

Research on Immobilized Ammonia-Oxidizing Bacteria by Sodium Alginate

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Ammonia-oxidizing bacteria were immobilized by sodium alginate and calcium chloride. The immobilization conditions and ammonia oxidation ability of the immobilized bacteria were investigated. The following immobilization conditions were found to be optimal: sodium alginate, 4.5%; calcium chloride, 2.0%; 2000 immobilized balls per 1000 immobilized medium; pH, 10; 110r/min and temperature, 30°C. The immobilized ammonia-oxidizing bacteria exhibited strong ammonia oxidation ability even after recycling for six times. The ammonia nitrogen removal rate of the immobilized ammonia-oxidizing bacteria reached 89.51% under the optimal immobilization conditions. When compared with non-immobilized ammonia-oxidizing, those immobilized by sodium alginate were superior with respect to preservation, recycle and ammonia oxidation ability.

Key words: Ammonia-oxidizing bacteria, Immobilization, Sodium alginate.

Ammonia-oxidizing bacteria belong to one of physiological subsets of nitrifying bacteria family (Woses *et al.* 1984). They have a role in the first rate-limiting step of nitrification (Deboer *et al.* 1991; Liu *et al.* 2004) and in the oxidation of amine to nitrites, and are widely used in the denitrification of industrial wastewater and soil (Hu *et al.* 2005; Yu *et al.* 2009). However, as ammonia-oxidizing bacteria are autotrophic, they have long generation time, slow growth rate, higher sensitivity, and can easily get eliminated (Werner *et al.* 2005). To address these limitations, cell immobilization technology has been developed. Immobilized cell technology, with obvious advantages, combines liquid fermentation and immobilized enzyme. The immobilized cells exhibit improved catalytic activity, shortened production time, lower production cost, increased yield, and wide application prospects.

(Chen *et al.* 1996; Lu *et al.* 1996). Many raw materials and synthetic polymers such as sodium alginate, polyacrylamide, agar, and polyvinyl alcohol (PVA) have been extensively applied in cell immobilization (Seung *et al.* 2005; Tsung *et al.* 2008). In the present study, ammonia-oxidizing bacteria were immobilized by sodium alginate to improve their characteristics and applications.

MATERIALS AND METHODS

Strains

The ammonia-oxidizing bacteria used in this study were screened from the activated sludge collected from a sewage treatment plant in China University of Mining and Technology (Xuzhou, China), and exhibited 98% homology with *Nitrosomonas* sp. GH22.

Methods

Culture medium

Simulation sewage medium (pH 8.0-8.2): 0.08% (NH₄)₂SO₄, 0.1% K₂HPO₄, 0.05% MgSO₄, 0.2% NaCl, 0.04% FeSO₄, and 1% CaCO₃.

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Immobilized medium (pH 7.0-7.2) contained 0.2% NH_4Cl , 0.1% K_2HPO_4 , 0.05% MgSO_4 , 0.04% FeSO_4 and 1% CaCO_3 .

Preparation of immobilized ammonia-oxidizing bacteria

The ammonia-oxidizing bacteria were cultured in simulation sewage medium. After allowing the culture to stand for some time, the supernatant was discarded. Subsequently, PVA and sodium alginate were added to the culture at a sodium alginate/culture ratio of 3:1 (v/v). By using an injector, the culture mixture was added to calcium chloride, and the bacteria were immobilized at 4°C for 6 h. Subsequently, the immobilized bacteria were washed with deionized water and inoculated into 250–1000 mL of immobilized medium at a concentration of 1–2 immobilized balls/mL immobilized medium at 24°C and 100 r/min for 12–20 days.

Determination method

The nitrite nitrogen content was determined by employing α -naphthylamine spectrophotometry and phenol disulfonic acid spectrophotometry, while the ammonia nitrogen content was evaluated by using phenol disulfonic acid.

RESULTS AND DISCUSSION

Immobilized medium optimization

Immobilized medium component was optimized as follows. The first immobilized medium only had ammonia sulfate and no sodium chloride; the second immobilized medium only had sodium chloride and no ammonia sulfate; the third immobilized medium only had ammonium chloride; the fourth immobilized medium was sterile water. All of the culture conditions were the same (Table 1).

Table 1. Effect of different immobilized medium on the formation of immobilized balls

immobilized medium	collapse	hardness	transparency	diameter	number of immobilized balls Cultured for 7 days
1	yes	soften	transparent	shorten	zero
2	yes	soften	transparent	shorten	zero
3	no	little changed	translucent	invariable	not reduced
4	no	little changed	translucent	invariable	not reduced

Table 2. Effect of calcium chloride concentration on the formation of immobilized balls

calcium chloride concentration/%	tailing	collapse	hardness	transparency	sphericity
1.6	no	yes	very soft	very transparent	no
1.8	no	yes	soft	transparent	no
2.0	yes	no	moderate	transparent	yes
2.2	yes	no	hard	translucent	yes
2.4	yes	no	hard	opaque	yes

Table 3. Effect of sodium alginate concentration on the formation of immobilized balls

sodium alginate concentration/%	tailing	collapse	hardness	transparency	sphericity
3.0	no	yes	very soft	very transparent	no
3.5	no	yes	soft	transparent	no
4.0	no	yes	moderate	transparent	yes
4.5	not obvious	no	moderate	translucent	yes
5.0	yes	no	hard	opaque	yes

The first and second immobilized mediums were not suitable for continued experiments. In other words, high concentration sulfate ion or sodion were not allowed to be contained in immobilized medium. The results were not the same as other references (Zheng *et al.* 2002; Zhang *et al.* 2006; Wang *et al.* 2007). Based on our experimental results (Table 1) and related documents (Wu *et al.* 1993), we explained reasonably as follows. Gelatinization means sodion of sodium alginate is replaced by calcium ion and become compact reticular formation if calcium ion exists in medium. Swelling rate of calcium alginate will increase and immobilized balls will dissolve if sodion exchanges with calcium ion. Because calcium sulphate is easier to deposit than calcium alginate, immobilized balls will also dissolve if sulfate ion exchanges with calcium ion. Therefore high concentration sulfate ion and sodion will

destroy calcified membrane. Here ammonia sulfate was replaced by ammonium chloride in no sodion immobilized medium.

Effect of calcium chloride concentration on the formation of immobilized balls

To determine the effect of calcium chloride concentration on the formation of immobilized balls, the following immobilization conditions were employed: 4.5% sodium alginate, 1.6%, 1.8%, 2.0%, 2.2% and 2.4% calcium chloride (Table 2).

As calcium chloride concentration less than 1.8%, immobilized balls were transparent and soft; as calcium chloride concentration more than 2.2%, immobilized balls were opaque, hard and trailing. While at a concentration of 2.0%, immobilized balls exhibited better transparency and hardness. 1.6% and 2.4% calcium chloride concentration were not suitable for immobilization and be removed.

Effect of sodium alginate concentration on the formation of immobilized balls

To determine the effect of sodium alginate concentration on the formation of immobilized balls, the following conditions were applied: 2.0% calcium chloride, and 3.0%, 3.5%, 4.0%, 4.5% and 5.0% sodium alginate (Table 3).

At less than 3.5% sodium alginate concentration, immobilized balls were transparent and soft; while at concentrations higher than 4.5%, immobilized balls were opaque, hard and trailing. A sodium alginate concentration of 4.5% produced immobilized balls that had better transparency and hardness. 3.0% sodium alginate concentration were not suitable for immobilization and be removed.

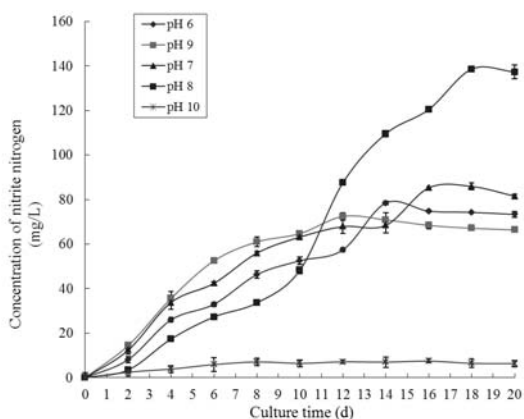


Fig. 1. Effect of pH on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria

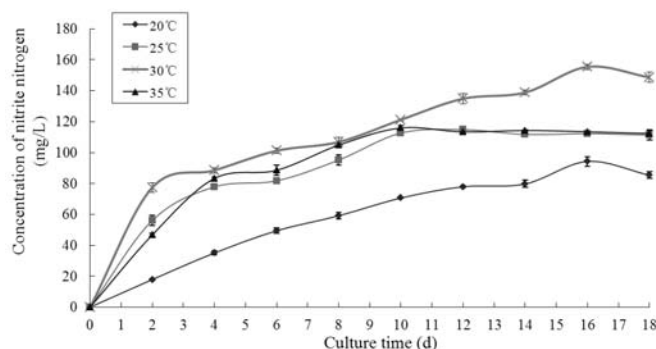


Fig. 2. Effect of temperature on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria

Effect of calcium chloride concentration on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria

To determine the effect of calcium chloride concentration on the ammonia nitrogen removal ability of the immobilized ammonia-

oxidizing bacteria, the following immobilization conditions were employed: 4.5% sodium alginate; 1.8%, 2.0% and 2.2% calcium chloride; and 500 immobilized balls. The reaction was carried out in 250 ml shake flask containing 250 ml of immobilized medium at pH 8.0, 30°C, and 110 r/min for 12 days.

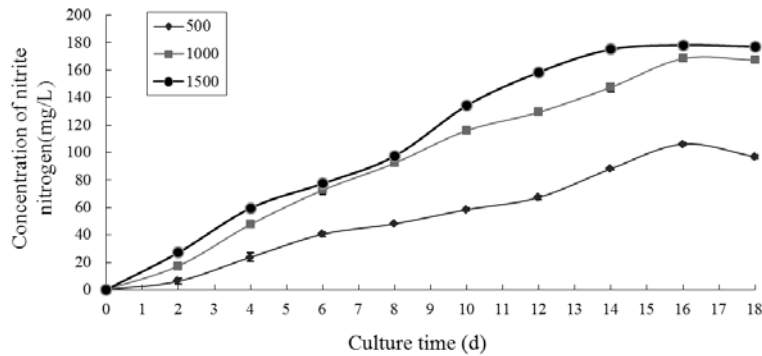


Fig. 3. Effect of inoculum concentration on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria

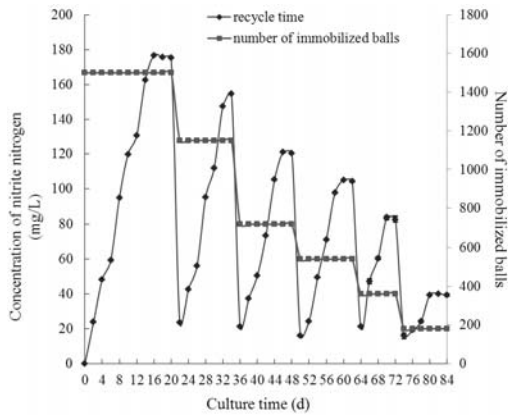


Fig. 4. Effect of recycling times on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria

The ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria under the above-mentioned conditions is presented as follows. When the concentration of calcium chloride is low, immobilized balls is soft and easily broken, when the calcium chloride concentration is higher, the microbial cell activity will be reduced due to the high osmotic pressure of salt which causes cell dehydration and microbial activity reduction. The nitrite nitrogen concentration increased with the increasing cultivation time. The optimal calcium chloride concentration was 2.0%, which produced the highest nitrite nitrogen concentration.

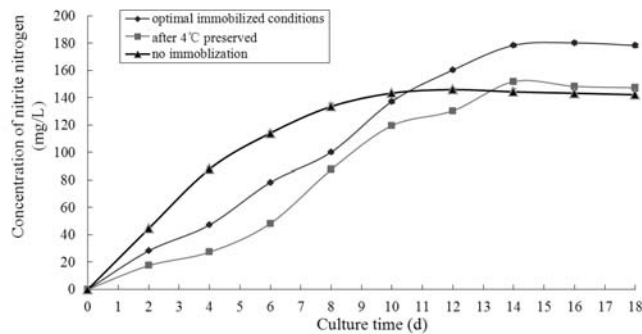


Fig. 5. Ammonium removal of no immobilized, immobilized and preserved at 4°C for one week ammonia-oxidizing bacteria

Effect of sodium alginate concentration on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria

To determine the effect of sodium alginate concentration on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria, the following immobilization conditions were employed: 2.0% calcium chloride; 3.5%, 4.0%, 4.5% and 5% sodium alginate; and 500 immobilized balls. The reaction was carried out in 250 ml shake flask containing 250ml of immobilized medium at pH 8.0, 30°C and 110 r/min for 12 days. The ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria under the above-mentioned conditions is shown as follows. Sodium alginate concentration affects the immobilized hardness. The higher concentration of sodium alginate, the harder immobilized strength. If the concentration is too high, immobilized cells won't grow. If the concentration is too small, immobilized balls be broken. The nitrite nitrogen concentration increased with the increasing cultivation time. The optimal sodium alginate concentration was 4.5%, which produced the highest nitrite nitrogen concentration.

Effect of pH on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria

To determine the effect of pH on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria, the following immobilization conditions were employed: 4.5% sodium alginate; 2.0% calcium chloride; and 1000 immobilized balls. The reaction was carried out in 500 ml shake flask containing 500 ml of immobilized medium, at different pH, 30°C and 110r/min for 20 days (Fig. 1). Strong acid and strong alkali will destroy all of the immobilized cells. Hydrogen ion affects the charge balance at cell surface and membrane permeability. Nutrients will unavailable by immobilized cells in strong acid culture medium. Nucleic acid and protein will be denaturation and the cell death in strong alkali culture medium. Nitrite nitrogen concentration increased with the increasing cultivation time. The optimal pH was 8, at which the highest nitrite nitrogen concentration was obtained.

Effect of temperature on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria

To determine the effect of temperature on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria, the following immobilization conditions were employed: 4.5% sodium alginate; 2.0% calcium chloride; and 1000 immobilized balls. The reaction was carried out in 500 ml shake flask containing 500 ml of immobilized medium at pH 8, different temperature, and 110 r/min for 18 days (Fig. 2). Metabolism of ammonia-oxidizing bacteria will slow and even inactive at high or low temperature. Nitrite nitrogen concentration increased with the increasing cultivation time. The optimal temperature was 30°C, at which the highest nitrite nitrogen concentration was obtained.

Effect of inoculum concentration on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria

To determine the effect of inoculum concentration on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria, the following immobilization conditions were employed: 4.5% sodium alginate; 2.0% calcium chloride; and 500, 1000 and 1500 immobilized balls. The reaction was conducted in 500 ml shake flask containing 500 ml of immobilized medium at pH 8, 30°C, and 110r/min for 18 days (Fig. 3). It is bad for immobilized ammonia-oxidizing bacteria development when inoculum is too high, because bacteria will compete with nutrition of culture medium. It is also bad when inoculum is too low, because nutrition of culture medium is not enough for immobilized ammonia-oxidizing bacteria metabolism. Therefore appropriate inoculum is good for immobilized ammonia-oxidizing bacteria. Nitrite nitrogen concentration increased with the increasing cultivation time. The nitrite nitrogen concentration was closed to 1500 immobilized balls' as 1000 immobilized balls were inoculated. In view of saving raw material, 1000 immobilized balls were used as the optimal inoculum in 500 ml immobilized medium.

Effect of recycling times on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria

To determine the effect of recycling times on the ammonia nitrogen removal ability of the immobilized ammonia-oxidizing bacteria, the following immobilization conditions were employed: 4.5% sodium alginate; 2.0% calcium

chloride; and 2000 immobilized balls. The reaction was conducted in 1000 ml shake flask containing 1000 ml of immobilized medium at pH 8, 30°C, and 110r/min for 18 days. Fresh immobilized medium was replaced periodically (Fig. 4).

If recycle times increase, the cells will gradually aging and metabolism slow down. When the immobilized cells grow, calcium alginate wall will expand and be easily broken. So if immobilized cells lose the protection, the cells will die. After the fifth recovery, the denitrifying capacity and activity of the immobilized ammonia-oxidizing bacteria were better preserved. Immobilized balls became big and broke constantly with increasing recycle times. After the sixth recovery, residual immobilized balls expanded too much to use.

Optimal immobilization conditions

The optimal immobilization conditions were as follows: 4.5% sodium , 2.0% calcium chloride, 2000 immobilized balls, pH of 8, and temperature of 30°C. The reaction was carried out in 1000ml shake flask containing 1000ml immobilized medium at 110r/min for 18 days (Fig. 5). Ammonia nitrogen removal results of no immobilized, immobilized and preserved at 4°C for one week.

Obviously, the ammonia-oxidizing bacteria that were immobilized under optimal conditions exhibited better ammonia nitrogen removal ability, when compared with the non-immobilized ones. Meanwhile immobilized ammonia-oxidizing bacteria still had strong ammonia nitrogen removal ability after preserving one week at 4°C. It means immobilized ammonia-oxidizing bacteria were good for preservation.

Capacity of ammoxidation of immobilized ammonia-oxidizing bacteria

Immobilized ammonia-oxidizing bacteria were cultured at the optimal conditions. Concentrations of nitrite nitrogen concentration, nitrate nitrogen concentration and ammonia nitrogen concentration were determined. Removal rate of ammonia nitrogen of immobilized ammonia-oxidizing bacteria reached 89.51%.

The ammonia-oxidizing bacteria that were immobilized under optimal conditions exhibited better ammonia nitrogen removal ability, when compared with the non-immobilized ones and were good for preservation. Optimal immobilization conditions were as follows: sodium alginate, 4.5%;

calcium chloride, 2.0%; and 2000 immobilized balls. The reaction was carried out in 1000ml shake flask containing 1000ml immobilized medium at pH 8, temperature 30°C, and 110r/min. Immobilized ammonia-oxidizing bacteria recycled six times under the optimal immobilization conditions. The ammonia nitrogen removal rate of the immobilized ammonia-oxidizing bacteria reached 89.51%.

Characteristics of the process of immobilization of immobilized ammonia-oxidizing bacteria by sodium alginate

The above experimental results showed that immobilized ammonia-oxidizing bacteria have strong resistance to adverse environment, insensitivity of temperature and pH changes, more stable, reusable, short reaction time, low cost and improved ammonia nitrogen removal ability.

Outlook of future research

Immobilized carriers and methods of immobilized ammonia-oxidizing bacteria need to be researched. For example set polyvinyl alcohol or microcapsule as the carriers and prepare immobilized cells using covalent or cross-linking method to determine the best immobilized carriers and methods. Quick preparation methods of immobilized cells should also be developed for continuous and industrialized operation. Immobilized ammonia-oxidizing bacteria of application should extended to small industrial scale.

CONCLUSIONS

Based on the findings obtained in the present study, the following conclusions could be drawn: 1. Hydrogen ion was necessary to immobilized ammonia-oxidizing bacteria for stable molecular structure of sodium alginate and molecular recognition between guluronic acid and mannuronic acid. The experimental results confirmed above theory, namely alkaline condition had greater influence on immobilized cell. The capacity of ammoxidation of immobilized ammonia-oxidizing bacteria was very low when pH was 10. 2. High and low culture temperature still had certain effect on immobilized ammonia-oxidizing bacteria. 3. Immobilized balls became big, outer calcific film became transparent, old culture medium became muddy with increased recycle times. It means ammonia-oxidizing bacteria isolated continuously

and had decreasing capacity of ammoxidation as increasing the number immobilized balls. 4. 4°C preservation had little effect on immobilized ammonia-oxidizing bacteria. It's convenient for industrialized production, preservation, transportation.

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