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High Rate of Water Biodenitrification Using Anthracite as Hyphomicrobium Denitrificans Carriers

Maryam Khakbaz Varkani[1], Mohsen Nosrati[2], Seyed Abbas Shojaossadati[3]
[1]*Department of Chemical Engineering, Tarbiat Modares University*
[2]*Department of Chemical Engineering, Tarbiat Modares University*
[3]*Department of Chemical Engineering, Tarbiat Modares University*

----------**ABSTRACT**----------
Pure culture of Hyphomicrobium denitrificans DSM 1869 was immobilized on anthracite and utilized for biological denitrification in 50-ml flasks employing methanol and acetic acid as carbon source. The results demonstrate that acetic acid was a suitable carbon source for H. denitrificans to remove high nitrate concentrations. The maximum denitrification rate was 233.1 mg NO_3-N/g MLSS.h and the highest NO_3-N removal efficiency was obtained when using C/N ratio of 4.0 and acetic acid as the carbon source. C/N ratio can significantly affect denitrification in different operational conditions. The low C/N ratios did not allow the denitrification process to be completed in case of high NO_3-Nconcentrations. High C/N ratio increased the rate of nitrate conversion when using acetic acid as a carbon source; but added a pollutant to denitrified water when using methanol as a carbon source. The results demonstrated that H. denitrificans was a suitable bacterium for denitrifying high NO_3-N concentrations.

Keywords *- Hyphomicrobium denitrificans DSM 1869, C/N ratio, Carbon source, Immobilized cells, Anthracite*

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I. INTRODUCTION

In numerous countries, groundwater is used as a source of drinking water, and high nitrate concentrations in groundwater present a potential risk to public health, particularly to infants [1, 2]. Major sources of nitrate in groundwater supplies include wastewater, fertilizers, and livestock farming [3, 4]. According to U.S. Environmental Protection Agency and European Community, the permissible concentration of nitrates in drinking water is 44.3 and 50 mg NO_3^-/l, respectively; although the recommended levels of nitrate is 25 mg NO_3^-/l according to European Community [5, 6]. Given that ingestion of high levels of nitrate may result to negative effects on human health, efficient and economic removal processes are required [7, 8]. Numerous methods with different performance and cost levels are available for the denitrification of drinking water. Ion exchange, reverse osmosis and biological denitrification are commonly utilized methods [9-12]. Biological denitrification is a proper technique for nitrate removal, owing to its lower operating and capital cost in comparison to physical-chemical processes [6, 13]. After the completion of biological denitrification process, no brines were left and the microbes and microbial flocs that were left as a "waste product" can be reutilized for water biodenitrification process; and it is the most significant advantage of biological denitrification. When the availability of oxygen is limited, considerable changes occur in the energy metabolism of denitrifying bacteria. Aerobic respiration is replaced by anaerobic respiration during which oxygen is substituted by an alternative electron acceptor. After oxygen depletion, nitrate is the first compound to be reduced. Nitrate reduction is not observed at an oxygen concentration above 0.2 mg/l [14, 15].

By biological denitrification, nitrate in the water is converted into gaseous nitrogen via a number of steps. In biological denitrification, which is known as nitrate respiration, the reaction sequence of this process is demonstrated in equation (1) [16, 17].

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (1)$$

One of the challenges in denitrification process is residual carbon sources; so reducing *C/N* ratio can eliminate this problem [15]. Denitrifiers belong to a biochemically and taxonomically diverse group of facultative anaerobes and are commonly found in natural soil and water. Approximately 146 types of bacteria, mainly heterotrophs, are capable of utilizing nitrogen oxides (nitrate and nitrite) as electron acceptors and produce mainly N_2 as reduction product. Biological denitrification of drinking water with heterotrophic microorganisms has been widely applied owing to its high efficiency and low cost [15, 18, 19].

In several researches, activated sludge of "water resource recovery facilities" has been employed as denitrifying bacteria by specific denitrification rates of 20 to 38 mg $NO_3^- - N$ /g MLSS.h [20, 21]. Many bacteria are only able to perform one or two steps of equation (1). It has been reported that the reduction of nitrate to nitrite was greatly influenced by nitrate-reducing bacteria like *Micrococcus spp.*, *Vibrio spp*., *Staphylococcus carnosus* and *Escherichia coli*. This was attributed to the production of nitrate reductase during the growth of these microorganisms, which causes nitrite accumulation. Being more toxic than nitrate, nitrite can further react with secondary amines in acidic conditions to form carcinogenic nitrosamines. Several studies have demonstrated that a direct relationship exists between nitrosamine and human cancers [22].

Using immobilized microorganism for denitrification has several advantages in comparison to processes involving suspended biomass. Some of these advantages include reducing bioreactor size and allowing increased flow rates. Various materials have been utilized as supports to fix microorganisms (e.g. sand, gravel, plastics, etc.) which are characterized by their high adsorption capacity and their irregular shape [9, 15, 23, 24].

In this study, anthracite is employed as a support for bacteria because of its proper size (2-3 mm), availability in most water treatment plants and surface specifications. It is also aimed to investigate effects of three different parameters on the biodenitrification employing immobilized cells of *Hyphomicrobium denitrificans* DSM 1869. These parameters include initial nitrate concentration, carbon source including methanol and acetic acid and *C/N* ratio. Nitrate and nitrite concentration, pH and COD were investigated during the experiments.

II. METHODS

2.1 Growth medium and microorganism

The required nutrient for biosynthesis comprises large amounts of C, H, O, N and P; minor amounts of K, Na, Mg, Ca and Fe and trace amounts of Mn, Zn, Cu, Co and Mo. Accordingly, the mineral salt medium (MSM) used for enrichment of *H. denitrificans* contained methanol 0.6% (v/v), NH_4SO_4 1750 mg/l, $MgSO_4.7H_2O$ 100 mg/l, $FeSO_4.7H_2O$ 20 mg/l, $Na_2HPO_4.H_2O$ 6140 mg/l, KH_2PO_4 680 mg/l, $ZnSO_4.7H_2O$ 1.5 mg/l, $CaCl_2.2H_2O$ 20 mg/l, $CoCl_2.6\ H_2O$ 0.6 mg/l, $CuSO_4.5H_2O$ 0.04 mg/l, $MnSO_4.5H_2O$ 5 mg/l, $Na_2MoO_4.2H_2O$ 0.04 mg/l and H_3BO_3 0.2 mg/l. pH of medium was 7.0 and all the chemicals utilized for denitrification experiments were of analytical grade.

H. denitrificans DSM 1869 was obtained from German Culture Collection. After subculture at least three times in the mentioned MSM, the culture was made on mineral salt agar (MSA) slant, and maintained at 4ºC in refrigerator. *H. denitrificans* was transferred to MSM and inoculated cultures were incubated at 37°C for 24 h in incubator. The cells were harvested by centrifugation at 10,000×g for 15 min, and the pellets were washed twice with phosphate buffer (pH 7) and the final individual cell counts of 10^5~10^6 CFU/ml were resuspended in pH 7 buffer phosphate.

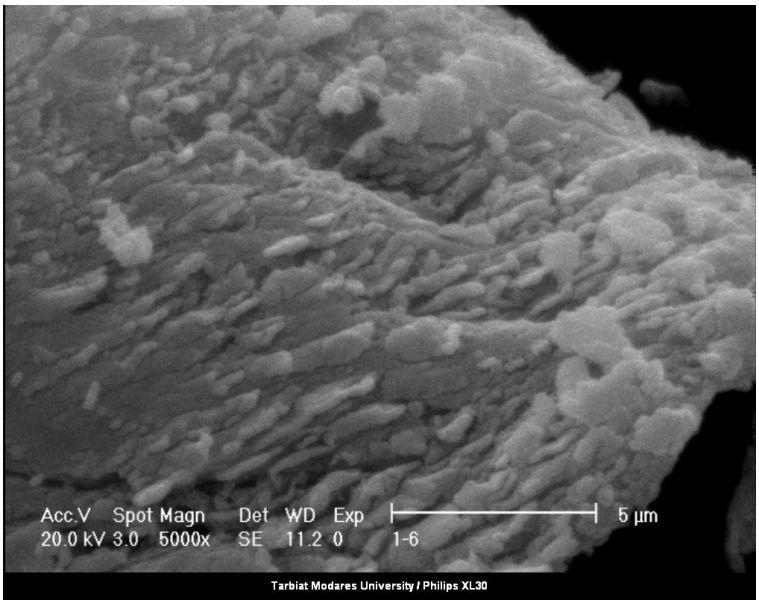
2.2 Artificial raw water and carbon source

The synthetic contaminated water was prepared from distilled water employing potassium nitrate (KNO_3) as the contaminant. Nitrate concentrations were 50, 75, 100, 150, 200, 250, 300 and 400 mg/l. Methanol and acetic acid were employed as the only carbon source and their concentrations were adjusted to give *C/N* weight ratios of 1.2, 2.6 and 4.0 by fixing the amount of KNO_3 (sole nitrogen source) and carbon source.

2.3 Cell Immobilization

In this study, anthracite was employed for cell immobilization. To have a uniform particle size, anthracite was passed through US standard mesh No. 8 sieve (2.36 mm nominal sieve opening) and was thereafter washed and dried in oven at 70°C for 1 h. A sample of 5 g anthracite and 47 ml MSM was added to 250 ml Erlenmeyer flask and was sterilized in autoclave at 121°C and 1.2 atm. An amount of 47 ml sterile MSM was inoculated with 3 ml bacterial culture and incubated in shaker incubator with shaking at 80 rpm and 30°C for 21 days.

After 21 days, the biofilm formed on anthracite (Fig. 1) and total 5 g media with the biofilm was transferred to 50 ml flasks.



Fgure1. Scanning electron microscopy (SEM) of immobilized *Hyphomicrobium denitrificans* on anthracite

2.4 Experimental set-up

The experimental system employed in the denitrification tests was 50 ml flasks. The anoxic condition was maintained by passing nitrogen gas (N_2) through the flasks. Mixing in the flasks was obtained by a magnetic mixer, which operated with a fixed speed of 80 rpm. Flasks were sealed with rubber plugs to maintain anoxic condition, and N_2 gas generated in reactions was discharged via exhaust needles installed in rubber plugs. All experiments were carried out in three replicates.

2.5 Analytical Methods

An amount of 0.5 ml samples were collected at fixed time intervals (each 6 hour) from the flasks in addition, were centrifuged at 8000 rpm for 15 min. $NO_3^- - N$ concentration was analyzed by ultraviolet spectrophotometric method employing a spectrophotometer of type CARY 100 Conc at a wavelength of 220 nm. Colorimetric method indicated in the Standard Methods for the Examination of Water and Wastewater was employed for the analysis of $NO_2^- - N$ concentrations [25].

Chemical oxygen demand (COD) was measured according to the closed reflux, colorimetric method that was suggested in the Standard Methods for the Examination of Water and Wastewater. An amount of 2.5 ml of the sample was treated with 1.5 ml digestion solution (which was a mixture of 10.216 g/L potassium dichromate, 167 ml concentrated sulphuric acid and 33.3 g/L mercuric sulphate) and 3.5 ml concentrated sulphuric acid, and digested at 150°C for two hours employing a thermo reactor (Hach model: DRB200). After cooling, the absorbance of the samples was read at 600 nm using a spectrophotometer (CARY 100 Conc). The concentration was determined with the aid of a calibration curve. Samples were centrifuged and filtered before analysis [25]. All chemicals used were of analytical grade and obtained from major retailers.

III. RESULTS AND DISCUSSION

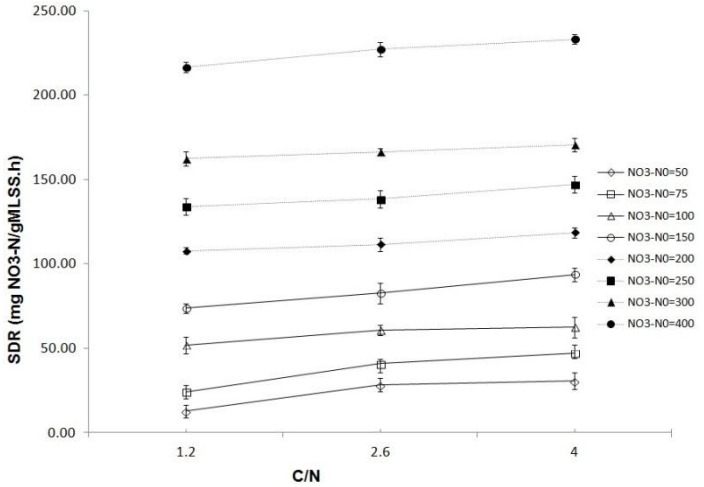
3.1 Effect of C/N ratio and carbon source on specific denitrification rate

C/N ratio is a key factor influencing the efficiency of denitrification. Denitrification tests were performed to determine optimum *C/N* (w/w) ratio for different initial $NO_3^- - N$ and carbon source concentrations. In this regard, an optimal (usually much lower) influent *C/N* ratio must be defined to obtain a suitable carbon concentration in denitrified water. The *C/N* ratios used were 1.2, 2.6 and 4.

Numerous carbon sources can be utilized in denitrification process. Nevertheless, for the denitrification of drinking water, sources are limited to simple and easily degradable carbon like methanol and acetic acid [15, 24]. Variation of the specific denitrification rate (SDR) with *C/N* ratios is demonstrated in Fig. 2.

$$SDR = \frac{\left(NO_3 - N\right)_0 - \left(NO_3 - N\right)_T}{MLVSS \times T} \qquad (2)$$

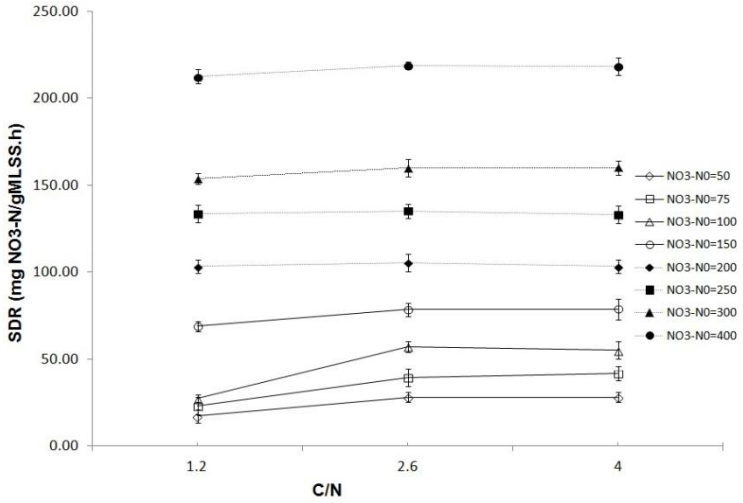
where SDR is the specific denitrification rate (mg $NO_3^- - N$ /g MLSS.h), $(NO_3^- - N)_0$ is the initial $NO_3^- - N$ concentration (mg $NO_3^- - N$ /l), $(NO_3^- - N)_T$ is concentration of $NO_3^- - N$ (mg $NO_3^- - N$ /l) after T hours and MLSS is mixed liquor suspended solids (g) showing biofilm mass on anthracite.



Fgure2. Variation of the specific denitrification rate with *C/N* ratio (acetic acid as carbon source)

A varying degree of nitrate reduction (from40.0 to 98.7% removal) was found. When using acetic acid as a carbon source, the maximum specific denitrification rate was 233.1 mg $NO_3^- - N$ /g MLSS.h for $(NO_3^- - N)_0$=400 mg/l and *C/N* ratio=4.0. It can be seen from Fig. 2 that by increasing the *C/N* ratio, the specific denitrification rate increased slightly and higher nitrate conversions could be achieved for different $(NO_3^- - N)_0$ concentrations.

By using methanol as a carbon source, the maximum specific denitrification rate was 218.75 mg $NO_3^- - N$ /g MLSS.h for $(NO_3^- - N)_0$=400 mg/l and *C/N* ratio=2.6. It can be seen from Fig. 3 that by increasing the *C/N* ratio from 1.2 to 2.6, the specific denitrification rate increased for different $(NO_3^- - N)_0$ concentrations. However, by increasing the *C/N* ratio from 2.6 to 4.0 for $(NO_3^- - N)_0$ of more than 150 mg/l, specific denitrification rate decreased or did not increase significantly. The decline in specific denitrification rate showed that high concentration of nitrate could suppress the activity of reductase enzymes, which converts NO_3^- to NO_2^-, NO_2^- to NO and NO to N_2O [26, 27].



Fgure3. Variation of the specific denitrification rate with *C/N* ratio (methanol as carbon source)

The higher specific denitrification rate for acetic acid may be attributed to the carbon and energy values of acetic acid, which indicate immediate assimilation by microbial cells. Acetic acid does not need preliminary processing and channels directly to the PHB formation reaction. Acetic acid can directly go through Tricarboxylic Acid (TCA) cycle to generate the reducing power required for PHB formation. Thus, acetic acid would be a more direct carbon source than methanol for denitrification process. Specific denitrification rates in different operational conditions changed from 12.8 to 233.1 mg $NO_3^- - N$ /g MLSS.h. The specific denitrification rates of this study were significantly higher than those obtained by other researchers. Buttiglieri et al. [20] observed the maximum nitrate removal rate close to 20 mg $NO_3^- - N$ /g VSS.h when utilizing ethanol as the only carbon source in a membrane bioreactor. The highest specific denitrification rate gained by Eldyasti et al. (2012) was about 38 mg $NO_3^- - N$ /g VSS.h in a fluidized bed bioreactor [21]. The results demonstrated that *H. denitrificans* DSM 1869 was capable of utilizing nitrates as fast as possible and was a suitable bacteria for denitrification process.

3.2 Effect of C/N ratio and initial NO3-N concentration on COD reduction

In different operational conditions (Table 1), nitrate removal was carried out simultaneously by COD decrease and nitrate was not trapped in the cells or anthracite (Fig. 4). The highest COD reductions appeared in lowest *C/N* ratios (Runs 1 to 8) when using acetic acid as carbon source (Fig. 4a). By increasing *C/N* ratio (comparing Runs 1 to 8 with Runs 9 to 16 and Runs 17 to 24), nitrate removal efficiency increased although COD reduction decreased. *C/N* ratio is a very critical parameter because if there is lack of carbon source in low *C/N* ratios, the bacterial activity would decreased and if carbon source are in excess, an organic pollutant would be added to the denitrified water. When utilizing methanol as a carbon source, increasing the *C/N* ratio and initial $NO_3^- - N$ concentration do not significantly affect COD reduction (Fig. 4b). Increasing the initial $NO_3^- - N$ concentration inhibited nitrite reductase enzyme. When nitrite could not be transformed to NO, the sequences of equation (1) would not be completed and there would be accumulation of carbon source in the medium [19, 28, 29]. As a result, COD reduction will decrease and COD of denitrified water will increase.

Table1. Different operational conditions

C/N ratio	1.2							
NO3-N	50	75	100	150	200	250	300	400
Carbon Source	Acetic Acid							
Run	1	2	3	4	5	6	7	8
Carbon Source	Methanol							
Run	25	26	27	28	29	30	31	32

C/N ratio	2.6							
NO3-N	50	75	100	150	200	250	300	400
Carbon Source	Acetic Acid							
Run	9	10	11	12	13	14	15	16
Carbon Source	Methanol							
Run	33	34	35	36	37	38	39	40

C/N ratio	4.0							
NO3-N	50	75	100	150	200	250	300	400
Carbon Source	Acetic Acid							
Run	17	18	19	20	21	22	23	24
Carbon Source	Methanol							
Run	41	42	43	44	45	46	47	48

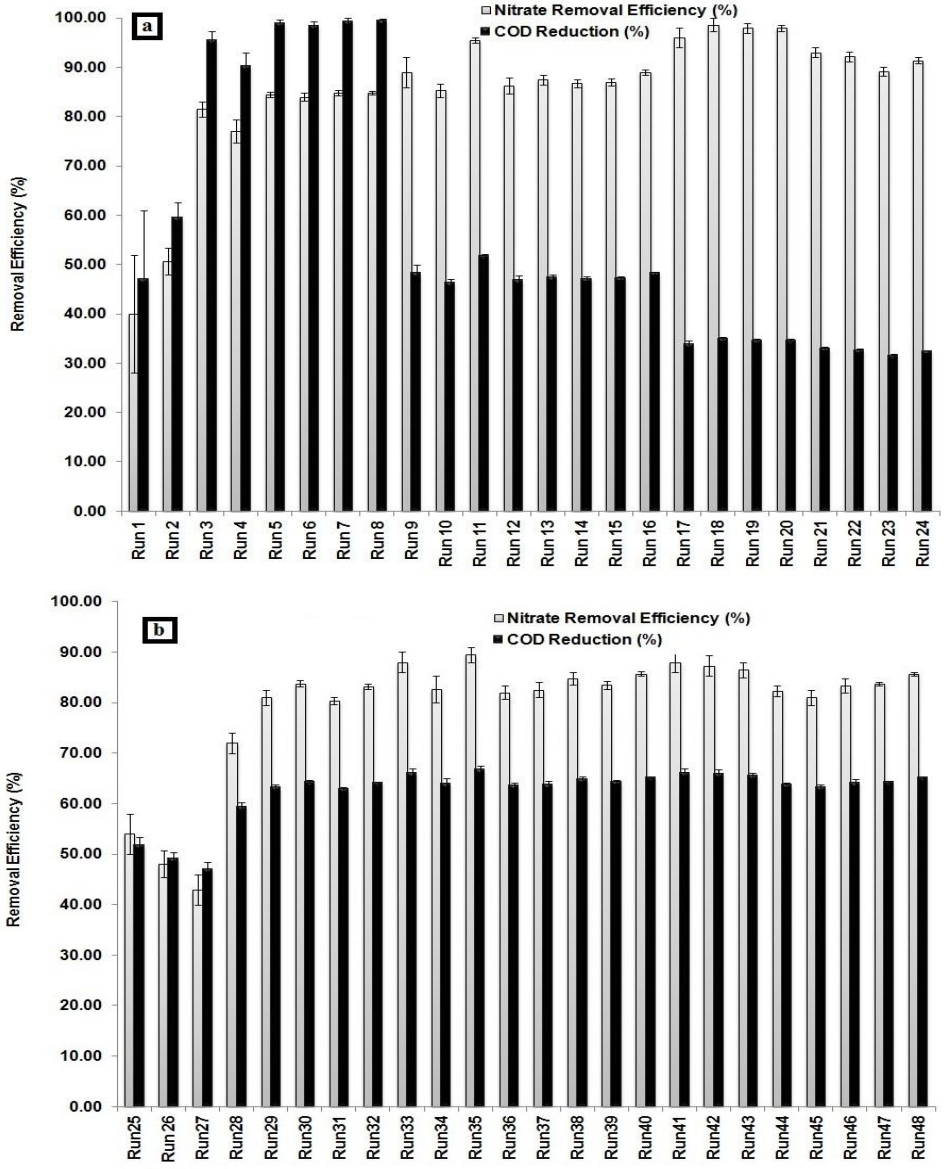


Figure3. Comparing nitrate removal efficiency and COD reduction: a) acetic acid as carbon source, b) methanol as carbon source

3.3 Nitrate and nitrite accumulation

Biological denitrification comprises a sequence of enzymatic reactions leading to the evolution of nitrogen. In this process, microorganisms first reduce nitrates to nitrites and then produce nitric oxide, nitrous oxide, and finally nitrogen gas.

The pathway for nitrate reduction is shown in equation (1). To reduce the risk to public health and given that nitrite is more toxic than nitrate; the nitrite formed must be minimized. With increase in ($NO_3^- - N$)$_0$ concentration, the nitrite accumulation increased for both carbon sources.

The accumulation of nitrite ions may also be attributed to the slowing of enzyme activity (nitrite reductase) [1, 2]. Figures 5 and 6 demonstrate that when carbon was insufficient, it would be quickly consumed for the first step and no more carbon would be left for the other steps [15, 23]. Nevertheless, the nitrite accumulation decreased by increasing the *C/N* ratio due to the adequate carbon sources available for *H. denitrificans*.

The lower final nitrite concentration utilizing acetic acid as an electron donor demonstrated that carbon source could have a significant effect on the production and accumulation of nitrite.

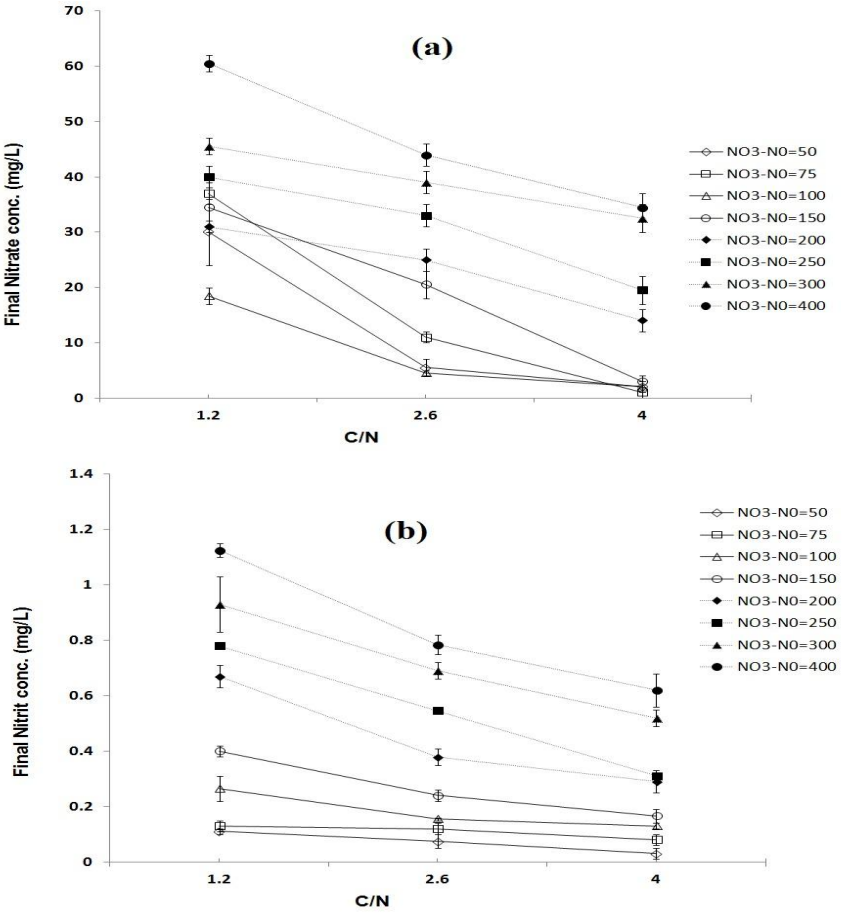


Figure.4 Final Nitrate (a) and Nitrite (b) concentration at different *C/N* ratios (acetic acid as carbon source)

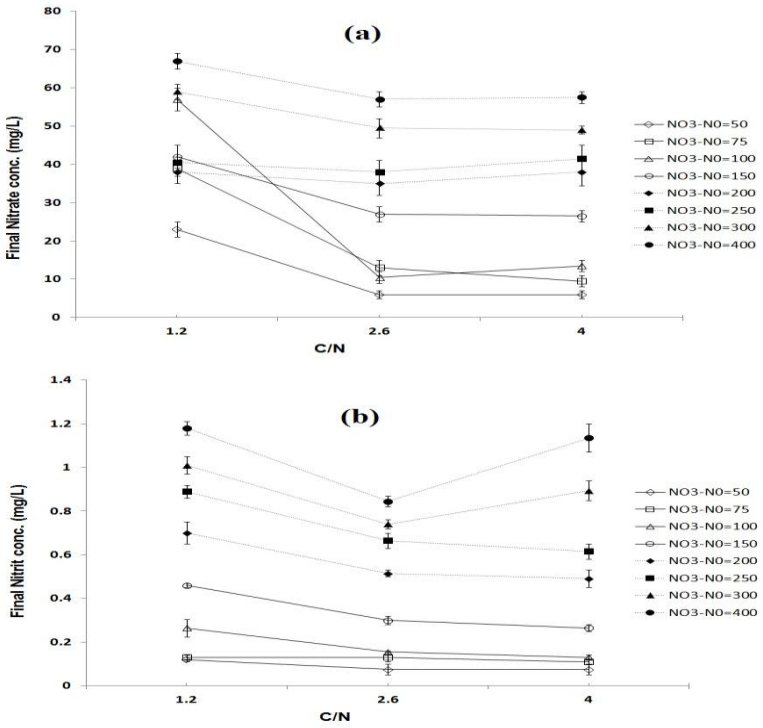


Figure5. Final Nitrate (a) and Nitrite (b) concentration at different *C/N* ratios (methanol as carbon source)

No matter the nature of the pathways, the amount of $NO_2^- - N$ formed was much less than $NO_3^- - N$ reduced at all times (Fig. 7), indicating that the majority of $NO_3^- - N$ was biologically reduced into nitrogen gas, as expressed in equation (1).

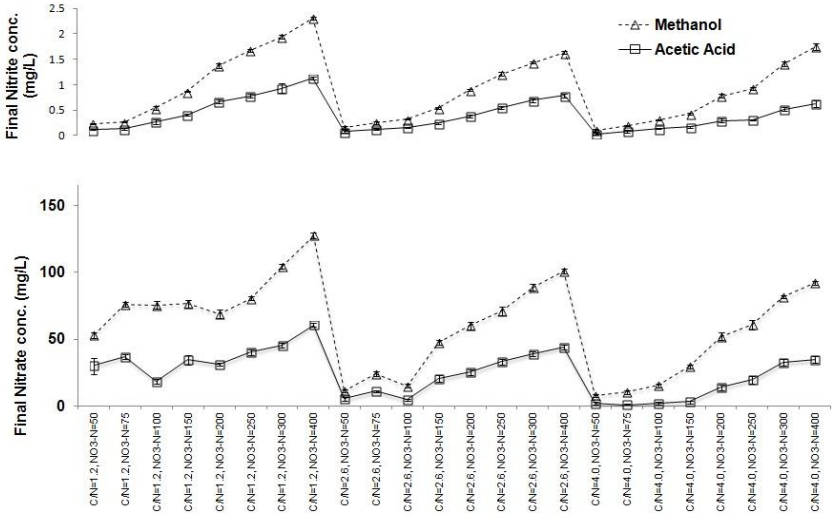


Figure6. Comparing NO_3-N consumption and NO_2-N production in different operational conditions

3.4 Nitrate removal efficiency

In order to optimize the amount of carbon source to achieve efficient $NO_3^- - N$ removal, the efficiency of the system was studied at varying operational conditions. As demonstrated in Fig. 8, nitrate removal efficiency was markedly affected by nitrate initial concentration, which indicated that high nitrate concentrations (400 mg/l) decreased nitrate removal efficiency. The reduction in nitrate removal efficiency at higher $NO_3^- - N$ concentration may be due to lack of soluble carbon compared to high $NO_3^- - N$ loading and inhibitory role of high nitrate concentrations. In other words, at higher nitrate concentrations, microorganism metabolism was inhibited, and low nitrate removal efficiency was observed, because not all steps of nitrate reduction (equation (1)) were completed.

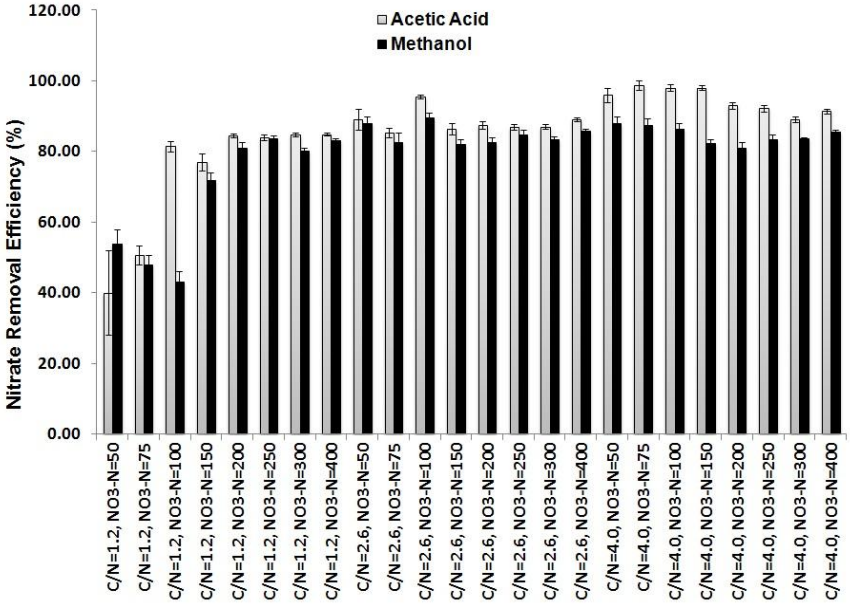


Figure7. Nitrate removal efficiency at different *C/N* ratios and $(NO_3^- - N)_0$ concentrations

The optimum pH for biological denitrification should be kept in the range of 7.0-9.0. For methanol carbon source, pH values were found to increase during the denitrification process. For acetic acid, the pH did not change significantly. Nevertheless, pH was 7.3 to 8.1 during all denitrification tests and accordingly, the effluent pH met the water quality standard (6.5-8.5 for drinking use). Undoubtedly, the pH in this study did not result in the lowering of denitrification performance. During all denitrification experiments, measured dissolved oxygen was between 0.1 to 0.2 mg/l, indicating that anoxic condition was prepared for denitrification process.

IV. CONCLUSIONS

The results demonstrated that biodenitrification of high nitrate concentrations was possible with pure strain of *H. denitrificans* DSM 1869 and addition of an external carbon source. Acetic acid was an effective and safe source of carbon and energy for *H. denitrificans*, especially for high nitrate concentrations. Inhibitory effect of high nitrate concentration was solved due to the carbon and energy value of acetic acid as carbon source. The results of this research demonstrated that using *H. denitrificans* was a promising technology that could compete successfully with systems that use activated sludge of "water resource recovery facilities", which may have many pathogens. In this study, it was shown that *H. denitrificans* can use nitrate and nitrite as electron acceptors as fast as possible and produce N_2 as reduction product. The present study clearly indicated that the maximum specific denitrification rate (233.1 mg $NO_3^- - N$ /g MLSS.h) was observed at *C/N* ratio of 4.0, nitrate level of 400 mg/l and acetic acid as a carbon source. *C/N* ratio played a critical role in denitrification process. The optimum *C/N* ratio for two carbon sources was different indicating that the inhibitory effect of high nitrate concentration could be affected by carbon source utilized. If *C/N* ratio was low, carbon source would be used for the first step ($NO_3^- \rightarrow NO_2^-$) and denitrification process could not be completed. If carbon source were in excess, an organic pollutant would be left in denitrified water. Application of immobilized cells was very effective because the bacteria used, did not suspend in the medium and did not add any pollutant to water. Nitrite production may sometimes be observed, especially when methanol was utilized as carbon source.

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