Rapid Screening of Ammonia Oxidizing Bacteria in the Sewage

Cuijuan Zhao, Wenjun Song, Jiping Wei, and Bozhi Li

***Abstract*—The ammonia oxidizing to nitrite is a key step in biological nitrogen removal process in biological wasterwater treatment systems. In order to reduce the concentration of NH_4^+-N in the sewage. An novel and rapid method for screening of AOB from the sewage is described in this study, AOB were isolated from Haihe hospital and Jizhuangzi sewage treatment plant. Due to the using of 96-well ELISA plate and nzyme-labelling measuring instrument, the speed of screen was fasted and the time of screen was to be shorten. As a result, 7 efficient bacteria were screened, which ammonia nitrogen removal rate was from 70% to 80%. These results might provide useful and valuable information about the nitritation process, which plays an important role in biological wastewater treatment systems.**

***Index Terms*—Sewage, ammonia oxidizing bacteria, rapid screening, identification.**

I. Introduction

Increasing population, agricultural advancement and urbanization have resulted in higher nitrogen loading to rivers through aqueous wastes from several key industries, agricultural, hospital and domestic wastes [1], [2]. Nitrogen in wastewater is usually present as organic nitrogen or ions such as NH_4^+, NO_2^- and NO_3^-. The discharge of effluents containing high concentrations of nitrogen is undesirable because it causes excessive oxygen demand, causes eutrophication, harms the ecosystem, weakens the water body vitality, deprives water use applications [3] and increases the formation of nitrosamines which are carcinogenic [4], [5].

Biological nitrogen removal is an important part of wastewater treatment processes due to the significant impact of nitrogen compounds on the aquatic environment and on wastewater treatment plant [6], [7]. Biological nitrification–denitrification is the most frequently used process for removing nitrogen in wastewater [8]. Studies have shown that the composition and diversity of microbial communities is important in nitrogen transformation and removal processes [9], [10]. The process of nitrification–denitrification is a microbial process which comprises of two phases [11]. The first, known as nitrification, is an aerobic process carried out by Gram-negative, in which ammonium is oxidized to nitrite (NO_2^-) by means of ammonia oxidizing bacteria (AOB). Ammonia-oxidizing bacteria (AOB) play an importance role in nitrogen conversion in biological waste water treatment systems. AOB produce soluble microbial products and nitrous oxides, respectively, Subsequently nitrite is oxidized to nitrate (NO_3^-) using nitrite oxidizing bacteria (NOB). The AOB and NOB use CO_2 or inorganic carbon as a carbon source for the synthesis of cellular material [12] and ammonia or nitrite as an energy source [13]. The second phase is denitrification, an anoxic microbial process by which nitrates are reduced to molecular nitrogen sequential process. These nitrates are first converted to nitrites (NO_2^-), then to nitric oxide (NO) [14], [15], after that to nitrous oxide (N_2O) and finally to molecular nitrogen (N_2), which is released into the atmosphere.

In recent years, some new BNR processes [16]-[18]. such as single high ammonia removal over nitrite (SHARON) and anaerobic ammonium oxidation (ANAMMOX), have been investigated widely [19]-[23], in addition, *Nitrosomonas eutropha* was already considered have the ability for simultaneous nitrification and denitrification [24], [25]. The objectives of this study were to research an novel and rapid method for the screening of AOB from the sewage, it can gain efficient bacteria which can removal ammonia nitrogen from sewage, so as to contribute to the cure of severe ammonia pollution.

II. Materials and Methods

A. Materials and Instruments

Sample was taken from the Tianjin Haihe Hospital aeration tank and the Tianjin Jizhuangzi sewage treatment plant aeration tank of sewage, the sample in this experiment as the experimental material, the screening of the experiment from the Haihe Hospital strains begin to H, in turn in Arabic numerals1 to 100 naming, starting with J from the Jizhuangzi sewage treatment plant screening strains sequentially with Arabic numerals 1 to 100 be named (they may be named in a random order, no special meaning).

Greiner Microlon 96-well ELISA Plate (Germany), 96-well deep well culture plate, volley of rifle fire, SPECTRmax PLUS enzyme-labelling measuring instrument (the Molecular devices company of USA).

B. Experimental Flow Chart

1) The separation of Ammonia-oxidizing bacteria

The substrate used for the solid isolation medium contained (per liter): $(NH_4)_2SO_4$, 472 g; KH_2PO_4, 7.25 g; Na_2HPO_4, 11.32 g; glucose, 12 g; $MgSO_4$, 200 g; $CaCl_2$, 20 g; $NaHCO_3$, 85 g; trace elements A($FeSO_4$ 5 g, EDTA 5 g);

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trace elements B(EDTA 15 g, $ZnSO_4$ 7H_2O 4.3 g, CoCl 6H_2O 2.4 g, $MnCl_2$ 6.29 g, $CuSO_4$ 5H_2O 2.5 g, Na_2MoO_4 2H_2O 2.2 g, $NiCl_2$ 6H_2O 1.9 g, H_3BO_3 0.14 g); Agar, 17 g. The pH of the isolation medium was adjusted to about 8.0 by 5% Na_2CO_3 and HCl. The isolation medium were loaded to 500 ml flask, subpackage finished, 115 ℃ sterilization 25 min. Sewage samples were diluted gradient to 10^{-4}, 10^{-5}, 10^{-6} times, in triplicate, 80 ul diluents were coated to ammonia oxidation bacteria solid isolation medium respectively, 30 ℃ cultivation 48h. The plates coating separate colony count in the 30-300 were selected, which were crossed to solid isolation medium the stars pure until a single colony, a single colony was marked on the flat plate and marked a total of 86 single colonies.

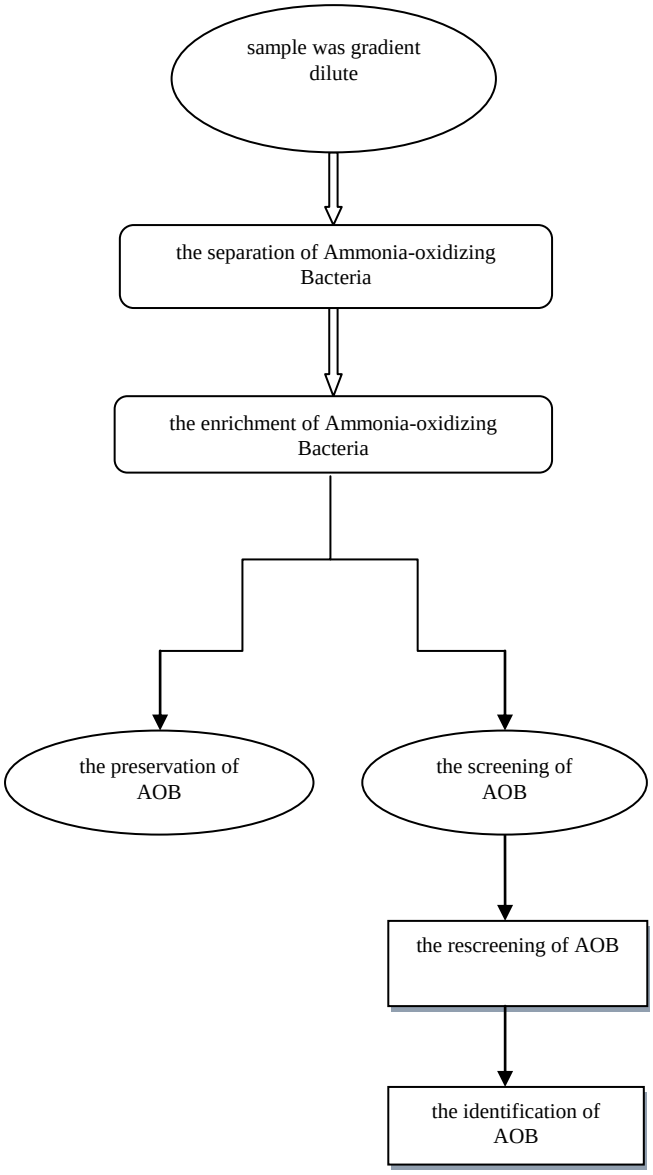

Fig. 1. Quick screening procedure of ammonia-oxidizing bacteria

2) The enrichment of Ammonia-oxidizing bacteria

The substrate used for the enrichment medium contained (per liter): $(NH_4)_2SO_4$, 472 g; KH_2PO_4, 7.25 g; Na_2HPO_4, 11.32 g; glucose, 24 g; $MgSO_4$, 200 g; $CaCl_2$, 20 g; $NaHCO_3$, 85 g; trace elements A; trace elements B. (the pH of the enrichment medium was adjusted to about 8.0 by 5% Na_2CO_3 and HCl. The enrichment medium were loaded to 2.0 ml screw frozen storage tube, each frozen storage tube added 0.6 ml enrichment medium, subpackage finished, would be tube cover screw down into frozen storage box, 115 ℃ sterilization 25 min). Single colony was inoculated to the enrichment medium with inoculating loop, they were cultivated on a rotary shaker at 170 rpm, 30 ℃ and 24h.

3) The screening of ammonia-oxidizing bacteria

a) Micro-well-plate chromatogram assay

The substrate used for the screening of medium contained (per liter): $(NH_4)_2SO_4$, 472 g; KH_2PO_4, 7.25 g; Na_2HPO_4, 11.32 g; CH_3COONa, 80 g; $MgSO_4$, 200 g; $CaCl_2$, 20 g; $NaHCO_3$, 85 g; trace elements A; trace elements B. (the pH of the enrichment medium was adjusted to about 8.0 by 5% NaOH and CH_3COOH, the identification medium was subpackaged to 96-well deep well culture plate, each well was added to the enrichment medium 200ul, subpackage finished, 115 ℃ sterilization 25 min). Then 20ul enrichment broth were added to every well by volley of rifle fire, then sterilized municipal wastewater as a comparative sample, municipal sewage concentrations of NH_4^+-N, COD were 31 mg/L and 184 mg/L, respectively. 200 ul municipal wastewater were added to corresponding well, 20 ul enrichment broth were inoculated to municipal sewage. The concentration of nitrite was measured according to Griess reagent colorimetric. It can identified dozens of strain and even hundreds of strain ammonia oxidation bacteria simultaneously by this method, thus fast and high throughput of ammonia oxidizing bacteria identification was achieved.

b) Spectroscopy

100 ul solution was added to 96-well ELISA plate from 96-well plate with volley of rifle fire, and then absorbance was measured at 550nm wavelength by using the enzyme-labelling measuring instrument, recorded value, calculated nitrite nitrogen concentration according to the standard curve.

C. Ammonia Oxidation Strains Preservation

The equal volume of 40% glycerol was added to the strain enriched tube remaining bacterial suspension, shocked uniformly and placed -20 ℃ refrigerator.

D. The Rescreening of Ammonia-Oxidizing Bacteria

The bacteria were rescreening from the beginning of the screen, enrichment bacteria liquid was added to 96-well deep well culture plate which contained rescreening medium by volley of rifle fire, they were cultivated on a rotary shaker at 170 rpm, 30 ℃and 24h, then their function was determined, the bacteria liquid was added to 96-well ELISA plate in triplicate, NH_4^+-N, NO_2^--N and NO_3^--N concentrations were measured. All inhibition experiments were performed in triplicate. Determination of ammonia nitrogen was by Nessler's reagent spectrophotometry [26], determination of nitrite was with Griess reagent colorimetric method [27], determination of nitrate by ultraviolet spectrophotometry [28], and then calculated the removal rate of ammonia nitrogen according to the appraisal medium initial ammonia nitrogen concentration.

E. The Identification of Ammonia-Oxidizing Bacteria

DNA was extracted from 1 g to 1.5 g (wet weight) of homogenized sediment sludge sample according to the

procedure. Fragments of genes were amplified using the following primer set: BOXAIR (5'-CTACGGCAAGGC GACGCTGACG-3'), PCR amplification was conducted in a total volume of 25 μl containing 2.5 μl of 10×PCR buffer (containing 1.5 mM $MgCl_2$), 2 mM of each deoxynucleotide triphosphate, 0.13 U of Taq polymerase (Sangon, Shanghai, China), 25 pM of each primer and 10–100 ng DNA. For amplification of the environmental samples, 400 ng μl-1 BSA was added to the reaction mixtures. PCR for AOB was conducted by subjecting the samples to the following conditions: initial denaturation at 95 ℃ for 7 min, followed by 30 cycles of 92 ℃ for 30 s, 52 ℃ for 60 s, and 65 ℃ for 8 min, with a final extension at 65 ℃ for 16 min.

III. Results and Discussion

A. The Screening of Strains

86 strains were separated by isolation medium, these 86 strains were cultured in 96-well deep well culture plate , the concentration of nitrite was determined with Griess reagent colorimetric method, color change was shown (Fig. 2).

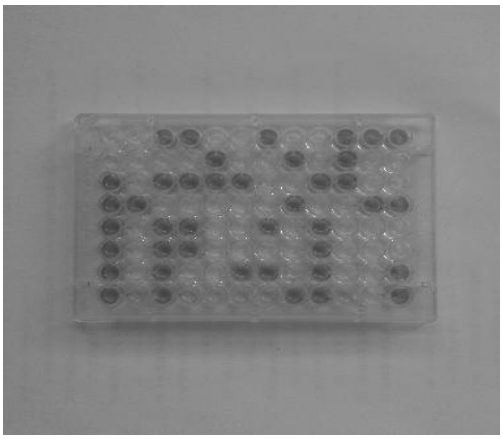

Fig. 2. The result of screening.

The concentration of nitrite grew to or above 0.2 mg/L, which nitrification activity was much higher than others, then the 48 strains were screened by this rapid method, they were numbered H-3, H-4, H-7, H-10, H-11, H-12, H-17, H-20, H-22, H-25, H-27, H-28, H-29, H-30, H-33, H-34, H-37, H-38, H-44, H-47, H-48, H-49, H-51, H-52, H-55, H-57, J-1, J-3, J-4, J-9, J-13, J-14, J-16, J-19, J-20, J-22, J-25, J-26, J-28, J-33, J-34, J-37, J-38, J-41, J-42, J-47, J-48 and J-49 respectively, their concentration of nitrite were shown (Table 1).

B. The Rescreening of Strains

The 48 strains which were from the first screening were rescreening, they were inoculated to the rescreening of medium, which contained 98 mg/L NH_4^+-N, concentrations of NH_4^+-N, NO_2^--N and NO_3--N were measured, the concrete numerical values were shown (Table 2), then calculated ammonia nitrogen removal, 7 efficient bacteria were screened, they were H-17, H-30, H-37, J-1, J-13, J-41 and J-49, their ammonia nitrogen removal rate were 70.41%, 73.47%, 82.65%, 84.69%, 70.41%, 80.61% and 84.69% respectively. These efficient bacteria will provide useful and valuable information about the nitritation process, which plays an important role in biological wastewater treatment systems.

TABLE I: The Concentration of Nitrite

strains	NO_2^--N （mg/L）	strains	NO_2^--N (mg/L)
H-3	0.225	J-1	0.294
H-4	0.241	J-3	0.236
H-7	0.263	J-4	0.241
H-10	0.218	J-9	0.237
H-11	0.264	J-13	0.283
H-12	0.229	J-14	0.219
H-17	0.283	J-16	0.267
H-20	0.216	J-19	0.281
H-22	0.209	J-20	0.239
H-25	0.271	J-22	0.208
H-27	0.228	J-25	0.227
H-28	0.269	J-26	0.264
H-29	0.237	J-28	0.273
H-30	0.286	J-33	0.251
H-33	0.253	J-34	0.218
H-34	0.282	J-37	0.267
H-37	0.293	J-38	0.236
H-38	0.249	J-41	0.291
H-44	0.217	J-42	0.213
H-47	0.238	J-47	0.254
H-48	0.246	J-48	0.269
H-49	0.263	J-49	0.294
H-51	0.254		
H-52	0.247		
H-55	0.228		
H-57	0.265		

TABLE II: The Ammonia Nitrogen Removal Rate of Strains

strains	NH_4^+-N （mg/L）	NO_2^--N （mg/L）	NO_3^--N （mg/L）	NH_4^+-N removal efficiency(%)
H-7	47	0.263	0.412	52.04
H-11	45	0.264	0.397	54.08
H-17	29	0.283	0.376	70.41
H-25	39	0.271	0.381	60.20
H-28	41	0.269	0.377	58.16
H-30	26	0.286	0.398	73.47
H-33	50	0.253	0.357	48.98
H-34	31	0.282	0.369	68.38
H-37	17	0.293	0.403	82.65
H-49	47	0.263	0.395	52.04
H-51	49	0.254	0.375	50.00
H-57	44	0.265	0.357	55.10
J-1	15	0.294	0.390	84.69
J-13	29	0.283	0.409	70.41
J-16	42	0.267	0.388	57.14
J-19	33	0.281	0.382	66.33
J-26	45	0.264	0.361	54.08
J-28	38	0.273	0.398	61.22
J-33	52	0.251	0.375	46.94
J-37	42	0.267	0.384	57.14
J-41	19	0.291	0.416	80.61
J-47	49	0.254	0.425	50.00
J-48	41	0.269	0.393	58.16
J-49	15	0.294	0.371	84.69

C. The Identification of Efficient Bacteria

7 efficient bacteria was identified by DNA isolation,

amplification, sequencing analysis, the electropherogram of 7 efficient bacteria was shown (Fig. 3). After sequencing analysis, as a result, H-17, H-30, H-37, J-1, J-13, J-41 and J-49 was *Betaproteobacteria* , *Gammaproteobacteria*, *Nitrosomonas eutropha*, *Nitrosospira sp*, *Micrococcus sp*, *Bacillus sp*, *Providencia rettgeri*.

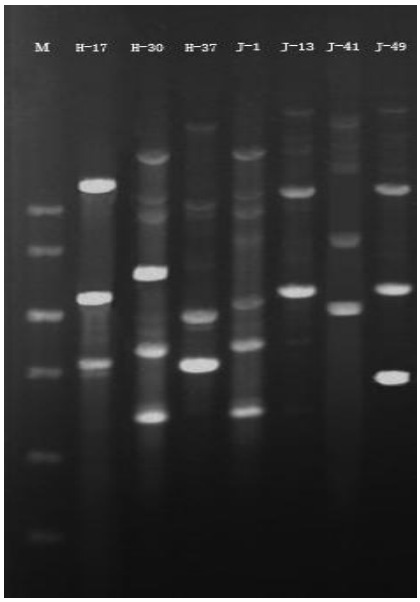

Fig. 3. The electropherogram of efficient

IV. Conclusion

In this work we establish a rapid and efficient method for screening of AOB from the sewage, this new method can be used for sewage treatment plant on a large scale, moreover it can obtain a reasonably high success rate in isolating AOB. The purpose of this study is to hope that by the screening of AOB and genetics research, it can gain efficient bacteria which can removal ammonia nitrogen from sewage, so as to contribute to the cure of severe ammonia pollution.

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