

Original Research Article

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Nitrification Potential of *Achromobacter xylosoxidans* Isolated from Fresh Water Finfish Farms of Kerala, India

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ABSTRACT

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A heterotrophic strain ACM-1, isolated from *Pangasius* spp fish farms showed an ability to convert ammonium to nitrate under aerobic conditions. Based on the molecular phylogenetic analysis 16S rRNA gene sequence ACM-1 matched similarity to *Achromobacter xylosoxidans* (100%). ACM-1 showed amplification for Beta-proteobacterial 16S AOB gene (465 bp) confirming the characteristic feature of ammonia oxidation. Sodium pyruvate (1%) was confirmed as the probable carbon source for the luxurious growth of *Achromobacter xylosoxidans* among the six carbon sources tested. At 30°C, 5% preculture and 120 rpm in shaker, ACM-1 could able to remove 100% ammonium within 36h. The ammonium removal rate was reported to be 1.3 NH₄⁺-N mg L⁻¹h⁻¹ at 30°C. The rate of ammonium removal at different temperature (20°C, 25°C, 37°C) ranged between 0.9 to 1.33 NH₄⁺-N mg L⁻¹ h⁻¹. The nitrite produced during this experiment remained at constant rate and nitrate concentration increased after the initial hours of experiment. Therefore, the results demonstrate that *Achromobacter xylosoxidans* ACM-1 can remove ammonium in batch cultures and could be applied in aquaculture farms as a biological nitrifier.

Introduction

Over the decades, Aquaculture as a traditional practice has evolved to science based activity to meet the demands of food security as well as national economy. During 2016, world aquaculture production has projected to 73.8 mt with US\$160.2 billion as an estimated first-sale value (FAO.2016). Total world aquaculture production accounted for 44.1% during 2014 and 31.1 % as compared in 2004.

Feed is a crucial input for Semi –intensive and intensive aquaculture and usually contributes over 40-50% production costs but nearly 36% of the feed is excreted in the form of organic waste (Brune *et al.*, 2003). The protein requirement of fish is about two to three times higher than that of mammals. Ammonium is one of the end products of protein metabolism (Walsh and Wright, 1995). In water, ammonia occurs in two forms which together are called total ammonia

nitrogen, or TAN. Chemically, these two forms are ionized ammonia (NH_4^+), un-ionized ammonia (NH_3). Haywood (1983) observed that unionized ammonia is 300–400 times more toxic than ionized ammonium. The unionized ammonia is more toxic than ionized ammonia because it is lipid soluble and consequently traverses biological membranes more readily than the charged and hydrated NH_4^+ ions (Korner *et al.*, 2001). The proportion of TAN that exists in the toxic form varies with increasing water, pH and temperature. Driving sources of ammonia nitrogen into the earthen ponds are mainly through inputs such as inorganic fertilizers such as urea, mineralization of fish waste and organic matter decomposition (Boyd, 2001). In intensive as well as Semi-intensive aquaculture systems, about 20–40% of nitrogen in feed are utilized and the remaining percentage of nitrogen can be regarded as inflow in to the pond sediment followed by mineralization (Folke and Kautsky, 1989). The lethal and sub-lethal effects of unionized ammonia in sensitive fish are such as gill damage, blood oxygen-carrying capacity reduction, lack and depletion of adenosine triphosphate (ATP) in the brain, and the liver and kidneys malfunction (Ariello *et al.*, 1981; Camargo and Alonso 2006; Lang *et al.*, 1987; Russo 1985; Tomasso *et al.*, 1980). The 96-hr LC_{50} of ammonia for rainbow trout is between 0.32 to 0.93 mg L^{-1} ; cutthroat trout, 0.50–0.80 mg L^{-1} ; freshwater prawns, 2.00–2.50 mg L^{-1} ; school prawns, 1.39 mg L^{-1} ; southern white shrimp, 0.69–1.20 mg L^{-1} ; pacific white shrimp, 1.20–2.95 mg L^{-1} (Boyd, C.E., 2013). In most cases, the acceptable level of unionized ammonia in aquaculture systems is 0.025 mg L^{-1} (Chen *et al.*, 2006). Biological removal of ammonia in earthen ponds takes place by several processes includes algal assimilation, bio-filtration, bio-floc technology and nitrification (Hargreaves, 1998). Nitrification is a major process in the nitrogen (N) cycling, including a two step

process, the oxidation of ammonia to nitrite and subsequently nitrite to nitrate (Prosser, 1989). Ammonia-oxidizing bacteria (AOB) are obligate chemolithoautotrophs, they use ammonia for respiration as well as for cell synthesis and carbon dioxide as the only carbon source (Chain *et al.*, 2003). Autotrophic AOB are exceedingly slow growing with doubling time of 7–8 h under ideal conditions (Watson, 1971), more than 26 h for nitrite bacteria and 60 h for nitrate bacteria (Shilo and Rimon, 1982) under *insitu* conditions making their isolation difficult, time-consuming. Interestingly heterotrophic nitrifiers such as *Rhodococcus* spp (Chen *et al.*, 2012), *Alcaligenes faecalis* (Joo *et al.*, 2005), *Bacillus* spp. LY (Zhao *et al.*, 2010a) *Bacillus methylotrophicus* (Zhang *et al.*, 2012), *Pseudomonas stutzeri* (Zhang *et al.*, 2011) *Acinetobacter calcoaceticus* (Zhao *et al.*, 2010) were reported widely under various environments. Therefore, an attempt was made to isolate, characterize the ammonia oxidizing bacteria from aquaculture farms and to estimate their ammonia oxidation rates at various temperatures.

Materials and Methods

Sediment samples were collected randomly from four *Pangasius* spp fish farms at four corners and centre, maintained in ice and transported with in 4 h to the laboratory. The fish ponds varied in size from 0.2-0.5 ha with a stocking density of 8000-12000 fingerlings/ha.

All the four fish farms were fed with chicken poultry waste as a supplementary diet at the rate of 3-4 %body weight without any aeration facilities. From each farm, sediment samples were mixed and subsamples in triplicate were taken for analysis of ammonium, nitrate, dry weight, total carbon, pH and isolation of ammonia oxidizing bacteria (AOB).

Determination of physico-chemical analysis

Sediment samples were processed for the estimation of mineral nitrogen (ammonium, nitrite, nitrate) following calorimetric method (Bollmann *et al.*, 2011; Kandeler and Gerber 1988; Keeney DR, Nelson DW, 1982; Shand *et al.*, 2008). Initially, 3–4 g sediment samples were mixed with 2 M KCl in the ratio 1:10, and were kept in shaking condition for 1 h at 200 rpm, centrifuged at 7500g for 10 min. The supernatant was used for mineral nitrogen analysis. All the sediment samples were kept for drying for 24 h at 110°C to determine the dry weight. Organic carbon was determined using Walkley and Black method (1934). pH was estimated by suspending sediment in water (1:2.5 ratios) using pH meter.

Isolation and characterization of AOB from sediment

Ten grams of sediment was enriched in the 100 ml mineral salt media (Verhagen and Laanbroek, 1991) containing (NH₄)₂SO₄ 3.3g L⁻¹, NaCl, 0.585 g L⁻¹, KH₂PO₄ 0.054 g L⁻¹, KCl 0.075 g L⁻¹, CaCl₂·2H₂O 0.147 g L⁻¹, MgSO₄·7H₂O, 0.049 g L⁻¹, trace elements solution 1 ml L⁻¹ (Verhagen and Laanbroek, 1991), 0.04% Bromothymol blue solution, 5 ml L⁻¹. HEPES buffer as a buffering agent was added to the media four times to the final ammonium concentration. The final pH of the media was adjusted to 7.8. The flasks were incubated in dark condition at 25 °C for 10-15 days and samples were monitored for every 4 days for reduction in pH as well as color change of MSM media from blue to red. Cultures that consumed around 80% or more of the NH₄⁺ were transferred into fresh medium at weekly intervals for 5 weeks. One ml of young culture was serially diluted to 10²–10⁶ in mineral salt medium with 10 mM NH₄⁺ and 1ml diluted cultures were transferred to 100 ml of MSM media with 2%

agar using pour plate method. Plates were sealed with parafilm and incubated for 3-4 months at 30± 2°C. Representative colonies were picked, transferred into the MSM broth with 10mM of ammonium and incubated under shaking condition for 1-2 weeks. Positive cultures showing oxidation of ammonium were purified onto the MSM media with 2% agar and purified colonies were transferred on to the Trypticase soya agar slants. Biochemical identification of isolates was performed as suggested by the Bergey's Manual of Systematic Bacteriology (Garrity *et al.*, 2004).

Betaproteobacterial AOB amplification using PCR

Bacterial DNA as a template for 16SrRNA and AOB amplification was isolated by boiling method. Young culture (18-20 h) was centrifuged at 10,000 rpm for 10 min, supernatant was discarded and the pellet was resuspended in 500 µl sterile molecular grade water. The whole mixture was centrifuged at 10,000 rpm for 10 min, supernatant was discarded and pellet was resuspended with 200 µl molecular grade water. The mixture was subjected to boiling at 95°C in a water bath for 10 min, later cooled at –20°C for 10 mins. The mixture was centrifuged at 8,000 rpm for 1min and aliquots of 2 µl of template DNA were used for PCR. For screening of AOB, PCR amplification was performed employing to CTO-189F and CTO-654R primers. The PCR protocol was followed as described by (Kowalchuk *et al.*, 1997) and PCR products were visualized using 2 % agarose.

Nitrification activity of the isolates using batch culture assay

To observe the effects of isolates on ammonia removal, 100ml of MSM media with 50 mg L⁻¹ of ammonium was prepared in 250ml

conical flask and autoclaved. For estimation of the microbial growth, 5 % of culture was grown on MSM media (pH-7) with addition of 1% sucrose, glucose, lactose, sodium acetate, trisodium citrate, sodium pyruvate at 30°C for 4 days under shaking conditions. For nitrification studies, the medium was then incubated aerobically with constant speed of 120rpm at four different temperatures of 20°C, 25°C, 30°C, 37°C for approximately 4 days. A 2ml of aliquots were periodically examined for determination of ammonium, nitrite and nitrate. A control without culture was used during the experiment period.

Analytical methods

The O.D of the culture was estimated using UV-Spectrophotometer at 600nm. In addition, samples were centrifuged at 8000 rpm for 10 min at 4°C, further supernatants were filtered using 0.22µ filter and filtrates were used for chemical analysis. The ammonium concentration was measured at 660nm using indophenols blue method (Kandeler and Gerber 1988) whereas nitrite and nitrate assay was carried using calorimetric method at 540nm (Keeney and Nelson 1982); (Shand *et al.*, 2008). For estimation of ammonium (0.1 to 10 ppm), nitrite (0.1 to 1 ppm) and nitrate (0.1 to 10 ppm) a standard curve is plotted, intercept and slope are recorded.

Molecular identification of isolate using 16SrDNA sequencing

The most promising AOB isolate showing potential nitrification rate was identified by 16SrDNA gene sequencing. Bacterial 16SrDNA amplified using universal bacterial primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-AAGTCGTAACAAGGTAACC-3') (Turner *et al.*, 1999). Bacterial 16SrRNA amplification was carried out using universal primers 8F and 1492 R

primers. The PCR conditions were initial denaturation at 95°C for 3 mins and 30 cycles of 95°C for 2 min, 52°C for 1 min, 68°C for 1.30 min and final extension at 68°C for 7 min. The amplified products (approx 1500bp) were purified and sequenced on automated ABI Sequencer. The 16SrDNA sequence was compared with other bacteria by using BLASTn (<https://blast.ncbi.nlm.nih.gov/blast>). 16SrDNA reference sequences obtained from Genbank database was aligned using multiple sequence alignment software CLUSTAL X and sequence similarities were determined. A phylogenetic tree was constructed using Mega 7.0.

Statistical analysis

All the data during this experiment were analysed using Microsoft excel. The Nitrification ratio was calculated as $(C_0 - C_n)/h$. C_0 is initial concentration of mineral nitrogen compounds. C_n is final concentration of mineral nitrogen compounds. Time of ACM-1 during the treatment is noted as h .

Results and Discussion

The Physico-chemical parameters of the four farm sediments were mentioned in table 1. The temperature of the farms ranged from 28.3 °C to 30.4 °C, sediment pH 6.7-7.1, salinity 0.1-0.14 ppt, organic carbon 0.94-1.07, and ammonium from 64.0-117.5 ($\mu\text{g g}^{-1}$), nitrate from 4.70 – 11.70 ($\mu\text{g g}^{-1}$).

Isolation and identification of AOB from fish farms

A total of 112 bacterial isolates (28 isolates per farm) were picked from the four farms. Among them, 12 isolates were found to be utilizing ammonium within 5 days time interval. All the 12 isolates were gram negative, short rod, motile, oxidase, catalase positive and indole and urease negative.

Table.1 Physico-chemical parameters of four fish farm sediments

Farm	Temperature (°C)	pH	Salinity (ppt)	NH ₄ ⁺ (µg/gm) d.wt	No ₃ ⁻ (µg/gm) d.wt	Organic carbon
1	30.4 ± 0.20	6.96 ± 0.11	0.1 ± 0.03	66.8 ± 2.95	4.70 ± 0.82	0.83 ± 0.045
2	28.3 ± 0.41	6.89 ± 0.07	0.1 ± 0.05	117.5 ± 5.63	11.7 ± 1.71	0.94 ± 0.06
3	29.3 ± 0.26	6.76 ± 0.15	0.1 ± 0.02	64.0 ± 3.97	6.73 ± 0.26	1.07 ± 0.110
4	29.5 ± 0.5	7.16 ± 0.03	0.2 ± 0.03	72.6 ± 1.35	5.43 ± 0.39	0.95 ± 0.105

Fig.1 A 465 bp product targeting AOB specific Bacterial 16S rRNA detected in ACM-1
A: negative control, B: Positive isolate, C: Test isolate 1; D: Test isolate 2; L: Ladder (100bp)

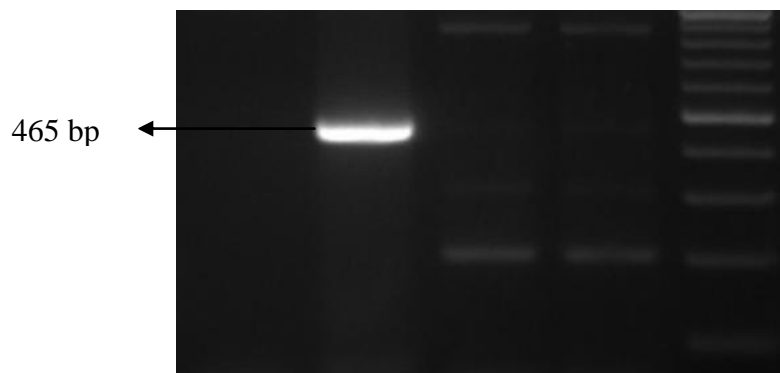


Fig.2 Phylogenetic tree based on the 16S rDNA sequence of strain ACM-1(4 8F 12281-5) with closely related sequences. The numbers at the forks indicate the bootstrap values in percentage

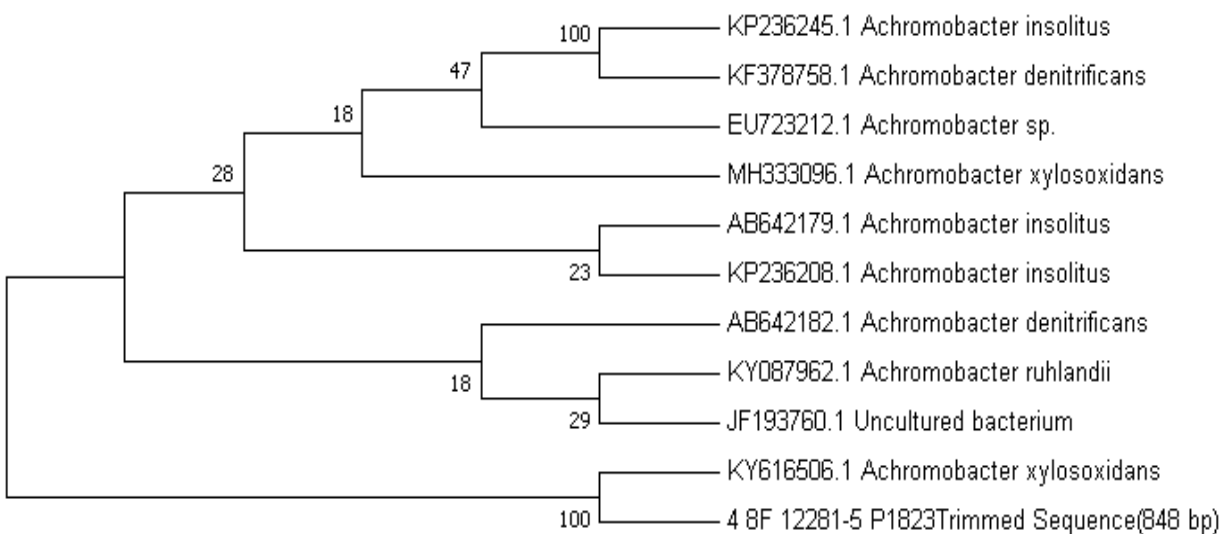


Fig.3 Cell growth and removal of ammonium by ACM-1in MSM containing system at 30°C.
 Symbols: diamonds, $\text{NH}_4^+\text{-N}$; Squares, cell growth at OD_{600}

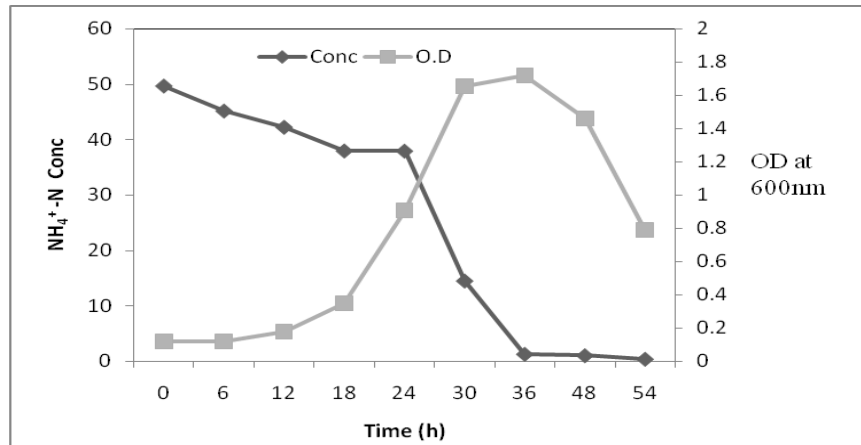


Fig.4 Cell growth and accumulation of Nitrite by ACM-1in MSM containing system 30°C.
 Symbols: diamonds, $\text{NO}_2^-\text{-N}$; Squares, cell growth at OD_{600}

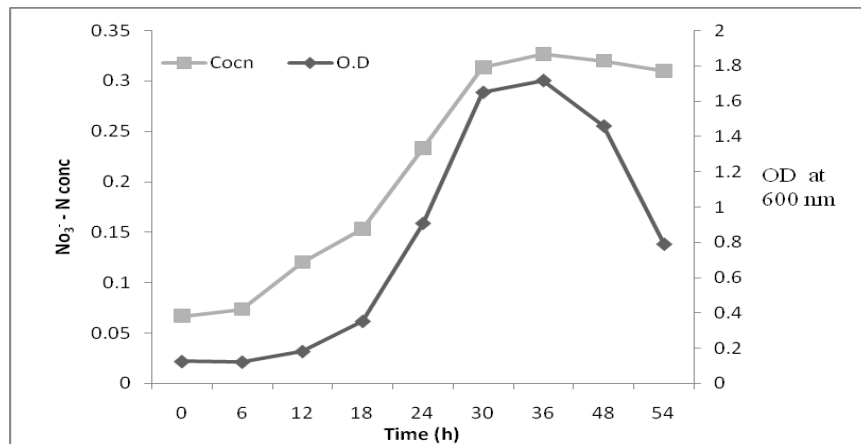
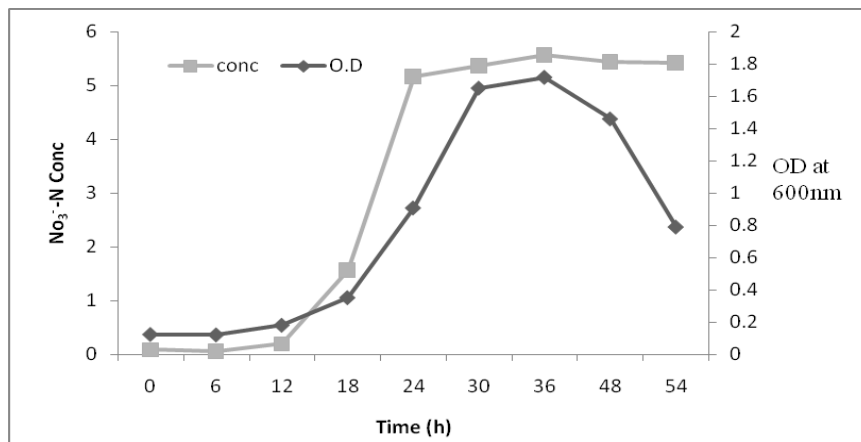


Fig.5 Cell growth and accumulation of Nitrate by ACM-1in MSM containing system at 30°C
 Symbols: diamonds, $\text{NO}_3^-\text{-N}$; Squares, cell growth at OD_{600}



All the isolates didn't show acid production from lactose, maltose, mannitol, sucrose and low utilization of glucose was observed. Twelve isolates showed positive for 16S AOB gene amplification (465 bp) with CTO189F and CTO454R primers as represented in (Fig.1) and were presumed to be ammonia oxidizing bacteria. The 16S rDNA nucleotide sequence of strain ACM-1 was closely related (100%) to *Achromobacter xylosoxidans* from NCBI gene bank data base. A phylogenetic tree was constructed based on closely related 16S rDNA sequences (Fig. 2). Morphological, biochemical and molecular analysis resembles that ACM-1 is *Achromobacter xylosoxidans*.

Estimation of nitrification activity

Isolates checked for the process of nitrification under different carbon sources at 30°C. The specific growth rate of ACM-1 on sodium pyruvate, trisodium acetate and sodium citrate was 0.35 h⁻¹, 0.27 h⁻¹ and 0.22 h⁻¹ whereas on glucose, moderate growth was recorded but ACM-1 didn't show any significant growth rate on sucrose, lactose. The growth at O.D₆₀₀ and ammonia removal rate of ACM-1 at different temperatures in the MSM medium was studied under shaking condition. ACM-1 was capable to grow observed at 20°C, 25°C, 30°C, 37°C. Cell growth and nitrifying efficiency of ACM-1 showed significant results at 30°C when compared to the 20°C, 25°C, 37°C. Cell growth and nitrification activity of ACM-1 at 30°C during this study was elaborated. Initially, after 2h of incubation when 1% sodium pyruvate was supplemented to the media, ACM-1 started growing, continued exponential phase for 30hr further reached to stationary phase by 36 hr as OD₆₀₀ increased from 0.014 to 1.172 and then it started the decline phase after 36 h. These results revealed that probable source of carbon to ACM-1 is sodium pyruvate and therefore the heterotrophic nature of the strain was

confirmed. The specific growth rate of the strain at different temperatures ranged from 0.18–0.55 h⁻¹. Robertson *et al.*, (1995) reported the specific growth rate of *Thiosphaera pantotropha* about 0.28–0.45 h⁻¹ under various growth conditions. The growth rates of *A. faecalis* No. 4 was reported to be 0.2 h⁻¹ as reported (Joo *et al.*, 2005) and 0.3 h⁻¹ was reported in *Bacillus* MS30 at initial ammonium concentration of about 70 ppm (Mevel and Prieur, 2000). Based on the comparison between these heterotrophs, the growth rate of *Achromobacter xylosoxidans* is at high rate as well as high utilization of carbon. ACM-1 completely oxidized initial ammonium (50 mg L⁻¹) within 36 h and the nitrification ratio for NH₄⁺ -N mg L⁻¹h⁻¹ with the ammonium removal rate was 1.38 ± 0.44 NH₄⁺ -N mg L⁻¹h⁻¹ as shown in Figure 3. *Pseudomonas alcaligenes* AS-1 showed the oxidation of NH₄⁺ -N at the rate 1.15 mg L⁻¹ h⁻¹ (Su *et al.*, 2006) where as the ammonium removal rate of *Bacillus* sp. LY reported at 0.43 mg L⁻¹h⁻¹ (Zhao *et al.*, (2010 a,b). Therefore, results appear to be *Achromobacter xylosoxidans* reported a high rate of ammonium removal when compared to *Bacillus* sp LY and *Pseudomonas alcaligenes* AS-1. Nitrification products (NO₂⁻-N and NO₃⁻-N) were detected during the experimental period. There was slow increase in nitrite (NO₂⁻-N) concentration of about 5ppm till the isolate reached the log phase (0-24 hr) and later it almost remained at constant rate during the experimental period (Fig. 4). Chen *et al.*, (2006) reported that nitrite production by *Rhodococcus* spp was about 5.5-6 ppm during 10 h incubation which mostly resemble to the present study. The increase in the detection of nitrate concentration was observed during the 0-16 hr of growth phase and there is no significant difference in the nitrate (NO₃⁻-N) production during the stationary and decline phase (Fig. 5). Therefore, low levels of NO₂⁻-N and NO₃⁻-N accumulation was observed during the

investigation period. The ammonium removal rate of *A. xylosoxidans* at different temperatures in the MSM medium was investigated during the study. At 25°C, 30°C, 37°C the ammonia removal rates showed similar pattern of ammonia removal 0.90, 1.33 and 1.19 NH₄⁺-N mg L⁻¹h⁻¹ except at 20°C. It is observed that NH₄⁺-N was reduced from 50 mg L⁻¹ to 1.89 ± 1.21 mg L⁻¹ after 72 h of incubation with the nitrification ratio of 0.66 mg L⁻¹ h⁻¹ NH₄⁺-N at 20°C.

There is no significant difference observed in the ammonia removal patterns during 30°C and 37°C incubation whereas ammonia removal rate at 20°C and 25°C was observed at lesser extent. The ammonia removal rate was recorded highest at 30°C in comparison to 25°C and 37°C. Therefore, experimental results suggest that *Achromobacter xylosoxidans* is capable to oxidize NH₄⁺-N and releases NO₂⁻-N and NO₃⁻-N during growth phase conditions.

In conclusion, Nitrifying bacteria plays a major role in conversion of ammonium to nitrite and helps in maintaining the water quality of the fresh water ponds. It is well known that fishes cultured in pond waters of greater than 1.5 mg/N is toxic to fishes. However the toxicity varies between the species of the fishes reared and size of the animals affected. In freshwater aquaculture system, the acceptable level of unionized ammonia is 0.0025 ppm (Chen *et al.*, 2006). Probably, aerating the pond water can reduce the ammonia levels but the nitrifying bacteria will definitely oxidize the toxic unionized ammonium to non-toxic nitrate. Therefore, the results of the present study indicate that *Achromobacter xylosoxidans* might be a preferred as nitrifying bacteria to oxidize ammonium as well as to reduce nitrite in fish ponds.

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