JOURNAL OF PURE AND APPLIED MICROBIOLOGY, APRIL 2014. Vol. **8**(2), p. 847-854

# Performance of the Immobilized Aerobic Denitrification Bacteria

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(Received: 06 October 2013; accepted: 28 December 2013)

**This paper aims to evaluate the nitrogen removal performance and the stability of the immobilized aerobic denitrification bacteria. The microbial immobilization method with polyvinyl alcohol (PVA) and sodium alginate (SA) as an immobilizing material are used to entrap the aerobic nitrifying bacteria *P. denitrification DL23*, and for testing performance and the activity of the immobilized beads. Two experiments will be conducted for the examinations of these performances by the researcher. The study of the stability of the particles reveals that 8h is the best cross-linking time with aspect of the mass transfer performance and the mechanical stability; the study of the performance of denitrification indicates that the effect of embedding extended the suitable range, which involves pH and temperature. The denitrification rate has remained above 70% in the process of 7 times' uses, indicates that the stable nitrogen removal of the immobilized bacteria for 14 days at least. The experimental result has a guiding role to the practices of immobilized aerobic denitrification.**

**Key words:** Nitrogen removal, Immobilization, Aerobic denitrification, *P. denitrification* DL23.

The Nitrogen pollution has caused the eutrophication, which is becoming one of the major water pollution problems in China water body. Biological denitrification has been used successfully in removing nitrates from wastewater to prevent eutrophication. The convention nitrogen removal theory presents nitrogen removal comes to wastewater only after the two processes: nitrification and denitrification, while the nitrogen removal process can only be conducted under nearly an exclusively anaerobic or microaerophilic trait (Zumft, 1997), so the immobilization technique only used for the nitrification bacteria. But some aerobic denitrifiers have been found useful towards nitrogen removal process. In 1980s, the aerobic denitrification bacteria *Thiosphaera pantotropha* (also called as *Paracoccus pantotrophus*) was isolated by Robertson firstly (Robertson, 1983). In recent years, more strains were reported with the further research.

With the development of wastewater treatment techniques, the widely access to immobilization has enabled people to use entrapped beads for the nitrogen removal. Embedding immobilization technique means that, the free cells or enzyme is to be pitched at the limited space which becoming the "immobilized cell" or "immobilized enzyme" by physical and chemical method. The immobilization technology has already appeared in 1960s, and in the 1969, the immobilized enzyme technology primary was used in production (Li Gang, 2002). In the following decades, the immobilized technology has been developed and applied rapidly and widely. It offers a promising potential for the improvement of the efficiency of bioprocess. Compared with free bacteria, immobilized bacteria has several

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advantages (Wang, 2002): (1) it can increase the biodegradation rate through a higher cell loading; (2) the bioprocess can be controlled more easily; (3) the continuous process can take place at a high dilution rate without washout; (4) the catalytic stability of biocatalysts as well as the tolerance against toxic compounds can be improved.

Due to immobilized cells being used for wastewater treatment, it needs to have excellent mechanical strength, mass transfer performance and the favorable performance of nitrogen removal. Cross-linking time is the main factor of the influences on mechanical strength and mass transfer performance of the immobilized beads, thus, the first part study is to investigate the effect of the across-linking time on mechanical strength and mass transfer performance of the immobilized cells to the determination of the suitability conditions. The foundation of the immobilized beads applications lies in the performance of nitrogen removal is, therefore, another part study is to examine the immobilization effect on the activity of the denitrification bacteria.

**Medium**

The DM(Liang *et al*., 2011) medium with the following composition was used for the main culture of the bacteria (per liter of deionized water): 13g of $C_4H_4Na_2O_4 \cdot 6H_2O$, 0.1g of $MgSO_4 \cdot 7H_2O$, 7.9g of $Na_2HPO_4 \cdot 12H_2O$, 1.5g of $KH_2PO_4$, 3g of $KNO_3$,2mL of trace element solution in 1L of distilled water (pH: 7.0~7.3). The NI medium contained(per liter of deionized water): 1.5g of $NH_4Cl$, 11g of Na-succinate$\cdot 6H_2O$, 0.1g of $MgSO_4 \cdot 7H_2O$, 6.7g of $Na_2HPO_4 \cdot 12H_2O$, 1g of $KH_2PO_4$, 2mL of trace element solution 1L of distilled water (pH: 7.0~7.3). The trace element solutions included 50.0g of EDTA, 2.2g of the $ZnSO_4$, 5.5g of $CaCl_2$, 2.06g of $MnCl_2 \cdot 4H_2O$,5.0g of $FeSO_4 \cdot 7H_2O$,1.1g of $(NH_4)_6Mo_7O_2 \cdot 7H_2O$,1.57g of $CuSO_4 \cdot 5H_2O$,1.61g of $CoCl_2 \cdot 6H_2O$. ·6H 2 O, 1.61; pH 7.0.

## EXPERIMENTAL

**Enrichment culture and strain isolation**

The activated sludge was taken from a secondary sedimentation tank at the Wenchang Waste Water Treatment Plant (Harbin, China), and acclimatized in the sequencing batch reactor (SBR). After that, 1 ml of the fresh sludge sample was taken from the SBR, inoculated in 100 ml Erlenmeyer flask with 9ml DM medium, and added several sterilized glass beads. The flask was inoculated at 28°C on a rotary shaker at 180 rpm. The final bacterial suspensions was diluted and streaked on DM medium agar plates, cultured at 28°C for 2~3 days. After repeating 5~6 times, the pure bacteria were isolated. Then all obtained strains were transferred into 250 ml flasks with 25ml DM and NI medium, cultured at 28°C and 160 rpm for 48 h. The concentrations of $NO_3^-$-*N*, $NO_2^-$-*N* and $NH_4^+$-*N* were measured at the end of the culture. The bacterium with the highest $NO_3^-$-*N*, $NO_2^-$-*N* and $NH_4^+$-*N* removal efficiency was obtained (Yang *et al*., 2011).

**Bacterial identification and physiological and biochemical characteristics**

The 16S rRNA gene sequences of the strains were amplified by PCR with primers named BSF8/20 (5'-AGAGTTTGATCCTGGCTCAG-3') and BSRl541/20(5'-AAGGAGGTGATC CAGCCG CA-3'). The 16S rRNA sequences used for the phylogenetic analysis were derived and compared with the the available data in GenBank by using BLAST (M adueno *et al*, 2011). Micro-biochemical tubes (Tianhe Co. Ltd., China) were used to examine physiological and biochemical characteristics of these strains.

**Immobilization methods**

The Immobilized cells were harvested in the cultured for 24h after bacteria of suspension by centrifugation ((3000 r/min, 5min) and washing with sterile deionized water, then obtained the bacteria 5g which were suspended with 10 mL physiological saline (5g/10mL). The embedding medium is prepared as mixed liquor of 6% of polyvinyl alcohol and 0.6% of sodium alginate, stirred and heated in the water bath at 90!. Mixed liquor of an embedding medium and bacterial suspension is taken to immobilize aerobic denitrifying bacterial cells, and the weight ratio of the embedding medium to bacterial suspension is 1: 10. And then the mixture was dropped into the crossing-link agent that saturated baric acid and 5% $CaC1_2$ solution, kept for 4 to 12 h to get beads (LI Chao-min *et al*., 2006). The beads were washed with physiological saline solution for 2 times, after that suck up the surface moisture and store at 4°C until further use. The process of immobilization is shown in figure 1.

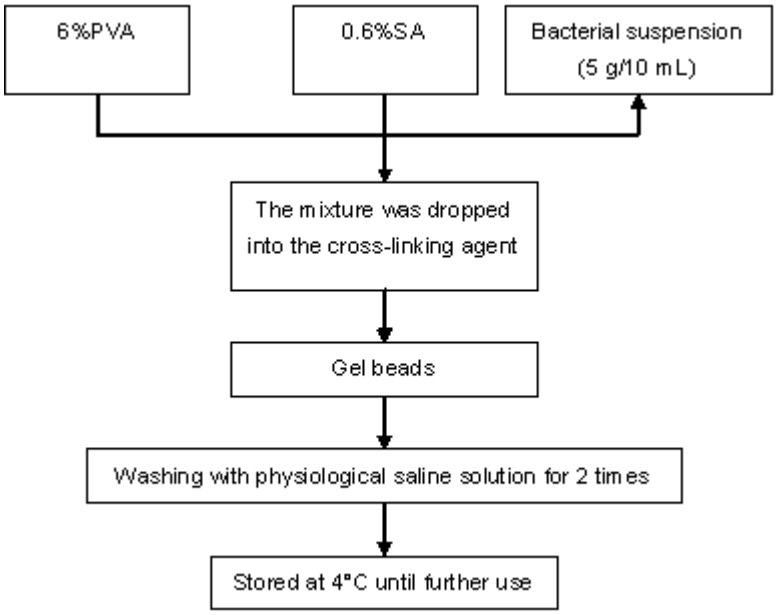


**Fig. 1.** The process of immobilization

**The immobilized particles performance test**

**Assay of the immobilize particle expandability**

The method is to pick and choose 20 similar immobilization particles, soak them in sterile deionized water and keep for 24h, measure all particles' diameter and average, and compare the mean value with the average diameter of the particles not soaked.

**Assay of the immobilized mechanical strength**

50 balls in similar dimension are chosen and placed into the 500ml beaker with 100 ml water filled, stirred for 5h at a rate of 500 r / min~3000 r / min. Evaluating the balls' mechanical strength by observing the changes of the diameter and the damage of the balls.

**Assay of the immobilization mass transfer performance**

To determine the immobilization particles' mass transfer performance, the steps are performed as follows: choose similar dimensions particles into the equal amount blue ink, observe the color of the particles that as indicators to qualitative judgment, at the same time.

**Determination of denitrification performance of immobilized beads**

To examine denitrification capacity of the immobilized balls batch text was set up three replicates for each treatment compared with the ability of nitrogen removal for free cells. The bacteria were harvested in the cultured for 24h after bacteria of suspension by centrifugation (3000 r/min, 5min), is then divided it in two with equal amount, the one is embedded by the former method, the other one preserved in the 0.9% saline that inoculation was 2% (v/v) and the bacterial suspension of OD600 around 1.5. The embedded bacteria inoculation was equal amount of the free bacteria. The culture is composed of sodium succinate as carbon source, C/N ratio of 10, at 37°C for 48 h and 160 rpm.

**The effect of pH on embedded bacteria**

This experiment intended to study the effect of pH on denitrification performance of the embedded bacteria under the same conditions that were cultured at 37°C for 48h and 160 rpm in the 500ml Erlenmeyer flask with 50 ml DM except at five different levels of pH i.e., 5, 6, 7, 8 and 9. The pH was measured using a pH meter. $NO_3^-$-*N* and *TN* was measured. The free bacteria suspension was repeated the above step as a control. Moreover, bacteria suspension by centrifugation (3000 r/min, 5min) before measures the nitrogen. $NO_3^-$-*N* was measured by ultraviolet spectrophotometry and $NO_2^-$*N* was measured by the N-(1-naphthalene)-diaminoethane photometry method. *TN* was determined by *UV* spectrophotometry, respectively (SEPA, 2002).

**The effect of temperature on embedded bacteria**

To observe the effects of incubation temperature on the immobilized balls, the temperature was adjusted to 20, 24, 28, 32 and 40°C. *DL-23* has low nitrogen removal rate at 20°C. Thus, this trial focuses on the adaptability of the low temperature after immobilization. The same culture condition follows the former except for the pH 6.0. Then *TN* was measured. The condition is that cultured immobilized particles in the 500 ml Erlenmeyer flask with 50 ml DM at 20°C for 60h, and 160 rpm. Measuring the nitrogen removal efficiency was repeated 3 times and averaged.

**Determination of the operational stability of the immobilization particles**

The operational stability of the immobilization particles is investigated by continuous nitrogen removal texts. The initial concentration of $NO_3^-$-N was 280 mg / L, C/N 10 at 37°C for 160 rpm runs the cycle for 48 h. Filtered off the immobilized beads after the end of each cycle, and then washed with distilled water and filtered. Running the next cycle under the same conditions until its removal rate of $NO_3^-$-N was significantly lower.

## RESULTS

**Isolation and identification of denitrifying bacteria**

After incubation, a total of 25 pure bacteria colonies were obtained from Wenchang Waste Water Treatment Plant, the aerobic denitrification and heterotrophic nitrification abilities of 10 isolates among colonies are tabulated in Table 1. Among them, one purified isolates showing higher denitriûcation efûciency was obtained and named as *DL-23*. So it was selected for further studies. The result of physiological and biochemical tests about strains *DL-23* is shown in Table 2. The 16S rRNA gene sequence of *DL-23* shared 100% similarity with *P. denitrificans DSM413* by comparing with data available in GenBank(Figure3). Thus, we designated *DL-23* as *P. denitrificans*. The phylogenetic tree showed that the strain *DL-23* was closely related to *P. denitrificans DSM413*.

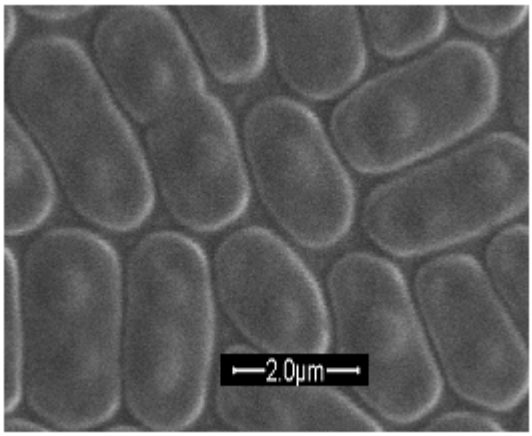


**Fig. 2.** Scanning electron micrograph of strain *DL-23*

**Table 1**. Taxonomical characteristics of DL-23

| Test | Result | Test | Result |
|---|---|---|---|
| Gram's | - | Oxidase | + |
| Growth temperature (°C) | 15~45 | Catalase | + |
| Growth pH | 6.0~11.0 | Amylase | - |
| $H_2S$ production | - | Dextrose | + |
| M-R reaction | + | Methanol | + |
| V-P reaction | + | Maltose | + |
| Gelatin liquefaction | - | Mannose | + |
| Sorbierite | + | Ethanol | + |
| Citrate | - | Phaseomannite | + |
| Casein hydrolysate | + | Mannite | + |
| Colony morphology | Round | Margin | Regular |
| Elevation | Raised | Surface | Smooth |
| Density | Opaque | Pigment Light | yellow |
| Shape | Rod | Size | Short |
| Arrangement | single | | |

(+),positive reaction; (“), negative reaction.

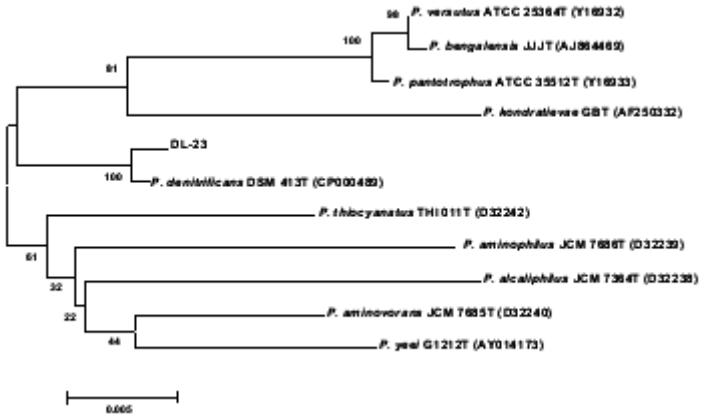


**Fig. 3.** Phylogenetic tree of *DL-23* based on a comparison of the 16S rDNA gene sequence

**Table 2.** The nitrogen removal ability of 10 bacteria isolates

| Culture condition | Isolated number | $NO_3^-$-N(420mg/L) | | | $NH_4^+$-N(285mg/L) | |
|---|---|---|---|---|---|---|
| | | $OD_{600}$ | $NO_2^-$-N | $NO_3^-$-N | $OD_{600}$ | $NH_4^+$-N |
| Carbon Source: Succinate C/N ratio: 15 Shaking Speed: 160 rpm Temperature: 28 °C | *DL-2* | 2.2 | 45 | 27 | 2.8 | 12 |
| | *DL-4* | 1.1 | 167 | 3.2 | 3.4 | 0 |
| | *DL-7* | 1.3 | 122 | 18 | 3.1 | 8 |
| | *DL-8* | 1.9 | 71 | 0 | 2.7 | 0 |
| | *DL-12* | 1.6 | 89 | 12 | 3.5 | 13 |
| | *DL-16* | 1.8 | 55 | 23 | 3 | 0 |
| | *DL-17* | 2.2 | 62 | 0 | 2.8 | 0 |
| | *DL-20* | 2.3 | 31 | 0 | 3.1 | 0 |
| | *DL-23* | 2.5 | 17 | 0 | 2.9 | 0 |
| | *DL-25* | 2.3 | 42 | 0 | 3.3 | 0 |

**The immobilized particles performance test**

The stability of embedding particles in the different cross-linking time shows in Table 1. With the extension of cross-linking time, the mechanical strength becomes better, the expansibility also increases, on the contrary, the mass transfer capacity of the beads cross-linked for 8h and 12h is better than 24h (Table 3). The reason for this result is that the strength of beads is increased with the extension of cross-linking time, and its internal structure becomes dense which enhance the mass transfer resistance of the reaction substrates and nutrient, so the activity of microbial cells is decreased. It can be concluded that 8h is the best cross-linking time for the mass transfer performance and mechanical strength.

**Table 3**. The stability of embedded bacteria under different crosslinking times

| 0.6%SA 6%PVA | 8 h | 12 h | 24 h |
|---|---|---|---|
| Sphericity | Good | Good | Good |
| Operation difficulty | General | General | General |
| Expansibility | + + | + + | + + + |
| Mass transfer performance | + + + | + + + | + + |
| Mechanical strength | + | + + | + + + |

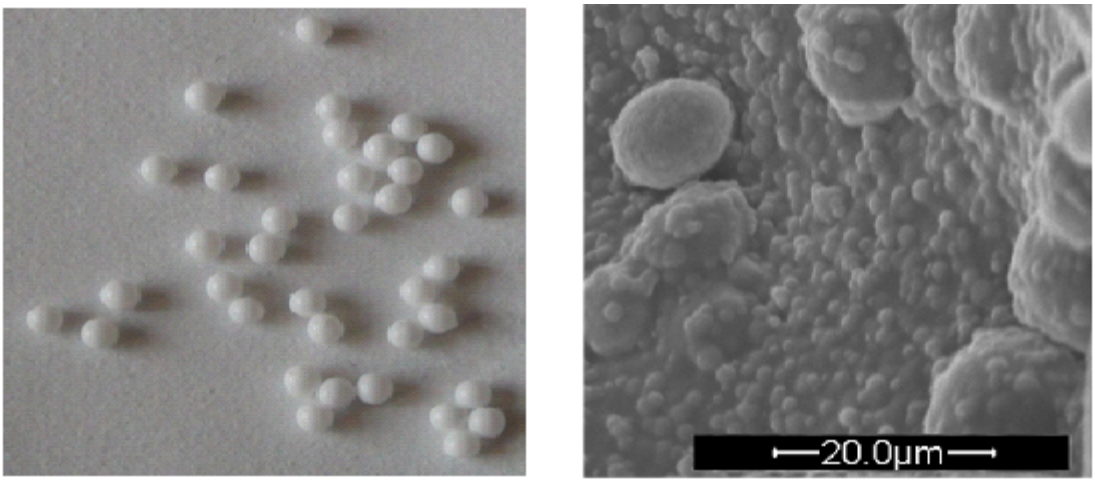


**Fig. 4.** Immobilized ball appearance and scanning electron microscopy

**The effect of pH on embedded bacteria**

Theoretically, the pH range of reaction system for the immobilized bacteria is wider than the free bacteria which are more sensitive. At pH 5, the bioactivity of the free cells is not showed, but the immobilized bacteria demonstrate bioactivity: $NO_3^-$-N and TN removal efficiency, the removal rate of $NO_3^-$-N and TN are 25%, 7.4%, respectively; the pH between 6~8, the removal efficiency of the entrapped bacteria is decreased compared with the free bacteria. It indicates that there are two main reasons: one is that some cells maybe lose its bioactivity during the embedding process; another one is that the certain mass transfer resistance comes from the particles' internal structure, so the transport rate between bacteria and reaction substrate is reduced; at pH 9, the efficiency removal of the free bacteria as controls, the embedded bacteria is increased which the removal rate of $NO_3^-$-N and TN are increased by14% and 9%, respectively (Fig. 5). In conclusion, the efficiency removal has certain reduced at pH between 6~8 after being embedded, but it causes extension of the pH range and the performance of nitrogen removal has significantly improved at pH 5 or 9.That is to say, the embedded bacteria were acid resistant, also showed resistance at alkaline.

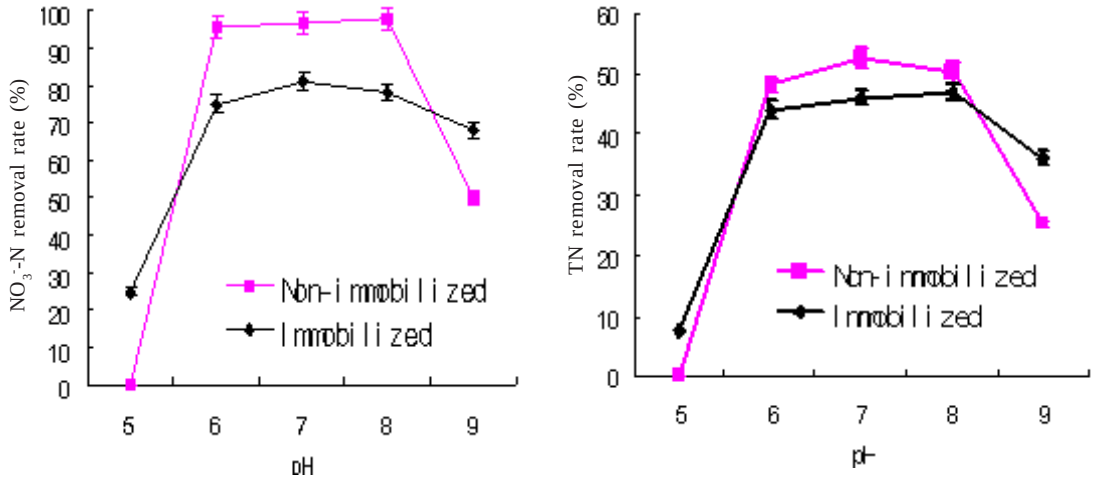


**Fig. 5.** Influence of pH value on the denitrification of free and immobilized bacteria

**The effect of temperature on embedded bacteria**

The test indicates that, with free bacteria in low bioactivity at the 20°C, the reaction produces nitrite nitrogen to be accumulated that results in decreasing removal rate of TN. Figure 6 shows that, compared with free bacteria, the embedded bacteria acquired more tolerance to low temperature environments of 20 °C and 24 °C, and the TN removal rate increased 4% and 3.7% respectively. At 28~37 °C, TN removal rate was decreased. When the embedded bacteria at the high temperature conditions of 42 °C, the removal rate of TN was higher than that of free bacteria. The results showed the denitrification has reduced the temperature degree influences on the strain *DL-23* to be reduced, and enlarged the temperature limit to be expanded.

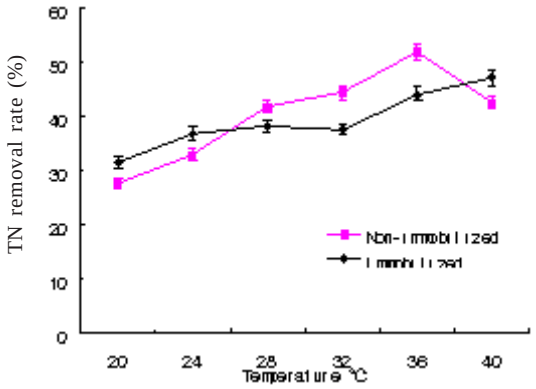


**Fig 6.** The nitrate reduction of free and immobilized bacteria at 20°C

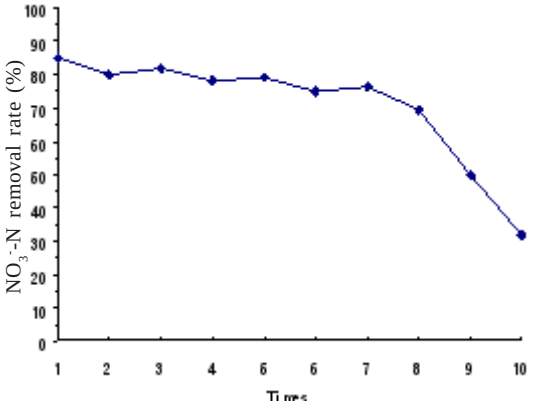


**Fig. 7.** Recycled use of the immobilized bacteria

**Determination of the operational stability of the immobilization particles**

In figure 7, with the increasing frequency of use, the immobilized bacteria denitrification rate is decreased and fluctuated obviously. It is possibly because there are some differences in the beginning of the denitrification of the internal bacteria of immobilization in every cycle. At the former 7 times of the use process, the denitrification rate has remained above 70%, which indicates that the stable nitrogen removal of the immobilized bacteria for a minimum of 14 days.

In practical application, one of the advantages of immobilized particles is that it can be reused, which could reduce the procedure and the cost. The result of continuous removal nitrogen test of the embedded particles demonstrates that immobilization provides a better microenvironment for the bacteria, and lower the external environment interference for the denitrification, which will be more convenient to play an effective role in the aerobic denitrification.

## CONCLUSIONS

In this study, one aerobic highest denitrifying bacteria *DL-23* was isolated and identiûed as *P. denitrificans DSM413*. Immobilization tests indicated that polyvinyl alcohol (PVA) is a promising type of fixed material, which is both economical and non-toxic. It is very suitable for microbial immobilization. But the drawback of using PVA beads is their high tendency to agglomerate due to the large number of the Hydroxyl groups which give rise to cohesion automatically. However, sodium alginate can improve the shortcoming of PVA gel, making the sphericity well and preventing it from obvious adhesion phenomenon when used in combination. Mechanical strength is related to the service life of immobilized beads; therefore, it becomes extremely important to study the mechanical strength of beads.

In this test, the immobilized beads obtained after the crosslinking time optimization

tests have the advantages of high mechanical strength, high activity and good stability. 8 hours is the best optimized condition with the entrapped particles hard, the shape in regular sphere and the expansibility not obviously in the different cross-linking time. But the expansibility is not obvious in the different cross-linking time. The tolerance of pH is enhanced after *DL-23* being immobilized. The denitrification rate is raised under pH 5and pH 9. By embedded, its inhibition of denitrification is reduced in the range of its application both at the low and high temperature. It shows the advantage of immobilized denitrifying bacteria with good operational stability, and continuous effective nitrogen removal of up to 14 days.

By comparing the temperature and pH effect on the activity of the non-immobilized bacteria and immobilized bacteria, it shows advantage after immobilization, but the average nitrogen removal rate of immobilization particles is declined by 10%~15%. It may be caused by the entrapped carrier's diffusion resistance on the substrate, which influenced the bacteria growth and the metabolism. Therefore, the immobilization method still has to be improved to enhance the mass transfer performance and regain its high-efficient denitrification characteristics.

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