# Effects of Seed Culture and Attached Growth System on the Performance of Anammox Hybrid Reactor Treating Nitrogenous Wastewater

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***Abstract*—The start-up of anammox (anaerobic ammonium oxidation) process in hybrid reactor delineated four distinct phases i.e. cell lysis, lag phase, activity elevation and stationary phase. Cell lysis phase was marked by death and decay of heterotrophic denitrifiers resulting in breakdown of organic nitrogen into ammonium. Lag phase showed initiation of anammox activity with turnover of heterotrophic denitrifiers, which is evident from appearance of $NO_3$-N in the effluent. In activity elevation phase, anammox became the dominant reaction, which can be attributed to consequent reduction of $NH_4$-N into $N_2$ with increased $NO_3$-N in the effluent. Proper selection of mixed seed culture at influent $NO_2^-$ /$NH_4^+$ ratio (1:1) and hydraulic retention time (HRT) of 1 day led to early start up of anammox within 70 days. Pseudo steady state removal efficiencies of $NH_4^+$ and $NO_2^-$ were found as 94.3% and 96.4% respectively, at nitrogen loading rate (NLR) of 0.35 kg N/$m^3$d at an HRT of 1 day. Analysis of the data indicated that attached growth system contributes an additional 11% increase in the ammonium removal and results an average of 29% reduction in sludge washout rate. Mass balance study of nitrogen indicated that 74.1% of total input nitrogen is converted into $N_2$ gas followed by 11.2% being utilized in biomass development. Scanning electron microscope (SEM) observation of the granular sludge clearly showed the presence of cocci and rod shaped microorganisms intermingled on the external surface of the granules.**

***Keywords*— Anammox, hybrid reactor, startup, granulation, nitrogen removal, mixed seed culture.**

## I. Introduction

THE wastewater generated from various industries i.e. slaughter house, pharmaceuticals, coke oven, tanneries and fertilizer wastewaters including landfill leachate and digestate from anaerobic digesters of manure processing contains varying amount of $NH_4$-N concentrations ranging from 220 to 2195 mg/l[1]-[7]. The conventional treatment system for removal of $NH_4$-N from industrial effluents involves either use of ammonium stripper or two stage nitrification/ denitrification system. The application of ammonium strippers are limited due to very less stripping efficiency varying from 50-65% and associated problem of air pollution. The two stage nitrification and denitrification system is cumbersome due to various operation and maintenance problems. Huge amount of biological sludge generated in the process also leads to the problem of their disposal. The failure of either of the stage affects the overall efficiency and stability of the process. Due to various constraints and other problems, these conventional processes are techno-economically infeasible.

The Anammox is one such microbial process that has changed the traditional concept of biological nitrogen removal. The process facilitates direct oxidation of ammonium nitrogen under anaerobic conditions with nitrite as an electron acceptor without addition of any external carbon sources. This can prove to be a novel and promising alternative to the conventional nitrogen removal processes because of the significant reduction of aeration costs, exogenous electron donor saving and low sludge production. However, Dexiang et al. [8] revealed that the anammox process underlies limitations at low inorganic carbon concentrations, but bicarbonate concentration of 2g/l also inhibited anammox activity. The process can find application in full-scale treatment of different kinds of ammonium-rich wastewater such as sludge digested liquor, landfill leachate, coke oven waste water, pharmaceuticals, etc. Tang et al. [9] achieved maximam nitrogen removal rate (NRR) of 0.0020 kg/($m^3$d) at HRT of 10.1 h when anaerobic granular sludge was used as an innoculum in UASB reactor (upflow anaerobic sludge blanket). While, Ma et al. [10] investigated the performance of UASB reactor inoculated with activated sludge and reported NRR of up to 2.28–5.13 kg/($m^3$d) at an HRT of 0.28-0.15 h, in an anammox bioreactor treating low strength wastewater [11]-[13]. Studies conducted by Chen et al. [14] achieved total NRR of 18.65 kg N/($m^3$d) at a NLR of 22.87 kg N/($m^3$d) using anaerobic granular sludge as an inoculum in Expanded Granular Sludge Bed (ESGB) reactor. Zheng et al. [15] started up successfully with nitrifying granular sludge in a UASB (upflow anaerobic sludge blanket) reactor with volumetric NLR and NRR of 1.41 and 1.39 kg N/($m^3$d) respectively. Similarly, Ni et al. [16] achieved steady

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state with ammonia and total nitrogen removal efficiencies around 85% and 72% respectively, after an operation of 180 days adopting a membrane aerated biofilm reactor.

Inspite of several advantages and its increasing popularity, start-up and grannualtion of the anammox cultures has its own difficulties. Extremely slow growth rates and difficulty in isolating the anammox bacteria in pure cultures are the two bottlenecks of the anammox process [17]. The autotrophic bacteria involved in the anammox process are characterized by low growth rates (0.072/d measured at 32°C) and low biomass yield generated per $NH_4$-N consumed (0.11-0.13g/g) as previously described [18]-[22]. The best reported duplication time for anammox bacteria is 7-22 days [23]-[24]. While this slow growth rate is an advantage due to less quanity of sludge being generated in the process, it becomes a handicap for startup or enrichment periods resulting into long start-up time and development of poor quality of granular sludge. The start-up of anammox process has been studied using different reactor configurations, such as UASB, circular stirred tank reactor (CSTR,) anaerobic membrane bioreactor (AnMBR), sequential batch reactor (SBR), and membrane sequential batch reactor (MSBR). However, the use of hybrid configuration of reactor can prove to a most suitable configuration owing to high sludge retention and good granulation extensively reported by many researchers [25]-[27]. In the above context, the present study emphasizes on investigating the Effects of seed culture and attached growth system on the performance of anammox hybrid reactor (AHR) treating nitrogenous wastewater.

## II. Materials and Methods

### *A. Experimental Setup of AHR*

The experimental setup of AHR is shown in Fig. 1. The reactor was fabricated of transparent acrylic plastic with an internal diameter of 10 cm and height 65 cm. The total working volume was 5 litres. Corrugated polyvinyl chloride (PVC) pipes of length 2.25 cm and diameter 2.25 cm were used as filter media. Total 55 nos. of PVC carriers were added to the reactor to constitute an AGM. The sludge blanket in the lower half of the reactor constitutes suspended growth system while filter media in the upper part provides attached growth for the microorganisms. To assess the effect of AGM, two outlets (Outlet I and II), were provided, one above and the other below the filter media, for facilitating the collection of samples (Fig. 1 (a)). The reactor was also completely covered with black cloth to avoid the growth of phototrophic organisms and oxygen production [28].

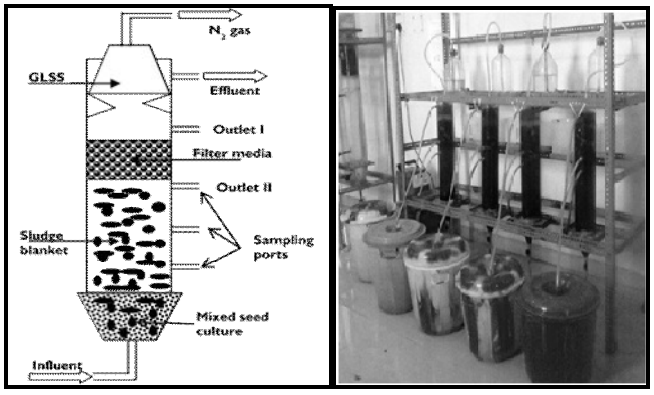



Fig. 1: (a) Schematic diagram of AHR (b) Experimental set-up of AHR

### *B. Seed Culture*

Many researchers [25], [29], [30] reported that combination of activated sludge and anoxic granular sludge resulted in early startup of anammox process. So, mixture of anoxic and conventional activated sludge obtained from Durgapur Coke Oven Plant was used for the purpose of study. The mixed seed culture was black in color with total suspended solid (TSS) and volatile suspended solid (VSS) concentration of 26.7 g/l and 5.93 g/l, respectively.

### *C. Synthetic wastewater*

The composition of enrichment medium used was adopted from van de Graaf et al. [31]. It contains (mg/l): $(NH_4)_2SO_4$, 330; $NaNO_2$, 345; $KHCO_3$, 500; $KH_2PO_4$, 27.2; $MgSO_4.7H_2O$, 300; $CaCl_2.2H_2O$, 180, and 1 ml trace element solutions I and II. Trace element solution I contained (per litre demineralized water): EDTA, 5 g ; $FeSO_4$, 5 g and trace element solution II contained (per litre demineralized water) : EDTA, 15 g ; $ZnSO_4.7H_2O$, 0.43 g ; $CoCl_2.6H_2O$, 0.24 g ; $MnCl_2.4H_2O$, 0.99 g; $CuSO_4.5H_2O$, 0.25 g; $NaMoO_4.2H_2O$, 0.22 g; $NiCl_2.6H_2O$, 0.19 g; $NaSeO_4.10H_2O$, 0.21g; $H_3BO_4$, 0.014g. Ammonium and nitrite were supplemented to mineral medium as required in the form of $(NH_4)_2SO_4$ and $NaNO_2$, respectively at an HRT of 24 hours. The influent concentrations of ammonium and nitrite were both elevated from 35 to 175 mg/l. Influent pH was maintained at 7.5 and fresh synthetic waste water was prepared daily.

### *D. Analytical Methods*

Effluent samples were collected and analysed on daily basis for $NH_4$-N, $NO_2$-N and $NO_3$-N as per the standard methods [32]. The volume of gas liberated was measured by water displacement method while its composition was analysed through Gas Chromatograph (GC) equipped with thermal conductivity detector (TCD) using DPK Spherocarp column. The GC was operated at injector and detector temperatures of 120ºC and 150ºC, respectively. $H_2$ @ 20 ml/min was used as a carrier gas. SEM (Scanning electron microscopy) was adopted as a tool to study the morphology of granules formed in anammox process.

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## III. Results and Discussion

### A. Start-up of anammox bioreactor

The performance of the AHR during start-up was monitored for 90 days as shown in Fig. 2. The influent concentration of ammonium and nitrite nitrogen were both kept at 35 mg/l to maintain an optimal $NH_4/NO_2$ ratio 1:1. The NLR was maintained constant at 0.07 kg $N/m^3d$ at an HRT of 24 hr during this period. The start-up of annomox reactor delineated four distinct phases i.e. cell lysis phase, lag phase, exponential/log phase and stationary phase. In the cell lysis phase, due to lack of organic substrate in the feed, the heterotrophic denitrifiers do not receive continuous and long-term supply of substrate (organics), which resulted in their autolysis due to adverse conditions and prolonged starvation as described by Tang et al. [33]. The decay of heterotrophic denitrifiers results in breakdown of organic nitrogen into ammonium which is evident from the increase in ammonium concentration in the effluent from 35 mg/l to 44.3 mg/l during the intial start-up phase (1-20 days). No ammonium removal was obtained during this phase. This phase was also marked by prominent removal of nitrite because of the enhanced activity of denitrifiers which used $NO_2$ as substrate while nitrate production was almost nil (Fig. 2-3) indicating absence of anammox activity in the reactor. Chamchoi and Nitisoravut [20] postulated that the change of seed sludge might cause the turnover of bacteria, and the former dormant bacteria might be killed, resulting in cell lysis and breakdown of organic nitrogen to ammonium. Similar phenomenon was also reported in anammox startup studies conducted by various others researchers [9], [20], [34].

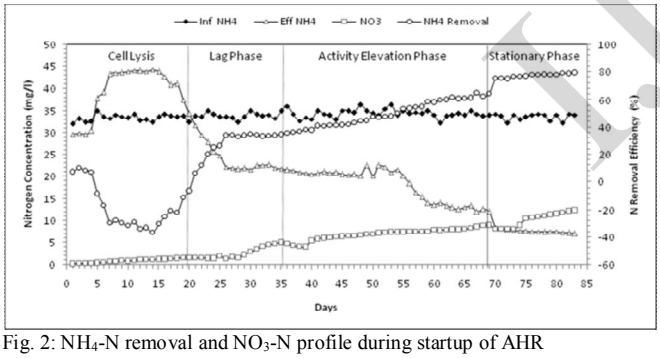



Fig. 2: $NH_4$-N removal and $NO_3$-N profile during startup of AHR

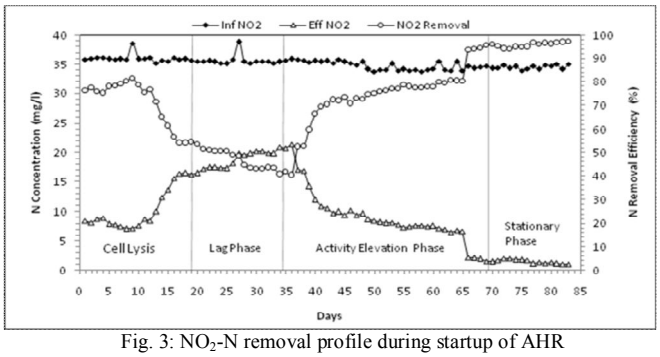



Fig. 3: $NO_2$-N removal profile during startup of AHR

In lag phase, the anammox activity in the reactor slowly enhanced which is evident from the decrease in effluent $NH_4$-N concentration from 44.3 mg/l to less than 35 mg/l from 21$^{st}$ day onwards indicating turnover of heterotrophic denitrifiers by this period and intitation of growth of anammox bacteria which is also indicated by decrease in nitrite removal efficiency(NRE) from 51.5 to 40.3%. This phase continued till 36 days and was marked by slow growth of anammox bacteria which is indicated by gradual increase in $NH_4$ removal efficiency and increase in effluent nitrate concentration from 1.5 to 5.0 mg/l during the reactor operation from 21$^{st}$ day onwards. This is a transition phase where the activities of denitrifiers are suppressed followed by setting-up of anammox conditions in the reactor which is favorable for the growth of anamox bacteria.

Continous and sharp increase in the ammonium and nitrite removal was observed during activity elevation phase (36-70 days). In this phase, anammox activities progressively enhanced and anammox became the dominating reaction in the reactor instead of denitrification and cell lysis, which is indicated by increase in ammonium removal and NRE from 35% to 75.2% and 40.3 to 95.0 % respectively. This is also evident from further increase in nitrate concentration from 5 to 9.4 mg/l from 36$^{th}$ day onwards which is an indicator of anammox reaction during start-up phase.

The nitrogen removal performance finally reached a relative stationary phase, which might be due to establishment of psuedo steady state conditions in the reactor. In this phase, ammonium removal and NRE though increased slightly from 75.2 to 79.4% and 95.0 to 97.1% respectively and then became almost stablised. The similar trend was observed in case of effluent nitrate concentration which slighly increased from 9.4 to 12.4 mg/l and later got stablised (Fig. 2).

Nitrite was effectively consumed by anammox bacteria as a significant ammonium removal was revealed and thus anammox emerged as a vital bio-process in the reactor. The anammox process was successfully started up from mixture of anaerobic sludge and conventional activated sludge using hybrid bioreactor within 70 days which was evident from almost constant removal of $NH_4$-N and $NO_2$-N indicating successful establishment of anammox reaction from 70$^{th}$ days onwards. The start-up period

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found in our case was much shorter than that reported by others [29], [34], [35] which can be attributed to the use of mixed culture of anaerobic and aerbic seed sludge used as inoculum. The removal efficiencies of $NH_4^+$ and $NO_2^-$ i.e. 79.4% and 97.1%, respectively, observed during this period is comparable with the values reported in the literature [9], [25]. Shortage of the substrate might be one of the major causes pertaining to lower values of $NH_4^+$ removal efficiency whereas consumption of nitrite by anammox bacteria led to higher values of $NO_2^-$ removal efficiency.

Start-up study delineated four distinct phases in our study as reported by other researchers [33], [36] whereas three phases were also reported by several other researchers [30], [34]. omitting the lag phase growth of anammox bacteria during the start-up. This may be due to fact that these researchers have increased the influent $NH_4$-N concentration after establishment of anammox process without establishing the pseudo steady state in the reactor.

Under constant HRT and influent conditions, increase in effluent pH (7.74-8.15) indicates improvement of anammox performance. At the end of the start-up period (stationary phase) the effluent pH of 8.15 was found constant indicating pseudo steady state condition. Similar observations were reported by several other researchers [36]-[38]. Complete absence of hydroxide alkalinity with progressive reduction of bicarbonate alkalinity from 307 to 181 mg/l and increase in pH value from 7.74 to 8.15 observed during the initial three phases indicated successful establishment of anammox process [39]. The reduction in the alkalinity may be attributed to consumption of bicarbonates by anammox bacterial population for their respective growth and development as described by Shalini et al. [40].

*B. Acclimation of AHR*

After establishment of anammox activity in the reactor, the influent $NH_4$-N was gradually increased from 50 mg/l to 175 mg/l in step-wise manner from 84$^{th}$ day onwards for acclimation of anammox biomass to higher concentration without changing the HRT. The NLR during this phase varied from 0.1 to 0.35 kg N/m$^3$d. The performance profile of the reator during acclimation phase is shown in Fig. 5-6. It can be seen from the figure that during each step increase in the influent ammonium concentration, the anammox performance registered little upset which is evident from sharp decrease in ammonium(5-10%) and NRE nearly 3-5% initially for 3-4 days which later got stablised. A sharp decline in ammonium removal efficiency (ARE) from 84.9% to 82% can be seen on 136$^{th}$ day when the reactor switched to influent ammonium concentration from 75 to 125 mg/l which got recovered after 3-4 days. However, the ARE kept on increasing with each step increase in the influent ammonium concentration till it reached a almost constant value. The swithing of reactor to every step increase was only done till the reactor got a ARE of > 80%. The reactor attained pseudo steady state removal of 93.8% on 156$^{th}$ day at influent ammonium concentration of 175 mg/l. The reactor showed good tolerance to substrate concentration ($NH_4$-N) even as high as 175 mg/l which is evident from nearly complete removal of $NO_2$-N and significant production of $NO_3$ which increased remarkably from 9.75 to 33.1 mg/l at the end of acclimation phase. It was observed that with the increase in nitrogen loading rate, the nitrogen removal performance of the reactor significantly improved. The maximum NRR of 0.33 kg N/m$^3$d was achieved at maximum NLR of 0.35 kg N/m$^3$d at an HRT of 1 day. Nitrogen removal efficiencies of $NH_4^+$ and $NO_2^-$ during this period were recorded around 94.3% and 96.4% respectively. The higher values of $NH_4^+$ and $NO_2^-$ removal efficiencies achieved in acclimation phase compared to start-up phase may be attributed to the high NLR (0.35 kg N/m$^3$d). Bagchi et al. [35] established anammox process in continuously stirred tank reactor and achieved maximum NRR of 0.216 at maximum NLR of 0.231kg N/m$^3$d. Tang et al. [9] successfully started up anammox process in UASB reactor and achieved maximam NRR of 2.09 kg N/m$^3$d at a NLR of 2.11 kg N/m$^3$d using anaerobic granular sludge as an innoculum. Variation in the NRR as observed by various researchers may be attributed to availability of the substrate and seeding sludge conditions during start-up of the reactor.

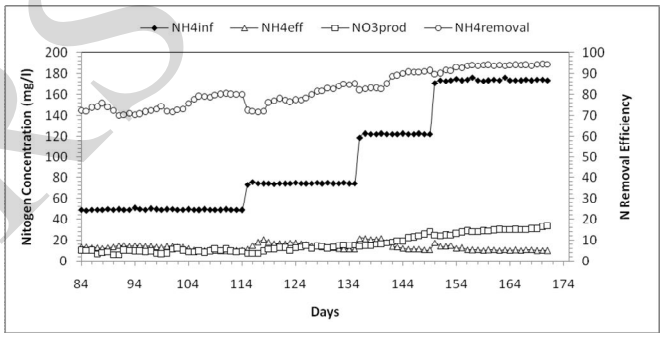



Fig. 5: Nitrogen removal profile ($NH_3$-N) during acclimation of AHR

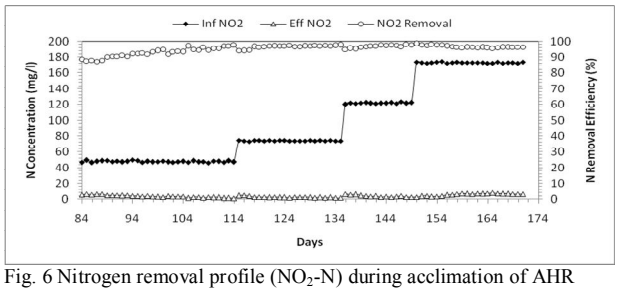



Fig. 6 Nitrogen removal profile ($NO_2$-N) during acclimation of AHR

Similar pH and alkalinity profile was observed during acclimation phase.

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*C. Contribution of attached and suspended growth system towards nitrogen removal*

The results indicated that the attached growth system contributed additional 11% ammonium removal (Fig. 8) and reduced the sludge washout rate by 29% (Fig. 9). Duan et al. [25] also used hybrid reactor configuration and reported an additional 5% ARE on account of attached growth system. The additional ammonium removal achieved in our study might be attributed to the presence of mixed seed culture which augmented the bacterial performance manyfolds..

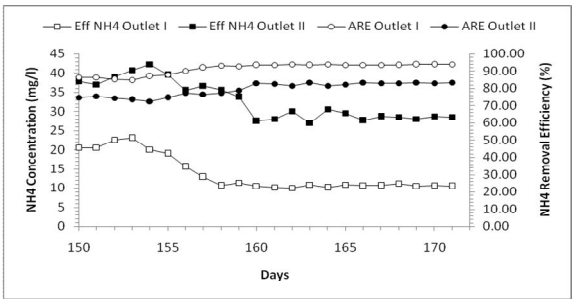



Fig. 8: Effluent $NH_4$-N concentration comparison of outlets I and II

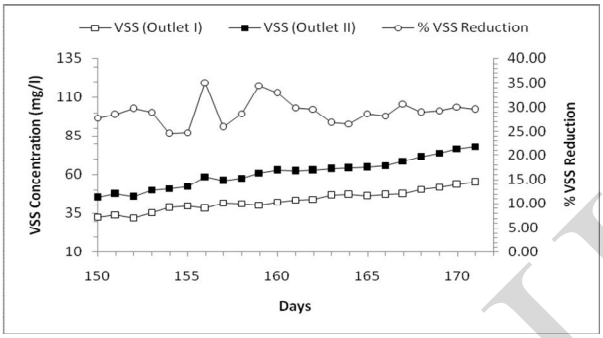



Fig. 9: Percentage VSS reduction and Effluent VSS comparision of outlets I and II

*D. Mass balance of $N_2$ in AHR*

Mass balance study was conducted to study the overall stoichometry of nitrogen conversion and its distributions in Anammox process. Table 2 showed the nitrogen mass balance derived using Eq. (1) on the basis of pseudo steady state performance data of the reactor.

$$N_{inf} = N_{eff} + N_{gas} + N_{syn} \quad (1)$$

where $N_{inf}$ and $N_{eff}$ represent the influent and effluent nitrogen concentrations, respectively, and $N_{gas}$ is nitrogen transferred to the produced dinitrogen gas while $N_{syn}$ denotes nitrogen being utilized for cell synthesis.

TABLE 2
MASS BALANCE OF $N_2$ IN AHR

| N removal paths | Input TN, g/d | Output N, g/d | % of Input TN |
|---|---|---|---|
| Influent Nitrogen | **1.737** | | |
| $NH_4$-N remaining in the effluent | | 0.049 | 2.82 |
| $NO_2$-N remaining in the effluent | | 0.031 | 1.81 |
| $NO_3$-N produced in the effluent | | 0.165 | 9.49 |
| Removal as $N_2$ gas | | 1.288 | 74.1 |
| N removal through biomass yield | | 0.196 | 11.2 |
| **Total** | **1.737** | **1.729** | **99.42** |

The study depicted that out of the total input $N_2$ in the reactor, 11.2% was used to synthesize cells, while 9.49% was converted to nitrate. The fraction of $N_2$ as the end product of Anammox accounts for 74.1% and becomes the most dominant nitrogen species in the effluent of AHR. Effluent $NH_4$-N accounts for 2.82% and 1.81% was converted into effluent $NO_2$-N and rest 0.58% remained unaccounted losses from the reactor. Mass balance study conducted by Ni et al. [16] reported only 68.5% of input nitrogen was converted to dinitrogen gas, while 15.4% accounted for effluent $NO_3$-N. Higher percentage conversion of nitrogen and lower nitrate production observed in our study may be attributed to use of mixed seed culture and hybrid reactor configuration.

*E. Morphological Study of Anammox Granules*

Enhanced biomass retention and good granulation are the key factors which govern the process performance and stability of bioreactor. The morphology of the granules demonstrated presence of heterogeneous bacterial population consisting of both cocci and rod shape along with few clusters of filamentous microorganism (Fig. 10 (a)). Tang et al. [41] also demonstrated similar morphology of anammox bacteria in UASB reactor. A few stayed separate, while most tended to grow in cluster as reported by other researchers [21], [42]. The surface morphology showed heterogeneous and rough surface with irregular projections. SEM images of granular sludge depicted that the granules were almost spherical in shape with an average size of 1.5-2.0 mm. The size distribution of the granules observed in our study is comparable with the values reported (0.01-2.00 mm) in literature [25], [35]. Interstitial void/cavities, which are usually found embedded in the mesh connecting several microbial cell clusters, were also seen on the surface. These interstitial void/cavities serve as channels for transport of substrate, metals and gases through the granules.

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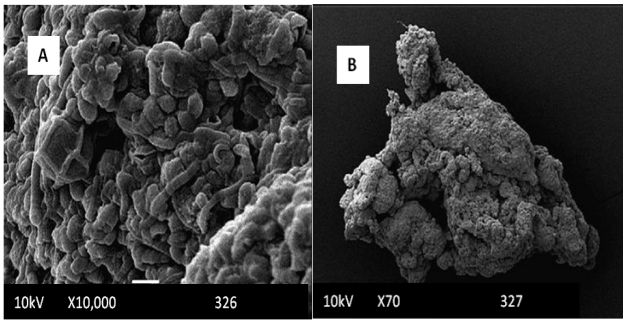



Fig. 13 (a) Cluster Of Cocci And Rod Shaped Bacteria In Anammox Granular Sludge (B) SEM Photos Of Granular Sludge In AHR

## IV. Conclusion

The study concluded that early start-up of anammox process can be achieved using mixed seed culture of anoxic and activated sludge using an AHR within 70 days. The start-up of anammox process can be described by four distinct phases, cell lysis phase, lag phase, activity elevation phase and stationary phase. Ammonium removal efficiencies of 94.3% can be attained at a NLR of 0.35 kg $N/m^3d$ and HRT of 1 day. Progressive increase in effluent nitrate concentration accompanied with high NRE serves as good indicator of anammox performance. The study revealed that attached growth system contributes an additional 11% increase in the ammonium removal and results an average of 29% reduction in sludge washout rate. Out of the total nitrogen supplement, 74.1% accounted for $N_2$ gas production and 11.2% got converted into biomass. Low biomass yield and high substrate conversion into gases as end products make the process superior than other conventional treatment systems. SEM study of biomass indicated development of well compacted granules (1.2-1.5 mm).

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