African Journal of Biotechnology Vol. 10(36), pp. 6985-6990, 18 July, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1855
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Heterotrophic nitrification and aerobic denitrification bacterium isolated from anaerobic/anoxic/oxic treatment system

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Accepted 25 March, 2011

A special strain of *Alcaligenes denitrificans,* designated as WY200811, with heterotrophic nitrification and aerobic denitrification ability was novel isolated from an anaerobic/anoxic/oxic treatment system and characterized. The average ammonia nitrogen (NH_4^+-N) and TN (total nitrogen) removal rates of the strain were 0.026 and 0.028 gN/g·Cell·h. Under the environmental conditions of 22% DO and C/N ratio of 5, the ultimate efficiencies of nitrification and denitrification were 57.0 and 57.3%, respectively. The pH in the medium was maintained between 6.0 and 7.0. The novel strain can rapidly reduce the ammonia to nitrogen gas without nitrite accumulation. The WY200811 can be used in a full-scale anaerobic/anoxic/oxic treatment system for efficiency in heterotrophic nitrification and aerobic denitrification.

Key words: Heterotrophic nitrification and aerobic denitrification, *Alcaligenes denitrificans,* nitrogen balance.

INTRODUCTION

The biological nitrogen removal is a two-step process that is normally carried out by different groups of bacteria. Ammonium is aerobically nitrified by autotrophic bacteria and then, the nitrate and nitrite is anaerobically converted to nitrogen gas by heterotrophic bacteria (Robertson et al., 1988). It is generally claimed that, only strictly anaerobic denitrification can occur and that only autotrophic nitrifying bacteria can effectively carry out nitrification. However, it has been reported that *Thiosphaera pantotropha* is capable of both heterotrophic nitrification and aerobic denitrification, yielding N_2 as the final product (Robertson and van Niel, 1988). After *T. pantotropha* was isolated, many other bacteria that have heterotrophic nitrification and aerobic denitrification abilities were isolated too, such as *β-proteobacteria, Paracoccus denitrificans, Alcaligenes faecalis, Comamonas* sp. (Ahn, 2006; Su et al., 2001; von Munch et al., 1996). The aerobic nitrification-denitrification bacteria are abundant in wastewater and sludge environments, such as night soil, sewage sludge and piggery wastewater (Joo et al., 2005a; Su et al., 2006; Moir et al., 1996).

The flow chart of heterotrophic nitrification and aerobic denitrification is shown in Figure 1. The reductases for the heterotrophic nitrification and aerobic denitrification have been isolated and characterized. These include, ammonia monooxygenase (AMO) (Stouthamer et al., 1997), hydroxylamine oxidase (HAO) (Otte et al., 1999), periplasmic nitrate reductases (NAP) (Berks et al., 1995), nitrite reductases (NIR) (Moir et al., 1993), nitric oxide reductase (NOR) (Fujiwara and Fukumori, 1996) and nitrous oxide reductase (NOS) (Rasmussen et al., 2002).

According to literatures, the efficiencies of nitrification and denitrification bacteria increase strongly as the ratio of C/N increase. When the C/N ratio is less than 10, the highest efficiency is obtained at 25 to 35°C and under a pH value of 6 to 8 (Khardenavis et al., 2007; Chen et al., 2003; Yu et al., 2008). The changes in the efficiency of nitrogen removal of nitrification-denitrification bacteria were studied when different carbon sources were provided (Modin et al., 2007). The rate of heterotrophic nitrification and aerobic denitrification by *Nitrosomonas eutropha* in a fermentor with O_2 and NO_2 (or NO) was studied too (Zar and Bock, 1998). Many species of

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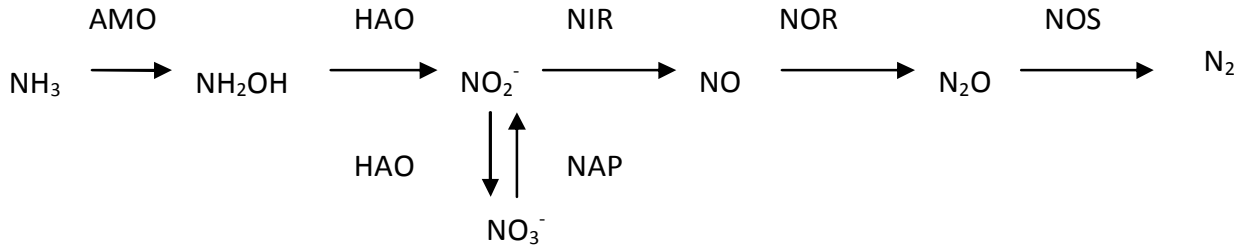

Figure 1. Flow chart and reductase of heterotrophic nitrification and aerobic denitrification.

heterotrophic nitrification and aerobic denitrification bacteria were isolated and characterized to study the treatment system (Zar and Bock, 1998; Joo et al., 2005b; Kim, et al., 2005). The common characteristics of heterotrophic nitrification and aerobic denitrification bacteria can be used to figure out the most efficient reaction conditions for the bacteria. The application of aerobic nitrification and denitrification is significant, but the species of heterotrophic nitrification and aerobic denitrification bacteria isolated are too scarce to permit the study of the common characteristics of bacteria performing both functions. The isolation of more species will assist the studies of such similarities. The objective of the study reported was to isolate and characterize a novel aerobic nitrification and heterotrophic denitrification bacterium from anaerobic/anoxic/oxic treatment system.

MATERIALS AND METHODS

Enrichment and isolation of the bacterium

The sludge (50 ml) obtained from the anaerobic/anoxic/oxic treatment system was inoculated into an enrichment liquid medium to grow the bacteria at 32°C. After 28 days of enrichment with repeated inoculation every fourth day, the growing cells were spread on the nitrification medium plates with 1.5% (w/v) nutrient agar. The enrichment medium contained (per l) yeast extracts 1.0 g, peptone 5.0 g, KNO_3 1.0 g.

Pure isolates were obtained by repeated streaking on fresh nutrient agar plates 3 times from the enrichment liquid medium. The isolates were identified as denitrification bacteria (Lin et al., 2007). Then, isolates were spread by streaking on characterization medium to estimate the ability to nitrify.

The medium used for identification of denitrification contained (per l) KNO_3 2.0 g, $NaNO_2$ 2.14 g, KH_2PO_4 1.50 g, $Na_2HPO_4 \cdot 12H_2O$ 10.50 g, $MgSO_4 \cdot 7H_2O$, 0.20 g and 2 ml mineral solution. The mineral solution was the same as Berks (Berks et al., 1994), the medium was sterilized by autoclaving for 20 min at 121°C.

Characterization of isolate

The medium used for the characterization of the isolate, contained (per l) sodium citrate 2.14 g, NH_4Cl, 0.40 g, KH_2PO_4 1.50 g, $Na_2HPO_4 \cdot 12H_2O$ 10.50 g, $MgSO_4 \cdot 7H_2O$ 0.20 g and 2 ml mineral solution. The medium was sterilized by autoclaving for 20 min at 121°C.

The nitrification and denitrification efficiencies of heterotrophic nitrification and aerobic denitrification bacteria are high when sodium citrate or glucose is the carbon source, the C/N ratio is between 5 and 10, the temperature is 30 to 32°C and the dissolved oxygen (DO) is saturated at 22 to 30% (von Munch et al., 1996; Joo et al., 2005a). In the present study, sodium citrate was used as carbon source, the C/N ratio was 5, the temperature was 32°C and the DO was 22%.

The initial composition of the substrate was as follows: NH_4^+-N, 105.0; NO_2^--N, 0.0; NO_3^--N, 0.0; TN, 115.0; C/N, 5, all values were in mg/l. Bacterial suspensions (5% w/v or v/v inoculums) of the isolates were inoculated into 500 ml flasks containing 200 ml of sterilized medium. Upper part of the flask was charged with oxygen at a purity of 98% (v/v) under a 100 kPa pressure. The pH value of the medium was 7 initially and once the bacteria were incubated into the flask they were under 150 r/min and 32°C for 96 h. The samples were taken from the flasks and measured at an interval of 8 h from the beginning of incubation. The DO, pH, biomass nitrogen, NH_4^+-N, NO_2^--N, NO_3^--N and total nitrogen were recorded. Gas left in the flasks was injected into the 20% (w/w) H_2SO_4 for measuring the NH_3.

To investigate changes in the cell density of the strain, the optical density (OD) of the bacterial suspension in the flask was determined at 520 nm. To demonstrate that the strains accomplished both nitrification and denitrification, NH_4Cl was used as the main nitrogen source in the medium. The other nitrogen source in the medium was EDTA, as the sole organic nitrogen source. The nitrification rate was calculated from the change in ammonium, nitrate and nitrite concentration in suspension. The denitrification rate was estimated from the change in ammonium concentration, the nitrite concentration produced, the reduction in the nitrate concentration and the TN in the bacterial suspension. The nitrogen balance for the flask culture was tested to estimate the nitrogen lost in the medium after the experiment. The TN in the cell was tested for the calculation of biomass nitrogen and the amount of nitrogen gas was calculated as the N lost from the flask (Frunzke and Meyer, 1990).

Phenotypical and chemotaxonomical characterization

Agar plate streaking and gram staining were made to obtain macroscopic and microscopic features of isolate. The cell size and morphology of isolate were also determined microscopically (E200 microscope, Nikon, Japan).

The isolate was identified by sequencing the 16S rDNA purified by a TIANamp bacteria DNA kit. The universal primer: F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and downstream primer R1522 (5'-AAGGAGGTGATCCAGCCGCA-3') were used to amplify their 16S rDNA by polymerase chain reaction (PCR) protocol. The 16 S rDNA gene sequence was determined by Guangzhou Top Genomics, Ltd. Finally, the 16S rDNA sequence of the strain was

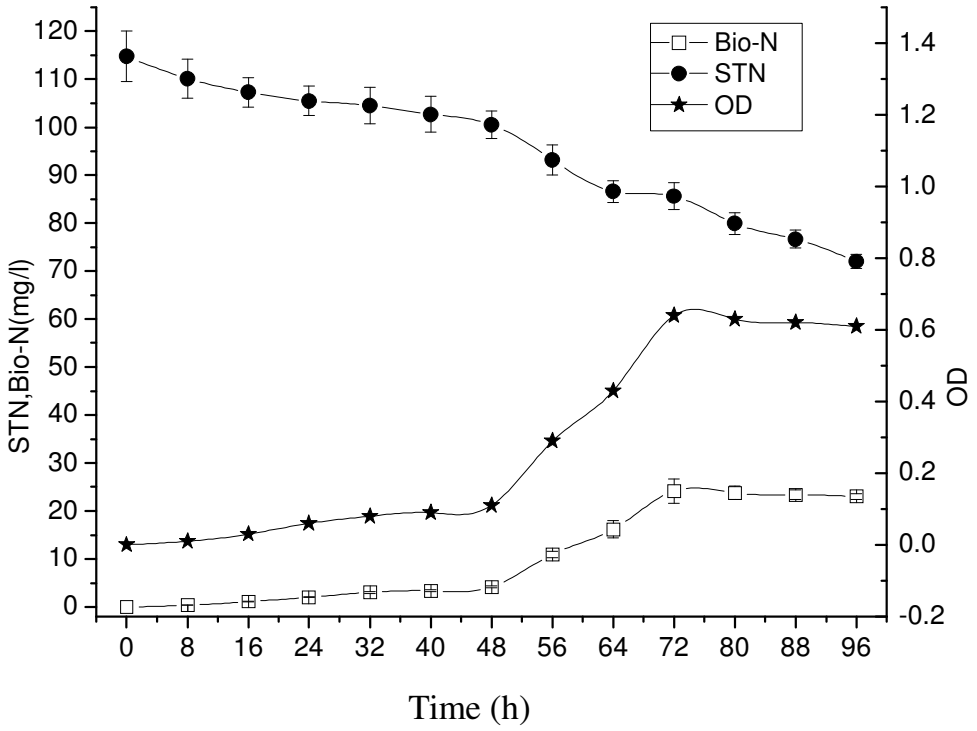

Figure 2. Changes in OD, STN and Bio –N of the characterization.

compared with other microorganisms by way of BLAST (http: //www.ncbi.nlm.nih.gov/BLAST/Blast. cgi).

Analytical methods

The bacterial suspension was centrifuged at 6000 r/min for 10 min and then, the supernatant was decanted and the residue was washed twice with distilled water. The water from the washing steps were combined with the supernatant. The aqueous and the pellet were determined with the TN separately. The TN of the cell pellet was the Bio-N and the nitrogen in the supernatant was the liquid TN (LTN). The LTN included NH_4^+-N, NO_2^--N, NO_3^--N, organic nitrogen (Org-N) and Bio-N. The Bio-N was the total nitrogen in the cell pellet. STN was the total nitrogen in the bacterial suspension. The Org-N was calculated as the difference between the LTN and NH_4^+-N, NO_2^--N, and NO_3^--N. The following relationship was applied:

(1) STN=LTN + Bio -N;
(2) LTN= NH_4^+-N + NO_3^--N + NO_2^--N + Org-N.

TN and NO_3^--N were determined by the ultraviolet adsorption methods. The pH value was determined with pH meter. DO was measured using a dissolved oxygen meter. The growth of isolates was examined by a spectrophotometer set at a wavelength of 520 nm (722 spectrophotometer; Shanghai Precision and Scientific Instrument Co. Ltd., China). The NH_4^+-N concentrations of the medium were determined using standard methods (APHA, 1992). The estimation of NH_4^+-N was performed by the Nessler's reagent method, which monitored the absorbance at 420 nm. Nitrate was estimated by the ultraviolet (UV2300, Shanghai Tech. Co., Ltd., China) adsorption and alkaline potassium persulfate digestion method (APHA, 1992). The nitrite content was estimated by the N-(1-naphthalene)-ethylene method (APHA, 1992), which monitored the absorbance at 540 nm.

RESULTS AND DISCUSSION

OD, DO and pH changes in medium

A strain was isolated and named WY200811. After inoculation into the characterization medium, the bacterial suspension slowly increased in OD during the first 48 h. Then, the cell population increased rapidly over the next 24 h, but the OD was almost unchanged over the final 24 h. These results show that, the number of new bacteria and bacteria decomposition was roughly the same, so the number of bacteria reached the maximum OD reading. The changes in OD, STN and Bio-N of the medium are presented in Figure 2.

The DO in the medium changed between 0.9 and 1.2 mg/l. The pH in the medium was maintained between 6.0 and 7.0. The lowest pH (6.0) appeared at the same time that DO was lowest and when NO_3^--N was highest. This phenomenon shows that, nitrification caused pH and DO to decrease in the medium. The changes in DO, STN and Bio-N are shown in Figure 2 (the dates of pH and DO are not shown in Figure 2).

Characteristics of isolates

The changes in NH_4^+-N, NO_2^--N, NO_3^--N, LTN and TN are shown in Figure 3. The concentration of NH_4^+-N decreased significantly during the first 48 h and was linked to denitrification or the oxidation of NH_4^+-N. A similar

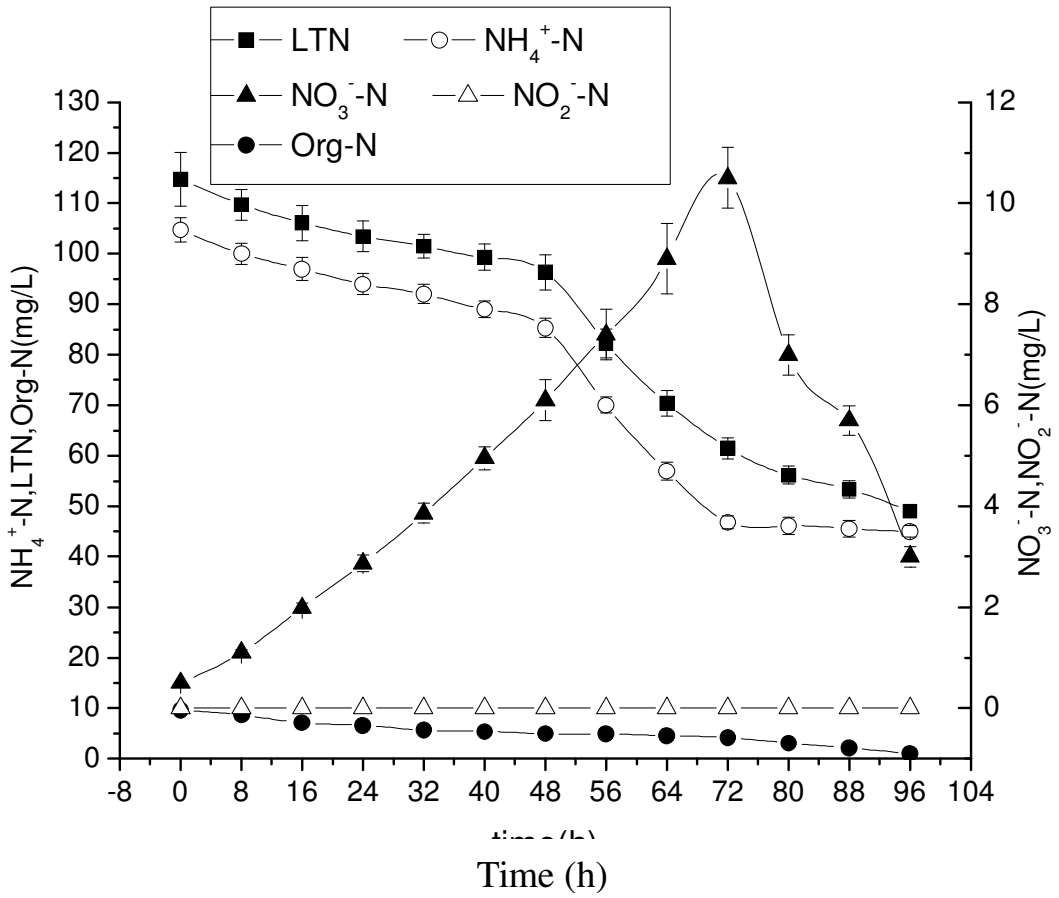

Figure 3. Changes in NH_4^+ -N, NO_2^- -N, NO_3^- -N, Org -N and LTN of the characterization.

pattern was observed for the total nitrogen in LTN and Org-N. Interestingly, the concentration of NO_3^--N increased during this period because NH_4^+-N was nitrified to NO_3^--N. During the period of bacterial growth (from 48 to 72 h), the concentrations of NH_4^+-N, LTN and NO_3^--N decreased more rapidly. The exponential growth phase began only 48 h after inoculation; hence, the rapid change in NH_4^+-N, TN and NO_3^--N was greatest after 48 h. The fastest decrease of LTN and fastest increase in Bio-N occurred between 48 and 72 h after inoculation, as shown in Figure 3. The STN concentration did not change so significantly because the nitrogen used to synthesize the biomass nitrogen was removed from the liquid to solid and was not released as gas.

Although, NO_3^--N increased significantly, the concentration of NO_2^--N was insignificant throughout the experiment, a phenomenon previously noted by others (Su et al., 2006). With nitrification and denitrification occurring concurrently, nitrate began to accumulate without any nitrite build-up (Berks et al., 1994). The 4.6% decrease in OD during the final 24 h showed that, bacterial cells had started to break down. The Org-N was almost exhausted after 96 h. The nitrification-denitrification proceeded faster than Org-N decomposition. In other words, the breakdown of the bacterial cells did not cause an increase in Org-N and LTN. However, the conversion of Org-N to NH_4^+-N caused the NH_4^+-N to slow down. The increase in Bio-N paralleled the increase in OD; when the OD reached the top (0.64), the concentration of Bio-N was greatest (24.1 mg/l).

The removal efficiency of NH_4^+-N was 18.6% after 48 h, increased to 55.3% after 72 h, then the rate declined until it reached about 57.0%, the ultimate nitrification. The decrease in NH_4^+-N was concomitant with the increase in OD. The LTN decrease trend was similar to that of NH_4^+-N. The initial LTN removal efficiency in the medium was 57.3% and the ultimate efficiency of denitrification was therefore, 57.3%.

The degradation rate of NH_4^+-N was 0.08 gN/gCell·h during the first 48 h. The rate of NH_4^+-N decomposition during the 48 to 72 h period was faster than during the first 48 h; hence, the decomposition rate per unit bacteria was reduced to 0.067 gN/gCell·h due to an exponential increase in the number of bacteria. The degradation rate of NH_4^+-N was 0.003 gN/gCell·h in the last 24 h. In both cases, the degradation rate of NH_4^+-N was essentially the rate of nitrification.

During the first 48 h, LTN decreased at 0.125 gN/

Table 1. Nitrogen balance in the characterization (units: mg/l).

Initial STN	Final NH_4^+ -N	Final NO_3^- -N	Final NO_2^--N	Final Org-N[a]	Final Bio -N	Final STN	N lost [b] (%)
114.8 ± 2.4	45.0 ± 1.2	3.0 ± 0.2	0 ± 0	1.0 ± 0.1	23.0 ± 0.6	72.0 ± 1.5	37.3

[a] Calculated value; [b] N lost =100 x{(Initial STN) -(Final NH_4^+ -N) -(Final NO_3^- -N) -(Final NO_2^--N) -(Final Org -N) - (Bio -N)}/(initial STN)
N lost =100 x {(Initial STN) - (Final STN)}/(Initial STN).

gCell·h. From 48 to 72 h, the reduction of LTN was 0.069 gN/gCell·h that slowed down to 0.01 gN/gCell·h during 72 to 96 h. The change in the denitrification paralleled the LTN decrease. The change in the degradation rate of NH_4^+-N and LTN was due to bacteria growing at different stages during the reaction. When the bacteria were growing in the logarithmic phase, the decomposition rate was higher than bacteria in the lag period, the reason why all of the changes were synchronized with OD and Bio -N.

The Org-N concentration declined rapidly in the first 48 h and then, more gradually until it reached about 1 mg/l. The organic nitrogen in the medium was initially incurporated during preparation but also resulted from the breakdown of cells. The sustained reduction of organic nitrogen in the medium showed that the decomposition rate of organic nitrogen was faster than the dissolved rate: Org-N was decomposed and turned to NH_4^+-N or NO_3^--N faster than the Org-N dissolution. Table 1 presents the nitrogen balance in the medium after 96 h incubation; 42.8 mg/l of nitrogen (37.3% of initial STN) in the medium was converted to gas after 96 h of incubation. These results showed that, the strain was capable of heterotrophic nitrification and aerobic denitrification. The concentration of NH_4^+-N in the 20% (w/w) H_2SO_4 solution was maintained at 0 mg/l, confirming that no ammonia gas escaped from the medium. Therefore, the NH_4^+-N decrease in medium was caused by bacterial degradation. The denitrification efficiency of WY200811 is higher than most of heterotrophic nitrification and aerobic denitrification bacteria isolated to date. For example, the denitrification efficiency of *T. pantotropha ATCC 35512* at 96 h is 27.3% (Su et al., 2001), while *A. faecalis* sp. no. 4, exhibits a maximum ammonium removal rate of 3% in 60 h with a high NH_4^+-N concentration of 1200 mg/l (Joo et al., 2005a). In contrast, 13.5% of the ammonium was removed by *Pseudomonas* sp. isolated from piggery wastewater within 58 h (Su et al., 2006) and the removed ammonium was about 80.7% (w/w) for the *Pseudomonas stutzeri* strains (Wen et al., 2010).

Taxonomic identification of isolate

The WY200811 was Gram-negative, rod-shaped and identified as *A. denitrificans* (97% identity).

In conclusion, the new strain *A. denitrificans* WY-200811 was isolated from sludge obtained from an A^2/O wastewater treatment plant. The cell population increased significantly in liquid medium with NH_4^+-N as the main nitrogen source; NO_3^--N increased at first and then, decreased significantly. The 37.3% of N was converted to N_2 gas in the flask. The ultimate efficiencies of nitrification and denitrification were 57.0 and 57.3%, respectively. Thus, the WY200811 strain is capable of high efficiency heterotrophic nitrification and aerobic denitrification. Because the WY200811 strain with high heterotrophic nitrification and aerobic denitrification capability was isolated from anaerobic/anoxic/oxic treatment system, the ways to improve the aerobic nitrification and denitrification efficiency of the treatment system can be deduced from the key factors affecting reaction efficiency of WY200811. Use of the immobilized *A. denitrificans* WY200811 cells for in anaerobic/anoxic/oxic treatment system to promote the efficient removal of ammonia will be investigated further in our laboratory.

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