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Identification of Low-temperature Oligotrophic and Heterotrophic Nitrifying Bacteria and Safety Tests with ICR Mice

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Abstract.- Five strains of heterotrophic nitrifying bacteria were isolated from Songhua River in northern China in winter. Through ammonia degradation experiment, the strain Y7 demonstrates the ability to convert ammonium to nitrogen under oligotrophic conditions at low temperature. The average ammonium removal rate was approximately 0.075±0.007 mg NH_4^+-N/L·h during the 48-hour observation period and 66.70±3.06% of ammonium removal was achieved in the end. The morphological and physiological characteristics and 16S rDNA sequence of strains were analyzed. The results showed that strain Y7 was belonging to *Acinetobacter* bacteria. Acute and chronic toxicity tests and Ames bacterial mutagenecity experiment were also conducted. No mortality occurred and none of the mice exhibited any clinical sign on cage-side observation. Blood and blood biochemical indicators of liver, kidney, spleen changed after contamination showed that various organs were not damaged and indicated that the strain Y7 is safe for animals. This study provided more information for the construction of nitrogen flora in the low-temperature oligotrophic environment.

Keywords: Low temperature, heterotrophic nitrifying bacteria, ICR mice, Safety.

INTRODUCTION

Urbanization in China has greatly affected the water environment. The use of agricultural pesticides and nitrogen containing detergents has increased the discharge of nitrogen from wastewater. With large amount of ammonia and nitrate nitrogen injected into the surface water, nitrogen balance of the original water has broken and the water environment in peri-urban areas and groundwater has been seriously affected. In recent years, eutrophication caused to algal blooms and red tides, has resulted in significant water supply disruption and economic loss. The report of Chinese Environment in the year 2012 showed that the top ten state-controlled sections in the basin were highly polluted with ammonia, major indicator of pollutants (Ministry of Environmental Protection, 2012). The oxidation of ammonia to nitrate by nitrifying bacteria under aerobic conditions has been widely used in sewage treatment (Zhu *et al*., 2008). However, since nitrifying bacteria cannot grow in low temperature their role in drinking water treatment is limited. Several studies have shown that when the temperature, decreases, nitrification rate also reduces, resulting in accumulation of nitrite in the sewage water (Papen and Von Berg, 1998; Fisher *et al*., 1956; Eylar and Schmidt, 1959; Doxtader and Alexander, 1966; Whire and Johnson, 1982). The denitrification is mainly completed by autotrophic nitrifying microorganisms. Recently heterotrophic microorganisms such as bacteria, actinomycetes, fungi and even algae have been reported to be involved in the nitrification process (Chen *et al*., 2012; Zhang *et al*., 2011, 2012; Zhao *et al*., 2012). However, studies on the nitrifying bacteria indicated that this nitrification was initially confined to the ammonia concentration of 20 mg/L or more, at 20-38°C and C/N ratio of 10-15 (Chen and Ni, 2011; Zhang *et al*., 2012; Zhao *et al*., 2010).

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The strain for nitrification of ammonia in the oligotrophic environment has not been reported at low temperature. The temperature in northeast region of China is extremely low during winter, which is only about 5°C. Hence, normal nitrifying bacteria cannot be used to remove ammonia due to the low temperature in winter. Screening of heterotrophic nitrifying bacteria adapted to the cold oligotrophic environment, is therefore, very important to remove ammonia from the cold source water.

The safety assessments are necessary before using these bacteria. Many studies have focused on the toxicological aspects of functional bacteria in food engineering. For example, several studies have focused on the safety of *Yarrowia lipolytica* (Engel, 1972; DeGroot *et al.*, 1971, 1976). In these studies dried biomass of *Yarrowia lipolytica* fed to rats and mice at dietary levels up to 30% did not exert harmful effect for two years and over three generations. However, the toxicity of heterotrophic nitrifying bacteria has not been studied.

In this study, five heterotrophic nitrifying bacteria were isolated from the Songhua River in winter and were screened for ammonia degradation. The safety tests were also conducted to provide more information for production of nitrogen flora in the low-temperature oligotrophic environment.

MATERIALS AND METHODS

Culture medium

Enrichment medium contained 5g/L peptone, 5g/L yeast extract and 8g/L NaCl. Heterotrophic nitrification medium consisted of the following components: 0.382g/L NH_4Cl, 1.0g/L CH_3CH_2ONa, 0.05g/L $MgSO_4 \cdot 7H_2O$, 0.2g/L K_2HPO_4, 0.12g/L NaCl, 0.01g/L $MnSO_4$ and 0.01g/L $FeSO_4$. The pH of above medium was adjusted to 7.0-8.2. A solid medium was prepared by adding agar powder into the liquid medium (1.5-2g of agar powder per 100mL). The liquid medium was sterilized in an autoclave at 121°C for 30min before using and then cooled down to room temperature. The solid medium was poured into a plate on a clean bench after sterilization (Huang *et al*., 2013).

Screening and purification of low-temperature heterotrophic nitrifying bacteria

The low-temperature oligotrophic heterotrophic nitrifying bacteria (*Acinetobacter*. Y7) were isolated from fresh water of Songhua River (Northeast China) during winter. Fresh water (10 ml) from the Songhua River was poured into a conical flask containing 250 mL sterilized rich medium 0.5 g/L of ammonia nitrogen. The conical flask was placed on a shaking incubator at 2°C for 15 days cycle. Before a new cycle, 10 % of culture solution was inoculated into fresh rich medium. The removal rate of ammonia nitrogen was detected at regular intervals during each cycle. Ammonium removal was measured to screen the bacterial cultures.

The culture was diluted and spread on the heterotrophic nitrifying medium and incubated at 2°C. When the colonies appeared, a single colony was picked for streaking. In order to ensure the purity of the strain, purification and separation was conducted 3 times.

The separated pure strains were inoculated into the rich medium and cultured in the shaking incubator at 2°C at 140 r/min for 3 days. After that, an appropriate amount of culture solution was centrifuged and the pellet was washed several times with sterile saline under sterile conditions. The bacterial pellet was suspended in sterile saline and inoculated into sterilized ammonia nitrogen solution (5 mg/L), pH 7.6. It was then cultured in the shaking incubator at 2°C and 140 r/min. Some culture was taken out at regular intervals, centrifuged and the supernatant used for measuring ammonia nitrogen concentration.

Identification of bacterial morphology and analysis of colonies

A set of culture plates with heterotrophic nitrification medium was prepared. A single colony was picked for streaking and then the plates were placed (upside down) in the incubator at 2°C. After the colonies had grown on the plates their characteristics were observed with naked eyes. Gram staining (Claus, 1992; Ahmad and Shakoori, 2013) was done and the bacterial morphology observed under the microscope.

A colony was sent to Shanghai Shenggong Company for DNA nucleotide analysis. The sequences obtained were compared in the NCBI (National Center for Biotechnology Information) and had similar sequence to MAGE4.0 software which received the same sequence through the phylogenetic differentiation method (NJ).

Acute toxicity test on ICR mice

Ninety, six week old ICR (Institute of Cancer Research) mice, half male and half female, weighting 18±2 g were purchased from Harbin Veterinary Research Institute and divided into three groups A, B and C, each of 30 mice. The purified heterotrophic nitrification bacteria were inoculated in a liquid medium under aerobic conditions for 5 days. The cells were collected by centrifugation, then washed 3 times with sterile saline. Group A and B were injected intraperitoneally 0.4 ml bacterial culture as 600 mg/kg and 1200 mg/kg body weight respectively, while group C was the control group.

Chronic toxicity on ICR mice

The purified bacterium was inoculated at low temperature of heterotrophic nitrification aerobic liquid medium for 5 days. The cells were collected by centrifugation and then washed 3 times with sterile saline and configured to 1.0×10^{11} CFU/mL (group a), 1.0×10^{10} CFU/mL (group b), 1.0×10^{9} CFU/mL (group c) and 1.0×10^{8} CFU/ml (group d) bacterial suspension respectively. One hundred mice, half male and half female, weighing 15±5 g were equally divided into five groups, labeled A, B, C, D and E. Group A to D were the experimental groups and group E was the control group. The mice were fed with bacterial suspension each day for three consecutive months. Daily cage-side observations focused on skin/fur, eyes, mucous membranes, respiratory and circulatory systems, motor activity and behavioral pattern.

Pathology detection

Heparin tubes were used to detect red blood cells count, white blood cells count, platelets count, and hemoglobin (content from the mice eyes. Automatic biochemical analyzer was used to measure the total protein, globulin, albumin, albumin/globulin, aspartate aminotransferase, alanine aminotransferase, glucose, blood urea nitrogen, creatinine, triglyceride, total cholesterol, alkaline phosphatase, lactate dehydrogenase, creatine kinase and other indicators.

The liver, kidneys, and spleen of mice were dissected out of the body, rinsed with saline and fixed in 4% formaldehyde. The histological sections were cut after paraffin embedding. The sections were stained, sealed with the neutral resin and observed under a light microscope.

Ames test

The Ames test (Ames *et al*., 1975; Maron and Ames, 1983) was performed to evaluate mutagenic properties of Strain Y7. A cytotoxicity assessment was performed to determine the appropriate dose range for the assay. The standard plate incorporation method was adopted. The heterotrophic nitrifying bacteria cultured cryogenic liquid was set as the experimental groups with four concentrations (100, 200, 400 and 800µl/dish, respectively). Apart from the above groups, another set of spontaneous revertant and positive control group (TA98 with DEXON, TA100 with sodium azide) was set. The experiment group was under constant temperature, then fresh enrichment broth of TA98 or TA00 100 µl was added. The mixture was quickly poured into the underlying material, turned evenly to distribute in the underlying flat material and was observed for 48h in incubation. Experiments were performed in triplicate and repeated once. The findings were used to calculate the average number of revertant colonies.

Data analysis and statistics

The data was expressed as Means±SD (standard deviation of means). Data were analyzed by one-way ANOVA with Tukey's HSD test ($P < 0.05$) using SPSS 19.0 software.

RESULTS AND DISCUSSION

Identification and characteristics of strain Y7

The strain Y7 had the highest removal rate of ammonia nitrogen. Bacteria grew well on the plate (Fig. 1) formed rounded, smooth, whitish and opaque colonies with neat edge. Strain Y7 was Gram-positive, nonspore-forming and brevi-

bacterium with the size of approximately 1.2 to 1.4-1.5 µm.

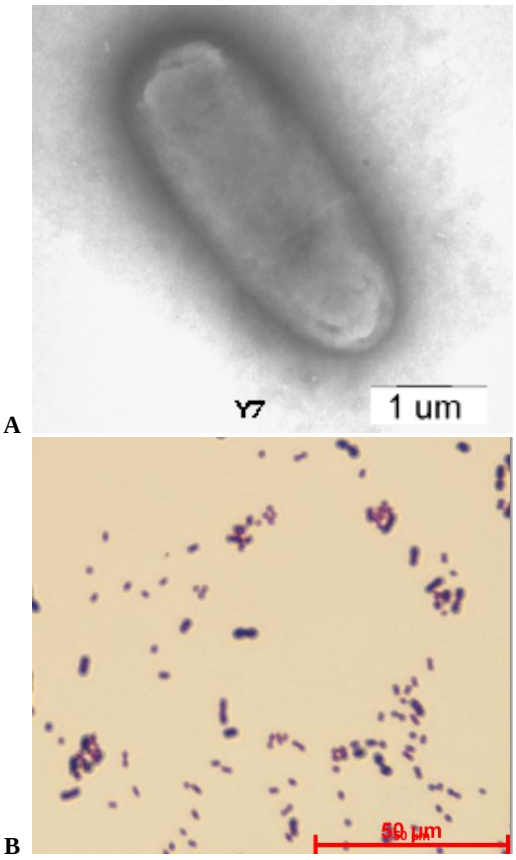

Fig. 1. A, Strains morphology and B, strains of bacteria form.

Partial 16S rRNA gene (1328 bp) of strain Y7 was amplified and sequenced. The homologous sequence in GenBank was obtained with Blast tools provided by NCBI (National Center for Biotechnology Information). A phylogenetic tree was constructed based on partial 16S rDNA sequence of Y7 using neighbor joining method with Bootstrap values of 1000 replications (Fig. 2). The results indicated that strain Y7 was closely related to members of genus *Acinetobacter*, and showed the highest similarity (98%) to *Acinetobacter* sp. Pi 4 (KC843489). Some members of *Acinetobacter* have heterotrophic nitrogen removal capability. Therefore, the isolated strain Y7 was identified to be an *Acinetobacter* species and heterotrophic bacterium.

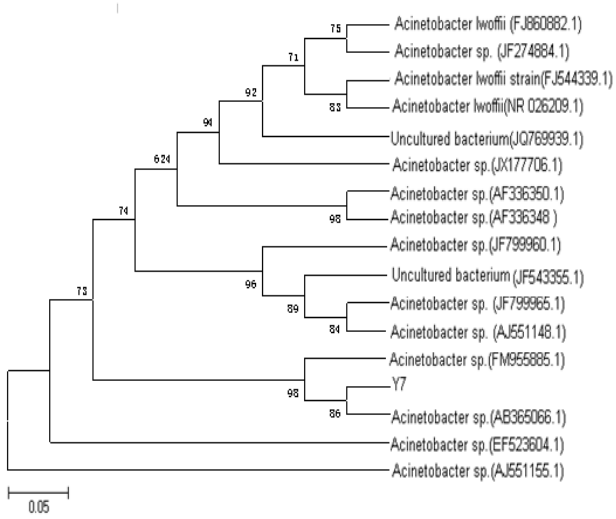

Fig. 2. Phylogenetic dendrogram based on 16S rRNA gene sequence analysis.

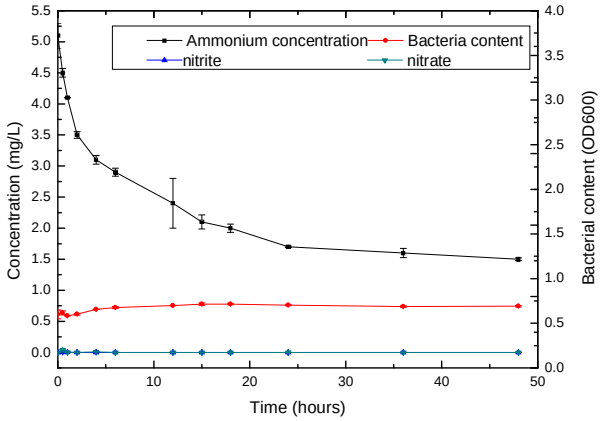

Fig. 3. Ammonium removal characteristics of *Acinetobacter* sp. Y7 at 2°C.

Ammonia degradation at low temperature

Normally, low ambient temperature can slow down microbial growth and inhibit the function of ammonium oxidizers. In this study, *Acinetobacter* sp. Y7 grew well under oligotrophic conditions and could utilize ammonium at 2°C. The ammonium removal characteristics of strain Y7 in shaking cultures is shown in Figure 3. With the initial ammonia concentration of 5mg/L, the concentration of ammonium decreased dramatically within the 48-hour observation period. As shown in Figure 3, the ammonium removal rate was 0.37±0.13 mg NH_4^+-N/L·h in the initial 6 h and then increased rapidly.

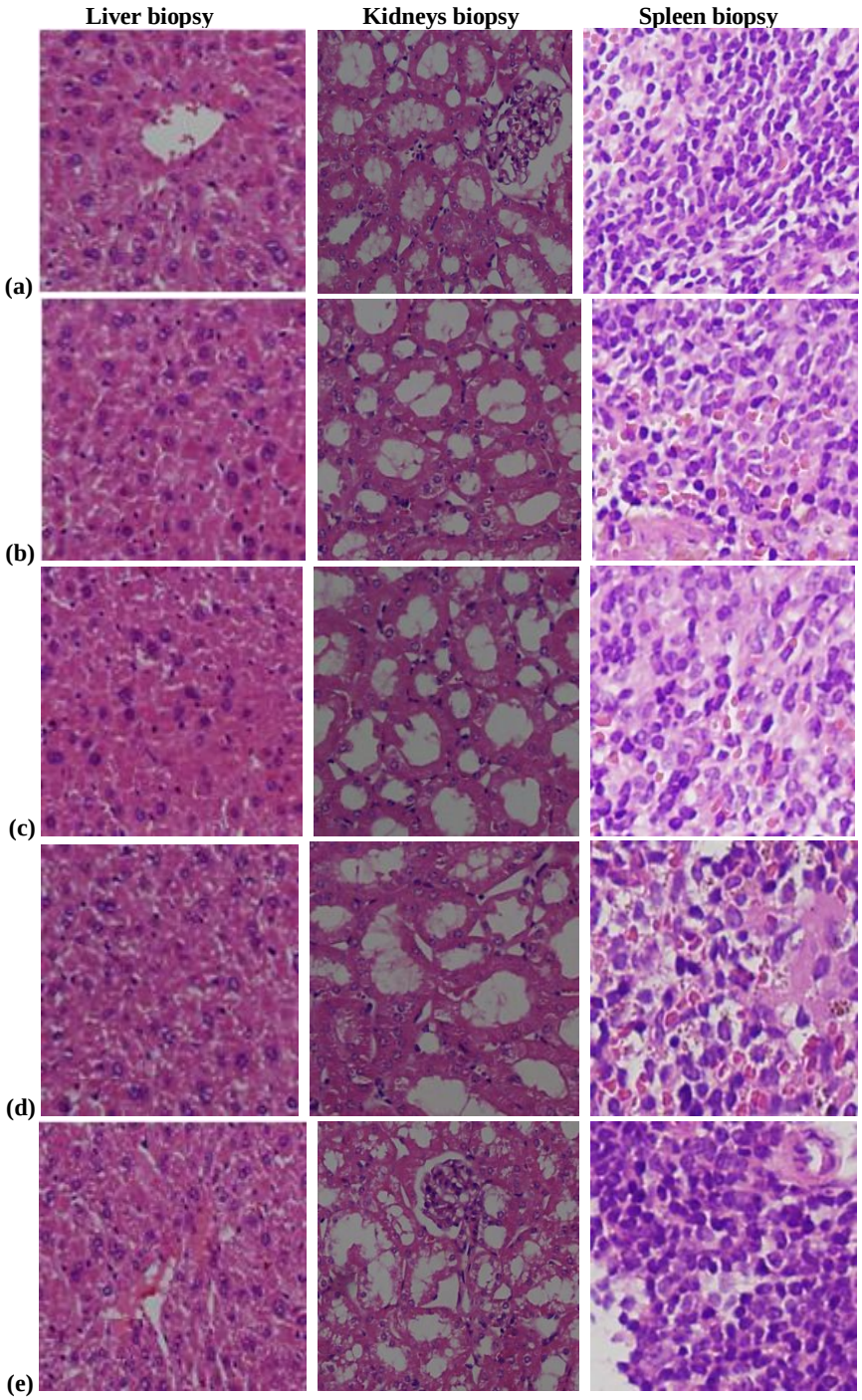

Fig. 4. Liver, kidneys, spleen biopsy in (a)-(d) experimental group and (e) control group (×40).

A final 66.70±3.06% of ammonium removal was achieved after 24 h of incubation. During the whole process, the average ammonium removal rate was approximately 0.075±0.007 mg NH_4^+-N/L·h (the whole test time was 48 h, and the value for 24h is 0.13±0.007 mg NH_4^+-N/L·h). The heterotrophic nitrification phenomenon was found in the soil nitrification process as early as in the late 19th and

early 20th century. In recent years, great progress has been made in heterotrophic nitrification at low temperature. In our previous study, isolated Strain Y16 had a similar characteristics for ammonium removal, with an average rate of 0.092 mg NH_4^+-N/L·h at 2°C (the test time was 36 h) (Huang *et al*., 2013). A newly isolated *Microbacterium* sp. SFA13 showed a higher nitrification rate of 0.11 mg NH_4^+-N/L·h at 5°C (In the initial 30 min, 11.39% NH_4^+-N was removed, and the removal rate was 1.16 mg NH_4^+-N/L·h.) (Zhang *et al*., 2013). Another *Pseudomonas stutzeri* YZN-001 had a notable ability to remove ammonium (0.3 mg NH_4^+-N/L·h) at 4°C (Zhang *et al*., 2011). At the same time, the accumulation of nitrite nitrogen was not detected and only trace accumulation of nitrate was observed during the ammonium removal process by strain Y7. These results indicated that the removal of ammonium by strain Y7 was mainly due to bacterial assimilation and simultaneous conversion into nitrogenous gas. Similar result has been found for several strains (Zhang *et al*., 2011; Huang *et al*., 2013), which was able to oxidize ammonium with no accumulation of nitrite and nitrate.

Toxicity of acute dose of strain Y7

Table I shows hematological values of mice after administration of acute dose of y7 bacteria. The results revealed no statistically significant differences among different groups. It is worth noting that the parameters of the differential blood count in group B were relatively higher compared to the control group and group A. However, all values in group B were still in the normal range. The difference between group B and the control group was not statistically significant. The biochemical parameters also remained unaffected by the acute infection treatments. The observed increased in aspartate aminotransferase and creatinine in group B were considered to be fortuitous, since there was no evidence of a relationship between dose and response, and since there were no corresponding changes observed in group C. Therefore, there was no significant difference among these groups.

Intragastric infection of Strain Y7

Mice were gavaged with Strain Y7 and observed continuously for three months. During the intragastric infection experiments, no mortality occurred and none of the mice (including treated and untreated) exhibited any clinical sign on cage-side observation. Food and water consumption as well as body weight gain did not differ among the treated groups (data not shown).

No abnormalities were detected in liver, kidneys, spleen or other organs of the mice (Fig. 4). Liver cells of the mice still kept their polygonal shape and normal structure in test groups and control group. Renal corpuscle and renal tubules of the mice worked normally. Spleen is the largest peripheral immune organ of the mammal. As the dose of the heterotrophic nitrification bacteria increase, the spleen still kept its normal color and contained a large amount of lymphocytes and macrophages. No spleen swelling occurred. These results showed that, it is safe to give the mice low-temperature heterotrophic nitrification bacteria (*Acinetobacter*.Y7) below the dose of 1.0×10^8 CFU per mouse via oral.

Mutagenic effect of strain Y7

Table III shows the number of reverse mutated-colonies caused by checked samples. Ames test on back mutation action of the strain using both TA_{98} and TA_{100} strains showed negative results. There were no revertants exceeding three times the background average either with or without the metabolic activation system. In addition, no dose-dependent increase was observed in revertants.

CONCLUSIONS

A novel heterotrophic nitrifying bacterium, *Acinetobacter* sp. Y7 was isolated from oligotrophic source water. In the initial ammonia concentration of 5mg/L, low temperature and oligotrophic conditions, ammonia degradation rate at 30min was 26.7%, higher than the rates obtained from normal oligotrophic strains of heterotrophic nitrification bacteria. The safety assessment of *Acinetobacter* sp.Y7 showed that the amount of bacteria below 1.0×10^8 CFU/Kg was safe, and the mutagenicity also did not appear. These results proved that *Acinetobacter* Y7 is non- pathogenic to animals and can be used to develop probiotics strains for treating micro-polluted source water in cold regions.

Table I.- Hematological and biochemical values in the mice acute toxicity study

Category	RBC ($\times10^9$/L)	WBC ($\times10^9$/L)	HGB (g/L)	PLT ($\times10^9$/L)	LYM (%)
Group A	7.3±0.2	5.6±0.7	138±4	764±125	0.83±0.2
Experimental group B	7.0±0.3	5.7±0.6	135±5	755±123	0.87±0.4
Experimental group C	7.6±0.3	5.8±0.2	141±3	776±112	0.90±0.2
Prob>F	0.30	0.22	0.10	0.10	0.10

Values are Mean±SE. Significance change at $p < 0.05$. HGB, hemoglobin, LYM, lymphocytes; PLT, platelets; RBC, red blood cell count; WBC, white blood cells.

Table II.- Biochemistry values in the mice acute toxicity study.

Parameters	Control (n=30)	600 mg/kg (n=30)	1200 mg/kg (n=30)
Alanine aminotransferase (IU/L)	62.89±6.12	59.38±5.83	64.22±5.13
Aspartate aminotransferase (IU/L)	125.64±11.56	130.64±9.36	128.26±7.30
Albumin (g/L)	34.46±1.67	34.16±1.08	31.16±3.08
Globulin (g/L)	24.44±4.5	23.56± 3.5	25.53±2.7
A/G	1.36± 0.34	1.46± 0.38	1.42±0.72
Urea (mmol/L)	5.86 ± 0.08	6.07 ± 0.12	5.93 ± 0.22
Creatinine (μmol/L)	35.68±4.34	40.01±5.39	38.45±3.33
Glucose (μmol/L)	11.67 ± 1.76	8.60 ±1.96	9.89 ± 2.14
Triglyceride (mmol/L)	0.56± 0.13	0.47±0.16	0.56±0.15
Cholesterol (mmol/L)	1.87 ±0.09	1.91 ± 0.10	1.89± 0.03

Table III.- The result of Ames Test on test sample (number of reverse mutated-colonies/Plate, x±sd, n=6)

Samples	Concentration (μl/plate)	TA_{98}	MR	TA_{100}	MR
Y7	100	32.6±10.0	1.09	165.0±19.3	1.11
	200	26.8±5.3	0.90	155.7±34.0	1.05
	400	29.6±3.2	0.99	155.2±19.5	1.05
	800	26.8±3.5	1.00	171.3±25.8	1.16

* MR value = number of revertant colonies / the number of spontaneous revertant colonies, MR ≥ 2 and as a positive result.
Values are mean±SE. Significance at $p < 0.05$.

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REFERENCES

AMES, B.N., MCCANN, J. AND YAMASAKI, E., 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutat. Res*., **31**: 347-364.

AHMAD, M.S. AND SHAKOORI, A.R., 2013. Isolation, molecular characterization and toxicity of cry1C gene harboring *Bacillus thuringiensis* from different habitats and localities of Pakistan. *Pakistan J. Zool.*, **45**: 261-271.

CHEN, P., LI, J., LI, Q.X., WANG, Y., LI, S., REN, T. AND WANG, L., 2012. Simultaneous heterotrophic nitrification and aerobic denitrification by bacterium *Rhodococcus* sp. CPZ24. *Bioresour. Technol.*, **116**: 266-270.

CHEN, Q. AND NI, J., 2011. Heterotrophic nitrification-aerobic denitrification by novel isolated bacteria. *J. Ind. Microbiol. Biotechnol.*, **38**: 1305-1310.

CHEN, Q. AND NI, J., 2012. Ammonium removal by Agrobacterium sp. LAD9 capable of heterotrophic nitrification-aerobic denitrification. *J. Biosci. Bioeng.*, **113**: 619-623.

CLAUS, D., 1992. A standardized Gram staining procedure. *World J. Microbiol. Biotechnol*., **8**:451-452.

DE GROOT, A.P., TIL, H.P. AND FERON, V.J., 1971. Safety evaluation of yeast grown on hydrocarbons. III. Two-year feeding and multigeneration study in rats with yeast grown on gas oil. *Fd. Cosmet. Toxicol.,* **9**:787–800.

DE GROOT, A. P., 1976. Safety evaluation studies with SCP. *PAG Bull*., **6**: 41–4.

DOXTADER, K.G. AND ALEXANDER, M., 1966. Nitrificationsoil by heterotrophic microorganisms soil. *Sci. Soc. AmeL Proc*., **30***:* 351-355.

ENGEL, C., 1972. Safety evaluation of yeast grown on hydrocarbons. In: *Proteins from hydrocarbons* (ed. Gounelle de Pontanel H) Academic Press, London, pp. 53–81.

EYLAR, O.R. AND SCHMIDT, E.L., 1959. A survey of heterotrophic microorganisms from soil for Ability to form nitrate and nitrate. *Microbiology, **20***: 473-481.

FISHER, T., FISHER, E. AND APPLEMAN, M.D., 1956. Nitrite production by heterotrophic bacteria. *Gen. Microbiol.,* **14**: 238-247.

HUANG, X., LI, W., ZHANG, D. AND QIN, W., 2013. Ammonium removal by a novel oligotrophic Acinetobacter sp. Y16 capable of heterotrophic nitrification-aerobic denitrification at low temperature. *Bioresour. Technol.,* **146**: 44-50.

MARON, D.M. AND AMES, B.N., 1983. Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.,* **113**: 173-215.

MINISTRY OF ENVIRONMENTAL PROTECTION, 2012. *People's Republic of China,* 4-15.

PAPEN H. AND VON BERG, R., 1998. A most probable number (MPN) method for the estimation of cell numbers of heterotrophic nitrifying bacteria in soil. *Plant and soil*, **199**: 123-130.

WHIRE, J.P. AND JOHNSON, G.T., 1982. Anatoxin production correlmed with nitrification in Aspergiuus flavus group species. *Mycologia.,* **74**: 718-723.

ZHAO, B., AN, Q., HE, Y.L. AND GUO, J.S., 2012. N_2O and N_2 productio during heterotrophic nitrification by *Alcaligenes faecalis* strain NR. *Bioresour. Technol.,* **116**: 379-85.

ZHANG, D., LI, W., HUANG, X., QIN, W. AND LIU, M., 2013. Removal of ammonium in surface water at low temperature by a newly isolated *Microbacterium* sp. Strain SFA 13. *Bioresour. Technol.,* **137**: 147-152.

ZHANG, Q.L., LIU, Y., AI, G.M., MIAO, L.L., ZHENG, H.Y. AND LIU, Z.P., 2012. The characteristics of a novel heterotrophic nitrification-aerobic denitrification bacterium, Bacillus methylotrophicus strain L7. *Bioresour. Technol.,* **108**: 35-44.

ZHANG, J., WU, P., HAO, B. AND YU, Z., 2011. Heterotrophic nitrification and aerobic denitrification by the bacterium *Pseudomonas stutzeri* YZN-001. *Bioresour. Technol.,* **102**, 9866-9869.

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