Tạp chí Khoa học và Công nghệ **53** *(6) (2015) 730-740*

DOI: 10.15625/0866-708X/53/6/5317

NITROGEN REMOVAL EFFICIENCY OF DENITRIFYING BACTERIA ISOLATED FROM AQUACULTURE PROCESSING WASTEWATER IN NITRATE-RICH ENVIRONMENT

Nguyen Hoai Huong*, Huynh Van Thanh

HCMC University of Technology (HUTECH), 475A Dien Bien Phu St., D. Binh Thanh. HCMC

*Email: *nh.huong@hutech.edu.vn*

Received: 12 November 2014, Accepted for publication: 3 September 2015

ABSTRACT

Denitrifying bacterial strain III-8, isolated from aquaculture processing wastewater and selected based on their ability to reduce nitrate and nitrite without ammonification was identified by traditional culture methods combined with 16S rRNA gene sequencing and searching the Genbank. As the results, III-8 was identified as *Achromobacter xylosoxidans*. Factors affecting denitrification efficiency such as NaCl concentration, biofilm carrier presence and microbial cell density were investigated in an experimental moving bed biofilm reactor (MBBR) system using artificial wastewater as well as aquaculture processing wastewater. In artificial wastewater with a loading of 140 mg ml^{-1} $N\text{-}NO_3^-$, strain III-8 showed a nitrogen removal efficiency up to 100 % in the salt free medium and 88 % in 3 % salt concentration biofilm carrier containing system with an initial cell density of 10^8 cfu ml^{-1}. In aquaculture processing wastewater with the same loading, the average nitrogen removal rate after 36 hours of treatment was found to be of 9.1 gN m^{-3} h^{-1} with 100 % efficiency. The high initial microbial density could be secured by aerobic propagation in a proper culture medium.

Keywords: denitrification, microbial density, moving bed biofilm reactor (MBBR), nitrite accumulation.

1. INTRODUCTION

Nitrate-rich effluent is the product of ineffective nitrogen removal processes due to the simple application of aerobic activated sludge process which stopped after the ammonification followed by nitrification [1]. In aquaculture sector, ammonia decrease is one of the most important objectives, therefore almost microbial preparations used in treatment aquaculture effluent contain ammonium assimilating microorganisms and/or nitrifying bacteria which could lead to the accumulation of NO_2^- and NO_3^- in the aquatic environment. Complete removal of nitrogen requires denitrification or anammox processes, both of them occur under anoxic conditions. Anammox technology is now still under research attempting to be applied for centralized wastewater treatment systems. Besides, conventional nitrogen removal *via*

nitrification followed by denitrification technology continue to be the focus of many improvement research projects based on their simplicity and efficiency [2]. With respect to aquaculture wastewater and nitrate-rich lakes, denitrification process is still the unique solution for nitrogen removal up to now. In Vietnam, bioaugmentation has been an emerging trend in environmental management in recent years and therefore, preliminary results on denitrifier isolation have been obtained [3, 4].

Denitrifiers (which are also called respiratory nitrate reducers) are bacteria which mainly reduce nitrate to nitrite under anoxic condition, then reduce nitrite to NO then N_2O and finally to N_2. As the results, nitrogen is transferred from aquatic environment into the atmosphere. From aquaculture processing wastewater of an agricultural wholesale market, we have isolated and successfully selected several denitrifiers which demonstrated to remove more than 90 % nitrate nitrogen without nitrite accumulation and ammonification, as required in National technical regulation on the effluent of aquatic products processing industry QCVN 11:2008/BTNMT [4].

In this study, we investigated influencing factors on the nitrogen removal efficiency and the conversion rate of a selected denitrifier strain III-8. In order to use this bacterial strain in wastewater treatment practices, identification is a step for risk assessment purpose which cannot be omitted.

2. MATERIALS AND METHODS

2.1. Materials

Bacterial strain III-8 was isolated from aquaculture processing wastewater of an agricultural wholesale market and selected based on its nitrogen removal efficiency *via* denitrification pathway [4]. Experimental wastewater was collected from wastewater treatment unit belonging to Food Processing Plant Tan Phu Trung, Seafood Joint Stock Company No 1. Lot C2-1, D4 Street, National Road 22, Tram Bom Hamlet, Tan Phu Trung Ward, Cu Chi District, Ho Chi Minh City.

2.2. Methods

2.2.1. Identification of denitrifier strain III-8

Morphological, physiological and biochemical characteristics of strain III-8 such as Gram stain, endospore presence/ absence, motility, catalase, oxidase, oxidation/ fermentation (O/F) tests were determined according to routine microbiological methods. Its other biochemical characteristics were analyzed using API 20NE Kit (Biomerieux) according to the manufacturer's recommendation and the results were obtained based on APIWEB search. Its biofilm formation capacity was investigated using crystal violet to stain adhering cells to the test tube walls at first, then 30 % acetic acid to wash the stained cells, and the washed suspension was finally measured at 550 nm [5].

The 16S rRNA gene sequencing was carried out by NamKhoa Biotek. DNA from the pure culture was extracted and 16S rRNA gene was amplified using universal primers 16S-F: *AGA GTT TGA TCC TGG CTC AG* and 16S-R: *ACG GCT ACC TTG TTA CGA CTT* with the following thermal regime: after an initial denaturation at 95 °C for 5 min, 40 cycles were carried out including denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension

at 72 °C for 1 min. The final extension was performed at 72 °C for 10 min. The reaction products were separeted in agarose gel by horizontal electrophoresis to verify bands with a size of 550 bp. The DNA purification was performed using The DNA sequencing was performed on 3130 Genetic Analyzer (Applied Biosystems). A search for strains from Genbank (NCBI) with 16S rRNA genes identical more than 99 %, compared with the obtained sequence using program BLASTN 2.2.28+ was performed.

2.2.2. Factors influencing the denitrification activity of the selected strain

Anoxic reactor with biofilm carriers: 100 ml glass flasks with 80 ml of experimental medium were connected to a plastic syringe to obtain bacterial metabolite gas. Every flask contained 5 g of biofilm carriers which were cut from plastic drinking straws with the following parameters: height 1.5 cm, diameter 0.6 cm, weight 0.0294 g, specific surface areas 19.23 $m^2 kg^{-1}$ and 1177 $m^2 m^{-3}$ to mimic the moving bed bioreactor (MBBR) [6].

Inoculum: The selected strain III-8 was propagated from a slant with Giltay nitrate medium on a shaker with 150 rpm. Bacterial density was determined by optical density measurement at 600 nm and using its cell standard curve. Inoculation ratio was performed at 10 %.

Nitrate-rich synthetic wastewater was prepared as follows: Giltay medium with the replacement of citric acid by sodium acetate and asparagine by peptone (it was called medium MT3). $N\text{-}NO_3^-$ concentration was fixed at 140 mg l^{-1} for all experiments.

Effect of different salt concentrations: Nitrogen removal efficiency was determined under the conditions described above in the same flasks without biofilm carriers with the addition of 0 %, 3 %, 5 %, 7 % NaCl.

Effect of biofilm carriers: Nitrogen removal efficiency was determined under the conditions described above in the same flasks with and without biofilm carriers.

Effect of initial bacterial density: Nitrogen removal efficiency and conversion rate were determined under the conditions described above with initial bacterial density of 10^5, 10^6, 10^7, 10^8 cfu ml^{-1} inoculated into flasks containing biofilm carriers.

In all experiments, free bacterial biomass (if it was the case) was analyzed based on optical density measurement at 600 nm. Samples were taken after a certain period of time. $N\text{-}NO_3^-$ was determined colorimetrically at 410 nm after its complexation with phenol disulfonic acid, $N\text{-}NO_2^-$ was quantified by colorimetric measurement at 520 nm after its complexation with Griess reagent, $N\text{-}NH_4^+$ was analyzed through reaction with Nessler reagent and measured at 430 nm on spectrophotometer Spectro UV-VIS 2500 (Labomed Inc.).

Nitrogen removal efficiency was evaluated based on nitrate reduction activity without nitrite accumulation and ammonification and calculated according to formula (2.1):

$$H\ \% = [1-(N\text{-}NO_3^-fin+N\text{-}NO_2^-fin+N\text{-}NH_4^+fin)/(N\text{-}NO_3^-ini+N\text{-}NO_2^-ini+N\text{-}NH_4^+ini)]\times 100 \quad (2.1)$$

Average nitrogen conversion rate was calculated according to formula (2.2):

$$V_{ave} = [(N\text{-}NO_3^-ini+N\text{-}NO_2^-ini+N\text{-}NH_4^+ini)-(N\text{-}NO_3^-fin+N\text{-}NO_2^-fin+N\text{-}H_4^+fin)]/T \quad (2.2)$$

where, $N\text{-}NO_3^-fin$, $N\text{-}NO_2^-fin$, $N\text{-}NH_4^+fin$ consecultively are final concentrations of $N\text{-}NO_3^-$, $N\text{-}NO_2^-$, $N\text{-}NH_4^+$ (measured after a certain period of time in flasks inoculated with bacteria) and $N\text{-}NO_3^-ini$, $N\text{-}NO_2^-ini$, $N\text{-}NH_4^+ini$ are initial concentration of $N\text{-}NO_3^-$, $N\text{-}NO_2^-$, $N\text{-}NH_4^+$, gN m^{-3}; T = conversion time, h.

Experiments for study on factors influencing nitrogen removal efficiency were carried out with 3 repetitions. The data were subjected to analysis of variances (ANOVA) with $p < 0.05$ to assess the difference in each parameter among treatments using software Statgraphics Centurion XV (Statpoint Technologies, Inc.).

3. RESULTS AND DISCUSSIONS

3.1. Bacterial identifiation

In the previous paper, we have already presented the isolation and selection of potential denitrifiers from aquaculture processing plants and aquaculture wholesale markets [4]. In this study, we investigated strain III-8 which proved to be the most potent denitrifier under experimental conditions without nitrite accumulation and ammonification. This is Gram negative, separate rods without endospore formation, motile, monotrichous, catalase positive, oxidase positive, oxidative but non fermentative (O^+/F^-), indol negative, Methyl Red negative, Voges-Proskauer negative, citrate positive, and biofilm formation positive. Biochemical test using API20 NE kit arranged strain III-8 into the species *Achromobacter xylosoxidans* with 97.4 % similarity. 16S rRNA gene sequencing of strain III-8 and a Blast search of available data in the Genbank database showed its high similarity (> 99 %) with *Achromobacter xylosoxidans* (Table 1). These combined results allowed us to conclude that strain III-8 belongs to species *Achromobacter xylosoxidans*.

According to the literature on the genera *Achromobacter/ Alcaligenes*, *Achromobacter xylosoxidans* is one of potential denitrifiers, with the former name *Alcaligenes xylosoxidans* or *Pseudomonas denitrificans* and classified as a safety level 2 organism [7]. These bacteria are essentially isolated from the soil, water environment, plants and are capable to degrade different kinds of organic compounds as well as to inhibit the plant pathogens. Recently, *Achromobacter xylosoxidans* has been also isolated from the respiratory tract of persons with cystic fibrosis (Table 1). Therefore, to ensure the biosafety requirement, the use of this bacterial strain shoud be carried out in centralized wastewater treatment plants and the effluent should be well disinfected prior to discharge into the environment. 16S rRNA gene sequence of the strain of interest was submitted to Genbank (NCBI) and can be retrieved with accession number KF534510.

Table 1. Comparison results of 16S rRNA gene sequences.

No	Retrieved records	Compared nucleotide number (bp)	Identity percentage (%)	Source/ Features	Accession number
1	*Ach. xylosoxidans* TPL14	512	99.8	Endophytic strain in Chinese cabbage, antimicrobial activity against pathogens	EU373389
2	*Ach. xylosoxidans* 53B	499	99.8	Bacterial strain isolated from a PAHs wastewater treatment plant	EF396325
3	*Ach. xylosoxidans*	511	99.6	Plant pathogen virulence silencer	DQ414679
4	*Ach. xylosoxidans* G5	510	99.4	Thiodiglycol degrader	EF186004

5	*Ach. xylosoxidans* NFRI-A1	508	99.4	Aflatoxin biosynthesis inhibitor	AB161691
6	*Ach. xylosoxidans* A8	510	99.2	Haloaromatic acid degrader	CP002287
7	*Ach. xylosoxidans* subsp. *xylosoxidans*	508	99.2	From cystic fibrosis patient	AF411021
8	*Ach. xylosoxidans* subsp. *xylosoxidans* A19	508	99.0	Ortho chlorobenzoate degrader	AF439314

3.2. Factors influencing the denitrification activity of the selected strain

The greatest challenge of bioaugmentation application in pollution treatment is that isolated microorganisms cannot survive in the real environmental conditions. Therefore, the next step after the isolation and selection of microorganisms from natural environments - the investigation on factors influencing treatment efficiency and conversion rate is an important bridge to scale up the bioaugmentation from the laboratory conditions to pilot experiments using real wastewater. In this study, we investigated the effect of salt concentration, the role of biofilm carriers and the initial microbial density on the nitrogen removal efficiency from synthetic nitrate-rich wastewater and then aquaculture processing plant wastewater.

3.2.1. Effect of salt concentrations

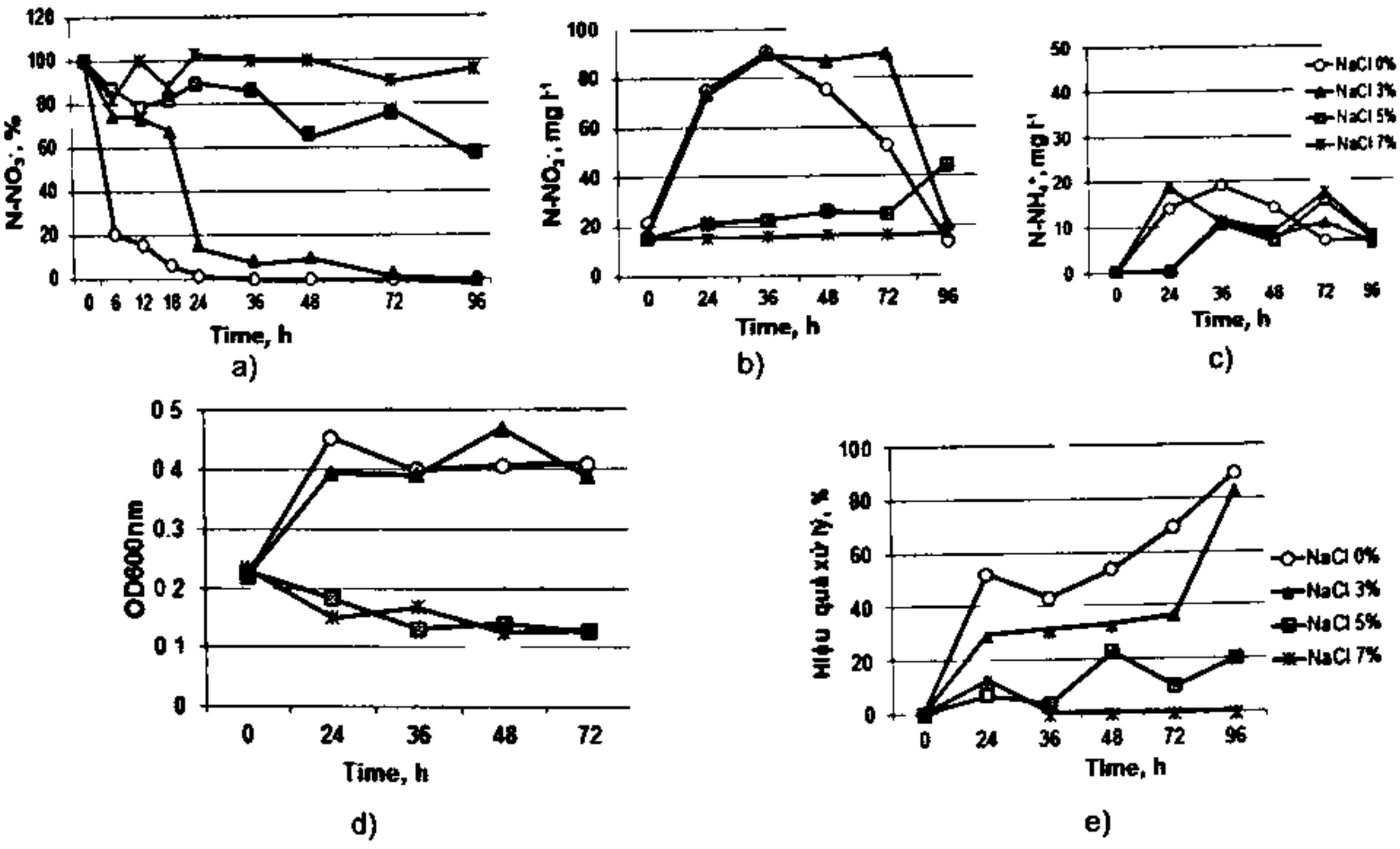

Figure 1. Effect of salt concentrations on denitrification kinetics; a) $N\text{-}NO_3^-$ change against the time, b) $N\text{-}NO_2^-$, c) $N\text{-}NH_4^+$, d) Effect of salt concentrations on bacterial growth, e) Nitrogen removal efficiency change against the time.

Aquaculture processing wastewater usually contains high salt concentrations. Most of microorganisms are salt intolerant and hence, they are restricted in real environment application. The effect of salt concentrations on bacterial growth and denitrification process is presented in Figures 1. Figures 1a, 1b, 1c show that at salt concentrations of 5 % and 7 %, almost $N\text{-}NO_3^-$ was not reduced, after the adaption period, $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$ appeared at low concentrations. This was compatible with the results shown in Figure 1d, namely, at 3 % NaCl, strain III-8 showed the growth as high as at 0 % NaCl; at salt concentration higher than 5 %, almost bacteria could not grow. 3 % NaCl did not significantly inhibit the bacterial growth, but reduced $N\text{-}NO_3^-$ slower than in the case without the salt, in spite of the same range of $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$ accumulation. $N\text{-}NO_3^-$ concentration decreased only after 24 h, leading to the increase of N removal efficiency which reached the same level of 90 % after 96 h as for salt free environment. (Figure 1e).

Thus, strain III-8 was able to growth and reduce nitrate in rather high salinity environment with tolerable salt concentration of 3 %. According to Ghevariya et al., 2011, *Ach. xylosoxidans* was reported to degrade chrysene at 1.5 M NaCl, equivalent to 8.7 % [8]. Hence, salt tolerance of *Achromobacter xylosoxidans* is one of prominent advantages of this species in comparison to other potential denitrifiers.

3.2.2. Adhesion ability to carriers

To increase the contact surface between bacteria and the medium, plastic carriers were used to mimic moving bed biofilm reactor (MBBR) used in wastewater treatment. Biofilm formation of strain III-8 was demonstrated through the adhesion of cells stained by crystal violet to the test tube walls (Figure 2a). To quantify the biofilm, the optical density at 550 nm was obtained from destaining solution of this test tube with acetic acid, meanwhile to quantify the free biomass, the optical density at 600 nm of the suspension of cells which were not attached to the test tube wall was measured. From the ratio between these two optical densities (Figure 2b), it could be assumed that in the real aquaculture processing wastewater, strain III-8 would be able to adhere well to carriers.

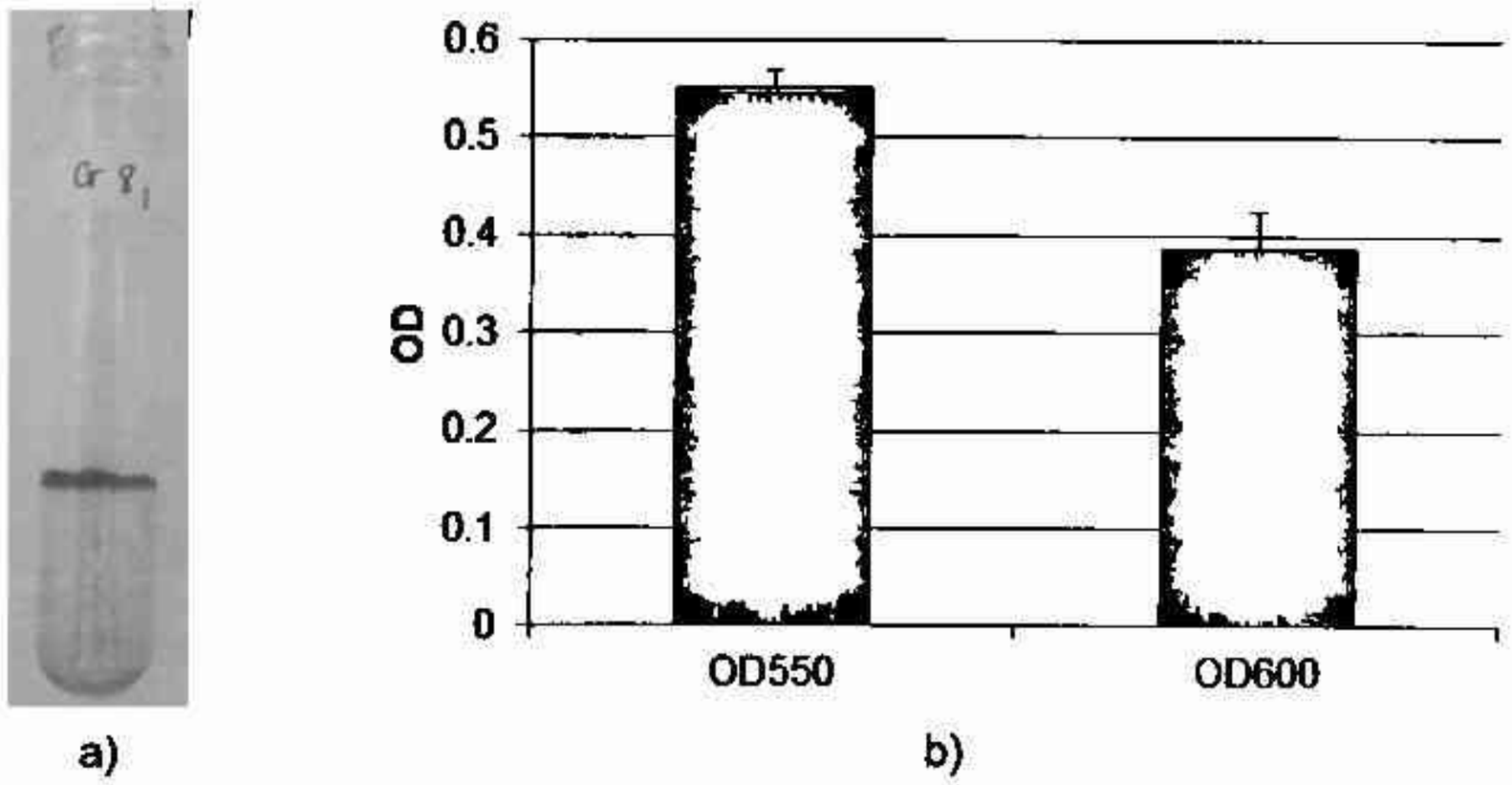

Figure 2. Biofilm formation ability of strain III-8, a) Biofilm formation on the test tube wall, b) Optical densities of attached cells to the test tube wall measured at 550 nm (OD550) and of free cells measured at 600 nm (OD600).

Results of the following experiment (Figure 3) demonstrated that in the carrier model reactor, the nitrogen conversion occurred faster than in that without carriers for both media 0 % NaCl and 3 % NaCl, leading to nitrogen removal efficiency of 100 % after 24 h for medium without NaCl and 88 % after 48 h for medium containing 3 % NaCl. $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$ also decreased sharply after 48 h in the carrier reactor model, even for 3 % NaCl medium. Hence, moving bed biofilm reactor (MBBR) was the proper system for anoxic treatment thanks to the possibility to increase the contact between microorganisms and wastewater for purpose of energy savings from agitation [6].

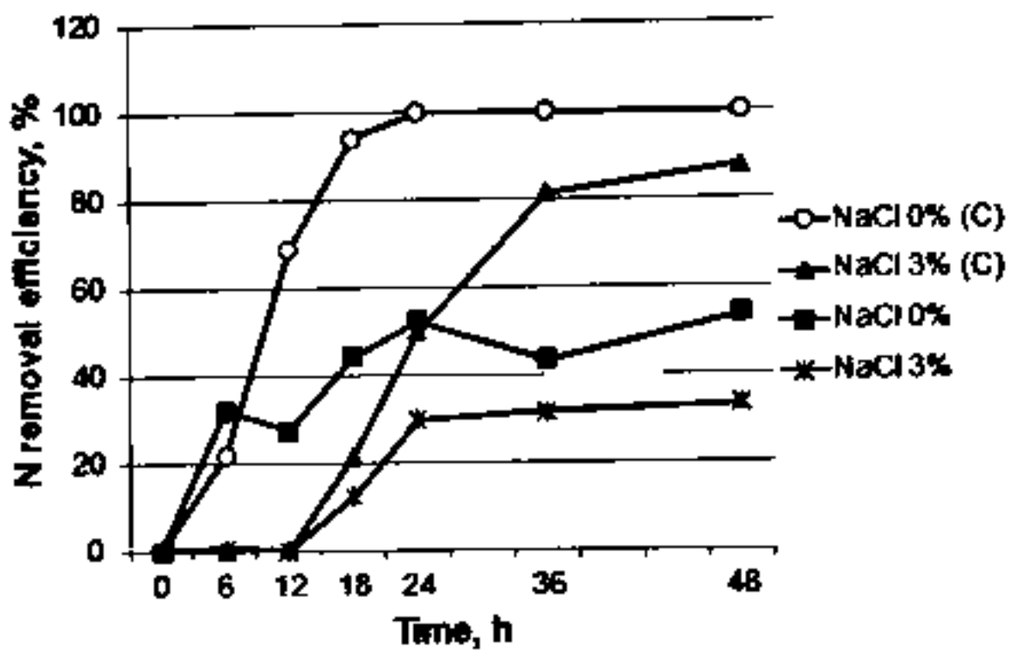

Figure 3. Effect of biofilm carriers on denitrification; a) Nitrogen removal efficiency, b) $N\text{-}NO_2^-$ and NH_4^+ accumulated in case of with and without carriers (C) after 48 h of treatment.

3.2.3. Effect of initial inoculation density

In order to successfully apply bioaugmentation, the control of initial inoculation density, especially in anoxic condition, was necessary since the lack of oxygen restricted the growth. However, high inoculum ratio increased the treatment cost. In this experiment, the initial inoculation density was adjusted to 10^8, 10^7, 10^6 và 10^5 cfu ml^{-1}. Experiment results are shown in Figure 4.

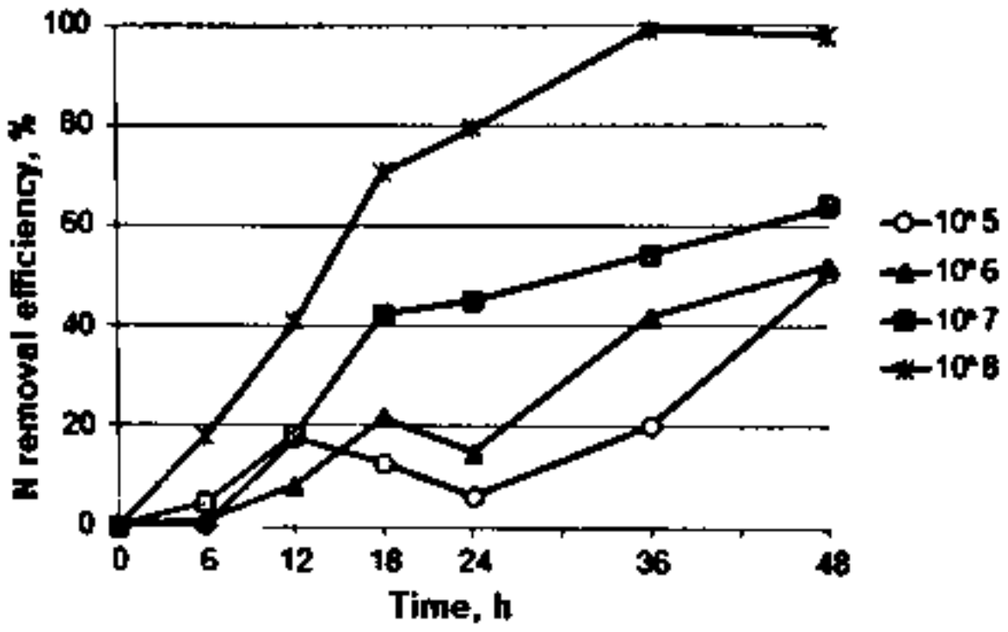

Figure 4. Effect of initial inoculation density on denitrification; a) Nitrogen removal efficiency and b) Conversion rate.

Indeed, significant effect of initial inoculation density on denitrification efficiency was seen, its low density led to longer lag phase and lower final biomass in comparison with high

density at 10^8 cfu ml^{-1}. At this high bacterial density, evidence that the nitrate reduction occurred rapidly was large released gas volume and nearly 100 % of nitrogen removal efficiency after 36 h without $N\text{-}NO_2^-$ and $N\text{-}NH_4^+$ accumulation. These results are similar to those obtained during the selection of potential denitrifiers which was described in the previous paper [4]. In this study, average conversion rate was 5.25 gN m^{-3} h^{-1}, comparable to 0.15 kgN m^{-3} day^{-1}, equivalent to 6.25 gN m^{-3} h^{-1} from other studies on MBBR [6].

3.2.4. Application of bioaugmentation in nitrate-rich aquaculture processing wastewater treatment

The composition of collected aquaculture processing wastewater was analyzed, its $N\text{-}NO_3^-$ loading was adjusted to 140 mg l^{-1}, to which sodium acetate was added so that C:N ratio = 12, the bacteria were inoculated at a density of 10^8 cfu ml^{-1} to 5 liter bioreactor with biofilm carriers with the specific surface area of 1177 m^2 m^{-3}. Treatment kinetics, nitrogen removal efficiency and average conversion rate were monitored after 12, 24, 36 and 48 h and presented in Figure 5.

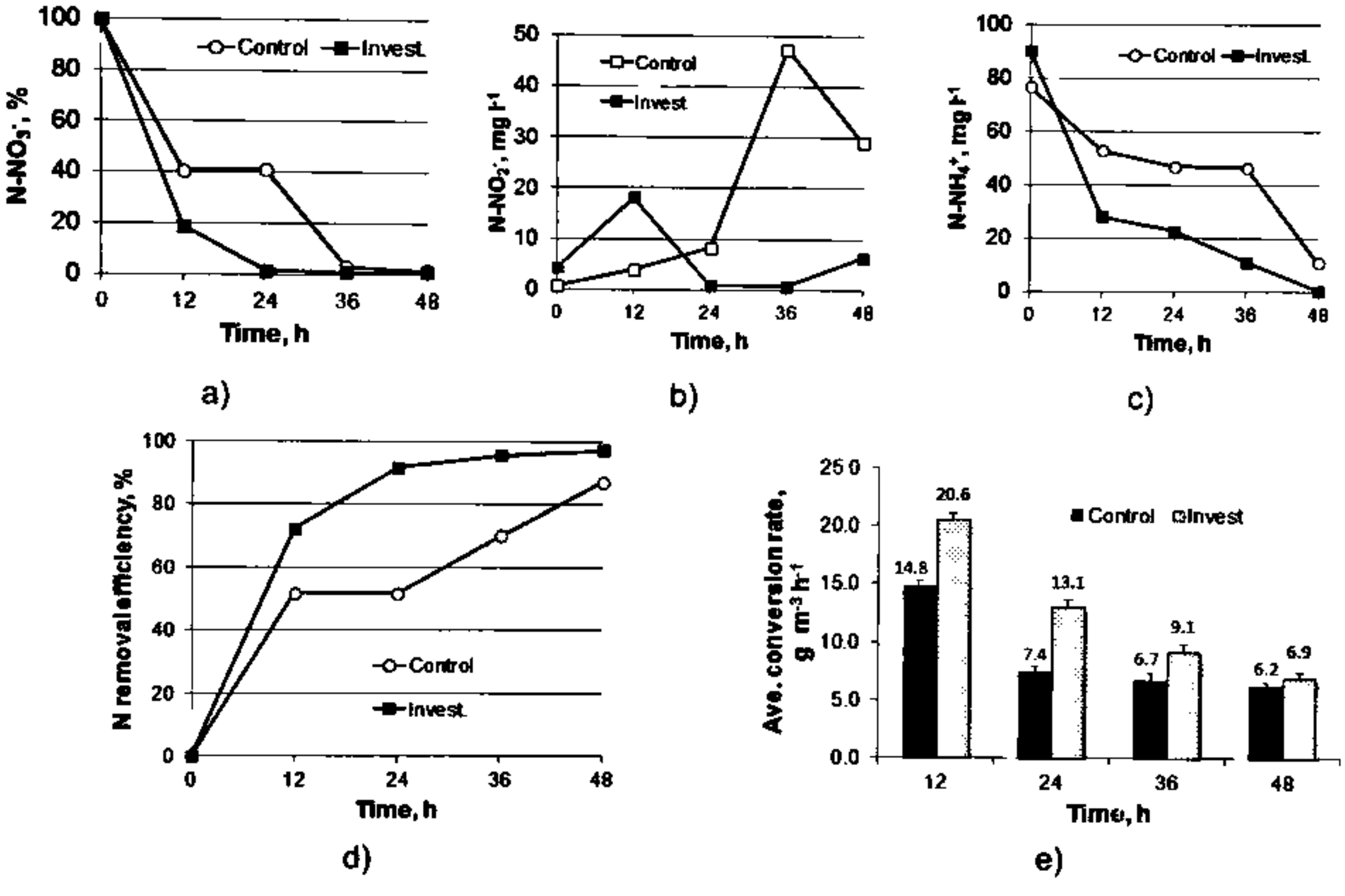

Figure 5. Aquaculture processing wastewater treatment in experimental model of MBBR, a) Kinetics of $N\text{-}NO_3^-$ removal, b) Kinetics of $N\text{-}NO_2^-$ conversion, c) Kinetics of $N\text{-}NH_4^+$ conversion, d) Kinetics of nitrogen removal efficiency, e) Denitrifying rate in the course of time.

The control sample was supplemented with wastewater so that the composition was similar to that of the investigated sample, except that the inoculum was omitted. Figure 5 shows that in the control sample, denitrifying bacteria existing previously in the wastewater were activated and hence, after 36 h under experimental conditions similar to those of investigated sample, $N\text{-}NO_3^-$ decreased totally to 0, however, $N\text{-}NO_2^-$ accumulated 50 mg l^{-1} after 36 h and still remained 30 mg l^{-1} after 48 h. Similarly, $N\text{-}NH_4^+$ in the control sample

remained at high level after 36 h, and decreased to nearly 10 mg l^{-1} only after 48 h. Meanwhile, in the investigated sample, $N\text{-}NO_3^-$ and $N\text{-}NO_2^-$ decreased to 0 after 24 h of treatment, $N\text{-}NH_4^+$ reduced to 10 mg l^{-1} after 36 h, meeting the requirements for type A effluent according to National technical regulation on the effluent of aquatic products processing industry QCVN 11:2008/BTNMT. The nitrogen removal efficiency of the investigated sample reached > 90 % after 24 h and nearly 100 % after 36 h of treatment. The denitrifying rate of both the control and the investigated samples were found rather high, however, in the investigated sample it was higher than that in the control, especially during the first 12 hour period. With regards to the average denitrifying rate in the time period for totally nitrogen removal, namely 48 h for the control and 36 h for the investigated sample, its value was found 9.1 gN m^{-3} h^{-1} in the investigated sample, compared to 6.2 gN $m^{-3}h^{-1}$ in the control. It could be explained that in the wastewater collected from the seafood processing plant, certain denitrifiers probably already existed. Under proper conditions such as appropriate C/N ratio, limited dissoved oxygen, the denitrification process occurred. However, due to the insufficiency of denitrifiers to dominate the microbial population, the nitrogen removal was not complete, leading to nitrite and ammonium accumulation. This experiment demonstrated that *Ach. xylosoxidans* III-8 well adapted to real seafood processing wastewater and can be applied for aquaculture processing wastewater treatment in general.

3.2.5 Insurance of inoculum during the course of denitrification

It was found earlier that the denitrifier inoculum at density of 10^8 cfu ml^{-1} should be ensured for successful nitrogen removal and it is very high density. To investigate the conditions to ensure the inoculum, experiment on biomass propagation under aerobic and anoxic conditions was carried out.

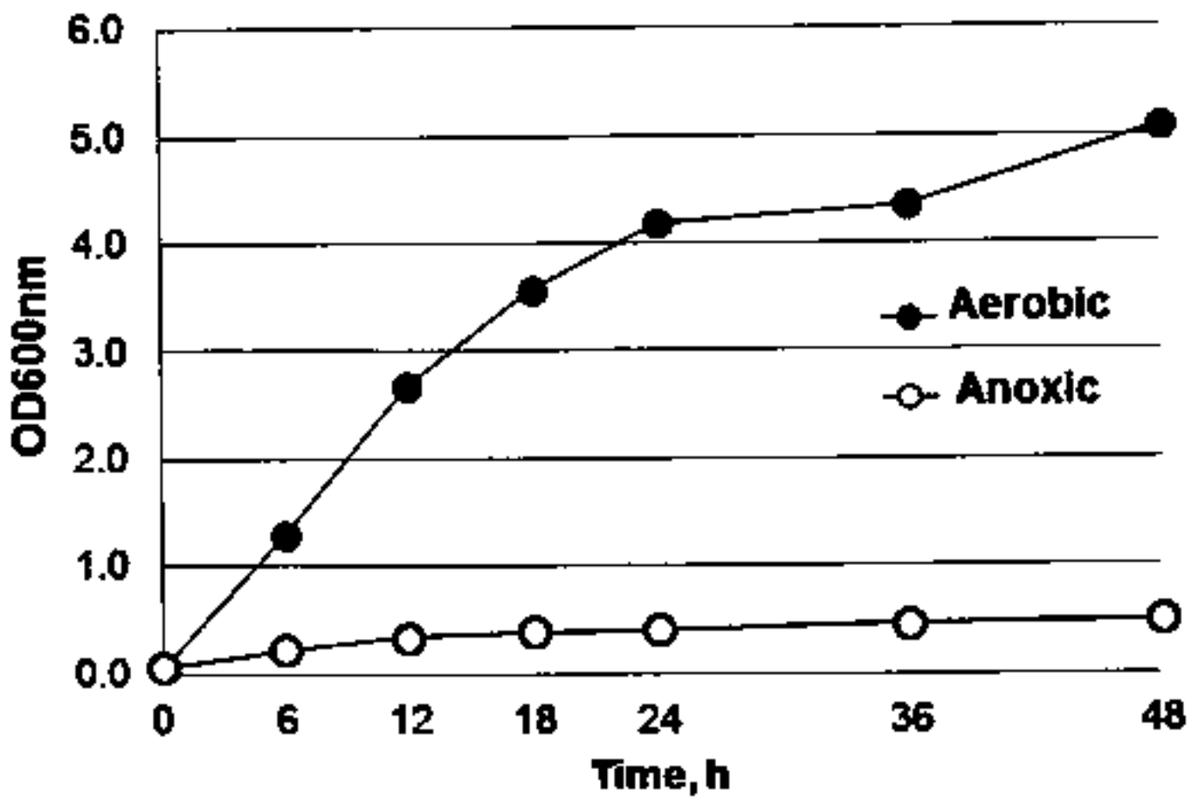

Figure 6. The growth curve of strain III-8 obtained under aerobic and anoxic conditions.

Figure 6 displayed the optical density at 600 nm against the time to evaluate the growth under both aerobic and anoxic conditions, among which the former is for biomass propagation and the latter is for denitrification. Under the aerobic condition, the bacterial growth was rather high with the generation time t_d = 1.3 h, compared to t_d = 3.1 h under anoxic condition. The denitrification occurs under anoxic condition, meaning that the biomass generated during this process is not high. As the result of this experiment, it was proposed that the bacterial biomass can be propagated under aerobic condition and then added to nitrate – rich treatment systems

which work under anoxic conditions. Alternatively, consecutive aerobic - anoxic systems can be used to ensure the bacterial inoculum for the denitrification process.

4. CONCLUSIONS

This study confirmed that strain III-8 isolated from aquaculture processing wastewater belongs to the species *Achromobacter xylosoxidans* - one of potential denitrifiers, which can be applied in bioaugmentation for nitrogen removal systems in centralized treatment units. In order to reach 100 % of nitrate removal efficiency after 36 h of treatment for a loading of N-NO_3^- 140 mg l^{-1} without nitrite and ammonium accumulation and with the average treatment rate of 9.1 gN m^{-3} h^{-1}, it was necessary to maintain the denitrifier density at 10^8 cfu ml^{-1} in a MBBR type with specific surface area of 1177 m^2 m^{-3}. The tolerable salt concentration for *Ach. xylosoxidans* III-8 was 3 %.

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TÓM TẮT

HIỆU QUẢ XỬ LÍ NITƠ TRONG MÔI TRƯỜNG GIÀU NITRATE CỦA VI KHUẨN PHẢN NITRATE PHÂN LẬP TỪ NƯỚC THẢI CHẾ BIẾN THỦY SẢN

Nguyễn Hoài Hương*, Huỳnh Văn Thành

Trường Đại học Công nghệ TP. Hồ Chí Minh (HUTECH), 475A Điện Biên Phủ, Quận Bình Thạnh, TP. HCM

*Email: *nh.huong@hutech.edu.vn*

Vi khuẩn phản nitrate III-8, phân lập từ nước thải chế biến thủy sản và chọn lọc theo khả năng loại trừ N-nitrate, không tích lũy N-nitrite và không sinh N-amôn được định danh bằng phương pháp truyền thống kết hợp giải trình tự gene rRNA 16S và so sánh trên Genbank. Kết quả cho thấy chủng III-8 thuộc loài *Achromobacter xylosoxidans*. Các yếu tố ảnh hưởng đến hiệu quả xử lí nitơ từ nitrate như nồng độ muối, giá thể bám dính và mật độ vi sinh vật được khảo sát trong mô hình moving bed biofilm reactor (MBBR) sử dụng nước thải nhân tạo và nước thải từ nhà máy chế biến thủy sản. Trong nước thải nhân tạo, chủng III-8 có khả năng xử lí nitrate tải trọng 140 mg ml^{-1} $N\text{-}NO_3^-$ trong môi trường 0 và 3 % NaCl có giá thể bám dính và mật độ vi sinh vật ban đầu là 10^8 cfu ml^{-1} với hiệu quả xử lí tương ứng 100 % và 88 %. Trong nước thải chế biến thủy sản ở cùng tải trọng $N\text{-}NO_3^-$, vận tốc xử lí trung bình sau 36 giờ để đạt 100 % hiệu quả là 9,1 gN m^{-3} h^{-1}. Mật độ vi khuẩn ban đầu cao có thể được bảo đảm bằng tăng sinh hiếu khí trong môi trường dinh dưỡng thích hợp.

Từ khóa: biofilm, mật độ vi sinh vật, MBBR, phản nitrate, tích lũy nitrite.