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# Anaerobic ammonia removal in presence of organic matter: A novel route

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**Abstract**

This study describes the feasibility of anaerobic ammonia removal process in presence of organic matter. Different sources of biomass collected from diverse eco-systems containing ammonia and organic matter (OM) were screened for potential anaerobic ammonia removal. Sequential batch studies confirmed the possibility of anaerobic ammonia removal in presence of OM, but ammonia was oxidized anoxically to nitrate (at oxidation reduction potential; ORP = $-248 \pm 25$ mV) by an unknown mechanism unlike in the reported anammox process. The oxygen required for oxidation of ammonia might have been generated through catalase enzymatic activity of facultative anaerobes in mixed culture. The oxygen generation possibility by catalase enzyme route was demonstrated. Among the inorganic electron acceptors ($NO_2^-$, $NO_3^-$ and $SO_4^{2-}$) studied, $NO_2^-$ was found to be most effective in total nitrogen removal. Denitrification by the developed culture was much effective and faster compared to ammonia oxidation. The results of this study show that anaerobic ammonia removal is feasible in presence of OM. The novel nitrogen removal route is hypothesized as enzymatic anoxic oxidation of $NH_4^+$ to $NO_3^-$, followed by denitrification via autotrophic and/or heterotrophic routes. The results of batch study were confirmed in continuous reactor operation.


*Keywords:* Anaerobic; Ammonia; Denitrification; Catalase; Mixed culture; Wastewater

## 1. Introduction

Nitrogen compounds ($NH_4^+$-N, organic bound N and $NO_3^-$) are major pollutants, which enter water bodies through aqueous wastes from several key industries (e.g. fertilizer, fish canning, refinery, tannery), agricultural run-off and domestic wastes [1]. As nitrogen pollution has become a cause for concern, many countries have enforced stringent nitrogen discharge standards in recent years. As a result, development of economical and sustainable techniques for reducing the nitrogen content from wastewaters has attracted a great deal of attention [2,3]. Processes such as single reactor high activity ammonia removal over nitrite (SHARON), anaerobic ammonia oxidation (ANAMMOX), completely autotrophic nitrogen removal over nitrite (CANON), de-ammonification and the nitrification–denitrification by methanotrophs, have emerged as promising technologies. A comprehensive review and descriptions of above new nitrogen removal processes are available in literature [4–7].

So far, only uses of inorganic electron acceptors like $NO_3^-$, $NO_2^-$ and $SO_4^{2-}$ [2,8,9] have been reported for anaerobic ammonia oxidation (anammox) and it was considered as an autotrophic process by a group of planctomycete bacteria [10]. $NO_2^-$ was found to be preferable and optimal electron acceptor compared to nitrate in anammox [11]. However, recently, oxidation of organic (acetic and propionic) acids by bonafide anammox bacteria was reported [12,13] and thereby exhibiting versatility of anammox bacteria. It has also been reported that presence of organic matter (OM, expressed as COD) adversely affects anammox [14] and co-existence of anammox culture and denitrifiers during start-up could slow down anaerobic ammonia removal [7]. More recently, metabolic diversity of *Nitrosomanas* strains for anaerobic ammonia removal was reported, but the source of $O_2$ for the oxidation of ammonia under anoxic conditions remained unknown [15]. The superoxide radical and the hydrogen peroxide (reactive oxygen species) are inevitable reactive byproducts of biological metabolism in oxidative stress conditions [16]. The presence of enzymes (superoxidase, peroxidase and catalase) defending the cells of anaerobic bacterium against reactive oxygen species (ROS) has been reported in the recent past [17,20]. Of these enzymes, catalase enzyme can reduce hydrogen peroxide to water and oxygen. As early as

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Table 1
The start-up conditions of batch study for screening of biomass

| Batch tag | Type of sludge | Volatile fraction of sludge used (%) | $NH_4$-N (mg/L)[a] | $NO_2^-$ (mg/L) | $NO_3^-$ (mg/L) | $SO_4^{2-}$ (mg/L) | COD (mg/L) | Volatile suspended solids (g) |
|---|---|---|---|---|---|---|---|---|
| AR1 | Anaerobically digested cow dung | 70.4 | 135 ± 0.4 | 0 | 0.68 ± 0.004 | 89 ± 0.2 | 640 ± 18 | 2.9 ± 0.1 |
| AR2 | Flocculent sludge from activated sludge (extended aeration) process treating tannery effluent. | 48.3 | 181 ± 0.5 | 130 ± 2.2 | 1 ± 0.005 | 164 ± 0.3 | 640 ± 18 | 0.45 ± 0.02 |
| AR3 | Anaerobically digested cow dung | 70.4 | 235 ± 0.7 | 130 ± 2.2 | 0.73 ± 0.004 | 89 ± 0.2 | 704 ± 22 | 2.9 ± 0.1 |
| AR4 | Anaerobic sludge from UASB reactor treating sewage | 46.31 | 111 ± 0.3 | 130 ± 2.2 | 0.31 ± 0.002 | 89 ± 0.2 | 320 ± 12 | 2.8 ± 0.1 |
| AR5 | Sludge collected from a clogged Sewer pipe | 42.1 | 108 ± 0.3 | 130 ± 2.2 | 0.23 ± 0.002 | 89 ± 0.2 | 640 ± 18 | 1.6 ± 0.1 |

[a] Initial ammonia variation is due to prior presence of different concentrations of ammonia presented in sludge.

in 1932, it was reported that nitrogen gas was generated via an unknown mechanism during fermentation in the sediments of lake Mendota, USA [21]. Very recently similar observations were found for the direct formation of nitrogen gas from ammonium (in the absence of oxidized nitrogen compounds) in fresh water sediments [22]. Many of the ammonia containing wastewaters are not free from OM and purely autotrophic anammox is not suitable in such cases [14]. However, not much work has been carried out to explore the possibility of anaerobic ammonia removal in presence of OM.

The objective of present work was to screen and develop mixed anoxic cultures (collected from diverse ecosystems containing OM), capable of carrying out anaerobic ammonia removal and denitrification simultaneously in presence of OM and/or inorganic electron acceptors. Such anoxic mixed culture can be applied for simultaneous removal of carbon and nitrogen compounds from wastewaters. An attempt was also made to understand the underlying mechanism of anaerobic ammonia removal in such mixed habitat in presence of OM.

## 2. Materials and methods

### 2.1. Mineral media for anaerobic ammonia removal

The mineral medium composition used (unless specified) throughout this study was (in g): $NaHCO_3$, 0.7372; $K_2HPO_4$, 0.1717; $CaCl_2{\cdot}2H_2O$, 0.3; $MgSO_4{\cdot}7H_2O$, 0.2; $FeCl_2$, 0.00464; EDTA, 0.00625; dissolved in 1 L of distilled water. One milliliter per liter of trace element solution was added to the above medium. The composition of trace element solution was (in g): $H_3BO_4$, 0.5; $ZnCl_2$, 0.5; $(NH_4)_6MO_7O_{24}{\cdot}4H_2O$, 0.5; $NiCl_2{\cdot}6H_2O$, 0.5; $AlCl_3{\cdot}6H_2O$, 0.5; $MnCl_2{\cdot}4H_2O$, 0.5; $CoCl_2{\cdot}4H_2O$, 0.5; $NaSeO{\cdot}3.5H_2O$, 1.0; $CuSO_4{\cdot}5H_2O$, 0.5; dissolved in 1 L of distilled water. Predetermined amount of ammonia (using $NH_4Cl$) and selected electron acceptor (either $NO_2^-$, $NO_3^-$ or $SO_4^{2-}$) were added as per requirement of each experiment. All chemicals were analytical reagent (AR) grade supplied by 'Qualigens' (India). Clean 'Borosil' (India) make glassware was used for reagents preparation and volume measurements.

### 2.2. Seed biomass for screening

Four sludges were collected from diverse ecological background for screening study as per the start-up conditions given in Table 1. All biomass were collected in clean plastic vessels to their full capacity without having any head space for air, sealed; thereafter immediately transported to the laboratory and preserved in refrigerator at 4 ± 0.1 °C untill further use.

### 2.3. Batch studies

#### 2.3.1. Screening of biomass for anaerobic ammonia removal process

This work was carried out as an initial step towards development of sulphidogenesis cum anaerobic ammonia removal process for the biological treatment of effluents containing carbon, sulphur and nitrogen compounds. Accordingly, while developing anaerobic ammonia removal process, any process, which supports simultaneous removal of carbon and nitrogen from effluents, was encouraged, rather than developing only a pure reported autotrophic anammox process.

Five, 500 mL glass bottles (DURAN, Germany), closed tightly with rubber septum; with gas release arrangement through a water seal, were used for initial set of batch studies (AR1 to AR5 in Table 1). Seventy-five milliliters of each biomass (known volatile suspended solids) was added to 425 mL mineral media in each bottle. The pH was then adjusted to 7.5 ± 0.1 and purged with pure $N_2$ for 3 min. After addition of ammonia and nitrite (except in AR1), samples were drawn for analysis at start-up conditions as given in Table 1. Then purging was again carried out for 1 min and the bottles were sealed with rubber septum to maintain anoxic condition. Batch experiments were carried out for 3 weeks and the final analyses of $NH_4^+$, $NO_2^-$ and COD were carried out at the end of experiment. Experiments were conducted at ambient liquid temperature of 30–32 °C. Mixing

was carried out by means of magnetic stirrer (Remi Equipments Ltd., India).

### 2.3.2. Confirmation of anaerobic ammonia removal process by enriched biomass

The settled biomasses from promising batch reactors (AR1, AR2 and AR3) were selected for further enrichment using sequential batch operation. Two cycles of operation (first cycle for 1 week and second cycle for 3 days) were carried out by feeding 100 mg/L $NH_4^+$ and 130 mg/L $NO_2^-$ in mineral media to AR2 and AR3, respectively. Neither $NH_4^+$ nor $NO_2^-$ was added to AR1, as it contained residual ammonia and only first cycle was operated for complete $NH_4^+$ removal. The other experimental conditions were same as in the screening tests. Further, batches AR2 and AR3 were selected and maintained in non-mechanical mixing conditions to ensure that aerobic nitrification did not take place. These two batch reactors were operated for another 40 days, with initial concentrations of 140 mg/L $NH_4^+$, 100 mg/L $NO_2^-$, 100 mg/L $NO_3^-$, respectively, and resazurin indicator added to each reactor. Anoxic condition was crosschecked by measuring oxidation-reduction potential (ORP) using a calibrated Cyber Scan pH (1100) meter in mV mode. Samples were withdrawn periodically from these reactors and analyzed for residual $NH_4^+$, $NO_2^-$ and $NO_3^-$.

### 2.3.3. Effectiveness of inorganic electron acceptors in anaerobic ammonia removal process

The adapted biomasses of cow dung and tannery sludge were subjected to three cycles of anaerobic ammonia removal process in sequential batch mode in anoxic conditions. Experiments were conducted in 250 mL capacity Erlenmeyer conical flasks with 100 mL mineral medium. Before the start of experiment, adapted biomass was added to 100 mL mineral medium, with selected electron acceptor and known concentrations of ammonia (electron donor). Weekly analyses for electron donor ($NH_4^+$) and electron acceptors ($NO_2^-$, $NO_3^-$, $SO_4^{2-}$) were carried out.

*2.3.3.1. First cycle.* The start-up conditions of first cycle are shown in Table 2 (section A). In this set of experiments, CD-Blank and TR-Blank were chosen to study anaerobic ammonia removal process in presence of OM (available through endogenous respiration) without any external supply of electron acceptors. Batch experiments were conducted for 2 weeks in an orbital shaker incubator (Remi Equipments Ltd.) at 120 rpm and at 30 °C. Representative samples of adapted cow dung and tannery sludge used in first cycle were dried at 103 °C, powdered and preserved in a desiccator for elemental carbon analysis using CHNS/O analyzer (Perkin-Elmer, USA), along with dried biomass of third cycle.

*2.3.3.2. Second and third cycles.* All the experimental conditions were the same as in the first cycle (Table 2, section A), except MLSS concentration. Centrifuged biomass from the corresponding previous cycle batch reactors were added to mineral media. The start-up MLSS concentrations in second cycle were 1250, 1100, 700, 850, 1350, 1500, 1500 and

Table 2
Start-up conditions of batch studies

| First cycle of experiments for effect of various electron acceptors (section A) | | | | | | | To identify autotrophic and heterotrophic growth in mixed culture (section B) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Batch tag | pH | $NH_4^+$ | $NO_2^-$ | $NO_3^-$ | Total nitrogen | $SO_4^{2-}$ | MLSS | Batch tag | pH | $NH_4^+$ | $NO_2^-$ | $NO_3^-$ | Total nitrogen | $SO_4^{2-}$ | MLSS | COD |
| CD-Blank | 8 ± 0.05 | 100 ± 0.3 | 0 | 0 | 77.78 ± 0.2 | 87 ± 0.2 | 1485 ± 20 | Blank (abiotic) | 7.5 ± 0.05 | 150 ± 0.43 | 0 | 0 | 116.7 ± 0.3 | 90 ± 0.2 | 0 | 0 |
| CD-$SO_4$ | 8 ± 0.05 | 100 ± 0.3 | 0 | 0 | 77.78 ± 0.2 | 380 ± 1 | 1485 ± 20 | CD-Blank | 7.5 ± 0.05 | 150 ± 0.43 | 0 | 0 | 116.7 ± 0.3 | 90 ± 0.2 | 30.3 ± 0.1 | 0 |
| CD-$NO_3$ | 8 ± 0.05 | 100 ± 0.3 | 0 | 133 ± 0.7 | 107.8 ± 0.4 | 87 ± 0.2 | 1485 ± 20 | CD-B-C | 7.5 ± 0.05 | 150 ± 0.43 | 0 | 0 | 116.7 ± 0.3 | 90 ± 0.2 | 30.3 ± 0.1 | 564 ± 26 |
| CD-$NO_2$ | 8 ± 0.05 | 100 ± 0.3 | 133 ± 2.2 | 0 | 118.3 ± 0.9 | 87 ± 0.2 | 1485 ± 20 | CD-$SO_4$ | 7.5 ± 0.05 | 150 ± 0.43 | 0 | 0 | 116.7 ± 0.3 | 423 ± 0.9 | 30.1 ± 0.1 | 0 |
| TR-Blank | 8 ± 0.05 | 100 ± 0.3 | 0 | 0 | 77.78 ± 0.2 | 87 ± 0.2 | 1507 ± 20 | CD-$NO_3$ | 7.5 ± 0.05 | 150 ± 0.43 | 0 | 315 ± 1.50 | 187.8 ± 0.6 | 90 ± 0.2 | 30 ± 0.07 | 0 |
| TR-$SO_4$ | 8 ± 0.05 | 100 ± 0.3 | 0 | 0 | 77.78 ± 0.2 | 380 ± 1 | 1507 ± 20 | CD-$NO_2$ | 7.5 ± 0.05 | 150 ± 0.43 | 525 ± 8.7 | 0 | 235.3 ± 2.3 | 90 ± 0.2 | 30.4 ± 0.1 | 0 |
| TR-$NO_3$ | 8 ± 0.05 | 100 ± 0.3 | 0 | 133 ± 0.7 | 107.8 ± 0.4 | 87 ± 0.2 | 1507 ± 20 | TR-Blank | 7.5 ± 0.05 | 150 ± 0.43 | 0 | 0 | 116.7 ± 0.3 | 90 ± 0.2 | 37.7 ± 0.2 | 0 |
| TR-$NO_2$ | 8 ± 0.05 | 100 ± 0.3 | 133 ± 2.2 | 0 | 118.3 ± 0.9 | 87 ± 0.2 | 1507 ± 20 | TR-B-C | 7.5 ± 0.05 | 150 ± 0.43 | 0 | 0 | 116.7 ± 0.3 | 90 ± 0.2 | 37.7 ± 0.2 | 564 ± 26 |
| Blank (abiotic) | 8 ± 0.05 | 100 ± 0.3 | 0 | 0 | 77.78 ± 0.2 | 87 ± 0.2 | 60 ± 20 | TR-$SO_4$ | 7.5 ± 0.05 | 150 ± 0.43 | 0 | 0 | 116.7 ± 0.3 | 430 ± 1.0 | 37.1 ± 0.2 | 0 |
| | | | | | | | | TR-$NO_3$ | 7.5 ± 0.05 | 150 ± 0.43 | 0 | 315 ± 1.50 | 187.8 ± 0.6 | 90 ± 0.2 | 37.1 ± 0.2 | 0 |
| | | | | | | | | TR-$NO_2$ | 7.5 ± 0.05 | 150 ± 0.43 | 525 ± 8.7 | 0 | 235.3 ± 2.3 | 90 ± 0.2 | 38 ± 0.2 | 0 |
| | | | | | | | | CD-B-HDNR | 7.5 ± 0.05 | 150 ± 0.43 | 0 | 782 ± 3.6 | 293.3 ± 1.1 | 90 ± 0.2 | 37.7 ± 0.2 | 564 ± 26 |
| | | | | | | | | CD-B-TDNR[a] | 8 ± 0.05 | NA | 0 | 1100 ± 5 | <248.4 | 270 ± 0.7 | 37.7 ± 0.2 | 0 |
| | | | | | | | | TR-B-HDNR | 7.5 ± 0.05 | 150 ± 0.43 | 0 | 782 ± 3.6 | 293.3 ± 1.1 | 90 ± 0.2 | 37.7 ± 0.2 | 564 ± 26 |
| | | | | | | | | TR-B-TDNR[a] | 8 ± 0.05 | NA | 0 | 1100 ± 5 | <248.4 | 270 ± 0.7 | 37.7 ± 0.3 | 0 |

All units in mg/L except pH. CD, Adapted cow dung based sludge; TR, adapted tannery based sludge; MLSS, mixed liquid suspended solids with volatile fraction = 65–75%; NA, not analysed; Blank (abiotic), Blank with Resazurin indicator; CD-B, CD-Blank; HDNR, heterotrophic denitrification; TDNR, *Thiobacillus* denitrification.

[a] Batch operated on *Thiobacillus denitrificans* media.

1500 mg/L in CD-Blank, CD-$SO_4$, CD-$NO_3$, CD-$NO_2$, TR-Blank, TR-$SO_4$, TR-$NO_3$, TR-$NO_2$ reactors, respectively. The start-up MLSS concentrations in third cycle were 950, 700, 750, 800, 850, 1350, 1400 and 1450 mg/L in CD-Blank, CD-$SO_4$, CD-$NO_3$, CD-$NO_2$, TR-Blank, TR-$SO_4$, TR-$NO_3$, TR-$NO_2$ reactors, respectively. Experiments were conducted for 1 week and 2 weeks in the case of second and third cycles, respectively. In the third cycle, biomass from CD-Blank was divided into equal parts and used in two different batch reactors, namely, CD-Blank and CD + Sucrose. Sucrose (COD = 632 mg/L) was added to the CD + Sucrose reactor to study the effect of externally supplied OM in total nitrogen removal. Soluble COD was also monitored along with other electron acceptors during initial and final phases of third cycle experiment. At the end of the experiment, 10 mL representative samples were drawn from all batch reactors (except CD + Sucrose); centrifuged, dried at 103 °C and percentage of carbon in biomass were determined.

#### 2.3.4. Evidence for autotrophic and heterotrophic nitrogen removal

Two milliliters supernatant was taken from each reactor of third cycle and added to respective 100 mL capacity serum bottle maintained in anoxic conditions, each containing 100 mL of mineral medium, with different electron acceptors. The inorganic electron acceptors were added as per stoichiometric requirements for anammox process [2,8,9]. Reactors were monitored for biomass growth, $NH_4^+$, $NO_2^-$, $NO_3^-$, $SO_4^{2-}$ and COD. Batch experiments were also conducted to obtain the evidence for autotrophic (in *Thiobacillus* denitrifying (TDNR) medium) and heterotrophic denitrification in the mixed culture. This study was conducted by using supernatant (2 mL) drawn from CD-Blank and TR-Blank reactors. The chemical composition of TDNR medium was (in g): $Na_2S_2O_3{\cdot}5H_2O$, 5; $K_2HPO_4$, 2; $KNO_3$, 2; $NaHCO_3$, 1; $MgSO_4{\cdot}7H_2O$, 0.6; $NH_4Cl$, 0.5; $FeSO_4{\cdot}7H_2O$, 0.01, dissolved in 1 L of distilled water. The base mineral medium composition used for heterotrophic denitrification was same as that used for anaerobic ammonia oxidation. However, sucrose (COD = 564 mg/L), $NO_3^-$ (782 mg/L), $NH_4^+$ (150 mg/L) and $K_2HPO_4$ (80 mg/L) were supplemented to the medium to promote heterotrophic denitrification. The start-up conditions of the experiments for evidence of autotrophic and heterotrophic nitrogen removal is are given in Table 2 (section B). Experiments were conducted for duration of 1 month in ambient conditions for ammonia removal and 2 weeks for denitrification with occasional mixing. Analyses of samples were performed once in a week.

#### 2.3.5. Evidence of oxygen generation by catalase enzyme activity

Serum bottles containing ammonia and nitrite in mineral medium and adapted cow dung and tannery sludge, respectively, were prepared in anoxic condition with resazurin indicator. The bottles were sealed with butyl rubber septum with aluminum cover and put for incubation at ambient temperature without any mechanical mixing. A blank serum bottle containing ammonia and nitrite in mineral medium with resazurin indicator, but without sludge was kept along with other two serum bottles. The oxygen generation possibility by catalase enzyme route was qualitatively verified by observing the appearance and disappearance of pinkish color of redox indicator in sludge containing bottles. Catalase enzyme activity of adapted biomass was also quantified as per the procedure given in Section 2.5.1.

### 2.4. Evidence of anaerobic ammonia removal with digested cow dung sludge in continuous reactor

In order to verify the anaerobic ammonia removal process in presence of organic matter observed in batch studies, a continuous reactor of 1.2 L liquid capacity was fabricated using acrylic cylinder. The reactor was having internal diameter of 50 mm and height of 700 mm, with plastic rings (bulk density = 150 kg/$m^3$; porosity = 90%, total area available for microbial attachment = 2975 $cm^2$; calculated by multiplying surface area of one ring by number of rings) filled in 1.0 L volume. The schematic of experimental set up is shown in Fig. 1. The reactor was initially seeded with 500 mL of anaerobically digested cow dung with 100 mL of adapted cow dung biomass for anaerobic ammonia removal with total initial VSS of 17.6 g. The reactor was operated in recycle mode through feed vessel for development of anaerobic ammonia removal culture at hydraulic retention time (HRT) of 1 h. Feeding of $NH_4^+$ in mineral medium

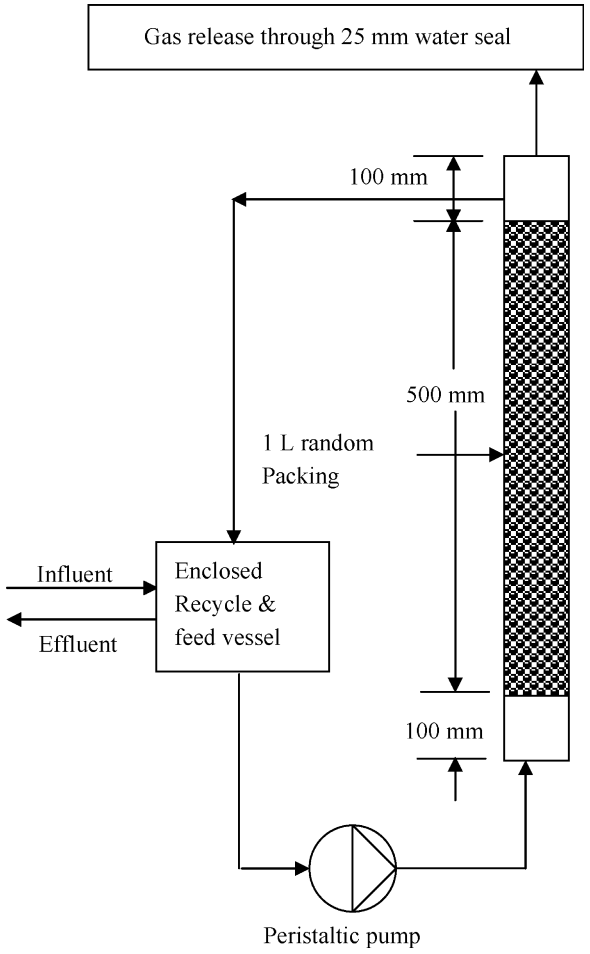


Fig. 1. Schematic of start-up phase of anaerobic ammonia removal reactor.

(without any external addition of electron acceptor) was carried out to feed vessel at intervals whenever $NH_4^+$ removal was substantial. The oxidation reduction potential maintained inside the reactor was $-225 \pm 25$ mV. Initially, $NH_4^+$ concentration was 100 mg/L ($NH_4^+$loading rate = 0.15 kg $NH_4^+/m^3$/day) for 25 days. Then from 26 to 50 days $NH_4^+$concentration was increased to 150 mg/L ($NH_4^+$loading rate = 0.23 kg $NH_4^+/m^3$/day), followed by a shut down period from 51st to 64th day. Reactor was re-started on 65th day, with $NH_4^+$concentration of 225 mg/L ($NH_4^+$loading rate = 0.34 kg $NH_4^+/m^3$/day) and operated until 80 days.

### 2.5. Analytical techniques

All physical–chemical parameter analyses were conducted as per Standard Methods [23]. $NH_4^+$, $NO_2^-$, $NO_3^-$ and $SO_4^{2-}$ were analyzed by Ion chromatography (DIONEX, USA) with ED50 electrochemical detector and results are processed with integrated Chromeleon software. COD was measured by closed reflux method using HACH (Loveland, USA) COD digestor. Elemental carbon analysis of dried biomass samples was carried out using Perkin-Elmer 2400 series CHNS/O analyzer. Absorbance for biomass growth was measured by UV–vis spectrophotometer (8500 series, TECHCOMP Ltd., Hong Kong) and MLSS was determined from the calibration curve prepared with known MLSS and corresponding absorbance. ORP was measured using double junction platinum ORP electrode connected to a calibrated Cyber Scan pH (1100) meter in mV mode (EUTECH Instruments, Singapore). ORP electrode was calibrated using Quinhydrone 86. Triplicate samples were analyzed on specific experiments and those values are expressed as average values with standard deviations in tables. However, figures are plotted with average values.

#### 2.5.1. Enzymatic assay of catalase and peroxidase

Catalase activity of developed cultures was tested using the following procedure [24]. A colony of bacteria was picked up from culture and transferred to a clean microscope glass slide containing a drop of water. A few drops of 3% $H_2O_2$ were added to the culture and the bubble generation was observed for 20 s. If bubbles appear within 20 s, the organism shows positive catalase activity. A blank in similar conditions without bacterial cells was also tested. The quantification of catalase activity in the extracted enzyme from mixed culture was determined by continuous spectrophotometric rate determination [25,26]. In this procedure, presence of catalase shows decrease in rate of absorbance at 240 nm with respect to blank test. The enzyme extraction procedure followed was by transferring the biomass to 50 mM monobasic phosphate buffer at pH 7 and centrifuged at 4 °C for 10 min at 10,000 × *g*. Then centrifuged biomass was re-suspended in 50 mM monobasic phosphate buffer at pH 7 (1 g biomass in 6 mL buffer). The biomass was lysed by sonication (five cycles of 60 s on and 60 s off at 175 W) (Sonics vibra cell) in ice bath. Then the suspension was centrifuged again at 4 °C for 10 min at 12,000 × *g* to get clear suspension of protein for immediate catalase enzyme activity measurements. The extracted enzyme was always kept at 4 °C. Presence of peroxidase in the extracted enzyme was tested by continuous spectrophotometric rate determination [27]. In this procedure, presence of peroxidase shows increase in rate of absorbance at 420 nm with respect to blank test.

## 3. Results and discussion

### 3.1. Screening of biomass for anaerobic ammonia removal process

Sludge collected from four different environmental backgrounds was used for screening the biomass for anaerobic ammonia removal process. The results obtained from the screening study are presented in Fig. 2a and b. It can be seen from these figures that significant ammonia removal occurred in reactors AR1, AR2 and AR3. There was removal of ammonium in AR1, though no external electron acceptor was added to this reactor. Here it is to be noted that $NO_2^-$ was not present in cow dung sludge and initial $NO_3^-$ presented (0.68 mg/L) was associated with cow dung biomass. It shows that cow dung sludge might have contained nitrifying bacteria. This $NO_3^-$ was not sufficient as per reported anammox reaction [2] for nitrogen removal. The $SO_4^{2-}$ available from the mineral medium might not have directly involved as per reported anammox reaction [9] as this reaction is not thermodynamically competent compared with sulphate reduction in presence of organic matter. Similar observation of direct formation of nitrogen gas from ammonium (in the absence of oxidized nitrogen compounds) in fresh water sediments was reported earlier also [22]. There are contradicting reports regarding occurrence of anaerobic ammonia removal in presence of OM. While Jetten et al. [15] mentioned anaerobic ammonia removal and nitrogen gas formation in presence of OM, Schalk et al. [28] reported that autotrophic anaerobic ammonia removal process was inhibited in presence of OM. However, the present study showed that anaerobic ammonia

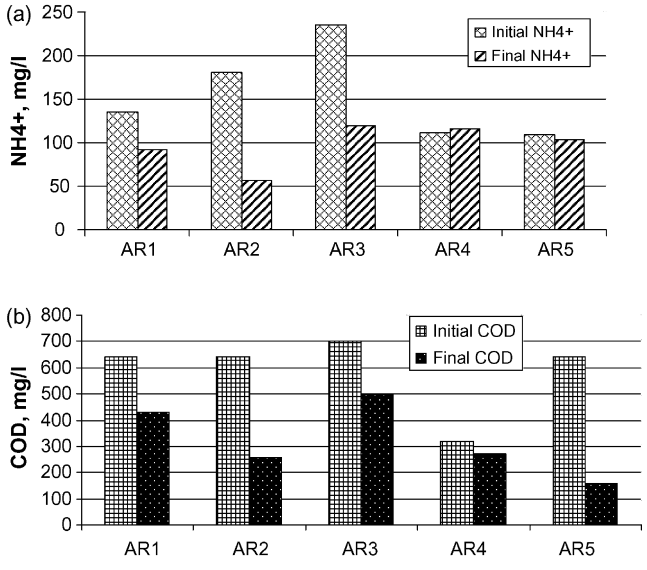


Fig. 2. Screening of biomass for anaerobic ammonia removal process. (a) Variation of ammonia concentration and (b) variation of COD.

removal culture could have co-habitation with heterotrophic culture. The possibility of such co-habitation is explained below.

The observation in this study is the anoxic oxidation of $NH_4^+$ into $NO_2^-/NO_3^-$. So cow dung sludge must have contained nitrifying bacteria as evident from the generation of nitrate in anoxic conditions through out the study. There are recent reports of such anoxic oxidation of $NH_4^+$ into $NO_3^-$ by oxides of Mn and Fe in anoxic sediments [29]. However, in the present study there was no source of oxides of Mn and Fe to supply oxygen for $NH_4^+$ oxidation. Hence, the present study hypothesizes enzymatic generation of oxygen when bacterial respiration takes place in an oxidative stress and/or in anoxic environment. From the nitrogen removal point of view, such anoxic oxidation of ammonia to nitrate and subsequent denitrification is thermodynamically feasible reactions in presence of OM along with anammox as evident from the following equations:

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+,$$
$$\Delta G^\circ = -349\,\text{kJ/M (nitrification)} \quad (1)$$

$$2NO_3^- + 1.25CH_3COOH \rightarrow 2.5CO_2 + N_2 + 1.5H_2O + OH^-,$$
$$\Delta G^o = -527.5\,\text{kJ/M (denitrification)} \quad (2)$$

$$5NH_4^+ + 3NO_3^- \rightarrow 4N_2 + 9H_2O + 2H^+,$$
$$\Delta G^o = -297\,\text{kJ/M (Anammox)} \quad (3)$$

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O,$$
$$\Delta G^o = -357\,\text{kJ/M (Anammox)} \quad (4)$$

So in anoxic ecosystems, when organic matter is available microbes may prefer reactions (1) and (2) and when organic carbon is limiting, reaction (3) or (4) may be preferable.

In all the batch reactors, $NO_2^-$ was removed completely, and there was appreciable removal of COD, which may be as a result of heterotrophic denitrification, anaerobic fermentation, methanogenesis and sulphate reduction. The stoichiometric ratios of $NO_2^-/NH_4^+$ of ammonia oxidation in AR2 and AR3 were 0.41 and 0.44, respectively. This is not matching with the stoichiometric ratio of autotrophic anammox (1.33) reported by earlier researchers [30,14]. Hence, this preliminary result shows that anaerobic ammonia removal in presence of organic matter in the system is not only by anammox (autotrophic) process but also by some other mechanisms of ammonia oxidation. AR2 and AR5 (Fig. 2b) were showing higher COD removal as the sludge sources were expected to have higher amount of undigested OM. It may be inferred from these preliminary results that anaerobic ammonia removal in presence of OM need not be fully autotrophic in nature and biomass for such anaerobic ammonia removal process can be developed from both aerobic and anaerobic sources of sludge. A possible reason for occurrence of anaerobic ammonia removal culture in flocculent aerobic or anaerobic sludge may be the long-term exposure of the mixed

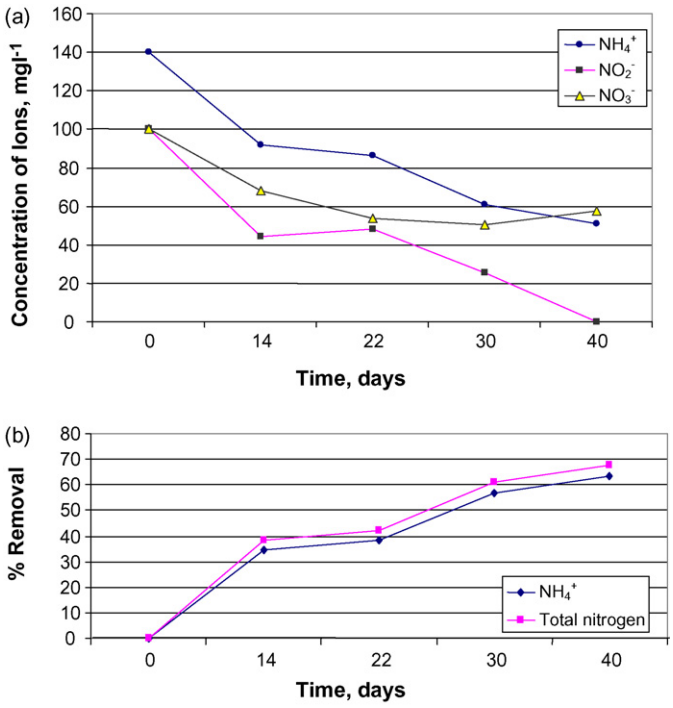


Fig. 3. Confirmation of anaerobic ammonia removal process in cow dung sludge {anoxic condition was verified by ORP ($-248 \pm 25$ mV) measurement and resazurin indicator; mass balance was closed for total nitrogen accounting in the system}. (a) Kinetics of ion concentration and (b) percentage removal of ammonium and total nitrogen.

culture to high concentrations of TKN/$NH_4$-N. Cow dung sludge slurry contains higher concentrations of TKN/$NH_4$-N. Flocs formation in aerobic sludge perhaps enhanced the chance of survival of anaerobic ammonia removal culture under oxygen stress conditions. The extended aeration process sludge (AR2) used was flocculent in nature and the sludge was exposed to TKN/$NH_4$-N presented in tannery effluent.

### 3.2. Confirmation of anaerobic ammonia removal by enriched biomass

Confirmation tests for anaerobic ammonia removal process by enriched biomass showed complete $NH_4^+$ removal in the first cycle within a week. However, $NH_4^+$ removal was $90.1 \pm 0.03$ and $78.9 \pm 0.07$% in AR2 and AR3, respectively, in the second cycle, in 3 days time. Such faster ammonia removal by pure reported anammox process is not expected, as the growth rate of reported anammox bacteria is very slow. In AR1, there was complete removal of $NH_4^+$ in absence of $NO_2^-$, which indicates a possibility of a different route for anaerobic ammonia removal in presence of OM as opposed to reported anammox process. Results of confirmation tests in AR3 (cow dung sludge in non-mechanical mixing conditions) are shown in Fig. 3a and b. In these tests, resazurin indicator confirmed anoxic condition at all times and ORP was $-248 \pm 25$ mV. In non-mixing conditions, $NH_4^+$ and $NO_2^-$ were consumed gradually and stoichiometric consumption ratio ($NO_2^-/NH_4^+$) was 0.44 at the end of the experiment. The percentage removal of total nitrogen (67.5%) was always slightly more than percentage

Table 3
Evidence of auto and heterotrophic growth of mixed anaerobic ammonia removing culture

| Batch tag | Batch description | Final MLSS (mg/L) | Percentage removal of $NH_4^+$ | Percentage removal of total nitrogen | Ratio of electron acceptor/$NH_4^+$ |
|---|---|---|---|---|---|
| Blank(A) | Abiotic blank | 23 ± 0.1 | 22.3 ± 0.1 | 22.3 ± 0.1 | 0 |
| CD-Blank | No external EA | 38.7 ± 0.2 | 30 ± 0.2 | 30 ± 0.2 | 0 |
| CD-B-C | Sucrose as EA | 203.4 ± 2.5 | 44.3 ± 0.3 | 44.3 ± 0.3 | 3.4 ± 0.15 |
| CD-$SO_4$ | $SO_4^{2-}$ as EA | 78.3 ± 0.4 | 37 ± 0.3 | 37 ± 0.3 | 0.44 ± 0.05 |
| CD-$NO_3$ | $NO_3^-$ as EA | 55 ± 0.3 | 32 ± 0.2 | 41 ± 0.3 | 0.7 ± 0.05 |
| CD-$NO_2$ | $NO_2^-$ as EA | 146 ± 0.8 | 40.2 ± 0.3 | 39.3 ± 0.3 | 1.35 ± 0.1 |
| TR-Blank | No external EA | 38.7 ± 0.2 | 30 ± 0.2 | 30 ± 0.2 | 0 |
| TR-B-C | Sucrose as EA | 211.5 ± 2.6 | 44.7 ± 0.3 | 44.7 ± 0.3 | 3.5 ± 0.15 |
| TR-$SO_4$ | $SO_4^{2-}$ as EA | 81.2 ± 0.4 | 38.2 ± 0.3 | 38.2 ± 0.3 | 0.5 ± 0.05 |
| TR-$NO_3$ | $NO_3^-$ as EA | 75 ± 0.4 | 34.1 ± 0.2 | 42.8 ± 0.3 | 0.7 ± 0.05 |
| TR-$NO_2$ | $NO_2^-$ as EA | 191.5 ± 1.0 | 43.5 ± 0.3 | 41.1 ± 0.3 | 1.28 ± 0.1 |

CD, Adapted biomass from cow dung based sludge; TR, adapted biomass from tannery sludge; EA, electron acceptor; range of initial MLSS = 30 ± 0.07 to 38 ± 0.2 mg/L.

removal of $NH_4^+$ (63.6%). However, ammonia and total nitrogen removal were occurring in anoxic conditions (Fig. 3b). Nitrite appears to be the preferred electron acceptor compared to nitrate (Fig. 3a). Nitrate was consumed initially, but later its rate of consumption reduced. The closed nitrogen (N) mass balance of data (Fig. 3) also showed that ammonia removal was not exclusively by anammox route (total N at start of batch = 81 mg, total $NO_2$-N consumed = 15.2 mg, total $NO_3$-N consumed = 4.8 mg, total $NH_4$-N consumed = 34.7 mg and total nitrogen at end of batch = 26.3 mg). Similar results were obtained when enriched biomass from tannery-based sludge (AR2) was used in non-mixing conditions. Stoichiometric consumption ratio ($NO_2^-/NH_4^+$) was 0.60 at the end of these experiments (results not shown). The results of above set of studies confirm the anaerobic ammonia removal process. It is also confirmed that, in presence of OM, anaerobic ammonia removal is not completely autotrophic in nature. Experiments were carried out to verify the possible presence of reported anammox bacteria along with bacteria responsible for anaerobic ammonia removal, as described in this study, in the enriched mixed culture. Results of this study are presented in Table 3 and discussed below.

Table 3 shows the evidence of autotrophic and heterotrophic growth of mixed anaerobic ammonia removing culture after 1 month of incubation at ambient temperature (33–35 °C). The MLSS values showed the evidence of growth and percentage of ammonia and total nitrogen removal was in correspondence with growth of cultures (there was corresponding increase of protein also). All electron acceptors (both inorganic and organic) were made use for growth and ammonia oxidation. It was observed that organic carbon and nitrite were more effective in anaerobic ammonia oxidation and in total nitrogen removal compared with other electron acceptors. The stoichiometric consumption of $NO_2^-$ and $SO_4^{2-}$ were found to be closely matching with autotrophic anammox as described by Strous et al. [8] and Polanco et al. [9]. Polanco et al. [9] reported possibility of anammox using $SO_4^{2-}$ in presence of OM, but there was no further report of anammox with $SO_4^{2-}$ as electron acceptor. The stoichiometric consumption of $NO_3^-$ (0.7) was more than that of autotrophic anammox (0.6). This increased consumption of $NO_3^-$ may be attributed to increased total nitrogen removal by denitrification in the mixed culture. The denitrification potential of mixed culture was tested in separate experiments. The ammonia oxidation in the system was not complete in these set of experiments. This might have been due to low concentrations of the pertaining microbes in the system. The presence of any reported anammox bacteria could not be verified by fluorescent in situ hybridization (FISH) technique using known oligonucleotide probes due to non-availability of facility.

### 3.3. Effectiveness of various electron acceptors on anaerobic ammonia removal

The effect of various inorganic electron acceptors on anaerobic ammonia removal and total nitrogen removal is presented in Table 4. Appreciable $NH_4^+$ oxidation occurred in all batch reactors. Although total nitrogen removal was high in the first cycle, there was reduction in the percentage removal of total nitrogen in many reactors in subsequent cycles, except when $NO_2^-$ was used as electron acceptor. Ammonia was oxidized to nitrate and was accumulating when the electron donor was limiting for denitrification. Limitation of the electron donor might have occurred due to the exhaustion of internal stored substrates, when the same biomass was subjected to endogenous respiration for a prolonged time in the sequential batch reactor (SBR) [31,32]. The denitrification using internally stored substrates as carbon and energy source in the absence of externaly supplied substrates was reported [32]. Recently, there have been reports on the accumulation of nitrate by nitrifiers in SBRs operating with limited external carbon source [33].

#### 3.3.1. Anaerobic ammonia removal with no external electron acceptor

Results from CD-Blank and TR-Blank reactors (Table 4) indicated that, in absence of any external electron acceptor, and in presence of OM, there was significant $NH_4^+$ oxidation. However, the total nitrogen removal was not commensurate in many of the cases (Table 4). This disparity may be due to anoxic oxidation of ammonia to nitrate and non-availability of electron donors for further denitrification, resulting in accumulation

Table 4
Effect of various electron acceptors on anaerobic ammonia oxidation and total nitrogen removal

| Batch tag | Cycle number | Final pH | Final $NH_4^+$ (mg/L) | Final $NO_2^-$ (mg/L) | Final $NO_3^-$ (mg/L) | Percentage removal of $NH_4^+$ | Percentage removal of total nitrogen | Percentage reduction of carbon from initial biomass |
|---|---|---|---|---|---|---|---|---|
| | 1 | 7.4 ± 0.05 | 58.9 ± 0.2 | 0 | 3.6 ± 0.02 | 41.1 ± 0.2 | 40.1 ± 0.2 | |
| CD-Blank | 2 | 7.8 ± 0.06 | 11.8 ± 0.04 | 0 | 3.3 ± 0.02 | 88.2 ± 0.04 | 87.2 ± 0.04 | |
| | 3 | 5.8 ± 0.05 | 29.2 ± 0.1 | 0 | 248 ± 1.2 | 72.7 ± 0.1 | 0 | 11.7 ± 0.04 |
| | 1 | 7.2 ± 0.05 | 21.3 ± 0.1 | 0 | 2.35 ± 0.01 | 78.7 ± 0.1 | 78 ± 0.1 | |
| CD-$SO_4$ | 2 | 7.4 ± 0.05 | 0 | 0 | 19.5 ± 0.1 | 100 | 94.3 ± 0.1 | |
| | 3 | 5.8 ± 0.04 | 22.9 ± 0.1 | 0 | 294.1 ± 1.4 | 77.1 ± 0.1 | 0 | 35.6 ± 0.12 |
| | 1 | 6.3 ± 0.04 | 9.8 ± 0.04 | 0 | 119.3 ± 0.6 | 90.2 ± 0.04 | 67.9 ± 0.06 | |
| CD-$NO_3$ | 2 | 7.3 ± 0.05 | 0 | 0 | 302.5 ± 1.4 | 100 | 36.6 ± 0.2 | |
| | 3 | 5.4 ± 0.03 | 54.1 ± 0.2 | 0 | 380.2 ± 1.8 | 45.9 ± 0.2 | 0 | 33.3 ± 0.11 |
| | 1 | 7.0 ± 0.05 | 13.6 ± 0.04 | 0 | 5.2 ± 0.02 | 86.4 ± 0.04 | 90 ± 0.05 | |
| CD-$NO_2$ | 2 | 7.6 ± 0.05 | 0 | 0 | 8.6 ± 0.04 | 100 | 98.4 ± 0.06 | |
| | 3 | 5.8 ± 0.05 | 19.3 ± 0.1 | 0 | 187.8 ± 0.9 | 80.7 ± 0.1 | 51.4 ± 0.2 | 21.3 ± 0.07 |
| | 1 | 6.9 ± 0.05 | 0 | 0 | 205.1 ± 1.0 | 100 | 40.4 ± 0.2 | |
| TR-Blank | 2 | 7.9 ± 0.06 | 30.8 ± 0.1 | 0 | 80.7 ± 0.4 | 69.2 ± 0.1 | 45.7 ± 0.2 | |
| | 3 | 6.6 ± 0.04 | 17.6 ± 0.1 | 0 | 213.6 ± 1.0 | 82.4 ± 0.1 | 20.4 ± 0.1 | 23.4 ± 0.08 |
| | 1 | 7.5 ± 0.05 | 49.9 ± 0.2 | 0 | 21.5 ± 0.1 | 50.1 ± 0.2 | 43.8 ± 0.2 | |
| TR-$SO_4$ | 2 | 7.9 ± 0.06 | 28.2 ± 0.1 | 0 | 75.2 ± 0.4 | 71.8 ± 0.1 | 50 ± 0.2 | |
| | 3 | 6.8 ± 0.04 | 24.6 ± 0.1 | 0 | 162.3 ± 0.8 | 75.4 ± 0.1 | 25.7 ± 0.2 | 19.9 ± 0.07 |
| | 1 | 7.0 ± 0.05 | 0 | 0 | 313.2 ± 1.5 | 100 | 34.3 ± 0.2 | |
| TR-$NO_3$ | 2 | 7.9 ± 0.06 | 33.2 ± 0.1 | 0 | 208.6 ± 1.0 | 66.8 ± 0.1 | 32.3 ± 0.2 | |
| | 3 | 6.7 ± 0.05 | 38.7 ± 0.1 | 36.4 ± 0.6 | 233.3 ± 1.1 | 61.3 ± 0.1 | 12.9 ± 0.1 | 18.9 ± 0.06 |
| | 1 | 8.0 ± 0.06 | 68.1 ± 0.2 | 12 ± 0.2 | 10.7 ± 0.05 | 31.9 ± 0.2 | 50.1 ± 0.2 | |
| TR-$NO_2$ | 2 | 8.0 ± 0.06 | 34.9 ± 0.1 | 0 | 9.7 ± 0.05 | 65.1 ± 0.1 | 75.2 ± 0.1 | |
| | 3 | 6.7 ± 0.04 | 0 | 0 | 162.1 ± 0.8 | 100 | 69 ± 0.2 | 17.7 ± 0.06 |
| CD-Blank + Sucrose | 3 | 6.8 ± 0.05 | 28.6 ± 0.1 | 0 | 102.5 ± 0.5 | 71.4 ± 0.1 | 41.8 ± 0.2 | |
| Blank (abiotic) | 1–3 | 8.0 ± 0.06 to 8.1 ± 0.06 | 78 ± 0.2 to 81 ± 0.2 | 0 | 0 | 19 ± 0.2 to 21 ± 0.2 | 19 ± 0.2 to 21 ± 0.2 | |

Batch tag descriptions are same as in Table 2. Initial carbon in cow dung based sludge = 33.50 ± 0.11%, initial carbon in tannery based sludge = 27.48 ± 0.1%.

of nitrate. The pH was reduced in the reactors as a result of nitrification.

The mechanism of this anoxic oxidation of ammonia to nitrate can be postulated as nitrification by facultative nitrifiers, which may use the oxygen, released via catalase enzyme while destroying $H_2O_2$ as per following Eq. (5). Possibility of $H_2O_2$ formation during biological metabolism is explained below.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \quad (5)$$

The serum bottle tests with substrates for anaerobic ammonia removal and adapted sludges with redox indicator were changing to bluish-pink at intervals, where as blank bottle colour was maintained as blue. Such change of colour of redox indicator is possible when $O_2$ is released to bulk solution. In these bottles, ammonia was oxidised to nitrate and accumulated. The oxygen release might have occurred as a result of reaction (5).

The $H_2O_2$ formation is possible in presence of trace amount of oxygen (oxidative stress) by the use of oxidative enzymes of facultative organisms. The trace amount of oxygen (below detectable level) might be available at ORP of $-248 \pm 25$ mV in the experimental system. Blokina et al. [34] in their review reported many ways of formation of reactive oxygen species (ROS) in oxidative stress conditions. Of the ROS, $H_2O_2$ and superoxide ($O_2^-$) are both produced in a number of cellular reactions including the iron-catalysed Fenton reaction and by various enzymes such as lipoxygenases, peroxidases and NADPH oxidase. Dismutation of superoxide anion ($O_2^-$) by superoxide dismutase will yield $H_2O_2$. Due to its relative stability, the level of $H_2O_2$ is regulated enzymatically by catalase and peroxidase enzymes. Peroxidases, besides their main function in $H_2O_2$ elimination, can also catalyse $O_2^-$ and $H_2O_2$ formation by a complex reaction in which NADH is oxidized using trace amounts of $H_2O_2$, first produced by the non-enzymatic break down of NADH. Next $NAD^-$ radical formed reduces $O_2$ to $O_2^-$, some of which dismutates to $H_2O_2$ and $O_2$. Thus, peroxidase and catalase play an important role in the fine regulation of ROS concentration in the cell through activation and deactivation of $H_2O_2$ [34]. Hydrogen peroxide accumulation under hypoxic conditions has been shown in the roots and leaves of *Hordeum vulgare* and in wheat roots. The superoxide radical and the hydrogen peroxide are inevitable and reactive byproducts of biological metabolism and must be eliminated as soon as possible [16]. The presence of enzymes (superoxidase, peroxidase and catalase) defending the cells of anaerobic bacterium against reactive oxygen species (ROS) was recorded in the last few years [17–20]. The presence of such anti-oxidant defense of anaerobic bacterium shows that superoxide radical and $H_2O_2$ might be produced during anaerobic metabolism in a complex environment.

The enzyme extracted from adapted cow dung and tannery sludge showed peroxidase and catalase enzymatic activity. The catalase activity was estimated to be 30 and 26 units/mg protein/min for cow-dung and tannery sludge, respectively. One unit will decompose 1.0 μmol of $H_2O_2$ per minute at pH 7.0 at 25 °C. Even though the cultures show the good catalase activity, the amount of oxygen produced in the system depends on the amount of $H_2O_2$ produced in the oxidative stress and/or in anoxic conditions. This is a limitation of the system for self-enzymatic generation of oxygen for nitrification as per Eq. (1). However, the results of this study show the feasibility of anaerobic ammonia oxidation to nitrate by catalased enzyme route. This might be happening in nature in various oxic-anoxic interfaces where microbes are always subjected to oxidative stress conditions. For engineering application of such anaerobic ammonia oxidation to nitrate, we need do design suitable oxic-anoxic interfaces to maximize the $H_2O_2$ production. That may be a future challenge, but that possibility will lead to low to medium concentration of anaerobic ammonia removal in presence of organic matter, where reported anammox has a limitation to perform.

### 3.3.2. Inorganic anions as electron acceptors for anaerobic ammonia removal

It has been observed that (Table 4) presence of anions like $NO_3^-$, $NO_2^-$ and $SO_4^{2-}$ were not effective for complete autotrophic anammox in presence of OM as reported by earlier researchers [2,8,9]. The stoichiometric ratio of anammox reaction was not satisfied in all cases where OM was present. Also there was no consistency of stoichiometric ratio of each electron acceptor (like $NO_3^-$, $NO_2^-$ and $SO_4^{2-}$) to electron donor ($NH_4^+$) in three cycles of SBR operation. For example, in case of CD-$SO_4$, the molar ratio of $SO_4^{2-}$ to $NH_4^+$ was found as 1.4, 0.2 and 0 in cycles 1, 2 and 3, respectively. Among the anions studied, $NO_2^-$ was more effective in total nitrogen removal. Here also stoichiometric ratio of $NO_2^-/NH_4^+$ was not matching with reported value of 1.33. In case of CD-$NO_2$, the molar ratio was 0.6, 0.5 and 0.65 in cycles 1, 2 and 3, respectively. Presence of $SO_4^{2-}$ enhanced ammonia oxidation and nitrogen removal in comparison to nitrate. The possible reason may be enhanced sludge hydrolysis and availability of liquid phase OM in sulphidogenic environment [35]. Results of this study showed that presence of inorganic electron acceptor in presence of OM helped in anaerobic ammonia oxidation.

### 3.3.3. Anaerobic ammonia removal with external carbon as electron acceptor

Effect of external organic carbon in anaerobic ammonia removal and in total nitrogen removal was studied by CD-Blank and CD-Blank + Sucrose, in third cycle of operation. The results showed comparable performance with respect to ammonia removal; where as enhanced removal of total nitrogen occurred in presence of sucrose. The presence of external supply of OM might have helped heterotrophic denitrification as per Eq. (6) given below. There was more accumulation of $NO_3^-$ in CD-Blank, cycle 3 (due to limitation of easily biodegradable OM) compared to CD-Blank + Sucrose reactor (Table 4). This confirms co-existence of anaerobic ammonia removal microbes and heterotrophic denitrifiers in cow dung biomass.

The elemental carbon analysis of samples before starting the experiment and at end of third cycle showed a decrease in its carbon content (Table 4, last column), which might have resulted in an increase in liquid phase COD. This COD (carbon) contributed to respiration of mixed culture in anoxic environment.

Table 5
Evidence of auto and heterotrophic growth of denitrifying culture

| Batch tag | Batch description | Final MLSS (mg/L) | Final $NO_3^-$ (mg/L) | Percentage removal of $NO_3^-$ | Final COD (mg/L) | Final $SO_4^{2}$ (mg/L) |
|---|---|---|---|---|---|---|
| CD-B-HDNR[a] | Sucrose as ED | 372 ± 4.8 | 52.4 ± 0.3 | 93.3 ± 0.1 | 99 ± 16 | 95 ± 0.3 |
| CD-B-TDNR[b] | $S_2O_3^-$ as ED | 186 ± 2.3 | 0 | 100 | 0 | 2442 ± 8 |
| TR-B-HDNR[c] | Sucrose as ED | 395 ± 5 | 3.4 | 99.5 ± 0.1 | 42 ± 8 | 95 ± 0.3 |
| TR-B-TDNR[d] | $S_2O_3^-$ as ED | 186 ± 2.3 | 0 | 100 | 0 | 2339 ± 7.7 |

ED, Electron donor; range of initial MLSS = 30 ± 0.07 to 38 ± 0.2 mg/L.
[a] CD-B-HDNR: CD-Blank reactor adapted biomass for heterotrophic denitrification.
[b] CD-B-TDNR: CD-Blank reactor adapted biomass for *Thiobacillus* denitrification.
[c] TR-B-HDNR: TR-Blank reactor adapted biomass for heterotrophic denitrification.
[d] TR-B-TDNR: TR-Blank reactor adapted biomass for *Thiobacillus* denitrification.

There was reduction in pH in all the reactors where $NO_3^-$ accumulation was observed, which is an indirect evidence of nitrification.

The results from abiotic reactor showed 19–22% of ammonia reduction (Table 4). This reduction might be due to the precipitation of an unknown compound and/or possible volatilization of ammonia at around pH 8 and above. Since the final pHs in the reactors with biomass were less than 8, ammonia oxidation might have occurred by biochemical routes.

### 3.4. Evidence of heterotrophic/autotrophic denitrification

For complete nitrogen removal from the system, nitrate needs to be removed by denitrification. The partial and or complete removal of total nitrogen from the system might have taken place via heterotrophic and autotrophic denitrifications as per Eqs. (6) and (7).

$$2NO_3^- + 1.25CH_3COOH \rightarrow 2.5CO_2 + N_2 + 1.5H_2O + OH^-, \quad \Delta G^\circ = -1055\,\text{kJ/R} \tag{6}$$

$$8NO_3^- + 5HS^- + 3H^+ \rightarrow 4N_2 + 5SO_4^{2-} + 4H_2O, \quad \Delta G^\circ = -3721\,\text{kJ/R} \tag{7}$$

Studies were conducted to confirm the presence of auto/heterotrophic denitrifiers in mixed anaerobic ammonia removal culture. Results obtained after 2 weeks of operation of the reactors are presented in Table 5. From these results, it is evident that biomass enriched from both cow dung and tannery contained both heterotrophic and autotrophic denitrifiers and denitrification in mixed culture might have happened as per Eqs. (6) and (7). There was corresponding increase in biomass in the reactors. Though all the reactors were operated as per stoichiometric requirements of electron acceptors and donors for anaerobic ammonia oxidation and denitrification with same amount of seed mixed culture, the denitrification efficiency was higher and its rate was faster as compared to that of anaerobic ammonia removal. This may be due to the fact that denitrifiers have higher growth yield (yield coefficient of heterotrophs; $Y = 0.3$) compared to nitrifiers/anammox ($Y = 0.066 \pm 0.01$) bacteria [30]. Moreover, denitrification reactions are thermodynamically more favorable than ammonia oxidation reactions [7]. Results of this study showed the co-habitation of anaerobic ammonia removing and denitrifying cultures.

### 3.5. Performance of a continuous reactor for anaerobic ammonia removal

The performance of a continuous operating anaerobic ammonia removal reactor (seed: enriched cow dung sludge) in recycle mode during start-up shows (Fig. 4) that ammonia oxidation was possible without adding any external electron acceptor. However, ammonium oxidation efficiency decreased with respect to time and with increased ammonia loading. There was a decrease of COD availability in the reactor and correspondingly there was accumulation of nitrate in the reactor limiting total nitrogen removal. The reactor was started with 17.6 g of VSS (cow dung), which contained heterogeneous biomass along with undigested cow dung. Therefore, initially, there could have been sludge digestion, which resulted in higher soluble COD. With respect to time, the amount of biodegradable substance might have reduced, resulting in lower COD. Similar accumulation of nitrate was observed in the third batch study also when COD was limiting (Table 4, cycle number 3). The results of this continuous reactor operation for 80 days indicated the existence of anaerobic ammonia removal process in presence of organic matter. It was also observed that the process could be shut down for a short period (2 weeks: 50–64 days) without having any adverse effects on the process while restarting. The decrease in performance in the removal of ammonia after shutdown period could be due to increased ammonia loading.

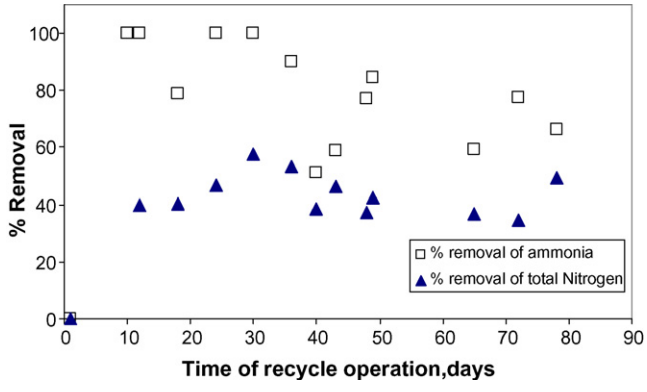


Fig. 4. Performance during start-up of anaerobic ammonia removal reactor.

## 4. Conclusions

Both anaerobically digested cow dung and flocculent type extended aeration process sludge contained ammonia could be potential sources for anaerobic ammonia removal in presence of OM. In presence of OM, nitrate was the preferred oxidation product of anaerobic ammonia oxidation at ORP of $-248 \pm 25$ mV. It is hypothesized that the oxygen required for anaerobic ammonia oxidation might have been obtained by catalase enzymatic activity of facultative anaerobes. Anaerobic ammonia removing culture could have co-habitation with heterotrophic culture and anaerobic nitrogen removal from the system was not completely by anammox process. Though role of anammox may be there in nitrogen removal, in presence of OM, denitrification might be the preferred pathway for nitrogen removal. Among the electron acceptors studied, nitrite was the most effective inorganic electron acceptor for anaerobic ammonia removal in presence of OM. Denitrification by the developed culture was much effective and faster compared to ammonia oxidation.

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