to its good properties, such as mechanical, physical, and chemical stability (Sumino et al., 1992). A new microorganism immobilization method involving preparation and gelation of WPU has been developed. The WPU gels provide greater mechanical strength and have the advantage of easy preparation without repetitive freezing and thawing, such as is necessary for PVA (Chen and Lin, 1994). WPU with carboxylate groups can be dispersed in water. The dispersed WPU emulsion is generally unstable and could flocculate when it mixes with the suspended cells with the addition of the promoter and initiator. That is, the WPU emulsion would be crosslinked to form a hard gel block. The proposed chemical structure is shown in Fig. 2. The WPU gels were cut into 3-mm cubes and Fig. 3a shows the shape of beads prepared using this method. As shown in Fig. 3b, each bead was filled with cells.

2.2 Acclimation of nitrobacteria immobilized in WPU to the synthetic wastewater

Because of the thermal efficiency of polymerization and the mass transfer resistance to species such as oxygen and ammonia ion, the nitrifying bacteria activity decreased during the process of immobilization. Thus, the acclimation process was necessary for the activity recovery of the immobilized nitrifying bacteria. Figure 4a shows the activity recovery of immobilized nitrifying bacteria in WPU pellets as determined by the ammonia removal rate. The immobilized nitrifying bacteria were acclimated to the synthetic wastewater in the up-flow inner circulation reactor. The volume of the immobilized pellets was 15% of the working volume of the reactor. Continuous experiments were conducted with temperature of 25°C, HRT of 8 hr, and DO of 3–5 mg/L. The initial ammonia

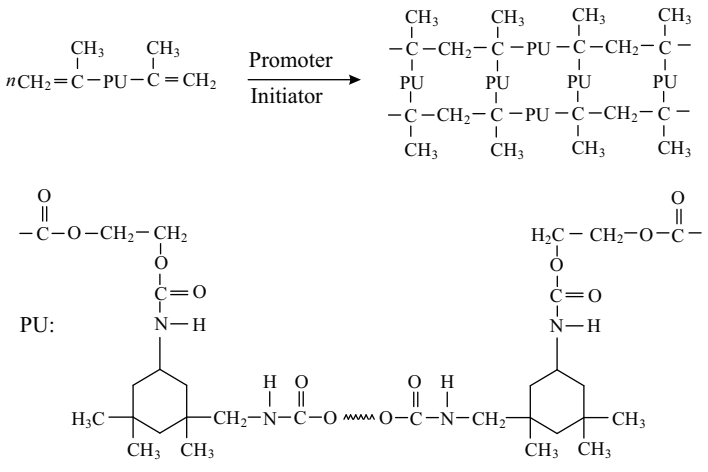

Fig. 2 Chemical structure of crosslinked WPU.

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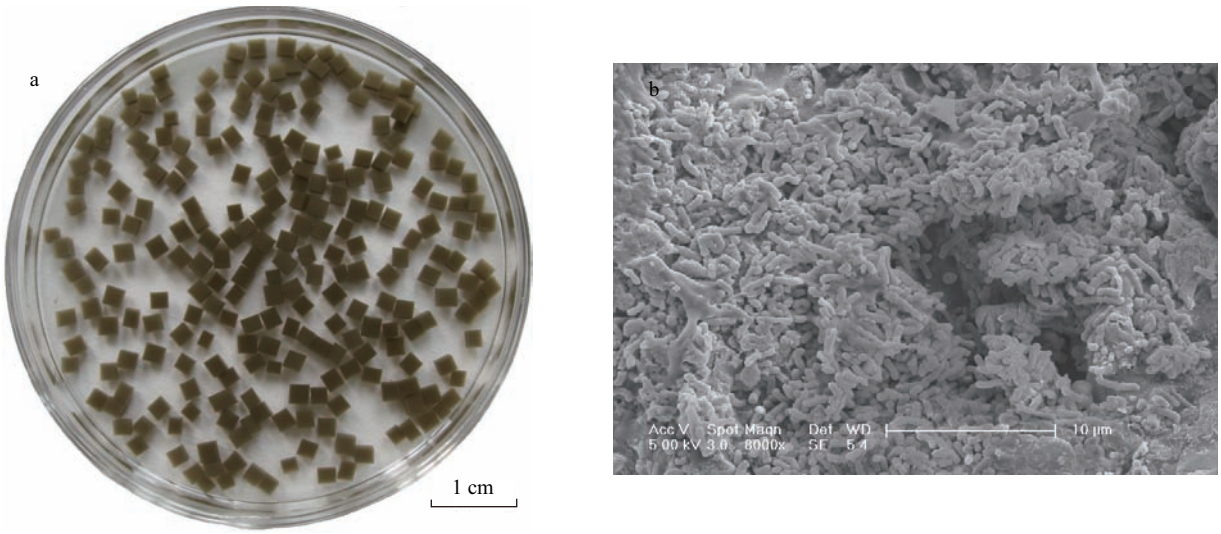

Fig. 3 Appearance of WPU-immobilized nitrobacteria pellets (a) and SEM micrograph of immobilized pellet surface (b).

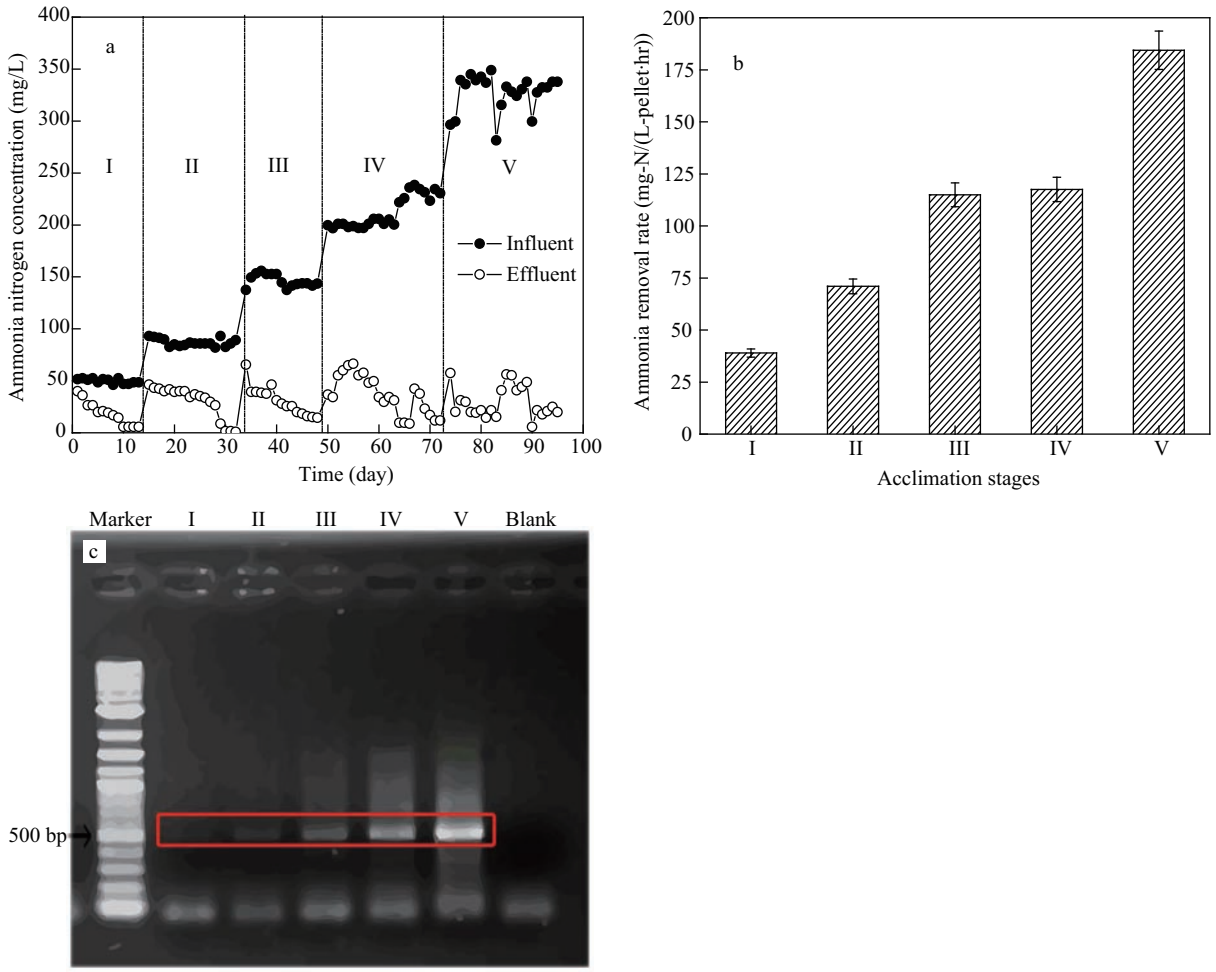

Fig. 4 Nitrification characteristics of WPU-immobilized nitrobacteria pellets with increasing ammonium loading (synthetic wastewater) (a), ammonium removal rate of WPU-immobilized nitrobacteria pellets at different acclimation stages (b), and gel picture of PCR amplifications (c). Experimental condition: 25°C, HRT 8 hr, DO 3–5 mg/L, initial ammonia nitrogen 40–360 mg/L, filling ratio of immobilized pellets 15%.

nitrogen concentration was 40 mg/L, which was gradually increased to 360 mg/L within 95 days. As shown in Fig. 4b, at the first stage of acclimation, the initial ammonia oxidation rate of the immobilized pellets was only 45 mg-N/(L-pellet·hr), which then increased to 72 mg-N/(L-pellet·hr) at the second acclimation stage, then increased to 118 and 123 mg-N/(L-pellet·hr) at the third and forth acclimation stages, respectively. The nitrification activity slowly recovered with operating time. However, it showed a dramatic increase and reached a maximum of 236 mg-N/(L-pellet·hr) in stage V. As the ammonia nitrogen removal rate reached to above 98% at the end

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of stage V, this indicated that stage V was the end of acclimation. The results also suggested that about 3 months were required for the recovery of activity of the immobilized nitrifying bacteria after the formation of the pellets. To estimate the amplified product base length by the strip on the Marker, as shown in Fig. 4c, the box marked region was the target bands, which was the gene fragment length of amplified ammonia oxidizing bacteria (about 500 bps). The intensity of the gel image light related to the amplified sample volume of the *amoA* gene fragment, that is, the stronger the band intensity was, the more ammonia-oxidizing bacteria there were. For samples collected from stage I to IV, the light intensity gradually grew stronger. These results confirmed that the quantity of the ammonia-oxidizing bacteria increased substantially during the acclimation. This phenomenon demonstrates that the immobilized pellets began to acclimate to the synthetic wastewater and could recover their activity and ammonia oxidation ability (Wijffels and Tramper, 1995).

2.3 Conversion of synthetic wastewater to the distillery wastewater system

After the acclimation, immobilized pellets were applied to the treatment of distillery wastewater in the up-flow inner circulation reactor. Nitrification characteristics in the treatment of distillery wastewater were evaluated. Figure 5 shows the time course of the concentrations of nitrogenous compounds in the influent and effluent in all the reactors. The influent ammonia concentration varied greatly with an average concentration of 75 mg/L, and the HRT of the reactor was kept at 3.7–5.6 hr. DO in the experiment was 1–3 mg/L from day 1–13, and 5–7 mg/L from day 14–29 (Fig. 5). In this experiment, two characteristics of nitrification were confirmed.

First, DO was an important factor in ammonia nitrogen removal. As shown in Fig. 6, sufficient DO in the water treatment system can improve the nitrification efficiency of immobilized cell pellets. When the DO concentration was less than 2 mg/L, the ammonia nitrogen removal rate

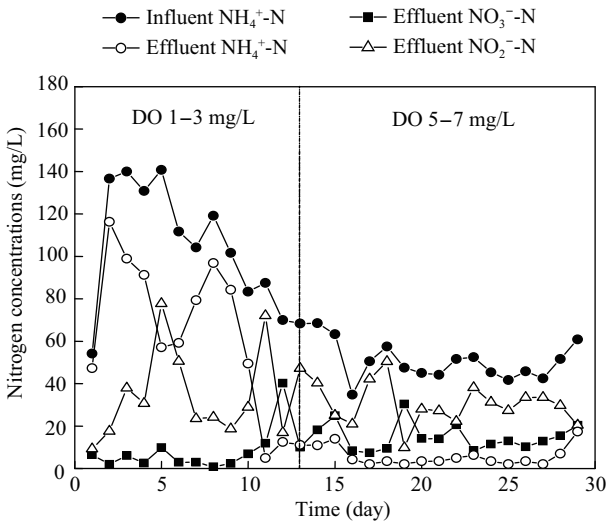

Fig. 5 Concentrations of nitrogenous compounds in the influent and effluents of corn distillery wastewater. Experimental condition: HRT 3.7–5.6 hr, 15–25°C.

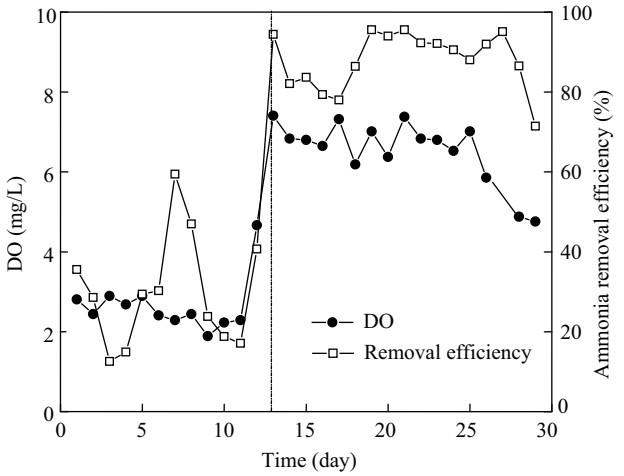

Fig. 6 Effect of DO on ammonia nitrogen removal efficiency.

was less than 20%. The ammonia nitrogen removal rate increased gradually as DO rose from 2 to 4 mg/L. When DO was 4 mg/L, the effluent ammonia concentration was lower than 10 mg/L and ammonia removal efficiency was 90%. When DO was higher than 4 mg/L, a low ammonium nitrogen concentration was detected in the effluent water; but the removal rate was stable at 90% when the DO further rose to 5–7 mg/L. The optimum DO concentration was 4 mg/L, taking account of energy consumption in the actual engineering project.

Second, an incomplete nitrification process occurred in the reactor. Most of the ammonium was oxidized to nitrite, not to nitrate. As shown in Fig. 5, the average effluent nitrite concentration was 32 mg/L, which was much larger than that of nitrate, 12 mg/L. According to the previous studies, nitritation is observed a low DO concentration is supplied (Garrido et al., 1997; Tokutomi, 2004; Antileo et al., 2006). However, in our experiment, this situation could not be changed when a higher DO level (5–7 mg/L) was applied in the later stage, indicating that a high DO level could not sharply increase the nitrate concentration in the effluent. This result indicated that nitrite oxidizers lost their activity possibly due to the following factors: free ammonium inhibition, the high alkalinity of distillery wastewater, and oxygen transfer resistance. Nitrite accumulation is one of the characteristics of this process and it also offers the possibility to achieve shortcut nitrification-denitrification. The concept of shortcut nitrogen removal is denitrifying nitrite to nitrogen gas before it oxidizes to nitrate. With this, up to 25% of the oxygen and approximately 40% of the carbon source can be saved in the biological nitrogen removal process (Bae et al., 2002). In practical application of this process, an anoxic denitrification tank would be placed in the final system to remove total nitrogen.

Moreover, the average COD of the influent and effluent were both about 150 mg/L (data not shown). Pellets embedded with nitrifying bacteria had no significant effect on the COD removal. The reason for this phenomenon was that the immobilized nitrifying bacteria had been acclimated to high-strength inorganic ammonia nitrogen wastewater and the majority of pores of the pellets were filled with nitrifying bacteria, with no space for het-

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erotrophic bacteria (Qiao et al., 2008).

After stabilization, the optimum HRT was obtained by increasing the flow rate. The operation was initiated with an ammonia concentration of 40 mg/L, DO of 4 mg/L and HRT of 1.2–3.5 hr. Considering HRT and the amount of ammonia removed, the ammonia removal rate and ammonia removal efficiency were calculated and are shown in Fig. 7. When HRT was between 1.2 and 2.5 hr, the ammonia nitrogen removal rate was unstable and the effluent ammonia nitrogen could not be stabilized below 15 mg/L. A short HRT led to a short nitritation time, which subsequently led to the instability of the bioreactor. When HRT was between 2.5 to 3.5 hr, the ammonia nitrogen removal efficiency was above 85% and effluent ammonia nitrogen was below 15 mg/L. The ammonia removal efficiency of the system operating with 2.3 hr of HRT was 86%, dropping to 60% for 1.2 hr of HRT. The removal rate reached its highest (162 mg-N/(L-pellet·hr)) when the HRT was 1.3 hr. However, the removal rate decreased with a further decrease in the HRT. A decrease in the HRT to less than 1.3 hr caused the removal efficiency to decrease drastically, indicating the limitations of external diffusion and wash-out of ammonia. Considering the limits of concentration of ammonia nitrogen emission and wastewater treatment system instability, it was appropriate to set the optimum HRT at 2.0–3.0 hr. When HRT was 2.0–3.0 hr, the effluent ammonia concentration was lower than 10 mg/L and the ammonia removal efficiency rate was 85%. No inhibitive material on the nitrification rate was found in the wastewater and no recognizable destruction of particles was observed during the period of operation.

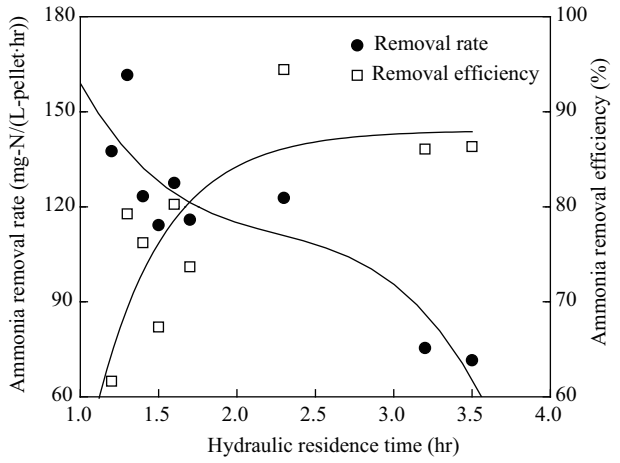

Fig. 7 Effects of HRT on ammonium removal rate and efficiency of WPU-immobilized nitrobacteria pellets. Experimental condition: initial ammonia concentration 40 mg/L, DO 4 mg/L.

3 Conclusions

The present method demonstrated that the WPU-immobilized pellets showed a great potential for the treatment of corn distillery wastewater. The removal of ammonia nitrogen was higher than 80% at HRT of 3 hr and DO of 4 mg/L, and continuous system operation was stable. High rates of nitrification were obtained with WPU-immobilized nitrifying bacteria pellets using our experimental setup. The highest ammonia removal rate, 162 mg-N/(L-pellet·hr), was observed when the HRT was 1.3 hr. Based on the developed nitrification process, the process was simple, economical and can be applied at the pilot-scale for distillery wastewater treatment systems. However, further studies on the composition of the nitrifying bacteria are required. Factors such as the concentration of dissolved oxygen, free ammonia concentration and pH value may affect accumulation of nitrite by immobilized pellets. In the future total nitrogen treatment, shortcut nitrification can be realized by controlling the factors to save energy consumption in the whole process.

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