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ORIGINAL PAPER

Mass production of nitrifying bacterial consortia for the rapid establishment of nitrification in saline recirculating aquaculture systems

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Abstract Two distinct nitrifying bacterial consortia, namely an ammonia oxidizing non-penaeid culture (AMO NPCU-1) and an ammonia oxidizing penaeid culture (AMOPCU-1), have been mass produced in a nitrifying bacterial consortia production unit (NBCPU). The consortia, maintained at 4°C were activated and cultured in a 2 l fermentor initially. At this stage the net biomass (0.105 and 0.112 g/l), maximum specific growth rate (0.112 and 0.105/h) and yield coefficients (1.315 and 2.08) were calculated respectively, for AMONPCU-1 and AMOPCU-1 on attaining stationary growth phase. Subsequently on mass production in a 200 l NBCPU under optimized culture conditions, the total amounts of NH_4^+–N removed by AMONPCU-1 and AMOPCU-1 were 1.948 and 1.242 g/l within 160 and 270 days, respectively. Total alkalinity reduction of 11.7–14.4 and 7.5–9.1 g/l were observed which led to the consumption of 78 and 62 g Na_2CO_3. The yield coefficient and biomass of AMONPCU-1 were 0.67 and 125.3 g/l and those of AMOPCU-1 were 1.23 and 165 g/l. The higher yield coefficient and growth rate of AMOPCU-1 suggest better energy conversion efficiency and higher CO_2 fixation potential. Both of the consortia were dominated by *Nitrosomonas*-like organisms. The consortia may find application in the establishment of nitrification within marine and brackish water culture systems.

Keywords Ammonia oxidizing consortia · Nitrifying bacteria · Mass production · Maximum specific growth rate · Nitrification · Yield coefficient

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Introduction

The most prominent requirement of any recirculating aquaculture system (RAS) is an efficient biofilter to prevent the accumulation of toxic metabolites such as ammonia and nitrite. High levels of ammonia and nitrite undermine commercial production objectives, as their toxic impacts are manifested through impaired growth or chronic diseases (Cheng et al. 2004; Svobodova et al. 2005). This is especially true in shrimp/prawn hatcheries where the daily specific excretion of ammonia by larvae and post-larvae is five fold higher than that of adults. To address this issue, fixed film biofilters are commonly employed for total ammonia nitrogen (TAN) removal (Seo et al. 2001; Shnel et al. 2002). Experience has shown that the biofilters in RAS require activation to achieve high bacterial growth (Valenti and Daniels 2000) due to the habitually low concentrations of nutrients in the system (Piedrahita 2003), where natural bacterial yield is too low, thus delaying colonization and biofilm formation. To remediate this situation and to establish nitrification rapidly in RAS, mass production of nitrifiers has become essential. However, this has been a challenge, owing to their slow growth rate (Voytek and Ward 1995).

Nitrification involves the simultaneous action of ammonia oxidizing bacteria that oxidize ammonia to nitrite

(nitritation, Eq. 1); and the nitrite oxidizing bacteria that oxidize nitrite to nitrate (nitratation, Eq. 2) (Wiesmann 1994).

$$NH_4^+ + 1.5\ O_2 \rightarrow\ NO_2^- + H_2O + 2H^+ \quad (1)$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad (2)$$

In nitritation, the oxidization of one mol of ammonia produces two protons (Eq. 1) and by means of the stoichiometric equations as shown in Eqs. 3–5, the rate of ammonia oxidation can be evaluated indirectly by monitoring the rate at which the protons are neutralized by the addition of a base, such as carbonate (Gernaey et al. 1998).

$$CO_3^{2-} + H^+ \leftrightarrow HCO_3^- \quad (3)$$

$$HCO_3^- + H^+ \leftrightarrow\ CO_2 + H_2O \quad (4)$$

$$NH_4^+ + 1.5\ O_2 + CO_3^{2-} \rightarrow NO_2^- + 2\ H_2O + CO_2 \quad (5)$$

Based on the above principle, Singh et al. (2007) developed two types of nitrifying bioreactors named Stringed Bed Suspended Bioreactors (SBSBRs) and Packed Bed Bioreactors (PBBRs) for application in shrimp/prawn hatcheries. These reactors function using nitrifying biofilms immobilized on plastic beads assembled in the form of cartridges. To generate the biofilm, two ammonia oxidizing consortia, AMOPCU-1 and AMONPCU-1, were developed by enrichment from shrimp culture systems (Achuthan et al. 2006) and immobilized on the substrata. However, to accomplish commercialization of the technology, the consortia need to be generated in larger quantities. In this study, an indigenous nitrifying bacterial culture production unit (NBCPU) was designed, fabricated, and applied for mass production of the consortia under optimized culture conditions. The consortia thus generated should find application in a variety of biofilters/bioreactors besides SBSBR and PBBR in RAS and in brackish and marine culture systems.

Materials and methods

Ammonia oxidizing bacterial consortia

Two nitrifying bacterial consortia, AMONPCU-1 and AMOPCU-1 developed by enrichment technique from shrimp culture systems under perpetual salinity regimes of 15 and 30 parts per thousand (ppt) respectively, were used in this study following optimization of culture conditions (Achuthan et al. 2006). The growth medium was prepared in seawater (salinity 15/30 ppt), supplemented with 10 mg/l substrate $(NH_4)_2\ SO_4$, 2 mg/l KH_2PO4 (Watson 1965) and incubated at an optimum temperature of 28°C and at pH 8.5. Prior to transferring the cultures to NBCPU, a preliminary characterization was accomplished by transmission electron microscopy (TEM). Aliquots of 10 ml each of the consortia were centrifuged at 6000*g* in a refrigerated centrifuge for 15 min. The pellets were washed with seawater of respective salinity and fixed in 2.5% glutaraldehyde prepared in seawater at 4°C overnight, post-fixed in 2% osmium tetroxide at 4°C for 2 h, washed repeatedly with seawater and dehydrated through an acetone series of 70–100%, embedded in epoxy resin, sectioned and stained with lead citrate and uranyl acetate and examined under an electron microscope (Morgagni 268-D, Netherlands).

Activation of stored consortia

The nitrifying bacterial consortia maintained under refrigeration (4°C) were brought to room temperature (26–28°C) and required aliquots were mixed with equal volumes of their respective growth media and kept on a rotary shaker operated at 100 rpm. The quantity of NH_4^+–N consumed (Solorzano 1969) and NO_2^-–N formed (Bendschneider and Robinson 1952) were monitored daily. The pH was maintained by supplementing sodium carbonate and evaporation loss was corrected with sterile distilled water. The incubation continued until the cultures actively started nitrification.

Culturing of the nitrifying consortia in 2 l fermentor

A 2 l capacity fermentor (New Brunswick, USA, Bioflow 2000) was used for scaling up the two ammonia oxidizing consortia. The detachable fermentor vessel with the respective growth medium was autoclaved at 10 psi for 10 min and reassembled. Optimum pH and temperature were auto-adjusted and the agitation was set at 200 rpm with an airflow rate of 1.0 l/min. Inocula were taken from the cultures that were activated on a rotary shaker. The fermentor vessel was kept covered, to prevent photoinactivation of monoxygenase. The NH_4^+–N consumed and NO_2^-–N formed were assayed once every 24 h. The pH was adjusted to optima by addition of 10% $Na_2\ CO_3$ through the base port. As consumption of NH_4^+–N progressed it was supplemented with fresh aliquots maintaining 10 µg/ml as the final concentration. Upon attaining stationary phase (characterized by the decline in the production of NO_2^-–N), the cultures were harvested and used for inoculating the 200 l fermentor, prior to which the biomass generated were determined gravimetrically. This was accomplished by passing a known quantity of the culture through a pre-weighed membrane (0.22 µm), drying at 80°C overnight, stabilizing in a desiccator and determining the difference as the biomass by dry weight. Subsequently, μ^{max} for each of the consortia at 28°C was estimated using a mathematical model of the quadratic

form fitted into the experimental data on log NO_2^-–N formed versus time by the method of least squares, using the statistical software SPSS (SPSS Inc., USA).

Mass production of ammonia oxidizing bacterial consortia

Designing and fabrication of the NBCPU

The NBCPU (Fig. 1) was designed based on the requirements for obtaining maximum biomass within the shortest duration possible and fabricated with locally available materials. A 200 l wide-mouthed polyethylene carboy fixed with a central 0.5 H.P AC/DC agitator motor (500 W, 0–500 rpm), served as the fermenter vessel. Inside the vessel, a coiled stainless steel pipe circulated water at constant temperature of 28°C from a thermocirculator (2500 W) to maintain the optimum temperature. A temperature probe and portals for addition of medium and inoculum were supplied. Aeration was provided through a stainless steel pipe, which delivered filtered, sterile air to the bottom centre through an air sparger. A drain line was placed at the base of the tank. The medium was subjected to the boiling temperature (100°C) for 30 min in a 100 l boiler (5000 W, stainless steel), and was passed through a sterile (autoclaved, 15 psi for 15 min) pipe to the fermentor vessel.

Before use, the fermentor vessel was rinsed with 70% ethanol (1 l) and after 30 min, washed with sterile tap water and drained off. The vessel was filled with the respective medium and aerated for 2 days through a cartridge filter (0.22 μm) and plated out on to ZoBell's agar and Saboraud dextrose agar prepared in aged seawater to record the presence of total viable bacterial and fungal populations, prior to inoculating with the nitrifiers. The inocula (stationary phase cultures) were introduced to a final concentration of 1% (v/v) and incubated under agitation at a rate of 100 rpm and with continuous aeration at 2 l/min, maintaining optimum culture conditions. The pH was maintained using aqueous 10% sodium carbonate and the temperature was regulated at 28°C by a thermocirculator. As the consumption of NH_4^+–N progressed it was supplemented with aliquots of fresh substrates at an exponential rate. This process was continued until the cultures attained stationary phase with daily monitoring of NH_4^+–N consumption and NO_2^-–N and NO_3–N production. Nitrate was estimated following the method of hydrazine sulfate reduction (Strickland and Parsons 1972). The initial and final biomass was measured gravimetrically. Based on the data generated, growth pattern, substrate consumption and product formation were determined. Alkalinity reduction due to hydroxylamine production was calculated based on the relationship 6.0–7.4 mg alkalinity

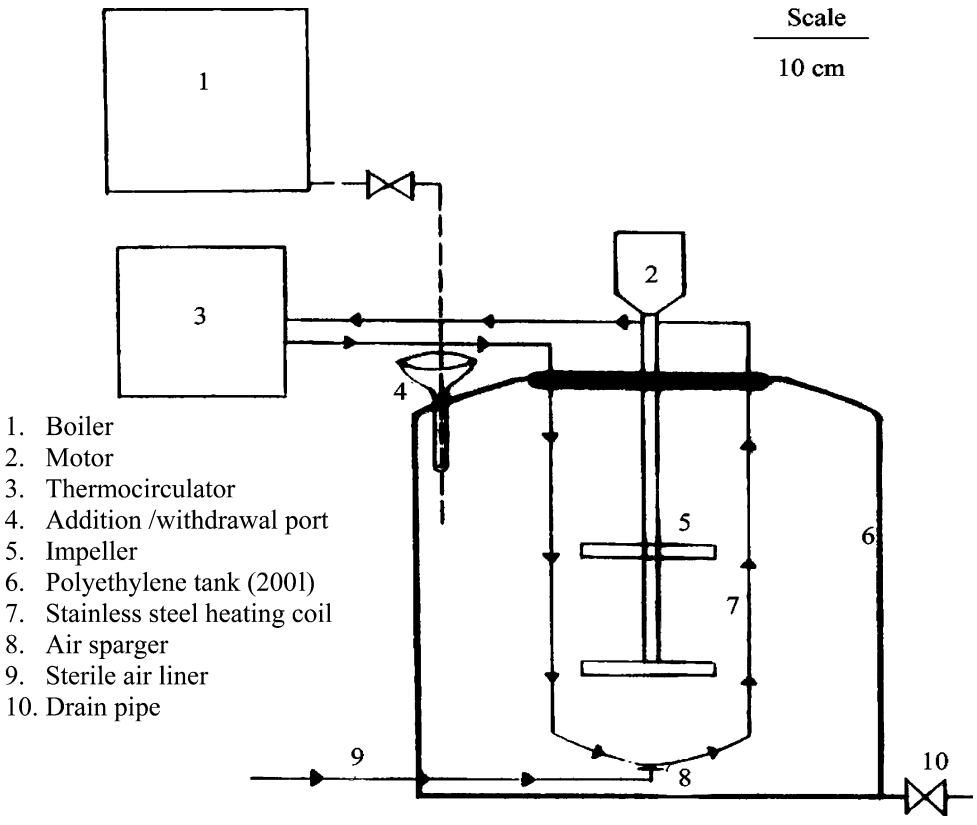

Fig. 1 Nitrifying Bacterial consortia Production Unit (NBCPU)

got consumed per milligram NH_4^+–N oxidized to NO_2^-–N (EPA 1975).

Yield coefficient/cell yield 'Y' of the consortia

The yield coefficient, 'Y' was determined as the ratio of weight of cells, or biomass generated, to the weight of substrate oxidized, or the ratio of the quantity of product formed to the quantity of substrate utilized (Sharma and Ahlert 1977). Cell yield or biomass generated was determined gravimetrically as described earlier.

Results

Characterization, activation and culturing of the consortia in 2 l fermentor

Transmission electron microscopy of the consortia as shown in Fig. 2 demonstrated characteristic cyst formation and cytomembranes resembling autotrophic nitrifies. Both the consortia were dominated by *Nitrosomonas*—like forms (0.55 × 0.43 µm), having intracytoplasmic membranes as flattened vesicles in the peripheral region of the cytoplasm besides cytoplasmic inclusion bodies resembling carboxysomes. This observation was supported by fluorescent in situ hybridization (FISH) employing fluorescent probes, specific for *Nitrosomonas* (unpublished). About 4–6 days were required for the cultures to become active after refrigeration for 5 years (Fig. 3). In both the consortia, the quantity of the product (NO_2^-–N and NO_3–N) formed was higher than that of the substrate (NH_4^+–N and NO_2^-–N) consumed. On amplification in a 2 l fermentor the product formation was still higher in magnitude than the substrate consumption (Fig. 4a, b). After 20–25 days culture, the consortia began to enter stationary phase. The net biomass yield, maximum specific growth rates and

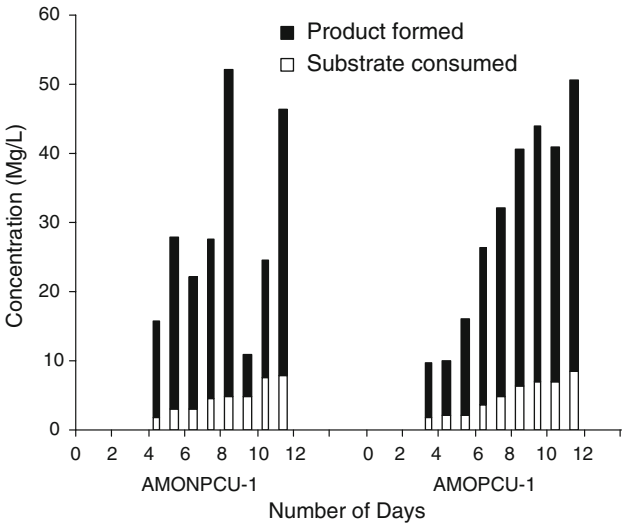

Fig. 3 The pattern of substrate consumption and product formation during activation of stored (4°C) nitrifying consortia at room temperature

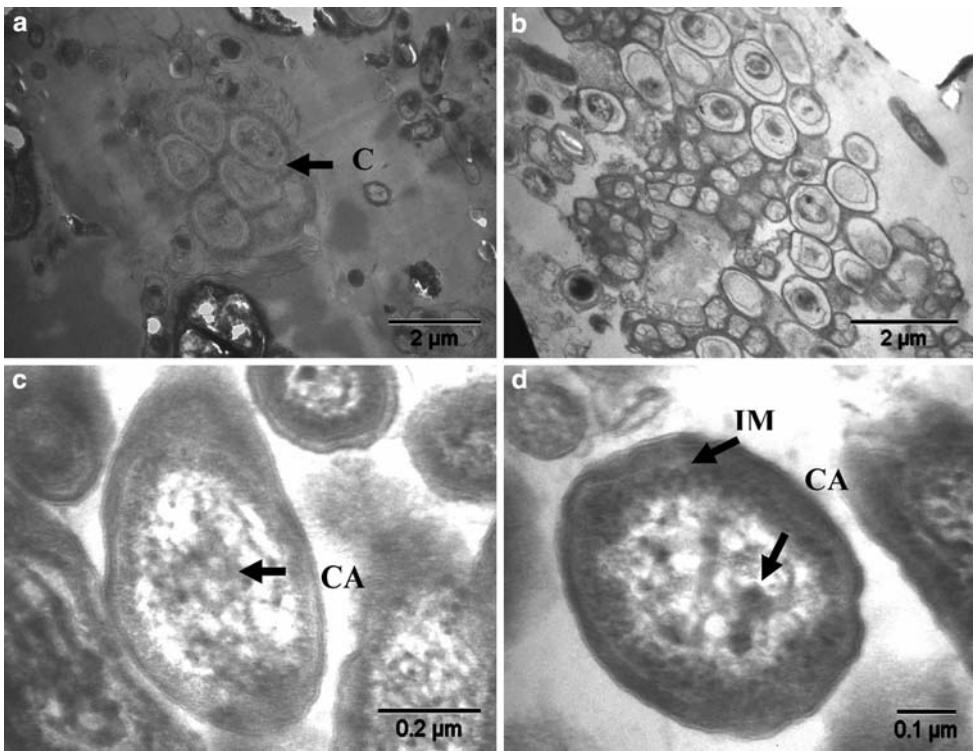

Fig. 2 Transmission electron micrographs of the nitrifying bacterial consortia. **a** and **b** Cysts in AMOPCU-1 and AMONPCU-1, **c** and **d** *Nitrosomonas*-like forms in AMOPCU-1 and AMONPCU-1). *G* Glycocalyx, *C* Cyst, *Ca* Carboxysomes, *IM* Intracytoplasmic membranes

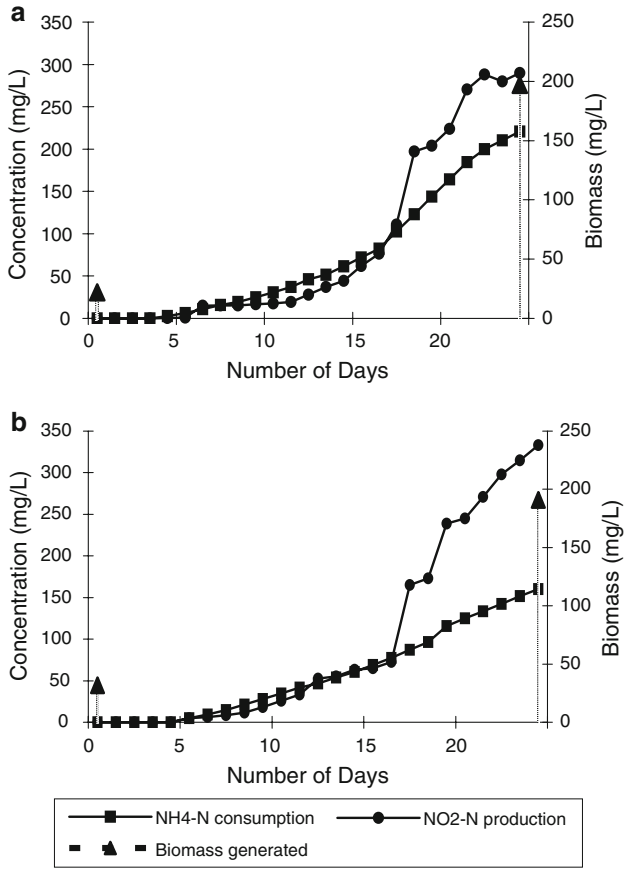

Fig. 4 **a–b** Growth curves of nitrifying consortia in 2 l fermentor

yield coefficients of the consortia during growth in 2 l fermentor are summarized in Table 1.

Mass production of the consortia in 200 l fermentor

On examining the total viable heterotrophic bacterial and fungal count prior to inoculation of the 200 l fermentor, around 15–20 CFU ml^{-1} of the former could alone be recovered. The substrate consumption and product formation during the growth of AMOPCU-1 and AMONPCU-1

Table 1 Growth and yield of ammonia oxidizing consortia, AMOPCU-1 and AMONPCU-1, during activation in 2 l fermentor and on mass production in 200 l fermentor

Nitrifying consortia	Biomass (g dry wt l^{-1}) at the day of harvest	μ_{max} (h^{-1})	Yield Coefficient (g product formed to g substrate utilized)
AMONPCU-1			
2 l	175.0 (25 d)	0.112	1.315
200 l	125.3 (160 d)		0.667
AMOPCU-1			
2 l	159.2 (25 d)	0.105	2.08
200 l	165.0 (260 d)		1.23

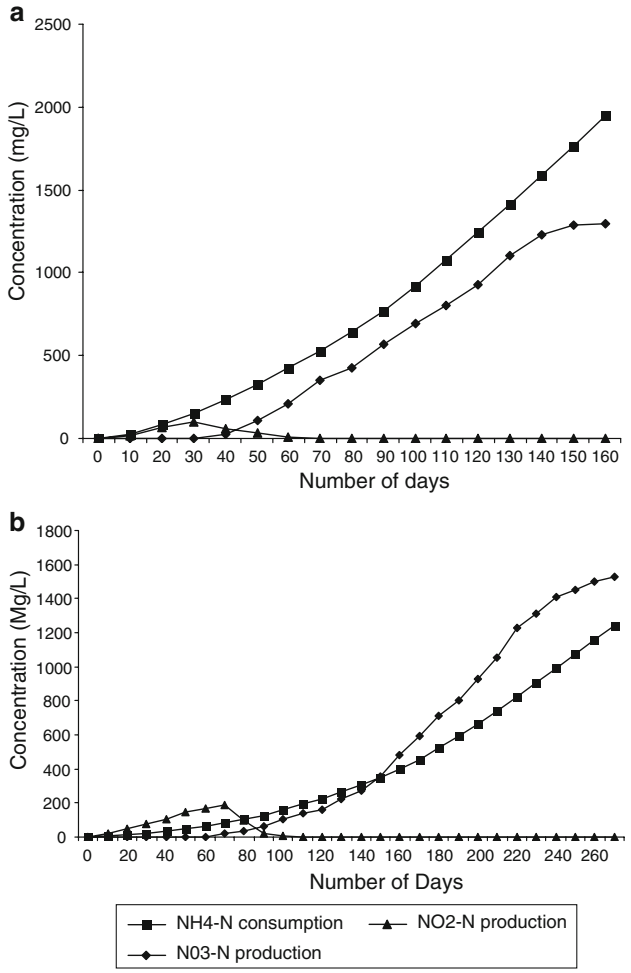

Fig. 5 **a–b**. Growth of nitrifying bacterial consortia in 200 l fermentor

in 200 l NBCPU are shown in Fig. 5a, b. The systems which started with 0.01 g/l residual NH_4^+–N consumed 1.24 and 1.95 g NH_4^+–N/l over a period of 270 and 160 days respectively, with a total corresponding output of 1.53 and 1.30 g NO_3–N/l. From the growth curve of AMOPCU-1 it could be inferred that until 70 days of incubation there was progressive build up of NO_2^-–N. Subsequently within another 20 days it rapidly declined and nitrate began to accumulate. After 90 days no residual nitrite could be detected and NH_4^+–N was found to be oxidized directly to NO_3–N. In a similar pattern, AMONPCU-1 exhibited a progressive build up of NO_2^-–N during the first 30 days, and subsequently within another 20 days it rapidly declined alongside the build up of NO_3^-–N, with no residual NO_2^-–N.

While the total NH_4^+–N removed by AMOPCU-1 and AMONPCU-1 was 1.24 and 1.95 g/l, respectively, for the culture period of 270 and 160 days, the total alkalinity reduction was by 7.45–9.19 and 11.69–14.42 g/l, respectively (Table 2). To compensate the alkalinity reduction,

Table 2 Alkalinity destroyed and carbonate used during mass production of ammonia oxidizing consortia AMOPCU-1 and AMONPCU-1

Consortia	Salinity (ppt)	pH	Total NH_4-N removed (g/l)	Total alkalinity destroyed (g)	Na_2CO_3 used (g)
AMPOCU-1 (Penaeid)	30	7.5	1.242	7.452–9.191	62.0
AMONPCU-1 (Non-penaeid)	15	8.5	1.948	11.688–14.415	78.0

the consortia were supplied with 62 and 78 g Na_2CO_3, respectively, during the period of culture.

Yield coefficient and biomass on mass production

Since the consortia attained equilibrium in the two step process of nitrification by converting NH_4^+–N directly to NO_3–N, without permitting residual NO_2^-–N to reach detectable levels, the yield coefficient was calculated from the quantity of NO_3–N produced. On attaining stationary phase, the yield coefficient of AMOPCU-1 was found to be 1.23 and that of AMONPCU-1 0.67. The net biomass yield of AMONPCU-1 at the end 160 days culture in 200 l fermentor was 125.3 g/l, while that of AMOPCU-1 at the end of 270 days was 165 g/l.

Discussion

Mass production of nitrifying bacterial cultures has always been a challenge due to their slow growth (Nejidat and Abeliovich 1994; Voytek and Ward 1995), and the difficulty in turbidometric growth determinations. The ultrastructure and FISH analysis of the consortia revealed the dominance of *Nitrosomonas* - like organisms, an important ammonia oxidizing bacteria (AOB) commonly found in biological wastewater treatment plants (Wagner et al. 1996). Although there are a few reports on the cultivation of pure cultures of nitrifiers (Tappe et al. 1996; Zart and Bock 1998; Chapman et al. 2006), attempts have never been made to scale up the process to large volumes. An important consideration for the design of mass production systems has been cost-effectiveness and accordingly, the medium optimized in this report was in seawater with addition of substrates, and carbonate to maintain optimum pH. Additionally, while fabricating the 200 l fermentor, locally available materials were used to reduce costs. As a specific requirement the fermentor vessel was kept opaque and well protected from sunlight, as visible and UV light have been reported to be lethal to some nitrifying organisms (Johnstone and Jones 1988; Diab and Shilo 1988). The overall heat treatment given to the medium could bring down the heterotrophic bacteria substantially facilitating cultivation of the nitrifying bacterial consortia.

While 4 to 6 days were required for activation of the consortia, culturing in a 2 l fermentor required 20–25 days. In both of these processes the quantity of the product formed was considerably higher than the substrate consumed as observed in our previous studies (Ramachandran 1998). The maximum specific growth rate of the ammonia oxidizers at a salinity of 30 and 15 ppt were 0.112 and 0.105/h, higher than the reported values of 0.014/h (Lawrence and McCarty 1970) and 0.092/h (Sharma and Ahlert 1977) for *Nitrosomonas.* In contrast, 0.032/h was considered the typical maximum specific growth rate at 20°C (Rittmann and Snoeyink 1984). Temperature has a strong effect on growth of nitrifiers, with the maximum growth rate at 30°C, three times higher than that at 20°C (USEPA 1993). In our studies the consortia were cultured at 28°C, and the possible reason for their higher specific growth rate values compared to the reported ones might be the comparatively higher temperature of incubation. Nevertheless, the values reported in the literature for ammonia oxidizers at higher temperatures had always been lower (0.043/ h) even at 30°C (Vadivelu et al. 2006), and (0.042/h) at 35°C (Van Hulle et al. 2004). The large variations in between the reported values and those of the present study might be due to the differences in the cultures used and the growth conditions provided as pointed out by Vadivelu et al. (2006) while investigating stoichiometric and kinetic characterization of *Nitrosomonas* sp. in mixed cultures.

The nitrifying bacterial consortia production unit (NBCPU) was successfully used for mass production of ammonia oxidizing consortia at salinities of 30 and 15 ppt where the two-step nitrification process could be established. It is an accepted principle that when populations of nitrifying bacteria are stabilized under constant conditions, residual nitrites will be at undetectable levels, with progressively increasing nitrate concentrations (Gray 1990). Similar observations were also made by Achuthan et al. (2006) during enrichment of the consortia from shrimp ponds. It has also been established that oxidation of nitrite to nitrate is more rapid than the preceding step (Stensel and Barnard 1992). This was especially true when the consortia AMOPCU-1 and AMONPCU-1 were grown in NBCPU, as no residual nitrite could be detected after 90 days of culturing, establishing the existence of population of nitritifiers and nitratifiers under equilibrium. This is in agreement with Bovendeur (1989) who demonstrated that nitratification (nitrite to nitrate) is slightly delayed and is subsequent to the development of nitritification (ammonia to nitrite) capability. The successful mass production of

AMOPCU-1 or AMONPCU-1 having nitritifiers and nitratifiers in equilibrium is advantageous in the practical sense that activation of the nitrifying bioreactors/biofilteres and establishment of rapid nitrification in RAS can be achieved with a single consortium.

During this production process the cultures AMOPCU-1 and AMONPCU-1 consumed comparatively less Na_2CO_3 for compensating the alkalinity reduction, adding little to the cost of production. There are reports that carbonate-based pH control yielded less cell density of *Nitrosomonas europea* (Chapman et al. 2006) as compared to bicarbonate-fed batch cultures. However, in the present study the addition of carbonate was sufficient to yield high biomass. Yield coefficients of AMOPCU-1 (1.23) and AMONPCU-1 (0.67) on mass production were comparatively high when compared to the estimated value (0.2) based on the thermodynamics of growth of ammonia oxidizers. In the practical sense there also have been reports of very low yield coefficient for ammonia (0.03–0.13) and nitrite (0.02–0.08) oxidizers (Sharma and Ahlert 1977). This is indicative of improved energy conversion efficiency and higher carbon dioxide fixation potentials of AMOPCU-1 and AMONPCU-1, primarily as the characteristics of tropical organisms, and secondarily may be due to their mixed culture configuration. While experimenting with nitrifying consortia developed from sewage in India, Ramachandran (1998) experienced a similar comparatively higher yield coefficient (0.1864–0.1939) for ammonia oxidizers and (0.1745–0.1986) nitrite oxidizers.

In the cultivation of *Nitrosomonas europea* pure cultures, batch-fed culturing with bicarbonate buffering yielded a culture density of 30 mg dry wt/l, whereas continuous culture under energy-limited growth conditions in a bench-scale bioreactor using a microfiltration membrane for high cell recycling produced final culture densities greater than 350 mg dry wt/l (Chapman et al. 2006). Conversely AMONPCU-1 yielded 125.3 g/l with in a period of 160 days and AMOPCU-1 yielded 165 g/l during 270 days of incubation. The characterization of the consortia showed dominance of autotrophic ntirifiers, while the higher yield coefficients suggested better energy conversion and higher CO_2 fixation potential of the consortia. The mass production process accomplished in these studies yielded sufficiently larger active biomass for immobilization in the bioreactors SBSBR and PBBR, with possible applications in marine and brackish water aquaculture systems, to establish instantaneous nitrification.

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