RESEARCH ARTICLE OPEN ACCESS

Screening and acclimation of efficient simultaneous nitrification and denitrification bacteria and their application in biotreatment of high ammonia pharmaceutical wastewater

Jinling Ma, Yu Chen, Suye Mao, Jinghui Sun, Zhitang Lu*
(College of Life Sciences, Hebei University and Key Laboratory of Microbial Diversity Research and Application of Hebei Province, Baoding 071002, China)

ABSTRACT
Simultaneous nitrification and denitrification (SND) bacteria can complete nitrification and denitrification processes under aerobic conditions simultaneously, which has some obvious advantages in comparison with traditional method for nitrogen removal, such as reducing energy consumption and construction cost. Three SND bacteria strains, YX3, YX4 and YX6 were isolated from a polluted river and identified as *Pseudomonas* spp. by phylogenetic analysis based on 16S rDNA sequencing. After cultivated in liquid heterotrophic ammoniation medium at 30°C by shaking at 150 rpm for 3 d, the NH_4^+-N removal rates of strains YX3,YX4 and YX6 were 93.50%, 91.50% and 91.00%, respectively; and the total nitrogen removal rates of YX3, YX4 and YX6 were reached 85.75%, 87.33% and 90.46%, respectively. The NO_3^--N removal rates of strains YX3, YX4 and YX6 were 87.24%, 89.88% and 88.73%, respectively, after cultivated in liquid denitrification medium at 30°C by shaking at 150 rpm for 3 d. These results show that all these strains were capable of simultaneous nitrification and denitrification. When strains YX3, YX4 and YX6 were faced to high ammonia pharmaceutical wastewater, NH_4^+-N concentrations decreased from 500±78.47 to 238.14±63.77mg/L, 155.82±79.95 mg/L and 214.62±92.69 mg/L, respectively, after cultured at 150 rpm and 30°C for 3 d. After four months of acclimation, the NH_4^+-N remove rates were improved significantly under the same culture conditions and the NH_4^+-N concentrations decreased linearly from 500±81.79 to 151.9±88.70mg/L, 94.73±58.66 mg/L and 114.49±56.84 mg/L of strains YX3, YX4 and YX6, respectively. All the strains showed rather steady features in bio-denitrification of high ammonia pharmaceutical wastewater after acclimation under laboratory conditions. This suggests that all the three strains have great application potential in high ammonia nitrogen pharmaceutical wastewater treatment.

Keywords **-** simultaneous nitrification and denitrification, nitrogen removal, acclimation, pharmaceutical wastewater, *Pseudomonas*

I. INTRODUCTION

Along with the development of industry and the growth of population, the environmental pollution is becoming more and more serious. A lot of nitrogen containing industrial wastewater and domestic sewage are not treated properly and discharged, which causes degradation of natural water qulity[1]. Especially many industrial processes, such as petroleum production, food processing, and various pharmaceutical processes, generate saline effluents containing inorganic nitrogen compounds, which are difficult to remove by traditional techniques [2].

In all of denitrification processes, the method of biological nitrogen removal is most cost-effective, low-impact in environment, and also most promising [3]. Conventional wastewater treatment systems for nitrogen removal are based on both aerobic nitrification and anaerobic or anoxic denitrification. This combination requires the spatial separation of nitrificationand denitrification units, or temporal separation of each process by alternating aeration and no aeration in the same unit[4]. However, in recent years, many researchers' reports proved the existence of simultaneous nitrification and denitrification (SND) phenomenon[5]. SND bacteria are a lot of hetero-trophic bacteria capable of both heterotrophic nitrification and aerobic denitrification, which can convert ammonia, nitrate and nitrite to N_2 gas by themselves [6]. SND may offer a potential to save the costs for the anoxic reactor or to reduce its size at least, providing that a considerable amount of denitrification takes place together with nitrification in the aeration tank. So the heterotrophic nitrification bacteria in the role of nitrogen wastewater treatment have caused wide public concern. To date, studies on heterotrophic nitrification and aerobic denitrification have focused on a low ammonium concentration that is discharged from domestic wastewater, and research on the treatment of high-strength ammonium wastewater is rare [7].

In this paper, we reported the isolation, screening and acclimation results of three SND strains, and their denitrification application in high ammonia high salt pharmaceutical wastewater under laboratory conditions.

II. MATERIALS AND METHODS

2.1 Samples

Sludge and water samples were collected from a seriously contaminated river, located at Jiaozhuang, Baoding, China, in July 9, 2013. Water indexes were listed in Table 1:

Table 1.Water indexes (TN, TP, COD)

Index	TN	TP	COD
Concentration (mg/m^3)	1651	198	652

*TN: total nitrogen; TP: total phosphorus; COD: chemical oxygen demand

2.2 Medium

The compositions of the heterotrophic ammoniation medium composed of (per liter): $(NH_4)_2SO_4$ 0.472g, Sodium citrate 4.902g, NaCl 2.5g, K_2HPO_4 5g, $FeSO_4•7H_2O$ 0.05g, $MnSO_4•4H_2O$ 0.05g, $MgSO_4•7H_2O$ 2.5g [8].

The nitration and nitrosation medium were identical to the heterotrophic ammoniation medium, except that $(NH_4)_2SO_4$ was replaced with the same amounts of $NaNO_3$ and $NaNO_2$, respectively. The media were sterilized by autoclaving for 20 min at 121°C.

2.3 Enrichment and isolation of heterotrophic ammoniation bacteria

For enrichment, 5 g of sludge or 5 mL of water samples were added to 95 mL heterotrophic ammoniation liquid medium within a 250 mL flask and cultured at 150 rpm and 30°C[9]. Cultures were enriched for 14 days, and during the enrichment, the enriched cultures were taken into a new liquid medium per 2 day to passage[10]. These cultures were plated onto sterilized heterotrophic ammoniation agar in order to confirm purity. The bacteria were streaked on fresh nutrient agar plates 3 times to obtain the pure isolate[11].

2.4 Screening of SND strains

The heterotrophic ammoniation isolates were inoculated into 250 mL flasks containing 100 mL of heterotrophic ammoniation liquid medium and incubated at 30°C by shaking at 150 rpm, and then the ability of nitrification were estimated[12]. Ammonia nitrogen (NH_4^+ -N) concentration was determined by Nessler's assay at a wavelength of 425nm (HJ 535-2009)[13]. Total nitrogen (TN) concentration was determined by alkaline potassium persulfate digestion-UV spectrophotomeric method of 220nm and 270nm (GB 11894-89) [14].

Pure isolates were inoculated into 250 mL flasks containing 100 mL of nitration and nitrosation liquid medium, incubated at 30°C by shaking at 150 rpm, and then estimate the ability of denitrification. Nitrate concentration was determined by spectrophotometric method with phenol disulfonic acid (GB 7480-87)[15]. The nitrite content was determined by the N-(1-naphthalene)-ethylene method, which monitored the absorbance at 540 nm (GB 7493-87)[16].

Nitrification and denitrification abilities of the isolates were determined by using the above methods, and the isolates with both functions were assumed as simultaneous nitrification and denitrification bacteria strains.

2.5 Acclimation of SND strains and treatment of high ammonia pharmaceutical wastewater

High ammonia wastewater with NH_4^+-N concentration about 500 mg/L and 4000 mg/L of COD was generated in large quantity by a pharmaceutical factory every day, and the sewage also contained about 30 000 ppm of NaCl. To use experimental strains in treatment of such wastewater, they were acclimated in the sterilized pharmaceutical wastewater from 0.25×, 0.5×, 0.75× to 1× of concentration continuously. The strains were shake-cultured in heterotrophic ammoniation liquid medium at 30°C and 150 rpm for 24h, and then they were inoculated into various wastewater samples in proportion of 10% (v/v). Each stage was repeated for 3 batches. All the acclimation and treatment were conducted at 30°C on a rotary shaker at around 150 rpm, and the concentrations of NH_4^+-N(HJ 537-2009), NO_3^--N and NO_2^--N, and COD_{Cr} (HJ/T 399-2007)were determined every 24h.

2.6 Phylogenetic identification of SND strains

The genomic DNA of tested SND strains was extracted with phenol-chloroform method[19]. 16S rDNA was amplified by polymerase chain reaction using universal forward primer 27F (5'-AGAGT TTGATCMTGGCTCAG-3') and reverse primer 1525R (5'-AGAAAGGAGGTGWTCCARCC-3')[20] with described procedure[21]. Purified PCR products were directly sequenced by Beijing Sunbiotech Corporation. Evolutionary distance matrices were calculated by using the method of Kimura 2-parameter and a Neighbour-joining tree was constructed using the Mega 5.1 program [22].

III. RESULTS

3.1 Isolation and screening of SND strains

After enrichment culture, 16 bacteria strains were isolated firstly on the heterotrophic ammoniation agar medium. Three strains, YX3, YX4 and YX6, with potential activity of heterotrophic nitrification were obtained by Nessler's assay determination. During characterization of hetero-trophic nitrifying bacteria, sodium acetate and ammonium sulfate were used as carbon and nitrogen source[23]. Table 2 showed the ammonia nitrogen and

total nitrogen removal rates variation of three strains during 3 days.

Table 2. The NH_4^+-N and TN removal rates of three strains in screening

Strains	NH_4^+-N remove rate (%)			TN remove rate (%)		
	1d	2d	3d	1d	2d	3d
YX3	94.00	95.30	93.50	65.62	70.50	85.75
YX4	89.73	88.64	91.50	68.84	76.49	87.33
YX6	89.28	93.24	91.00	70.60	86.33	90.46

The concentrations of NH_4^+-N and TN were significantly reduced in the initial three days. Strain YX3 removal rate of NH_4^+-N reached the highest value of 95.30% in the second day, TN removal rate reached the highest value of 85.75% in the third day; strain YX4 removal rates of NH_4^+-N and TN reached the highest in the third day, which were 91.50% and 87.33% respectively; strain YX6 removal rate of NH_4^+-N reached the highest value of 93.24% in the second day, and TN removal rate reached the highest value of 90.46% in the third day.

To assess the denitrification performance of the isolates, sodium nitrate and sodium nitrite were used as nitrogen source, respectively. After 3d of aerobic training at 30°C by shaking at 150 rpm, nitrate removal rates of strains YX3, YX4 and YX6 were 87.24%, 89.88% and 88.73% respectively, and nitrite removal rates were 84.40%, 88.39% and 89.33% respectively. NO_3^- and NO_2^-concentrations changes in culture medium of three strains during three days as showed in Fig 1 and Fig 2:

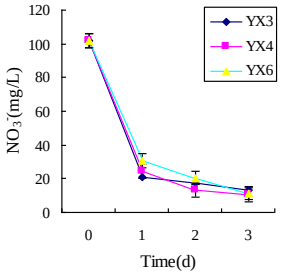

Fig.1 NO_3^- concentration changes in culture medium of three strains during three days

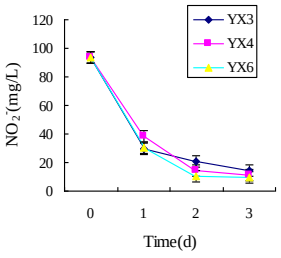

Fig.2 NO_2^- concentration changes in culture medium of three strains during three days

3.2 Denitrification efficiency of SND strains in treatment of pharmaceutical wastewater

Strains YX3, YX4 and YX6 were used in bio-treatment of high ammonia pharmaceutical wastewater collected from a factory located in Baoding China. The acclimated strains were tested comparing with the original wild isolates under the same conditions. All the experiments were performed by incubating the bacterial strains with pharmaceutical wastewater at 30°C and 150 rpm, and the NH_4^+-N concentration and COD_{Cr} were determined every 24 hours to assess their abilities to remove nitrogen and COD_{Cr} from the water. NH_4^+-N concentrations variations in pharmaceutical wastewater of wild and acclimated strains were showed in Fig 3.

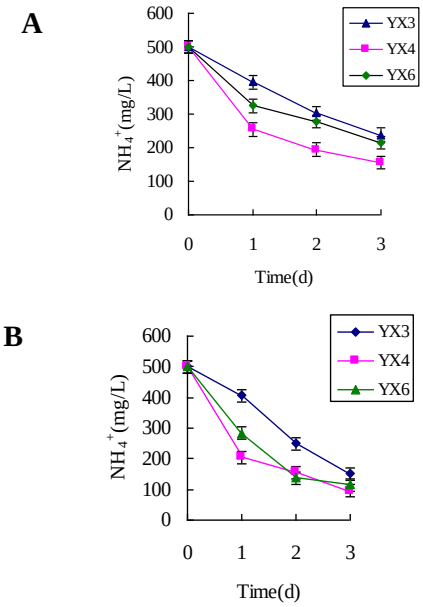

Fig.3 Comparison of NH_4^+-N concentrations changes in pharmaceutical wastewater during treated by wild and acclimated SND strains (A: before acclimation, B: after acclimation)

Fig 3 represented that three wild isolates could remove more than 52% of the NH_4^+-N from 500±78.47 mg/L NH_4^+-N containing pharmaceutical wastewater. Strains YX3, YX4 and YX6 reduced NH_4^+-N from 500±78.47 mg/L to 238.14± 63.77mg/L, 155.82±79.95 mg/L and 214.62±92.69 mg/L, respectively. After four months of acclimatization, all the three strains could remove more than 69% of the NH_4^+-N from 500±81.79 mg/L NH_4^+-N containing pharmaceutical wastewater, NH_4^+-N concentrations reached 151.9±88.70mg/L, 94.73±58.66 mg/L and 114.49±56.84 mg/L after treated by the acclimated strains YX3, YX4 and YX6, respectively. And nitrite and nitrate in wastewater were almost not detected during the treatment. Result clearly showed that the ammonia

nitrogen removal rates were improved after a short period of acclimation.

Variations of COD_{Cr} in pharmaceutical wastewater during treated by acclimated SND strains were also determined, and the results were showed in Fig 4.

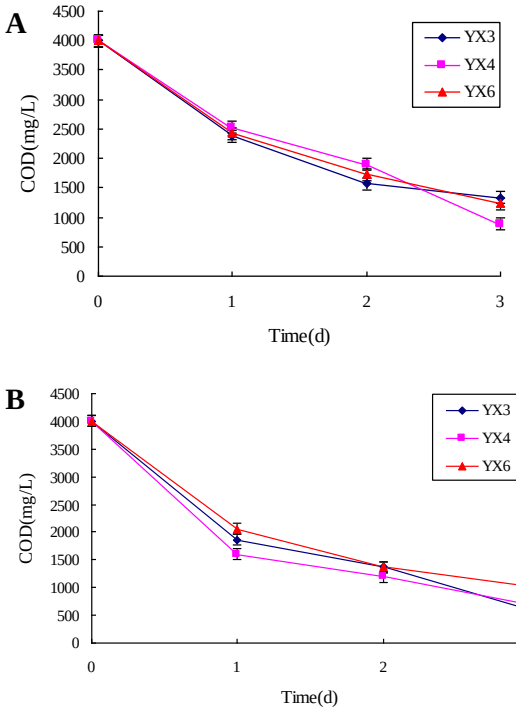

Fig.4 Comparison of CODCr concentrations changes in pharmaceutical wastewater during treated by wild and acclimated SND strains (A: before acclimation, B: after acclimation)

Fig 4 represented that three wild isolates could remove more than 65% of the COD_{Cr} from about 4000mg/L COD_{Cr} containing pharmaceutical wastewater. At beginning of the acclimation, COD_{Cr} concentration decreased linearly during the first 72 h, for example, YX3 removed COD_{Cr} from 4000±906.32 to 1388.12±391.28mg/L, YX4 decreased to 883.28±189.72.mg/L, YX6 decreased to 1233.93±236.61 mg/L. After four months of acclimatization, three strains could remove more than 74% of the COD_{Cr} from about 4000±1024.87 mg/L COD_{Cr} containing pharmaceutical wastewater. Specifically, COD_{Cr} concentrations reached 601.47±391.28mg/L, 690.64±146.86 mg/L, and 1028.46± 175.37 mg/L after treated by the acclimated strains YX3, YX4 and YX6, respectively. Results clearly showed that all the strains are able to effectively remove COD_{Cr} in pharmaceutical wastewater, and after a short period of acclimation, the ability of three strains COD_{Cr} removal rates were improved significantly.

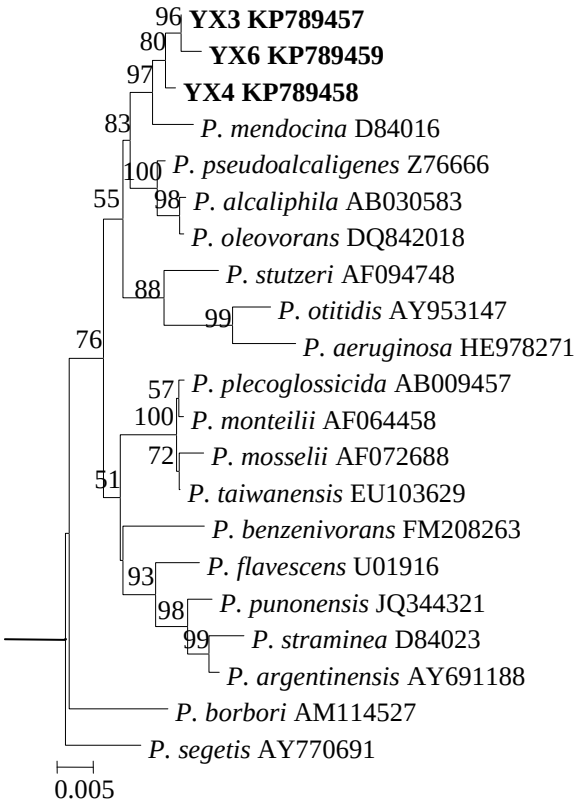

Fig 5. Neighbor-joining phylogenetic tree of strain YX3, YX 4 and YX6 and related *Pseudomonas* representatives based on almost complete 16S rDNA sequences. The tree was rooted using *Stenotrophomonas acidaminiphila* AMX19 (AF273080) as outgroup. Bootstrap analysis were carried out by re-sampling the data 1000 times, bootstrap values above 50% were indicated at the nodes.

3.3 Phylogenetic determination of the isolated strains

Determined 16S rRNA gene sequences were deposited in GenBank with accession numbers KP789457, KP789458 and KP789459 for strains YX3, YX4 and YX6, respectively. 16S rRNA gene sequence determination showed that strains YX3, YX4 and YX6 have similar sequences with the similarities more than 99%, and that they are clustered with the same neighbor *Pseudomonas mendocina* NCIB 10541^T (D84016), forming a distinct lineage within the genus *Pseudomonas* (Fig 5). The results indicated that the three strains belonged to the genus *Pseudomonas* and showed a close phylogenetic relationship to the species *Pseudomonas mendocina*. A phylogenetic tree including strains YX3, YX4 and YX6 and other type strains of the related species was given in Fig 5.

IV. DISCUSSION

The finding of simultaneously nitrifying-denitrifying nitrogen removing bacteria caused a brand new concept for nitrogen removal from wastewater since there are other studies reporting SND since the early 2000s. In this research, we obtained three strains that capable of simultaneous nitrification and denitrification. They could effectively remove ammonium from medium and wastewater. The removal rates of NH_4^+-N were all reached 90% in liquid heterotrophic ammoniation medium. When assess the denitrification performance of the isolates, nitrite and nitrate removal rates of three strains were all reached 80% in the corresponding media mentioned above. And denitrification efficiency of three strains were higher than most of heterotrophic nitrification and aerobic denitrification bacteria reported. For example, the denitrification efficiency of *Pseudomonas mendocina* at 90 h is 64% when concentration of NO_2^--N was 100mg/L in medium[24], and the removal rate of NO_3^--N by heterotrophic nitrification-aerobic denitrifier bacteria CPZ24 is 66.74% in aerobic denitrification[25]. Compared with domestic sewage, component of pharmaceutical wastewater was complicated, and contains some toxic substances, which increased the difficulty for microorganisms to remove nitrogen efficiently[26]. Three strains YX3, YX4 and YX6 demonstrated the strong adaptability to tolerate pharmaceutical wastewater and remove ammonia nitrogen from it. Ammonia nitrogen removal rates of wild strains YX3, YX4 and YX6 were about 60%-69% in high-strength ammonium and high salt pharmaceutical wastewater with ammonium concentrations reached ~500 mg/L NH_4^+-N and ~30000 ppm NaCl; after acclimation, the removal rates of ammonia nitrogen were reached 70%-80%. Results indicate that *Pseudomonas* spp. are potentially microbial resources in high ammonia nitrogen pharmaceutical wastewater treatment.

Although these strains reached a certain effect of denitrification in treating pharmaceutical wastewater, this work was carried out under laboratory conditions, the popularization and application of these strains will still need further study in pilot and plant scale.

Acknowledgements

This work was supported Hebei Provincial Key Discipline of Bioengineering (Hebei University).

REFERENCES

[1] M. D. Yuan and Y. F. Xin, Screening and Identifiication of One Strain of Heterotrophic Nitrification-aerobic Denitrifying Bacteria and Its Denitifying Activity. *Natural Science, 13(3), 2012, 339-343.*

[2] O. Lefebvre and R.Moletta,. Treatment of organic pollution in industrial saline wastewater: a literature review. *Water Res, 40, 2006, 3671–3682.*

[3] L. Robertson, E. Van Nie, R.M, Torremans and J. G. Kuenen, Simultaneous Nitrification and Denitrification in Aerobic Chemostat Cultures of *Thiosphaera antotropha*. *Appl Environ Microbiol , 54(11), 1998, 2812-2818*.

[4] J. L.Hee,, H. B. Jae and M.C. Kwang,. Simultaneous nitrification and denitrification in a mixed methanotrophic culture. *Biotechnology Letters, 23, 2001, 935–941*

[5] J. J. Liu, P. Wang, and H. Wang, Study on Denitrification Characteristics of a Heterotrophic Nitrification Aerobic Denitrifier. *Research of Environmental Sciences, 21(3), 2008, 121-127.*

[6] A.A. Khardenavis, J. Kapley and H. J. Purohit, Simultaneous nitrification and denitrification by diverse *Diaphorobacter* sp. *Appl Microbiol Biotechnol, 77, 2007, 403–409.*

[7] S. J Hung, H. Mitsuyo and S. Makoto, Heterotrophic nitrification and aerobic denitrification by a novel *Halomonas campisalis. Journal of Bioscience and Bioengineering, 2, 2005, 184–191.*

[8] Y. K. Lü, J. H.Yin, Y. X Liu and W. Q. Zhang, Isolation and identification of a heterotrophic nitrifier and its optimal conditions for nitrification. *CIESC Journal, 62(5), 2011, 1424-1443.*

[9] Y.H. Ahn, Sustainable nitrogen elimination biotechnologies: a review. *Process Biochem, 41, 2006, 1709-1721.*

[10] Y, Lin, Characterization of newly heterophic nitrifying bacterium. *Water Practice&Technology. 1(3), 2005,243-249.*

[11] Y. Lin, H. Kong, Y. He, B. Liu, Y. Inarmori and L. Yan, Isolation and characterization of a new heterotrophic nitrifying *Bacillus sp* Strain. *Biomed Environ. Sci, 20, 2007, 450-455.*

[12] Y. Wen, Y. Ren, C. Wei, K. Li, F. Lin and X. Chen, A study on nitrogen removal efficiency of *Psedomonas stutzeri* strains isolated from an anaerobic/anoxic/oxic wastewater treatment process. *African Journal of Biotechnology. 9(6), 2010, 869-873.*

[13] HJ 535-2009. *Determination of ammonia - Nessler's reagent spectrophotometry*. Environmental Science Press of China. 2005.

[14] GB 11894-89. *Determination of total nitrogen - Alkaline potassium persulfate digestion-UV spectrophotomeric method*. Environmental Science Press of China, 1989.
[15] GB 7480-87. *Determination of nitrate - spectrophotometric method with phenol disulfonic acid.* Environmental Science Press of China , 1987.
[16] GB 7493-87. *Determination of nitrite - N-(1-naphthalene)-ethylene method*. Environmental Science Press of China, 1987.
[17] HJ 537-2009. *Determination of ammonia nitrogen - Distillation-neutralization titration*. Environmental Science Press of China, 2009.
[18] HJ/T 399-2007.*Determination of the chemical oxygen demand fast digestion-spectrophotometric method*. Environmental Science Press of China, 2007.
[19] J. Marmur, A procedure for the isolation of deoxyribonucleic acid from microorganisms. *Journal of Molecular Biology, 3, 1961, 208-218.*
[20] D.J. Lane, 16S/23S rDNA Sequencing. In E. Stackebrandt, and M. Goodfellow(Ed), *Nucleic acid techniques in bacterial systematic,* (John Wiley & Sons, Chichester,, 1999)115-175.
[21] Z. Lu, Z, Liu, L, Wang, Y. Zhang, W. Qi and M. Goodfellow, *Saccharopolyspora falva* sp. nov. and *Saccharopolyspora thermopohila* sp.nov., novel actinomycetes from soil. *International Journal of Systematic and Evolutionary Microbiology, 51,2001, 319-325.*
[22] K, Tamura, D, Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, EGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28, 2011, 2731-2739.* DOI: 10.1093/molbev/msr121
[23] J. Su, F. Ma, J.Wang, S. Gao, L. Wei andW. Li, Nitrificatication and denitrification characteristics of new heterotrophic. *Journal of Tianjin University, 40(10), 2007, 1205-1208.*
[24] W. Zhang, Identification and Characteristics Analysis of Simultaneous Nitrifying-Denitrifying Bacteria and Nitrogen Removing. *Journal of Civial, Architectural &Environmental Engineering, (34)5, 2012, 136-140.*
[25] P.Z. Chen, L.G. Wang, Y.C. Wang, J. Li, W. Ding, T.Z. Ren and S.P. Li, Screening and Denitrification Characteristics of a Heterotrophic Nitrification-Aerobic Denitrifier Bacteria. *Environmental Science, (30)12, 2009. 3614-3618.*
[26] Chen, Z. F., Yi, L. H., Pu, Y. P., *et al*. (2007). Screening of a heterotrophic nitrifying bacterium strain and its optimal conditions for nitrogen removing. Journal of Southeast University, (37)3, 486-490.