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Nitrogen Removal by a Fungal Aerobic Denitrifier of *Penicillium* Strain

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Abstract: A kind of aerobic *Penicillium* that can remove ammonia, nitrite and nitrate was isolated through an improved bromothymol blue (BTB) selective culture medium method in this experiment and then the nitrogen removal by the strain was detailedly investigated. The results showed that this strain was able to make use of many kinds of organic carbon compounds as sole carbon source for the removal of the three types of inorganic nitrogen compounds but the way of removal was different. Ammonia was assimilated for forming cell components such as amino acid and protein, different from which, nitrite and nitrate were eliminated by the aid of dual assimilation and denitrification. When the three types of nitrogen coexist, the removal order was as follows: ammonia>nitrite>nitrate. Type of carbon source, initial nitrogen concentration and carbon nitrogen ratio (C/N) all had different effect on final solution pH, dry weight, nitrogen removal rate and removal ability of the strain. It was tested that non-polar organic carbon source containing -CH₃ group like sucrose was inclined to be used by the strain. When sucrose was carbon source, the optimum C/N of ammonia, nitrite and nitrate removal were separate 4-6, 8-12 and 12-16. In addition, it was demonstrated with calculation that the removal abilities of the above mentioned three nitrogen of the strain were about 50, 60 and 90 mg g⁻¹ respectively, showing its tremendous capability of nitrogen removal.

Key words: Aerobic denitrification, fungus, *Penicillium*, nitrogen removal, assimilation, nitrogen pollution

INTRODUCTION

Saccharides and proteins generated in the food production process and aquaculture are largely discharged into the water environment and then these compounds are decomposed into ammonia and other small molecular nitrogen compounds by organisms. Water body pollution and eutrophication caused by these nitrogen not only reduce its ornamental value but also endanger hydro-biological lives (Macilwain, 1995). Moreover, nitrite and nitrate can induce methemoglobinemia and cancer which influences severely on human health. Therefore, it is vital important to remove these nitrogen from water.

Now-a-days, biological denitrification technology is the only economically feasible and environmentally friendly method for the water treatment with large water volume and low absolute nitrogen concentration (Mateju *et al.*, 1992; Gupta and Gupta, 2001). Aerobic denitrification becomes a research hotspot since the discovery of aerobic denitrifying phenomenon and denitrifier bacteria. Its advantages have two main aspects as follows: first, aerobic denitrifiers can conduct denitrification under aerobic conditions, thus they are able to be used for nitrogen treatment of aerobic waters

like rivers, lakes and oceans. Second, organic carbon sources are provided as electron donors for denitrification, resulting in both COD and nitrogen elimination. Due to these superiorities, the screening techniques were extensively researched by researchers and an increasing number of excellent aerobic denitrifiers were isolated and studied (Kim *et al.*, 2005; Huang and Tseng, 2001). However, it was found from these studies that most bacterial denitrifiers can only use micro-molecule carbon and nitrogen compounds and the availability of macro-molecule carbon and nitrogen compounds was very low. This strict requirement of carbon source and nitrogen source imposes restrictions on the removal effect of nitrogen and COD in the real water environment. Comparatively speaking, fungi that have much better assimilation ability of complicated nitrogen and carbon compounds are more promising microbes in the nitrogen removal process. Many researchers revealed that some *Penicillium* such as *Penicillium canescens* had an excellent capacity of utilizing complicated nitrogen compounds (Aleksenko *et al.*, 1995). Renosto *et al.* (1981) successfully purified nitrate reductase from the mycelium of *Penicillium notatum*, verifying the real existing of nitrate reductase in the *Penicillium*. Furthermore, some

strains of *Penicillium* that have the ability of heterotrophic ammonia oxidation can translate ammonia into nitrite and nitrate (Wang and Yu, 2010). Even, ammonia would become the regulator of the uptake system of *Penicillium chrysogenum* inducing the nitrate absorption by mycelium at the shortage of ammonia (Glodsmith *et al.*, 1973). These discoveries indicated that *Penicillium* was a kind of potential excellent nitrogen remover. This paper successfully isolated a *Penicillium* strain named ST2 that had the capacity of aerobic denitrification and then its nitrogen removal ability was comprehensively studied under different conditions which would provide a theoretical foundation for the practical application of this strain.

MATERIALS AND METHODS

Microorganism: Kiaochow bay locates in the shore of Yellow Sea of Shan Dong province of China and many cities are around the bay. Therefore, it became one of the most nitrogen polluted area in China because many nitrogen compounds were input into the bay along with the running water of many seaside rivers around the bay. The HouJia river that was heavily polluted by agricultural effluent and domestic sewage was one of the seaside rivers of Kiaochow bay. The sludge was collected from this river in May 2011 and the denitrifier was isolated from the substrate sludge through the serial dilution method and the improved bromothymol blue (BTB) selective culture medium method. The pollution index of the supernatant of the substrate sludge was showed in Table 1. The improved BTB medium consisted of 0.01 g L⁻¹ bromothymol blue (BTB, dissolved in 0.5 mL ethyl alcohol), 20 g L⁻¹ agar, 1 g L⁻¹ NaNO₃, 1 g L⁻¹ KH₂PO₄, 0.15 g L⁻¹ NaCl, 8 g L⁻¹ CH₃COONa.3H₂O, the pH of it was adjusted to 6.5, then the medium was autoclaved at 121°C for 20 min. According to the concentration gradient, the cell supernatant of the substrate sludge was diluted 10³, 10⁴...10⁹ times by deionized water. Then, 0.2 mL homogeneous diluent was pipetted by a micropipettor and subsequently coated BTB selective culture medium. After that, these culture

dishes were incubated at 30°C for seven days in the electro heating standing temperature cultivator. Several distinct different single fungus colonies were observed after five days and some parts of BTB medium had changed from green to blue which suggested that these fungi were possibly relating to possessing denitrifying ability. Soon afterwards, these fungal cells were picked out and purified by successive streak transfer on BTB medium. Afterwards, this fungus was inoculated into LB liquid medium which was cultured in the constant temperature incubator with 160 r min⁻¹ of rotating speed at 30°C for three days. After done, some drops of nitrite chromogenic reagent were added into LB medium, this strain was proved having aerobic denitrification ability if the color of LB medium showed red. The nitrite chromogenic reagent consisted 4-aminobenzene sulfonamide, 20 g; N-1-naphthyl ethylenediamine hydrochloride, 1 g; phosphoric acid, 50 mL; water, 250 mL and was diluted to 500 mL. The LB liquid medium contained NaNO₃: 1 g L⁻¹, KH₂PO₄: 1 g L⁻¹, NaCl: 0.15 g L⁻¹, CH₃COONa.3H₂O: 8 g L⁻¹, the pH of it was adjusted to 7.0, then the medium was autoclaved at 121°C for 20 min.

18S rDNA gene sequencing and morphological observation:

The DNA of the strain was extracted by a UNIQ-10 column type fungus DNA Isolation Kit and then was amplified through Polymerase Chain Reaction (PCR) with universal primers (NS1,5'GTAGTCATATGCTTGTCTC3' and NS6,5'GCATCACAGACCTGTTATTGCTC3'). The PCR reaction system was composed of 10 pM of template, 1 µL of primer up (10 µM), 1 µL of primer down (10 µM), 1 µL of dNTP mix (10 Mm each), 5 µL 10**Taq* reaction Buffer, 0.25 µL *Taq* (5 µ µL⁻¹) and then added water till 50 µL. Genes were amplified by first 5 min pre-denaturation at 98°C, then 35 cycles of denaturation at 95°C for 35 sec, being annealed at 55°C for 35 sec subsequently, elongation at 72°C for 40 sec, then final 8 min extension at 72°C. After that, the PCR products were purified and sequenced by a DNA sequencer (3730, ABI, USA). The matching of the closest sequences in the GenBank database depended on the online BLAST program and MycoBank. To confirm the genetic result, some cells were fetched from plating medium and then observed under microscope (Fluorescence Convert Microscope, LEICA, DMI3000B, Germany).

Carbon source utilization: The availability of different substrate was assessed by testing seven carbon source including sodium acetate, sodium citrate, glucose, soluble starch, sucrose, sodium oxalate and sodium succinate. The homogeneous spore suspension was prepared by

Table 1: The pollution index of the supernatant

Index	COD _{Cr}	TN	NH ₄ -N	NO ₃ -N	DO	TP	Cl ⁻	pH
Concentration (mg L ⁻¹)	57	6.33	0.875	4.53	5.9	0.134	187	8.6
Index	Cd	Cu	Zn	Ni	Pb	Mn	Cr	Hg
Concentration (mg L ⁻¹)	0.0041	N	N	0.012	0.0066	N	N	N

COD_{Cr}: Potassium dichromate method, Ammonia, reagent colorimetric method, DO: Dissolved oxygen meter (HengXin AZ8403, Taiwan), TN-TP: High-temperature digestion colorimetric method, Cl⁻: Silver nitrate titration method, pH: pH meter, Heavy metal, atomic absorption spectrometry, N: Undetected

transferring some spores from the plate medium into 10 mL aseptic water and then 0.5 mL of the prepared suspension was incubated into each above different carbon source medium. Subsequently, these mediums, were cultured in a shaking table with a stirring rate of 160 rpm at 30°C for seven days, then filtered. Thereafter, the nitrate removal rate, nitrite concentration and pH of the filtrate are analyzed. All experiments are carried out in the conical flask with standard volume of 250 mL. In addition, a ten-day time series experiment was carried out for investigating the nitrate and carbon source (COD_{cr}) removal ability of ST2 strain. One sample was taken out at the frequency of 1 time per day for analysis after inoculation. Each sample was performed in duplicate and the average value was used for plotting in the whole experiments. In order to get exact dry weight, filter paper and filtered filter paper were both oven dried at 80°C for 4 h and exposed to air until the weight became constant. The inoculation, culture and analytical methods of all next experiments were similar to the carbon source utilization experiment.

Effect of initial nitrogen concentration and C/N: Sucrose was set as carbon source and ammonia, nitrite, nitrate were set as nitrogen source in these two experiments. In the initial nitrogen concentration experiment, the concentration was set by the concentration gradient under the same C/N. In the C/N experiment, the C/N of ammonia medium was set as 4, 6, 8, 10 and 12 and the C/N of nitrite and nitrate medium were both set as 4, 8, 12, 16 and 20.

Nitrogen removal process: An eight-day time series experiment was carried out for making sure the utilization competition relationship of the three types of nitrogen by this strain. One sample was taken out at the frequency of 1 time per day for analysis after inoculation which was used for investigating the growth of denitrifying microorganisms and the removal situation of the three kinds of nitrogen.

RESULTS

Strain identification: ST2 strain was preliminary estimated as a kind of fungus because of the obvious villiform colony characteristic. There were many hyphae seen under the microscope, the shape of sporophore and conidium looked like a broom and the conidium appeared round shape and short chain. These distinguishing feature were the same as fungus *Penicillium*. In addition, the gene fragment homology result both indicated that this strain had a 100% similarity with the fungus

Penicillium by the identification program of GenBank and MycoBank. The follow-up study showed that ST2 strain grew faster in organic medium than in non-organic medium, it can grow between 10 and 45°C but the optimum temperature for growth was 30°C. The tolerable pH of the strain was between 3 and 9 and the best pH was between 6 and 8. Moreover, the strain can survive in the medium of up to 7% of salt concentration.

Carbon source utilization: As Table 2 shown, ST2 strain can make use of sodium acetate, glucose, soluble starch and sucrose but sodium succinate, sodium citrate and sodium oxalate were not used. It was revealed from the ammonia removal test that carbon source had a great influence on the removal rate. The lowest removal rate of 35.75% was achieved in the sodium acetate medium but the highest one, obtained in the solutions of soluble starch, was more than two times than the lowest value. However in the aspect of dry weight, glucose medium got the maximum value of 1.867 g L⁻¹ which was three times than that of sodium citrate medium. The dry weights of soluble starch and sucrose that were all more than two times than that of sodium citrate medium also appeared relatively high. Based on these experimental data, the ammonia removal abilities of ST2 were calculated as the highest of 58.65 mg g⁻¹ and lowest of 25.68 mg g⁻¹ in the sucrose and glucose medium respectively and the later was less than half of the former. Moreover, the other two ammonia removal abilities of sodium acetate and soluble starch also exhibited differently. The pH of sodium acetate sharply rose from 7 to 8.81 whereas that they all decreased significantly to about 2.8 in the other three solutions of non-polar organic carbon source. It was markedly that the acid-fast ability of ST2 strain was much stronger than the alkali-resistant ability, the strain cannot survive in the

Table 2: The carbon source utilization of the strain

Carbon source	Ammonia removal rate (%)	Dry weight (g L ⁻¹)	Ammonia removal ability (mg g ⁻¹)	Initial pH	Final pH
Sodium acetate	35.75	0.624	46.83	7.02	8.81
Glucose	58.65	1.867	25.68	6.81	2.79
Soluble starch	79.74	1.683	38.68	6.70	2.81
Sucrose	68.53	1.393	58.65	7.03	2.82

Carbon source	Nitrate removal rate (%)	Dry weight (g L ⁻¹)	Nitrate removal ability (mg g ⁻¹)	Initial pH	Final pH
Sodium acetate	5.74	0.587	15.95	6.72	9.03
Glucose	81.21	1.868	70.96	6.80	5.50
Soluble starch	64.53	1.685	62.47	7.52	7.07
Sucrose	99.66	1.744	93.23	7.57	6.40

Initial ammonia and nitrate concentration were 81.74 and 163.13 mg L⁻¹, respectively, Solution pH was controlled at approximately 7 and C/N was set as 6 and 30 for ammonia and nitrate, respectively, Each sample was performed in duplicate and the average value was used for this table, The errors were all controlled below 5%

solutions of strong acid ($\text{pH} < 3$) or strong basic ($\text{pH} > 9$). Obviously, carbon source type which firstly influenced the metabolic process of the strain then significantly changed the pH of solution. Moreover, no nitrite was detected in all ammonia mediums, showing that ammonia removal by ST2 strain was a typical assimilation process.

The similar result that carbon source had a great influence on the removal rate was found in the nitrate removal test. The nitrate removal rate of sodium acetate was the lowest of only 5.74% which was clearly due to the inhibitory action of alkaline because the solution pH quickly achieved the highest tolerable pH value of 9. On the contrary, the removal rates of other three non-polar carbon source were all more than 60% and sucrose solution got the maximum of nearly 100% which was most attributed to the relatively stable pH. Because even if carbon source type had discriminatory effect on pH change, the final pH of the three solutions were all inside the scope of 5.5-7.07 and this pH range evidently had no inhibiting effect on cell growth and denitrification. The dry weights of sodium acetate, glucose and soluble starch had little difference between ammonia and nitrate solutions. But in the sucrose medium, the dry weight of nitrate solution increased by 25.2% comparing with that of ammonia solution. Therefore, the variation of nitrate removal and dry weight can result in discrepant removal ability. The maximum ability was got in sucrose solution with the value of 93.23 mg g^{-1} which was almost six times than the ability using sodium acetate as carbon source. Furthermore, the strain also showed good nitrate removal

ability in the other two non-organic carbon source mediums and the removal ability in size order was: sucrose > glucose > soluble starch > sodium acetate. Meanwhile, ST2 strain also exhibited an excellent nitrite removal capacity. When nitrogen source was nitrate, nitrite was detected only in soluble starch solution with the low concentration of 0.06 mg L^{-1} among the four solutions in the present of sufficient carbon source.

In the nitrate and COD removal process (Fig. 1), nitrate concentration slowly declined from 80 to 68 mg L^{-1} as time goes on from the first day to the third day. A small number of cells was observed and the dry weights were all no more than 0.015 g L^{-1} . At the same time, COD and pH were also gently reduced from 6.8 to 6.4 g L^{-1} and from 7.46 to 7.21, respectively. Obviously from the above mentioned indexes, the nitrate and COD removal effect was not efficient at the early growth period. After one day and surprisingly, an explosion in growth of ST2 strain occurred and the dry weight climbed steeply to 0.875 g L^{-1} , the concentrations of nitrate and COD dramatically decreased and the removal rates of nitrate and COD exceeded 50 and 40%, respectively. Simultaneously, solution pH remarkably decreased from 7.21 to 6.55. These performance indicated that cell growth and denitrification activity started to work actually since then. Within the next few days, dry cell weight kept going up to about 1 g L^{-1} and then maintaining this weight hereafter. In the meantime, nitrate and COD concentration decreased continuously with time and the final removal rates were separately more than 99 and 90%. In the latter

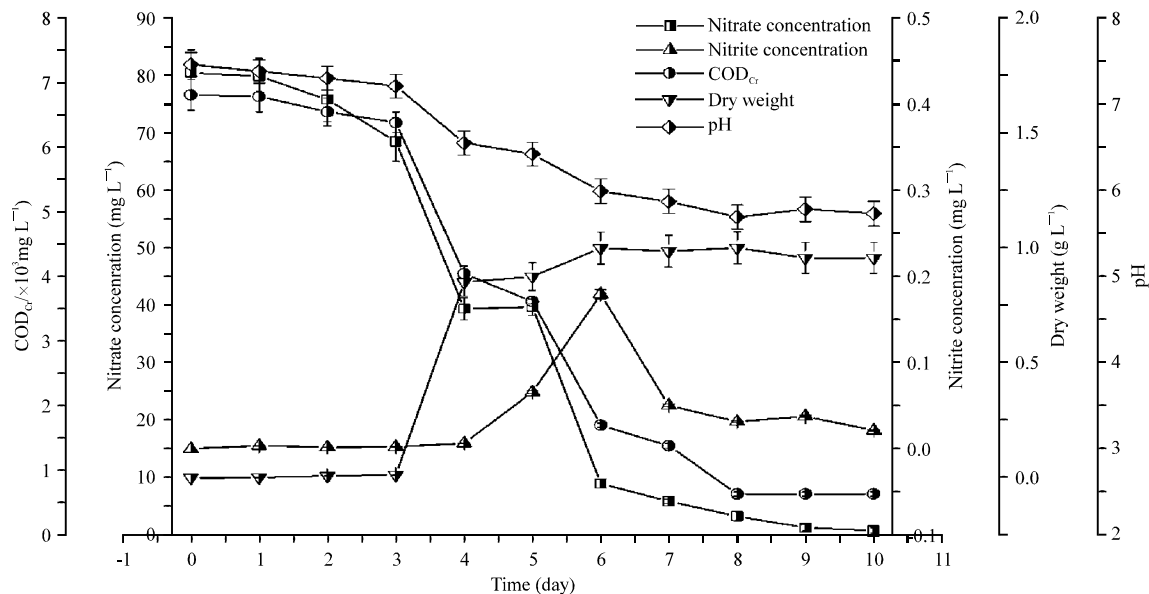


Fig. 1: Nitrate removal process, Initial nitrate concentration was 80.44 mg L^{-1} , initial COD was 6.81 g L^{-1} and initial pH was 7.46

half process, pH continued falling slowly to the minimum value of 5.7. In particular, a mass of nitrate and COD were eliminated in the condition of nearly unchanged cell dry weight which possibly mainly depended on denitrification. Nitrite was generated more and more in company with the increasing cells and denitrification activity in the preliminary and middle stage of the test and the nitrite accumulation climbed up from none to the maximum of 0.18 mg L⁻¹ after six days. Afterwards, nitrite concentration started falling when most nitrate was removed and it was soon consumed to below 0.05 mg L⁻¹.

The activity of periplasm reductase of aerobic bacteria denitrifiers was enhanced by the intermediate products of tricarboxylic acid cycle such as citrate and acetate, thus some researchers found that a higher denitrification efficiency was obtained using acetate as carbon source than using glucose (Oguz *et al.*, 2006; Sears *et al.*, 2000). However, this experimental result did not reflect this law. In contrast, sucrose showed up its excellent capacity of assimilation in the multiplicative process of the strain, indicating that sucrose was one of good choice of growth substratum and electron donors that easily being utilized by the strain. Even, the nitrogen removal rate and removal ability were both higher in sucrose medium than in sodium acetate medium which was so different from the conclusion of other learners. If sucrose and sodium acetate display their own advantage in the aspect of supporting strain growth and serving as electron donors respectively, the denitrification efficiency of the mixed carbon source solution will be certainly higher than that of single carbon source solution when the C/N of solutions are the same. In order to verify the above conjecture, another contrast experiment was designed and carried out. The result was listed in Table 3.

The result that obtained was different from the speculation. The nitrate removal rate was 43.19% in the mixed carbon source medium and the rate was much higher than the rate of 3.7% in sodium acetate medium whereas that it was less than half of the rate in sucrose medium. Analogously, the dry weight of mixed medium was 1.669 g L⁻¹ which was between the values of two single carbon source solutions. However, the dry weight

of mixed medium was far more than 0.394 g L⁻¹ of sodium acetate solution but it was weaker than 1.747 g L⁻¹ of sucrose medium. This difference was possibly ascribed to the different acid-alkali production process that was determined by carbon source type. The acid generated by utilizing sucrose neutralized the alkali generated by consuming sodium acetate which deregulated the alkalinity restriction of the use of sodium acetate and thus, most sodium acetate can be consumed by the strain. This explanation also can be confirmed by the pH change because the pH of mixed solution was between that of the two single carbon source solutions.

It had been proved from former experimental result that sucrose was an easy assimilative carbon source for this strain. So, if sodium acetate was a much easier carbon source that was used as electron donor for denitrification, the nitrate removal ability of the strain in the mixed solution was certainly higher than that in the single solution. However, this result was not the case. The removal ability of the strain in mixed carbon source solution, sodium acetate solution and sucrose solution were 41.82, 15.17 and 90.62 mg g⁻¹, respectively. Obviously, the removal ability of the strain in mixed carbon source solution was between the two single solutions. These consequences fully testified that sucrose and sodium acetate both showed no preference between the two different roles.

Effect of initial nitrogen concentration and C/N: The effect of initial nitrogen concentration was shown in Fig. 2. In the initial ammonia concentration test, a lower removal rate of 68.5% was obtained when the ammonia concentration was the highest of 81.7 mg L⁻¹ and the pH performed the greatest dropping to the lowest tolerable value of 2.82 which was the reason for the low removal rate. While, the rates of other four solutions all reached 100% and the final pH that decreased obviously fluctuated between 3.27 and 7.45 in all the four mediums. These pH did not reach the tolerable value which was contributed to the 100% ammonia removal rate. Cell dry weight increased with the increasing initial ammonia concentration and even they presented a good linear relation, indirectly indicating that the ammonia removal abilities of the strain changed little (47-63 mg g⁻¹) with the change of initial ammonia concentration.

All nitrites were eliminated in various initial nitrite concentration solutions but their pH changed little (6.6<final pH<7.4). No toxic action was observed even in the concentrated nitrite solution of 61 mg L⁻¹. Cell dry weight increased from about 0.14 to about 1 g L⁻¹ when the initial nitrite concentration increased from 7.62 to 61 mg L⁻¹, noteworthy, dry weight and initial nitrite

Table 3: The contrast experiment of nitrate removal by mixed or single carbon source

Carbon source	Initial pH	Final pH	Nitrate removal rate (%)	Dry weight (g L ⁻¹)	Nitrate removal ability (mg g ⁻¹)
Sodium acetate	7.04	8.76	3.7	0.394	15.17
Sucrose	7.12	6.64	89.71	1.747	90.62
Sodium acetate+sucrose	6.61	8.22	43.19	1.669	41.82

The C/N was set as 30 in single carbon source solution and the C/N of sodium acetate and sucrose were both set as 15 in the mixed carbon source solution. Initial nitrate concentration was 176.47 mg L⁻¹

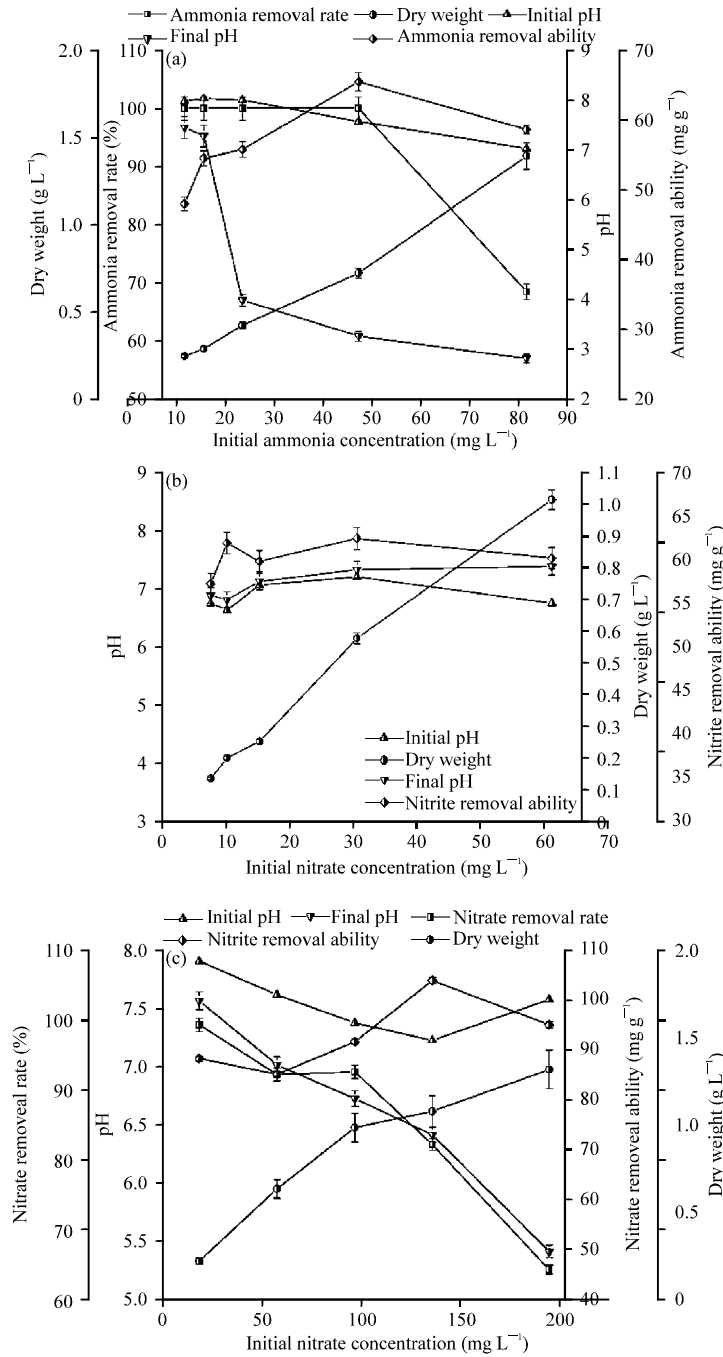


Fig. 2(a-c): Effect of initial nitrogen concentration, (a) Initial ammonia concentration was set according to concentration gradient as follows: 81.7, 47.4, 23.7, 15.8 and 11.9 mg L⁻¹, (b) Initial nitrite concentrations were 60.96, 30.48, 15.24, 10.16 and 7.62 mg L⁻¹, respectively and (c) Initial nitrate concentrations were 194.1, 135.8, 97.05, 58.23 and 19.41 mg L⁻¹, respectively, C/N of ammonia test was controlled at 12 and C/N of nitrite/nitrate were both controlled at 20

concentration showed up an excellent linear relation. Therefore, it was calculated that nitrite removal ability of the strain kept around 60 mg g⁻¹ which was hardly changed in relation to the initial nitrite concentration.

As for nitrate, all solution pH performed a slight downtrend and the higher the initial nitrate concentration, the greater the decrease amplitude and cell dry weight. The pH dropped the most from 7.57 to 5.41 when the initial

nitrate concentration was 194.1 mg L^{-1} . As the same, the heaviest cell dry weight of 1.31 g L^{-1} was also obtained in such high nitrate concentration. Calculated result showed that nitrate removal abilities of the strain were all more than 88 mg g^{-1} , demonstrating that ST2 strain was a good choice as a candidate of eminent nitrate removers. Strangely, the nitrate removal rate reduced with the increase of initial nitrate concentration because final pH and cell density were without any inhibitory action even in the medium of the highest initial nitrate concentration. Over 99% of removal rate was got when the initial nitrate concentration was 19.41 mg L^{-1} but only 64.2% was obtained in the solution of highest initial nitrate concentration. Obviously, strain growth and denitrification activity were considerably inhibited by too high nitrate concentration. No nitrite accumulation was discovered in all solutions, showing that nitrite removal efficiency was much higher than nitrate removal efficiency.

In the C/N experiment of ammonia shown as Fig. 3, pH of all solutions went an apparent decline, a fluctuating final pH of between 3.2 and 3.5 that was almost not changed by C/N were obtained, showing that the final pH had no restriction on cell growth. By combining the initial ammonia concentration results, it was found that the acid production amount was in relation to ammonia concentration but was unrelated with carbon source concentration. The removal rate of ammonia was only 67.77% with the C/N of 4, in such situation, carbon source definitely was insufficient for complete ammonia removal because 100% of the removal rates was attained when C/N = 6. Cell dry weight rose from 0.64 to 1.799 g L^{-1} when the C/N increased from 4 to 12 but the linear relation of dry weight and ammonia concentration was not dominant. Obviously, dry weight kept going up even when ammonia had already depleted, showing that the strain can use other type of organic nitrogen and residual carbon source for growth. Therefore, the dry weight increment was not completely provided by ammonia when carbon source was superfluous and a large error would occur if ammonia removal ability was calculated by dry weight and ammonia removal amount. The removal rate had already reached 100% when C/N = 6. In this particular case, the calculated ammonia removal ability 55 mg g^{-1} was the closest approached with the real value and this value was very close to the result of initial ammonia test. But the calculated ammonia removal ability was 31.6 mg L^{-1} when C/N = 12, existing big deviation.

All solution pH slightly increased from between 6.55 and 6.92 to between 7.22 and 7.59 when nitrogen source was nitrite and clearly the pH was not influenced by C/N. Nitrite removal rate and dry weight both increased

accordingly with the increasing C/N when C/N < 12. But when C/N ≥ 12 , nitrite removal rate achieved 100% and the dry weight was maintained around 1 g L^{-1} . It was discovered by the aid of calculating that the nitrite removal abilities were 77 and 65 mg g^{-1} when the C/N were 4 and 8, respectively and then, the nitrite removal abilities were uniform as 60 mg g^{-1} when C/N ≥ 12 . There was a possible reason other than measurement error for the above mentioned difference that the strain consumed more nitrite in the early phase of strain generation than in the later period of denitrification.

Like the result of nitrite test, pH performed weak rising was also inconspicuous with the C/N change in nitrate solution and all the final pH kept around 7. Nitrite removal rate and dry weight both increased with the increasing C/N. Over 99% of removal rate along with the maximum dry weight 1.03 g L^{-1} was got when C/N = 20. But the rate was 98.7% when the C/N was 16, so this C/N can be considered to be the optimum C/N for nitrate removal. It was calculated that the removal abilities all exceeded 100 mg g^{-1} in the condition of C/N ≤ 16 but the ability was inaccurate value of lower 85 mg g^{-1} when C/N was 20 which illuminated that the strain kept growing depending on other nitrogen compounds and surplus carbon source.

Nitrogen removal process: Figure 4 showed the nitrogen removal process. Ammonia concentration dipped a bit after one day, indicating that the strain started reproducing. But the concentrations of nitrite and nitrate had not changed, so the ammonia was preferentially assimilated by the strain. By this time, little biomass weighted 12.1 mg L^{-1} was got from the medium and the pH also decreased slightly which confirmed the ammonia utilization process by the strain. Beginning from the next day to the third day, the explosive growth had occurred and cell dry weight jumped to 1.5 g L^{-1} and simultaneously the pH evidently dropped from 7.1 to 6.36. At the moment, ammonia had been completely used up but nitrite and nitrate remained original value. After ammonia disappeared, nitrite concentration started falling and also were disappeared completely one day later. In the process, pH adversely rose from 6.36 to 7.06 and dry weight kept increasing to 1.881 g L^{-1} companying with still unchanged nitrate concentration. These changes of indexes also demonstrated the sustained consumed process of nitrite. From there, nitrate was just getting started being utilized and the concentration dropped from 98.9 to 36 mg L^{-1} in a day with the removal rate of 63.6%. Simultaneously, the dry weight continued to rise to the maximum of 3.1 g L^{-1} . In the next few days, nitrate concentration and the final removal rate kept approximate

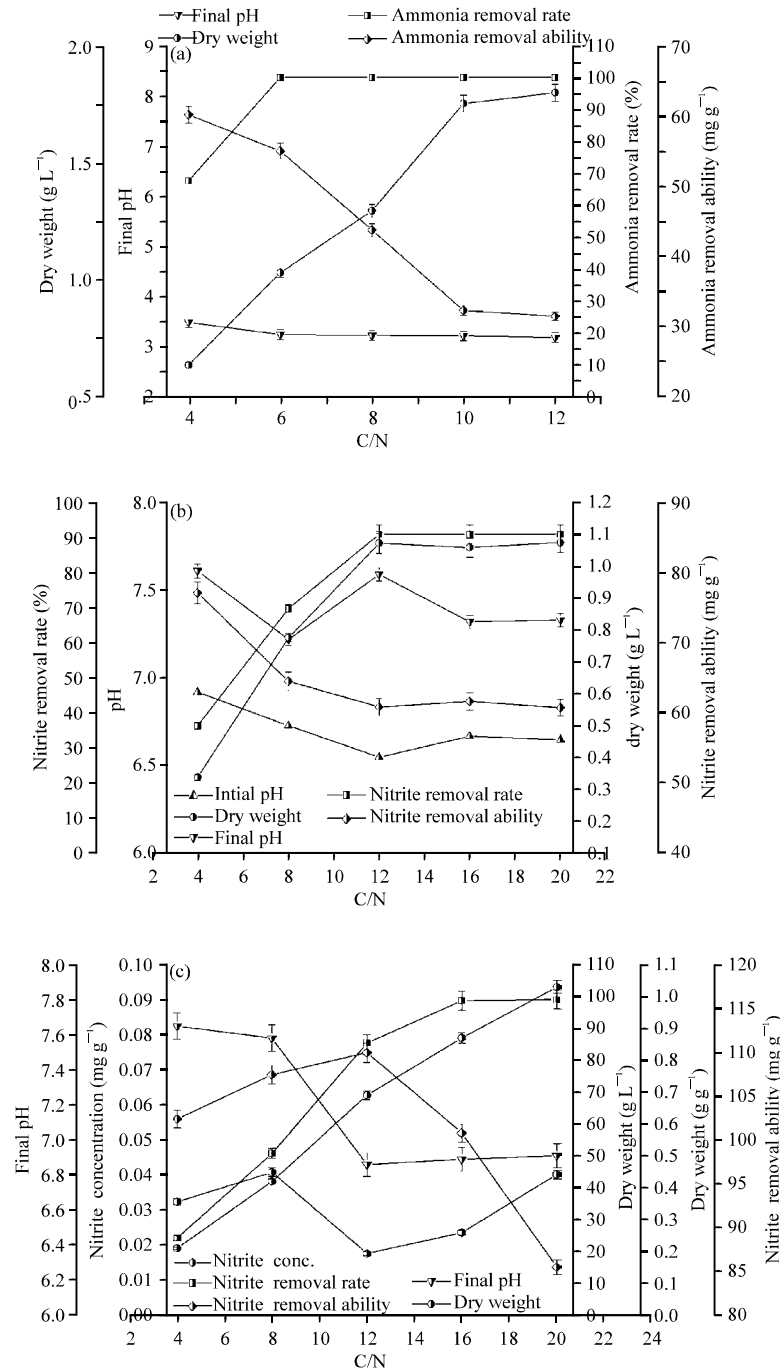


Fig. 3(a-c): Effect of C/N, (a) Initial ammonia concentration was 56.83 mg L⁻¹, (b) Initial nitrite concentration was 65.28 mg L⁻¹ and (c) Initial nitrate concentration was 88.84 mg L⁻¹

30 mg L⁻¹ and 70%, respectively. In addition, the pH again declined slightly to the final value of 6.88 and the dry weight had fluctuation according to time because of autolysis and carbon source reuse. Nitrate was not totally removed which was possibly due to insufficient carbon

source or over high cell density. In the whole process, the concentration of DO was between 3 and 7 mg L⁻¹, indicating that the strain had the capacity of removing the three types of inorganic nitrogen compounds under complete aerobic conditions.

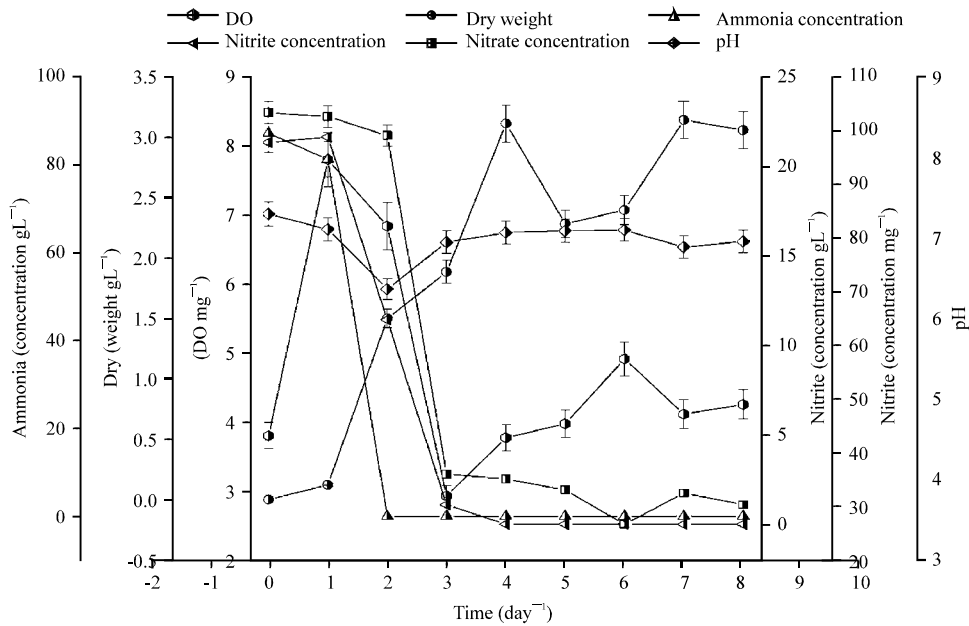


Fig. 4: Nitrogen removal process, Initial ammonia concentration was 87 mg L⁻¹, initial nitrite concentration was 21.3 mg L⁻¹ and initial nitrate concentration was 103.2 mg L⁻¹. Initial pH was 7.29

DISCUSSION

Based on the above morphological characteristic and genetic result, this strain was definitely identified as the fungus *Penicillium*. Carbon source has a dual function on aerobic denitrifier growth and denitrification process for the role of substrate and electron donor respectively, therefore, different molecular structure and molecular weight can result in total different cell biomass and nitrogen removal rate (Her and Huang, 1995). In a general way, carbon sources of small molecular weight and simple structure are liable to be assimilated by microorganism. For instance, it was revealed that *Alcaligenes faecalis* gave priority to utilize citrate and acetate whereas that glucose, sucrose and fructose were not consumed (Joo *et al.*, 2005). However, the different is that ST2 strain tends to utilize organic compounds containing the group of methyl. In addition, different carbon source can bring about different nitrogen removal rate, cell biomass, pH change and nitrogen removal ability of the strain and too high or too low pH could directly affect the activity of microbial assimilation and denitrifying enzyme, then causing the low removal efficiency. In this experiment, 2.8 and 9 were separately the lowest and highest pH that the strain can bear which was one of the most important reasons that led to low removal rates of ammonia and nitrate. It was also discovered that ammonia was translated into parts of cells such as amino acid and protein by the aid of assimilation rather than nitrification

but nitrite and nitrate removal mainly depended on assimilation and denitrification. The result of nitrite concentrations in the four nitrate solutions indicated that the type of carbon source has different effect on the activity of periplasm nitrate reductase and nitrite reductase which both influenced the nitrite accumulation (Ge *et al.*, 2012). It also showed from the result that the activity of the nitrite reductase was much stronger than that of the periplasm nitrate reductase. It's worth noting that the pH of solution decreased in denitrification the process when the carbon source was non-polar organic compound which is in inconformity to the traditional theory that denitrification is an alkali production process. Thus this strain can be considered as both an aerobic denitrifier and a pH regulator.

Figure 1 showed the whole denitrification process, from which it was drawn that dry weight increment and nitrite appearing were companied with the consumption of COD and nitrate which illustrated that carbon source was utilized not only as necessity for growth but also as electron donor for the denitrifying activity. Moreover, the nitrite accumulation rose first and fall later, the possible reason was that nitrite generation was more and more with the increasing cell biomass but after most nitrate had been removed, nitrite accordingly was soon wiped off.

Unlike the predicting outcome, it was inferred from the mixed carbon source experimental result that acetate should not be used directly serving as electron donor in denitrification process, furthermore, acetate was also not

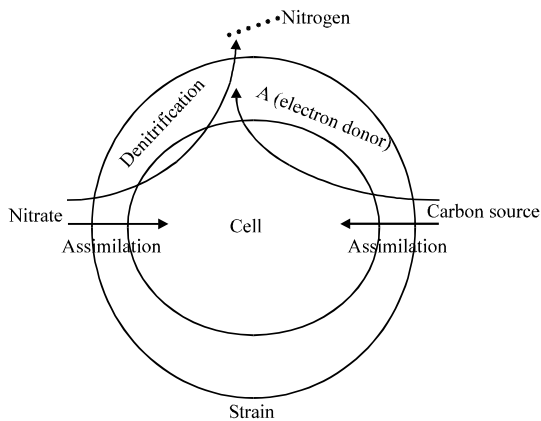


Fig. 5: The suppositional schematic diagram of assimilation and denitrification process by the strain

likely changed into some electron donor material that can be easily exploited. So acetate and sucrose must be translated into a kind of active substance that can be used as electron donors with the aid of the participation of related enzymes. The suppositional schematic diagram was shown as Fig. 5, on the one hand, nitrate and carbon source are assimilated as energy sources for cellular component and strain reproduction, on the other hand, carbon source is converted to a kind of materials named A that possesses much stronger denitrifying activity and then, nitrate is denitrified to nitrogen-containing gases using A as electron donor in the presence of denitrifying enzyme.

Nitrogen concentration has many aspects on microbial growth and nitrogen removal. Over high nitrogen concentration may directly poison the relative enzyme activities, then influencing the cell growth. Moreover, high nitrogen concentration can lead to more formation amount of acid or alkali which is also one of the restraining factors for the subsequent growth of the strain. It was obviously seen from the initial ammonia concentration test that ammonia assimilation process was an acid production process. The pH that already reached the lowest limiting value when initial ammonia concentration was 81.7 mg L^{-1} greatly inhibited the cell growth which possibly brought about the low ammonia removal rate. The ammonia removal ability of the strain had little difference in solutions of different initial concentration, the fundamental cause of which was that the ammonia assimilation process conducted similarly even in different initial concentration solutions. The 61 mg L^{-1} of nitrite concentration had no inhibitory action on strain growth and denitrification, showing that the strain had a good nitrite endurance capacity. In addition,

the whole course of denitrification was nearly a pH stable process and it can be derived from the value of the nitrite removal ability that the strain was a choice of a microorganism capable of good nitrite removal ability. In contrast, nitrate elimination was a weak acid yield process. The removal efficiency of nitrite was greater than that of nitrate which could probably be explained by the reason that the activity of nitrite reductase was higher than that of nitrate reductase. As a result, nitrite was preferentially denitrified rather than nitrate with the addition of enough carbon source. Besides, high nitrate concentration was likely found to reduce the cell biomass and nitrate removal efficiency.

C/N plays an important role in the aerobic denitrification and denitrifiers often require much larger proportion of carbon source in comparison with common microbes for strain growth and denitrification. However, an excess of carbon source could cause both COD and cost increase instead of enhancing denitrification efficiency (Sison *et al.*, 1996), on the contrary, insufficient carbon source may bring about the halfway denitrification and nitrite accumulation (Her and Huang, 1995). Therefore, an optimal C/N is necessary for denitrifier growth and denitrification. Whereas, due to the diversity of metabolic mechanism and electron transport pathway, the optimal C/N is different even if the different strain uses the same carbon source. Moreover, the given strain also possibly requires different optimal C/N using different carbon source. Owing to these reasons, an appropriate C/N of this strain is needed to be obtained by the test.

The best C/N of ammonia removal by the strain is between 4 and 6 from ammonia experiment. Ammonia were depleted when the C/N was higher than the best C/N. At this time, strain went on growing by making use of other types of nitrogen source and residual carbon source, so the cell dry weight persisted rising and the calculated ammonia removal ability of the strain kept decreasing. By combining the results of initial concentration and C/N test, the ammonia removal ability of the strain should be between 50 mg g^{-1} and 60 mg g^{-1} . The optimal C/N for nitrite removal lay between 8 to 12 and the removal ability maintained around 60 mg g^{-1} which was similar with the results of initial nitrite concentration. As a general rule, nitrate denitrification treatment demands more plenty of carbon source than nitrite denitrification treatment. It was tested that the optimum C/N for nitrate removal which fluctuated between 12 and 16 was higher than that for nitrite removal. The nitrate removal ability of the strain was worked out to be about 100 mg g^{-1} which can effectively represent the real nitrate removal ability of the strain involving in the previous research foundation.

From Fig. 4, it was drawn the conclusion that the utilized order of the three types of nitrogen was: ammonia>nitrite>nitrate which was also confirmed by the pH variation that it rose at beginning then declined. Nitrate was not completely eliminated in the later stage of the experiment and a short of carbon source or an over large of cell density were estimated to be the reasons. High DO concentration was measured in the whole nitrogen removal process, illuminating that this strain can simultaneously remove ammonia, nitrite and nitrate through assimilation and denitrification in the absolutely aerobic waters.

CONCLUSIONS

The new isolated fungus named ST2 was identified as a kind of *Penicillium*. Sodium acetate, glucose, soluble starch and sucrose among the seven tested carbon source were used by the strain which indicates that this strain is inclined to make use of non-polar organic carbon source containing methyl group for its assimilation and denitrification activity. When sucrose was set as carbon source, pH of ammonia solution dropped markedly but it almost maintained stable in nitrite or nitrate solutions. Initial nitrogen concentration and C/N both have effect on solution pH and nitrogen removal rate. Ammonia and nitrate were removed firstly and lastly respectively when the three kinds of inorganic nitrogen sources coexist. In the nitrate removal experiment, a large number of COD were consumed companying with nitrate eliminating and less accumulation of nitrite was detected even under the condition of lack of carbon source. Therefore, this strain is an excellent microbial inoculum applicable for nitrogen and COD removal.

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