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High nitrite removal capacity of an aerobic denitrifier *Klebsiella oxytoca* DF-1 isolated from aquaculture ponds in coastal mudflats

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The aims of this study were to help the development of the models describing denitrification in the recirculation aquaculture systems and to contribute to the knowledge on the role of heterotrophic bacteria in aquaculture nutrient removal systems in mudflats along the coast. Six aerobic denitrifying strains were isolated by bromothymol blue (BTB) plate technique from the fish farm sediments in mudflat along the coast. The strain DF-1 was selected for the aerobic denitrification studies as it was highly effective in removing nitrite compared with other strains; it lowered the nitrite nitrogen (nitrite-N) concentration from 10 mg/L to zero in 20 h. The aerobic denitrification by the strain DF-1 occurred at significantly higher rates of nitrite-N degradation than the positive controls (sterile screening media) under different pH, temperature, and various concentrations of nitrite and salinity. The rate of nitrite-N degradation was 99.3, 97.7, 99.5, 96.5, and 91.8% when sucrose, glucose, sodium acetate, sodium succinate and potassium sodium tartrate, respectively, were used as the carbon source. The strain DF-1 contained the *nir*K gene as indicated by the amplification of nitrite reductase. The strain DF-1 was identified as *Klebsiella oxytoca* by the morphological observation and 16SrRNA gene analysis.

Key words: Coastal pollution, high nitrogen risk, aerobic denitrification, *Klebsiella oxytoca*, nitrite nitrogen degradation rate, aquaculture.

INTRODUCTION

Aquaculture in mudflats along the coast contributes significantly to the world food supply. This process could be of particular importance in countries with scarce arable land but high population, such as China. The combined water surface area for aquaculture in mudflats was estimated at 122,000 ha in Jiangsu Province, China (Blancheton, 2000; Adachi et al., 2002).

Nitrite is an important component in the nitrogen cycle, but it has become a matter of great concern for intensive aquaculture industry in recent years. The nitrite in the water rapidly increased because the substantial amounts of uneaten feedstuff and aquatic animal excretion are gene-

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rated in the process (Lin and Wu,1996; Gupta et al.,1997; Bell et al., 2000; Shen et al., 2006; Song et al., 2001). The presence of nitrite in wastewater discharge is undesirable for several reasons: although not as toxic to fish and many other aquatic organisms as ammonia or nitrate that directly affect the animal immune system (Li et al., 2005, 2008), the presence of nitrite and nitrate ions in drinking water is a potential health hazard (Grguric et al., 2000; Fang et al., 2010). It threatens the quality of water used for household purposes by many farm families (Lee et al., 1996; Ka et al., 1997). Nitrite accumulates in the closed systems and could cause fish diseases outbreak and result in huge economic losses (Maag and Vinther, 1996; Ole et al., 2009; Kshirsagar et al., 1995). Thus, removal of nitrite nitrogen to decrease its accumulation is required, with due attention paid to economics (Stouthamer, 1988; Lukow and Diekmann, 1997; Ozeki et al., 2001; Franco et al., 2001; Obaja et al., 2003; Braker et al., 1998, 2000, 2001; Joo et al., 2006; Santoro et al., 2006; Diep et al., 2009).

Physical, chemical and biological methods are used in aquaculture for removing nitrite, but physical and chemical methods cannot remove nitrite without secondary pollution and residues. Hence, the best way to remove nitrite is via the biological methods (Van et al., 2006). Denitrification was considered an anaerobic process by previous investigators (Wang et al., 2002); however, it is essential to keep a certain concentration of dissolved oxygen (DO) in the high-density culture, thus requiring a continuous supply of oxygen (Zheng et al., 2002), resulting in the growth and the rate of denitrification by anaerobic denitrifying bacteria being suppressed. Therefore, more attention needs to be paid to aerobic denitrifying bacteria. Robertson and Kuenen (1990) were the first to report on aerobic denitrifiers in 1980s. They isolated *Thiosphaera pantotropha* (now reclassified as *Paracoccus denitrificans*), *Pseudomonas spp*. and *Alcaligenes faecalis* from an anaerobic wastewater treatment reactor. Since then, more organisms that can perform aerobic denitrification under different partial pressure of air in the environment were reported (Zumft et al., 1997): for example, *P. denitrificans* can perform aerobic denitrification at 90% air saturation (Vollack et al., 1999; Valentin and Elena, 2006). Several other aerobic bacteria, for example, *Microvirgula aerodenitrificans* (Timmermans and Van, 1983), *Paracoccus sp*. (Takaya et al., 2003) and *Pseudomonas sp*. (Syu, 2001) were reported to remove nitrite under different air saturation, and more and more isolates were applied in the wastewater treatment systems.

The present work describes the characterization of a denitrifying bacterium isolated from the fish farm sediments in the coastal mudflats and the quantification of aerobic and anoxic nitrogen removal. The strain immobilization by different support materials was also assessed. Such studies will help the development of the models describing denitrification in the real recirculation aquaculture systems and contribute to the knowledge on the role of heterotrophic bacteria in aquaculture nutrient removal systems in coastal mudflats.

MATERIALS AND METHODS

Samples and culture media

The sediments were collected from fish ponds, Dafeng district, Jiangsu province, China (April, 2011) into sterile conical flasks and stored at 4°C until the strains were isolated. Water in the fish ponds had pH 8.3 (pH meter PB-10 Sartorius) and conductivity 6.8 mS/cm at 18°C (conductivity meter, Spectrum Technologies, Inc.).

The culture media were as follows [GB7493-87(1987)]: Bromothymol blue (BTB) medium: 0.1% (w/v) L-asparagine, 0.1% KNO_3, 0.1% KH_2PO_4, 0.005% $FeCl_2 \cdot 6H_2O$, 0.02% $CaCl_2 \cdot 2H_2O$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.368% NaCl, 1 mL of BTB per litre of the medium [1% in ethanol], 2% agar, pH 7.0; Screening medium (SM): 0.284% sodium succinate, 0.0015% $NaNO_2$, 0.136% KH_2PO_4, 0.027% $(NH_4)_2SO_4$, 0.1% yeast extract (Difco), 0.019% $MgSO_4 \cdot 7H_2O$, 0.368% NaCl, 1 mL of a trace element solution(Jayakumar et al., 2004) per litre of the medium , pH 8.2; Denitrification medium (DM): 0.472% sodium succinate, 0.0015% $NaNO_2$, 0.15% KH_2PO_4, 0.042% Na_2HPO_4, 0.06% NH_4Cl, 0.5% Casamino Acids [Difco], 0.368% NaCl, 2 mL of a trace element solution per litre of the medium , pH 8.2. All the media were sterilised 20 min by 0.11Mpa,121°C.

Isolation of denitrifying bacteria

A 20-mg sediment sample from fish ponds was transferred to sterile 500-mL conical flasks containing 200 mL SM liquid medium with sealing membrane and incubated at 30°C for 36 h (130 rotations per minute), followed by drawing 2 mL of solution from each flask and transferring to three sterile conical flasks that contained 200 mL SM liquid medium. These subculturing procedures were repeated three times. Thereafter, 0.1 mL solution was taken from each flask and spread on solid LB medium. The colonies were picked out and plate-streaked individually. Those purified colonies were incubated on the BTB medium plates at 30°C for 2 days, and the blue colonies were picked out for further assay. They were isolated and re-screened three times by spreading on solid DM medium and incubited at 30°C for 24 h. The colonies were then picked out and plate-streaked individually for the next experiment.

Assay of nitrite nitrogen (nitrite-N) degradation rate of isolates

Each isolate of 2-mL liquid culture (A600=0.600) was placed in a 500-mL conical flask that contained 198 mL liquid DM (denitrification medium) and cultured in an incubator shaken with 130 rotation per minute at 30°C for 24 h. At the end of the culture period, liquid samples of 10 mL were taken and centrifuged at 8000×*g* for 10 min, then assayed for the concentration of nitrite-N in the supernatant by spectrophotometric method according to GB 7493-87 (1987). The nitrite-N degradation rate of isolates was as follows:

$$\text{Nitrite-N degradation rate (\%)} = [T_{end} - T_{original}] / T_{original} \times 100\%$$

Where, $T_{original}$ is the Nitrite-N concentration before the culture; T_{end} is the Nitrite-N concentration at the end of the culture

Assay of the N_2-production capacity of isolates

Each isolate of 50 µL liquid culture (A600=0.600) was transferred to 5 mL liquid DM and incubated as described above. Recordings of the amount of N_2 produced were done daily.

Influences of different pH, temperature, carbon source and salinity on the nitrite-N degradation rate and the growth of the strain DF-1

The sterile DM was selected as the positive control. The degradation rate of nitrite-N was determined under different pH, temperature, carbon sources and salinity. The pH was set at 3, 5, 7, 9 or 11; the temperature was set at 15, 20, 25, 30, 35 or 40°C; the carbon sources were sucrose, lucose, lactose, sodium acetate, sodium succinate, sodium citrate or potassium sodium tartrate; the salinity of artificial seawater (w/v) was set at 0, 5, 10, 15, 20, 25 or 30 g/L. The 1-mL aliquot of activated DF-1 was added to 99 mL of sterile DM and cultured for 24 h on a shaker (130 rotations per minute) with three repetitions. At the end of the culture, the absorbance of the media supernatant was measured spectrophotometrically after centrifuging at 8,000×*g* for 10 min.

Identification of strain DF-1

The screened pure strain DF-1 was identified primarily on the basis of colony morphology and Gram Stain as well as microscopic examination of its smear. The template DNA from strain DF-1 was obtained from cells grown on DM by using genomic DNA extraction kit. The 16S rRNA gene was amplified by a polymerase chain reaction (PCR) thermal cycler with each general primer F(5′AGAGTTTGATCCTGGCTCAG3′)(10 μL), R(5′GGTTACCTTGTTACGACTT3′)(10 μL), 2 μL reaction buffer, 0.4 μL 10mM dNTPs, 0.2 μL Taq polymerase , 2 μL DNA plates and 15.4 μL sterilized water to achieve a final volume of 50 μL. The PCR conditions were: initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 20 s, 50°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 10 min. The PCR products were sent to Huada Co., Ltd (Shanghai,China) for RNA sequencing.

The partial sequences were searched against GenBank using the Advanced BLAST similarity search option accessible from the homepage at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The MEGA 5.0 version software was used to check alignment and instruct the phylogenetic tree.

Amplification of the nitrite reductase gene sequences of strain DF-1

The primer pairs of nirK1F-nirK5R for nirK and nirS1F-nirS6R for nirS were used to amplify the fragments of the nirK and nirS gene of strain DF-1 (Song et al., 2011). The primer pairs were nirSCd3aF:5′AACG(C/T)(C/G)AAGGA(A/G)AC(C/G)GG3′,nirSR3cd :5′GA(C/G)TTCGG(A/G)TG(C/G) GTCTT(C/G)A(C/T)GAA'; NirK-F1aCu: 5′ATCATGGT(C/G)CTGCCGCG3′, NirK-R3Cu: 5′GC CTCGATCAG(A/G)TTGTGGTT3'. The PCR conditions were according to the method of Braker et al. (2000). Aliquots of 10 μL of the reactions were analyzed by electrophoresis on 2% (w/v) agarose gels followed by 15 min of staining with SYBR Green I (DingGuo Co., Ltd. Shanghai, China). Bands were visualized by UV excitation.

Statistical analysis

All statistics were performed using SPSS for Windows version 11.0. The results were shown as the average of at least three independent experiments. Variation was expressed as SD. Student's t-test and analysis of variance (ANOVA) were applied to detect the significant differences among the treatments. Duncan's multiple range test was used to determine significant differences among the means ($P \leq 0.05$).

Table 1. The relative assessment of the rate of production of nitrogen from screening medium by isolates.

Time/day	DF-1	DF-2	DF-3	DF-4
1	-	-	-	-
2	+	-	-	-
3	++	+	+	-
4	+++	+	+	-
5	++++	++	++	+
6	+++++	++	+++	+

"-''means no aerogenesis and "+''means aerogenesis and the number of pluses is proportional to increased rate of nitrite decomposition.

RESULTS AND DISCUSSION

Isolation of the aerobic denitrifying strains

Six strains were isolated from the sediment samples of the fish ponds under aerobic conditions, and then the four strains (Table 1; Figure 1) that could remove the nitrite-N were selected according to their BTB plate's reaction. Finally, strain DF-1 with the highest efficiency to remove nitrite among them was selected.

The growth of strain DF-1 and degradation of nitrite

The concentration of nitrite- N declined with strain DF-1 growth (Figure 2). Strain DF-1 could degrade the nitrite-N from 6.5 mg/L to zero in 20 h.

Effects of pH, temperature, carbon source and salinity on the degradation of nitrite-N by strain DF-1

Denitrification by strain DF-1 reached the peaks at pH pH 9.0 (Figure 3); its nitrite-N degradation rate reached 96% at pH 3.0 and was maintained at 97-99% at pH 7.0-9.0, but reached only 23% at pH 5.0 and 41% at pH 11.0. The growth (absorbance) of strain DF-1 increased with the pH rising and reached the peak at pH 9.0, and then reduced at pH 11.0.

Denitrification by strain DF-1 increased with the temperature rising and reached the peak (approximately 100%) at 35°C (Figure 4); when the temperature continued to raise, the rate of nitrite-N degradation by strain DF-1 dropped to 89%. Similarly to nitrite degradation, the absorbance of strain DF-1 increased with the temperature rising, reached the peak at 35°C and then decreased.

Strain DF-1 maintained high denitrification at salinity from 0 to 5 whereas nitrite-N degradation rate gradually decreased when the salinity concentration increased. At salinity of 15‰, nitrite-N degradation rate was almost 0. The absorbance of strain DF-1 increased from 0.3 to 0.36 when salinity increased from 0 to 5, but then decreased

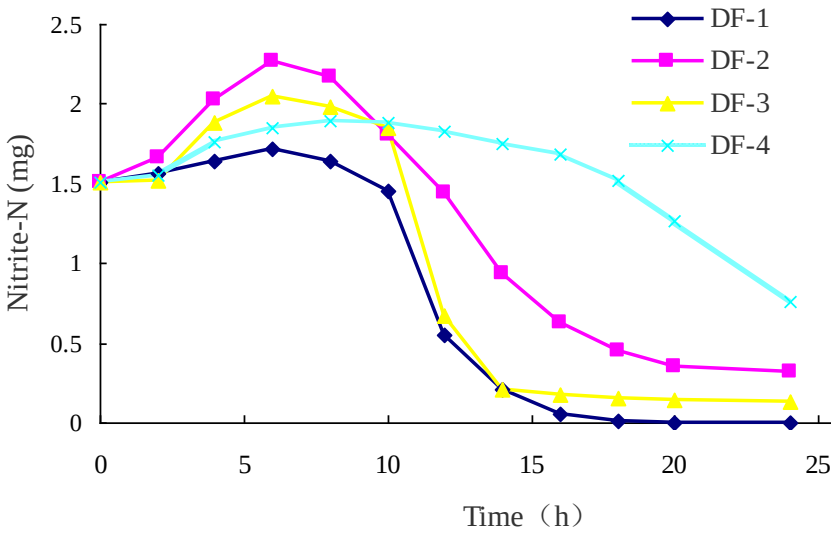

Figure 1. Time-course of nitrite degradation by various strains.

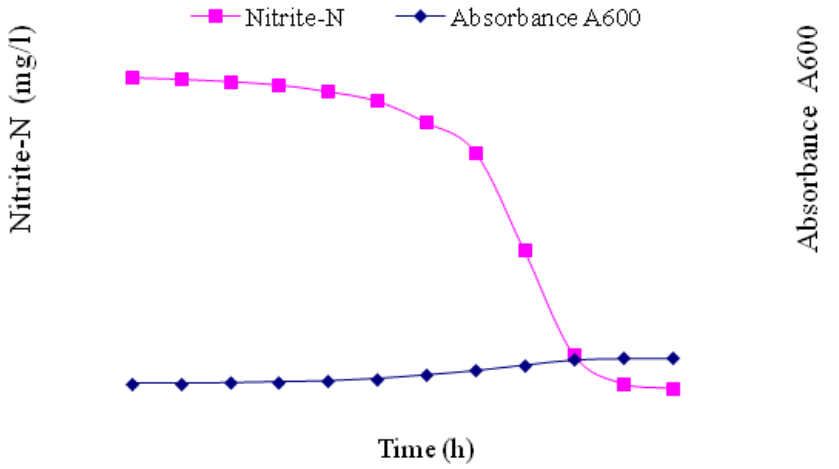

Figure 2. Dynamics of strain DF-1 growth (absorbance A600) and degradation of nitrite.

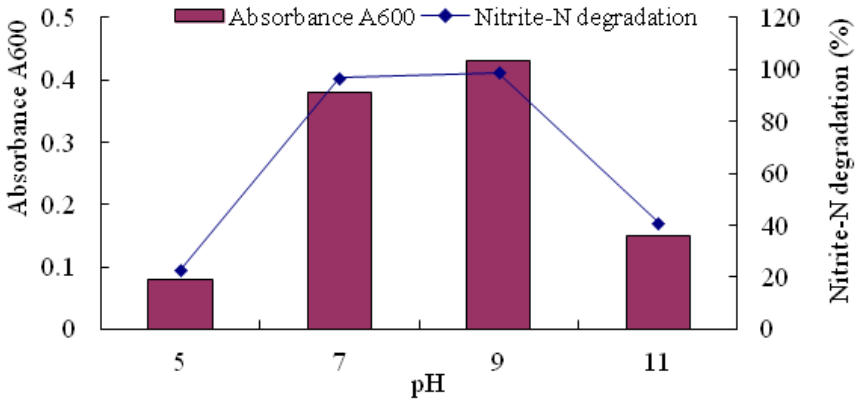

Figure 3. Dynamics of DF-1 growth (absorbance A600) and degradation of nitrite under different pH conditions.

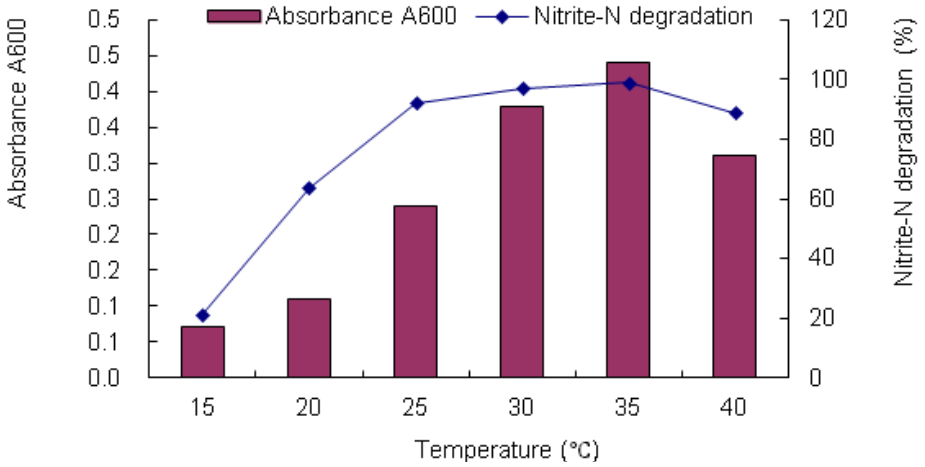

Figure 4. Dynamics of DF-1 growth (absorbance A600) and degradation of nitrite under different temperatures.

Table 2. Carbon source suitability for growth and nitrite degradation by strain DF-1.

Carbon source	Absorbance A600	Nitrite-N concentration before the culture (mg/L)	Nitrite-N concentration at the end of the culture (mg/L)	Nitrite-N degradation (%)
Sucrose	0.53	10.011±0.021	0.069±0.132	99.3
Glucose	0.31	10.009±0.013	0.233±0.125	97.7
Lactose	0.06	10.004±0.032	8.010±0.157	20.1
Sodium acetate	0.58	9.999±0.025	0.051±0.146	99.5
Sodium succinate	0.36	10.002±0.047	0.354±0.133	96.5
Sodium citrate	0.08	10.011±0.031	8.026±0.141	19.8
Potassium sodium tartrate	0.23	10.000±0.026	0.823±0.098	91.8

to 0 at salinities from 15 to 30‰.

Strain DF-1 grew well and nitrite-N degradation rate reached 99% when sucrose or sodium acetate was a carbon source (Table 2). With sucrose, glucose, sodium acetate, sodium succinate or potassium sodium tartrate, the rate of nitrite-N degradation was high (92-99%). However, with lactose or sodium citrate, the capacity for denitrification worsened, and the rate of nitrite-N degradation was 20%. Interestingly, when the carbon source was glucose, sodium succinate or potassium sodium tartrate, strain DF-1 had the rate of nitrite-N degradation as high as on sucrose or sodium acetate, but did not grow nowhere near as well (Table 2; Figure 5).

Identification of strain DF-1

The BLAST program was used to identify homologous sequences in the GenBan database. The sequence was aligned with multiple alignment program CLUSTAL W. The MEGA5.0 was used to construct a phylogenetic tree shown in Figures 6 and 7. 16SrRNA identification revealed that strain DF-1 has 98% homology with *Klebsiella oxytoca* (Figure 6), which was supported also by morphological observations.

Amplification of nitrite reductase

Amplification of the nitrite reductase structure genes nirS and nirK from strain DF-1 is shown in Figure 7. The nirK amplification product was 482 bp, while the nirS amplification production was negative. The result indicated that strain DF-1 had nitrite reductase structure of nirS gene.

Primary production is generally limited by the balance between additions of nitrogen (N) fixed via biological and industrial means, and its removal by microbial anaerobic respiration. Heterotrophic denitrification, in which nitrate (NO_3^-) and nitrite (NO_2^-) are reduced stepwise to the gaseous products nitric oxide (NO), nitrous oxide (N_2O) and N_2, has long been considered the dominant process for returning N to the atmosphere. The efficiency of aerobic denitrifying bacteria in removing nitrogen during wastewater treatment was a frequent topic of research, but some biological characteristics were neglected, example pathogenicity. Most isolates were Gram- negative bacteria with potential pathogenicity (Read and Fernandes, 2003) or poor stress tolerance and low denitrifi-

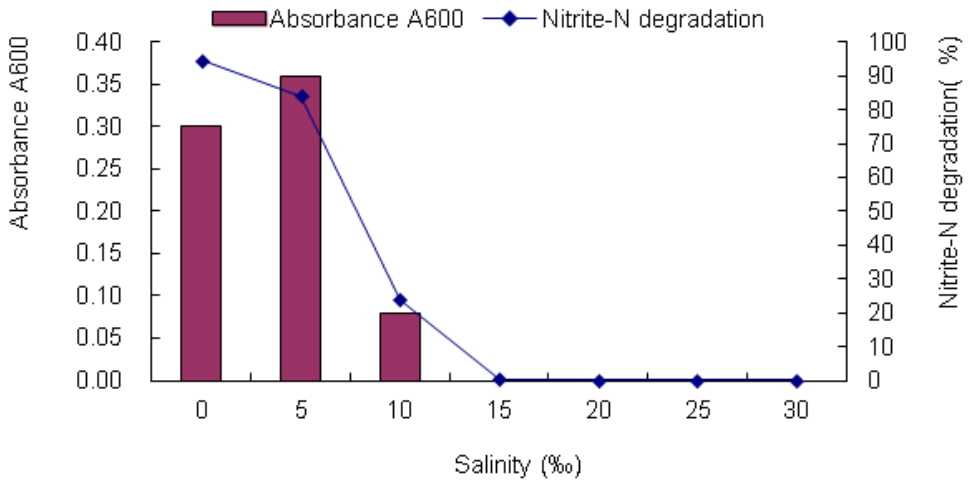

Figure 5. Dynamics of DF-1 growth (absorbance A600) and degradation of nitrite under different salinities

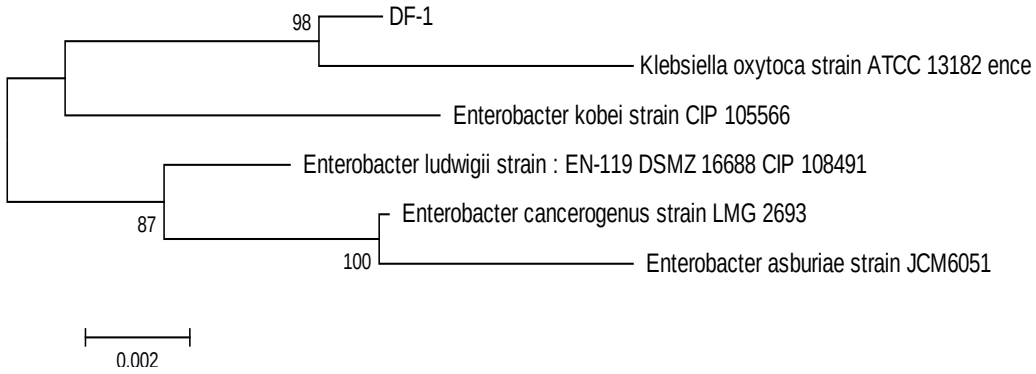

Figure 6. Phylogenetic tree generated from an alignment of the 16 SrRNAs.

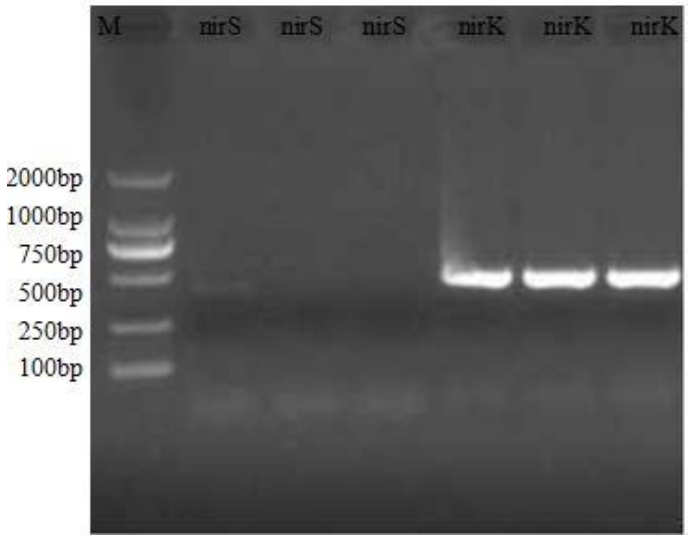

Figure 7. Amplification profiles of nirS and nirK genes from strain DF-1.

cation efficiency (Ryosuke et al., 2000). Therefore, few isolates used in the pollution water treatment were truly suitable for aquaculture industry. In this study, aerobic denitrification strain DF-1 with high efficiency was characterized, which might be a good probiotics used in the intensive culture. It may also help us understand the principles of improving water quality under sufficiently aerated conditions.

The optimum pH was neutral or slightly alkaline for denitrifying bacteria reductase. When pH deviated from this optimum, the activity of denitrifying bacteria could be reduced (Robertson and Kuenen, 1984; Robertson et al., 1988). In the present study, strain DF-1 was adapted to a broad pH range. The nitrite-N removal rate was approximately 100% at pH 7.0 to 9.0 (Figures 1 to 3).

The activity of denitrification reductase would be inhibited by high or low temperature (Otani et al., 2004). In the present study, the optimum temperature of strain DF-1 was within the range of 25-35°C, with the nitrite-N degradation rate reaching 90% when temperature ranged between

25 and 40°C. The rate was still more than 85% at 40°C, which contrasts with published reports (Pai et al., 1999) on other strains that almost completely lost denitrification capacity at the same temperature. Hence, strain DF-1 could tolerate high temperature and is therefore suitable for nitrite degradation in summer when nitrite usually accumulates in the culture ponds.

One of the main factors affecting bacterial growth is the availability of carbon sources. The type of carbon source used seemed to affect final biomass yields only after extended cultivation periods. High nitrite-N degradation (>91%) was obtained with sucrose, glucose, sodium acetate, sodium succinate or potassium sodium tartrate, in agreement with the findings of other authors (Otani et al., 2004). However, strain DF-1 showed low denitrification when lactose or sodium citrate was used as a carbon source. This capacity has a significant practical use, as nitrate/nitrite loads can be initially reduced aerobically, when biomass is forming in an aerated tank, and then the remaining load can be anoxically converted to N_2 in a denitrification tank using the external inputs of specific carbon sources. Also, some nitrate might be reduced by aerobic denitrification simultaneously with nitrification in nitrification basins. This opens up different operation perspectives for nitrogen removal processes in the recirculating seawater treatment systems. Anoxically, strain DF-1 showed typical denitrifying behavior, sequentially converting nitrate to nitrite and gaseous products.

Halophilic bacteria mainly include *Haloarcula*, *Halobacterium*, *Haloferax* of *Archaea* and *Bacillus* as eubacterium. The reductase activity of denitrifying bacteria could be inhibited by salinity. The results in this study indicated that DF-1 could maintain high nitrite-N degradation rate in salinity from 0 to 10. Therefore, strain DF-1 could be used for nitrite-N degradation both in high- and low-salinity water (Figures 4 to 7).

Klebsiella oxytoca can rapidly produce acid and gas from fermentation of glucose. Adachi et al. (2002) isolated from sweet potato a strain of *Klebsiella oxytoca* with the capacity to fix nitrogen. The strain of *Klebsiella oxytoca* BAS-10 isolated by Franco et al. (2001) had strong heavy metal resistance. Ryosuke et al. (2000) isolated extracellular polysaccharide AZ-9 from the fermentation broth of *Klebsiella oxytoca* TMN3, and found its anti-arthritis activity in mice. Other applications of *Klebsiella oxytoca* were also reported, such as processing propionitrile, and producing 2,3-butanediol and 1,3-propanediol. In the study presented here, strain DF-1 was identified as *Klebsiella oxytoca* according to the 16 SrRNA sequence analysis.

There were many reports on the composition of and correlation among genes for nitrite reductase. Dissimilatory nitrite reduction is catalysed by two structurally different but functionally equivalent enzymes which are thought to be mutually exclusive in a given strain, *nirK* encodes a copper-containing nitrite reductase while the *nirS* product contains cytochrome *cd*1. Nitrite reductase functional genes were polyphyletic and closely related to the denitrification properties of bacteria. Consequently, analyses focused on nitrite reductases have been used to characterize denitrifying communities in a variety of marine environments, instead of 16S rRNA-based methods. In the present study, the result indicated that nirK nitrite reductase gene existed in stain DF-1 (Figure 7).

CONCLUSIONS

In conclusion, strain DF-1 could remove the nitrite-N in the aerobic conditions and can be suitable as potential probiotics for intensive aquaculture. Studies such as the one presented in this article can help the development of models describing denitrification in real recirculation aquaculture systems in the coastal mudflats. Other aspects to be investigated in further research with strain DF-1 include characterization and quantification of denitrification gaseous products, besides the evaluation of other metabolic traits with impact on bioreactor improvement, such as the capacity of simultaneous nitrogen and phosphorus removal.

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